MULTILOCUS SEQUENCE TYPING OF *BRUCELLA* ISOLATES FROM THAILAND

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Abstract. Although brucellosis outbreaks in Thailand are rare, they cause abortions and infertility in animals, resulting in significant economic loss. Because *Brucella* spp display > 90% DNA homology, multilocus sequence typing (MLST) was employed to categorize local *Brucella* isolates into sequence types (STs) and to determine their genetic relatedness. *Brucella* samples were isolated from vaginal secretion of cows and goats, and from blood cultures of infected individuals. *Brucella* species were determined by multiplex PCR of eight loci, in addition to MLST based on partial DNA sequences of nine house-keeping genes. MLST analysis of 36 isolates revealed 78 distinct novel allele types and 34 novel STs, while two isolates possessed the known ST8. Sequence alignments identified polymorphic sites in each allele, ranging from 2-6%, while overall genetic diversity was 3.6%. MLST analysis of the 36 *Brucella* isolates classified them into three species, namely, *B. melitensis*, *B. abortus* and *B. suis*, in agreement with multiplex PCR results. Genetic relatedness among ST members of *B. melitensis* and *B. abortus* determined by eBURST program revealed ST2 as founder of *B. abortus* isolates and ST8 the founder of *B. melitensis* isolates. ST 36, 41 and 50 of Thai *Brucella* isolates were identified as single locus variants of clonal cluster (CC) 8, while the majority of STs were diverse. The genetic diversity and relatedness identified using MLST revealed hitherto unexpected diversity among Thai *Brucella* isolates. Genetic classification of isolates could reveal the route of brucellosis transmission among humans and farm animals and also reveal their relationship with other isolates in the region and other parts of the world.

Keywords: *Brucella* sp, multilocus sequence typing, multiplex PCR typing, phylogenetic tree, e-BURST, Thai isolates

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INTRODUCTION

Brucellosis is one of the most important zoonotic diseases that resulting in serious economic losses on animal farm and public health. It causes abortion in animals and causing acute febrile illness, undulant fever in humans, which

may progress to a more chronic form lead to severe debilitation (Nicola *et al,* 2008). In domestic animals, the disease occurs as a chronic infection that results in placentitis and abortion in pregnant females or orchitis and epididymitis in males (Corbel, 1997; Xavier *et al,* 2010). Human brucellosis is considered as a life-threatening debilitating disease characterized by weakness, fever, malaise, arthritis, osteomyelitis, endocarditis or meningoencephalitis (Christopher *et al,* 2010). The infection is widely distributed to the high endemic regions, such as the Mediterranean, the Middle East, China, Mongolia, Latin America and parts of Asia (Noutsios *et al,* 2012).

Brucella are gram-negative, facultative intracellular pathogens. The traditional classification of *Brucella* species is largely based on its preferable hosts, antigenic differences, phenotypic characteristics and minor basis of biochemical characteristics methods (Moreno *et al,* 2002; Banai and Corbel 2010). There are six classical *Brucella* species: *B. abortus* (bovine), *B. melitensis* (ovine and caprine), *B. suis* (porcine), *B. ovis* (ovine), *B. canis* (canine) and *B. neotomae* (desert wood rat). Three out of six species, *ie*, *B. melitensis*, *B. abortus* and *B. suis* represent a significant public health concern. In addition, there were *B. ceti* isolated from marine mammals, with cetaceans (dolphin, porpoise, and whale species) and *B. pinnipedialis*, with the various seal species as the preferred hosts. The recently identified novel *B. inopinata* isolated from a wound associated with infection of the implanted breast (Groussaud *et al,* 2007; De *et al,* 2008; Cloeckaert *et al,* 2011).

Multilocus sequence typing (MLST) has a number of advantages, *viz* high discriminatory power at species level over other types of molecular techniques, such as 16S rRNA phylogenetic markers, resolution of which sometimes is insufficient at the species level for some microbial populations (Glaeser and Kämpfer 2015). MLST technique involves PCR amplification followed by DNA sequencing of selected housekeeping genes. This approach has been applied broadly to microbial typing and epidemiological studies at both local and global levels of population structure and phylogenetic relationships (Enright and Spratt 1999, Urwin and Maiden, 2003). The first application of MLST for phylogenetic analysis of genus *Brucella* was published in 2007, and examined partial DNA sequences of the nine housekeeping genes from 160 isolates (Whatmore *et al*, 2007). Overall genetic diversity confirmed uniformity of this genus, which possesses only 1.5% polymorphic sites, representing 27 distinct sequence types (STs). Clustering data confirmed close vicinity of *B. canis* with *B. suis* biovar 3 and 4, and marked difference with *B. suis* biovar 5. The marine strains are tightly clustered. An extended MLST method was developed by amplifying and sequencing longer sequences, which allowed differentiation and genotyping of *Brucella* isolates (Chen *et al,* 2011). More recently, MLST was used to investigate etiology of human brucellosis incidence in three provinces of China (Chen *et al,* 2013).

Human brucellosis in Thailand has been considered as a rare disease, with the first case reported in 1970 (Visudhiphan and Na-Nakorn, 1970). No additional cases were found until in 2003, 38 cases of human brucellosis were reported, affirming that brucellosis is a re-emerging disease and is becoming a serious public health threat for Thailand (Manosuthi *et al,* 2004). Brucellosis in Thailand is an occupational infec-

tion associated with closed contact with infected animals. The majority of reported cases, from Kanchanaburi Province (Chuawong and Prasitpol, 2008), Nakhon Sawan Province (Tonghong, 2007) and Prachuap Khiri Khan Province (Tikunrum, 2008), were associated with *B. melitensis* from goat. Patients were either rural farmers in close contact with infected goat herd or those consuming unpasteurized goat dairy products. The majority of animal brucellosis cases were reported from Nakhon Si Thammarat and Kanchanaburi Provinces in the same period (Wongphruksasoong *et al*, 2012).

