

## Isolation and Identification of *Brucella melitensis* in Goats

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**Abstract:** Isolation of *Brucella melitensis* is the standard gold of identification and confirmation of animal brucellosis. However in Malaysia, *Brucella* sp., infection of goat was increasing recently and there is no evidence for diagnosis of the serover of *Brucella* sp., that cause the disease in goat population except the detection of serological methods. Isolation and identification of *Brucella melitensis* have been done by bacteriological methods in addition to conventional Polymerase Chain Reaction (PCR) and Real-time PCR for detection of *Brucella melitensis* from samples collected from vaginal swabs on suspected farm. In conclusion, four isolate have been got out of 300 vaginal samples and all isolate is belong to *Brucella melitensis* server 1. The Real-time PCR is the easy and save method for confirmation of brucellosis in goats population.

**Key words:** Goat, Brucellosis, isolation, Real-time PCR, PCR, goats

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### INTRODUCTION

The most reliable and the only unique method for diagnosing animal brucellosis is isolation of *Brucella* species (Alton *et al.*, 1988). A definite diagnosis requires the isolation of *Brucella* sp. from blood, bone marrow or other tissues. However, cultural examinations are time-consuming, hazardous and not sensitive. Thus, clinicians often rely on the indirect proof of infection (Al-Dahouk *et al.*, 2003; Bounaadja *et al.*, 2009).

The bacteriological diagnosis of *Brucella melitensis* can be made by microscopic examination of stained smears from vaginal swabs, placentas or aborted fetuses (Stamp's method). However, morphologically related organisms such as *Brucella ovis*, *Chlamydomphila abortus* or *Coxiella burnetii* can cause misleading diagnoses. Therefore, isolation of *B. melitensis* on appropriate culture media is recommended for accurate diagnosis (Garin-Bastuji *et al.*, 2006). Thus, taking vaginal swabs and milk samples is the best way to isolate of *B. melitensis* from sheep and goats. The spleen and lymph nodes (iliac, supramammary and prefemoral) are the best sites for obtaining samples for isolation of *Brucella melitensis* during post-mortem examination (Marin *et al.*, 1996).

Recently, the Polymerase Chain Reaction (PCR) were shown to be a valuable method for detecting DNA from different fastidious and non-cultivable agents (Brikenmeyer and Mushahwar, 1991). Although, there are several studies on *Brucella*-DNA detection by PCR from

pure culture (Fekete *et al.*, 1990; Herman and Herman, 1992), there is no substantial evidence to indicate the presence of brucellosis in goats in Malaysia but then there were no studies being done on the diagnosis of brucellosis in these small ruminants in the past years and there has been no report of strong serological reactor or the presence of *B. melitensis* in goats in Malaysia until 2007 (Bahaman and Bejo, 2007). Except annual Department of Veterinary medicine (DVS) report for brucellosis eradication programs and Bahaman *et al.* (2009) were reported infection of goats with *Brucella melitensis* by In house Rose Bengal Plate Test (RBPT) and other serological test.

Diagnosis of brucellosis is vital for early situation of proper therapy as untreated cases may progress to chronic stage. Though, the demonstration of the causative agent in blood is considered as the most conclusive test in the diagnosis of brucellosis, isolation of *Brucella* organism by blood culture is relatively low (Jain and Tilak, 2008).

The diagnosis of brucellosis is based on a detailed history obtained from the patients and the isolation of *Brucella* sp. from blood cultures are accepted as the standard method. The definitive diagnosis of brucellosis requires isolation of the bacterium from blood, bone marrow or tissue samples. Although, the sensitivity of blood culture method varies depending on the individual laboratory practices, quantity of bacteria in blood and the methods used, it changes between 15-70%. Bone marrow

culture is considered as the gold standard for the diagnosis of brucellosis since, the relatively high concentration of *Brucella* in reticuloendothelial system enables the detection of the organism (Aliskan, 2008).

## MATERIALS AND METHODS

**Vaginal swabs samples:** About 300 vaginal swab samples were collected from goats and transported in *Brucella* broth as transport media.

**Bacterial isolation:** The vaginal swabs were cultured on *Brucella* agar with *Brucella* supplement (Pronadisa®) then it was incubated in 37°C for 5 days. The plates were examined after 3 days and then it was observed in each day to observe the development of the bacterial colonies. *Brucella* sp. were identified by morphological, cultural and biochemical characteristics such as oxidase, urease, CO<sub>2</sub> requirement, H<sub>2</sub>S production, nitrate reduction, growth in the presence and absence of thionin and basic fuscine (20 µg mL<sup>-1</sup>).

