MULTIPLE-LOCUS VARIABLE-NUMBER TANDEM REPEAT ANALYSIS OF *BRUCELLA* ISOLATES FROM THAILAND

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Abstract. Brucellosis-induced abortion can result in significant economic loss to farm animals. Brucellosis can be transmitted to humans during slaughter of infected animals or via consumption of contaminated food products. Strain identification of *Brucella* isolates can reveal the route of transmission. *Brucella* strains were isolated from vaginal swabs of farm animal, cow milk and from human blood cultures. Multiplex PCR was used to identify *Brucella* species, and owing to high DNA homology among *Brucella* isolates, multiple-locus variable-number tandem repeat analysis (MLVA) based on the number of tandem repeats at 16 different genomic loci was used for strain identification. Multiplex PCR categorized the isolates into *B. abortus* (n = 7), *B. melitensis* (n = 37), *B. suis* (n = 3), and 5 of unknown *Brucella* spp. MLVA-16 clustering analysis differentiated the strains into various genotypes, with *Brucella* isolates from the same geographic region being closely related, and revealed that the Thai isolates were phylogenetically distinct from those in other countries, including within the Southeast Asian region. Thus, MLVA-16 typing has utility in epidemiological studies.

Keywords: *Brucella*, MLVA-16 typing, tandem repeat unit, Thailand

INTRODUCTION

Brucellosis is the most widespread zoonosis in the world and is of major public health and economic importance (Pappas *et al*, 2006). *Brucella* spp infect several important livestock species, such as cattle, goat, pig, sheep, and water buffalo (Di Giannatale *et al*, 2008). Main sign of infection in all animal species is abortion or premature expulsion of the fetus. In humans, the disease can induce undulant fever, malaise, and myalgia, and sometimes is associated with serious complications, such as encephalitis, meningitis, peripheral neuritis, spondylitis, suppurative arthritis, and vegetative endocarditis (Borriello *et al*, 2013). The disease can also

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occur in a chronic form affecting various organs and tissues (Franco *et al,* 2007). The pathogen can be transmitted to humans through consumption of contaminated or untreated milk or dairy products, or by direct contact with infected animals (Borriello *et al,* 2013). As no effective human vaccine is currently available, prevention and control of brucellosis rely on its early diagnosis, adequate antibiotic treatment, proper animal husbandry and hygienic products.

Because of genetic homogeneity within the genus *Brucella*, subtyping isolates remains a challenge. In 1965, genus *Brucella* contained three classic species, namely, *B. abortus* (associated host: cattle), *B. melitensis* (sheep and goat), and *B. suis* (swine) (Olsen and Palmer, 2014). However, since the early 1960s, at least **7** new species have been identified in the genus *Brucella*: *B. ovis*, isolated from a ram in 1952 (Simmons and Hall, 1953), *B. canis* from placental and fetal tissues of aborted beagle pups (Carmichael and Kenney, 1968), *B. neotomae* from desert wood rat (Stoenner and Lackman, 1957), *B. ceti* predominantly from porpoise and dolphin (Foster *et al,* 2007), *B. pinnipedialis* predominantly from seal (Foster *et al,* 2007), *B. microti* from vole and wild red fox (Scholz *et al,* 2009), and *B. inopinata* from an infected human breast implant (Scholz *et al,* 2010). Further isolates have been recovered from wild Australian rodents (Tiller *et al,* 2010) and from a human patient with chronic destructive pneumonia (Tiller *et al,* 2010), which was suggested to be a novel lineage of *B. inopinata*. Most recently by Hofer *et al* (2012), two atypical *Brucella* strains were isolated from two foxes in eastern Austria and placed in the genus *Brucella*, but molecular analysis of *recA* and *omp2a/b* indicated that they are novel species, distinct from other *Brucella* species, including *B. microti* (Al Dahouk *et al,* 2012). Other potential new strains, with characteristics typical of *Brucella*, have recently been recovered from two stillborn baboons (*Papio* sp) (Schlabritz-Loutsevitch *et al,* 2009) and from an African bullfrog (*Pyxicephalus edulis*) (Eisenberg *et al,* 2012).

Development of strain-typing methods is essential for investigating sources of epidemic events. Multiple-locus variable-number tandem repeat analysis (MLVA), based on the variability in copy numbers of tandem repeat units in several loci, has discriminatory potential for genotyping and epidemiological trace-back assessment (Al Dahouk *et al,* 2007). This assay is capable of discriminating *Brucella* isolates originating from a restricted geographic region, confirming its potential utility as an epidemiological tool (Garcia-Yoldi *et al,* 2007; Kattar *et al,* 2008; Marianelli *et al,* 2008).

MLVA-16 assay, based on 16 loci containing tandem repeats, including eight moderately variable minisatellites and eight highly polymorphic microsatellites (Le Fleche *et al,* 2006; Al Dahouk *et al,* 2007), were used in this study to characterize the diversity of *Brucella* strains isolated from vaginal swabs of farm animals and from culture collections obtained from Thai infected patients.

MATERIALS AND METHODS

Sample collection

Vaginal swabs and milk specimens were collected from cattle and goats on farms in central Thailand: Kanchanaburi, Nakhon Pathom, Nakhon Sawan, Prachuap Khiri Khan, Ratchaburi, and Saraburi. Vaginal swabs were placed in Cary Blair transport medium (Oxoid, Hampshire, UK) and maintained at 4°C during transportation.

Human isolates were obtained from culture collection of the Medical Bacteriology Group, Department of Medical Science, National Institute of Health (NIH), Bangkok, Thailand. All strains were derived from human blood cultures obtained from various Thai provinces, which had been sent to NIH, Thailand for bacterial identification.

Ethical approval to collect vaginal secretions from goats and cows was obtained from the Faculty of Tropical Medicine Animal Care and Use Committee, Mahidol University (FTM-ACUC; 008/2007). A material transfer agreement permitted analysis of *Brucella* strains isolated from patients, which had been sent for identification to the Department of Medical Sciences, NIH, Ministry of Public Health, Bangkok, Thailand. All the samples were anonymized, except for the province of each hospital from which the isolate was collected, which was retained.

***Brucella* culturing**

A portion of each vaginal swab was streaked onto *Brucella* agar [trypticase soy agar with antibiotic supplement (Oxoid, Hamshire, UK) and 5% horse serum (Gibco, Gaitherberg, MD)]. The other vaginal swab portion and the milk samples were enriched in biphasic agar (Brucella agar slant overlaid with tryptic soy broth) for 5-7 days and bacterial film from the agar slant was restreaked on Brucella agar. All cultures were incubated for 5 days under 5% CO_2 at 37°C. Single colonies were preliminarily identified as *Brucella* spp by gram-negative coccobacilli appearance, which were positive in an oxidase test (Yagupsky, 1999). The putative *Brucella* strains were propagated on Brucella agar plates and a proportion were kept as glycerol stocks at -70°C, and the remaining were placed in normal saline, sedimented and stored at -70°C until used.

Multiplex PCR assay

Genomic DNA was extracted from bacterial cell pellet using a commercial DNA extraction kit (Omega Bio-Tek, Norcross, GA), according to the manufacturer's instructions, and stored at 4°C until analyzed. Stock *Brucella* strains (denoted B1-B4) in our culture collection were investigated for species. Eight primer pairs (Table 1) (López-Goñi *et al*, 2008) were in a PCR mixture of 25 µl containing 12.5 µl of JumpStart™ REDTaq® ReadyMix™ (Sigma, St Louis, MO), 8 µl of primer mix (10 pmol/µl), and 2 µl of DNA template. Thermocycling was conducted in a Mastercycler Nexus instrument (Effpendorf, Hamberg, Germany) as follows: 95°C for 5 minutes; followed by 34 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; then a final heating at 72°C for 7 minutes. Amplicons were separated by 1.5% agarose gel-electrophoresis and visualized by ethidium bromide staining. *Brucella* spp were assigned based on the PCR profiles (Table 2) according to López-Goñi *et al* (2008).