Brucella spp were characterized by DNA homology of > 90% identity among each species, based on DNA hybridization experiments, and thus the traditional view of *Brucella* taxonomy is that of a monospecies (Verger *et al,* 1985; O'Callaghan and Whatmore, 2011). However, in the past 20 years molecular typing has been developed to differentiate members of this genus and to understand their epidemiology (Whatmore, 2009).

Although brucellosis outbreaks in both humans and animals in Thailand during 2007-2008 were reported, there is no report on the genetic diversity of *Brucella* spp. This study was conducted to understand the genetic relatedness of isolates from humans and from animals where the outbreaks occurred. The genetic relationships among local *Brucella* isolates derived from human and animal origin were compared with isolates from other countries, based on available MLST strategy (Whatmore *et al,* 2007).Identification of *Brucella* isolates based on sequence types could be used to trace transmission routes and determine prevalence among humans and animals, which will benefit public health control and prevention.

MATERIALS AND METHODS

Sample collection

Twenty-one *Brucella* isolates from humans during 2005-2009 were obtained from the Medical Bacteriology Group, Department of Medical Science, National institute of Health, Thailand; and 27 *Brucella* strains were isolated from cattle and goat in farms located in six provinces of central Thailand, namely, Kanchanaburi, Nakhon Pathom, Nakhon Sawan, Prachuap Khiri Khan, Ratchaburi, and Saraburi. Four *Brucella* stock cultures from Microbiology and Immunology Department, Faculty of Tropical Medicine, Mahidol University, Bangkok were included.

Collection of specimens from farm animals was performed using a protocol approved by the Ethical Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University. Human isolates were obtained from the culture collection of the Medical Bacteriology Group, Department of Medical Science, National Institute of Health (NIH), Bangkok under a material transfer agreement. All of these strains were derived from human blood cultures from various Thai provinces, which had been sent to NIH for bacterial identification. Subjects were anonymized but source provinces were retained.

Brucella culturing

Brucella were isolated from vaginal swab and milk by culturing on Brucella agar [(trypticase soy agar with antibiotic supplement (BAS; Oxoid, Hampshire, UK) and 5% horse serum (Gibco, Gaitherberg, MD)] for 3 days at 37°C. Vaginal swab and milk samples also were cultured in Biphasic agar (Brucella agar slant overlayed with tryptic soy broth) for 3-4 days, and a number of bacterial films on agar slant were re-streaked on Brucella

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.

agar. All cultures were incubated for 3-4 days under 5% CO_2 atmosphere at 37°C. Single colony was preliminary screened as *Brucella* spp by determining for gram-negative cocco-bacilli with positive oxidase test. These putative *Brucella* strains were propagated on Brucella agar plate to obtain bacterial cells for subsequent DNA analysis. Each strain was kept in 15% glycerol stock at -70°C.

Multiplex PCR

Genomic DNA was extracted from bacterial cell pellet using a commercial genomic DNA extraction kit (Omega bio_tek, Gaitherberg, GA) stored at 4°C until used. Eight primer pairs PCR, described by López-Goñi *et al* (2008) were used (Table 1). The multiplex PCR was performed in a 50-µl mixture containing 25 µl of JumpStart REDtaq ReadyMix (Sigma, St Louis, MO), 1 µl of 8 pairs of primer (10 pmol/µl) (16 µl mixture), 3 µl of DNA template and distilled water to make a total volume of 50 µl. Thermocycling was performed in a Mastercycler Nexus instrument (Effpendorf, Upsala, Sweden) 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 step at 72°C for 7 minutes. Amplicons were analyzed by 1.5% agarose gel-electrophoresis and ethidium bromide staining.

MLST assay

MLST was based on nine genomic loci of *Brucella* spp using primers listed in Table 2 (Whatmore *et al*, 2007). PCR was prepared in 25-µl mixture containing 12.5 µl of JumpStart REDtaq ReadyMix (Sigma, St Louis, MO), 1 µl of a pair of primers

Table 2
Primers used in multilocus sequence typing of *Brucella* sp.

Gene / locus	Putative function		Primer sequence (5′-3′)	Length (bp)
gap	Glyceraldehyde 3-phosphate dehydrogenase	Forward	YGCCAAGCGCGTCATCGT	589
		Reverse	GCGGYTGGAGAAGCCCCA	
aroA	3-Phosphoshikimate1-carboxyvinyltransferase	Forward	GACCATCGACGTGCCGGG	565
		Reverse	YCATCAKGCCCATGAATTC	
glk	Glucokinase	Forward	TATGGAAMAGATCGGCGG	475
		Reverse	GGGCCTTGTCCTCGAAGG	
dnaK	Chaperone protein	Forward	CGTCTGGTCGAATATCTGG	470
		Reverse	GCGTTTCAATGCCGAGCGA	
gyrB	DNA gyrase B subunit	Forward	ATGATTTCATCCGATCAGGT	469
		Reverse	CTGTGCCGTTGCATTGTC	
trpE	Anthranilate synthase	Forward	GCGCGCMTGGTATGGCG	486
		Reverse	CKCSCCGCCATAGGCTTC	
cobQ	Cobyric acid synthase	Forward	GCGGGTTTCAAATGCTTGGA	422
		Reverse	GGCGTCAATCATGCCAGC	
omp25	25 kDa outer- membrane protein	Forward	ATGCGCACTCTTAAGTCTC	490
		Reverse	GCCSAGGATGTTGTCCGT	
int-hyp	Upstream and extreme 5′ of hypothetical protein (BruAb1_1395)	Forward	CAACTACTCTGTTGACCCGA	430
		Reverse	GCAGCATCATAGCGACGGA	

Y=C/T; K= G/T; M=A/C; S=G/C.