**Serotyping:** All *Brucella melitensis* local isolate which got from this study have been sub-cultured and lyophilized by lyophilizer and sent to serotyping under biohazard safety condition shipmen. Typing was kindly performed by Veterinary Laboratories Agency (VLA-UK) by standard procedures. Lyophilized pure culture of four isolates were sent to the *Brucella* Reference Research Statutory and Exotic Bacterial Department, Veterinary Laboratory Agency, New Haw, Addlestone, Surrey, United Kingdom for typing to biovar level.

### Identification of *Brucella melitensis* isolates DNA extraction from samples

**Biochemical test:** Phenotyping of *Brucella melitensis* is the most reliable technique to diagnosis of *Brucella* sp. Bacterial ability to grow in media containing thionin (20 µg mL<sup>-1</sup>) and basic fuchsin (20 µg mL<sup>-1</sup>) was done. Growth in Triple Sugar Iron (TSI) agar test, oxidase, Urease activity test, nitrate reduction and CO<sub>2</sub> requirement was carried out on the suspected colony to identify local isolate (Jang *et al.*, 2004 ; Nielsen and Duncan, 1990).

### Conventional Polymerase Chain Reaction (PCR)

**DNA extraction:** The DNA was extracted from bacterial culture using Promega kit (Promega, USA). Briefly, 1 mL overnight *Brucella* culture was transferred to an eppendorf tube. Samples were centrifuged at 16,000×g for 2 min to pellet the cells. The supernatant was removed and 600 µL of nucleolysis solution was added. The cells were re-suspended by gently pipeting. The samples were

incubated at 80°C for 5 min to lyse the cells; then it was cooled to room temperature. About 3 µL of RNase solution was added to the cell lysate. The tube was inverted 2-5 times to mix. The sample was incubated at 37°C for 15-60 min. The sample was cooled to room temperature. About 200 µL of protein precipitation solution was added to the RNase-treated cell lysate. The sample was vortexed vigorously at high speed for 20 sec to mix the protein precipitation solution with the cell lysate. The sample was incubated on ice for 5 min and it was centrifuged at 16,000×g for 3 min. The supernatant containing the DNA was transferred to a clean 1.5 mL microcentrifuge tube containing 600 µL isopropanol. The sample was mixed gently by inversion until the thread-like strands of DNA form a visible mass. Sample was centrifuged at 16,000×g for 2 min then the supernatant was poured off carefully and the tube was drained on clean absorbent study. About 600 µL of room temperature 70% ethanol was added and the tube was inverted gently several times to wash the DNA pellet. The sample was centrifuged at 16,000×g for 2 min. The ethanol was aspirated carefully. The tube was drained on clean absorbent study and the pellet allowed to air-dry for 10-15 min. 100 µL of DNA rehydration solution was added to the tube. The DNA was rehydrated by incubating the solution overnight at 4°C. The purity and concentration was measured.

**Conventional PCR protocol:** A 1 mL overnight culture was transferred into sterile cups and DNA was extracted according to the manufacturer's protocol with a Wizard genome purification kit (Promega, USA) for gram negative bacteria as previous. The upstream primer that is derived from the 3' end of the genetic element IS711. Whereas, the downstream primer was derived from signature sequences of *Brucella melitensis* P1 (5'-CATGCGCTATGTCTGGTTAC-3') and P2 (5'-AGTGTTCGGCTCAGAA TAATC-3') were used to amplify a 252 bp fragment (Redkar *et al.*, 2001). For DNA amplification, 24 µL of reaction mixture was added to 1 µL of purified DNA. The master mixture for the PCR included the following: 1x buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM each deoxynucleoside Triphosphate (dNTP) (Qiagen, Germany), 25 µM each primer and 2 U of Taq DNA polymerase (Qiagen, Germany). PCR amplifications were performed with a Bio-rad temperature cyler (Mycycler Bio-Rad California, USA). The primary PCR amplification consisted of an initial denaturation step at 95°C for 5 min; 34 cycles at 95°C for 1.15 min, 45°C for 2 min and 72°C for 2 min and a final elongation phase at 72°C for 5 min. After the 1st reaction, 1 µL of the amplified product was added to 24 µL of the master mixture. Each time a PCR was

performed, a positive control (*Brucella melitensis* 16 M reference strain DNA) was run and a reagent control (all PCR reagents without DNA) to evaluate the success of amplification and the purity of the reagents, respectively. The product of the PCR was electrophoresed with 1 µL of gel loading buffer (concentrated) through a 2% agarose gel at 75 V for 1.30 h in 1×TBE buffer (Mbiotech, Korea). Molecular size markers 100 bp (Promega, Madison, Wis.) were run concurrently. The gel stained with ethidium bromide (0.5 µg mL<sup>-1</sup>) was examined under UV light for the presence of a 252 bp band and photographed by Bio-rad Gel doc. system for documentation and determination of expected band.

