DEVELOPMENT OF IN-HOUSE ROSE BENGAL PLATE TEST FOR DIAGNOSIS OF BRUCELLOSIS IN CATTLE AND GOATS

MOHAMMED SANI YAHAYA

FPV 2016 7

DEVELOPMENT OF IN-HOUSE ROSE BENGAL PLATE TEST FOR DIAGNOSIS OF BRUCELLOSIS IN CATTLE AND GOATS

By

MOHAMMED SANI YAHAYA

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2016

COPYRIGHT

DEDICATION

This thesis is especially dedicated to:

My beloved parents**,**

ALHAJI YAHAYA INUWA

And

HAJIYA TALATU INUWA

My beloved wife and children**,**

HAJIYA SHEMSIYYA HAMISU

MOHAMMED SANI YAHAYA (BOY)

HALIMATU SADIYA (SIDIYA)

Who always pray, supported and encourage me to do the best.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

DEVELOPMENT OF IN-HOUSE ROSE BENGAL PLATE TEST FOR DIAGNOSIS OF BRUCELLOSIS IN CATTLE AND GOATS

By

MOHAMMED SANI YAHAYA

May 2016

Chairman : Associate Professor Siti Khairani Bejo, PhD
Faculty : Veterinary Medicine

Brucellosis is endemic in South East Asia. Cattle and goats are considered as main livestock in Malaysia due to increase local demand for their milk and meat. This study was designed to develop an in-house Rose Bengal plate test (LRBPT), using local *B. melitensis* isolate and to determine the analytical and diagnostic performance characteristics of the LRBPT, using serum samples from goats and cattle respectively. The performance of LRBPT was compared to commercial RBPT produced by commercial producers. Comparison was performed using compliment fixation test (CFT) as the gold standard. The isolates were reconfirmed using colony morphology, biochemical test and PCR amplification of 16S RNA. All isolates were identified as *B. melitensis* and revealed a similar pattern to the reference strain 16M. Therefore one isolate was chosen as antigen for development of LRBPT.

The sensitivity and specificity was calculated using CFT as the gold standard. Out of 1063 goat sera analysed 364(34.24%), 335(31.51%), and 373(35.08%) were positive by LRBPT, commercial RBPT-*B. melitensis* (cRBPT-*B.melitensis*), and CFT respectively. The sensitivity calculated for the LRBPT compared with CFT was 90.1% while cRBPT-*B. melitensis* was 85.0%. However, the specificity of the LRBPT was lower (95.9%), than the cRBPT-*B. melitensis* (97.4%). Similarly, the positive predictive value (PPV) and negative predictive value (NPV) of the LRBPT are 92.3%, and 94.7%, respectively, compared to that of cRBPT-*B.melitensis* which is 94.6%, and 92.3% respectively. Furthermore, it was observed that the LRBPT has a better value of NPV (94.7%) than that of the cRBPT- *B. melitensis* NPV (92.3%). However the cRBPT- *B. melitensis* has a higher value of PPV (94.6%), than LRBPT (92.7%).

The performance of the LRBPT was also investigated using serum samples collected from cattle. The sensitivity and specificity was calculated using cRBPT-*B. abortus* as the reference or gold standard. The study found that out of 1000 cattle sera analysed 304(30.4%), 282(28.2%), and 208(20.8%) were positive by LRBPT, cRBPT- *B. melitensis* and cRBPT-*B.abortus* respectively. Nevertheless the LRBPT

(88.9%) is still more sensitive compared to cRBPT-*B. melitensis* (84.1%). While the cRBPT-*B. melitensis* has higher specificity (86.5%), than LRBPT (85.0%). Furthermore, it was also observed that the PPV of the LRBPT is lower (60.9%) when compared to that of cRBPT-*B. melitensis* (62.1%). Similarly the NPV of the LRBPT is also higher (96.7%) than that of cRBPT-*B. melitensis* (95.4%).

High sensitivity and low cost LRBPT compared to cRBPT-*B. melitensis* test kit was successfully developed. It was here by recommended that this diagnostic test was suggested to replace the available cRBPT-*B. melitensis* which is relatively more expensive and less sensitive in detection of brucellosis in cattle and goats. It could also be used for epidemiological surveillance of goat and cattle brucellosis in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains.

PEMBANGUNAN *IN-HOUSE ROSE BENGAL PLATE TEST* UNTUK DIAGNOSIS BRUCELLOSIS DALAM LEMBU DAN KAMBING

Oleh

MOHAMMED SANI YAHAYA

Mei 2016

Pengerusi : Profesor Madya Siti Khairani Bejo, PhD
Fakulti : Perubatan Veterinar

Brucellosis adalah penyakit endemik di Timur Selatan Asia. Lembu dan kambing dianggap sebagai ternakan utama di Malaysia oleh kerana permintaan tempatan yang tinggi untuk susu dan daging. Kajian ini bertujuan untuk membangunkan LRBPT, menggunakan isolat tempatan *B. melitensis* serta untuk menentukan prestasi analisis dan diagnostik LRBPT, menggunakan sampel serum daripada kambing dan lembu. Prestasi LRBPT dibandingkan dengan RBPT komersial yang dihasilkan oleh pengeluar komersial. Perbandingan dilakukan dengan menggunakan CFT sebagai 'gold standard'. Isolat telah dikenalpasti semula menggunakan morfologi koloni, ujian biokimia dan tindak balas rantai polimerase (PCR) yang mengamplifikasi RNA 16S. Semua isolat telah dikenal pasti sebagai *B. melitensis* dan menunjukkan corak yang sama dengan *B. melitensis* 16M rujukan. Oleh itu, satu isolat telah dipilih sebagai antigen untuk pembangunan LRBPT.