(10 pmol/µl), 3 µl of DNA template and distilled water to make a total volume of 25 µl**.** Thermocycling was performed as described above. Amplicons were analyzed as described above, and subjected to purification using Geneaid gel/PCR DNA fragment Kit (Geneaid Biotech, New Taipei City, Taiwan). Each purified PCR product was then inserted into plasmid vector pSC-A using Stratagene PCR Cloning Kit (Agilent Technologies; Stratagene Products Division, La Jolla, CA). Plasmid inserts were sequenced (1st Base, Singapore) using M13 forward and reverse primers of the cloning plasmid. Sequences were deposited with GenBank and accession numbers are listed in Table 4.

The raw sequence data of each allele of the *Brucella* isolates were edited with Demo-Sequencer software version 4.5 (http://www.genecodes.com/sequencher-feature). Comparison analysis of the isolate sequence with those available in MLST database (Whatmore *et al*, 2007), was performed using Mega 5 (Tamura *et al*, 2011). Distinct allele of each locus was assigned based on multiple alignments among other former allele member available in the database. Arbitrary numerical designation for unique allelic types from all nine loci was constructed and sequence type (ST) was then assigned. Allelic profiles and sequence data were also imported into the ST analysis and recombination test (START) package (Jolley *et al*, 2001) was employed to determine % GC content, and the degree of selection based on dN/dS

Table 3
Amplicon profiles of eight genes used in multiplex PCR identification of *Brucella* spp.

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

[a]Samples ID derived from human source: DMST9; animal source: Pra kogmilk, Kan Yim-V, Kan Yim-M.
[b]Samples ID derived from human source: DMST17, DMST4, DMST2, DMST10, DMST1, DMST3, DMST14, DMST11, DMST13, DMST15, DMST16, DMST19, DMST6; animal source: NakswS16, NakptE37swab, NakswS25, RatR-55, Sar29S, NakswS24, Sar29M, Nakpt F25milk, Nakpt L5swab, Nakpt E74swab, Sar43S, Rat R-13, Sar34S.
[c]Samples ID derived from human source:DMST8, DMST18, DMST21.
[d]Laboratory strains: B1, B2, B3. Gene identities and amplicon sizes are listed in Table 1.

(average frequencies of synonymous substitutions per potential synonymous site (d_S) and nonsynonymous substitutions per potential nonsynonymous site (d_N) was calculated by the method of Nei and Gojobori (1986). A phylogenetic tree was constructed using concatenated nucleotide sequences of all nine loci with MEGA 5 software, and percent bootstrap confidence level of internal branch was calculated from 500 resamplings of the original data.

RESULTS

Identification of *Brucella* sp by multiplex PCR

There were 52 *Brucella* isolates, 21 derived from humans, 24 from caprine, 3 from bovine and 4 from bacterial stock kept in the laboratory. Multiplex PCR based on eight pairs of primers of López-Goñi *et al* (2008) targeting 8 housekeeping genes revealed that 37 isolates were *B. melitensis* (15 from humans and 22 from caprine), 7 isolates of *B. abortus* (2 from humans, 2 from caprine and 3 from bovine) and the remaining 3 isolates of *B. suis* (from humans) (Table 3). The four laboratory strains had multiplex PCR profiles similar to *B. abortus* S-19 vaccine strain (Table 3). Complete sequences for all nine housekeeping genes were successful in only 36 isolates.

MLST of *Brucella* spp

Using MLST scheme of Whatmore *et al* (2007), nine loci of 36 Thai *Brucella* isolates were sequenced and their sequence

Table 4
Genes, allele type number and sequence types (ST) of *Brucella* species and strains based on multilocus sequence typing.

Gene/locus Species and strain	Host and source	ST	Allelic type profile /Gene bank accession number[a]								
			gap	*aroA*	*glk*	*dnaK*	*gyrB*	*trpE*	*cobQ*	*Omp25*	*Int-hyp*
B. abortus 544	Biovar1	1	2	1	1	2	1	3	1	1	1
	Not known		AM694191	AM694192	AM694193	AM694194	AM694195	AM694196	AM694197	AM694198	AM694199
B. abortus 5/93	Bovine	2	2	1	2	2	1	3	1	1	1
	UK		AM694290	AM694291	AM694292	AM694293	AM694294	AM694295	AM694296	AM694297	AM694298
B. abortus	Bovine	3	6	1	2	2	1	3	1	1	1
03/4923-239	Turkey		AM694335	AM694336	AM694337	AM694338	AM694339	AM694340	AM694341	AM694342	AM694343
B. abortus 870	Biovar6	4	2	1	2	2	2	3	1	1	1
	Not known		AM694344	AM694345	AM694346	AM694347	AM694348	AM694349	AM694350	AM694351	AM694352
B. abortus S19	Vaccine strain	5	2	1	1	2	1	4	1	1	1
			AM694353	AM694354	AM694355	AM694356	AM694357	AM694358	AM694359	AM694360	AM694361
B. abortus	Biovar3	6	5	7	10	7	6	3	7	1	1
Tulya	Not known		AM694371	AM694372	AM694373	AM694374	AM694375	AM694376	AM694377	AM694378	AM694379
B. melitensis	Rough strain	7	3	5	3	2	1	5	2	10	2
B115	Not known		AM694398	AM694399	AM694400	AM694401	AM694402	AM694403	AM694404	AM694405	AM694406
B. melitensis	Biovar2	8	3	2	3	2	1	5	3	8	2
63/9	Not known		AM694416	AM694417	AM694418	AM694419	AM694420	AM694421	AM694422	AM694423	AM694424
B. melitensis	Biovar3	9	3	2	3	2	1	5	3	9	2
Ether	Not known		AM694506	AM694507	AM694508	AM694509	AM694510	AM694511	AM694512	AM694513	AM694514
B. melitensis	Ibex	10	3	2	3	5	1	5	2	10	2
F12/01	UAE		AM694515	AM694516	AM694517	AM694518	AM694519	AM694520	AM694521	AM694522	AM694523
B. melitensis	Human	11	3	2	3	2	1	5	3	10	2
UK31/99	UK		AM694533	AM694534	AM694535	AM694536	AM694537	AM694538	AM694539	AM694540	AM694541
B. melitensis	Human	12	3	2	3	2	1	5	2	10	2
UK19/04	UK		AM694551	AM694552	AM694553	AM694554	AM694555	AM694556	AM694557	AM694558	AM694559
B. ovis REO	Not known	13	1	3	9	2	1	3	4	3	1
			AM694578	AM694579	AM694580	AM694581	AM694582	AM694583	AM694584	AM694585	AM694586
B. suis RT1	Equine	14	1	6	4	1	4	3	5	2	1
	Croatia		AM694740	AM694741	AM694742	AM694743	AM694744	AM694745	AM694746	AM694747	AM694748
B. suis 79/194	Hare	15	1	2	7	1	3	3	5	2	3
	Czechoslovakia		AM694812	AM694813	AM694814	AM694815	AM694816	AM694817	AM694818	AM694819	AM694820

