https://scilett-fsg.uitm.edu.my/
Vol. 17(1) JANUARY 2023

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v17i1.19029

Phylogenetic Analysis and Comparative Genomics of *Brucella melitensis* in Human Brucellosis: A Review

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Accepted: 10 December 2022; Published: 21 January 2023

ABSTRACT

Brucellosis is a significant zoonotic disease caused by certain species of *Brucella* and has a wide range of clinical severity in humans. *Brucella melitensis* is the most virulent human species associated with acute and chronic *Brucella* disease. The genome of *B. melitensis* is remarkably conserved, with less than 3% variations between strains from diverse geographical locations. Hence, whole genome sequencing (WGS) analysis has provided broad information on genomic variations of this species and to understand the epidemiology and geographical origin among different strains. This review paper discussed the availability of WGS data could allow for the screening of multiple genomes, identify the SNP variations and detect the emergence of novel virulence genes that could be associated with pathogenicity mechanisms among *B. melitensis*. The phylogenomic analysis allows the clustering of known strains into five clades, which corresponds well with clinical and geographical features. Recent methods such as core genome multi-locus sequence typing (cgMLS) and multi-locus variable-number tandem-repeat analysis (MLVA) have allowed the study of large epidemiological datasets with high resolution. As *Brucella* genomes are highly conserved at the species, comparative genomic analysis has provided high-quality information and allowed for high-resolution analysis of the phylogenomic relationship of the species. The current review presents an overview of highlights emergence of novel virulence genes and the evolutionary path of *B. melitensis* in phylogenomic analysis, which allow us to conduct trace-back studies and correlate genotypes with the geographical origin of the strains.

Keywords: *Brucellosis, Brucella melitensis, whole genome sequencing, phylogenetic, multiple-locus sequence typing*

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Vol. 17(1) JANUARY 2023

ISSN: 1675-7785
eISSN: 2682-8626

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INTRODUCTION

Brucellosis is a significant zoonotic disease affecting humans and animals and is responsible for substantial economic losses of livestock in many areas of the world. The incidence rate for human brucellosis reported by World Health Organization (WHO) is an estimated 500,000 new cases annually [1]. The disease is transmitted to humans by direct contact with infected animals or by consumption of unpasteurised infected dairy products [2, 3]. The bacteria cause brucellosis from the genus *Brucella* [4], of which four species, i.e. *Brucella abortus, Brucella melitensis, Brucella suis* and *Brucella canis*, are significant in both animal and human infections. Among these species, *B. melitensis* is considered the most virulent in humans and is associated with acute and chronic brucellosis, which may progress to more severe symptoms such as endocarditis [5].

B. melitensis is a Gram-negative bacterium and facultative intracellular pathogen associated with natural animal reservoirs such as sheep and goats [6]. In humans, *B. melitensis* infection causes many clinical diseases in children and adults, typically presenting symptoms with arthralgia, pyrexia (undulant fever) and fatigue. A retrospective study from Turkey found that almost 70% of human brucellosis cases were between ages 13 to 44, and arthralgia (73.7% of cases) was the most common reported symptoms, followed by pyrexia (72.2%) and fatigue (71.2%) [7]. However, some infected patients may be asymptomatic or have a mild and self-limited febrile illness. In addition, *B. melitensis* has a shallow infectious dose of 10 to 100 bacteria cells. Thus, humans are easily infected with this species via the respiratory tract or mucosal route by the aerosol-carrying *B. melitensis*. Brucellosis is associated with occupational exposure groups, including veterinarians, veterinary assistants, farmers, abattoirs, hunters, and laboratory workers who handle live cultures of *B. melitensis* [8]. In a review by Pereira et al. [8], laboratory-acquired brucellosis was reported in 183 individuals over the past 20 years. The majority was from exposure to *B. melitensis*.

