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Türk Hijyen ve Deneysel Biyoloji Dergisi

The role of *Brucella abortus* strains in the abortion etiology of domestic ruminants in the Cukurova region, Turkey and molecular epidemiological characteristics

Türkiye'nin Çukurova bölgesinde evcil ruminantların düşük etiyolojisinde *Brucella abortus* suşlarının rolü ve moleküler epidemiyolojik özellikleri

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ABSTRACT

Objective: Brucellosis caused by bacteria of the *Brucella* genus is a globally important zoonotic disease. *B. abortus*, which usually causes abortion and infertility in cows, can cross-infect ruminants and also cause chronic disease in humans. Controlling outbreaks in ruminants is essential to prevent economic losses and human disease due to bovine brucellosis. Since classical phenotypic methods alone are insufficient in monitoring epidemic strains of *B. abortus*, genotypic surveillance has become crucial today. We aimed to determine the role of *B. abortus* strains in the abortion etiology of domestic ruminants in the Cukurova region, Türkiye, reveal the phylogenetic relationships of the isolated strains using molecular methods and examine the availability of these methods in this field.

Methods: One hundred eighteen abortion samples were included in the study. Following the isolation and identification of *B. abortus*, the clonal relationship between these isolates was investigated by Pulse-Field Gel Electrophoresis (PFGE) and Multi-Locus Variable Number Tandem Repeat Analysis (MLVA) methods.

ÖZET

Amaç: *Brucella* cinsi bakterilerin sebep olduğu bruselloz dünya çapında önemli zoonotik bir hastalıktır. Genellikle ineklerde düşüğe ve kısırlığa sebep olan *B. abortus* ruminantlar arasında çapraz enfeksiyon yapabilmekte, insanlarda da kronik hastalık oluşturabilmektedir. Ruminantlarda salgınların kontrol altına alınması sığır brusellozuna bağlı ekonomik kayıpların ve insanlardaki hastalığın önlenmesi için esastır. Salgın suşlarının izlenmesinde klasik fenotipik yöntemler tek başına yeterli olmadığından günümüzde genotipik sürveyans önem kazanmıştır. Çalışmamızda, Türkiye'nin Çukurova bölgesinde evcil ruminantların düşük etiyolojisinde *B. abortus* suşlarının rolünün belirlenmesi, izole edilen suşların filogenetik ilişkilerinin moleküler yöntemlerle ortaya çıkartılması ve bu yöntemlerin bu alanda uygulanabilirliğinin sorgulanması amaçlanmıştır.

Yöntem: Çalışmaya 118 düşük örneği dahil edilmiştir. *B. abortus* izolasyonu ve identifikasyonunu takiben bu izolatlar arasındaki klonal ilişki Pulse-Field Gel Electrophoresis (PFGE) ve Multi-Locus Variable Number Tandem Repeat Analysis (MLVA) yöntemleri ile araştırılmıştır.

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Results: *B. abortus* was detected in 17 (14.4%) of 118 abortion cases examined. The distribution of *B. abortus* isolates among cattle, goats and sheep was 72.2% (13/18), 5.9% (3/51) and 2.0% (1/49), respectively. The 14 (82.4%) of *B. abortus* isolates were biovar 3. A single cluster and four pulsotypes (HGDI=0.5662) were determined by the PFGE, five types (HGDI=0.6838) by the MLVA-16, and six genotypes (HGDI=0.7132) by evaluating these two methods together. Genotype 1, which consists of nine (52.9%) isolates, six obtained from cattle and three from goats, was predominant.

Conclusion: Although the discriminative powers of the PFGE and MLVA methods were not high enough when a small number of samples were examined, the discriminatory power of MLVA-16 was determined to be higher than PFGE in determining the regionally predominant *B. abortus* genotypes. Moreover, the use of both together was even more effective. Since using the two methods together in epidemiological studies requires additional costs and effort, it was concluded that the predominant genotypes could be determined, especially during epidemic periods and evaluated in vaccine studies. Additional examination of human clinical specimens in epidemic areas will further strengthen the results to be obtained. Thus, our study will provide essential data for the studies to be performed in order to control brucellosis outbreaks, which constitute a serious public health problem.

Key Words: Abortion, *Brucella abortus* , brucellosis, epidemiology, MLVA, PFGE

Bulgular: İncelenen toplam 118 düşük vakasından 17 (%14,4)'sinde *B. abortus* tespit edilmiştir. Sığır, keçi ve koyunlardaki *B. abortus* izolatlarının dağılımı sırasıyla %72,2 (13/18), %5,9 (3/51) ve %2,0 (1/49) idi. *B. abortus* izolatlarının üçü biyotiplendirilememişken, 14'ünün (%82,4) biovar 3 olduğu tespit edilmiştir. PFGE ile tek küme ve 4 pulsotip (HGDI=0,5662), MLVA-16 ile 5 tip (HGDI=0,6838), iki yöntem birlikte değerlendirildiğinde de 6 genotip (HGDI=0,7132) belirlenmiştir. Altısı sığırdan, üçü de keçiden elde edilmek üzere 9 (%52,9) izolatı barındıran genotip 1'in baskın olduğu görülmüştür.