B. suis RT19	Porcine France	16	4	2	7	1	3	3	5	2	3
			AM695010	AM695011	AM695012	AM695013	AM695014	AM695015	AM695016	AM695017	AM695018
B. suis 63/252	Caribou USA	17	1	6	4	1	5	3	5	2	4
			AM695073	AM695074	AM695075	AM695076	AM695077	AM695078	AM695079	AM695080	AM695081
B. suis 63/198	Reindeer Fmr USSR	18	1	6	4	1	5	3	5	2	5
			AM695100	AM695101	AM695102	AM695103	AM695104	AM695105	AM695106	AM695107	AM695108
B. suis 513	Not known	19	1	2	4	6	1	3	5	2	1
			AM695109	AM695110	AM695111	AM695112	AM695113	AM695114	AM695115	AM695116	AM695117
B. canis 79/92	Canine Germany	20	1	6	4	1	5	3	5	6	4
			AM695145	AM695146	AM695147	AM695148	AM695149	AM695150	AM695151	AM695152	AM695153
B. canis F7/05A	Canine South Africa	21	1	6	4	1	5	3	5	5	4
			AM695172	AM695173	AM695174	AM695175	AM695176	AM695177	AM695178	AM695179	AM695180
B. neotomae 65/196	Desert Wood Rat, USA	22	1	2	5	2	1	6	5	4	1
			AM695199	AM695200	AM695201	AM695202	AM695203	AM695204	AM695205	AM695206	AM695207
Brucella sp VLA04.72	Porpoise UK	23	1	4	8	4	1	2	5	2	1
			AM695361	AM695362	AM695363	AM695364	AM695365	AM695366	AM695367	AM695368	AM695369
Brucella sp 39/94	Common Seal, UK	24	1	2	6	2	1	2	5	2	1
			AM695397	AM695398	AM695399	AM695400	AM695401	AM695402	AM695403	AM695404	AM695405
Brucella sp 55/94	Otter UK	25	1	2	4	2	1	2	5	2	1
			AM695424	AM695425	AM695426	AM695427	AM695428	AM695429	AM695430	AM695431	AM695432
Brucella sp UK1/2000	Striped Dolphin,UK	26	1	2	4	2	1	2	6	7	1
			AM695559	AM695560	AM695561	AM695562	AM695563	AM695564	AM695565	AM695566	AM695567
Brucella sp F5/99	Bottlenosed Dolphin, USA	27	1	2	4	3	1	1	5	2	1
			AM695613	AM695614	AM695615	AM695616	AM695617	AM695618	AM695619	AM695620	AM695621
B. suis DMST8	Human Petchabun, Th	28	1	6	4	1	5	3	9	11	4
			KM196772	KM196664	KM196808	KM196736	KM196844	KM196952	KM196700	KM196916	KM196880
B. suis DMST18	Human Chanthaburi, Th	29	1	6	4	10	9	10	13	14	4
			KM196781	KM196673	KM196817	KM196745	KM196853	KM196961	KM196709	KM196925	KM196889
B. melitensis DMST17	Human Chanthaburi, Th	30	1	12	3	2	1	5	3	8	2
			KM196780	KM196672	KM196816	KM196744	KM196852	KM196960	KM196708	KM196924	KM196888
B. abortus Pra kog milk	Bovine Prachuap Khiri Khan, Th	31	2	1	2	2	1	5	1	1	1
			KM196791	KM196683	KM196827	KM196755	KM196863	KM196971	KM196719	KM196935	KM196899
Brucella sp *Brucella* sp B2	Lab stock, Bangkok, Th	32	2	1	2	11	1	4	15	1	1
			KM196785	KM196677	KM196821	KM196749	KM196857	KM196965	KM196713	KM196929	KM196893
Brucella sp B3	Lab stock, Bangkok, Th	33	2	1	14	2	1	4	16	1	1
			KM196786	KM196678	KM196822	KM196750	KM196858	KM196966	KM196714	KM196930	KM196894

Table 4 (Continued).