MLVA-16 genotyping assay

MLVA-16 genotyping was performed as described by Le Fleche *et al* (2006) and modified by Al Dahouk (2007). In brief, PCR amplification was performed as described above but with 16 primer pairs (Table 3) divided into three panels, namely, panel 1 for species identification and containing primers that bind eight minisatellite loci (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, and Bruce55), panel 2A containing primers that bind three microsatellite loci (Bruce18, Bruce19, and Bruce21), and those of panel 2B bind five microsatellite loci, Bruce04, Bruce07, Bruce09, Bruce16, and Bruce30), and panel 2B containing

Table 1
Primers used in multiplex PCR determination of *Brucella* sp.

No.	Primer[a]	Putative function of target gene	DNA sequences (5′-3′)	Length (bp)
1	BMEI0998F	Glycosyltransferase (*wboA*)	ATCCTATTGCCCCGATAAGG	1,682
	BMEI0097R		GCTTCGCATTTTCACTGTAGC	
2	BMEI0535F	Immunodominant antigen (*bp26*)	GCGCATTCTTCGGTTATGAA	450
	BMEI0536R		CGCAGGCGAAAACAGCTATAA	
3	BMEII0834F	Outer membrane protein (*omp31*)	TTTACACAGGCAATCCAGCA	1,071
	BMEII0843R		GCGTCCAGTTGTTGTTGATG	
4	BMEI1436F	Polysaccharide deacetylase	ACGCAGACGACCTTCGGTAT	794
	BMEI1435R		TTTATCCATCGCCCTGTCAC	
5	BMEII0428F	D-Erytrulose1-phosphate dehydrogenase (*eryC*)	GCCGCTATTATGTGGACTGG	587
	BMEII0428R		AATGACTTCACGGTCGTTCG	
6	BR0953F	ABC transporter binding protein	GGAACACTACGCCACCTTGT	272
	BR0953R		GATGGAGCAAACGCTGAAG	
7	BMEI0752F	Ribosomal protein S12 (*rpsL*)	CAGGCAAAGCCTCAGAAGC	218
	BMEI0752R		GATGTGGTAACGCACACCAA	
8	BMEII0987F	Transcription regulator	CGCAGACAGTGACCATCAAA	152
	BMEII0987R		GTATTCAGCCCCCGTTACCT	

[a]Based on *B. melitensis* (BME) and *B. suis* (BR) genome sequences.

Table 2
Brucella species-specific genes used in the study.

Specific gene/locus	*wboA*	*omp31*	Poly saccharide deacetylase gene	*eryC*	*Bp26*	ABC transporter binding protein gene	*rpsL*	Transcriptional regulator (CRP family) gene
B. abortus[a]	+	-	+	+	+	-	-	+
B. melitensis[b]	+	+	+	+	+	-	-	+
B. suis[c]	+	+	+	+	+	+	-	+
B. abortus S19 Vaccine strain[d]	+	-	+	-	+	-	-	+
B. ovis[e]	-	+	+	+	+	-	-	+

[a]Human source: DMST7, DMST9; animal source: Kog milk, Yim-M, Yim-V, A18 swab. [b]Human source: DMST1, DMST2, DMST3, DMST4,DMST5, DMST6, DMST10, DMST11,DMST12, DMST13, DMST14, DMST15, DMST16, DMST17, DMST19; animal source**:** 29M, 29S**,** 34S, 43S, S24, S19, S16, S25, R-14, R-55, R-13, R-48, A19 swab, P1 swab, L5-milk, L5-swab, L6-milk, F18 swab, F25 milk, E37 swab, E74 swab, P9 swab. [c]Human source: DMST8**,** DMST18, DMST21. [d]B1, B2, B3, B4. [e]Human source: DMST20; animal source: R18.

Table 3
Primer pairs used in MLVA-16 genotyping of *Brucella* spp.

VNTR-16M[a]	Name	Chr	Forward primer (5′-3′)	Reverse primer (5′-3′)
Panel 1				
BRU1322_134bp_408bp_3u	Bruce06	1	ATGGGATGTGGTAGGGTAATCG	GCGTGACAATCGACTTTTTGTC
BRU1134_18bp_348bp_4u	Bruce08	1	ATTATTCGCAGGCTCGTGATTC	ACAGAAGGTTTTCCAGCTCGTC
BRU211_63bp_257bp_2u	Bruce11	1	CTGTTGATCTGACCTTGCAACC	CCAGACAACAACCTACGTCCTG
BRU73_15bp_392bp_13u	Bruce12	2	CGGTAAATCAATTGTCCCATGA	GCCCAAGTTCAACAGGAGTTTC
BRU424_125bp_539bp_4u	Bruce42	1	CATCGCCTCAACTATACCGTCA	ACCGCAAAATTTACGCATCG
BRU379_12bp_182bp_2u	Bruce43	1	TCTCAAGCCCGATATGGAGAAT	TATTTTCCGCCTGCCCATAAAC
BRU233_18bp_151bp_3u	Bruce45	1	TCCTTGCCTCTCCCTACCAG	CGGGTAAATATCAATGGCTTGGA
BRU2066_40bp_273bp_3u	Bruce55	1	TCAGGCTGTTTCGTCATGTCTT	AATCTGGCGTTCGAGTTGTTCT
Panel 2A				
BRU339_8bp_146bp_5u	Bruce18	2	TATGTTAGGGCAATAGGGCAGT	GATGGTTGAGAGCATTGTGAAG
BRU324_6bp_163bp_18u	Bruce19	2	GACGACCCGGACCATGTCT	ACTTCAC CGTAACGTCGTGGAT
RU329_8bp_148bp_6u	BBruce21	2	CTCATGCGCAACCAAAACA	GATCTCGTGGTCGATAATCTCATT
Panel 2B				
BRU1543_8bp_152bp_2u	Bruce04 orTR6	1	CTGACGAAGGGAAGGCAATAAG	CGATCTGGAGATTATCGGGAAG
BRU1250_8bp_158bp_5u	Bruce07	1	GCTGACGGGGAAGAACATCTAT	ACCCTTTTTCAGTCAAGGCAAA
BRU588_8bp_156bp_7u	Bruce09 or TR8	1	GCGGATTCGTTCTTCAGTTATC	GGGAGTATGTTTTGGTTGTACATAG
BRU548_8bp_152bp_3u	Bruce16	2	ACGGGAGTTTTTGTTGCTCAAT	GGCCATGTTTCCGTTGATTTAT
BRU1505_8bp_151bp_6u	Bruce30 or TR2	1	TGACCGCAAAACCATATCCTTC	TATGTGCAGAGCTTCATGTTCG

[a]Variable number of tandem repeat: nomenclature_repeat unit size (size from genome of *B. melitensis* 16M strain). Chr, chromosome.

Table 4
Brucella spp and hosts found in Thailand.

	Host				
Brucella sp	Human	Caprine	Bovine	Laboratory stock	Total
B. melitensis	15	22[a]	-	-	37
B. abortus	2	2[b]	3[c]	-	7
B. suis	3	-	-	-	3
Unidentified *Brucella* sp	1	-	-	4	5
Total	21	24	3	4	52

[a]Four isolates from Saraburi, 4 from Nakhon Sawan, 4 from Ratchaburi, and 10 from Nakhon Pathom. [b]Strain R18 from Ratchaburi has profile similar to S19 vaccine strain and A18 swab, isolated from Nakhon Pathom, had typical *B. abortus* profile. [c]One Kog milk strain was from Prachuap Khiri Khan and strains Yim-M and Yim-V were from Kanchanaburi.

primers that bind the most variable loci [thereby given a lower weight in a clustering analysis (Al Dahouk *et al*, 2007)]. Amplicons were separated by 3% agarose gel- electrophoresis as described above. Gel images were recorded in a Syngene gel documentation instrument (Frederick, MD) using GeneRuler™ 100-bp Plus DNA ladder (Thermo Scientific) as standard molecular size markers. Sizes of amplicons from alleles at each locus were also confirmed using an Agilent 2100 high-resolution capillary electrophoresis Bioanalyzer (Santa Clara, CA) according to the manufacturer's instructions. The sizes of amplicons generated from each locus were converted into tandem repeat units according to procedures and database of Le Fleche *et al* (2006) and Al Dahouk *et al* (2007). The species of each *Brucella* isolate obtained from multiplex PCR was used to predict the tandem repeat units based on the size of amplicons (alleles) derived from each locus, because the prediction from repeat units is more reliable when the *Brucella* species is known.