Sonuç: PFGE ve MLVA yöntemlerinin ayırt edici güçleri az sayıda örnek incelendiğinde yeterince yüksek bulunmasa da, bölgesel hakim *B. abortus* genotiplerinin belirlenmesinde MLVA-16'nın ayırt edici gücünün PFGE'ye göre daha yüksek olduğu, ikisinin birlikte kullanılmasının ise daha da etkili olduğu belirlenmiştir. İki yöntemin epidemiyolojik çalışmalarda bir arada kullanılmasının ek maliyet ve zahmet gerektirmesi sebebiyle, özellikle salgın dönemlerinde baskın genotiplerin tespit edilerek aşı çalışmalarında değerlendirilebileceği kanaatine varılmıştır. Salgın bölgelerinde insan klinik örneklerinin de ek olarak incelenmesi elde edilecek sonuçları daha da güçlendirecektir. Sonuç olarak çalışmamız, ciddi bir halk sağlığı sorunu oluşturan bruselloz salgınlarının kontrol altına alınabilmesi için yapılacak çalışmalara önemli veri sağlayacaktır.

Anahtar Kelimeler: Düşük, *Brucella abortus*, bruselloz, epidemiyoloji, MLVA, PFGE

INTRODUCTION

Brucella abortus is a Gram-negative, non-spore, non-motile, intracellularly-located bacterium and shows interest in the reticuloendothelial system. It causes brucellosis, a zoonotic disease of worldwide importance, and leads to animal reproductive problems. *B. abortus* usually settles in the uterus and mammary gland tissue in pregnant cows, causing abortion and infertility, while the crucial symptoms of the disease in infected bulls are orchitis associated with seminal vesiculitis and epididymitis (1). Although *B. abortus* is predominant in cattle, cross-infection between ruminants is also observed

(2). The infectious agent has an affinity for the uterus containing high concentrations of erythritol, especially in the last trimester of pregnancy, and preferentially proliferates in placental trophoblasts. Due to the absence of erythritol in the human placenta, it is thought that this bacterium does not play a role in the abortion or preterm birth etiology in humans (3). In humans, the infection is formed by contact with infected animals or the consumption of unpasteurised dairy products, causing a long-lasting disease. The general symptoms of human brucellosis are weakness, high fever, anorexia, myalgia and arthralgia (4). These cause physical inadequacy and loss of labour, and treatment expenses also cause a significant economic loss (5). Therefore, controlling and preventing brucellosis in animals is essential to eradicating the disease in humans (6).

Despite the eradication programs, including vaccines for the prevention and elimination of the disease in ruminants, brucellosis is still common worldwide (5-7). It continues to be a significant problem for Turkey and other Middle Eastern countries (8). Therefore, it is clinically essential to monitor epidemic strains to get the epidemics under control, prevent the spread by identifying the sources of infection and contribute to the selection of regional anti-brucellosis strategies. However, identifying *Brucella* species and biovars using classical phenotypic methods is insufficient to monitor epidemic strains. In the genotyping surveillance follow-up of brucellosis, the Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) method, which is successfully used in the clonal typing of fastidious microorganisms such as *M. tuberculosis* (9), is also used in addition to the Pulse-Field Gel Electrophoresis (PFGE) method (10,11), which is accepted as the gold standard for bacterial typing due to its high distinctiveness (12).

We aimed to determine the role of *B. abortus* strains in the abortion etiology of domestic ruminants in the Cukurova region, Turkey, to reveal the phylogenetic relationships of the isolated strains by using the PFGE and MLVA methods and thus investigate the availability of these methods in this field.