Sensitiviti dan spesifisiti telah dikira dengan menggunakan CFT sebagai 'gold standrad'. Daripada 1063 sera kambing yang telah dianalisis; 364 (34.24%), 335 (31.51%), dan 373 (35,08%) masing-masing adalah positif oleh LRBPT, komersial RBPT-*B. melitensis* (cRBPT-*B. melitensis*) dan CFT. Sensitiviti dikira untuk LRBPT berbanding dengan CFT adalah 90.1% manakala cRBPT-*B. melitensis* adalah 85.0%. Walau bagaimanapun, spesifisiti LRBPT adalah lebih rendah (95.9%), daripada cRBPT-*B. melitensis* (97.4%). Begitu juga, nilai ramalan positif (PPV) dan nilai ramalan negatif (NPV) daripada LRBPT masing-masing adalah 92.3%, dan 94.7% berbanding dengan cRBPT-*B. melitensis* yang menunjukkan 94.6% (PPV) dan 92.3% (NPV). Tambahan pula, didapati LRBPT mempunyai nilai NPV yang lebih baik (94.7%) berbanding dengan NPV untuk cRBPT-*B. melitensis* (92.3%). Namun cRBPT-*B. melitensis* mempunyai nilai yang lebih tinggi PPV (94.6%), daripada LRBPT (92.7%).

Prestasi LRBPT juga diuji dengan menggunakan sampel serum yang dikumpul daripada lembu. Sensitiviti dan spesifisiti dikira menggunakan cRBPT- *B. abortus* sebagai rujukan atau 'gold standard'. Kajian mendapati bahawa daripada 1000 sera

lembu dianalisis 304 (30.4%), 282 (28.2%), dan 208 (20.8%) adalah masing-masing positif oleh LRBPT, cRBPT- *B. melitensis* dan cRBPT- *B. abortus*. Walaupun demikian, LRBPT (88.9%) masih lebih sensitif berbanding cRBPT- *B. melitensis* (84.1%). Manakala, cRBPT- *B. melitensis* mempunyai spesifisiti yang lebih tinggi (86.5%) berbanding LRBPT (85.0%). Tambahan pula, ia juga diperhatikan bahawa PPV untuk LRBPT adalah lebih rendah (60.9%) berbanding dengan yang cRBPT- *B. melitensis* (62.1%). Namun NPV untuk LRBPT lebih tinggi (96.7%) berbanding dengan cRBPT- *B. melitensis* (95.4%).

Ujian diagnostik LRBPT mempunyai sensitiviti yang tinggi dan kos yang rendah berbanding cRBPT- B. melitensis telah berjaya dibangunkan dalam kajian kini. Adalah disyorkan agar ujian diagnostik ini digunakan untuk menggantikan ujian cRBPT-B.melitensis yang sedia dimana harganya lebih mahal dan kurang sensitif dalam mengesan brucellosis dalam lembu dan kambing. Ia juga boleh digunakan untuk pengawasan epidemiologi brucellosis pada kambing dan lembu di Malaysia.

ACKNOWLEDGEMENTS

All thanks and praise is due to Almighty Allah who has granted me the privilege of sustenance until date. The completion of this thesis was made possible via invaluable support granted by many. I would like to acknowledge my gratitude to the entire members of my supervisory committee; Associate Professor Dr. Siti Khairani Bejo, Professor Ariff M. Omar, and Associate Professor Dr. Zunita Zakaria. I am grateful for the time and effort you have all patiently committed in supporting and guiding me throughout the period of my study. The thoughtful and constructive suggestions and ideas rendered by the committee have been of greatest importance to this study. Besides, I would like to express my special thanks to the chairman of my supervisory committee; Associate Professor Dr. Siti Khairani Bejo working with her has been an extremely rewarding endeavor.

My profound gratitude also goes to all my postgraduate colleagues and the laboratory staff of Bacteriology Laboratory, Faculty of Veterinary Medicine. I do appreciate the love and understanding extended to me during the period of my study. Most importantly I would like to express my heart-felt appreciation for the inestimable sacrifice and effort of my parents for their entire sacrifice on my educational pursuit since childhood to date is highly acknowledged. Also acknowledge of the love and well wishes of my relatives, family and friends for their love concern and prayers throughout the period of my study.

Finally behind every successful man is a woman they say; I therefore acknowledge the sacrifice and inestimable support of my wife, who has been all alone for the period of the study. Last but not the leastt my appreciation goes to my beloved children Mohammed Sani Yahaya (Boy) and Sadiya Mohammed Sani. May Allah reward you all beyond measures Ameen.

I certify that a Thesis Examination Committee has met on 04 May 2016 to conduct the final examination of Yahaya Mohammed Sani on his thesis entitled "Development of In-House Rose Bengal Plate Test for Diagnosis of Brucellosis in Cattle and Goats" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Abdul Rahman bin Omar, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Mohd Zamri b Saad, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Kasing Apun, PhD
Professor
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 June 2016

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science**.** The members of the Supervisory committee were as follows:

Siti Khairani-Bejo, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohamed Ariff Omar, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Zunita Zakaria, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of graduate studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work
- quotations, illustrations and citations have been duly referenced
- the thesis has not been submitted previously or concurrently for any other degree at any institutions
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be owned from supervisor and deputy vice –chancellor (Research and innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software

Signature: _____ Date: _____

Name and Matric No.: Mohammed Sani Yahaya , GS40922

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature:	_____	Signature:	_____
Name of Chairman of Supervisory Committee:	Associate Professor Dr. Siti Khairani Bejo	Name of Member of Supervisory Committee:	Professor Dr. Mohamed Ariff Omar