Genetic diversity index determination

In order to obtain genetic diversity index for all *Brucella* isolates in this study, copy numbers of tandem repeats at each of the 16 loci were analyzed using V-DICE (VNTR DIversity and Confidence Extractor) program (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). A polymorphism index was determined, based on Simpson's diversity output data, which measures the variation among the numbers of repeats at each locus, ranging from 0.0 (no diversity) to 1.0 (complete diversity). The precision of the diversity index (DI) is expressed with the 95% confidence interval (CI).

Data analysis

Tandem repeat units for the 16 loci of *Brucella* isolates predicted from their allele sizes were considered as datasets. BioNumerics version 6.6 (Applied Math, Austin, TX) was used to analyze the datasets and to generate a dendrogram. General information for each isolate was recorded, *viz*, sample name, type of infected host, province of the source farm. Archived tandem repeat profiles for *Brucella* strains from other countries (Her *et al*, 2009; Maquart *et al*, 2009; Lista *et al*, 2011) were added to our Thai dataset. These character data were subjected to a clustering analysis, based on unweighted pair group method

Table 5
Repeat unit data from MLVA-16 locus among *Brucella* isolates in Thailand and strains from MLVA database.

Laboratory ID#	Sample ID	Host	Source	Multiplex PCR species identification	Position in MLVA cluster	Repeat unit data set of MLVA-16 locus															
						6	8	11	12	42	43	45	55	18	19	21	4	7	9	16	30
[a]DMST 1	DMST 21526	human	Chon Buri	*B. melitensis*	A	1	6	4	13	3	3	3	2	5	22	8	5	4	4	7	7
						156*	379	359	391	412	254	158	237	143	186	165	175	152	128	188	159
[a]DMST 2	DMST 22192	human	Samut Prakan	*B. melitensis*	A	1	6	4	13	3	3	4	2	5	22	8	5	4	4	7	7
						155	379	363	384	418	193	167	238	145	186	168	178	154	133	186	161
[a]DMST 3	DMST 23233	human	Chaiyaphum	*B. melitensis*	A	1	5	4	14	3	3	4	2	6	22	8	6	4	5	8	7
						142	363	361	400	421	195	172	237	150	189	168	182	154	137	198	156
[a]DMST 4	DMST 23234	human	Chai Nat	*B. melitensis*	A	1	6	4	14	3	3	4	2	6	23	9	16	6	5	7	8
						146	381	361	406	398	195	174	237	152	190	170	264	162	141	182	170
[a]DMST 5	DMST 23564	human	Chai Nat	*B. melitensis*	A	1	6	4	14	3	3	4	2	6	23	9	16	6	5	7	7
						146	385	361	409	398	195	174	237	154	190	170	268	164	143	182	154
[a]DMST 6	DMST 23565	human	Sa Kaeo	*B. melitensis*	A	1	6	4	14	3	3	4	3	6	23	9	12	6	6	6	9
						145	385	359	388	398	195	179	275	154	192	172	236	164	148	172	175
[a]DMST 7	DMST 23727	human	Chaiyaphum	*B. abortus*	A	3	6	4	13	2	3	4	4	8	23	9	5	8	6	4	7
						408	386	352	364	311	195	179	273	173	193	170	178	186	145	160	156
[a]DMST 10	DMST 24387	human	Sa Kaeo	*B. melitensis*	A	1	5	3	16	3	3	4	3	6	23	9	12	5	6	7	9
						143	371	326	431	396	195	179	275	154	198	177	234	154	148	186	178
[a]DMST 11	DMST 24734	human	Uttaradit	*B. melitensis*	A	1	5	3	16	3	3	4	3	6	23	9	7	5	7	10	7
						143	365	323	431	398	195	179	275	154	198	179	192	158	151	207	156
[a]DMST 12	DMST 25484	human	Suphan Buri	*B. melitensis*	A	1	6	3	16	3	3	4	3	6	25	9	11	5	6	9	9
						144	385	315	434	398	195	179	236	150	201	172	229	158	149	200	178
[a]DMST 13	DMST 26165	human	Uttaradit	*B. melitensis*	A	1	6	3	16	3	3	4	3	5	25	9	7	5	7	10	7
						144	378	323	431	398	195	177	275	148	203	172	199	158	151	207	156
[a]DMST 14	DMST 26346	human	Chanthaburi	*B. melitensis*	A	1	6	3	16	3	3	4	3	5	25	9	15	5	6	8	9
						145	387	323	431	398	195	174	275	143	201	179	257	156	149	196	177
[a]DMST 15	DMST 27015	human	Kanchanaburi	*B. melitensis*	A	1	6	3	16	3	3	4	3	4	25	9	7	4	6	9	9
						144	385	320	437	398	229	169	275	139	200	175	193	154	146	204	178
[a]DMST 16	DMST 27016	human	Kanchanaburi	*B. melitensis*	A	1	6	3	16	3	3	4	3	4	25	9	7	4	5	10	9
						142	384	323	437	398	190	167	275	137	200	170	192	147	143	205	177
[a]DMST 17	DMST 27020	human	Chanthaburi	*B. melitensis*	A	1	6	3	12	3	3	2	2	4	21	9	6	6	8	7	9
						142	385	318	388	398	196	126	237	135	183	170	183	166	164	184	175

