

## Isolation and molecular characterization of *Brucella melitensis* from seropositive goats in Peninsula Malaysia

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**Abstract.** A study was carried out to isolate *Brucella melitensis* using established bacteriological and PCR techniques in *Brucella* seropositive goats in farms in Selangor, Negeri Sembilan, Melaka and Pulau Pinang. *Brucella melitensis* was isolated from 7 of 134 reactors with the highest isolation from the vaginal swabs (57.14%) followed by the spleen (28.57%), uterine fluid (14.29%). No *Brucella* was isolated from the lymph nodes. PCR confirmed all the seven isolates as *B. melitensis* and isolates were phylogenetically related to other isolates from India, Iran, and Israel but most closely related to isolates from Singapore.

### INTRODUCTION

Brucellosis is endemic in many developing countries and is caused by *Brucella* species that affect man, domestic and some wild animals, and marine mammals (Bhatia & Narain, 2010; Seleem *et al.*, 2010). An estimated 500,000 new human cases were reported annually worldwide (Pappas *et al.*, 2006). It is the second most important zoonosis after rabies and has gained prominence over the years since its discovery on the island of Malta (Seleem *et al.*, 2010; Abubakar *et al.*, 2012). Eight species of *Brucella* are recognized to affect terrestrial animals which include: *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella neotomae*, *Brucella canis*, *Brucella ovis*, *Brucella microti* and *Brucella inopinata* (Scholz *et al.*, 2010). Marine mammals are affected by *Brucella ceti* and *Brucella pinnipedialis* (Foster *et al.*, 2007). As with

many other zoonotic or potentially zoonotic diseases, the bacteria infect mainly occupationally exposed individuals such as farmers and veterinary personnel (Pappas, 2010). Humans usually contract the infection through ingestion of the organism (such as through infected food and drinking infected milk) or by contamination of mucous membranes and abraded skin (Falenski *et al.*, 2011).

In Malaysia, previous work has reported the isolation of *B. melitensis* in livestock (Al-Garadi *et al.*, 2011a). Bamaiyi *et al.* (2011) recently reported the detection of *B. melitensis* antibodies among humans working closely with animals especially goats in some parts of the peninsula. The Department of Veterinary Services, Malaysia conducts a national sero-surveillance program to monitor the prevalence of *B. melitensis* among goat and sheep populations. In this program, reactors

confirmed positive using the standard laboratory complement fixation test (CFT) are required to be culled (with compensation) in an effort to eradicate the infection among the goat populations. The present paper describes the isolation followed by confirmation via PCR of *B. melitensis* from previously confirmed seropositive goats.

## MATERIALS AND METHODS

### Bacteriological culture

Samples were collected from 134 goats in farms located in Selangor, Negeri Sembilan, Melaka and Pulau Pinang states of Malaysia during several culling exercises following brucellosis active sero-surveillance program. Specimens collected for isolation from each goat included: spleen, lymph nodes, uterus and vaginal swabs. These specimens were taken to the Bacteriology Laboratory of the Faculty of Veterinary Medicine, Universiti Putra Malaysia where the laboratory protocols were carried out under the biosafety chamber for bio-hazardous material. The protocols were performed as outlined by OIE (2009) and Jang *et al.* (2008). Briefly, thin smears were made from the specimens on clean microslides and these were stained with modified Ziehl-Neelsen (ZN) stain. Specimens from positive smears were inoculated into *Brucella* agar (Laboratorios CONDA, Spain) added with *Brucella* supplements (Laboratorios, CONDA, Spain) and incubated at 37°C for 3-7 days. Suspect colonies based on morphology and microscopic examination was further sub-cultured for another 3-7 days to obtain pure isolates. Biochemical tests which include Triple Sugar Iron agar (TSI), Sulfide-Indole-Motility (SIM), Urease Nitrate broth, Oxidase test, Basic Fuchsin tolerance (20g/ml), Thionin tolerance tests (20g/ml) were performed and isolates were categorized as *Brucella melitensis* (Jang *et al.*, 2008). DNA was extracted from the positive cultures using a commercial kit (Promega Inc. USA).

### Polymerase Chain Reaction (PCR)

The reaction mixture consisted of final concentrations as follows: 1×PCR buffer,

1.5mM MgCl<sub>2</sub>, 200 μM of the dNTP's, 1.0 μM of each primer, 2.5 U of Taq Polymerase (Bioron®, Germany). DNA amplification reactions was performed at these conditions; incubation at 90°C for 5 mins, denaturation at 95°C for 1.15 mins, annealing at 48°C for 2 mins, elongation at 72°C for 2 mins and extension at 72°C for 5 mins and the expected product size was 252bp. The oligonucleotide sequence (IS711 *Brucella melitensis*) for the primer was:

Forward: 5'CAT GCG CTA TGT CTG GTT AC 3' ;

Reverse: 5'ATG GTT TCG GCT CAG AAT AATC 3' (Redkar *et al.*, 2001).