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Zunita Zakaria

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
APHA	Animal and plant Health Agency
ASe	Analytical Sensitivity
Asp	Analytical Specificity
AUC	Area Under Curve
Bp	Base pair
cRBPT-*BA*	Commercial RBPT-*B. abortus*
cELISA	Competitive enzymes-linked immunoassay
cRBPT-*Bm*	Commercial RBPT-*B. melitensis*
CFT	Compliment Fixation Test
DNA	Deoxyribonucleic acid
DSe	Diagnostic Sensitivity
DSp	Diagnostic Specificity
ELISA	Enzymes-linked immunosorbent assay
FAO	Food and Agricultural Organization
FAO-APHCA	FAO- Animal Production and health Commission for Asia and the Pacific
FN	False Negative
FP	False Positive
FPSR	False Positive Serological reaction
H_2S	Hydrogen sulphide
iELISA	Indirect enzymes-linked immonosorbent assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K	Kappa Value
LRBPT	In-house Rose Bengal plate test
LPS	Lipopolysaccharide

ml	Milliter
µL	Micro liter
NPV	Negative Predictive Value
OMP	Outer Membrane Protein
OIE	Office International Opizootis
O-LPs	O-lipopolysaccharide
OPS	O-polysacchride
PH	Hydrogen ion concentration
PCR	Polymerase chain reaction
PPV	Positive Predictive Value
PFGE	Pulsed-Field gel electrophoresis
PI	Performance Index
RBPT	Rose Bengal plate test
R-LPs	Rough lipopolysaccharide
ROC	Receiver Operating Curve
SLPs	Smooth lipopolysaccharide
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Introduction

Brucellosis is an economically important disease in production animals worldwide caused by *Brucella species* (Godfroid *et al.,* 2011). Brucellae are Gram-negative, facultative intracellular bacteria that infect many species of animals and man. Ten species are recognized within the genus *Brucella specie.* However, there are 6 "classical" species of the genus *Brucella* based mainly on differences in pathogenicity and host preference which include *B. abortus, B. melitensis, B. suis B. neotomae, B. ovis* and *B. canis*. The main pathogenic species of *Brucella*, worldwide are *B. abortus* and *B. melitensis* which cause abortion and infertility in their natural hosts (Banai and Corbel, 2010; Godfroid *et al.*, 2010). Bovine brucellosis is usually caused by *B. abortus* and less frequently by *B. melitensis* (OIE, 2009a). On the other hand, caprine brucellosis is mainly caused by *B. melitensis* and sporadic cases have been observed in goats due to *B. abortus* (OIE, 2009b). The presence of brucellosis in Malaysia was first confirmed with the isolation of *B. abortus* from large ruminants in 1950. Small ruminants' brucellosis was first reported in sheep between 1987 and 1991 using serological method (Bahaman *et al.,* 2007). The cases of brucellosis in goats have increased during the period 2000-2009 affecting all states in Malaysia, especially in 2004 where a significant surge in the sero-prevalence was 0.91% observed and the trend had continued into recent years (Bamaiyi *et al.,* 2015). Similarly, bovine brucellosis has been reported to be widespread among herds in Peninsular Malaysia with prevalence 21.8% (Anka *et al.,* 2013). The disease status of brucellosis due to *B. melitensis* in Malaysia has been shifted from confirmed infection but no clinical disease to disease presence, which means that the disease status started unaccustomed condition (OIE, 2014). The new status of brucellosis in Malaysia imposes an increase in demand for more surveillance programs to detect the infected animals within the herds and flocks.

The spread of the disease later instigated in nationwide brucellosis eradication program, which involved the testing and slaughter of seropositive animals and consequently resulted in a marked decline in the number of seropositive cattle (Bahaman et al., 2007).

This policy has a significant impact on the operational activities of the farms which consequently affect their economy.

The Laboratory diagnosis of brucellosis can be classified mainly into two categories, the direct methods that determine the presence of the bacteria such as bacterial isolation, and the indirect methods, mainly serological tests, which detect the immune response against the bacterial antigens such as Rose Bengal plate test (RBPT), Enzyme linked immunosorbent assay (ELISA) and complement fixation test (CFT) (Nielsen, 2002; Al Dahouk *et al.,* 2003; Poester *et al.,* 2010). Isolation

and identification of *Brucella* offers definitive diagnosis and is considered the gold standard method for diagnosis of brucellosis (Al Dahouk *et al.*, 2003; Bamaiyi *et al.*, 2014). However, this method is time consuming and needs skilled personnel in addition biohazard effect (Godfroid *et al.*, 2011; Poester *et al.*, 2010). Therefore, serological tests are normally performed which can offer fast and cost effective method for diagnosis in addition to less demand are needed with low individual risks comparing to bacterial isolation especially during the control programs of brucellosis (Nielsen and Yu, 2010; Poester *et al.*, 2010). The RBPT is considered as one of the most suitable screening tests that have been used for control of brucellosis (Garin-Bastuji *et al.*, 2006; OIE, 2009b, 2009a; Nielsen and Yu, 2010). However, positive reactions should be retested by ELISA or CFT to confirm the results (Nielsen, 2002; OIE, 2009a).

The Low sensitivity of commercial RBPT produced by VLA, UK has been reported by Shahaza *et al.* (2009). In this study in-house RBPT antigen that could be produced easily with low cost and simple methodology will be developed using local isolate of *B. melitensis*. The performance of the kit was compared with that of commercially available one using the CFT as the gold standard. Therefore, the objectives of the study are to:

1. Develop an in-house RBPT for detection of cattle and goats brucellosis.
2. Determine the analytical and diagnostic performance characteristics of the newly developed in-house RBPT among goats serum.
3. Determine the analytical and diagnostic performance characteristics of the newly developed in-house RBPT among cattle serum.

1.2 Research problem

Despite the importance of the disease both economically and for human health. Low sensitivity of commercial RBPT antigen for *B. melitensis* reported in previous studies in addition to high cost and time consuming orders of the commercial kits and reagents could impose obstacles in the way of control programs of bovine and caprine brucellosis.

1.3 Research hypothesis

The in-house RBPT has higher sensitivity and specificity than the commercial RBPT for diagnosis of cattle and goats brucellosis.

1.4 Significance of the study

Development of a simple and universal assay for detection of antibody to *Brucella* sp. in cattle and goats sera allows for better control of this disease and there by lead to quicker eradication.