[a]DMST 18 DMST 30490	human	Chanthaburi	*B. suis*	A	2	4	3	13	3	3	5	2	5	20	9	5	11	13	9	10
					255	352	675	355	398	256	185	237	143	190	173	195	168	200	167	160
[a]DMST 19 DMST 30491	human	Sa Kaeo	*B .melitensis*	A	1	4	2	13	3	3	2	2	5	23	9	7	4	8	7	9
					138	386	323	388	394	196	143	237	143	190	170	190	154	166	186	175
[a]DMST 20 DMST 30844	human	Phetchabun	*Brucella* spp	A	1	5	2	11	3	2	3	2	4	23	9	7	6	8	9	8
					139	373	326	364	397	255	150	237	144	195	177	195	165	165	198	165
29M	caprine	Saraburi	*B. melitensis*	A	1	6	4	11	3	3	4	2	6	21	9	14	8	3	4	9
					140	381	361	364	398	195	169	235	159	180	177	246	185	123	161	170
29S	caprine	Saraburi	*B. melitensis*	A	1	6	4	11	3	3	4	2	7	21	9	14	8	3	4	8
					144	379	361	364	398	195	171	237	161	180	171	246	185	121	159	169
34S	caprine	Saraburi	*B. melitensis*	A	1	5	4	11	3	3	4	2	7	21	9	14	8	3	3	8
					142	374	370	364	398	197	169	237	164	180	173	250	182	123	154	168
43S	caprine	Saraburi	*B. melitensis*	A	1	5	4	11	3	3	4	2	7	21	9	13	8	3	3	8
					142	368	370	364	398	197	171	237	165	180	175	240	185	121	148	167
S24	caprine	Nakhon Sawan	*B. melitensis*	A	2	4	4	11	3	3	4	2	7	20	9	6	9	3	4	5
					271	342	368	364	398	197	169	237	161	175	176	188	188	131	161	145
S19	caprine	Nakhon Sawan	*B. melitensis*	A	1	4	4	11	3	3	4	2	7	20	9	6	9	3	4	6
					144	340	386	364	398	196	167	237	159	173	176	185	188	124	158	148
S16	caprine	Nakhon Sawan	*B .melitensis*	A	1	4	4	11	3	3	4	2	6	19	9	5	9	3	4	6
					142	342	386	364	398	196	167	237	153	170	173	179	187	120	158	151
S25	caprine	Nakhon Sawan	*B. melitensis*	A	1	4	4	11	3	3	3	2	5	19	9	5	8	3	3	6
					142	346	390	364	396	196	152	237	144	168	176	177	183	120	156	149
R-14	caprine	Ratchaburi	*B. melitensis*	A	1	6	3	11	3	3	3	2	6	25	9	8	8	3	4	6
					138	378	322	364	397	195	150	236	155	202	171	202	182	120	158	146
R-55	caprine	Ratchaburi	*B. melitensis*	A	1	6	3	11	3	3	3	2	6	25	9	9	8	3	3	5
					140	381	328	364	398	195	152	236	152	206	170	206	183	125	154	141
R-13	caprine	Ratchaburi	*B. melitensis*	A	1	5	3	12	3	3	3	2	7	25	9	8	8	3	3	5
					141	374	344	381	398	196	152	238	160	204	175	204	153	123	153	141
R-48	caprine	Ratchaburi	*B. melitensis*	A	1	6	4	12	2	3	3	3	10	25	9	9	8	4	5	6
					143	387	359	388	285	196	152	275	188	206	172	206	185	134	171	152
A19 swab	caprine	Nakhon Pathom	*B. melitensis*	A	4	5	4	12	2	3	3	3	10	25	9	5	8	6	6	8
					545	371	386	388	288	190	152	275	182	207	175	172	180	150	172	167
P1 swab	caprine	Nakhon Pathom	*B. melitensis*	A	1	4	2	12	2	3	4	3	9	25	9	5	8	7	5	8
					143	361	396	388	288	195	164	275	178	209	174	176	182	154	171	166
L5-Milk	caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	11	3	2	4	2	7	25	9	9	8	6	8	9
					141	363	327	364	398	185	166	237	162	200	179	208	181	149	197	176
L5-Swab	caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	11	3	2	4	2	7	23	9	9	8	6	8	9
					141	369	324	364	398	185	164	237	160	194	176	208	182	147	197	175

Table 5 (Continued).

Laboratory ID#	Sample ID	Host	Source	Multiplex PCR species identification	Position in MLVA cluster	Repeat unit data set of MLVA-16 locus															
						6	8	11	12	42	43	45	55	18	19	21	4	7	9	16	30
L6-Milk		caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	12	3	2	4	2	7	25	9	9	8	7	8	9
						138	367	333	388	398	185	151	237	163	202	179	208	182	151	195	176
F18 swab		caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	11	3	2	3	2	6	23	9	8	8	5	8	9
						147	359	320	364	398	254	152	237	153	192	173	204	187	140	195	175
F25 milk		caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	11	3	2	3	2	6	23	9	9	8	5	4	9
						147	367	320	364	398	185	152	237	156	194	170	205	182	141	160	175
E37 swab		caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	13	3	3	3	2	7	25	9	9	8	7	6	9
						147	365	325	396	398	193	152	237	165	200	176	206	183	151	175	178
E74 swab		caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	13	3	3	3	2	7	25	9	9	8	7	9	9
						149	368	328	396	398	195	152	237	163	200	176	206	182	151	204	178
P9 swab		caprine	Nakhon Pathom	*B. melitensis*	A	1	5	3	14	3	3	4	3	7	25	9	9	8	7	6	8
						141	371	334	408	398	195	158	275	165	214	176	206	182	155	175	167
[2]MM154	S596	human	Paris, France	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	5	4	8	6	9
[2]RR179	AUB BRUP-S24	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	5	5	8	7	9
[2]W173	AUB BRUP-S14	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	5	5	9	6	6
[2]W178	AUB BRUP-S23	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	5	40	8	5	5	7	5	6
[2]H233	BfR X	human	Bosnia	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	7	4	3	7	8
[2]W169	AUB BRUP-S11	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	3	4	3	7	6
[2]H234	BfR VII	human	Syria	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	4	4	3	4	4
[2]V221	BfR 62	human	Iraq	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	4	4	3	4	5
[2]W172	AUB BRUP-S13	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	4	4	3	7	4
[2]W171	AUB BRUP-S12	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	5	4	3	5	5
[2]W175	AUB BRUP-S20	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	2	3	2	4	40	8	8	3	3	5	4
[2]W177	AUB BRUP-S22	human	Lebanon	*B. melitensis*	B	1	5	3	13	3	3	3	2	4	40	8	5	4	3	8	4
[2]W176	AUB BRUP-S21	human	Lebanon	*B. melitensis*	B	1	4	3	13	3	2	3	2	4	40	8	8	4	3	8	5
[2]GG105	BfR 68	human	Tyrol, Germany	*B. melitensis*	B	1	5	3	13	2	2	3	2	3	42	8	4	4	3	7	6
[2]MM152	S594	human	Paris, France	*B. melitensis*	B	1	5	3	13	2	3	3	2	4	40	8	4	4	3	4	6
[2]MM153	S595	human	Poitiers, France	*B. melitensis*	B	1	5	3	13	3	2	2	2	6	10	8	7	4	3	5	6
[2]MM156	S219	human	Tarbes, France	*B. melitensis*	B	1	5	3	13	3	2	2	2	6	10	8	4	4	3	6	6
[2]MM158	S220	human	Agen, France	*B. melitensis*	B	1	5	3	13	3	2	3	2	5	10	8	3	4	3	5	5
[2]H232	BfR 20	human	Pakistan	*B. melitensis*	B	3	5	3	13	3	2	3	3	6	40	8	7	4	5	5	3