Gene/locus Species and strain	Host and source	ST	Allelic type profile /Gene bank accession number[a]								
			gap	*aroA*	*glk*	*dnaK*	*gyrB*	*trpE*	*cobQ*	*Omp25*	*Int-hyp*
Brucella sp B1	Lab stock, Bangkok, Th	34	2 KM196784	13 KM196676	2 KM196820	2 KM196748	1 KM196856	4 KM196964	1 KM196712	16 KM196928	1 KM196892
B. melitensis DMST 4	Human Chainat, Th	35	3 KM196770	2 KM196662	3 KM196806	2 KM196734	1 KM196842	4 KM196950	3 KM196698	8 KM196914	6 KM196878
B. melitensis DMST 2	Human Samut Prakan, Th	36	3 KM196768	2 KM196660	3 KM196804	2 KM196732	1 KM196840	7 KM196948	3 KM196696	8 KM196912	2 KM196876
DMST 10	Human Sa Kaew, Th	37	3 KM196774	2 KM196666	3 KM196810	2 KM196738	1 KM196846	8 KM196954	11 KM196702	12 KM196918	2 KM196882
B. melitensis Naksw S16	Caprine Nakhon Sawan, Th	38	3 KM196793	2 KM196685	3 KM196829	2 KM196757	1 KM196865	11 KM196973	3 KM196721	18 KM196937	2 KM196901
B. melitensis Nakpt E37swab	Caprine Nakhon Pathom, Th	39	3 KM196801	2 KM196693	3 KM196837	2 KM196765	1 KM196873	15 KM196981	3 KM196729	21 KM196945	2 KM196909
B. melitensis DMST 1	Human Chonburi, Th	40	3 KM196767	2 KM196659	3 KM196803	2 KM196731	7 KM196839	3 KM196947	8 KM196695	8 KM196911	2 KM196875
B. melitensis DMST 3	Human Chaiyaphum, Th	41	3 KM196769	2 KM196661	3 KM196805	8 KM196733	1 KM196841	5 KM196949	3 KM196697	8 KM196913	2 KM196877
B. melitensis DMST 14	Human Chanthaburi, Th	42	3 KM196777	2 KM196669	3 KM196813	9 KM196741	1 KM196849	5 KM196957	12 KM196705	8 KM196921	2 KM196885
B. melitensis Naksw S25	Caprine Nakhon Sawan, Th	43	3 KM196794	2 KM196686	3 KM196830	12 KM196758	1 KM196866	12 KM196974	3 KM196722	8 KM196938	8 KM196902
B. melitensis Rat R-55	Caprine Ratchaburi, Th	44	3 KM196795	2 KM196687	3 KM196831	13 KM196759	1 KM196867	5 KM196975	1 KM196723	19 KM196939	9 KM196903
B. melitensis Sar 29S	Caprine Saraburi, Th	45	3 KM196788	2 KM196680	15 KM196824	2 KM196752	1 KM196860	5 KM196968	3 KM196716	17 KM196932	2 KM196896
B. melitensis Naksw S24	Caprine Nakhon Sawan, Th	46	3 KM196792	2 KM196684	16 KM196828	2 KM196756	12 KM196864	5 KM196972	3 KM196720	8 KM196936	2 KM196900
B. melitensis DMST 11	Human Uttaradit, Th	47	3 KM196775	8 KM196667	11 KM196811	2 KM196739	1 KM196847	9 KM196955	3 KM196703	13 KM196919	2 KM196883
B. melitensis DMST 13	Human Uttaradit, Th	48	3 KM196776	9 KM196668	12 KM196812	2 KM196740	1 KM196848	5 KM196956	3 KM196704	8 KM196920	2 KM196884

B. melitensis	Human	49	3	10	3	2	8	5	3	8	7
DMST 15	Kanchanaburi, Th		KM196778	KM196670	KM196814	KM196742	KM196850	KM196958	KM196706	KM196922	KM196886
B. melitensis	Human	50	3	11	3	2	1	5	3	8	2
DMST 16	Kanchanaburi, Th		KM196779	KM196671	KM196815	KM196743	KM196851	KM196959	KM196707	KM196923	KM196887
B. melitensis	Caprine	51	3	14	3	2	10	5	3	8	2
Sar 29M	Saraburi, Th		KM196787	KM196679	KM196823	KM196751	KM196859	KM196967	KM196715	KM196931	KM196895
B. melitensis	Caprine	52	3	16	3	2	15	5	19	8	2
Nakpt F25milk	Nakhon Pathom, Th		KM196799	KM196691	KM196835	KM196763	KM196871	KM196979	KM196727	KM196943	KM196907
B. melitensis	Caprine	53	3	17	3	2	1	14	3	8	2
Nakpt L5swab	Nakhon Pathom, Th		KM196800	KM196692	KM196836	KM196764	KM196872	KM196980	KM196728	KM196944	KM196908
B. melitensis	Caprine	54	3	18	3	2	1	5	20	8	2
Nakpt E74swab	Nakhon Pathom, Th		KM196802	KM196694	KM196838	KM196766	KM196874	KM196982	KM196730	KM196946	KM196910
B. abortus	Human	55	7	1	2	2	1	3	10	1	1
DMST 9	Chanthaburi, Th		KM196773	KM196665	KM196809	KM196737	KM196845	KM196953	KM196701	KM196917	KM196881
B. melitensis	Human	56	8	6	13	2	1	5	3	8	2
DMST 19	Sa Kaew, Th		KM196782	KM196674	KM196818	KM196746	KM196854	KM196962	KM196710	KM196926	KM196890
B. suis	Human	57	9	2	4	1	1	3	14	15	4
DMST 21	Nakhon Phanom, Th		KM196783	KM196675	KM196819	KM196747	KM196855	KM196963	KM196711	KM196927	KM196891
B. melitensis	Caprine	58	10	2	3	2KM196754	11KM196862	5KM196970	KM196718	KM196934	KM196898
Sar 43S	Saraburi, Th		KM196790	KM196682	KM196826						
B. melitensis	Caprine	59	11	2	3	14	13	5	3	8	2
Rat R-13	Ratchaburi, Th		KM196796	KM196688	KM196832	KM196760	KM196868	KM196976	KM196724	KM196940	KM196904
B. abortus	Bovine	60	12	1	2	15	1	3	1	1	1
Kan Yim-V	Kanchanaburi, Th		KM196797	KM196689	KM196833	KM196761	KM196869	KM196977	KM196725	KM196941	KM196905
B. abortus	Bovine	61	13	15	2	2	14	13	18	20	1
Kan Yim-M	Kanchanaburi, Th		KM196798	KM196690	KM196834	KM196762	KM196870	KM196978	KM196726	KM196942	KM196906
B. melitensis	Human	8	3	2	3	2	1	5	3	8	2
DMST 6	Sa Kaew, Th		KM196771	KM196663	KM196807	KM196735	KM196843	KM196951	KM196699	KM196915	KM196879
B. melitensis	Caprine	8	3	2	3	2	1	5	3	8	2
Sar 34S	Saraburi, Th		KM196789	KM196681	KM196825	KM196753	KM196861	KM196969	KM196717	KM196933	KM196897

