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Molecular epidemiological characteristics of *Brucella* spp. isolated from human and animal brucellosis

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ABSTRACT

Brucellosis is the most common zoonotic disease in the Mediterranean region where Turkey is located. Therefore, particularly in this region, it is crucial to follow molecular surveillance methods for the control and eradication of this disease agent, which threatens both human and animal life. We have conducted a molecular-based surveillance study for detecting the origin of the possible reservoir in human brucellosis and transmission of *Brucella* spp. between humans and ruminants. For the detection of clonal relationships, we isolated *Brucella* spp. from human and ruminants diagnosed with brucellosis and evaluated them by PFGE and MLVA methods. We found 10 subclusters in PFGE and 22 subclusters in MLVA-16. Human and ruminant isolates were found to co-exist in MLVA-16-m1 and PFGE-a8, a9, a10 subcluster by both methods. In conclusion, the results revealed that these subcluster isolates that co-exist some ruminant and human isolates can be more effective in infecting humans.

Key words: Brucella, MLVA, Molecular surveillance, PFGE.

INTRODUCTION

Brucellosis is the most common zoonotic disease in the Mediterranean region, as well as being encountered in Central Asia, Middle East, America, and Africa. Every year, an estimated 500,000 new cases are reported. *Brucella abortus, Brucella melitensis, Brucella ovis, Brucella suis, Brucella canis,* and *Brucella neotomae* are the etiologic agents of brucellosis (Day *et al*., 2012; Christopher *et al*., 2010; Kumar *et al*., 2010). These agents are transmitted from animal products and animal reservoir to humans by direct or indirect contact. *Brucella* infections and epidemics cause substantial economic losses due to infertility and abortion in domestic animals. The mortality of human brucellosis is low but complications may affect any organ system.

The disease may present as a chronic, localized infection or delayed convalescence and may relapse (Kaur *et al*., 2017; WHO, 2006).

Despite eradication programs involving vaccines for the reduction and elimination of the disease in ruminants, brucellosis is still seen all over the world (Olsen *et al*., 2005). The addition of epidemiological surveillance studies to these programs is an important requirement (Sing, 2015). Especially, molecular-surveillance analyses include the investigation of genotypes, spread and primary source of infections to easily control outbreaks. One of these analyzes, PFGE, has been proposed by Jensen *et al*. (1999), Chen *et al*. (2014) and Bahmani *et al*. (2017) for investigating the epidemiology of brucellosis because of its high reproducibility and discriminatory power. Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) has high discriminatory power particularly to distinguish *Brucella* which has more than 95% DNA/DNA homology. Fleche *et al*. (2006) and Santis *et al*. (2011) have reported that MLVA is a valuable, user-friendly and infectiously safe method for epidemiological analysis of *Brucella* spp.

This study aimed to detect the origin of the possible animal reservoir of human brucellosis and transmission of *Brucella* spp. between humans and ruminants by molecular methods. For this purpose, *Brucella* spp. isolated from human and ruminants were assessed by PFGE and MLVA for the detection of clonal relationships.

MATERIALS AND METHODS

This study was conducted between January 2012 and January 2014 with a total of 50 samples from the liver, spleen and stomach content of aborted fetuses of goats (9), cattle (9), and sheep (32). The materials were obtained from Adana Veterinary Control and Research Institute, Turkey. In addition, 25 human *Brucella* strains isolated from the

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patients' venous blood samples at Cukurova University, Faculty of Medicine, Department of Microbiology were included in the analyses.

Brucella strains isolated from the ruminant and human samples using *Brucella*-selective agar with 7% horse serum and supplement (Oxoid, Basingstoke, UK) were identified based on colonial morphology, microscopic appearance and biochemical properties in VITEK II (bioMérieux). For conventional biotyping of *Brucella* spp., CO_2 requirements, production of urease, H_2S, sensitivity to basic fuchsin and thionin, agglutination with A and M monospecific sera, and susceptibility to Tbilisi phage were investigated by Adana and Pendik Veterinary Control and Research Institute. The identified isolates were stored at Cukurova University, Faculty of Medicine, Department of Microbiology.

Brucella DNA samples were prepared by *boiling* and *freeze*-thawing processes without using chemical reagents. All *Brucella* strains were identified using AMOS-ERY-PCR with primers specific to *B. abortus, B. melitensis, B. ovis, B. suis* according to method outlined by Ocampo-Sosa *et al.* (2005).