**Detection of *Brucella melitensis* by Real-time-PCR:** All local isolate and reference strain was subjected into Real-time PCR using a specific primers for *Brucella melitensis* for detection *IS711* gene to established the rapid technique for diagnosis and confirmation of *Brucella melitensis* local isolate. The primers P1 (5'-CATGCGCTATGT CTGGTTAC-3') and P2 (5'-AGTGTTCGCTCAGAATAATC-3') were used to amplify a 252 bp fragment (Redkar *et al.*, 2001). Real-time PCR was performed on DNA extracted from bacterial culture.

Two microliters of the DNA suspensions were used in the Real-time PCR reactions. About 0.5 µL (0.5 µM) from each the forward primer and reverse primer which have previously been described (Redkar *et al.*, 2001). About 12.5 µm SYBRR green master mixes was used (Qiagen, Germany) and 9.5 µL RNase free water to give a final volume of 25 µL. Amplification was carried out on the opticon Real-time PCR detection system (Biorad, USA), PCR parameters consisted of a single 5 min incubation step at 95°C followed by a 30 sec step at 65 8°C and by 34 cycles consisting of 30 sec at 65°C, 15 sec at 95°C and 30 sec at 65°C.

To confirm that the amplification signals were the result of specific target amplification, the real-time amplification was followed by melt curve analysis (from 60-95°C in 20 min with increments of 0.5°C). Non-template controls (NTC, consisting of H<sub>2</sub>O) were used as negative controls for amplification.

**Characterization of *Brucella melitensis* isolates:** *Brucella* sp. is differentiable by Polymerase Chain Reaction (PCR) and phenotyping procedure (Gandara *et al.*, 2001; Meikle *et al.*, 1989).

**Negative staining:** Negative staining is a rapid technique to differentiate between *Brucella melitensis* and

*Y. enterocolitica* by shape and cell morphology. The sample have been mixed with negative stain by equal portion on a parafilm sheet and the samples was applied to the grids and the excess was removed by touching a filter study to the edge of the grid after draying for 15-30 min the grid can be examined as usual by electron microscopy (Bozzola and Russell, 1999).

Cell morphology was examined using a Philips electron microscopy with cells grown for 2 days at 37°C on standard nutrient agar. Negative staining (with 1% uranyl acetate) was performed.

**Sequencing *IS711* region of *Brucella melitensis* isolates:** The *IS711* gene was amplified from five isolates of *Brucella melitensis* and *Brucella melitensis* 16 M reference strain after cultivation of *Brucella* sp. at 37°C for 48 h.

DNA have been extracted by DNA extraction kit (Promega, USA) and amplified by using conventional PCR protocol as previously described. PCR product was separated by electrophoresis system and the gene was present on predicted size.

The gels were cut by sharp blades in specific fragment size and re-purified the gene product from the gel by using Promega DNA purification system (Promega, USA). The re-purified product have been checked for purity and integrity by using agarose electrophoresis methods to confirm the re-purification.

The samples were sent for sequencing and the BLASTN query result of genes amplified for local *Brucella melitensis* isolates were compared with gene sequences of *IS711* obtained from GenBank under accession number (ref/NC 003317.1) for *IS711* region and *Brucella melitensis* 16 M reference strain.

## RESULTS AND DISCUSSION

**Bacterial isolation:** A total of four isolates were obtained from 300 vaginal samples and the Veterinary Laboratories Agency (VLA)-UK confirmed isolates were *Brucella melitensis* biotype 1.

**Identification of *Brucella melitensis* isolates:** The local isolates were appeared the ability to grow on media containing 20 µL basic fuchsin but no on thionin media, the confirmation includes biochemical, dye tolerance was showing in Table 1.

