Kafkas Univ Vet Fak Derg
20 (3): 411-415, 2014
DOI: 10.9775/kvfd.2013.10305

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Single Nucleotide Polymorphism Analysis of the *rpoB* Gene Region for Genotyping of *Brucella melitensis* Strains Isolated from Field in Turkey [1]

Murat SAYAN [1] Sevil ERDENLİĞ GÜRBİLEK [2]

[1] *This study has been presented in XVI. Turkish Clinic Microbiology and Infection Diseases Congress, 13-17 March 2013, Antalya - TURKEY*

[1] Clinical Laboratory, PCR Unit, Faculty of Medicine, University of Kocaeli, TR-41000 Kocaeli - TURKEY
[2] Department of Microbiology, Faculty of Veterinary Medicine, University of Harran, TR-63200 Şanlıurfa - TURKEY

Makale Kodu (Article Code): KVFD-2013-10305

Summary

We previously described the first molecular characterization of *Brucella* isolates in Turkey that were examined by single nucleotide polymorphisms (SNPs) at the *rpoB* locus of *B. melitensis* strains isolated from adult and pediatric patients. However, the molecular typing of *B. melitensis* strains causing animal infections in Turkey has not been previously investigated. The aim of this study was to evaluate SNP analysis of *rpoB* gene of *B. melitensis* from field isolates in Turkey and to try to find out one of the most appropriate methods other than conventional method for long term evaluation of epidemiological studies. Thirty-two *B. melitensis* strains isolated from Marmara, Aegean, Mediterranean, Central Anatolia and Eastern Anatolia regions of Turkey were investigated together with 3 reference strains. According to *rpoB* sequencing results, three distinct genotypes (SNP type 1, type variant 2 and type 2) were recognized. SNP technique characterized the strains at the molecular levels independently from *B. melitensis* biovars. Our study showed that SNP analysis has a better discriminatory capability in identification of *B. melitensis* strains compared to classical method. In conclusion, it was suggested that SNP analysis could be useful as a molecular epidemiological method to determine relationships between *B. melitensis* isolates and might aid in effective surveillance and control method for brucellosis particularly in conjunction with a national databases.

Keywords: *Brucella melitensis, Molecular typing, Single nucleotide polymorphism, Sequence analysis*

Türkiye'de Sahadan İzole Edilmiş *Brucella melitensis* Suşlarının Genotiplendirilmesinde *rpoB* Gen Bölgesi Tek Nükleotit Polimorfizm Analizi

Özet

Ülkemizde ilk kez *Brucella* izolatlarının moleküler karakterini ortaya çıkardığımız önceki çalışmada erişkin ve çocuk hastalardan izole edilen *B. melitensis* izolatlarında *rpoB* geni tek nükleotit polimorfizm (SNP) analizi ile değerlendirilmişti. Ülkemizde daha once hayvan enfeksiyonlarından izole edilen *B. melitensis* suşlarında SNP analizi ile genotiplendirme değerlendirilmiş değildir. Bu çalışmada, *B. melitensis* saha suşlarının moleküler tiplendirmesinde *rpoB* geninin SNP ile analizinin değerlendirilmesi ve epidemiyolojik çalışmalarda kullanılabilecek uygun tiplendirme yönteminin tanımlanması amaçlanmıştır. Araştırmada referans suşlar ile birlikte Marmara, Ege, Akdeniz, Orta Anadolu ve Güneydoğu Anadolu bölgelerimizden izol edilmiş toplam 32 *B. melitensis* saha suşu çalışıldı. *rpoB* geni sekanslarının değerlendirilmesi sonrasında *B. melitensis* saha suşlarında üç moleküler tip tanımlandı; SNP tip 1, SNP tip 2 ve SNP variant tip 2. SNP analiz tekniği *B. melitensis* suşlarını biyovar özelliklerinden bağımsız bir şekilde moleküler olarak tiplendirmektedir. Çalışmamız, epidemiyolojik olarak SNP analizinin *B. melitensis* suşlarını tanımlamada klasik yönteme göre yüksek ayrım gücüne sahip olduğunu göstermektedir. Sonuç olarak SNP analizinin *B. melitensis* izolatları arasındaki ilişkiyi saptayacak faydalı bir moleküler epidemiyolojik metot olduğu ve bu yöntemin brusellozun kontrolü ve etkili bir surveyansına yönelik hazırlanacak ulusal bir brusella veritabanının oluşturulması halinde katkı sağlayacağı görüşüne varılmıştır.