[3]GG102	KBa156	cattle (Korean native)	South Korea	*B. abortus*	B	4	5	4	12	2	3	3	3	6	42	8	4	4	3	3	6
[3]GG103	KBa155	cattle Korean native)	South Korea	*B. abortus*	B	4	5	4	12	2	4	3	3	6	42	8	4	4	3	3	6
[3]RR186	KRef09	cattle	USA	*B. abortus*	B	4	5	4	12	2	3	3	3	6	42	8	3	7	3	3	5
[3]J206	SC1	cattle	Brazil	*B. abortus*	B	4	5	4	12	2	2	3	3	6	44	8	3	4	3	5	6
[3]MM159	KRef04	cattle	England	*B. abortus*	B	4	5	4	12	2	2	3	3	5	42	8	3	5	3	4	5
[3]V214	KRef06	cattle	Africa	*B. abortus*	B	3	5	3	12	2	2	3	3	7	42	8	3	6	3	3	3
[2]MM155	S901	human	Lyon, France	*B. melitensis*	B	3	5	3	13	1	1	3	3	7	42	8	9	5	6	6	3
Yim-M		bovine	Kanchanaburi	*B. abortus*	C	3	4	2	12	4	2	3	3	6	20	6	2	5	7	3	6
						380	381	348	364	285	196	152	275	188	206	170	168	132	133	167	135
Yim-V		bovine	Kanchanaburi	*B. abortus*	C	3	4	2	12	4	2	3	3	6	20	6	2	5	7	3	6
						378	381	339	366	288	196	152	275	178	206	178	206	198	132	159	135
A18 swab		caprine	Nakhon Pathom	*B. abortus*	C	3	4	2	12	4	2	3	3	5	20	6	2	6	9	3	6
						544	368	393	388	288	195	162	275	180	209	170	176	183	156	172	167
[b]B1		bacteria stock	Bangkok	*Brucella* spp	C	3	4	2	13	4	2	3	3	5	18	6	2	5	7	3	6
						542	378	372	365	277	195	150	275	167	189	176	176	192	123	149	155
[b]B2		bacteria stock	Bangkok	*Brucella* spp	C	3	4	2	13	4	2	3	3	5	18	6	2	5	7	3	6
						413	377	336	365	294	194	149	275	167	189	177	176	192	124	151	153
[b]B3		bacteria stock	Bangkok	*Brucella* spp	C	3	4	2	13	4	2	3	3	5	18	6	2	5	7	3	6
						545	379	402	364	276	252	150	275	161	189	174	176	192	126	149	155
[b]B4		bacteria stock	Bangkok	*Brucella* spp	C	3	4	2	13	4	2	3	3	5	18	6	2	5	7	3	6
						542	379	372	355	276	196	167	275	175	189	176	188	192	123	148	156
R-18		caprine	Ratchaburi	*Brucella* spp	C	3	4	2	13	4	2	3	3	5	18	6	2	5	7	3	6
						143	387	359	388	285	196	152	275	188	206	172	206	185	134	171	152
[2]TT76	R1Sp	unknown	Spain	*B. melitensis*	C	3	4	2	13	4	2	3	3	5	36	6	2	6	7	3	6
Kogmilk		bovine	Prachaub Khiri Khan	*B. abortus*	C	3	4	2	13	4	2	4	3	6	18	6	2	6	7	3	6
						424	368	374	366	275	185	171	274	181	178	173	168	200	131	154	138
[a]DMST 9	DMST 23965	human	Chanthaburi	*B. abortus*	C	3	4	3	14	4	2	3	3	7	18	6	2	5	8	3	6
						395	365	323	406	314	185	152	273	188	197	175	185	175	149	167	155
[2]MM137	S244	cattle	Central, Kenya	*B. melitensis*	C	3	4	3	13	4	2	3	3	8	36	6	2	4	6	3	6
[2]MM138	S245	cattle	Central, Kenya	*B. melitensis*	C	3	4	3	13	4	2	3	3	8	36	6	2	4	6	3	6
[2]MM139	S81	cattle	Central, Kenya	*B. melitensis*	C	3	4	3	13	4	2	3	3	8	36	6	2	4	6	3	6
[2]MM141	S82	cattle	Central, Kenya	*B. melitensis*	C	3	4	3	13	4	2	3	3	8	36	6	2	4	6	3	6
[2]MM142	S201	cattle	Central, Kenya	*B. melitensis*	C	3	4	3	13	4	2	3	3	8	36	6	2	4	6	3	6
[2]TT75	R32	(commercial)	Spain	*B. melitensis*	C	3	4	2	13	4	2	3	3	9	36	6	2	4	6	3	6

Table 5 (Continued).

Laboratory ID#	Sample ID	Host	Source	Multiplex PCR species identification	Position in MLVA cluster	Repeat unit data set of MLVA-16 locus															
						6	8	11	12	42	43	45	55	18	19	21	4	7	9	16	30
[2]V215	R5	sheep	South Africa	*B. melitensis*	C	3	4	2	13	4	2	3	3	8	36	6	2	4	6	4	6
[2]TT74	R26	(commer-	Spain	*B. melitensis*	C	3	4	2	13	4	2	3	3	8	36	6	2	5	5	3	6
		cial)																			
[2]TT77	BCCN#92-87	sheep	Spain	*B. melitensis*	C	3	4	2	13	4	2	3	3	8	36	6	2	5	6	3	6
MM126		human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM127	S22	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM128	S23	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM129	S211	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM131	S212	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
MM132		human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM133	S230	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM134	S72	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM135	S73	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[2]MM136	S243	human	Callao, Peru	*B. melitensis*	C	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[a]DMST 21	DMST 31267	human	Nakhon	*B. suis*	C	3	4	3	13	3	2	3	3	5	21	6	2	5	8	3	8
			Phanom			244	346	669	328	397	196	186	237	135	195	170	195	154	172	177	165
[a]DMST 8	DMST 23728	human	Phetchabun	*B. suis*	C	3	4	3	13	3	2	3	3	6	21	6	2	5	10	3	8
						264	354	726	391	397	237	186	237	166	192	187	197	168	209	170	156
[2]MM151	S202	human	Essonne, France	*B. melitensis*	C	3	4	3	14	5	2	3	3	6	36	5	2	4	6	5	4
[2]RR184	FH 2208	red fox	Austria	*B. microti*	D	4	5	12	13	5	2	5	6	10	8	9	8	6	7	11	5
[2]RR185	FK 21908	red fox	Austria	*B. microti*	D	4	5	12	13	5	2	5	10	6	8	9	10	6	9	11	5
[2]KK122	M621/99/2	gray seal	Scotland	*B. pinnipedialis*	D	3	5	6	13	3	2	5	4	7	44	9	6	6	4	3	3
[2]J207	100V	sheep	Brazil	*B. ovis*	D	3	5	2	10	1	1	5	2	3	8	9	8	4	13	13	2
[2]MM148	BCCN#77-7	sheep	Nice, France	*B. ovis*	D	3	5	2	10	1	1	5	2	3	8	9	8	6	13	9	2
[2]MM149		sheep	Rennes, France	*B. ovis*	D	3	5	2	10	1	1	5	2	3	8	7	7	6	10	8	2
[2]LL41		swine	Ribatejoe	*B. suis*	D	2	5	8	9	5	1	5	5	6	19	9	8	5	15	2	6
Used in Fig 2																					
[2]S-25		swine	Badajoz, Spain	*B. suis*		2	5	8	9	5	1	5	5	6	38	9	2	5	19	2	6
[2]S-97		swine	Croatia	*B. suis*		2	3	6	10	4	1	5	2	4	38	9	2	7	8	5	3
[2]BCCN#87-57		human	Canada	*B. suis*		2	3	9	11	3	1	5	2	4	40	9	5	5	10	10	3
[2]REF 1330		swine	USA	*B. suis*		2	3	6	10	4	1	5	2	4	19	9	6	6	5	5	3

[2]REF Thomsen	swine	Denmark	*B. suis*	2	4	8	14	6	1	5	2	6	22	9	9	9	18	2	4
[2]REF 40	reindeer	Former USSR	*B. suis*	2	3	9	11	3	1	5	2	5	18	9	4	5	9	6	3
[4]ATCC 23445 (NC_010169.1)			*B. suis*	2	4	8	14	6	1	5	2	6	43	9	9	9	18	2	4
[4]1330			*B. suis*	2	3	6	10	4	1	5	2	4	38	9	6	6	5	5	3
[4]A13334			*B. abortus*	4	5	4	12	2	3	3	3	6	43	8	4	4	3	3	6
[4]ATCC 23457 (NC_012441.1)			*B. melitensis*	1	5	3	12	2	2	3	2	4	41	8	8	4	3	5	4
[4]HB07-12	sheep	Hebei, China	*B. melitensis*	1	5	3	13	2	2	3	2	4	40	8	5	4	3	7	6
[4]KBa0143	cattle (dairy)	South Korea	*B. abortus*	4	5	4	12	2	3	3	3	6	42	8	4	4	3	3	6
[4]S152	human	Callao, Peru	*B. melitensis*	3	4	2	13	5	2	3	3	7	36	6	2	5	7	4	4
[4]BCCN#77-72	sheep	Nice, France	*B. ovis*	3	5	2	10	1	1	5	2	3	8	9	8	6	13	9	2
[4]100V2	Sheep	Brazil	*B. ovis*	3	5	2	10	1	1	5	2	3	8	9	8	4	13	13	2
[4]REF 23082		USA	*B. abortus*	4	5	4	12	2	3	3	3	6	42	8	3	7	3	3	5
[4]ATCC 23365 (NC_010103.1)			*B. canis*	2	3	9	11	3	1	5	2	5	40	9	7	6	10	7	3
[4]HSK A52141 (NC_016778.1)			*B. canis*	2	3	9	11	3	1	5	2	5	40	9	5	5	7	8	3

[a]National Institute of Health (NIH), Medical Bacteriology Group, Department of Medical Science in Thailand. [b]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. [1]Lista *et al* (2011). [2]Maquart *et al* (2009). [3]Her *et al* (2009). [4]Database from http://minisatellites.u-psud.fr. *Amplicon size (bp). #Used in Fig 1.

using arithmetic averages (UPGMA) with a categorical similarity coefficient. Maximum parsimony was used to draw a clustering tree, with 200 bootstrap simulations, and the data were treated as categorical.