The *Brucella* genome has more than 90% genetic similarity across species and is highly conserved at the genus level and all major species of *Brucella* [3, 4, 9]. Despite the high genetic similarity among the various *Brucella* spp. several approaches like multiple locus sequence typing (MLST), multiple-locus variable-number tandem-repeat (MLVA) and whole genome sequencing (WGS) have been successfully used to analyse phylogenetic patterns and their association with the geographic origin of the host. A large number of whole genome sequencing data have been generated on *Brucella* spp. in the last decade. As of July 2022, a total of 1,055 genome assemblies of *Brucella* spp. and 388 genome assemblies of *B. melitensis* were available in NCBI. Following the availability of increasing the WGS of *B. melitensis* genomes generated recently, we have reviewed comparative genomic analysis has provided high-quality information on the emergence of novel virulence genes, presence of antimicrobial genes (AMR) and variation genes and allowed for high-resolution analysis of the phylogenomic relationship of the species.

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Genome Sequence of Brucella melitensis

The genome sequence of the reference strain *B. melitensis* 16M was first described by DelVecchio et al. [10] with the genetic material contained in two circular chromosomes of 2.11 Mbp (chromosome I) and 1.18 Mbp (chromosome II), as shown in Figure 1. Chromosome I encodes genes involved in core metabolic processes such as transcription, translation and protein synthesis. In contrast, chromosome II carries the genes involved in membrane transport, regulation and energy metabolism [3]. In addition, both chromosomes share similar GC content and have the same housekeeping gene distribution pattern [10, 11].

The genomic features of *B. melitensis* reported from different geographical locations are similar. For instance, Karthik et al. [12] reported the genome of *B. melitensis* from 17 Indian strains to be approximately 3.2 Mbp in size with 57.24% GC content. Similar findings among 21 *B. melitensis* strains from Austrian Human Brucellosis and four strains of *B. melitensis* were isolated from patients of Brucellosis in Malaysia with approximately 3.2 – 3.3 Mbp genome size and GC content of 57.2 [13, 14]. Several studies showed larger differences in the number of protein-coding genes from annotated *B. melitensis* strains. Tan et al. [15] reported 3355 and 3408 protein-coding genes as compared to a study from Karthik [12] reported 2632 protein-coding genes in the genomes of local *B. melitensis* strains

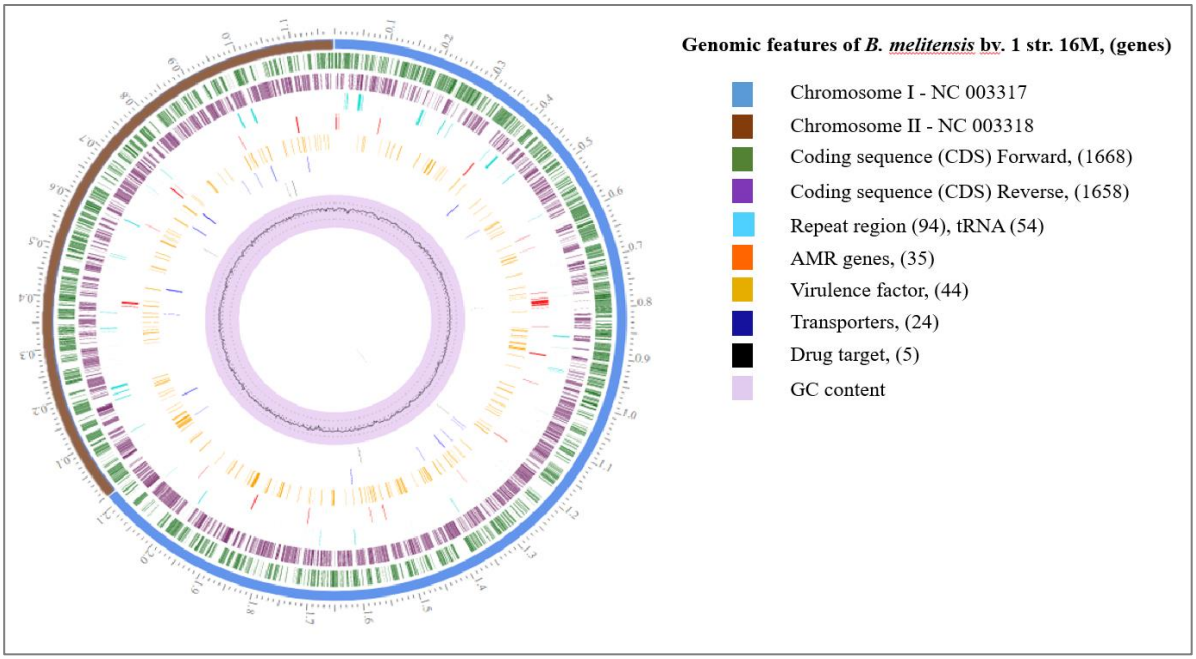

Figure 1: Circular alignment representation of *B. melitensis* strain 16M genomes (generated using PATRIC)