All *Brucella* strains were identified as *Brucella melitensis* by biochemical characteristics such as positive oxidase and Urease, negative H<sub>2</sub>S, no Co<sub>2</sub> requirement and growth in the present of basic fuchsin and thionin (20 mg mL<sup>-1</sup>).

**Polymerase Chain Reaction (PCR):** The conventional PCR is highly specific and all *Brucella* strains were identified as *B. melitensis* by conventional PCR which successfully amplified 252 bp by specific shown as in Fig. 1.

**Detection of *Brucella melitensis* by Real time-PCR:** The results indicated that IS711 Real-time PCR assay is a specific and sensitive tool for detection of *Brucella* sp. infections in goats.

The successful Real-time PCR has been achieved with high efficiency with CT value mean  $12.8 \pm 0.8$  as shown in Fig. 2. The melting temperature curve indicates

Table 1: Showed the biochemical test results and dye tolerate for the four *Brucella melitensis* local isolate from vaginal swabs

Staining/biochemical test	<i>B. melitensis</i>
Modified ziehl-neelsen stain	Acid fast, red coccobacilli
Growth in triple sugar iron	+
CO <sub>2</sub> requirement	-
H <sub>2</sub> S	-
Oxidase	+
Nitrate reduction	+
Urease	+
Growth on basic fuschin tolerate	+
Thionin tolerate	-

Table 2: Real-time PCR result from *Brucella melitensis* local isolate and *Brucella melitensis* 16 M reference strain showing CT and Tm value

Description	C (t)	Tm
Positive control	12.29	82.5
1	13.38	82.0
2	12.27	82.0
3	13.91	82.0
4	12.16	82.0
Non template control	N/A	74.5

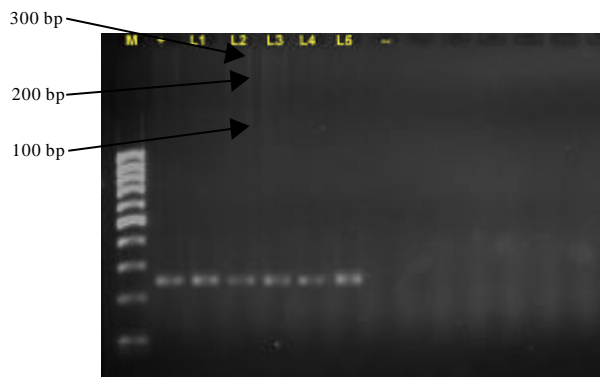


Fig. 1: Show the expected band of *Brucella melitensis* from local isolate. Lane M is 100 bp marker, lane+*Brucella melitensis* 16 M reference strain as a positive control, lane L1-L4 local isolate from goats farm in current study. Lane L5 local isolate from sheep in previous study Lane was negative control

there is no noisy curved on the specific melting temperature which it is around 82 as shown in Fig. 3 and Table 2.

**Characterization of *Brucella melitensis* isolates**

**Negative staining:** Coccobacilli or sporadically formed short rods arranged individually or in irregular clusters. The cell shape, morphology and diameter are similar to *Brucella melitensis* 16 M reference strain as in Fig. 4 and clearly distinguished from *Brucella abortus* and *Yersinia enterocolyctica* 0:9 reference strains Fig. 5.

**Sequencing of IS711 region of *Brucella melitensis* isolates:**

The analyzed gene sequences showed high percent similarity when compare to the GenBank database.

The amplification products for all isolate were consistent with the predicted size as in Fig. 6 and 7. Phylogenetic tree and sequencing blast result were

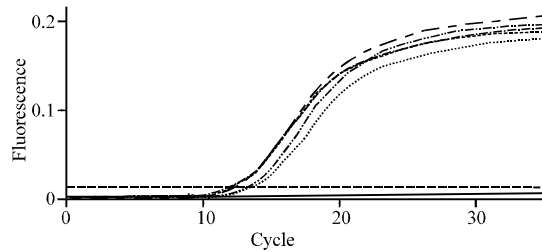


Fig. 2: Successful PCR amplification curve of all *Brucella melitensis* local isolate from 1-4 and *Brucella melitensis* 16 M reference strain which are up the threshold line (---) and non template control under the serrated line which indicated there is no amplification occurred