Positive and negative controls used were 16M reference strain (Veterinary Laboratories Agency, Weybridge, UK) and distilled water, respectively. The amplified PCR product was separated using electrophoresis in 1.5% agarose gel at 85volts for 1 hour. The gel was stained in ethidium bromide (0.5mg/ml) for 10 min and de-stained with distilled water and then examined under UV light using the GelDoc (AlphaImager) programme (Figure 1). The DNA bands were carefully cut with a sterile blade using a UV variable intensity transilluminator (312nm) and sequenced based on the IS711 gene. The sequences were deposited in the GenBank with the following accession numbers obtained: JN561153; JN561154; JN561155; JN561156; JN561157; JN561158; JN561159.

Single Nucleotide Polymorphism (SNP) was used for genotyping. The sequences were aligned and compared with other available partial sequences at NCBI Blast, and the phylogenetic tree was created using the Neighbour joining method of Saitou & Nei (1987) using the software CLC Genomics WorkBench 3.6.5.

## RESULTS

From 509 available specimens of spleen, vaginal swabs, lymph nodes and uterine fluid taken from 134 goats, seven isolates from seven different goats were confirmed as *B. melitensis* (Table 1 and Figure 1). The

phylogenetic analysis (Figure 2) revealed that the sequences were most closely related to *B. melitensis* from Singapore, India, Iran and Palestine, Israel.

#### DISCUSSION

The isolation rate from the sampled seropositive animals was low (5.2%) but is in agreement with the reported low isolation rate by other laboratories elsewhere (Poester *et al.*, 2010). This is not surprising because of the slow growing and fastidious nature of the pathogen (Seleem *et al.*, 2010). The highest isolation rate was via vaginal swabs,

consistent with the report that vaginal swabs is one of the best specimens for the isolation of *B. melitensis* because the vagina excretes copious amounts of the organism for several weeks after abortions (Garin-Bastuji *et al.*, 2006). Although the lymph nodes are one of the best sites for isolation of *Brucella* at post-mortem (Garin-Bastuji *et al.*, 2006; Zowghi *et al.*, 2008) no isolates were recovered from the lymph nodes in this study. This may be due to the low number of lymph node samples taken or the infection stage of the animals (Ahmed *et al.*, 2010; Seleem *et al.*, 2010). During the acute stage of infection, a significant number of *Brucella* isolates can be recovered from the lymph nodes but as

Table 1. Number of seropositive goats positive for *B. melitensis* isolation

State	No. of animals sampled	No. positive (%)	Sample type (number)
Selangor	56	3 (%)	Vaginal swab (1) and spleens (2)
Negeri Sembilan	29	2 (%)	Vaginal swab (1) and uterine fluid (1)
Melaka	40	2 (%)	Vaginal swabs (2)
Pulau Pinang	9	0	No isolate
Total	134	7 (5.22%)	

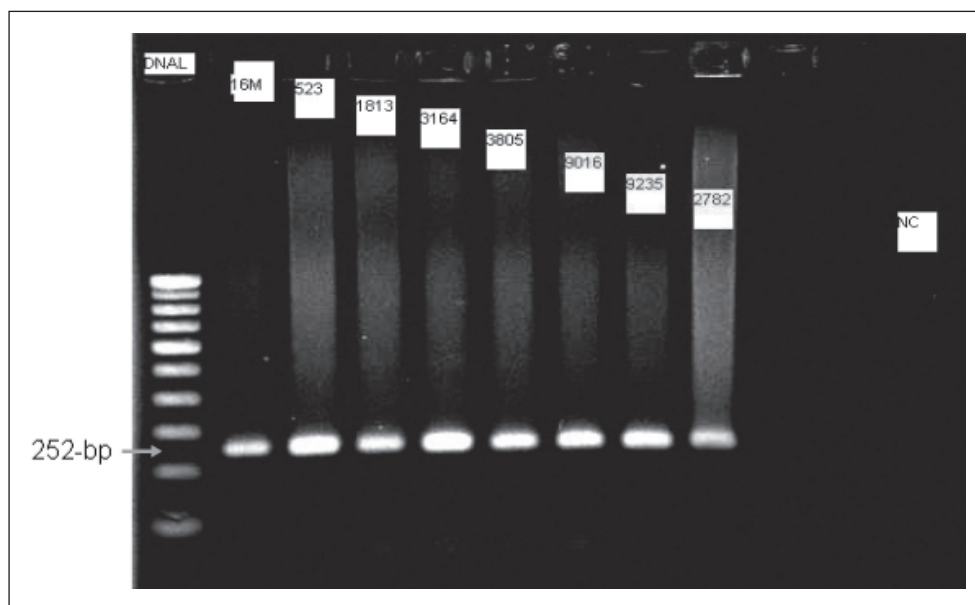


Figure 1. Seven isolates confirmed as *B. melitensis* from seropositive goats in Malaysia. 16M=Positive control; 523 and 1813=Melaka; 3164, 3805 and 2782=Selangor; 9016 and 9235=Negeri Sembilan; PCR product size=252-bp