REFERENCES

Abernethy, D. A., Menzies, F. D., McCullough, S. J., McDowell, S. W. J., Burns, K. E., Watt, R., Gordon, A. W., Greiner, M., and Pfeiffer, D. U. (2012). Field trial of six serological tests for bovine brucellosis. *The Veterinary Journal, 191*(3), 364-370.

Abdoel, T., Dias, I. T., Cardoso, R., and Smits, H. L. (2008). Simple and rapid field tests for brucellosis in livestock. *Veterinary Microbiology, 130*(3-4), 312-319.

Adone, R., Muscillo, M., La Rosa, G., Francia, M., and Tarantino, M. (2011). Antigenic, Immunologic and Genetic Characterization of Rough Strains *B. abortus* RB51, *B. melitensis* B115 and *B. melitensis* B18. *PloS one, 6*(10), e24073. Retrieved from doi:10.1371/journal.pone.0024073

Affi, M. M., Abdul-Raouf, U. M., El-Bayoumy, E. M., Montasser, A. M., and Mohamad, H. A. (2011). Isolation and Biotyping of *Brucella melitensis* from Upper Egypt. *Journal of American Science, 7*(3), 653-659.

Ahmed, I. M., Khairani-Bejo, S., Hassan, L., Bahaman, A. R., and Omar, A. R. (2015). Serological diagnostic potential of recombinant outer membrane proteins (rOMPs) from *Brucella melitensis* in mouse model using indirect enzyme-linked immunosorbent assay. *BMC Veterinary Research, 11*(1), 275.

Al Dahouk, S., Tomaso, H., Nöckler, K., Neubauer, H., and Frangoulidis, D. (2003). Laboratory-based diagnosis of brucellosis-a review of the literature. Part I: Techniques for direct detection and identification of *Brucella* spp. *Clinical Laboratory, 49*(9-10), 487-505.

AL-Garadi, M. A., Khairani-Bejo, S., Zunita, Z., and Omar, A. R. (2011). Isolation and identification of *Bucella melitensis* in goats. *Journal of Animal and Veterinary Advances, 10*(8), 972-979.

Al-Majali, A. M. (2005). Seroepidemiology of caprine Brucellosis in Jordan. *Small Ruminant Research, 58*(1), 13-18.

Al-Majali, A. M., Majok, A. A., Amarin, N. M., and Al-Rawashdeh, O. F. (2007). Prevalence of, and risk factors for, brucellosis in Awassi sheep in Southern Jordan. *Small Ruminant Research, 73*(1-3), 300-303.

Al-Talafhah, A. H., Lafi, S. Q., and Al-Tarazi, Y. (2003). Epidemiology of ovine brucellosis in Awassi sheep in Northern Jordan. *Preventive Veterinary Medicine, 60*(4), 297-306.

Alton, G. (1987). Control of *Brucella melitensis* infection in sheep and goats-a review. *Tropical Animal Health and Production,, 19*(2), 65-74.

Alton, G. G. (1990). *Brucella melitensis*. In K. H. Nielsen and J. R. Duncan (Eds.), *Animal brucellosis* (pp. 383–409). Florida, USA: CRC Press, Boca Raton.

Aras, Z., and Ateş, M. (2011). The first report of isolation and molecular characterisation of *Brucella melitensis* Rev-1 vaccine strain from an aborted sheep fetus in Turkey. *Small Ruminant Research, 95*(2-3), 150-159.

Alonso-Urmeneta, B., Marín, C., Aragón, V., Blasco, J. M., Díaz, R., and Moriyón, I. (1998). Evaluation of lipopolysaccharides and polysaccharides of different epitopic structures in the indirect enzyme-linked immunosorbent assay for diagnosis of brucellosis in small ruminants and cattle. *Clinical and Diagnostic Laboratory Immunology, 5*(6), 749-754.

Anka, M. S., Hassan, L., Adzhar, A., Khairani-Bejo, S., Mohamad, R. B., and Zainal, M. A. (2013). Bovine brucellosis trends in Malaysia between 2000 and 2008. *BMC Veterinary Research, 9*(1), 230.

Bahaman, A. R., Joseph, P. G., and Khairani-Bejo, S. (2007). A review of the epidemiology and control of brucellosis in Malaysia. *Jurnal Veterinar Malaysia, 19*(1), 1-6.

Bamaiyi, P. H., Hassan, L., Khairani-Bejo, S., and Zainal Abidin, M. (2014). Updates on brucellosis in Malaysia and Southeast Asia. *Malaysia Journal of Veterinary Research, 5*, 71–82.

Bamaiyi, P. H., Hassan, L., Khairani-Bejo, S., ZainalAbidin, M., Ramlan, M., Adzhar, A., Abdullah, N., Hamidah, N. H. M., Norsuhanna, M. M., and Hashim, S. N. (2015). The prevalence and distribution of *Brucella melitensis* in goats in Malaysia from 2000 to 2009. *Preventive Veterinary Medicine*(0).

Banai, M. (2002). Control of small ruminant brucellosis by use of *Brucella melitensis* Rev. 1 vaccine: laboratory aspects and field observations. *Veterinary Microbiology, 90*(1), 497-519.

Banai, M., and Corbel, M. (2010). Taxonomy of *Brucella*. *Open Veterinary Science Journal, 4*(1), 85-101.

Blasco, J. M. (1997). A review of the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. *Preventive Veterinary Medicine, 31*(3), 275-283.

Blasco, J. M. (2006). Existing and future vaccines against brucellosis in small ruminants. *Small Ruminant Research, 62*(1-2), 33-37.

Blasco, J. M., and Molina-Flores, B. (2011). Control and eradication of *Brucella melitensis* infection in sheep and goats. *Veterinary Clinics of North America: Food Animal Practice, 27*(1), 95-104.

Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P. G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C. M., Olliaro, P., Peeling, R. W.,

and Ramsay, A. (2008). Evaluation of diagnostic tests for infectious diseases: general principles. *Nature Reviews Microbiology, 8*, S17-S29.

Benkirane, A. (2006). Ovine and caprine brucellosis: World distribution and control/eradication strategies in West Asia/North Africa region. *Small Ruminant Research, 62*(1-2), 19-25.

Bundle, D. R., Cherwonogrodzky, J. W., Caroff, M., and Perry, M. B. (1987). The lipopolysaccharides of *Brucella abortus* and *B. melitensis*. *Annales de l'Institut Pasteur/Microbiologie, 138*(1), 92-98.

Buyukcangaz, E., and Sen, A. (2007). The first isolation of *Brucella melitensis* from bovine aborted fetus in Turkey. *Journal of Biological and Environmental Sciences, 1*(3), 139-142.

Cardoso, P., Macedo, G., Azevedo, V., and Oliveira, S. (2006). *Brucella* spp noncanonical LPS: structure, biosynthesis, and interaction with host immune system. *Microbial Cell Factories, 5*(1), 13. Retrieved from http://www.microbialcellfactories.com/content/5/1/13 website:

Carvalho Neta, A. V., Mol, J. P., Xavier, M. N., Paixao, T. A., Lage, A. P., and Santos, R. L. (2010). Pathogenesis of bovine brucellosis. *The Veterinary Journal, 184*(2), 146-155.

Celebi, O., and Atabay, H. I. (2009). Seroepidemiological investigation of brucellosis in sheep abortions in Kars, Turkey. *Tropical animal health and production, 41*(1), 115-119.

Cherwonogrodzky, J., Dubray, G., Moreno, E., and Mayer, H. (1990). Antigens of *Brucella*. In K. H. Nielsen and J. R. Duncan (Eds.), *Animal brucellosis* (pp. 19-64). Florida, USA: CRC Press, Boca Raton.

Cloeckaert, A., Baucheron, S., Vizcaino, N., and Zygmunt, M. S. (2001). Use of recombinant BP26 protein in serological diagnosis of *Brucella melitensis* infection in sheep. *Clinical and Diagnostic Laboratory Immunology, 8*(4), 772-775.

Cloeckaert, A., De Wergifosse, P., Dubray, G., and Limet, J. N. (1990). Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. *Infection and Immunity, 58*(12), 3980-3987.

Cloeckaert, A., Kerkhofs, P., and Limet, J. N. (1992). Antibody response to *Brucella* outer membrane proteins in bovine brucellosis: immunoblot analysis and competitive enzyme-linked immunosorbent assay using monoclonal antibodies. *Journal of Clinical Microbiology, 30*(12), 3168-3174.

Cloeckaert, A., Vizcaíno, N., Paquet, J.-Y., Bowden, R. A., and Elzer, P. H. (2002). Major outer membrane proteins of *Brucella* spp.: past, present and future. *Veterinary Microbiology, 90*(1–4), 229-247.

Corbel, M. J. (1997). Brucellosis: an overview. *Emerging Infectious Diseases, 3*(2), 213-221.

Corbel, M. J. (2006). Brucellosis in humans and animals (pp. 28-35). Geneva, Switzerland: World Health Organization.

Crowther, J. R. (2009). *Methods in Molecular Biology: The ELISA Guidebook* (2 ed.). New York, USA: Humana Press.

Department Veterinary Services. (2015). Malaysia: Number of Livestock, 2012-2013. Retrieved 6.4.2015, from Department Veterinary Services http://www.dvs.gov.my/en/statistik

Díaz, A. E. (2013). Epidemiology of brucellosis in domestic animals caused by *Brucella melitensis*, *Brucella suis* and *Brucella abortus*. *Revue scientifique et technique (International Office of Epizootics), 32*(1), 43-51, 53-60.

Díaz-Aparicio, E., Aragón, V., Marín, C., Alonso, B., Font, M., Moreno, E., Pérez-Ortiz, S., Blasco, J. M., Díaz, R., and Moriyón, I. (1993). Comparative analysis of *Brucella* serotype A and M and *Yersinia enterocolitica* O: 9 polysaccharides for serological diagnosis of brucellosis in cattle, sheep, and goats. *Journal of Clinical Microbiology, 31*(12), 3136-3141.

Erganis, O., Huseyin Hadimli, H., Solmaz, H., and Corlu, M. (2005). Comparison of Rose Bengal plate test antigens prepared from *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. *Bulletin of the Veterinar Institute in Pulawy, 49*(2), 165–167

FAO. (2010). *Brucella melitensis* in Eurasia and the Middle East (Vol. 10, pp. 5-36). Rome: FAO Animal Production and Health Proceedings.

Fawcett, T. (2006). An introduction to ROC analysis. *Pattern Recognition Letters, 27*(8), 861-874.

Ferreira, A. C., Cardoso, R., Dias, I. T., Mariano, I., Belo, A., Preto, I. R., Manteigas, A., Fonseca, A. P., and De Sá, M. I. C. (2003). Evaluation of a modified Rose Bengal test and an indirect Enzyme-Linked Immunosorbent Assay for the diagnosis of Brucella melitensis infection in sheep. *Veterinary Research, 34*(3), 297-305.

Gall, D., Nielsen, K., Vigliocco, A., Smith, P., Perez, B., Rojas, X., and Robles, C. (2003). Evaluation of an indirect enzyme-linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep. *Small Ruminant Research, 48*(3), 173-179.

Garin-Bastuji, B., Blasco, J. M., Marín, C., and Albert, D. (2006). The diagnosis of brucellosis in sheep and goats, old and new tools. *Small Ruminant Research, 62*(1-2), 63-70.

García-Yoldi, D., Marín, C. M., de Miguel, M. J., Muñoz, P. M., Vizmanos, J. L., and López-Goñi, I. (2006). Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clinical Chemistry, 52*(4), 779-781.