MATERIAL and METHOD

Isolation and Identification of *B. abortus* strains

The 118 abortion samples, which were taken from 51 goats, 49 sheep and 18 cattle, and sent to the laboratory of Adana Veterinary Control Institute between 2013 and 2014 for examination, were included in the study. Each sample was inoculated on two *Brucella* selective agar media (Oxoid, Basingstoke, UK) supplemented with 7% horse serum. These media were incubated at 37 ºC for four days, one under aerobic conditions and the other in an incubator containing 10% CO_2. Phenotypic species identifications of *B. abortus* isolates were performed in automatised VITEK 2 system (BioMerieux, Durham, North Carolina, USA). Biotype determinations were carried out in Istanbul Pendik Veterinary Control Institute Reference Laboratory by using classical typing techniques, including CO_2 maintenance, H_2S production, urease activity in Christensen's urea agar and Rustagian-Stuart urea broth, the reproduction in the medium containing basic fuchsin (MilliporeSigma, MA, USA) and thionine (Merck, Germany) in concentrations of 20 µg/ml and 40 µg/ml, respectively (dye sensitivity test), lysis with Tbilisi (Tb) and Berkeley (Bk) phage, and agglutination tests with monovalent A and M antiserum. Reference strains of *B. abortus* (bv 1: 544, ATCC 23448; bv 2: 86/59, NC 10501; bv 3: Tulya, ATCC 23448) were used as control strains for the determination of species and typing.

The species identification of *B. abortus* isolates was confirmed in the laboratory of the Medical Microbiology Department, Cukurova University Faculty of Medicine, using the AMOS-ERY-PCR method developed by Ocampo-Sosa AA et al. (13). DNA was extracted by the freeze-thawing method.

The isolates were stored in brain heart infusion broth supplemented with blood and gelatin stabs at -80 ºC and were recultivated by inoculating in 5% sheep blood agar when needed.

Determination of Clonal Relationship

The clonal relationship between the isolates was examined using PFGE and MLVA-16 methods in the laboratory of the Medical Microbiology Department, Cukurova University, Faculty of Medicine. The discriminatory power of the tests (HGDI: Hunter-Gaston diversity index) was calculated using the site http://insilico.ehu.es/mini_tools/discriminatory_power/index.php (Access date: 01.03.2022). *B. abortus* S19 reference vaccine strain was used as a control.

Determination of Clonal Relationship by PFGE Method

XbaI restriction enzyme (New England Biolabs, MA, USA) was used to restrict the chromosomal DNA in agarose gel prepared from isolates, as previously described by Cespedes et al. (14). Electrophoresis was completed in the CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium) (Switch time: 0.5-10 s, 6 V/cm, 14 °C, 24 h, 0.5xTBE). The gel was stained with five µg/ml ethidium bromide, and DNA bands were visualised using the Gel Logic 1500 Imaging System (Kodak Company, NY, USA). The dendrogram of the obtained PFGE band profiles was created using the Gel Compar II version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) software program with the Unweighted pair group method with mathematical averaging (UPGMA) process. The relationship between the isolates was calculated according to the "Dice" similarity coefficient based on the bands, with a tolerance of 1.5%. Strains with 85% band similarity were considered in distinct clonal-related clusters. The clusters were named with capital letters (A, B, C, etc.).

Determination of Clonal Relationship by MLVA-16 Method

After DNA extraction from the isolates, 12 PCR assays, four duplex PCR's (Bruce6-12, Bruce45-55, Bruce8-42, and Bruce11-43) and eight singleplex PCR's (Bruce4, Bruce7, Bruce9, Bruce16, Bruce18, Bruce19, Bruce21, and Bruce30), were performed as previously described to determine the specific number of VNTR (Variable Number Tandem Repeat) units of 16 loci (15-17). The primer sequences used are shown in Table 1. Amplicons were subjected to electrophoresis through 2.5-4.5% (wt/vol) agarose (MilliporeSigma,

Table 1. Primer sequences used in the MLVA-16 method

Locus	Forward Primer 5'-3'	Reverse Primer 5'-3'
Bruce04	CTGACGAAGGGAAGGCAATAAG	CGATCTGGAGATTATCGGGAAG
Bruce06	ATGGGATGTGGTAGGGTAATCG	GCGTGACAATCGACTTTTTGTC
Bruce07	GCTGACGGGGAAGAACATCTAT	ACCCTTTTTCAGTCAAGGCAAA
Bruce08	ATTATTCGCAGGCTCGTGATTC	ACAGAAGGTTTTCCAGCTCGTC
Bruce09	GCGGATTCGTTCTTCAGTTATC	GGGAGTATGTTTTGGTTGTACATAG
Bruce11	CTGTTGATCTGACCTTGCAACC	CCAGACAACAACCTACGTCCTG
Bruce12	CGGTAAATCAATTGTCCCATGA	GCCCAAGTTCAACAGGAGTTTC
Bruce16	ACGGGAGTTTTTGTTGCTCAAT	GGCCATGTTTCCGTTGATTTAT
Bruce18	TATGTTAGGGCAATAGGGCAGT	GATGGTTGAGAGCATTGTGAAG
Bruce19	GACGACCCGGACCATGTCT	ACTTCACCGTAACGTCGTGGAT
Bruce21	CTCATGCGCAACCAAAACA	GATCTCGTGGTCGATAATCTCATT
Bruce30	TGACCGCAAAACCATATCCTTC	TATGTGCAGAGCTTCATGTTCG
Bruce42	CATCGCCTCAACTATACCGTCA	ACCGCAAAATTTACGCATCG
Bruce43	TCTCAAGCCCGATATGGAGAAT	TATTTTCCGCCTGCCCATAAAC
Bruce45	ATCCTTGCCTCTCCCTACCAG	CGGGTAAATATCAATGGCTTGG
Bruce55	TCAGGCTGTTTCGTCATGTCTT	AATCTGGCGTTCGAGTTGTTCT