[a]Upper number refers to allele profile and lower number to GenBank accession number. Gene/locus is identified in Table 2. ST1 – ST 17 are based on Whatmore *et al* (2007) classification and ST 28 - ST61 are from this study. Th, Thailand.

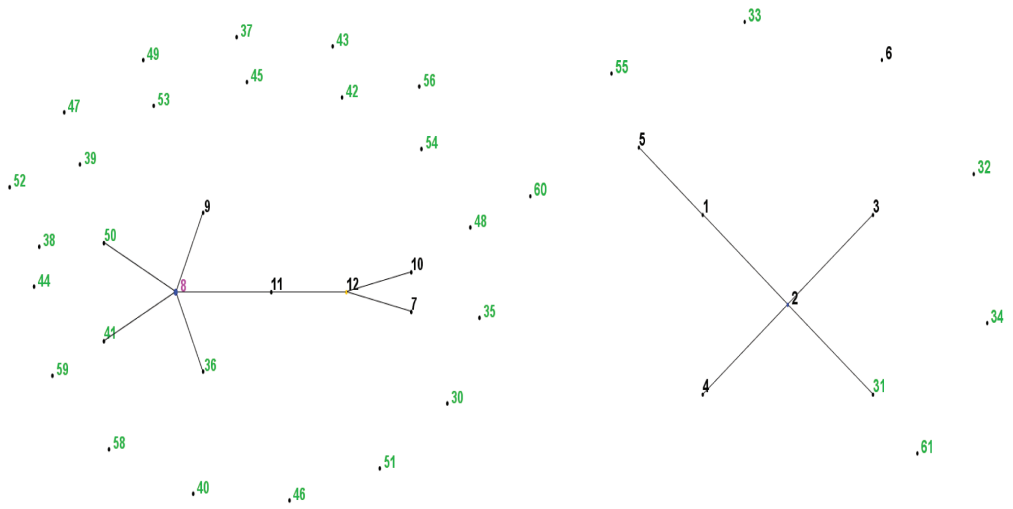

Fig 1–Population snapshot of *Brucella* ST profiles using comparative eBRUST. A) *B. melitensis* clonal complex (CC) 8 having ST8 as founder. B) *B. abortus* CC2 having ST2 as founder. Black and green letter indicates strains from dataset of Whatmore *et al* (2007) and this study, respectively.

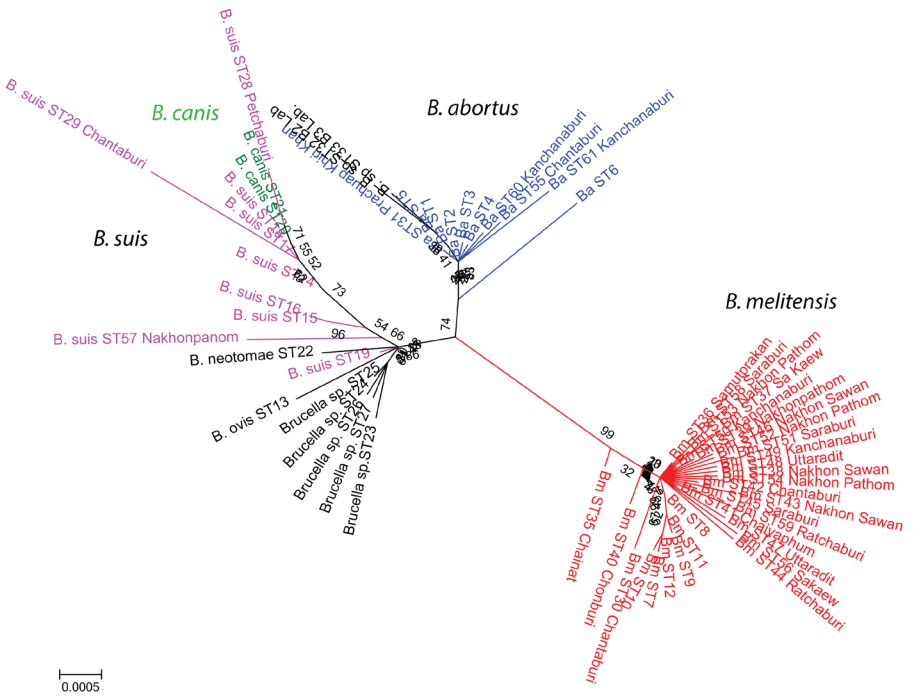

Fig 2–Phylogenetic tree of *Brucella* spp. The phylogenetic tree was inferred using the Maximum Likelihood method based on Tamura 3-parameter model. Analysis involved 61 nucleotide sequences with a total of 4,396 positions in the final dataset. Number represents percent similarity.