Garin-Bastuji, B., Blasco, J. M., Grayon, M., and Verger, J.-M. (1998). *Brucella melitensis* infection in sheep: present and future. *Veterinary Research, 29*(3-4), 255.

Garin-Bastuji, B., Blasco, J. M., Marín, C., and Albert, D. (2006). The diagnosis of brucellosis in sheep and goats, old and new tools. *Small Ruminant Research, 62*(1-2), 63-70.

Garin-Bastuji, B., Hummel, N., Gerbier, G., Cau, C., Pouillot, R., Da Costa, M., and Fontaine, J.-J. (1999). Non specific serological reactions in the diagnosis of bovine brucellosis: experimental oral infection of cattle with repeated doses of *Yersinia enterocolitica* O:9. *Veterinary Microbiology, 66*(3), 223-233.

Garrido-Abellan, F. (2001). Brucellosis in sheep and goats (*Brucella melitensis*). In R. Ahl (Ed.), *Report of the Scientific Committee on animal Health and Animal Welfare* (pp. 1-89). Santa Fe – Spain: European Commission

Godfroid, J., Scholz, H. C., Barbier, T., Nicolas, C., Wattiau, P., Fretin, D., Whatmore, A. M., Cloeckaert, A., Blasco, J. M., Moriyon, I., Saegerman, C., Muma, J. B., Al Dahouk, S., Neubauer, H., and Letesson, J. J. (2011). Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. *Preventive Veterinary Medicine, 102*(2), 118-131.

Gupta, V. K., Verma, D. K., Singh, S. V., and Vihan, V. S. (2007). Serological diagnostic potential of recombinant outer membrane protein (Omp31) from *Brucella melitensis* in goat and sheep brucellosis. *Small Ruminant Research, 70*(2-3), 260-266.

Jacques, I., Olivier-Bernardin, V., and Dubray, G. (1998). Efficacy of ELISA compared to conventional tests (RBPT and CFT) for the diagnosis of Brucella melitensis infection in sheep. *Veterinary Microbiology, 64*(1), 61-73.

Joseph, P. G. (1987). Brucellosis in Malaysia, Technical Report No. 4 (pp. 1-22). Ministry of Agriculture Malaysia, Department of Veterinary Services: Veterinary Reseach Institute, Ipoh.

Kahler, S. C. (2000). *Brucella melitensis* infection discovered in cattle for first time, goats also infected. *Journal of the American Veterinary Medical Association, 216*(5), 648.

Kaltungo, B. Y., Saidu, S. N. A., Sackey, A. K. B., and Kazeem, H. M. (2015). Sero-prevalence of brucellosis in sheep in North Senatorial District of Kaduna State, Nigeria. *Asian Pacific Journal of Tropical Disease, 5*(2), 163-168.

Khairani-Bejo, S, & Ardhy-Adman Bahaman, AR. (2006). Investigation of canine brucellosis in Klang Valley Malaysia. *Journal of Animal and Veterinary Advances, 5*, 42-44.

Kumar, S., Tuteja, U., Kumar, A., and Batra, H. V. (2008). Expression and purification of the 26 kDa periplasmic protein of Brucella abortus: a reagent for the diagnosis of bovine brucellosis. *Biotechnology and Applied Biochemistry, 49*(3), 213-218.

Letesson, J. J., Tibor, A., Van Eynde, G., Wansard, V., Weynants, V., Denoel, P., and Saman, E. (1997). Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology, 4*(5), 556-564.

MacMillan, A. (1997). Investigation of the performance of the Rose Bengal plate test in the diagnosis of *Brucella melitensis* infection in sheep and goats. *World Animal Review (FAO)*. MacMillan, A. (1990). Conventional serological tests. In K. H. Nielsen and J. R. Duncan (Eds.), *Animal brucellosis* (pp. 153-197). Florida, USA: CRC Press, Boca Raton.

Mathew, C., Stokstad, M., Johansen, T. B., Klevar, S., Mdegela, R. H., Mwamengele, G., Michel, P., Escobar, L., Fretin, D., and Godfroid, J. (2015). First isolation, identification, phenotypic and genotypic characterization of *Brucella abortus* biovar 3 from dairy cattle in Tanzania. *BMC Veterinary Research, 11*, 156.

McGiven, J., Taylor, A., Duncombe, L., Sayers, R., Albert, D., Banai, M., Blasco Martínez, J. M., Elena, S., Fretin, D., and Garin-Bastuji, B. (2011). The first international standard anti-*Brucella melitensis* serum. *Scientific and Technical Review of the Office International des Epizooties, 30*(3), 809-819.

Minas, A. (2006). Control and eradication of brucellosis in small ruminants. *Small Ruminant Research, 62*(1-2), 101-107.

Moreno, E., and Moriyón, I. (2006). The genus *Brucella*. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrant (Eds.), *The prokaryotes* (Vol. 5, part 1, section 3.1, pp. 315-456). New York: Springer-Verlag.

Munoz, P. M., Marin, C. M., Monreal, D., Gonzalez, D., Garin-Bastuji, B., Diaz, R., Mainar-Jaime, R. C., Moriyon, I., and Blasco, J. M. (2005). Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. *Clinical and Diagnostic Laboratory Immunology, 12*(1), 141-151.

Naletoski, I., Kirandziski, T., Mitrov, D., Krstevski, K., Dzadzovski, I., and Acevski, S. (2010). Gaps in brucellosis eradication campaign in sheep and goats in Republic of Macedonia: lessons learned. *Croatian Medical Journal, 51*, 351-356.

Nicoletti, P. (2010). Brucellosis: past, present and future. *Contributions. Section of Biological and Medical Sciences. Macedonian Academy of Sciences and Arts, 31*, 21-32.

Nielsen, K. (2002). Diagnosis of brucellosis by serology. *Veterinary Microbiology, 90*(1), 447-459.