MLVA-16 PCR was performed as previously described by Al Dahouk *et al.* (2007). The primer was grouped as three panels. Panel 1 primers were combined into four duplex PCR sets (Bruce 6-12, Bruce 45-55, Bruce 8-42, and Bruce 11-43). Panel 2A and 2B primers were used in a singleplex PCR set. *B. melitensis* ATCC 23458 reference strain was used as the control. After 12% polyacrylamide gel electrophoresis, the DNA fragments were evaluated by the 10 bp and 50 bp DNA ladder (Thermo Fisher Scientific, Invitrogen™), and their size was determined. The number of repeats corresponding to the fragment size was detected based on a table in MLVABank (NCSR, 2013). A dendrogram was generated using the unweighted pair-group method based on the calculation of arithmetic averages and the Dice coefficient with software created by Bikandi *et al.* (2004). If the Dice coefficient correlation was 100%, the isolates were considered to be clonally related. The isolates were clustered in these methods and the discriminatory power (D value) of each MLVA (MLVA-8, MLVA-11, MLVA-16) technique was calculated using Hunter and Gaston's generalized formula with software created by Bikandi *et al.* (2004).

Macrorestriction analysis of isolates was performed by the XbaI-PFGE method as previously described by Cespedes *et al.* (2011). The XbaI enzyme (New England Biolabs, Mass., USA) was used to restrict chromosomal DNA, and PFGE was run in a CHEF-DR II apparatus (Bio-Rad Laboratories, Calif., USA), with pulses ranging from 0.5 to 10s at 6 V/cm and 12°C for 24 h. Gel images were exported to Gelcompar II software (Applied Maths, SintMartens-Latem, Belgium) for analysis. The comparisons were undertaken using the band-based Dice coefficient. Dendrograms were generated using the unweighted pair-group method by arithmetic averaging with 1% position tolerance. The isolates were considered to be clonally related if the Dice coefficient correlation was more than 80%. Lastly, the discriminatory power of the PFGE technique was calculated using Hunter and Gaston's generalized formula with software created by Bikandi *et al.* (2004).

RESULTS AND DISCUSSION

Although zoonotic diseases caused by the agents of *Brucella* species are relatively common in Turkey, there are only a limited number of studies that examined the clonal relationships of strains to establish an epidemiological database. Therefore, this study aimed to obtain wide-angle epidemiological data for Brucellosis, a major problem for humans and particularly ruminants. It is thought that this study will provide added value for the "single world, single medicine, single health" concept, which is especially important for the control of zoonotic diseases.

In this study, 66 *B. melitensis* and 9 *B. abortus* isolates were identified to be biovar 3 by biotyping method and 731-bp AMOS-ERY-PCR products. Karagul *et al.* (2017), Samadi *et al.* (2010), Sammartino *et al.* (2005), and Xiao *et al.* (2015) have reported, *B. melitensis* biovar 3 predominates almost exclusively in the Mediterranean countries and Middle East.

The similarity percentage of the PFGE pattern of the *B. melitensis* isolates was 93.3. In *Xba*I-PFGE, these isolates were grouped into cluster A containing 10 subclusters (a1-a10) based on a cut off value of 90% and showed in Fig 1. The largest subcluster was a10 consisted of 2 ruminant and 15 human isolates. The ruminant isolates were distributed among 10 subclusters (a1-a10) and 25 human isolates were in three subclusters (a8, a9, and a10). The results showed that subclusters a1 to a7 contained only ruminant isolates. The *B. abortus* isolates were grouped under three subclusters of cluster B (b1-b3) with 94.1% similarity. The D value in Table 1 was determined as 0.873.

Brucella melitensis and *Brucella abortus* isolates were also grouped using the MLVA-8, MLVA-11 and MLVA-16 methods. According to the results, the *B. melitensis* isolates were divided into genotypes 43, 44, 57 and 59 by MLVA-8 and genotypes 110, 112, 117 and 125 by MLVA-11. The *B. abortus* isolates were grouped in a single cluster by MLVA-8 and MLVA-11. The 66 *B. melitensis* isolates that belonged to genotypes 43, 44, 57 and 59 were in the East Mediterranean group. Similar finding was reported in Turkey by Kiliç *et al.* (2011). Al Dahouk *et al.* (2007) showed that the East Mediterranean group containing these genotypes is geographically clustered in countries, such as Turkey, Iraq, Syria, Lebanon, Germany, Israel, Switzerland, Greece, France, and Bosnia.

In MLVA-16, five primers (Bruce 4, Bruce 7, Bruce 9, Bruce 16, and Bruce 30) were used differently from the MLVA-11 method. The gene regions targeted by these primers are highly polymorphic. These primers are named as 2B panel. The discriminatory power of panel 2B was high in this study. The MLVA-16, these isolates were grouped in 22 subclusters (m1-m22) and showed in Fig 2. Thirty-two *B.melitensis* isolates containing all human isolates were in cluster m1. However, *B.abortus* isolates were grouped in six subclusters (Ab1-Ab6).