MA, USA) gels with ethidium bromide at 100 V for 2-4 h, depending on their size. After electrophoresis, the gels were visualised by an imaging system (Kodak Company, NY, USA). The 10 bp and 50 bp DNA ladders (TrackIt-InvitrogenTM, Thermo Fisher Scientific, MA, USA) were used to determine the band size.

According to the obtained MLVA band sizes, the numbers of repeated VNTR units were detected using the table published in the MLVA database (Fig. 1) (http://mlva.u-psud.fr/*Brucella*/spip.php?article93, Accessed: 21.10.2014 and 05.07.2022). The resulting band profiles were evaluated with the Unweighted pair group method with mathematical averaging (UPGMA) using the site "http://insilico.ehu.es/dice_upgma/" (Access date: 01.03.2022). In addition, the "Dice" similarity coefficient based on bands was used to determine the relationship between strains, and a dendrogram was created. Accordingly, strains with 80% band similarity were considered in clonally related clusters.

***Brucella* table for alleles assignement [allele calling convention to convert alleles size (bp) into number of repeats (u)]**

Panel 1 (agarose2%)														
bruce06-BRU1322_134bp_408bp_3u	140 (1u)	274 (2u)	**408 (3u)**	542 (4u)										
bruce08-BRU1134_18bp_348bp_4u	312 (2u)	330 (3u)	**348 (4u)**	366 (5u)	384 (6u)									
bruce11-BRU211_63bp_257bp_2u	**257 (2u)**	320 (3u)	383 (4u)	509 (6u)	635 (8u)	698 (9u)	887 (12u)	1013 (14u)	1076 (15u)					
bruce12-BRU73_15bp_392bp_13u	[b]302 (7u)	[b]317 (8)	327-332 (9u)	342-347 (10u)	362 (11u)	377 (12u)	**392-397 (13u)**	407-411 (14u)	422 (14u)	437 (14u)	452 (14u)			
bruce42-BRU424_125bp_539bp_4u	164 (1u)	289 (2u)	414 (3u)	**539 (4u)**	664 (5u)	789 (6u)	914 (7u)							
bruce43-BRU379_12bp_182bp_2u	170 (1u)	**182 (2u)**	194 (3u)											
bruce45-BRU233_18bp_151bp_3u	133 (2u)	**151 (3u)**	169 (4u)	187 (5u)										
bruce55-BRU2066_40bp_273bp_3u	193 (1u)	233 (2u)	**273 (3u)**	313 (4u)	353 (5u)	393 (6u)	433 (7u)		[a]553 (10u)					
Panel 2A (agarose3%)														
bruce18-BRU339_8bp_146bp_5u	130 (3u)	138 (4u)	**146 (5u)**	154 (6u)	162 (7u)	170 (8u)	178 (9u)	[a]186 (10u)						
bruce21-BRU329_8bp_148bp_6u	140 (5u)	**148 (6u)**	156 (7u)	164 (8u)	172 - 175 (9u)									
Panel 2B (agarose3%)														
bruce04-BRU1543_8bp_152bp_2u	144 (1u)	**152 (2u)**	160 (3u)	168 (4u)	176 (5u)	184 (6u)	192 (7u)	200 (8u)	208 (9u)	216 (10u)	224 (11u)	232 (12u)	240 (13u)	248 (14u)
	256 (15u)	264 (16u)	272 (17u)	280 (18u)	288 (19u)	296 (20u)	304 (21u)	312 (22u)	320 (23u)		360 (28u)			
bruce07-BRU1250_8bp_158bp_5u	134 (2u)	142 (3u)	150 (4u)	**158 (5u)**	166 (6u)	174 (7u)	182 (8u)	190 (9u)	198 (10u)	206 (11u)	214 (12u)	222 (13u)	230 (14u)	
	246 (16u)													
bruce09-BRU588_8bp_156bp_7u	124 (3u)	132 (4u)	140 (5u)	148 (6u)	**156 (7u)**	164* (8u)	172 (9u)	180 (10u)	188 (11u)	196 (12u)	204 (13u)	212 (14u)	220 (15u)	228 (16u)
	236 (17u)	244 (18u)	252 (19u)	260 (20u)	268 (21u)	276 (22u)	284 (23u)	292 (24u)						
bruce16-BRU548_8bp_152bp_3u	144 (2u)	**152 (3u)**	160 (4u)	168 (5u)	176 (6u)	184 (7u)	192 (8u)	200 (9u)	208 (10u)	216 (11u)	224 (12u)	[a]232 (13u)	240 (14u)	248 (15u)
	[b]254 (16u)		[b]270 (18u)											
bruce30-BRU1505_8bp_151bp_6u	119 (2u)	127 (3u)	135 (4u)	143 (5u)	**151 (6u)**	159 (7u)	167 (8u)	175 (9u)	183 (10u)	191 (11u)	199 (12u)			
Previous Panel 2A member														
bruce19-Bru324_3bp_163bp_36u	76 (7u)		82 (9u)		[a]88 (11u)		**163 (36u)**		169 (38u)	[c]172 (39u)	[c]175 (40u)	178 (41u)		184 (43u)
	187 (44u)	190 (45u)	193 (46u)		202 (49u)									