Nielsen, K., Gall, D., Smith, P., Bermudez, R., Moreno, F., Renteria, T., Ruiz, A., Aparicio, L., Vazquez, S., Dajer, A., Luna, E., Samartino, L., and Halbert, G. (2005). Evaluation of serological tests for detection of caprine antibody to *Brucella melitensis*. *Small Ruminant Research, 56*(1-3), 253-258.

Nielsen, K., and Yu, W. (2010). Serological diagnosis of brucellosis. Contributions. Section of Biological and Medical Sciences. *Macedonian Acad. Sci. Arts, 31*, 65-89.

Nimri, L., and Batchoun, R. (2011). Genetic homogeneity of clinical isolates of *Brucella Melitensis*: a single ribotype. *Webmed Central Microbiology, 2*(1), 1-7. Retrieved from http://www.webmedcentral.com/article_view/1508 website:

Ocholi, R. A., Kwaga, J. K. P., Ajogi, I., and Bale, J. O. O. (2004). Phenotypic characterization of *Brucella* strains isolated from livestock in Nigeria. *Veterinary Microbiology, 103*(1–2), 47-53.

OIE. (2009a). Office International des Épizooties, Bovine brucellosis,chapter 2.4.3. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (pp. 1-35). Paris, France: OIE.

OIE. (2009b). Office International des Épizooties, Caprine and ovine brucellosis (excluding *Brucella ovis*), chapter 2.7.2. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (pp. 1-10). Paris, France: OIE.

OIE. (2013). Office International des Épizooties, principles and methods of validation of diagnostic assays for infectious diseases, chapter 1 . 1 . 5 . In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (pp. 1-17). Paris, France: OIE.

OIE. (2014a). Office International des Épizooties, development and optimisation of antibody detection assays, chapter 3 . 6 . 1 . In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (pp. 1-13). Paris, France: OIE.

OIE. (2014b). World Animal Health Information Database (WAHID) of World Organization for Animal Health Retrieved 16.1.2015, 2015, from http://www.oie.int/wahid

Petrie, A., and Watson, P. (2006). *STATISTICS FOR VETERINARY AND ANIMAL SCIENCE* (2 ED.): BLACKWELL.

Poester, F. P., Nielsen, K., Samartino, L. E., and Yu, W. L. (2010). Diagnosis of Brucellosis. *The Open Veterinary Science Journal, 4*(1), 46-60.

Reyes, R. E., Andrade, A. A., González, C. R., Herrera, M. O., and Jiménez, R. C. (2012). *Mechanisms of O-Antigen Structural Variation of Bacterial Lipopolysaccharide (LPS)*: INTECH Open Access Publisher.

Robinson, A. (2003). Guidelines for coordinated human and animal brucellosis surveillance *156* (pp. 15-38). Rome: Food Agriculture Organization of the United Nations(FAO).

Robles, C. A., Nielsen, K., Gall, D., and Willems, P. (2009). Evaluation of three different antigens in an indirect enzyme-linked immunoassay for the detection of antibodies against *Brucella abortus* SRB51 in vaccinated heifers. *Veterinary Immunology and lmmunopathology, 127*(1-2), 153-155.

Sam, I. C., Karunakaran, R., Kamarulzaman, A., Ponnampalavanar, S., Syed Omar, S. F., Ng, K. P., Mohd Yusof, M. Y., Hooi, P. S., Jafar, F. L., and AbuBakar, S. (2012). A large exposure to *Brucella melitensis* in a diagnostic laboratory. *Journal of Hospital Infection, 80*(4), 321-325.

Samartino, L. E., and Enright, F. M. (1993). Pathogenesis of abortion of bovine brucellosis. *Comparative Immunology, Microbiology and Infectious Diseases, 16*(2), 95-101.

Samartino, L. E., and Enright, F. M. (1996). *Brucella abortus* differs in the multiplication within bovine chorioallantoic membrane explants from early and late gestation. *Comparative Immunology, Microbiology and Infectious Diseases, 19*(1), 55-63.

Schoonjans, F., Zalata, A., Depuydt, C., and Comhaire, F. (1995). MedCalc: a new computer program for medical statistics. *Computer methods and programs in biomedicine, 48*(3), 257-262.

Seleem, M. N., Boyle, S. M., and Sriranganathan, N. (2008). *Brucella*: a pathogen without classic virulence genes. *Veterinary Microbiology, 129*(1-2), 1-14.

Seleem, M. N., Boyle, S. M., and Sriranganathan, N. (2010). Brucellosis: a re-emerging zoonosis. *Veterinary Microbiology, 140*(3-4), 392-398.

Shahaza, O., Khairani-Bejo, S., Zunita, Z., & Bahaman, A.R. (2009). In-House Rose Bengal Plate Agglutination Test (RBPT) for a Rapid Diagnosis of Brucellosis in Goats in Malaysia. *International Journal of Tropical Medicine, 4*(3), 116-118.

Stevens, M. G., Olsen, S. C., and Cheville, N. F. (1995). Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. *Veterinary Immunology and Immunopathology, 44*(3), 223-235.

Thavaselvam, D., Kumar, A., Tiwari, S., Mishra, M., and Prakash, A. (2010). Cloning and expression of the immunoreactive *Brucella melitensis* 28 kDa outer-membrane protein (Omp28) encoding gene and evaluation of the potential of Omp28 for clinical diagnosis of brucellosis. *Journal of Medical Microbiology*, *59*(Pt 4), 421-428.

Xavier, M. N., Paixao, T. A., Hartigh, A. B., Tsolis, R. M., and Santos, R. L. (2010). Pathogenesis of *Brucella* spp. *Open Veterinary Science Journal*, *4*(1), 109-118.

Yaeger, M., & Holler, L. D. (2007). Bacterial causes of bovine infertility and abortion. *Current therapy in large animal theriogenology. 2nd ed. Missouri, USA: Saunders Elsevier*, 389-399.