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Virulence factor genes

One exciting aspect of *B. melitensis* is the high 97% similarity at the genome level. Thus, the variability of this genome is found only in 3% of their content compared to other Gram-negative bacteria, such as *Salmonella enterica* serovar *Typhimurium*, which can vary from 2% to 20% of the genome size [9, 16]. *B. melitensis* does not possess many classical virulence factors such as capsule, exotoxin, cytolysin, pili, or fimbriae [10]. Instead, genetic determinants for pathogenicity in *B. melitensis* are mainly genes involved in intracellular survival, lipopolysaccharide (LPS) synthesis, secretion system, iron uptake and motility/flagellar genes [3].

Many *B. melitensis* genes have been identified as potential virulence factors using WGS technology to search virulence factor databases and provide information on how these genes differ between strains. Major virulence factors, such as the *virB* operon gene from the type IV secretory system, play an essential role in intracellular survival by adhering to the strains to the host cell and cell entry, as in Table 1. The type IV secretory system has been reported in 43 strains of *B. melitensis* in Egypt [17]. Similar findings from studies of comparative genomics of Indian isolates of *B. melitensis* strains indicate the presence of predicted virulence genes, including *virB* genes, *BvrR*/*BvrS*, and LPS genes in all strains except strain 2007BM-1, which lacks the *ricA* and *wbkA* genes, whilst studies from complete genome sequencing in clinical *B. melitensis* strains isolated in Russia revealed *virB* genes and LPS group had high-level expression [12, 18].

The genomic data of *B. melitensis* generated in the past 20 years have increased the understanding of the genetic variation of virulence factors that are important in humans and animals. However, understanding virulence factor and pathogenicity mechanism among *B. melitensis* or other species of *Brucella* need more emphasise since there is still a gap in this field. Analysing whole genomic sequencing needs complete metadata with genomic information such as clinical data regarding disease severity, treatment failures on demographics and information on relapse patients.

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Table 1: The virulence factor genes involved in the reference strain of *B. melitensis* 16M from the virulence factor database (VFDB)

Classification of virulence factor	Genes	Total genes
Intracellular survival	*rickA, cgs*	2
Intracellular survival, Intracellular entry, Immuno-modulatory activity	*wbkB, wbkC. Pgm, wzm, wzt, pmm, manAoAg, gmd, manCoAg, per, wbkA*	11
Secretion system, Type IV secretion system, Intracellular survival and replication	*virB3, virB1, virB8, virB5, virB7, virB9, virB10, virB11, virB4, virB6, virB2*	11
Immune evasion, WXXXE motif protein	*btpA, btpB*	2
Miscellaneous	*wbpZ, lpsA, wboA, lpxE, lpsB/lpcC,wbpL, fabZ, lpxC, htrB, kdsB, lpxD. wbdA, acpXL, virB1, kdsA, lpxB, lpxA, acpXL*	18

Antimicrobial resistance genes

Due to the highly infectious nature of *B. melitensis* strains, it is important to have an adequate and prompt treatment plan to manage human brucellosis. The recommended antibiotic treatment by WHO, a combination of doxycycline with either rifampicin or streptomycin for a minimum of 21 days, is still being used today. *Brucella* species show susceptibility to a broad range of antimicrobial agents. However, several studies have described resistance or intermediate resistance to rifampicin and trimethoprim/sulfamethoxazole in areas where these antibiotics are commonly used [19]. A review study from Wareth et al. [20] reported that more than half of the studies (22/40) among human brucellosis in the Middle East and North African countries were resistant to rifampicin. Similar findings were reported from a study in Malaysia, where 11 (26%) out of the 41 *Brucella* isolates displayed intermediate resistance or resistance to rifampicin [21]. Rifampicin is also used to treat other bacterial infections, such as *Staphylococcus epidermidis*; however, a study from Malaysia reported 16.7% found resistance to rifampicin from clinical samples of the Coagulase-Negative Staphylococci (CoNS) samples [22].

