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Identification and Differentiation of Malaysian *Brucella* Isolates Based on *rpoB* Gene Sequence Analysis

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Abstract

Brucella sp. causes brucellosis in both humans and animals. Identification of this pathogen remains controversial due to the instability of the phenotypic characteristics among *Brucella* isolates which complicates the identification and subdivision of this genus down to the species level and biovar determination. In this study, we report the potential of *rpoB* gene sequence analysis as an alternative tool in the identification and differentiation of *Brucella* sp. in Malaysia. The *rpoB* gene marker was able to distinguish 21 *Brucella* isolates into 10 distinct *RpoB* marker patterns namely RpoB 1 to RpoB 10. Two isolates clustered into RpoB 1 pattern and 12 isolates clustered into RpoB 2 pattern were identified as *Brucella suis* and *Brucella melitensis*, respectively, by *rpoB* gene sequence and phylogenetic tree analysis. Three strains with RpoB 3 and one from RpoB 4 pattern were suggested to be *Brucella melitensis* as its *rpoB* sequences were closely related and almost identical to the three published *Brucella melitensis* (Accession number CP001851.1, CP002459.1 and CP001488.1). The remaining four isolates from RpoB 5 until RpoB 8 patterns were identified as *Brucella melitensis* but probably with different biovar. RpoB 9 and RpoB 10 patterns belong to the published *Brucella abortus* (Accession number AM040264 and CP003176.1) and *Brucella canis* (Accession number CP003174 and CP000872.1), respectively. *B. melitensis* was the most common species in the study indicating that human brucellosis cases in Malaysia is probably affected mainly by this species. In conclusion, this study shows that the *rpoB* marker is potentially be implied to identify and differentiate local *Brucella* sp. in Malaysia.

Keywords: Brucellosis; *Brucella*; *RpoB*; Codon; Phylogenetic tree

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Introduction

Brucella species is a highly infectious bacterium which can infect humans and animals causing the disease called brucellosis. Humans may be infected from direct contact with contaminated livestock or infected wildlife or *via* ingestion of contaminated milk products, commonly from goats. Laboratory-associated exposure to aerosols generated from handling of *Brucella* isolates [1-4] have also been reported, and is one of the leading laboratory-acquired infections worldwide [5,6]. Brucellosis cases in humans have been reported in many countries including Iran [7], Saudi Arabia [8], West and Central Africa [9], Latin America, Asia, European Union, the Balkan Peninsular [10], Nigeria [11], Turkey [12], China [13] and Tanzania [14]. Brucellosis among humans and animals are often associated with *Brucella melitensis*, *Brucella abortus* and *Brucella suis*, and *Brucella* sp. are preliminary identified based on their host preference [15-17]. A new rapid, robust and accurate molecular method to identify *Brucella* genus to species level is clinically important for brucellosis outbreak investigations, patient management and eventually to improve the eradication program in the areas of endemic brucellosis [18].

Culture and isolation of *Brucella* sp. remains as the gold standard for diagnostic confirmation [4]. However, the difficulties of this conventional method as the species is a slow-growing bacterium, fastidious and additionally its instability in biochemical assays, causing potential misidentification of the pathogen [1,19]. Presumptive identification of *Brucella* genus is made based on morphologic, culture and serologic observations of the isolates [17,20]. Distinction and subdivision of the genus into species are based on the type of host preference, oxidative metabolism pattern and phage sensitivity tests. For the classification of a biotype of the species, methods used are Carbon Dioxide (CO_2) requirement, Hydrogen Peroxide (H_2O_2) production, dye sensitivity and reaction with monospecific antisera A and M antigens [21]. However, all these methods are time consuming,

Table 1: *Brucella* isolates obtained from 21 cases of human brucellosis in Peninsular Malaysia.