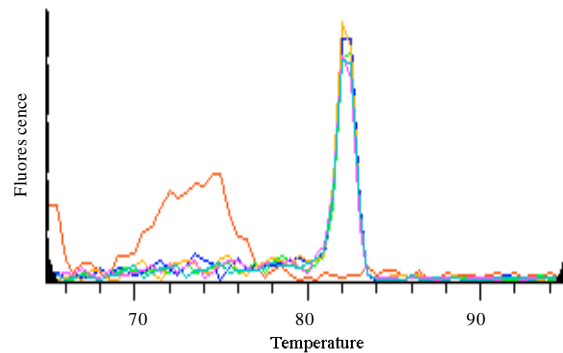


Fig. 3: Melting curve graph showing the melting temperature of all local isolate and *Brucella melitensis* 16 M reference strain with mean of  $82 \pm 0.5$

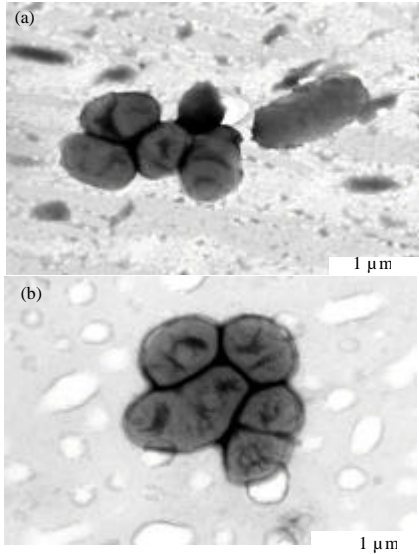


Fig. 4: Transmission electron microscopy of the *Brucella melitensis* local strain (a-21.5 K) stained with negative staining shows an organism with undistinguished morphology from *Brucella melitensis* 16 M reference strain (b-27.5 K)

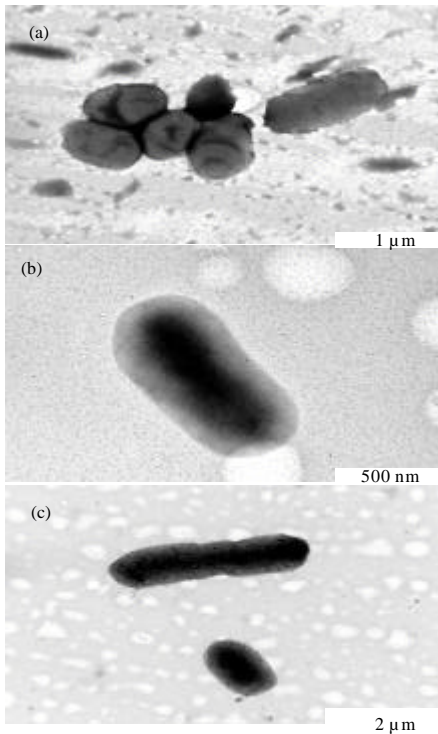


Fig. 5: Transmission electron microscopy of the *Brucella melitensis* local strain (a-21.5 K) isolate after negative staining shows an organism with distinguished morphology from *Brucella abortus* (b-46 K) and *Yersinia enterocolitica* reference strain (c-12.5 K)

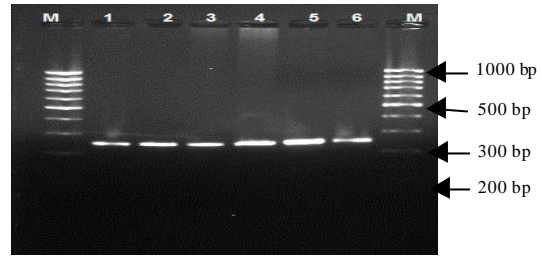


Fig. 6: PCR Product after re-purification IS711 region from agarose gel with expected band (252 bp) of *Brucella melitensis* 16 M reference strain (1), 4611 *Brucella melitensis* local isolate (2), *Brucella melitensis* local isolate (3), *Brucella melitensis* local isolate (4). *Brucella melitensis* local isolate (5) and (M) is 100 bp DNA marker

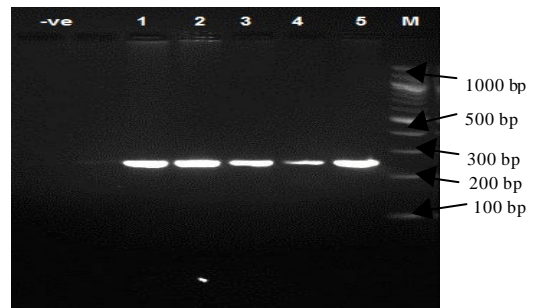


Fig. 7: Successful PCR product with expected band (252 bp) after amplification IS711 region of *Brucella melitensis* 16 M reference strain (1, 6), 4611 *Brucella melitensis* local isolate (2), *Brucella melitensis* local isolate (3), *Brucella melitensis* local isolate (4). *Brucella melitensis* local isolate (5) and (M) is 100 bp DNA marker