Anahtar sözcükler: *Brucella melitensis, Moleküler tiplendirme, Tek nükleotit polimorfizmi, Sekans analizi*

İletişim (Correspondence)
+90 414 3183918
serdenlig@harran.edu.tr

INTRODUCTION

An important component for recognition and control disease outbreaks is identification of the reservoir and mode of transmission of the infectious agents involved. This often requires the establishment of relationship among the pathogens isolated during the outbreak. Because each species of microorganism comprises almost limitless number of strains, identification of an organism to the species level is not sufficient for most of molecular epidemiological works. Strain typing, which is the method mostly used in order to establish a relationship among organisms belonging to the same species, is generally required.

Genotypic characterization is important for patient management and may be used to trace sources of *Brucella* infection and to distinguish between relapse and re-infection [1-3]. If re-infection is observed, the patient should be further educated to avoid consumption of unpasteurized dairy products and contact with infected animals. If it relapses, treatment options may need to be reconsidered. Hence, control measures can be implemented very early and further spread of the disease may be prevented [4,5].

Rapid and accurate typing procedures are crucial for epidemiologic surveillance, investigation of outbreaks, and follow-up of a control program. Many molecular typing methods commonly used for the subtyping of isolates of other bacterial species are not appropriate for routine typing of *Brucella* strains, and none has proven to be fully satisfactory for epidemiological trace-back investigations of brucellosis [5,6]. Insertion sequence based typing and polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) are the examples of such methods [7,8]. In recent studies investigating reference and clinical *Brucella* isolates, the utility of the *rpoB* gene, encoding the DNA-dependent RNA polymerase β subunit (RNAP), for genotyping *Brucella* strains via a single nucleotide polymorphism (SNP) based method were examined [9-11].

Brucellosis is endemic and approximately 10.000 human brucellosis cases are reported annually in Turkey. The reported incidence is 150 cases per 1 million inhabitants [12]. Previous studies conducted in different regions of Turkey found that human brucellosis was almost exclusively caused by *B. melitensis,* accounting for 99% of the total cases, and *B. melitensis* biovar 3 was the biovar most frequently isolated in humans [13-16]. High resolution typing of *Brucella* isolates is important for epidemiological surveillance; investigation of outbreaks in regions of both low and high endemicity; and distinguishing cases of human reinfection from relapse; thereby influencing clinical therapeutic decisions [1]. In a recent publication, the MLVA-16_{UPSUD} assay was applied to investigate epidemiological relationships for the first time, among human brucellosis isolates collected from all regions of Turkey [17]. But, the molecular typing of *Brucella* strains isolated especially from animal infections has not been sufficiently investigated in Turkey. The aim of this study was to evaluate to SNP analysis of *rpoB* gene using field isolates of *B. melitensis* from animals and human beings from Turkey in order to assess the value of this analysis for epidemiological surveillance.

MATERIAL and METHODS

Brucella Strains

In this study, 32 *B. melitensis* field strains isolated various provinces from Marmara (Canakkale, Yalova, Kirklareli, Istanbul, Edirne, Bilecik, Kocaeli), Aegean (Afyon), Mediterranean (Adana), Central Anatolia (Ankara, Cankiri, Aksaray, Konya, Eskisehir, Kırsehir) and Eastern Anatolia (Erzurum) regions of Turkey were investigated together with 3 control reference strains. *B. melitensis* biovar 1 (16M; ATCC 23456), biovar 2 (63/9; ATCC 23457), and biovar 3 (Ether; ATCC 23458) were used as reference strains. All *Brucella* isolates were biotyped using the classical biotyping procedures described by Alton *et al*.[18], i.e. CO_2 requirement, H_2S production, urea hydrolysis, agglutination with monospecific antisera, dye sensitivity and phage typing.