No.	Isolates/Accession no.	Species	Biovar	Brucellosis cases
1	[a]Brc3-09	*Brucella melitensis*	2	Sporadic cases
2	[a]Brc4-10	*Brucella melitensis*	3	Sporadic cases
3	[a]Brc5-10	*Brucella melitensis*	3	Sporadic cases
4	[a]Brc1-11	*Brucella melitensis*	1	Outbreak
5	[a]Brc2-11	*Brucella melitensis*	3	Outbreak
6	[a]Brc3-11	*Brucella melitensis*	3	Outbreak
7	[a]Brc4-11	*Brucella melitensis*	3	Outbreak
8	[a]Brc7-11	*Brucella melitensis*	3	Outbreak
9	[a]Brc9-11	*Brucella melitensis*	3	Outbreak
10	[a]Brc11-11	*Brucella melitensis*	3	Outbreak
11	[a]Brc23-11	*Brucella melitensis*	2	Outbreak
12	[a]Brc27-11	*Brucella melitensis*	3	Outbreak
13	[a]Brc28-11	*Brucella melitensis*	3	Outbreak
14	[a]Brc29-11	*Brucella melitensis*	2	Outbreak
15	[a]Brc30-11	*Brucella melitensis*	3	Outbreak
16	[a]Brc31-11	*Brucella melitensis*	2	Outbreak
17	[a]Brc32-11	*Brucella melitensis*	2	Outbreak
18	[a]Brc33-11	*Brucella melitensis*	3	Outbreak
19	[a]Brc34-11	*Brucella melitensis*	1	Outbreak
20	[a]Brc36-11	*Brucella melitensis*	2	Outbreak
21	[a]Brc39-11	*Brucella melitensis*	3	Outbreak
22	[b]AE009516	*Brucella melitensis* 16M	1	Outbreak
23	[b]CP001851.1	*Brucella melitensis* M5-90	Genome sequence is ~99.6 % similar to *Brucella melitensis* 16M	
24	[b]CP002459.1	*Brucella melitensis* M28	Genome sequence ~99.6 % similar to *Brucella melitensis* 16M	
25	[b]CP001488.1	*Brucella melitensis* ATCC 23457	Genome sequence ~99.6 % similar to *Brucella melitensis* 16M	
26	[b]AM040264	*Brucella abortus* 2308	Genome sequence is identical to *Brucella abortus* Biovar 1	
27	[b]CP003176.1	*Brucella abortus* A13334	Genome sequence is identical to *Brucella abortus* Biovar 1	
28	[b]CP000911.1	*Brucella suis* ATCC 23445	Genome sequence is identical to *Brucella suis* Biovar 1	
29	[b]CP002997.1	*Brucella suis* 1330	1	
30	[b]CP003174	*Brucella canis* HSK A52141	No Biovar available	
31	[b]CP000872.1	*Brucella canis ATCC 23365*	No Biovar available	

[a] Local isolates; [b] Reference isolates from the Gene Bank database with the accession numbers

complex and potentially hazardous to laboratory personnel [6]. Moreover, instability in some of the phenotypic characteristics among *Brucella* isolates complicates the identification and subdivision of the *Brucella* to species level and their biovar determinations [20,21]. Regarding *Brucella* serological tests, though they are sufficient for differentiating between active and non-active brucellosis infection, the test often shows cross-reactivity with other bacteria [22].

PCR-based methods has recently been employed to identify *Brucella* sp. including multiplex PCR, Real-time PCR as well as for biotyping [5,6,23]. Multilocus Enzyme Electrophoresis (MLEE) on 99 *Brucella* isolates covering almost all important *Brucella* sp. revealed that the genome composition of the members was highly similar, indicating limited genetic diversity among the genus [24]. Other studies have also shown that *Brucella* members share ~90% genomic similarity, which significantly reduces the genetic variation and hyper-variability regions among the species itself and this complicates the identification of *Brucella* isolates to species level [24-26].

The detection of *rpoB* gene by PCR was reported to be useful in differentiating *Brucella* into species [21]. The *rpoB* gene is essential for sustaining prokaryotic cell function and offers useful phylogenetic information especially for bacterial species delineation [27-29]. The *rpoB* gene is also used as an alternative indicator for protein-coding genes in prokaryotes and comparison of its codon nucleotides within the targeting positions gave important identity to the *Brucella* sp. [30]. It was also reported that the identified Single Nucleotide Polymorphisms (SNPs) in the specific gene sequences can be used as biomarkers for epidemiological investigation of brucellosis [21,31]. The use of targeted codons at specific positions within the *rpoB* gene sequences also help in the identification of *Brucella* isolates by generating highly specific results [32,33]. Therefore, this study was conducted to determine the usefulness of the *rpoB* gene in the identification and differentiation of *Brucella* species isolated from human brucellosis cases in Malaysia.