Generally, antibiotic susceptibility testing of AMR in *B. melitensis* using live cultures is rarely used for diagnosis or surveillance due to the highly contagious nature of the strains and the requirement for BSL3 laboratory facilities. A Study from Schaeffer et.al [14] used a genetic approach, i.e. detection of the *mprf* gene conferring resistance to defensins in *B. suis* from WGS data. The *mprF* gene is an integral membrane protein that modifies the membrane surface and acts

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as a defence system in most pathogenic *Brucella* spp. including the reference strain *B. melitensis* 16M. Antibiotic resistance genes in the genome of the 16M strain can be screened by comparing genomes sequences with known AMR genes in curated databases such as the Comprehensive Antibiotic Resistance Database (CARD), as in Table 2.

In addition, studies from the comparative analysis of AMR among *B. melitensis* strains in Norwegian have reported resistance to rifampicin (*rpoB*), trimethoprim (*folA*) and fluoroquinolone (*gyrA*, *gyrB*, *parC* and *parE*) genes [19]. Therefore, using WGS data to screen for the presence of AMR genes may be a convenient alternative, although it is more definitive than in vitro antimicrobial susceptibility testing.

Table 2: Prediction of antimicrobial resistance gene (AMR) in *B. melitensis* strains using comprehensive antibiotic resistance database (CARD)

Strain	ARO term	Resistance mechanism	Gene	AMR Gene Family	Drug class
16M and MY1483/09	adeF	antibiotic efflux	*gyrA*	resistance-nodulation-cell division (RND) antibiotic efflux pump	a fluoroquinolone antibiotic, tetracycline antibiotic
	B. suis mprF	antibiotic target alteration	*mprF*	defensin resistant mprF	Peptide antibiotic

Genome phylogeny analysis of B. melitensis

Multi-locusMulti-locus variable-number tandem-repeat analysis (MLVA), multi-locus sequence typing (MLST) and Pulsed-Field Gel Electrophoresis (PFGE) are methods commonly used for molecular typing and genotyping *Brucella* spp. These approaches were developed mainly for epidemiological studies in outbreak investigations of human and animal brucellosis. However, these molecular typing techniques must produce sufficient information and are not completely discriminatory between *Brucella* spp. strains since the genetic analysis do not cover the bacteria's entire genome. Subsequently, several numbers of PCR- based typing methods such as AMOS-PCR, Bruce-ladder and variable number tandem repeats (VNTR) were developed for identifying and genotype *B. melitensis* and have been used until today [23-25]. Over the past decade, whole genome sequencing (WGS) analysis has been used widely for entire sequencing genomes of *Brucella* strains.

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The first study of whole genome-based phylogeny and divergence of *Brucella* spp. was described by Foster et al. [26], which included a phylogenetic tree of evolution relationship for five *Brucella* spp. namely *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis*. In this study, they found that the *B. ovis* lineage was first separated from other species of *Brucella*, resulting in the species becoming a common ancestor. This ancestor produced two distinct groups, representing *B. suis* and *B. canis* in one group and *B. melitensis* and *B. abortus* in another group. Several phylogenomic analyses were developed on the evolutionary path of *B. melitensis* using whole genome SNP-based, MLST and core genome MLST (cgMLST). The approaches using genome-wide SNPs are considered to give better resolution and higher amounts of information because they cover intergenic regions in the whole genome compared to the MLST [3].

SNP-based phylogenetic analysis

The genotyping of *B. melitensis* using clade-specific SNPs was first reported by Foster et al. [27] using a molecular invasion probe (MIP) from the SNPs data among 30 strains of *B. melitensis*. Although the approach is useful for rapid genotyping into groups of species level, high-resolution genetic analysis among *B. melitensis* strains using whole genome SNPs is more appropriate for understanding the epidemiology and distribution of strains circulating in a region.