Polymerase Chain Reaction

Bacterial nucleic acid was extracted from cultures by magnetic-particle technology on the BioRobot EZ1 (QIAGEN GmbH, Hilden, Germany) instrument. Two PCR tests were carried out to amplify two specific regions of the *rpoB* gene. The regions of the *B. melitensis* genome covering the biovar reference strain specific codons (Cd); Cd 629, Cd 985, Cd 1249, Cd 1309 were amplified using primers as described by Marianelli *et al*.[19]. Primers rBseq7 and - 4143rB gave a 1254 bp-long fragment and the primers +1418rB and rBseq5 gave a 738 bp-long fragment. PCR amplifications were carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany) using Quantitect SYBR Green PCR mix (QIAGEN GmbH, Hilden, Germany). Amplifications were initiated by denaturing the sample for 15 min at 94°C, followed by 40 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 90 s. After the last cycle, samples were incubated for an additional 10 min at 72°C. Three micro liters of each reaction mixture were analyzed by electrophoresis through a 1% agarose gel.

rpoB Sequencing

All PCR products were purified using the High Pure PCR Products Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and directly sequenced with the ABI PRISM 310 Genetic Analyzer equipment using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc, Piscataway, USA). Primers rBseq7, rBseq9, +1418rB [9] and an additional designed primer, rBseq8-AS:

(5'-TGGATCTTGCGCTTCACAG-3'), were used for sequencing. The electropherogram were assembled by Vector NTI v5.1 (InforMaxTM InvitrogenTM life science software, Frederick, MD 21704, USA) software based on the published *rpoB* sequence of *B. melitensis* 16M. All consensus sequences were generated, and then compared to the published *B. melitensis* 16M *rpoB* gene for detection of nucleotide diversity [19].

RESULTS

According to *rpoB* sequencing results 3 distinct genotypes (SNP type 1, type variant 2 and type 2) were recognized *(Table 1)*. Four strains (27, 30, 184 and 212) had no miss-sense mutations at Cd 629, Cd 985, Cd 1249 and Cd 1309 showing a genotype identical to that of the *B. melitensis* biovar 1 reference strain 16M. Except one strain(212), rest of the three isolates were classically typed as biovar 3 at the *Brucella* Laboratory of Pendik Veterinary Control Institute *(Table 1)*.

Twelve strains had miss-sense mutations at Cd 629, Cd 985 and Cd 1309 with a genotype identical to the *B. melitensis* biovar 2 reference strain 63/9. In the case of the remaining 16 strains, miss-sense mutations were found only at Cd 629 and Cd 1309, and no miss-sense mutation was found at Cd 985. Therefore these strains are different from any of the reference *Brucella* strains. However given their apparent closer relationship to the *B. melitensis* biovar 2 reference strain sequence with regard to the *rpoB* gene analysis they were named as variant 2 *(Table 1)*. Although most isolates were identified classically as biovar 3, no isolate was found to share the *rpoB* genotype of the biovar 3 reference strain Ether.

DISCUSSION

Phenotypic characteristics (e.g. biotyping, serotyping, antimicrobial susceptibility profiles) historically have been used to type strains, However, these methods often have disadvantages because of their inability to consistently discriminate between different strains, labor intensity, or lack of reproducibility. In contrast, certain molecular methods do not have these limitations and have increased strain typing capabilities.