Table 5
Genetic diversity and dN/dS ratio of nine employed in multilocus typing of Thai *Brucella* isolates

Locus	Number of alleles	Number of polymorphic site (%)	dN	dS	dN/dS	Mean percent GC content
gap	13	16 (2.7)	0.0028	0.0142	0.1972	58
aroA	18	21 (3.7)	0.0039	0.006	0.6623	62
glk	16	17 (3.6)	0.0071	0.0064	1.1066	63
dnaK	15	15 (3.2)	0.0038	0.0048	0.7791	61
gyrB	15	16 (3.4)	0.005	0.0084	0.5959	59
trpE	15	14 (2.9)	0.0043	0.0066	0.6557	58
cobQ	20	26 (6.1)	0.0085	0.0135	0.6313	59
omp25	21	24 (4.9)	0.0049	0.0192	0.2572	59
int-hyp	9	10 (2.3)	0.0077	0.0089	0.8582	56

dN, mean non-synonymous substitution per site; dS, mean synonymous substitution per site.

types were assigned (Table 4). Twenty-seven STs are known STs, but 34 isolates had a total of 34 novel STs (assigned ST28 - ST61). The number of allele types identified in *gap, aroA, glk, dnak, gyrB, trpE, cobQ, omp25*, and *int-hyp* was 13, 18, 16, 15, 15, 15, 20, 21 and 9, respectively. Overall, there were 159/4,396 (3.6%) polymorphic nucleotide sites among the nine loci. The dN/dS ratio for all 7 housekeeping genes was <1, indicating that the genes are under stabilizing selection except for *glk* (dN/dS = 1.1066) (Table 5). GC content of the various loci ranged from 56% (*int-hyp*) to 63% (*glk*) (Table 5) in comparison to overall genomic GC content of approximately 57.0% (Whatmore *et al*, 2007).

Population genetics of *B. melitensis* and *B. abortus*

An eBURST diagram was drawn to determine the evolutionary relationship among isolates of *B. melitensis*, *B. abortus* and reference strains. Population snapshot of *B. melitensis* ($n = 32$) in comparison with reference *B. melitensis* strains indicated that clonal complex 8 (CC8) has ST8 as founder (Fig 1A), and 5 single-locus variants (SLVs), namely ST9, 11, 41, 36 and 50 (Table 6). One SLV of the founder (ST11) has diversified to produce a double-locus variant (DLV), ST12, which has become subgroup founders of ST7 and 10. The size of the circles shows that the ST8 founder is also the most prevalent ST in this group (Fig 1A). The new STs found in our study were present as singletons ($n = 21$). In *B. abortus* population, a clonal complex 2 (CC2) was found (Fig 1B**)**.

B. abortus CC2 has four SLVs: ST1, 3, 4 from reference strains and ST31 from this study (Table 6). ST5 is a DLV and the remaining 7 (ST6, 32, 33, 34, 55, 60 and 61) are singletons. Population analysis of the other *Brucella* spp could not be performed due to the low numbers of isolates.

Assessment of genetic recombination and mutation events

Recombination or mutation event within *B. melitensis* and *B. abortus* populations were estimated to understand diversification of the bacteria by selecting clusters of isolates that have identical al-

Table 6
Allele profile among single locus variant members of clonal complex 8 (CC8) derived from *Brucella melitensis* isolates and clonal complex 2 (CC2) derived from *B. abortus* isolates.

	Location	Cluster member	ST type	Locus								
				gap	*aroA*	*glk*	*dnaK*	*gyrB*	*trpE*	*cobQ*	*omp25*	*int-hyp*
CC8												
B. melitensis (strains)												
63/9	Not known	Founder	8	3	2	3	2	1	5	3	8	2
Ether	Not known	SLV	9	3	2	3	2	1	5	3	9	2
UK31/99	UK	SLV	11	3	2	3	2	1	5	3	10	2
DMST2	Samut Prakan, Th	SLV	36	3	2	3	2	1	7	3	8	2
DMST3	Chaiyaphum, Th	SLV	41	3	2	3	8	1	5	3	8	2
DMST16	Kanchanaburi, Th	SLV	50	3	11	3	2	1	5	3	8	2
CC2												
B. abortus	UK	Founder	2	2	1	2	2	1	3	1	1	1
544	Not known	SLV	1	2	1	1	2	1	3	1	1	1
03/4923-239	Turkey	SLV	3	6	1	2	2	1	3	1	1	1
870	Not known	SLV	4	2	1	2	2	2	3	1	1	1
Pra kog milk	Prachuap Khiri Khan, Th	SLV	31	2	1	2	2	1	5	1	1	1

Th, Thailand.

Table 7
Genetic variation among single locus variant members of *Brucella melitensis* clonal complex 8 (CC8) and *B. abortus* clonal complex 2 (CC2).

B. melitensis CC8 (ST8 as founder)			Mutation	*B. abortus* CC2 (ST2 as founder)		Mutation
ST, locus, allele number				ST, locus, allele number		
Aligned position	259	399		Aligned position	70	
ST8, *omp25*, allele 8	C	C		ST2, *glk*, allele 2	G	
ST9, *omp25*, allele 9	T	T	two point mutations	ST1, *glk*, allele 1	T	single mutation
ST11, *omp25*, allele10	T	-	single mutation			
Aligned position	310			Aligned position	431	
ST8, *trpE*, allele 5	T			ST2, *gap*, allele 2	G	
ST36, *trpE*, allele 7	C		single mutation	ST3, *gap*, allele 6	T	single mutation
Aligned position	231			Aligned position	67	
ST8, *dnaK*, allele 2	G			ST2, *gyrB*, allele 1	C	
ST41, *dnaK*, allele 8	A		single mutation	ST4, *gyrB*, allele 2	A	single mutation
Aligned position	14			Aligned position	480	
ST8, *aroA*, allele 2	T			ST2, *trpE*, allele 3	A	
ST50, *aroA*, allele 11	C		single mutation	ST31, *trpE*, allele 5	C	single mutation

lelic profiles and identifying single locus variants that differ from founder profile (Feil *et al,* 2000). Sequences of non-identical alleles in all single locus MLST variants with their clonal founders were compared, and multiple nucleotide changes (>1) are assumed to be caused by recombination while single nucleotide differences, not found elsewhere in the database, are assumed to be due to *de novo* mutation. For *B. melitensis* CC8, *omp*25 locus demonstrated two positions of nucleotide change in SLV-ST9 when compared with founder ST8 (Table 7), suggesting a recombination event. SLV-ST11 had only a single mutation at this locus. For *trpE*, *dnaK* and *aroA* loci, each contained a single mutation at SLV- ST36, SLV- ST41 and SLV-ST50, respectively (Table 7). In *B. abortus* CC2, *glk*, *gap* and *gyrB* showed single nucleotide changes in SLV-ST1, SLV-ST3 and SLVs-ST4, respectively (Table 7).