RESULTS

Species of *Brucella* isolates determined by multiplex PCR

From 2009 to 2011, 300 vaginal swabs and 10 milk samples were collected from farms in central Thailand. Bacterial colonies grown on *Brucella*-selective agar and screened for gram-negative coccobacilli with positive oxidase test were propagated on Brucella-selective agar. Extracted bacterial DNA was subjected to multiplex PCR for *Brucella* species identification (Table 2). Twenty-two isolates from goats and 15 from humans were identified as *B. melitensis* (Table 4). Among the seven isolates identified as *B. abortus*, two were from caprines, two from humans (DMST 7 from Chaiyaphum and DMST 9 from Chanthaburi), and three from cows (Kog milk, Yim-V, and Yim-M). Three *B. suis* isolates were from humans (DMST 18 from Chanthaburi, DMST 8 from Phetchabun and DMST 21 from Nakhon Phanom). The multiplex PCR bands for DMST 20 (from Phetchabun) are similar to those of *B. melitensis*, but lacks the 1682 bp, and so was likely to be *B. ovis* (Table 4). The four reference *Brucella* strains (B1–B4) and R-18 strain have multiplex PCR profiles (bands at 152, 450, 794, and 1682 bp similar to that of *B. abortus* S-19 strain (Garcia-Yoldi, 2006).

Assignment of tandem repeat units for each allele size

Brucella strain signature was identified using an MLVA-16 typing scheme. The sizes of amplicons derived from 16 loci in all the isolates were determined by

Table 6
Simpson's diversity index (DI) for all loci of *Brucella* spp determined in the study.

Locus	DI			
	Whole population (n = 52)	*B. melitensis* (n = 37)	*B. abortus* (n = 11)	*B. suis* (n = 3)
Panel 1				
Bruce06	0.498	0.153	0.298	0.444
Bruce08	0.665	0.622	0.165	0
Bruce11	0.669	0.546	0.314	0.444
Bruce12	0.783	0.758	0.512	0.444
Bruce42	0.423	0.149	0.165	0
Bruce43	0.465	0.234	0.165	0.444
Bruce45	0.570	0.505	0.298	0.444
Bruce55	0.514	0.438	0.165	0.444
Panel 2A				
Bruce18	0.751	0.735	0.612	0
Bruce19	0.811	0.730	0.512	0.444
Bruce21	0.418	0.149	0.165	0.444
Panel 2B				
Bruce04	0.865	0.863	0.165	0.444
Bruce07	0.743	0.673	0.430	0.444
Bruce09	0.814	0.793	0.446	0.444
Bruce16	0.826	0.856	0.165	0.444
Bruce30	0.760	0.722	0.165	0.444

One isolate, DMST20, predicted to be *B. ovis* was not included for determination of diversity index.

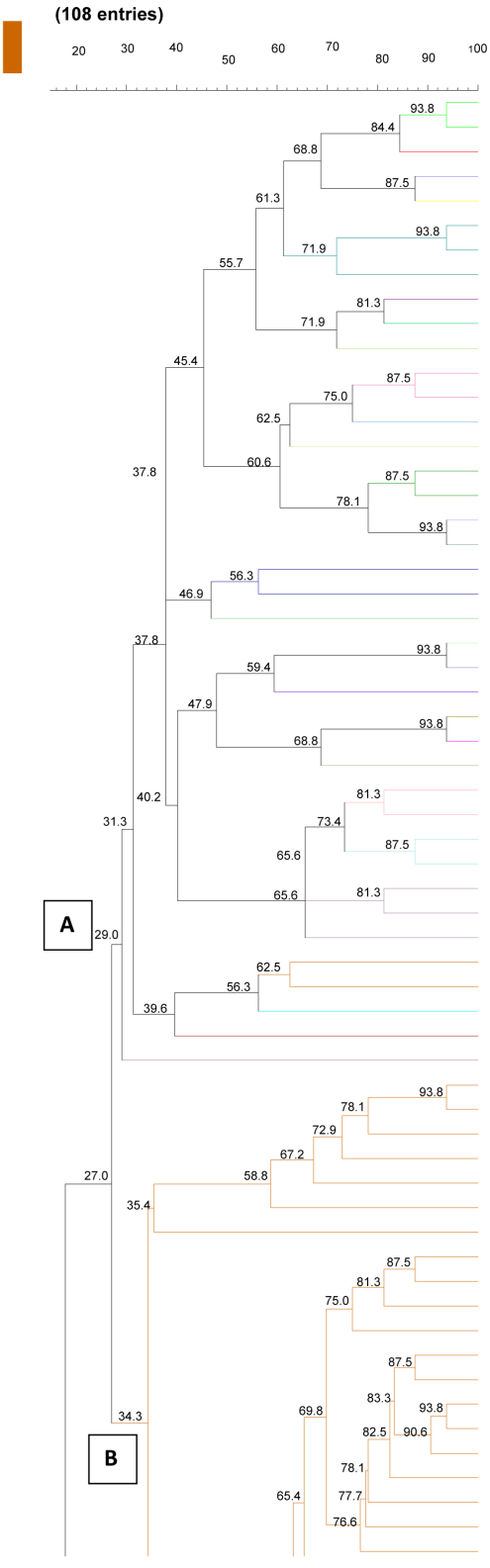
agarose gel- electrophoresis and capillary electrophoresis. The range of amplicon size for each allele was used to determine the number of tandem repeat units, based on data of Al Dahou *et al* (2007) and Le Fleche *et al* (2006). In this study, the variable allele types were predominantly found in Bruce 11, and the numbers of repeat units were higher for the loci of panel 2 than for those of panel 1 (Table 5). MLVA profiles of all the Thai *Brucella* isolates and some selected *Brucella* species and strains from other countries also are shown in Table 5.

Genetic diversity

Simpson's diversity index (DI) revealed that the variable allele types were predominantly found in Bruce 04 (DI = 0.865) (Table 6). The numbers of repeat units were higher for loci of panel 2 (DI = 0.418-0.865) than for those of panel 1 (DI = 0.423-0.783).

Clustering analysis based on MLVA-16 genotyping

The character dataset for the tandem repeat units at 16 loci in *Brucella* genome was subjected to a clustering analysis. A dendrogram was constructed using UPGMA protocol for 52 Thai isolates (Fig 1). Isolates from several countries (in Europe, Central and South America, and Southeast Asia), selected from the *Brucella* genotyping public database were included for comparison. The closely-