An example of phylogenomic analysis is shown in Figure 2 using the concatenated sequence genome data of 68 *B. melitensis* strains from the NCBI database. These genome sequences were distributed into five major phylogenetic clusters corresponding to clinically described genotypes [15, 28]. Genotype I represent the West Mediterranean strains associated with European countries, while genomes assigned to Genotype II are classified as Asian strains. Genotype III is represented by strains of African origin, and both genotypes IV and V consist of strains from the European and American clades. Several studies revealed that Genotype II has undergone divergence into nine subgenotypes, denoted as IIa - IIi, which separate the strains according to a diverse geographical area, including East Asia (China), Southeast Asia (Malaysia, Thailand), South Asia (India, Pakistan), Western Asia/ Near East (Iraq, Saudi Arabia, Turkey, Syria) and Europe (Norway, Portugal). *B. melitensis* strain UK3/06 is the sole representative of genotype IIa [28]. Recently, Zhao et al. [29] reported a new *B. melitensis* strain QH2019005 isolated from China was assigned to genotype IIj as a new subgenotype.

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Vol. 17(1) JANUARY 2023

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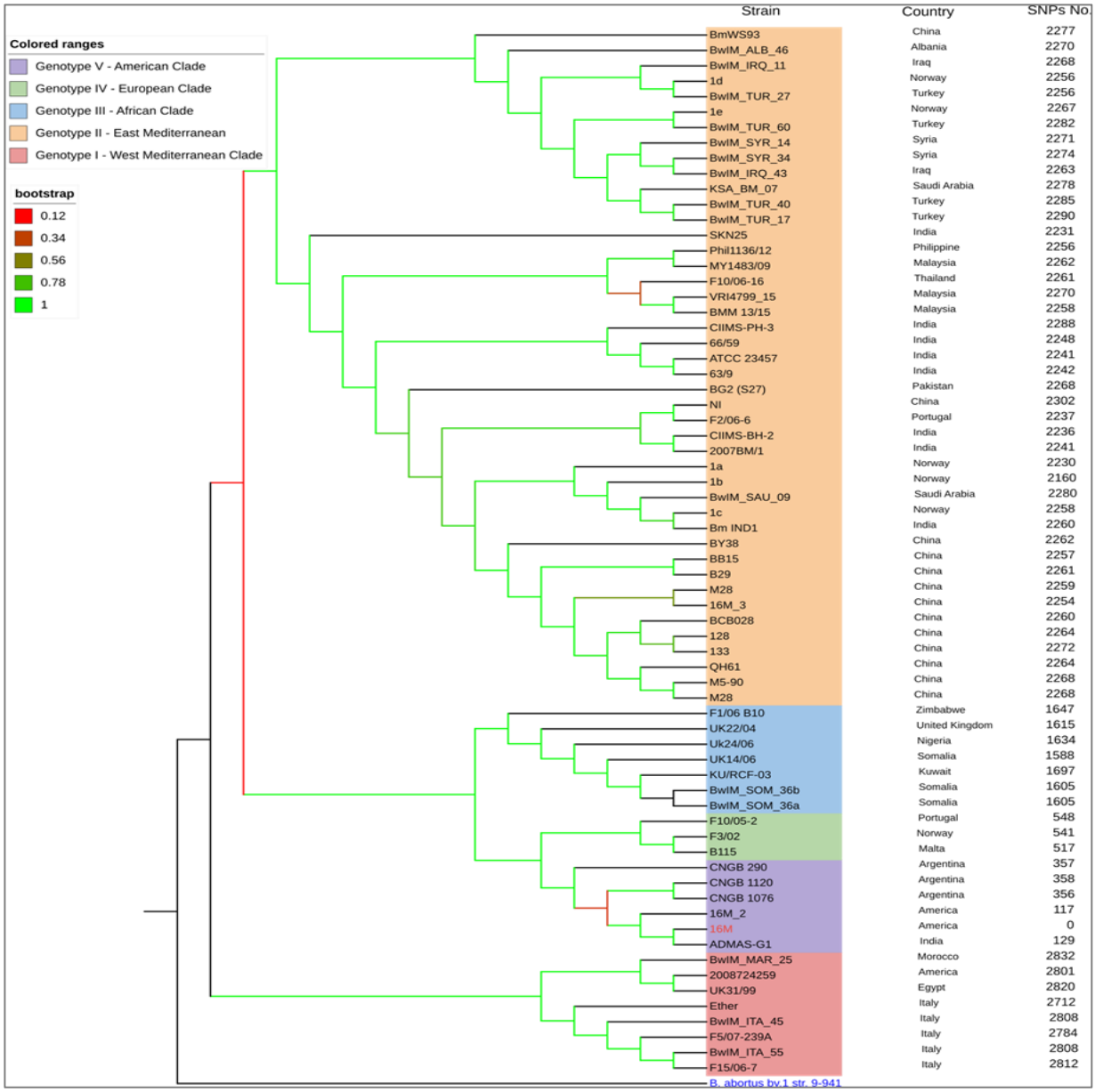