Materials and Methods

Brucella isolates

Brucella isolates obtained from 21 cases of human brucellosis in Peninsular Malaysia between January 2009 and December 2011 were randomly selected for this study (Table 1). The isolates were isolated from blood specimens and all of them were identified as *Brucella melitensis* (Biovar 1 (2 isolates), Biovar 2 (6 isolates) and Biovar 3 (13 isolates)) based on colony characterization on *Brucella* agar and conventional biochemical test including catalase, oxidase, urea hydrolysis, nitrate reduction, H_2S production and growth on thionine dye incorporated into trypticase soy agar at different concentrations [34-35]. The biovar determination of the isolates was performed based on antisera assay [36-38]. Confirmation of the *Brucella* sp. was by 16s rRNA PCR [39].

DNA template and PCR assay

DNA were extracted from the isolates using a QIAamp® DNA Mini Kit (Qiagen) and kept at - 80°C before further use. The forward and reverse primers used to detect the *rpoB* gene were 1rB (5' ATGGCTCAGACCCATTCTTTC 3') and 4134rB (5' TTATTCTGCCGCGTCCGGAA 3') respectively as described in other studies [21]. The PCR parameters were an initial denaturation step of 94°C for 2 min followed by denaturation at 94°C for 30s, annealing at 60°C for 30s and, elongation at 72°C for 3 min for 30 cycles. The PCR mixture contained 100 ng/µl of DNA template and 50 pmol of primers. The PCR product (4134 bp) was then further purified using a PCR purification kit (Qiagen) before sequencing.

Phylogenetic tree analysis

Phylogenetic analysis was carried out to determine the species of the *Brucella* isolates. A dendrogram was then constructed based on the *rpoB* gene nucleotide sequences of the 21 isolates by using an Unweighted Pair Group Method with Arithmetic mean (UPGMA) in FPQuest software (BioRad). All the isolates were clustered and connected based on their calculated genetic distance values and the confidence level of the generated cluster was 0.5 (default value). The interpretation of the genetic distance used in the analysis was as follows: "0" (0.0000) means the two population/species were genetically identical while "1" (1.0000) indicated that the two population/species were different [34,35]. Ten sets of the *rpoB* gene sequence from published strains of *Brucella melitensis* 16M (Accession number AE009516), *Brucella melitensis* M28 (CP002459.1), *Brucella melitensis* M5-90 (CP001851.1), *Brucella melitensis* ATCC 23457 (CP001488.1), *Brucella canis* HSK A52141 (CP003174), *Brucella canis* ATCC 23365 (CP000872.1), *Brucella suis* ATCC 23445 (CP000911.1), *Brucella suis* 1330 (CP002997.1), *Brucella abortus* 2308 (AM040264) and, *Brucella abortus* A13334 (CP003176.1) were included in the analysis to be compared with the consensus *rpoB* gene sequences of the 21 studied isolates. These 10 published strains were chosen based on their well-characterized *rpoB* gene. Details of the reference strains are summarized in Table 2.

rpoB gene sequence and data analysis

The quality of the nucleotide sequences of the *rpoB* gene for the 21 *Brucella* isolates was analyzed using Vector NTI Advance software (version 10.0, Invitrogen). Confirmation of Single Nucleotide Polymorphisms (SNPs) within the gene sequences was carried out by repeating the sequencing once more. Identification and characterization of nucleotide variations of the *rpoB* gene sequences for all the studied *Brucella* isolates including the published *Brucella* strains were performed at 22 positions target loci of the *rpoB* gene sequence. A detailed study on the *rpoB* gene sequences of *Brucella melitensis* 16M revealed that at these 22 target loci there were significant variations in the nucleotide sequences which can potentially be used as biomarkers for *Brucella* species differentiation. To determine whether the *rpoB* biomarker is reliable and can produce real-time results in differentiating *Brucella* strains to species level and possibly biovar, the *rpoB* gene sequences of the 21 *Brucella* isolates identified as *Brucella melitensis* by the conventional method were compared against *rpoB* gene sequences of identified and published *Brucella melitensis*, *Brucella abortus*, *Brucella canis* and *Brucella suis*.

Results

The full length of *rpoB* genes of the 21 human *Brucella* isolates were successfully amplified and sequenced. All of the isolates had the same *rpoB* gene nucleotide sequences with length of 4134 bp and a total of 1,378 amino acid sequences were deduced from the nucleotide sequences of each sample.