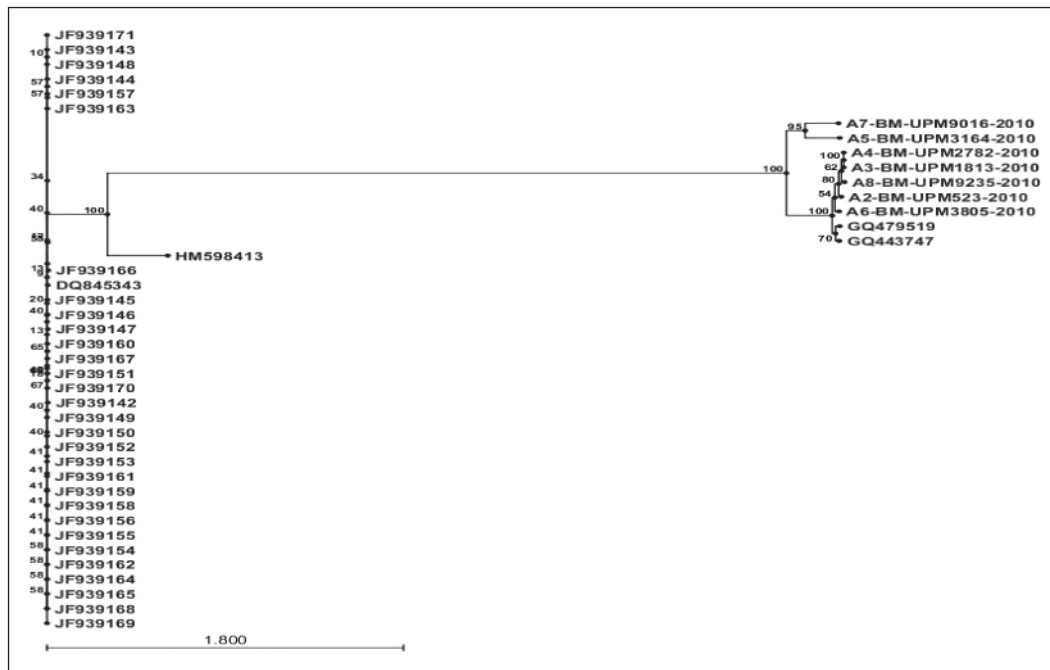


Figure 2. Dendrogram of *Brucella melitensis* isolates from Malaysia compared to others from other regions using the Neighbour Joining (NJ) method

the infection progresses, the recovery rate declines as the organism localizes in a variety of other tissues and organs (Enright, 1990).

PCR confirmed all cultures based on biochemical analyses as *B. melitensis*. The sensitivity and specificity of conventional PCR was reported to be 95.9% and 93%, respectively in blood samples of goats (Al-Garadi *et al.*, 2011b). In this study, using biochemically positive cultures, the sensitivity was 100%. PCR also has the added advantage of being able to simultaneously detect *B. abortus* and *B. melitensis* and differentiate them with high sensitivity and specificity (Mirnejad *et al.*, 2012).

The phylogenetic tree revealed that our isolates are genetically related to lineages and topotypes from other parts of Asia namely Singapore, India, Iran and Israel. This indicates a common ancestral evolutionary origin of the isolates (Hall, 2001). It is known that geographical locations play an important role in relationship between isolates with isolates from similar regions tending to be more closely related (Hall, 2001). The close homology with isolates from places like Israel and Iran indicates the wide

geographical distribution of this genotype (Muendo *et al.*, 2012). Isolates within Malaysia have a close relationship (>95%), with some level of variations not uncommon in geographically proximate samples (Griffiths *et al.*, 2010; Nagalingam *et al.*, 2012) as degrees of differences may occur due to factors such as transhumance, mutation, breed and species variability. These are in agreement with the findings of Al-Garadi *et al.* (2011a) that Malaysian isolates are closely related to each other. In a study in Turkey, many *B. melitensis* isolates were found to be closely related irrespective whether they originated from man or animals (Sayan *et al.*, 2009). The close homology between *Brucella* species and between strains in a species causes researchers to suggest that there should be only one species of *Brucella* and others should be considered biovars (DelVecchio *et al.*, 2002; Adrian, 2009).

The isolation and confirmation of *B. melitensis* from seropositive goats in Malaysia is of interest to the livestock industry due to the infectious nature of the organism and its economic impact on animal productivity. More proactive measures should

be taken to protect the goat populations from this zoonotic infection and also to protect the human populations especially those who work closely with animals. Different strains of *B. melitensis* in Malaysia may have originated from other Asian countries but this needs further study to establish the origins of the strains and their role in the epidemiology of brucellosis in Malaysia.

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