Besides, the Hunter-Gaston Discriminatory Index (HGDI) value was also calculated and tabulated in Table 2, and the most discriminative primers were found to be Bruce 16 (0.467), Bruce 7 (0.397), and Bruce 4 (0.374). Similarly, Kýlýç *et al.* (2011) concluded that the HGDI value of panel 2B was found to be high. Also, when the primers were evaluated one by one, the highest HGDI value was Bruce 16. Liu *et al.* (2017) and Tay *et al.* (2015) have reported that Bruce 4 and Bruce 16 had the highest values.

The D value was calculated to determine the discriminatory power of the methods. Data in Table 1 show that the D value for MLVA-8, MVA-11 and MLVA-16 was determined as 0.461, 0.461 and 0.808, respectively. The highest value was found in PFGE (0.873). Allardet-Servent *et al.* (1988) and Cespedes *et al.* (2011) have indicated that the DNA profile of *Brucella* isolates in PFGE analysis was similar to a high degree due to the >95% homology in the genus. However, we evaluated the isolates at the subcluster level, rather than the cluster level based on a cut off value of 100% by PFGE. Interestingly, PFGE had high discriminatory power despite only having 10 subclusters compared to the 22 subclusters revealed by MLVA-16. However, what really determined the high discriminatory power of PFGE was the division of the MLVA-16-m1 cluster into five subclusters by PFGE.

In the current study, when the MLVA-m1 cluster containing 25 *B. melitensis* isolates from human blood samples and seven *B. melitensis* isolates from various

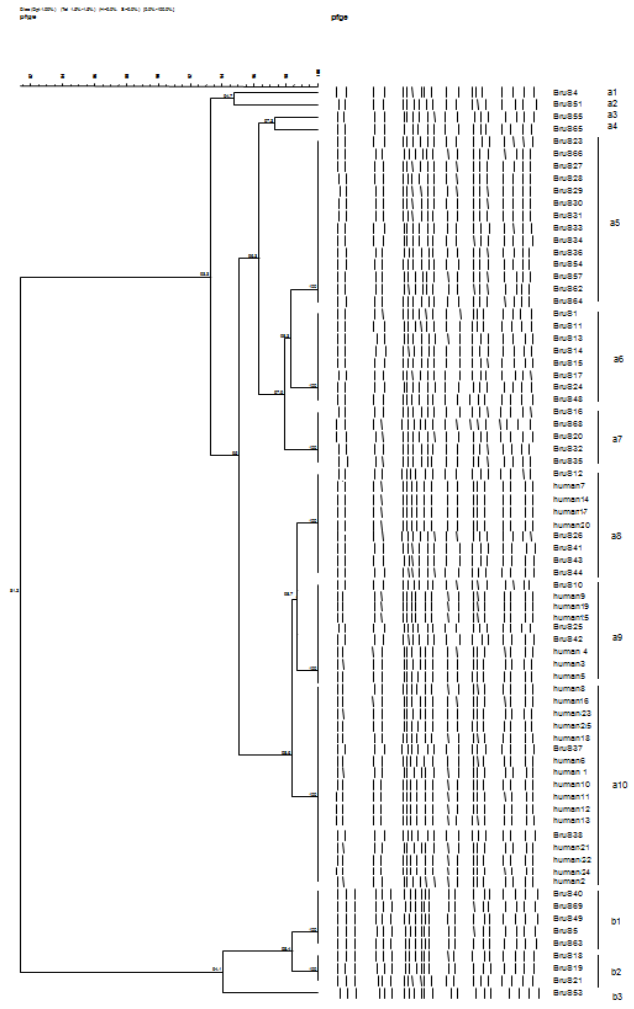


Fig 1: Dendrogram of the PFGE pattern of *Brucella* spp.