Key	Host	Source	Specie
L5-Milk	caprine	Nakhon Pathom	*B. melitensis*
L5-Swab	caprine	Nakhon Pathom	*B. melitensis*
L6-Milk	caprine	Nakhon Pathom	*B. melitensis*
F18 swab	caprine	Nakhon Pathom	*B. melitensis*
F25 milk	caprine	Nakhon Pathom	*B. melitensis*
E37 swab	caprine	Nakhon Pathom	*B. melitensis*
E74 swab	caprine	Nakhon Pathom	*B. melitensis*
P9 swab	caprine	Nakhon Pathom	*B. melitensis*
R-14	caprine	Ratchaburi	*B. melitensis*
R-55	caprine	Ratchaburi	*B. melitensis*
R-13	caprine	Ratchaburi	*B. melitensis*
S19	caprine	Nakhon Sawan	*B. melitensis*
S24	caprine	Nakhon Sawan	*B. melitensis*
S16	caprine	Nakhon Sawan	*B. melitensis*
S25	caprine	Nakhon Sawan	*B. melitensis*
29M	caprine	Saraburi	*B. melitensis*
29S	caprine	Saraburi	*B. melitensis*
34S	caprine	Saraburi	*B. melitensis*
43S	caprine	Saraburi	*B. melitensis*
DMST(17)	human	Chanthaburi	*B. melitensis*
DMST(19)	human	Sa Kaeo	*B. melitensis*
DMST(20)	human	Phetchaburi	*Brucella* spp
DMST (1)	human	Chon Buri	*B. melitensis*
DMST(2)	human	Samut Prakan	*B. melitensis*
DMST(3)	human	Chaiyaphum	*B. melitensis*
DMST(4)	human	Chai Nat	*B. melitensis*
DMST(5)	human	Chai Nat	*B. melitensis*
DMST(6)	human	Sa Kaeo	*B. melitensis*
DMST(12)	human	Suphan Buri	*B. melitensis*
DMST(14)	human	Chanthaburi	*B. melitensis*
DMST(15)	human	Kanchanaburi	*B. melitensis*
DMST(16)	human	Kanchanaburi	*B. melitensis*
DMST(11)	human	Uttaradit	*B. melitensis*
DMST(13)	human	Uttaradit	*B. melitensis*
DMST(10)	human	Sa kaeo	*B. melitensis*
A19 swab	caprine	Nakhon Pathom	*B. melitensis*
R-48	caprine	Ratchaburi	*B. melitensis*
P1 swab	caprine	Nakhon Pathom	*B. abortus*
DMST(7)	human	Chaiyaphum	*B. melitensis*
DMST (18)	human	Chanthaburi	*B. suis*
GG102	cattle (Kore.	Gyeonggi, South Ko.	*B. abortus*
GG103	cattle (Kore.	Kangwon, South Ko.	*B. abortus*
RR186	cattle	USA	*B. abortus*
J206	cattle	Brazil	*B. abortus*
MM159	cattle	England	*B. abortus*
V214	cattle	Africa	*B. abortus*
MM155	human	Lyon, France	*B. melitensis*
MM154	human	Paris, France	*B. melitensis*
RR179	human	Lebanon	*B. melitensis*
W173	human	Lebanon	*B. melitensis*
W178	human	Lebanon	*B. melitensis*
H233	human	Bosnia	*B. melitensis*
W169	human	Lebanon	*B. melitensis*

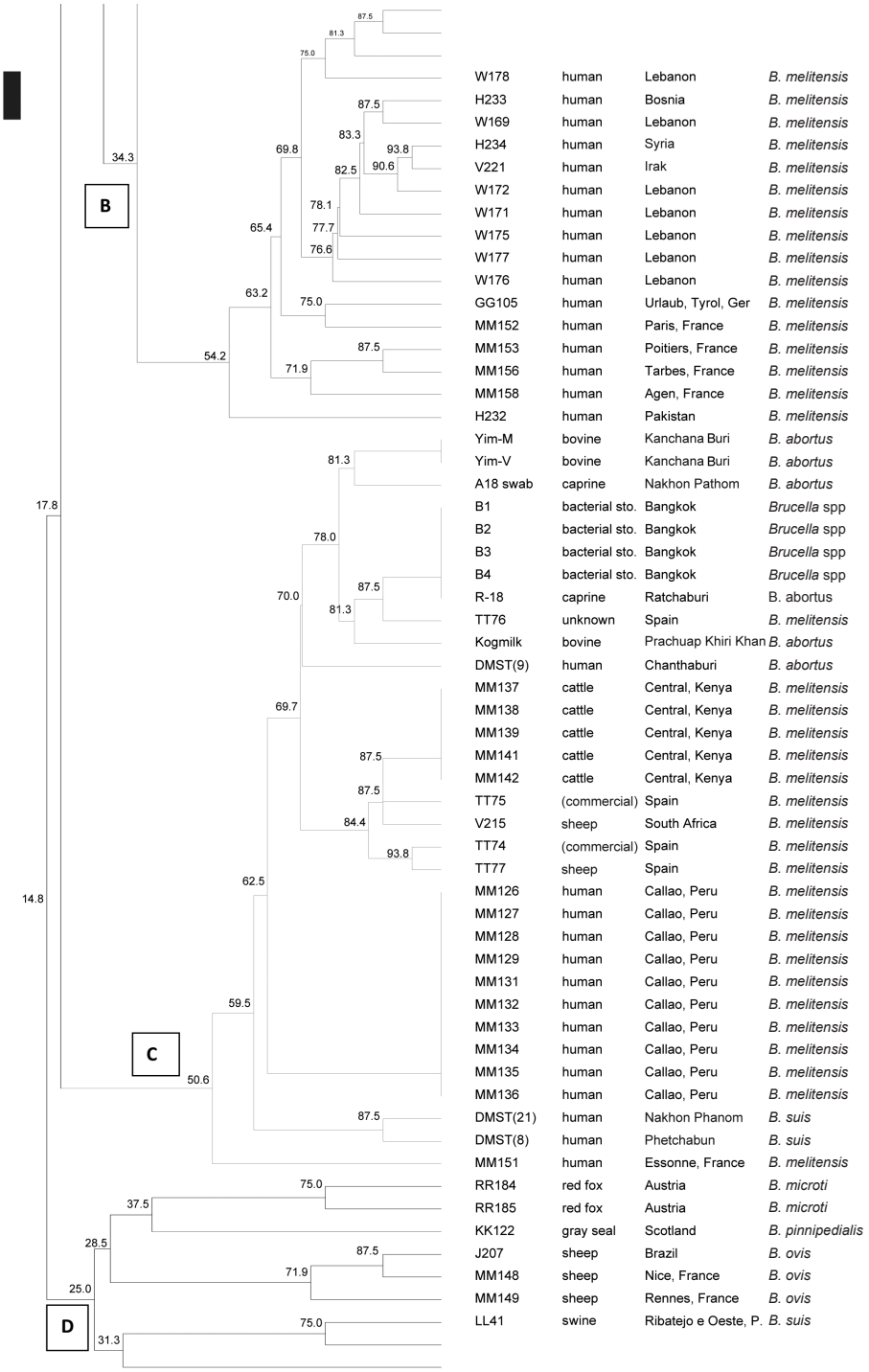
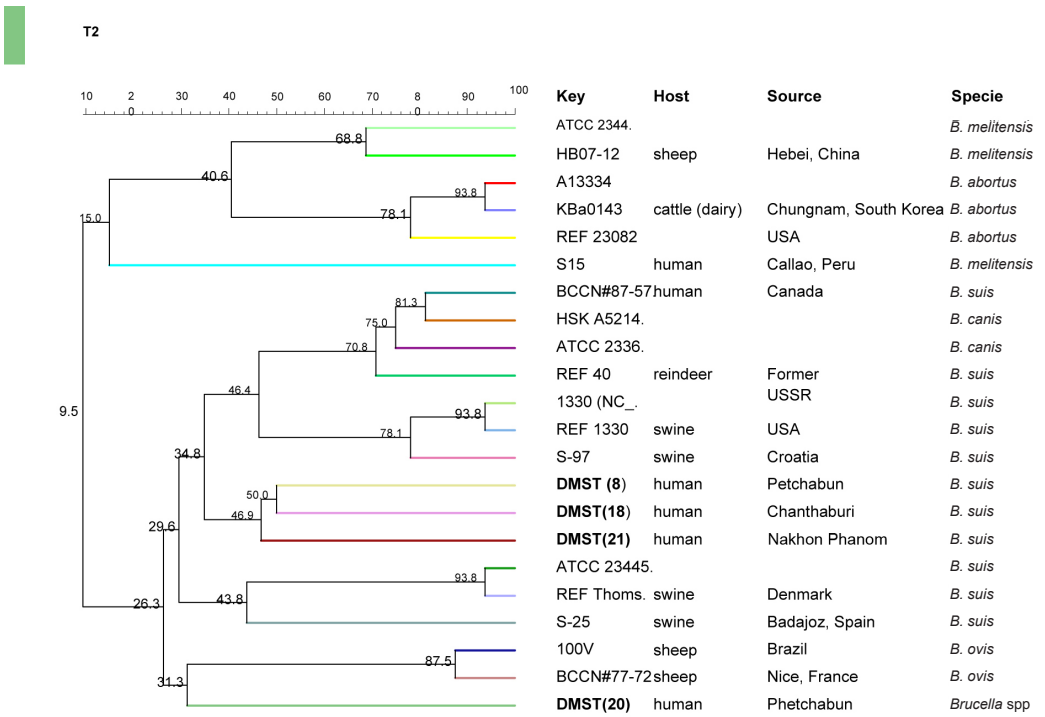