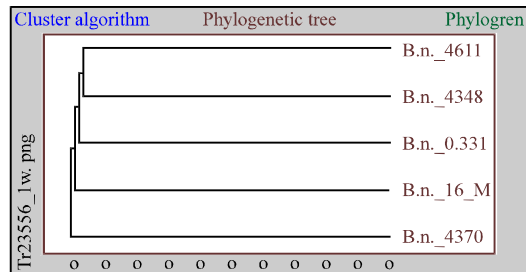


Fig. 8: Phylogenetic tree of the amplified (252 bp) sequences from IS711 region in *Brucella* species. The local isolate, *Brucella melitensis* is most closely related to *Brucella melitensis* 16 M comparing with GeneBank data and enrolled reference from *Brucella melitensis* 16 M showing all reference have 100% similarity in 252 bp of IS711 with *Brucella melitensis*

showed in Fig 8-10, subsequently. Isolation of *Brucella* sp. is usually difficult, hazardous, time consuming and unpractical, however the isolation of *Brucella* sp. is considered to be the only unequivocal method for the confirmation (Alton *et al.*, 1988).

The Serological test is the most suitable screening methods for goat's brucellosis whilst isolation of the

organisms is important to confirm the disease. It is sometimes important to analyse, multiple isolates within a given species to determine whether they represent a single strain or multiple strains. If a species of bacteria is isolated and cultivated in the laboratory, it is known as a strain. A single isolate with distinctive characteristics may also represent a strain. These typing methods are useful

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B.m* 4611 CATGCGCTATGCTCGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATTG
B.m* 4348 CATGCGCTATGCTCGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATTG
B.m* 0331 CATGCGCTATGCTCGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATTG
B.m* 16_M CATGCGCTATGCTCGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATTG
B.m* 4370 CATGCGCTATGCTCGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATTG
*****

B.m* 4611 TCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACAG
B.m* 4348 TCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACAG
B.m* 0331 TCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACAG
B.m* 16_M TCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACAG
B.m* 4370 TCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACAG
*****

B.m* 4611 CGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAATC
B.m* 4348 CGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAATC
B.m* 0331 CGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAATC
B.m* 16_M CGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAATC
B.m* 4370 CGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAATC
*****

B.m* 4611 TCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTGA
B.m* 4348 TCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTGA
B.m* 0331 TCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTGA
B.m* 16_M TCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTGA
B.m* 4370 TCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTGA
*****

B.m* 4611 GCCGAAACACTA
B.m* 4348 GCCGAAACACTA
B.m* 0331 GCCGAAACACTA
B.m* 16_M GCCGAAACACTA
B.m* 4370 GCCGAAACACTA
*****
    
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Fig. 9: Multiple sequence alignment of *Brucella melitensis* local isolates show 100% homology in all four isolate nucleotides (5'-3') rather than with *Brucella melitensis* 16 M reference strain (B.m.\*) *Brucella melitensis*

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Query 1      CATGCGCTATG-TCTGGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATT 59
|||||
Sbjct 1209648 CATGCGCTATGATCTGGTTACGTTGAATGCAGACACGCCCTAGGGGTGAATCTGGAAATT 1209589

Query 60     GTCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACA 119
|||||
Sbjct 1209588 GTCAGAAAGACAGTGCTTCGTCACGCTAGAGCGCTCGCTGCCATACTTGCAACAGTGACA 1209529

Query 120    GCGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAAT 179
|||||
Sbjct 1209528 GCGATAATTGCCGTTATTGGCTGGTGGCAGGGCGAAGATTGGCGGGTAAGCTATTCCAAT 1209469

Query 180    CTCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTG 239
|||||
Sbjct 1209468 CTCGCTATTGTTAATGGCGCTCTATTGGATATTACTGCTCTACCTTCTGTGGATTATTCTG 1209409