APPENDICES

Appendix A

Media used for bacterial identification

1. *Brucella* agar (BBL™)

Brucella agar powder 43 g
Distilled water 1 L
The agar was autoclaved at 121°C for 15 minutes.

2. *Brucella* broth (BBL™)

Brucella broth powder 28 g
Distilled water 1 L
The agar was autoclaved at 121°C for 15 minutes.

3. Basic fuchsin agar

Basic fuchsin powder 0.02 g
Distilled water 1 ml

- The mixture was boiled for 20 minutes to dissolve the dye.
- The prepared dye mixture (100 µl) were added into 100 ml of sterile *Brucella* agar.
- The agar was poured into sterile petri plate under sterile precautions at 20 ml per plate.

4. Thionin agar

Thionin powder 0.02 g
Distilled water= 1 ml

- The mixture was boiled for 20 minutes to dissolve the dye.
- The prepared dye mixture (100 µl) were added into 100 ml of sterile *Brucella* agar .
- The agar was poured into sterile petri plate under sterile precautions at 20 ml per plate.

Appendix B

Differential Staining

1. Gram stain:

- A clean microscope glass slide was prepared.
- A loop of *Brucella* broth containing *Brucella melitensis* was transferred and smeared onto (a).
- The smear was heat to fix.
- The glass slide was flooded with crystal- violet solution for one minute.
- The stain was washed with iodine solution.
- The iodine was left on the glass slide for two minutes
- Excess iodine was drained off and decolonization with acetone for one to three seconds was performed.
- The glass slide was rinsed using tap water.
- The glass slide was flooded with safranin solution for one minute.
- The glass slide was rinsed using tap water.
- The glass slide was dried and examined under light microscope. *Brucella species* were red, small coccobacilli bacteria.

2. Modified acid fast stain:

- A clean microscope glass slide was prepared.
- A loop *of Brucella* broth containing *Brucella melitensis* was transferred and smeared onto (a).
- Smear was heat to fix.
- The glass slide was flooded with diluted, carbol Fuschin for five minutes.
- The stain was washed away using tap water.
- The glass slide was flooded with 0.5 % acetic acid for one minute.
- The stain was also washed away using tap water.
- The glass slide was flooded with methylene blue for one minute.
- The stain was washed using tap water.
- The glass slide was dried and examined under light microscope. *Brucella species* were red, small coccobacilli bacteria.

Appendix C

Reagents for in-house RBPT

1. Rose Bengal 1%

Rose Bengal powder	1g
Distilled water	100 ml

The solution was kept in dark bottle at room temperature.

2. Phenol saline

Sodium chloride	9 g
Phenol	4 g
Distilled water	1000 ml

3. RBPT diluent

Sodium hydroxide	21.1 g
Phenol saline	353 ml
Lactic acid	95 ml
Phenol saline	Adjust to 1056 ml

- The sodium hydroxide was dissolved in 353 ml of phenol saline.
- Lactic acid was then added to the solution and the final volume was adjusted to 1056 ml by adding phenol saline.
- The solution was autoclaved at 121^{o}C for 15 minutes.

BIODATA OF STUDENT

Mohammed Sani Yahaya was born on 29th March, 1977 at Jahun Jigawa State Nigeria. He obtained his secondary school certificate in Science Secondary Kafin/Hausa Jigawa State, before joining Kano State Polytechnic (SOT) where he graduated with HND in Biology /Microbiology with lower credit in 2008. He served in General Hospital Akure Ondo State, Nigeria for the mandatory National Youth Corps. He got his first appointment in Federal Polytechnic Kazaure as a Technologist in 2006. He also obtained his Postgraduate Dilploma certificate in Biological Science Bayaro University Kano State Nigeria. He is presently pursing for MSc, Bacteriology in Universiti Putra Malaysia.

LIST OF PUBLICATIONS

Yahaya M. S, Khairani-Bejo S, Zunita Z and A.M. Omar .Development and validation of in-house Rose Bengal plate test for the diagnosis of brucellosis in goats. Submitted to Malaysian Journal of Veterinary Research, **(Under Review)**.

Yahaya M. S, Khairani-Bejo S, Zunita Z and A.M. Omar .Occurrence of Brucellosis in Cattle and goats in Malaysia: Submitted to Pertanika Journal of Scholarly Research Reviews, **(Under Review)**.

UNIVERSITI PUTRA MALAYSIA

STATUS CONFIRMATION FOR THESIS / PROJECT REPORT AND COPYRIGHT

ACADEMIC SESSION : _____

TITLE OF THESIS / PROJECT REPORT :

DEVELOPMENT OF IN-HOUSE ROSE BENGAL PLATE TEST FOR DIAGNOSIS OF BRUCELLOSIS IN CATTLE AND GOATS

NAME OF STUDENT : MOHAMMED SANI YAHAYA

I acknowledge that the copyright and other intellectual property in the thesis/project report belonged to Universiti Putra Malaysia and I agree to allow this thesis/project report to be placed at the library under the following terms:

1. This thesis/project report is the property of Universiti Putra Malaysia.
2. The library of Universiti Putra Malaysia has the right to make copies for educational purposes only.
3. The library of Universiti Putra Malaysia is allowed to make copies of this thesis for academic exchange.

I declare that this thesis is classified as :

*Please tick (√)

☐	**CONFIDENTIAL**	(Contain confidential information under Official Secret Act 1972).
☐	**RESTRICTED**	(Contains restricted information as specified by the organization/institution where research was done).
☐	**OPEN ACCESS**	I agree that my thesis/project report to be published as hard copy or online open access.

This thesis is submitted for :

☐	**PATENT**	Embargo from_____ until _____ (date) (date)

Approved by:

_____	_____
(Signature of Student) New IC No/ Passport No.:	(Signature of Chairman of Supervisory Committee) Name:
Date :	Date :

[Note : If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization/institution with period and reasons for confidentially or restricted.]