Fig 1–Dendogram of clustered MLVA-16 genotypes. The dendogram is constructed from MLVA-16 profiles of 21 Thai *Brucella* spp isolates from humans, 24 from caprine, 3 from bovine, 4 stock cultures of unknown origin, and more than 56 reference strains. The four columns next to the dendeogram indicated name of strain, host, source of sample and species assignment.



Key	Host	Source	Specie
ATCC 2344.			*B. melitensis*
HB07-12	sheep	Hebei, China	*B. melitensis*
A13334			*B. abortus*
KBa0143	cattle (dairy)	Chungnam, South Korea	*B. abortus*
REF 23082		USA	*B. abortus*
S15	human	Callao, Peru	*B. melitensis*
BCCN#87-57	human	Canada	*B. suis*
HSK A5214.			*B. canis*
ATCC 2336.			*B. canis*
REF 40	reindeer	Former USSR	*B. suis*
1330 (NC_.			*B. suis*
REF 1330	swine	USA	*B. suis*
S-97	swine	Croatia	*B. suis*
DMST (8)	human	Petchabun	*B. suis*
DMST(18)	human	Chanthaburi	*B. suis*
DMST(21)	human	Nakhon Phanom	*B. suis*
ATCC 23445.			*B. suis*
REF Thoms.	swine	Denmark	*B. suis*
S-25	swine	Badajoz, Spain	*B. suis*
100V	sheep	Brazil	*B. ovis*
BCCN#77-72	sheep	Nice, France	*B. ovis*
DMST(20)	human	Phetchabun	*Brucella* spp

Fig 2–Dendogram of clustered MLVA-16 genotypes to verify Thai *Brucella* isolates, DMST8, 18, and 21 as *B. suis*, and DMST20 as *B. ovis*.

related genetic profile of Thai strains, belong to *B. melitensis* were included in cluster A. *B. melitensis* isolated from caprine were clustered together and were in the distinct cluster from human isolates (DMST series). *B. melitensis* strains from other countries were in cluster B, together with strains of *B. abortus*, although the clade of *B. abortus* was separated from *B. melitensis*. The Thai isolates were distinct from the foreign isolates and located in a distinct A cluster. A number of additional strains of *B. melitensis* were included in the cluster, while the Thai strains of *B. abortus* were also included in this cluster C. Other *Brucella* spp, *ie*, *B. microti, B. pinnipedialis*, *B. suis*, and *B. ovis*, were included in cluster D.

Multiplex PCR classified DMST 8, 18, and 21 as *B. suis*, and DMST 20 as probably *B. ovis*. In order to confirm this classification, a cluster analysis of MLVA-16 dataset of these isolates was performed in comparison to many strains of *B. suis* and *B. ovis* strains from the public database, and the strains that were closely related to our Thai strains were selected and included in the dataset (Fig 2). The Thai *B. suis* isolates, DMST8, 18, 21 were clustered in a single clade next to *B. suis* isolates from swine in other countries. DMST 20 clustered with *B. ovis* strains isolated from sheep in Brazil and France.

DISCUSSION

Basic microbiological protocol used for primary screening of *Brucella* isolates is based on bacterial morphology of gram-negative coccobacilli, but for species

identification, multiplex PCR profiles at eight loci are required (Lopez-Goni *et al* (2008)). However, size of an allele and number of its repeat units are specific to a particular species (Le Fleche *et al* (2006). For instance, a large database of MLVA profiles for various strains of *B. melitensis* has allowed more reliable identification of *B. melitensis* (Al Dahouk *et al* (2007).

Thai *B. melitensis* strains (cluster A) were phylogenetically different from those of other countries (cluster B). Among the Thai *B. melitensis* strains, as expected, those from the same geographic region were located close to one another in the same cluster. Strains derived from caprine were located together and separated from strains derived from humans, suggesting that strains from human and non-human hosts were generically different. There was no instance of zoonotic *Brucella* transmission from animals to humans. *Brucella* infections in humans ought to be due to person-to-person transmission.

When *B. melitensis* DMST 6 and Sar34S strains were subjected to a multilocus sequence typing (MLST) analysis, the strains matched *B. melitensis* ST8 strain (Chawjiraphan *et al*, 2016). In MLVA-based analysis, these two strains clustered with *B. melitensis*, and according to host species and regional source. MLVA correctly assigned both DMST6 and Sar34S to *B. melitensis*, consistent with MLST strategy, indicating the reliability of this MLVA technique.

Multiplex PCR identified DMST 8, 18, and 21 as *B. suis*, and DMST 20 as *B. ovis*, so their MLVA-16 profiles were subjected to a clustering analysis with other *B. suis* and *B. ovis* strains available in the MLVA database. MLVA-based cluster analysis correctly placed multiplex PCR-identified *B. suis* strains among those isolated from swine.

Multiplex PCR identified DMST 20 (from human) as *B. ovis* and MLVA analysis placed the strain in the cluster containing strains that were often isolated from sheep, and it is therefore possible that the human source acquired the infection from sheep.

Tandem repeat units for each locus from the MLVA-16 panel were used to calculate Simpson's DI of *B. melitensis* samples only (n = 37). MLVA-16 profile for the loci of panel 1 had lower DI values than those of panel 2A or panel 2B, suggesting the loci of panel 1 are more conserved than those in panel 2. These results supported the selection of loci marker by Le Fleche *et al (*2006), who informed that markers of panel 1 were minisatellite loci with repeat units length above 9 bp, while markers of panel 2 were microsatellites of highly polymorphic octamers with 2-5 bp repeat unit.

The four stock *Brucella* strains and R-18 strain isolated from goats in Ratchaburi had multiplex PCR profiles similar to that of *B. abortus* strain S-19 vaccine strain, MLVA profile of which was clustered among the reference *B. abortus* strains from foreign countries, different from the C cluster of the Thai strains (data not shown). A possible explanation is that the vaccine strain had reverted to a viable form and was transmitted among other animals.

Yim-M and Yim-V strains isolated from cattle and A-18 swab specimen was from a goat but had multiplex PCR profile of *B. abortus*, and their MLVA profiles also were closely related. Goat in the same farm might have acquire *B. abortus* infection either from cow or the environment. Yim-M was isolated from milk and Yim-V from a vaginal swab from the same cow. The other two *B. abortus* isolates, Kog milk from a cow and human strain DMST 9, were clustered next to one another in the

dendrogram, and both were localized to the correct *B. abortus* cluster.

In conclusion, this study demonstrates that MLVA-16 strategy was able to classify *Brucella* isolates at the strain level, and also to cluster the species correctly, except that the Thai isolates of *B. abortus* and *B. melitensis* shared the same cluster. Although a limited number of *Brucella* isolates was included, this study reveals that the Thai *Brucella* strains are distinct from strains from other continents, and even other Asian countries. Moreover, *Brucella* strains associated with each host species were phylogenetically distinct. MLVA-16 typing, combined with multiplex PCR, should prove useful in *Brucella* diagnosis, epidemiology and control.

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Conflict of interests

The authors declare no conflict of interests.

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