The phylogenetic tree analysis of the 21 studied isolates and 10 published *Brucella* strains showed two distinct *rpoB* gene clusters, A1 and A2 (Figure 1). The A1 cluster contained *B. abortus* (strains A 13334 and 2308), *Brucella canis* (strains *HSK A52141* and *ATCC 23365*) and *Brucella suis* (strains *1330* and *ATCC 23445*) and two studied isolates, Brc3-09 and Brc4-10. Both of the isolates were from sporadic brucellosis cases in Malaysia. All the *Brucella* isolates in the A1 cluster were genetically related to each other at a genetic distance of <0.0024. The remaining studied *Brucella* isolates together with the three published *B. melitensis* strains (*M5-90*, *M28*, and *ATCC 2345*) were grouped into the A2 cluster. All the studied isolates in this cluster were from an outbreak except for Brc5-10 which was a sporadic case. All the *Brucella* isolates in the A2 cluster were genetically related to each other with genetic distances of between 0.0007 to 0.0046. Detailed analyses of the *rpoB* gene of the 21 *Brucella* isolates were performed on the 22 identified DNA loci in the full length of the *rpoB* gene sequences and all the nucleotides and amino acid variations

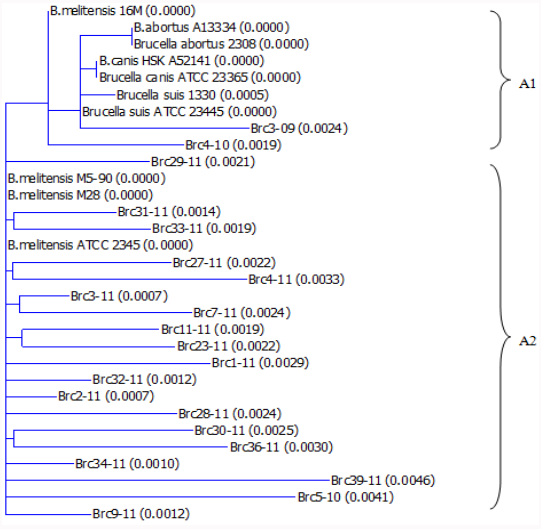

Figure 1: The phylogenetic tree of the 21 investigated *Brucella* isolates and 10 published strains (*Brucella melitensis* 16M, B. abortus 2308, *Brucella abortus* A13334, *Brucella suis* ATCC 23445, *Brucella suis* 1330, *Brucella melitensis* M5-90, *Brucella melitensis* M28, *Brucella melitensis* ATCC 2345, *Brucella canis* HSK A51241 and *Brucella canis* ATCC 23365). The *rpoB* sequence profile of *Brucella melitensis* 16M was used as the main reference strain to calculate genetic distances between the strains.

Table 2: Nucleotide and amino acid sequences in the rpoB gene of the investigated and published *Brucella* strains.

