UNIVERSITI PUTRA MALAYSIA

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By

TAKELE BELAY

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

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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR DIAGNOSIS OF *BRUCELLA MELITENSIS* INFECTION IN SHEEP

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August 2008

Chairman: **Siti Khairani Bejo, PhD**

Faculty: **Veterinary Medicine**

In this study, a SYBR Green based real-time PCR assay using primers specific to IS711 of *Brucella melitensis* was developed and its diagnostic utility was evaluated by comparing it with conventional PCR method for detection of *Brucella* DNA from cultured bacteria and experimentally infected mice samples. The study was first undertaken with cultured bacteria, *B. melitensis* 16M as a reference and two *B. melitensis* Malysian isolates. *Brucella abortus* 544 and *Yersinia enterocolitica* O:9 were also used for cross reactivity testing. Three of the *B. melitensis* were successfully detected using both optimized SYBR Green I real-time and conventional PCR. No amplification products were produced with these primers from strains of *B. abortus* and *Y. enterocolitica* O:9 indicating that the primer sets are specific only for *B. melitensis.* The specificity of the PCR products amplified by the primers from Malysian strains of *B. melitensis* were confirmed by sequence analysis. The detection limit of the real-time PCR was compared with conventional PCR by preparing serially ten-fold dilutions of *B.*

melitensis DNA. The real-time PCR was found to be 10-fold more sensitive than the conventional PCR, detecting up to 50 fg of genomic DNA.

The viability of using conventional and real-time PCR assays together with culture methods as potential diagnostic tools for the detection of *B. melitensis* in experimentally infected mice was also assessed over an 8-week period of infection. It was evident from our result that regardless of duration of infection and sample type (liver, lymph node, blood, spleen and serum) the diagnostic yield (sensitivity) was 100%, 96.7% and 62.5% for real-time PCR, conventional PCR and culture methods respectively. This indicated that the sensitivity of the PCR assays was far higher than that of the culture method. The specificity of both conventional and real-time PCR was 100%. When serum was used as PCR template, *B. melitensis* specific amplicon was detected only until day 45 post inoculation (p.i) using conventional PCR unlike the real-time PCR method whereby positive results were detected through out the study period. In all samples with positive real-time PCR results, *B. melitensis* specific amplicons were distinguished by their characteristic melting temperature ranged between 86.4 ^{o}C to 87.4 ^{o}C in melting curve analysis.

Analytical sensitivities of the different tissue types used in the study were also compared using threshold cycle (Ct) values of the real-time PCR. For this purpose, samples collected at day 30 p.i. were used and the most sensitive tissue sample amongst the tested samples was estimated from the standard curve obtained from cloned *B. melitensis* 16M. Accordingly, the most sensitive tissue was found to be serum (mean Ct=15.07) followed by lymph node (mean Ct=19.50), spleen (mean Ct= 24.2), liver (mean Ct=26.4) and whole blood (mean Ct=26.2). These results indicate that serum samples

followed by lymph node are more sensitive for the real-time PCR assay than the other samples tested in this study.

In conclusion, this present results suggest that the diagnostic yield of real-time PCR that uses serum samples was at least as sensitive as a similar PCR protocol that uses other tested tissue samples. The speed and technical simplicity of real-time PCR coupled with minimal risk of handling serum samples make it a useful alternative to blood / other tested tissue PCR for animals with suspected brucellosis and negative or doubtful serological test results. However, although the use of this real-time PCR assay as a supplemental diagnostic tool for detection and identification of *Brucella* organisms in clinical specimens could be recommended, the need for the assay to be validated with confirmed positive and negative clinical samples of its primary host animals, and verify its ability to differentiate the vaccinal strains from field strains, is desirable to achieve the utmost benefits from the SYBR Green I based real-time PCR assay.