The distribution of biovars may vary between localities or even within a locality and this can provide useful epidemiological information. However, in many instances a single biovar predominates and this makes the tracing of sources of infection difficult. There have been some limited studies examining the biovars associated with Turkish *B. melitensis* isolates. Almost all *B. melitensis* strains are reported to be biovar 3 or 1 as not 2. From January 1996 to May 2002, 243 brucellosis patients were admitted to Dokuzoguz and colleague's clinic. *Brucella spp.* were isolated from blood cultures of 54 patients out of 243 (22%). Eighty-three percent of the isolates were speciated as *B. melitensis,* and 17% as *B. abortus.* Among *B. melitensis* species, 35 (78%) were identified as biovar 3, and 10 (22%) as biovar 1 [14]. In the studies between the years 2002-2005, 41 out of 50 *B. melitensis* isolates from Central Turkey were demonstrated as biotype 3, predominantly [13,15]. In a study, 162 human brucella isolates collected from different parts of Turkey during an 8-year period (from 2001 to 2008) were evaluated by bacteriological, epidemiological, and molecular typing (MLVA-16) characteristics. A total of 162 *Brucella* isolates were identified as *B. melitensis* biovar 3 (161 isolates) and *B. abortus* biovar 3 (one isolate) [17]. In our recently published reports, 94 human *Brucella* isolates collected also in an 8-year period from the beginning of 2002 to the end of 2009 throughout Turkey were investigated. The isolates were identified at species and biovar levels by conventional methods. Except one isolate, all were identified as *B. melitensis* biovar 3 [16]. These findings indicated that *B. melitensis* biovar 3 is predominant biovar responsible for human brucellosis in Turkey.

In our previous study, we described the first molecular characterization of *Brucella* isolates in Turkey examining mutations by using SNP analysis of the *rpoB* gene region to type *B. melitensis* strains isolated from our adult and pediatric patients. Sixty two *B. melitensis strains of human and animal origin* isolated from various regions of Turkey were used in this study. It was found that 52 *B. melitensis* isolates represented biovar 3 and 10 isolates represented biovar 1 by using conventional biotyping procedures. Eight strains, which had no miss-sense mutations at Cd 629, Cd 985, Cd 1249 and Cd 1309 were identified as genotype 1 (shared with the biovar 1 reference strain). Six strains that had miss-sense mutations at Cd 629, Cd 985 and Cd 1309 were identified as genotype 2 (shared with the biovar 2 reference strain). In the other 48 strains, miss-sense mutations were found only at Cd 629 and Cd 1309, and no miss-sense mutation was found at Cd 985. Therefore, those strains were identified as variants of genotype 2 [10]. In the present study, SNP molecular method and conventional biotyping procedures were applied to 28 animal and 4 human isolates of *B. melitensis* obtained from five regions of Turkey. However, there were still same inconsistency between results of SNP and classical biotyping methods.

SNP analysis for *Brucella* genotyping was found to be promising for a couple of reasons: if one has the right potential molecular marker for genotyping, SNP analysis is easy to perform. Actually, SNP technique is characterized the strains at the molecular levels independently from *B. melitensis* biovars. The results of *rpoB* sequencing by SNP analysis were seemed to be very useful while comparing to biovar analysis by conventional methods. Because of the uncontrolled animal movements in borders of Turkey, it is always possible some exogeneous *B. melitensis* strains might enter into country. In these circumstances, SNP

Table 1. *rpoB sequencing results of B. melitensis strains according to single nucleotide polymorphism method in the study group*
Tablo 1. *Çalışma grubunda tek nükleotit polimorfizm yöntemine gore B.melitensis suşlarının rpoB dizileme sonuçları*