Figure 2: The phylogenetic tree of 69 genome *B. melitensis* retrieved from the NCBI database was constructed based on kSNP sequenced representation of the clustering within five genotypes. The phylogenetic tree was visualised using iTOL version 5

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Multi-locusMulti-locus Sequence Typing Genotypes (MLST)

MLST is frequently used to categorise *Brucella* species and isolates due to the high genomic homology within the genus. The *Brucella* MLST profile currently hosted at pubMLST (https://pubmlst.org/organisms/brucella-spp) was initially developed by Whatmore et al. [30] using sequences of nine informative housekeeping genes (the 9-locus scheme). Subsequently, 12 loci have been added to form the MLST 21-locus scheme [25]. Extensions to these schemes have been used to assign *Brucella* spp rapidly. Isolates within a specific geographical region, e.g. in Xinjiang, China, a study by Sun et al. [31] revealed that out of 50 *B. melitensis* isolates, 49 could be assigned to type ST8, and one isolate was assigned to a new sequence type, ST37. The prevalent MLST sequence types in Qinghai Province are ST7 and ST8 [32]. In Thailand, a study using eight additional gene loci with the 9-locus scheme showed that the prevalent sequence type for *B. melitensis* is ST8 and revealed a diverse range of other sequence types among the *Brucella* spp. strains isolated from humans [33]. In a study by Liu et al. [34] in the Guangxi Province, China, MLST revealed a shift in the prevalent sequence type from ST17 and ST21 to the recently emerging ST8. Thus, MLST can be helpful for local pathogen surveillance in monitoring and epidemiological studies and provide a convenient way to detect the presence of foreign or new emerging strains quickly and trace the transmission route.

Whole genome MLST (wgMLST)

The use of whole genome MLST (wgMLST) in *Brucella* spp. allowed us to assign sequence typing (ST) and cluster the genotype computationally faster using the entire assembly genome. Pelerito et al. [11] describe a study using wgMLST for retrospective analysis of the genetic relatedness among *B. melitensis* strains causing human brucellosis in Portugal from 2010 to 2018. The study showed phylogenetic clustering among 36 strains of *B. melitensis* have separated within genotypes II and IV, whilst strains in genotype IV were correlated with migrations among peoples sharing similar eating habits between countries in Portugal, Italy and Greece. Another study also reported that one strain (ADMAS-G1) among 17 strains of *B. melitensis* from India was assigned ST7 using wgMLST, while the rest were assigned ST8 [12]. The phylogenetic tree analysis showed the ADMAS-G1strain clade together with *B. melitensis* 16M and vaccine strain Rev. 1. Following the study, Janowicz et al. [35] reported the *B. melitensis* strains of the ST8 from whole genome analysis are mainly from East Mediterranean while ST7 are from the American linage. None of the *B. melitensis* strains from Asia was typed as ST7 as in the genome data in the *Brucella* PubMLST database. The application of wgMLST in the reference laboratory can be established as a technology transition for brucellosis surveillance. Thus, it facilitates any future outbreak to prevent the spreading of the disease.