Keywords: *Real-time PCR, SYBR Green, Brucella, Diagnosis, Mice, Culture*

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan Master Sains

PEMBANGUNAN SATU UJIAN PCR MASA NYATA UNTUK DIAGNOIS JANGKITAN *BRUCELLA MELITENSIS* DI DALAM BIRI-BIRI

Oleh

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Ogos 2008

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Dalam kajian ini, assai PCR masa nyata berasaskan SYBR Green dengan menggunakan primer yang khusus kepada IS711 bagi *B. melitensis* telah dibangunkan dan utiliti diagnostiknya telah dinilai dengan membandingkannya dengan kaedah PCR konvensyenal bagi mengesan DNA *Brucella* daripada bakteria berkultur dan sampel mencit yang telah dijangkiti. Kajian berkenaan dijalankan terlebih dahulu dengan bakteria berkultur, *B. melitensis* 16M sebagai rujukan dan dua isolat *B. melitensis* setempat. *Brucella abortus* dan *Y. enterocolitica* O:9 juga digunakan untuk pengujian kereaktifan silang. Tiga daripada *B. melitensis* telah berjaya dikesan dengan menggunakan PCR masa nyata SYBR Green I teroptimum dan PCR konvensyenal. Tiada produk amplifikasi dihasilkan dengan primer ini daripada strain *B. abortus* dan *Y. enterocolitica* O:9 yang menunjukkan bahawa set primer adalah khusus hanya untuk *B .melitensis.* Spesifisiti produk PCR yang dihasilkan oleh primer daripada strain *B. melitensis* akhirnya disahkan oleh analisis jujukan**.** Had pengesanan PCR masa nyata

dibandingkan dengan PCR konvensional dengan menyediakan pencairan pada tahap sepuluh kali ganda secara bersiri bagi DNA *B. melitensis.* PCR masa nyata didapati 10 kali ganda lebih sensitif daripada PCR konvensional, mengesan sehingga 50 fg DNA genomik.

Kebolehhidupan menggunakan assai PCR konvensional dan masa nyata bersama-sama dengan kaedah kultur sebagai alat diagnostik berpotensi bagi pengesanan *B. melitensis* di dalam mencit yang dijangkiti secara uji kaji juga telah dinilai sepanjang tempoh 8-minggu jangkitan. Jelas melalui keputusan kami bahawa tanpa menghiraukan tempoh jangkitan dan jenis sampel (hati, nodus limfa, darah, limpa dan serum), hasil diagnostik (kepekaan) masing-masing adalah 100%, 96.7% dan 62.5% untuk kaedah PCR masa nyata, PCR konvensional dan kultur. Ini menunjukkan kesensitifan assai PCR adalah jauh lebih tinggi daripada kaedah kultur. Spesifiti kedua-dua PCR konvensional dan masa nyata adalah 100%. Apabila serum digunakan sebagai templat PCR, amplikon khusus *B. melitensis* telah dikesan hanya sehingga 45 p.i dengan menggunakan PCR konvensional tidak seperti kaedah PCR masa nyata yang telah mengesan keputusan positif di sepanjang tempoh kajian berkenaan. Dalam semua sampel dengan keputusan PCR masa nyata yang positif, amplikon khusus *B. melitensis* telah dibezakan melalui suhu lebur yang jelas dengan julat antara 86.4 ^{o}C hingga 87.4 ^{o}C dalam analisis lengkung lebur.

Kesensitifan analisis bagi jenis tisu berlainan yang digunakan dalam kajian ini juga telah dibandingkan dengan menggunakan nilai kitaran ambang (Ct) PCR masa nyata. Untuk tujuan ini, sampel yang diambil pada hari 30 p.i. telah digunakan dan sampel tisu yang

paling sensitif daripada sampel yang diuji telah dianggarkan daripada lengkung standard yang diperoleh daripada 16M *B. melintesis* yang telah diklon. Seterusnya, tisu yang paling sensitif adalah serum (min Ct=15.07) diikuti dengan nodus limfa (min Ct=19.50), limpa (min Ct= 24.2), hati (min Ct=26.4) dan darah penuh (min Ct=26.2). Keputusan ini menunjukkan bahawa sampel serum dan diikuti dengan nodus limfa adalah lebih sensitif untuk assai PCR masa nyata daripada sampel lain yang diuji dalam kajian ini.

Kesimpulannya, keputusan ini menunjukkan bahawa hasil diagnostik PCR masa nyata yang menggunakan sampel serum adalah sekurang-kurangnya sama sensitif seperti protokol PCR serupa yang menggunakan sampel tisu lain yang diuji. Kelajuan dan keringkasan teknikal PCR masa nyata di samping risiko minimum yang terlibat dalam mengendalikan sampel serum menjadikannya alternatif berguna bagi PCR darah / tisu lain yang diuji untuk haiwan yang disyaki mempunyai brucellosis dan keputusan ujian serologi yang negatif atau meragukan. Namun, walaupun kami menyarankan penggunaan assai PCR masa nyata ini sebagai alat diagnostik tambahan untuk pengesanan dan pengenalpastian organisma *Brucella* dalam spesimen klinikal, keperluan untuk assai agar disahkan dengan sampel klinikal positif dan negatif bagi haiwan hos utamanya, dan menentusahkan kemampuannya untuk membezakan strain vaksin daripada strain lapangan, adalah dikehendaki untuk mencapai faedah tertinggi yang diingini daripada assai PCR masa nyata berasaskan SYB Green 1.