darker gray is the *in silico* inferred size in 16M genome sequence (NC_003317 and NC_003318) * observed size in Nouzilly-Orsay 16M reference strain
The indicated PCR product sizes are as obtained when using the primers published in Le Flèche et al. 2006 BMC microbiology. They need to be ajusted if alternative primers are used.
DNA sizing equipment must be calibrated by using reference strains as raw size estimates may need to be adjusted.
[a] *Alleles observed in B. microti isolates* [b] *Alleles observed in B. ceti isolates* [c] *Allele observed in B. inopinata*
Le Flèche et al. 2006 version 3.6 (last modified April 22 2013)
Version 3.6 minor format errors; Version 3.5 and 3.4 modifications concern the alleles size range for locus Bruce21-BRU329_8bp_148bp_6u: (172-175)bp encoded 9u;
for locus bruce12-BRU73_15bp_392bp_13u: (327-332)bp encoded 9u; (342-347) encoded 10u; (392-397)bp encoded 13u and all alleles larger than 407bp are called 14u;
locus Bruce19-BRU324_6bp_163bp_18u is renamed bruce19-BRU324_3bp_36u and new alleles size range is presented

Figure 1. Repeated unit numbers and amplicon sizes for allele identification by MLVA-16 method (http://mlva.u-psud.fr/brucella/spip.php?article93, Access date: 21.10.2014 and 05.07.2022)*The numbers above express the "b" or the Path coefficient

The study was approved by the Çukurova University, Faculty of Medicine Non-Invasive Research Ethics Committee (Date: 22.07.2022 and Number:50243401/2022-7).

RESULTS

B. abortus was isolated in 17 (14.4%, 95% confidence interval: 0.081-0.207) of 118 abortion cases in which fetal and maternal samples were analysed. The distribution of *B. abortus* isolates among cattle, goats and sheep was 72.2% (13/18, 95% confidence interval: 0.515-0.929), 5.9% (3/51, 95% confidence interval: 0.006-0.123) and 2.0% (1/49, 95% confidence interval: 0.019-0.060), respectively. Three (17.6%) isolates could not be typed, while 14 (82.4%) isolates were biovar 3. All of the isolates were also confirmed as *B. abortus* by AMOS-ERY-PCR.

Examination of the clonal relationship by the PFGE method determined that *B. abortus* isolates showed a similarity of more than 94.9% and were distributed into four pulsotypes (A1-4) within cluster A. A2 was the predominant pulsotype with 11 (64.7%) isolates. The HGDI value of the PFGE method was calculated as 0.5662 (Fig. 2).

Moreover, in the result of examining the clonal relationship of the isolates by the MLVA-16 method, five distinct genotypes were determined. Three alleles were detected at both loci of Bruce07 and Bruce30 in Panel-2B with HGDI values of 0.404 and 0.470, respectively. The remaining 14 loci demonstrated single alleles (HGDI=0). Thus, the discriminatory power of the MLVA-16 method was higher than the PFGE method (HGDI=0.6838) (Fig. 3 and Table 2).

The overall evaluation of the PFGE and MLVA-16 results clustered the *B. abortus* isolates in 6 distinct genotypes. Genotype 1, which contains 9 (52.9%) isolates, six from cattle and three from goats, was predominant (Table 3). Consequently, when these two methods were evaluated together to determine the clonal relationship, it was observed that the discriminative power increased slightly (HGDI=0.7132).