Isolates	*rpoB* marker Patterns	Codon Residues [b*Melitensis* 16M (AE009516)]																							
		¶	89	243	268	270	271	341	344	489	526	549	579	626	629	716	737	898	969	985	1201	1249	1309	1361
		# ¶¶	D GAT	D GAT	T ACG	E GAG	V GTC	E GAG	N AAC	M ATG	D GAC	T ACC	I ATC	V GTC	A GCG	P CCG	V GTT	P CCG	R CGC	A GCC	G GGC	M ATG	L CTG	L CTG
b *Brucella suis ATCC 23445* (CP000911.1)	Suis (RpoB 1)		*	*	ACC T	GAA E	GTG V	GAA E	*	*	GAT D	*	ATT I	GTG V	*	*	GTG V	*	*	GCG V	*	*	*	*
a Brc3-09			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	*	*	GTG	*	*	GCG	*	*	*	*
a Brc4-10			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	*	*	GTG	*	*	GCG	*	*	*	*
b *Brucella suis 1330* (CP002997.1)			*	*	ACC	GAA	GCG A	GAA	*	*	GAT	*	AAC N	GTG	*	*	GTG	*	*	GCG	*	*	*	*
b *Brucella melitensis M5-90* (CP001851.1)	*Melitensis* (RpoB 2)		*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG V	*	GTG	*	*	GTG	*	*	*	*
b *Brucella melitensis M28* (CP002459.1)			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
b *Brucella melitensis ATCC23457* (CP001488.1)			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc29-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc27-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc4-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc5-10			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc1-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc2-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc9-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc34-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc28-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc31-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc33-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc23-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	*	*	*	*
a Brc36-11	*Melitensis* (RpoB 3)		*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	GCG	TGG	*	TGG
a Brc32-11			*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GTG	GTG	*	GTG	*	*	GTG	GCG	TGG	*	TGG
a Brc7-11	*Melitensis* (RpoB 4)		*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	ATT	GAT D	GAT D	*	GTG	*	*	GTG	*	*	*	*
a Brc30-11	*Melitensis* (RpoB 5)		*	*	ACC	GAA	GTG	GAA	*	*	CAG Q	CGC R	GGC G	GTG	GTG	ATT I	GTG	AAA K	GAT D	GTG	*	*	GAA E	GAT D
a Brc3-11	*Melitensis* (RpoB 6)		*	*	ACC	GAA	GTG	GAA	*	*	CAG	CGC	GGC	GCG A	AAC N	ATT	GAT D	AAA	GAT	GAT D	GTG V	GGC G	GAA	GAT
a Brc39-11	*Melitensis* (RpoB 7)		*	*	ACC	GAA	GTG	GAA	*	*	GAT	*	TAT Y	GGC G	GGC G	ACC T	GGC G	GCG A	CCG P	CGC R	TGG W	CAT H	AGC S	GCG A
a Brc11-11	*Melitensis* (RpoB 8)		*	ATT I	CGC R	CGC R	AGC S	AAA K	*	TGC C	ACC T	CCG P	AGC S	AGC S	TAA STP	CGC R	CTG L	CGC R	GCG A	AGC S	GCG A	TGG W	TGG W	TGG W
b *B. abortus 2308* (AM040264)	*Abortus* (RpoB 9)		*	*	ACC T	GAA E	*	GAA E	*	*	GAT D	*	ATT I	*	*	*	GTG V	*	*	GCG A	*	*	*	*
b *Brucella abortus A13334* (CP003176.1)			*	*	ACC	GAA	*	GAA	*	*	GAT	*	ATT	*	*	*	GTG	*	*	GCG	*	*	*	*
b *Brucella canis A52141 b B.canis A52141* (CP003174)	*Canis* (RpoB 10)		*	*	GCG	ACC	GGC	GAA	*	*	GAT	*	ATT	ATT	*	*	GTG	*	*	GCG	*	*	*	*
b *Brucella canis ATCC 23365* (CP000872.1)			*	*	GCG	ACC	GGC	GAA	*	*	GAT	*	ATT	ATT	*	*	GTG	*	*	GCG	*	*	*	*

detected are shown in Table 1. Ten *rpoB* gene sequence patterns RpoB 1 to RpoB 10 were identified. Species-specific markers were observed for *Brucella melitensis*, *Brucella suis*, *Brucella abortus* and *Brucella canis*, and a new species classification based on DNA polymorphism at *rpoB* locus is proposed.

Two studied *Brucella* isolates, Brc3-09 and Brc4-10, had *rpoB* gene sequence patterns that were identical to the reference strain of *Brucella suis* (ATCC 23445) at nine DNA loci (Locus: 268 (Threonine, T), 270 (Glutamic acid, E), 271 (Valine, V), 341 (Glutamic acid, E), 526 (Aspartic acid, D), 579 (Isoleucine, I), 626 (Valine, V), 737 (Valine, V) and 985 (Valine, V)), and these isolates were clustered into RpoB 1. *Brucella suis* (CP002997) was also clustered in the RpoB 1 pattern as its *rpoB* gene sequence showed very small nucleotide variations to the gene sequence pattern which involved nucleotide substitutions at codon 271 (GTG to GCG) (mutation V271A), and at codon 579 (ATC to AAC) (mutation I579N).