Strain	Provience*	District	Village	Origin	Biovar	SNP type	Codon Residue										
							495-628	629	630-712	965-984	985	986-1062	1217-1248	1249	1250-1308	1309	1310-1364
							•	GCG	•	•	GCC	•	•	ATG	•	CTG	•
16M	-	-	-	Reference	1	1	•	•	•	•	•	•	•	•	•	•	•
63/9	-	-	-	Reference	2	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
Ether	-	-	-	Reference	3	3	•	•	•	•	•	•	•	ATA	•	•	•
184	Canakkale	Gelibolu	Findikli	Goat	3	1	•	•	•	•	•	•	•	•	•	•	•
283	Yalova	Termal	Akkoy	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
281	Kirklareli	Kofcar	Y.Kanara	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
212	Kirklareli	Pinarhisar	Y.Kasabasi	Goat	1	1	•	•	•	•	•	•	•	•	•	•	•
234	Erzurum	Askale	Gorkaynak	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
293	Istanbul	Silivri	Beyciler	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
227	Erzurum	Askale	Kukurtlu	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
236	Erzurum	Cat	Kaplica	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
229	Erzurum	Cat	Tuzlatasi	Cattle	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
285	Adana	Yumurtalik	Yahsiler	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
235	Erzurum	Olur	Kaledibi	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
230	Erzurum	Merkez	Tepekoy	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
248	Ankara	Ayas	Hacimemmi	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
260	Edirne	Merkez	Tayakadin	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
259	Canakkale	Gelibolu	Namaztepe	Sheep	1	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
280	Edirne	Havsa	Kabagac	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
F7	Istanbul	Hadımkoy	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
282	Bilecik	Bozuyuk	Kandilli	Lamb	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
38	Aksaray	Merkez	Sultanhani	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
F9	Istanbul	Omerli	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
162	Aksaray	Merkez	-	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
6	Ankara	Bala	Merkez	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
296	Cankiri	Eldivan	Saritarla	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
61	Afyon	Goynuk	B.Cobanlar	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
255	Kirsehir	Merkez	M.Uzunali	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
30	Konya	Merkez	Saricalar	Sheep	3	1	•	•	•	•	•	•	•	•	•	•	•
240	Eskisehir	Merkez	Satilmisoglu	Sheep	1	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
298	Cankiri	Cerkes	Orenli	Sheep	1	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
284	Bilecik	Osmaneli	Yesilcimen	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
27	Istanbul	Catalca	Incegiz	Sheep	3	1	•	•	•	•	•	•	•	•	•	•	•
4988	Kocaeli	Golcuk	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
6130	Istanbul	Kavakli	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•

** Provience from Marmara (Canakkale, Yalova, Kirklareli, Istanbul, Edirne, Bilecik, Kocaeli), Aegean (Afyon), Mediterranean (Adana), Central Anatolia (Ankara, Cankiri, Aksaray, Konya, Eskisehir, Kırsehir) and Eastern Anatolia (Erzurum) regions of Turkey*

technique may be used as a epidemiological tool in the light of national database in Turkey. Moreover, the SNP pattern of a *B. melitensis* strain proved to be stable by comparing both *Brucella* strains isolated from different patients within the same outbreak, and strains from the same patient before first-line therapy and after relapse despite antibiotic treatment [1,5].

SNP typing method is not congruent with phenotypic observations. Upon re-analysing the whole sample set one would expect to see *rpoB* SNP typing forming its own subgroups/genetic variants, that would not be homogenous with observed phenotypes [20]. As molecular typing is much more robust and it is not subject to interpretation when comparing to classical typing, outputs are repeatable and transferable across many laboratories. This is one of the major driving factors behind making molecular typing a confirmatory tool used alongside phenotypic typing.

In conclusion, our results provide proof of the different characteristics of SNP in genotyping of *B. melitensis* isolates that could not be differentiated by conventional microbiological methods. *rpoB* SNP typing can be used as a molecular epidemiological tool to determine relationships for *B. melitensis* isolates and might provide effective surveillance and control mechanisms in brucellosis in Turkey.

REFERENCES

1. Al Dahouk S, Hagen RM, Nockler K, Tomaso H, Wittig M, Scholz HC: Failure of a short-term antibiotic therapy for human brucellosis using ciprofloxacin. A study on *in vitro* susceptibility of *Brucella* strains. *Chemotherapy,* 51, 352-356, 2005.

2. Kattar MM, Jaafar RF, Araj GF, Le Fleche P, Matar GM, Rached RA: Evaluation of a multilocus variable-number tandem-repeat analysis scheme for typing Human *Brucella* isolates in a region of brucellosis endemicity. *J Clin Microbiol*, 46, 3935-3940, 2008.