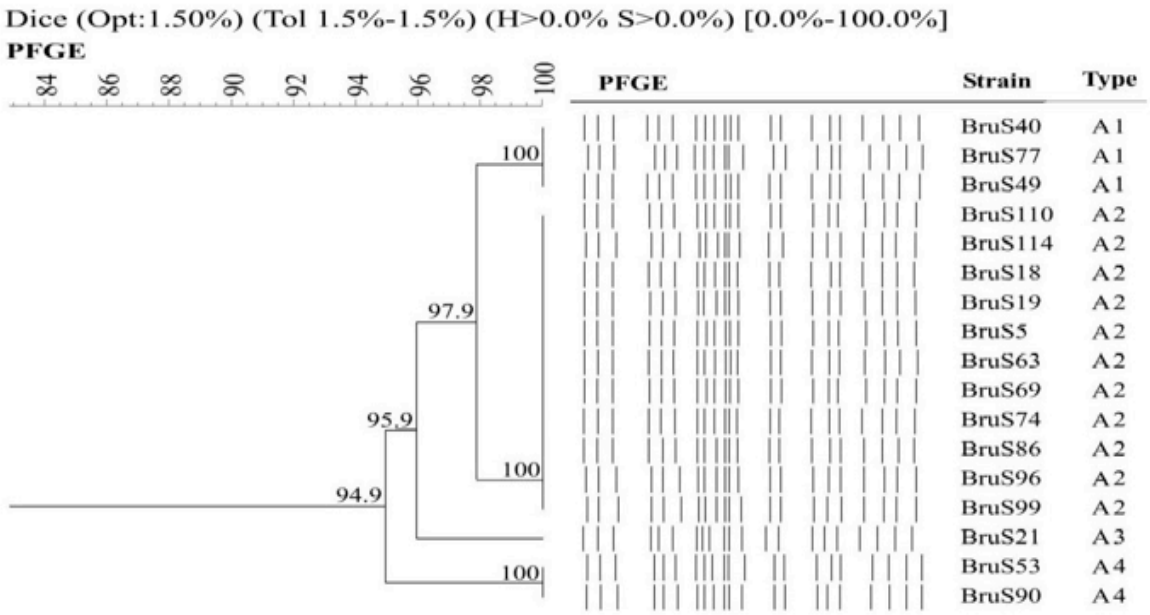

Figure 2. Dendrogram of 17 *B. abortus* isolates according to PFGE band profiles

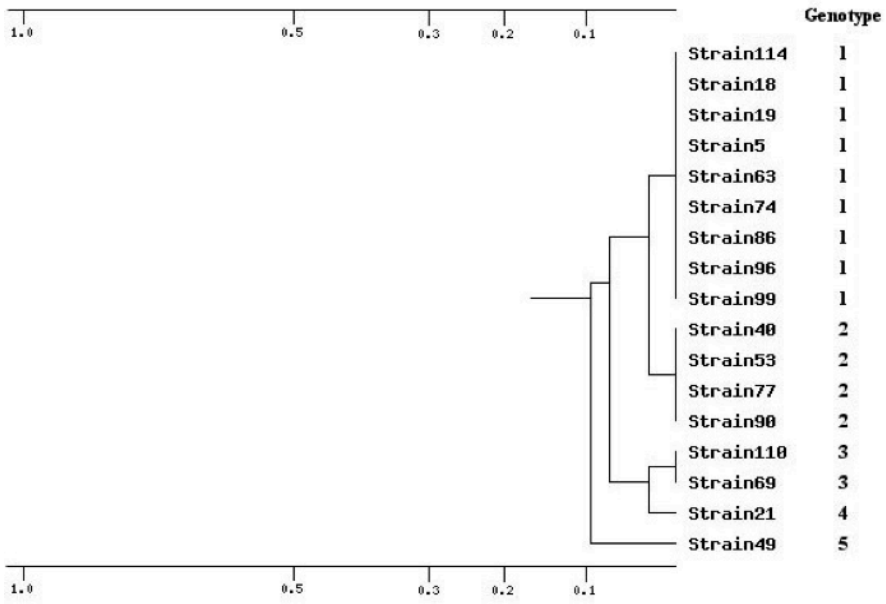

Figure 3. Dendrogram of 17 *B. abortus* isolates according to MLVA-16 band profiles

Table 2. Allele status and HGDI values of the total of 17 *B. abortus* isolates as a result of MLVA-16

Loci	No. of Alleles	No. of Repetitions (No. of Isolates)	HGDI*
Panel 1			
Bruce 06	1	2	0
Bruce 08	1	5	0
Bruce11	1	3	0
Bruce12	1	12	0
Bruce42	1	2	0
Bruce43	1	2	0
Bruce45	1	3	0
Bruce55	1	1	0
Panel 2A			
Bruce 18	1	6	0
Bruce 19	1	44	0
Bruce21	1	8	0
Panel 2B			
Bruce 04	1	2	0
Bruce 07	3	4 (3), 5(1), 6 (13)	0.4044
Bruce09	1	5	0
Bruce 16	1	5	0
Bruce 30	3	3 (12), 4 (1), 5 (4)	0.4706
Overall	5**		0.6838

*HGDI: Hunter-Gaston diversity index.