Kata kunci: *PCR masa-nyata, SYBR-Green, Brucella, diagnostik, mencit*, *kultur*

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Last but, by no means least, I'm grateful to the Netherlands Organization for International Cooperation in Higher Education (Nuffic) for providing me the scholarship.

I certify that Examination Committee has met on 16th October 2008 to conduct the final examination of Takele Belay Yazew on his Master of Science thisis entitiled "Development of a Real-time PCR for Diagnosis of Brucella melitensis Infection in Sheep" in accordance with Universiti Pertanian Malaysia (higher Degree) Act 1980 and University Pertanian Malysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of Examination Committee wer as follows:

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

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Date: 16 October 2008

DECLARATION

I here declare that the thesis is based on my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

Takele Belay

Date:

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
µg	Microgram
µM	Micromolar
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 260 nm
AMOS-PCR	*Abortus-melitensis-ovis-suis* PCR
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumine
CDC	Charge Coupled Device
cDNA	Complementary deoxyribonucleic acid
CFT	Complement Fixation Test
cfu	Colony forming units
CO_2	Carbon dioxide
Ct	Threshold cycle
CV	Coefficient of variation
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
df/dt	Derivations of fluorescence with respect to temperature
dGTP	Deoxyguanosine triphosphate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
DVS	Department of Veterinary Service
EDTA	Ethylenediaminetetraacetic acid disodium salt
ELISA	Enzyme-linked immunosorbent assay
EtdBr	Ethidium bromide
fg	Femto gram (10^{-15})
fig.	Figure
FOA	Food and Agriculture Organization of the United Nations
FRET	Fluorescence resonance energy transfer
g/mol	Gram per mole
h	Hour
IHK	Institue Haiwan Kluang
IMR	Institute of Medical Research
INF	Interferon
Ip	Intraperitonieally
IS	Insertional sequence
IU	International unit
L	Litter
LPS	Lipopolysaccharide
Mb	Mega base
$MgCl_2$	Magnisium chloride
min	Minute
mL	Millilitre

mM	Millimolar
mol	Mole
MZCP	Mediterranean Zoonoses Control Programme
ng	Nanogram (10^{-9})
NTC	No template control
O.D	Optical density
OIE	International Epizootic Office
omp	Outer membrane protein
ORF	Open reading frame
p.i	Post inoculation
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	Pico gram
pp	Pages
RBPT	Rose Bengal plate test
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolution per minute
s	Seconds
SD	Standard deviation
ssDNA	Single stranded DNA
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBE	Tris-base EDTA buffer

Tm	Melting temperature
UPM	Universiti Putra Malaysia
UV	Ultraviolet
V	Volt
v/v	Volume to volume ratio
VRI	Veterinary Research Institute
w/v	Weight to volume ratio
WHO	World Health Organization
H_2S	Hydrogen sulfoxide

CHAPTER 1

INTRODUCTION

Brucellosis, caused by Gram-negative facultative organisms belonging to the genus *Brucella*, is an infectious zoonotic disease that is associated with chronic debilitating infections in humans and reproductive failure in domestic animals (Corbel, 1997). Although brucellosis in livestock and transmission of infection to the human population has been significantly decreased following the instigation of effective vaccination-based control and slaughter programs in parts of the world, it remains an uncontrolled problem in regions of high endemicity such as the Mediterranean, Middle East, Africa, Latin America and parts of Asia. The most recent concern focuses on the potential use of *Brucella* species, primarily *B. melitensis*, as an agent of biological warfare because of the debilitating disease it causes. Widespread dispersal of aerosolized *B. melitensis* would pose a biological, agricultural, as well as an economical threat to all countries involved (Refai, 2002; Kaufmann *et al*., 1997).

The disease is caused by several *Brucella* species, which are aerobic, non-motile, Gram-negative, non-spore forming, and facultative intracellular coccobacilli. On the basis of 16S rRNA gene sequence comparisons, the genus *Brucella* is grouped in alpha-2 subdivision of the class *Proteobacteria* (Moreno *et al.,* 1990). The genus is divided according to cultural, metabolic, antigenic properties and host specificity in to six species: *B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis* (Corbel and Morgan, 1984). In addition, a new strain affecting marine mammals was recently