Twelve studied *Brucella* isolates; Brc29-11, Brc27-11, Brc4-11, Brc5-10, Brc1-11, Brc2-11, Brc9-11, Brc34-11, Brc28-11, Brc31-11, Brc33-11 and Brc2311 and three published *Brucella melitensis* strains were clustered into the RpoB 2 pattern. While two strains; Brc36-11 and Brc32-11 were observed in RpoB 3 pattern followed by one strain; Brc7-11 in RpoB 4 pattern (Table 2). RpoB 2 was observed as a predominant pattern for the *rpoB* gene of the studied *Brucella* isolates as 12 out of 21 (57%) strains were identified with this genotyping pattern. Interestingly, the three *B. melitensis* published *rpoB* gene also shared the RpoB 2 pattern with the studied *Brucella* isolates whereby their amino acid sequences at 10 identified loci were identical (Locus: 268 (Threonine, T), 270 (Glutamic acid, E), 271 (Valine, V), 341

(Glutamic acid, E), 526 (Aspartic acid, D), 579 (Isoleucine, I), 626 (Valine, V), 629 (Valine, V), 737 (Valine, V) and 985 (Valine, V)). All these studied *Brucella* isolates were from brucellosis outbreaks except for the Brc5-10 isolate.

The remaining four studied *Brucella* isolates; Brc30-11, Brc3-11, Brc39-11 and Brc11-11 were clustered into RpoB 5, RpoB 6, RpoB 7 and RpoB 8, respectively. All the isolates shared identical nucleotide sequences at least at four DNA loci of the respected gene (Locus: 268 (Threonine, T), 270 (Glutamic acid, E), 271 (Valine, V) and 341 (Glutamic acid, E)) except for the Brc11-11 isolate as its nucleotides were strikingly different compared to the other *Brucella* strains in this study. This suggests that the strain probably originated from a different region in Malaysia, or it was an imported strain. RpoB 9 and RpoB 10 were observed among the reference strains of *B. abortus* and *B. canis*, respectively. Nucleotide sequences of the 22 studied DNA loci of the *rpoB* gene were variable. None of the local studied isolates were detected as *Brucella abortus* and *Brucella* canis.

Detailed analysis of the nucleotides at the specific DNA loci in the *rpoB* gene sequence and its encoded amino acid residues of all the reference strains except *Brucella melitensis* 16M showed that *Brucella melitensis* and *Brucella suis* are highly similar. The only differences between these two species were nucleotide insertions at codon 629 (GTG) in *Brucella melitensis*. *Brucella abortus* was similar to *Brucella melitensis* whereby all its codon residues in the analyzed region were identical except for codons 271 (GTG), 626 (GTG) and 629 (GTG). While for *Brucella canis*, its nucleotides were strikingly different from the other *Brucella* species except at a few DNA loci (Table 2).

Discussion

Molecular genotyping can provide valuable insight into the identification and differentiation of *Brucella* sp., one of which is *rpoB* gene codon analysis. Codons at 22 targeted loci of the *rpoB* gene of *Brucella melitensis* 16M from position 89 to 1361 have been shown to be highly discriminative for *Brucella* species identification [21]. The deduced codons were able to generate distinctive *rpoB* marker patterns for *Brucella* sp. and identified four important species namely *abortus*, *melitensis*, *suis* and *canis*.

In our study, the *rpoB* gene analysis successfully detected Nucleotide Polymorphisms (SNPs) in the *rpoB* gene sequences of the isolates which are important for distinguishing species and possibly biovars. The *rpoB* gene marker at the 22 specific loci successfully differentiated *Brucella melitensis* into different molecular *RpoB* patterns which could be applied for biovar identification of the species. However, the *rpoB* gene patterns from our isolates did match with the biovar results obtained from the conventional methods. Similar findings were reported by another study on Turkish isolates [36]. This could be due to instability in the biochemical outputs displayed by members of the *Brucella* genus resulting in misidentification of species and biovar [33].

In a previous study, the strain relationship of 45 *Brucella melitensis* isolates from an outbreak and sporadic brucellosis cases in Malaysia was investigated using Multiple-Locus Variable-number tandem repeat (MLVA) [40]. In that study, the MLVA marker M05 was the predominant genotype pattern among the Malaysian *Brucella melitensis* isolates and about 80% of the species shared this pattern. Interestingly, in this study, five isolates with the MLVA M05 marker had different *RpoB* markers (RpoB 3, RpoB 4, RpoB 6, RpoB 7 and RpoB 8). These isolates showed variability in the *rpoB* gene sequence as nucleotide transversion, SNPs and nucleotide insertions observed at loci 489 and onwards of the gene. The genetic variations detected by the *rpoB* gene marker among the Malaysian *Brucella melitensis* revealed that the isolates are hetero species and this marker was probably the indicator for a biovar identification for the species and it will be more robust if more *Brucella suis* isolates should be tested to this *RpoB* biomarkers. Though the MLVA method is good in investigating strain relationships among Malaysian *Brucella melitensis*, however, the *rpoB* gene marker which is PCR-sequencing based method is observed as more discriminative to detect SNP in the *rpoB* gene sequences of this genus which their species homology exceeds 95% [25,39].