**Numbers of isolates belonging to the alleles: 9,4,2,1,1.

Table 3. The distribution of the six *B. abortus* genotypes determined as a result of the overall evaluation of MLVA-16 and PFGE methods

Genotype	MLVA-16 Type	Bruce06	Bruce08	Bruce11	Bruce12	Bruce42	Bruce43	Bruce45	Bruce55	Bruce18	Bruce19	Bruce21	Bruce4	Bruce7	Bruce9	Bruce16	Bruce30	PFGE Type	No. of Isolates	Source (No.)
1	**1**	2	5	3	12	2	2	3	1	6	44	8	2	6	5	5	3	**A02**	9	goat (3), cattle (6)
2	**2**	2	5	3	12	2	2	3	1	6	44	8	2	6	5	5	5	**A01**	2	cattle (2)
3	**2**	2	5	3	12	2	2	3	1	6	44	8	2	6	5	5	5	**A04**	2	cattle (2)
4	**3**	2	5	3	12	2	2	3	1	6	44	8	2	4	5	5	3	**A02**	2	cattle (2)
5	**4**	2	5	3	12	2	2	3	1	6	44	8	2	5	5	5	3	**A03**	1	sheep (1)
6	**5**	2	5	3	12	2	2	3	1	6	44	8	2	4	5	5	4	**A01**	1	cattle (1)

DISCUSSION

Including epidemiological surveillance studies in the planned control and eradication strategies to prevent the losses in the animal production system and infections in humans caused by *B. abortus*-induced brucellosis is crucial to reveal the regionally predominant genotypes. Thus, it is possible to monitor biovars and genotypes, vaccine strains and new potential species in the field, regulate vaccination policies by determining the source of the epidemic, especially during periods of increased abortion cases in domestic ruminants, and monitor the movement of bacteria in animal and human populations.

In the present study, fetal and maternal samples from 118 cases that resulted in abortion in domestic ruminants in the Cukurova region were examined to determine the role of *B. abortus* strains in the etiology of abortion and the phylogenetic relationships of isolated strains. As a result, *B. abortus* was detected in 14.4% of the examined abortion cases, and the distribution of *B. abortus* isolates among cattle, goats and sheep was 72.2%, 5.9%, and 2.0%, respectively. Furthermore, three out of the 17 *B. abortus* isolates could not be typed, while 14 (82.4%) isolates were found to be biovar 3. It is well known that *B. abortus* strains are predominant in cattle, but they may cause abortion in sheep and goats due to cross-infection among species (1,2,18,19). Although the biovar distribution varies according to the country and region, studies from Turkey show that biovar 3 is predominant among the *B. abortus* isolates identified in cattle (18,19). Biovar 3 is also prevalent in Italy (20), Iran (21) and many West African countries (22), while biovar 1 was commonly isolated in Nigeria (23), Zimbabwe (24) and Egypt (2,25). In the study examining 5203 Brucella field isolates from the abortion cases of domestic ruminants, mostly cattle (57.8%), between 2010 and 2015 throughout Turkey, Karagul et al. (19) identified 3024 (58.1%) isolates as *B. abortus*. Of them, 2872 were biovar 3, 120 were biovar 1, one was biovar 2, one was biovar 9, and 30 were vaccine strain S19. The researchers reported the distribution of *B. abortus* biovar 3 isolates among cattle, goats and sheep as 94.5%, 9.3% and 6.4%, respectively. These data are similar to our results (19).

To investigate the phylogenetic relationship of *B. abortus* isolates, we preferred the PFGE and the MLVA-16 methods, as mentioned above. It has been shown that the PFGE method, accepted as the gold standard in the epidemic analysis of hospital infections, can also be used to investigate the genotypic relationship between Brucella species. Due to its low discrimination and difficulty in use, the PFGE method has been replaced by the MLVA method, which has higher discriminatory power and

applicability, even though it has been more costly (14-17,26). There are a few studies in which different *Brucella* species were investigated together with these two methods worldwide (14,26). Our research is one of the rare studies in our country in which the clonal relationships of animal-origin *B. abortus* isolates were investigated by the PFGE and the MLVA methods.

In our study, 17 *B. abortus* isolates were distributed in a single cluster and four pulsotypes by the PFGE, five types by the MLVA-16, and six genotypes by an overall assessment of these two methods. By the MLVA-16, loci in all panels except Bruce07 and Bruce30 in Panel-2B contained a single allele (HGDI=0) and Bruce07 and Bruce30 had three alleles (HGDI values were 0.404 and 0.470, respectively).