Query 240    AGCCGAAACACT 251
|||||
Sbjct 1209408 AGCCGAAACACT 1209397
    
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Fig. 10: Blast result of *Brucella melitensis* sequence of 252 bp from IS711 region in hypothetical protein gene (5'-3') with *Brucella melitensis* 16 M chromosome I, complete sequence (ref[NC\_003317.1]) showed 99% homology with this part. The gap is 1/252 from primer designation

for infection control in hospital, epidemiological studies and understanding the pathogenesis of infection. Collection of vaginal sample is the easiest methods for sampling, however it is not usually reflected the true prevalence of the herd's infection. There are many factors conflicting isolation of *Brucella melitensis* in goats by vaginal samples which is including the stage of the diseases, time of abortion, excretion of Brucella in vaginal exudates or uterine exudates and the stage of the infection rather than the quantity of Brucella in the samples. Teixeira-Gomes *et al.* (2000) and Leyla *et al.* (2003) isolated *Brucella melitensis* from vaginal samples and aborted fetuses in sheep and goats and the result of this study in isolation of *Brucella melitensis* organisms from vaginal swabs from goats is consistent with the reports of these researchers.

Four *Brucella melitensis* isolates were isolated from vaginal samples. The isolates were able to grow on media containing 20 µL basic fuchsin but disable to grow in the present of thionin in the media. All biochemical test results were match with references strains except thionin result which match with identification made by Corbel (1991). Many *Brucella melitensis* isolated in India, Italy, Kuwait, Saudi Arabia, the Federal Republic of Germany and Zimbabwe were inhibited by thionin (20 micrograms mL<sup>-1</sup>) but not by basic fuchsin (20 micrograms mL).

It has often been reported that the sensitivity of culture method is often low depending on the diseases stage, Brucella species, culture medium, quantity of circulating bacteria and culture techniques employed (Marin *et al.*, 1996; Gamazo *et al.*, 1993). Phenotypic techniques, those that detect characteristics expressed by the microorganism and genotypic techniques, those that involve direct DNA-based analysis of chromosomal or extrachromosomal genetic elements. The conventional PCR need more time and hardly and hazard chemical was used (e.g., ethidium bromide) for staining the gel comparing with real-time one but it was use also here to establish the protocol for using IS711 Real-time PCR moreover to confirm, it is doubt result. For this reason, researchers propose the employment of IS711 Real-time PCR as a complementary tool in brucellosis screening programs and for confirmation of diagnosis in doubtful cases to establish the new biomarker.

The negative staining technique is quite simple, rapid and requires minimum of experience and equipment. Generation of a negative stained grid takes <4 min and even to provide a rapid clinical diagnosis with certain infection by indication of the present of bacteria. A good negative stain revealed the outline and form of structure that are spread out on a support film. The detection of

*Brucella melitensis* and differentiated it according to its morphological shape and size in a specific case history of abortion and characteristic clinical signs can be useful to confirmation of the infection by *Brucella melitensis* and is useful methods to differentiated it from other infection specially *Y. enterolytic*. *Brucella melitensis* is non motile bacteria and does not have flagella as in the figures of negative staining.

The mobile genetic element IS711 has proven a useful target for molecular characterisation based on the number and distribution of IS711 copies within the bacterial genomes. Sequencing data confirm that the actual size of the IS711 amplification products matched the predicted size of 252 bp. The sequencing of the amplified fragments for the IS711 gene from four isolates showed 100% homology. The PCR-sequencing based analysis of gene IS711 showed 100% homology to *Brucella melitensis* 16 M genome.

This 252 bp fragment was found to be present in all of the *Brucella melitensis* isolate tested. The IS711 fragment was showed 99% homology to (ref|NC\_003317.1) *Brucella melitensis* 16 M complete sequence in gene bank. The 1% contrast was shown from original primer designated by Redkar *et al.* (2001).

From sequencing analysis result, there is no mutation point in all isolate. The genetic homology and diversity between the local isolates itself and with reference strain would be established an idea to make good planning for control the disease and compare it with gene bank data to see the relative of that's local isolate with other global records especially with related with Malaysia goats exporting countries such as Thailand which have boarding with Malaysia, especially it is very close to the sampling place.

## CONCLUSION

In this study, *Brucella melitensis* serovar 1 is the corresponding of brucellosis in goats brucellosis in Malaysia which have severity in transmission of brucellosis to human been. The national measures for controlling need to improve the diagnostic programme in order to assess the control and spread of brucellosis. The eradication programme is should be consider the technique that can be detected the *Brucella melitensis* in low prevalence area to eradicated the brucellosis such as Real-time PCR.

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