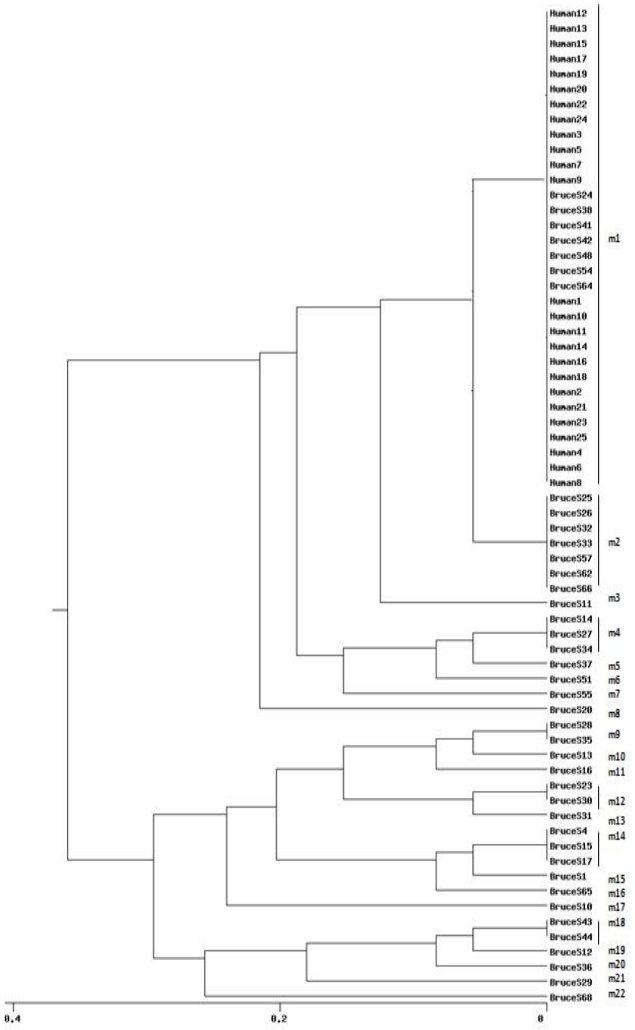


Fig 2: MLVA-16 results based on the dendrogram for *Brucella melitensis* spp.

Table 1: Comparison of MLVA and PFGE genotypes.

	PFGE type (isolate number)	MLVA 16type	MLVA 8 type	MLVA 11type	Bruce 04	Bruce 07	Bruce 09	Bruce 16	Bruce 30
D value	**0.873**	**0.808**	**0.461**	**0.461**					
B. melitensis biovar 3 (66 isolates)	a5(2) ,a6(2), a8(5), a9(7), a10(16)	m1	43	125	3	5	3	6	4
	a5(4),a7,a8,a9	m2	43	125	3	5	3	6	4
	a6	m3	43	125	3	5	3	6	3
	a5(2),a6	m4	43	125	3	4	3	5	5
	a10	m5	43	125	3	4	3	5	4
	a2	m6	43	125	3	4	3	6	4
	a3	m7	59	117	3	5	3	6	4
	a7	m8	44	110	3	5	3	6	4
	a5,a7	m9	44	110	2	5	3	5	4
	a6	m10	44	110	3	5	3	5	4
	a7	m11	43	125	3	5	3	5	4
	a5(2)	m12	57	112	4	5	3	5	4
	a5	m13	57	112	3	5	3	5	4
	a1,a6(2)	m14	43	125	3	5	3	5	4
	a6	m15	43	125	4	5	3	5	3
	a4	m16	43	125	4	5	3	5	4
B.abortus biovar 3(9 isolates)	a9	m17	43	125	3	6	3	5	3
	a8(2)	m18	57	112	3	5	3	6	4
	a8	m19	57	112	3	4	3	6	4
	a5	m20	57	112	2	4	3	6	4
	a5	m21	43	125	2	4	3	6	5
	a7	m22	43	125	3	4	3	5	5
	b1(2), b2(2)	Ab1	38	68	2	6	5	5	3
	b2	Ab2	38	68	2	5	5	5	3
	b1	Ab3	38	68	2	6	5	5	5
	b1	Ab4	38	68	2	4	5	5	4
	b3	Ab5	38	68	2	6	5	5	5
	b1	Ab6	38	68	2	4	5	5	3

Table 2: HGDI value of MLVA-16 primers.

Primer	HGDI value
Bruce 4	0.374
Bruce 6	0.214
Bruce 7	0.397
Bruce 8	0.102
Bruce 9	0.214
Bruce 11	0.026
Bruce 12	0.214
Bruce 16	0.467
Bruce 18	0.214
Bruce 19	0.214
Bruce 21	0
Bruce 30	0.362
Bruce 42	0.214
Bruce 43	0.171
Bruce 45	0
Bruce 55	0.214

ruminant clinical specimens were analyzed by PFGE, 25 human and three ruminant isolates were commonly found in subclusters a8, a9 and a10. These isolates were thought to be subtypes that could be better adapted to humans and should be more careful in terms of public health. These isolates are followed by molecular epidemiological methods, will contribute to the “one medical one health” and public health studies.

CONCLUSION

The analysis of *B. melitensis* and *B. abortus* isolates showed that the discriminatory power of PFGE and MLVA-16 is high, and these methods can efficiently identify the molecular patterns of the isolates in the study area. In addition, some MLVA and PFGE subclusters of *B. melitensis* biovar 3, which predominate in Turkey and East Mediterranean, are thought to be powerful zoonotic agents transmitted and adapted from ruminants to humans.

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