Another PCR based method, High-Resolution Melt analysis (HRM), is another option for differentiating *Brucella* sp. where the assay targets specific loci within the genomes of the species. The HRM method was able to identify six *Brucella* sp. including *Brucella melitensis*, *Brucella canis*, *Brucella neotomae*, *Brucella ovis*, *Brucella abortus* and *Brucella suis* from 153 *Brucella* isolates using seven gene markers (Bspp, Bcan, Bmar, Bmel, Bneo, Bsui and Boa) [33]. However, the assay was unable discriminate *Brucella suis* bv4 from *Brucella canis* which limits its use for biovar determination.

On the other hand, two isolates, Brc3-09 biovar 2 and Brc4-10 biovar 3, initially identified as *Brucella melitensis* by conventional methods were detected as *Brucella suis* by the *rpoB* gene marker. This genetic biomarker distinguished *Brucella suis* from *Brucella melitensis* by detecting nucleotide insertions at locus 629 (GTG) which gives an additional amino acid residue in the *rpoB* gene of *Brucella melitensis*. Limited genetic diversity shared by *Brucella suis* and *Brucella melitensis* makes this mutation an important marker for differentiating between these two species. *Brucella suis* was shown to have high similarity in genetic content and genome organization to *Brucella melitensis* [32,34]. MLEE analysis found that *Brucella suis* formed a cluster with *Brucella melitensis* and *Brucella canis*, all three having identical ribotypes and similar rRNA gene restriction fragment length polymorphisms [24]. Moreover, *Brucella* is a monospecific genus-group and most of its species diverged from a common ancestor due to the ability of the species to adapt to the host preferences and geographical niche of the isolates [35]. Changes in the genetic organization of the *Brucella* genome either by deletion-insertion, addition of unique nucleotides or mutation may lead to multiple progenies of *Brucella* sp with identical genetic properties. Given that the conventional *Brucella* sp. identification method often produces discordant results [33], the *rpoB* marker would probably be a good method to be used as a molecular biomarker for *Brucella* identification as it demonstrated higher discriminatory power in genotype analysis [37,40]. Moreover, this PCR-based method has advantages over culture especially for slow-growing bacteria as the method requires a very small amount of genomic material [41-53].

None of the investigated isolates were identified as *Brucella abortus* or *Brucella canis*, which suggests that these strains are not common causes of human brucellosis in Malaysia. This study also revealed that *Brucella melitensis* is the commonest species of *Brucella*, and the major cause of human brucellosis in Malaysia. Worldwide, *Brucella melitensis* is the most prevalent species causing human brucellosis including in Korea, Argentina and Southern China [37-40].

Conclusion

rpoB gene codon analysis using published *Brucella melitensis*

16M codon residues at the identified loci was able to distinguish clinical *Brucella* isolates obtained from human brucellosis cases in Malaysia to the genus level, and for some isolates, to species level. Each of the marker patterns represented by the investigated isolates and published strains were unique, its nucleotides differing from each other at specific codon positions of the *rpoB* gene sequence. The analysis provides a useful molecular marker pattern and can be used in identifying *Brucella* isolates in Malaysia. Further studies should be carried out on a larger number of isolates with a wider range of biovars to determine the specificity of this *rpoB* gene marker in biovar identification for each species.

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Ethics Statement

The research was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia, reference number NMRR-11-699-10195.

Author Contributions

A.M.N performed the experiments, analyzed and troubleshot the results and wrote the manuscript, S.M.G contributed in manuscript writing, J.A.Z and T.B.Y prepared genomic DNA of studied *Brucella* strains, R.H did identification on the studied *Brucella* strains and N.A had contributed in result analysis and manuscript writing.

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