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Core genome MLST (cgMLST)

A recent genomic approach based on core genome MLST (cgMLST) analysis has recently gained popularity for genotyping *B. melitensis* [36, 37, 38]. The cgMLST is a whole genome-based genotyping that compares gene-by-gene using a predefined set of core genes and provides more excellent resolution than traditional MLST typing. For example, a study on Portuguese isolates of *B. melitensis* was able to identify multiple genetic linkages with other strains using 164 loci-based cgMLST and give a higher resolution of discrimination among other strains [39]. Genome assemblies from the Italian *B. melitensis* strain also reported genotyping using cgMLST and were assigned the profile using 3704 target core genes [35]. However, it is essential to note that cgMLST has a superior genotyping resolution, mainly in large-scale epidemiological studies. Thus if a few sampling has been carried out, it may produce a low-quality sequence and lead to a misleading interpretation of genetic linkage analysis.

Multi-Locus Variable-Number Tandem-Repeat Analysis (MLVA)

DNA fingerprinting based on the number of tandem-repeat, namely MLVA, is also gaining popularity as a quick and straightforward method for molecular typing in *Brucella* spp. and other pathogenic bacterial species. The first MLVA-16 scheme was established by Le Flèche et al. [40] and has been used for epidemiological trace-back investigation, following which an MLVA-21 scheme based on 21 different loci was implemented to increase the resolution in the cladogram context [41]. MLVA is frequently used with MLST for fast resolution of *Brucella* isolates into the species and sequence types. For example, in Sun et al. [31] study, an 8-loci MLVA scheme (MLVA-8) was used to assign the 50 *B. melitensis* strains into genotypes 42 and 63, while the 16-loci MLVA-16 scheme assigned these isolates to 28 genotypes. In a study by Shevtsov et al. [42] in neighbouring Kazakhstan, MLVA-8 assigned a set of 128 *B. melitensis* strains to genotypes 42, 43 and 63, while MLVA-16 sorted these strains into 25 genotypes. An analysis using MLVA-16 on Egyptian *B. melitensis* strains showed that these strains can be clustered into 21 genotypes and are closely related to Italian strains [43]. However, the higher resolution of whole genomic sequencing analysis, such as SNP-based and cgMLST, was published to provide a more powerful tool for discriminating intraspecies of *Brucella* strains than the MLVA technique. Georgi et al. [44] reported that by using SNP based on WGS data, they could distinguish between 63 clinical isolates of *B. melitensis* from Germany into three distinct subclusters, all of which belong to the Eastern Mediterranean clade. In contrast, using MLVA could only discriminate 52 of the 63 isolates.

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CONCLUSION

Comparative genomic analysis of the *B. melitensis* genome has provided high-quality information and allowed for high-resolution analysis of the phylogenomic relationship of this species. Such data allow us to conduct trace-back studies and correlate genotypes with the geographical origin of the strains. Besides, the speciality genes among strains of *B. melitensis* also give a variety of functional genes such as predicted virulence gene, AMR gene, presence of prophage and variation gene, which give varies among strains. However, supporting data such as the pathogenicity mechanism and virulence factor of *B. melitensis* still needs to be fully understood, as well as the presence of the AMR gene. Combining these data will allow for the prediction of emerging pathogenic strains and facilitate their epidemiology analysis.

ACKNOWLEDGMENTS

The authors would like to acknowledge the support of the Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia, the Director General of Health, Malaysia and the Director of the Institute for Medical Research for providing the facilities and permission to publish this article.

AUTHOR'S CONTRIBUTION

Jama'ayah Zahidi carried out the research and wrote and revised the article. Faiz Foong Abdullah and Rohaidah Hashim conceptualised the central research idea and provided the theoretical framework. Faiz Foong also supervised the research progress, review, and revisions and approved the submission of the article.

CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted without any self-benefits or commercial or financial conflicts and declare the absence of conflicting interests with the funders.

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Vol. 17(1) JANUARY 2023

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Vol. 17(1) JANUARY 2023
ISSN: 1675-7785
eISSN: 2682-8626

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