Gökmen et al. (27) evaluated nine *B. abortus* strains isolated from ruminant fetal debris in our region using the MLVA and the PFGE methods. The researchers determined a single cluster and three pulsotypes by the PFGE, six types by the MLVA, and seven genotypes using two methods together to evaluate. In addition, three alleles were detected at both Bruce07 and Bruce30 loci and a single allele at the remaining loci. Along with these results, the numbers of repetitions at loci are also very similar to our results (27). As far as we know, no other study in our country applied these two methods together on *B. abortus* isolates.

In Chile, Cespedes et al. (14) evaluated the *B. abortus* isolates obtained from milk and fetal debris of ruminants using the PFGE and MLVA-16 methods, the same as our study, and determined that 69 *B. abortus* isolates were collected in a single cluster and distributed into two pulsotypes by the PFGE analysis. As a result of the MLVA of 71 *B. abortus* isolates, they established that the discriminatory power of the Panel-1 loci and the Panel-2A loci except Bruce19 was low. In contrast, the discriminatory power of the Bruce19 locus and the Panel-2B loci was high (HGDI>0.6). Although the discriminatory power of Panel-2B is generally high, as in our results, it is thought that the discriminatory power of other loci also emerges due to the more significant number of samples and regional differences (14).

Singh et al. (28) typed 11 *B. abortus* strains isolated from vaginal swabs and milk samples of ruminants in India using the MLVA-16 method and reported that all of them belonged to *B. abortus* biovar 1, gathered in a single cluster with 90% similarity and divided into five genotypes. The researchers detected three alleles at only two (Bruce07 and Bruce30) out of 16 loci and a single allele at the remaining 14 loci, similar to our study. Moreover, the repeat numbers at loci except for Bruce08, 12, 42, 43, 45, 18, and 21 and the discriminatory power (HGDI) values agree with our results (28).

In another study in India, Kumari et al. (29) typed 17 *B. abortus* isolates, of which 13 were field isolates and four reference strains, using the MLVA-15 method and reported that all of them belonged to *B. abortus* biovar 1 and were divided into eight clusters and 13 genotypes with 90% similarity. They determined three and four alleles at the Bruce07 and Bruce30 loci, respectively, two alleles at each of the Bruce12, 04, 09, 16, and 18 loci, and a single allele at the remaining loci. Despite the repeat numbers being similar to our results at the Bruce07 locus, they differed at Bruce30 and some other loci. Consequently, Bruce07 and Bruce30 loci appear more distinctive as in our results (29).

As a result of the MLVA-16 analysis in Egypt, Wareth et al. (25) distributed 20 *B. abortus* strains, two of which were vaccine strains, isolated from 18 ruminants, one dog, and one cat, into three clusters and nine different genotypes. The researchers determined three alleles at each Bruce06 and Bruce07 loci, two at each Bruce43, 18, 19, 04, 16, and 30, and a single allele at the remaining loci. The repeat numbers at the Bruce08, 12, 42, 45, and 21 loci are the same as in our study. Although the Bruce43, 18, 07 and 30 loci appeared congruent with our research, the numbers of alleles and repeats at the remaining loci differed (25).

By evaluating the results of PFGE and MLVA-16 assays together, we determined that genotype 1, including nine (52.9%) isolates, six of which were

obtained from cattle and three from goats, was predominant among six *B. abortus* genotypes. The fact that each of three *B. abortus* isolates, which were obtained from goats, existed in this dominant genotype points to cross-infection among ruminants and suggests that such regional predominant genotypes can be determined and used as prototypes in vaccine studies (1,2,18,19). Although brucellosis has been substantially eradicated among animals in some developed countries owing to eradication programs for the prevention and elimination of the disease in ruminants, including vaccination, it is still common throughout the world, including in Turkey (6-8,30). Besides, the risk it carries for human health increases the importance of the disease even more (5,6). Therefore, regular vaccination of animals is essential to control this disease (6,8).

In conclusion, although the discriminatory powers of the MLVA and the PFGE methods were not high enough when a small number of samples were examined, the MLVA-16 was found to be more discriminative than the PFGE in determining the regionally predominant *B. abortus* genotypes. Moreover, using these two methods together was even more effective. However, it requires additional cost and effort, so we believe the predominant genotypes can be determined and evaluated in vaccine studies, especially during epidemic periods. Additional examination of human clinical specimens in epidemic areas will further strengthen the results to be obtained. Thus, our study will provide essential data for the studies to be performed in order to control brucellosis outbreaks, which constitute a serious public health problem.

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ETHICS COMMITTEE APPROVAL

* The study was approved by the Çukurova University, Faculty of Medicine Non-Invasive Research Ethics Committee (Date: 22.07.2022 and Number:50243401/2022-7).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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