3. Marianelli C, Petruccab A, Pasquali P, Ciuchini F, Papadopouloub S, Cipriani P: Use of MLVA-16 typing to trace the source of a laboratory-acquired *Brucella* infection. *J Hosp Infect*, 68, 274-276, 2008.

4. Al Dahouk S, Nöckler K, Hensel A, Tomaso H, Scholz HC, Hagen RM: Human brucellosis in a nonendemic country: A report from Germany, 2002 and 2003. *Eur J Clin Microbiol Infect Dis*, 24, 450-456, 2005.

5. Al Dahouk S, Le Flèche P, Nöckler K, Jacques I, Grayon M, Scholz HC: Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Meth*, 69, 137-145, 2007.

6. Whatmore AM, Shankster SJ, Perrett LL, Murphy TJ, Brew SD, Thirlwall RE: Identification and characterization of variable-number tandem-repeat markers for biotyping of *Brucella* spp. *J Clin Microbiol*, 44, 1982-1893, 2006.

7. Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S: Molecular characterization of *Brucella* strains isolated from marine mammals. *J Clin Microbiol,* 38, 1258-1262, 2000.

8. Al Dahouk S, Tomaso H, Berninghoff PE, Splettstoesser WD, Scholz HC, Neubauer H: Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP). *Crit Rev Microbiol*, 31, 191-196, 2005.

9. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R: Molecular characterization of the *rpoB* gene in *Brucella* species: new potential molecular markers for genotyping. *Microbes Infect*, 8, 860-865, 2006.

10. Sayan M. Yumuk Z, Bilenoğlu O, Erdenliğ S, Willke A: Genotyping of *B. melitensis* by *rpoB* gene analysis and reevaluation of conventional serotyping method. *Jpn J Infect Dis*, 62, 160-163, 2009.

11. Sayan M: *Brucella melitensis* biovar 2 can not be detected by serological method in Turkey. Letter to Editor. *Turk Klin Tip Bilim Derg*, 29, 1351-1352, 2009.

12. Turkish Statistical Institute: Address-based population register system, December 31, 2008 census. Turkish Statistical Institute, Ankara, Turkey. www.turkstat.gov.tr, May, 2012.

13. Bodur H, Balaban N, Aksaray S, Yetener V, Akıncı E, Colpan A: Biotypes and antimicrobial susceptibilities of *Brucella* isolates. *Scand J Infect Dis*, 35, 337-338, 2003.

14. Dokuzoguz B, Ergönül O, Baykam N, Esener H, Kiliç S, Celikbas A: Characteristics of *B. melitensis* versus *B. abortus* bacteraemias. *J Infect,* 50, 41-45, 2005.

15. Tanyel E, Coban AY, Koruk ST, Simsek H, Hepsert S, Cirit OS: Actual antibiotic resistance pattern of *Brucella melitensis* in central Anatolia. An update from an endemic region. *Saudi Med J*, 28, 1239-1242, 2007.

16. Sayan M, Kılıc S, Uyanık MH: Epidemiological survey of rifampicin resistance in clinic isolates of *Brucella melitensis* obtained from all regions of Turkey. *J Infect Chemother*, 18, 41-46, 2012.

17. Kilic S, Ivanov IN, Durmaz R, Bayraktar MR, Ayaslioglu E, Uyanik H: MLVA genotyping of human *Brucella* isolates from Turkey. *J Clin Microbiol*, 49, 3276-3283, 2011.

18. Alton GG, Jones LM, Angus RD, Verger JM: Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris,1988.

19. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R: Genetic bases of the rifampicin resistance phenotype in *Brucella* spp. *J Clin Microbiol*, 42, 5439-5443, 2004.

20. Whatmore AM, Perret LL, MacMillan A: Characterization of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol,* 7, 34, 2007. DOI: 10.1186/1471-2180-7-34