Phylogenetic tree of strains with various STs

The 4,396 bp concatenated sequences of the nine loci (*gap-aroA-dnaK-gyrB-trpE-cobQ-omp25-int-hyp*) were used to construct a phylogenetic tree, based on a maximum likelihood algorithm, to examine the relationship among the STs. The phylogenetic tree of all *Brucella* isolates demonstrated that they belonged to three major groups, namely that of *B. melitensis*, *B. abortus* and *B. suis*/*B. canis*/*B. neotomae*/*Brucella* of unknown species (Fig 2). Among the 36 human and animal isolates in this study, species of which were predicted by multiplex PCR and each relevant species located in clusters generated from reference strains of known *Brucella* spp, the phylogenetic tree revealed that (i) Thai strains belonging to *B. melitensis* were individually divergent, (ii) laboratory strains, B1, B2 and B3 clustered with *B. abortus*, and (iii) isolate ST28_DMST8 was closely related to *B. canis* branch.

DISCUSSION

In Thailand, MLST method has not previously been used for genotyping *Brucella* isolates. Fewer than 10% of cases of human brucellosis was reported, mostly because of misleading clinical picture (Visudhiphan and Na-Nakorn, 1970; Laosiritaworn *et al,* 2007). MLST analysis will be helpful to gain more understanding genetic relationships and epidemiology among *Brucella* isolates in Thailand. To this end, genetic relatedness of nine target genes were determined based on the available MLST strategy for conclusive speciation among 36 *Brucella* isolates from humans and animals, of which 24 isolates were identified as *B. melitensis*, 7 as *B. abortus* and 3 as *B. suis*.

The high prevalence of *B. melitensis* detected in this study was in agreement with that in the brucellosis outbreak in Thailand during 2007-2008 (Danprachankul *et al,* 2009). In this current study of 2012-2014, animal brucellosis was found in five provinces, namely, Kanchanaburi, Nakhon Pathom, Nakhon Sawan, Ratchaburi and Saraburi. Two provinces, Kanchanaburi and Nakhon Sawan, are non-southern provinces that had the most recent (2007-2008) outbreak of human and/or caprine brucellosis (Danprachankul *et al,* 2009). Twenty-four new STs were identified among the isolates of *B. melitensis* from our study, while 1 known STs (ST8) was identified, the same as those in sheep from China, which revealed the majority of *B. melitensis* as ST 8, followed by ST7 (Ma *et al,* 2016). *B. abortus* was revealed as ST5, while *B. suis* as ST14. MLST analysis of *B. abortus* in India revealed 21 field-isolated strains as ST1, one field isolate as ST7 and another as ST8 (San-

karasubramanian *et al*, 2016). As report by Whatmore *et al* (2007), the majority of *B. suis* isolates belong to ST14, 15 and 16 and a few strains to ST18 and 19, while 3 *B. suis* strains in Thailand, DMST8, 18 and 21, were included in new ST types of 28, 29 and 57, respectively. Thus, in our study, taken together, more new *Brucella* STs were revealed, indicating the diversity of isolates in Thailand. *Brucella* strains in each area received different environmental stimulators that could possibly influence their genetic profiles, so isolates from Thailand that has no exchange to any other isolates, became different from foreign isolates.

An eBURST diagram was constructed to reveal the population structure of *B. melitensis* and *B. abortus*. For the former, the population consisted of three clonal complexes with ST8, ST12 and ST17 as founders. *B. melitensis* strains of ST8 were found previously in both Asia (India and Mongolia) and Europe Kosovo, Greece, Cyprus, and the United Kingdom (Whatmore *et al*, 2007), and also been identified in Thailand. It is possible that these *Brucella* strains may somehow be transmitted among these countries, especially among those within close geographical proximity to Thailand such as Mongolia, where there is a high incidence of brucellosis (Zhang *et al*, 2010). ST members that behave as SLV in each clonal complex have been analyzed for recombination, substitutions and point mutation. For SLV members of CC2, each has a single nucleotide substitution in one locus, thus making each SLV diverse from their founder. As for SLV members of CC8, three have single mutations and one member has a recombination. The existence a clonal population structure was supported by sequence alignment of SLV members, in comparison to their founder, which showed that the majority has diverged. Furthermore, other unrelated STs, both of reference STs and new STs, were individually distributed along the eBURST diagram. These observations indicate that the population structure of *Brucella* isolates in Thailand are diverse.

In the case of *glk*, its dN/dS ratio was 1.1066, indicating that this gene was subjected to positive selection. It is possible that *glk* (encoding glucokinase) might be involved with pathogenic potential for stimulation of infection. Glucokinase could play a key role in bacteria survival, which might help to account for its positive selection. The six remaining loci encoding housekeeping genes (*gap, aroA, dnaK, gyrB, trpE*, and *cobQ*) were under neutral selection.

In conclusion, in this study we have expanded existing knowledge of *Brucella* population in Thailand. The allelic profile and ST information have enlarged the central database for MLST of *Brucella* spp. The data should be of particular use for molecular typing, evolutionary biology and global epidemiology of *Brucella* in our country and the Southeast Asian region. This would allow improvement in the current management strategies to control brucellosis.

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