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RESEARCH ARTICLE

The mismatched isolation of Brucella strains from nomic hosts

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Primer konaklardan izole edilen uyumsuz Brucella türleri

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Öz

Amaç: Bu çalışmada Kafkas Üniversitesine ait Brucella suş koleksiyonunun bir bölümü çeşitli yöntemler ile değerlendirilmiştir.

Gereç ve Yöntem: Suşların orjini abort hikayesi olan sığır ve koyunlar oluşturudu. Otuz adet *Brucella* suşu (16'sı koyun ve 14'ü sığır orjinli) rastgele seçildi ve bu çalışmaya dahil edildi. Standardize fenotipik karakterizasyona ilaveten suşların moleküler tiplendirmesi için cins spesifik Real Time PCR ve tür spesifik Bruce-Ladder PCR uygulandı.

Bulgular: Onaltı koyun örneğinin 15'i *B. melitensis* ve 14 sığır örneğinin 13'ü *B. abortus* olarak identifiye edildi. İlginçtir ki, koyun aborte fötusten izole edilen bir suş *B. abortus* ve sığırdan izole edilen bir suş *B. melitensis* olarak tanımlandı. Bununla birlikte, koyun fötusundan elde edilen bir suş bütün fenotipik özellikleri yönünden *Brucella* cinsine benzer karakterde olmasına rağmen genotipik olarak (ne Real Time PCR ne de *Bruce-Ladder* PCR) *Brucella* spp. olarak doğrulanamadı ve ileride karakterize edilmek üzere *Ochrobactru*m spp. şüpheli olarak saklandı.

Öneri: Bu çalışma belli konaklar arasında çapraz *Brucella* infeksiyonunun sıradışı olarak nitelendirilebileceğini fakat zamanla bunun olağan olabileceğini göstermektedir. Bu şekildeki çalışmalar, *Brucella* cinsinin patojenitesini, konak spesifitesini ve gelişimini daha iyi anlamaya yardımcı olacaktır.

Anahtar kelimeler: *Brucella,* saha suşlar, sğır, koyun, moleküler karakterizasyon

Abstract

Aim: A small part of field *Brucella* strain collection of Kafkas University was evaluated by different methods in this study.

Materials and Methods: The strains were originated from cattle and sheep which had a history of abortion. Thirty field *Brucella* strains (16 were originated from sheep and 14 were from cattle) were randomly selected and included in the study. In addition of standardized phenotypical characterization, genus specific real-time PCR and species specific *Bruce-Ladder* PCR were conducted for molecular typing.

Results: Out of 16 sheep and 14 cattle samples 15 and 13 were identified as *B. melitensis* and *B. abortus*, respectively. Interestingly one strain was characterized as *B. abortus* isolated from aborted sheep foetus and the other as *B. melitensis* from aborted cattle milk sample. Furthermore one strain from aborted sheep foetus was unable to be confirmed as *Brucella* spp. by genotypically (neither by real-time PCR nor *Bruce-Ladder* PCR) though all phenotypic features were similar to *Brucella* genus and kept under strict conditions for future characterization on suspicion of *Ochrobactrum* spp.

Conclusion: These report alleges that *Brucella* cross-infection among certain hosts ceased to be extraordinary and gained an usual face over time. These kind of efforts would help in further understanding the evolution, host specificity and pathogenicity of the genus *Brucella*.

Keywords: *Brucella*, field strains, cattle, sheep, molecular characterization

Introduction

Brucella spp. are the causative agents of brucellosis, an infectious disease that affects various species of animals and can be transmitted to humans through direct contact with infected animals, indirectly by the ingestion of raw milk products, and during the handling of strains or infected material in the laboratory (Otlu et al 2008, Aras et al 2009). *Brucellosis* is endemic in Turkey and causes severe economic losses in livestock. Studies in various parts of the country indicate that the disease is widespread among cattle and sheep populations (İyisan et al 2000, İça et al 2014). *Brucellosis* in farm animals have been reported in East Anatolian Region in where Kars is the most important city with the huge animal population and substantial amount cases of given disease (Ünver et al 2006, Şahin et al 2008, Çelebi et al 2011, Büyük et al 2011).

Currently the *Brucella* genus consists of 12 species with validly published names, based on host preferences, phenotypic differences and pathogenesis (Godfroid et al 2011, Whatmore et al 2014, Scholz et al 2016). While a broad host range generally exists for *Brucella* species, *Brucella* infection follows a very strict, host-related hierarchy of pathogenicity (Adam 2002). It is known that the principal species of *Brucella* in cattle and sheep are *B. abortus* and *B. melitensis,* respectively. Some individual biotypes has limited scale and cross-infection among host species is very rare in natural condition. Inappropriate management may allow the disease to be transferred to a heterologous host such as cattle and sheep. Unexpected isolation of related strains from both hosts are likely and may also causes occasional infections in animals resulting in abortions (Ocholi et al 2005, Büyükcangaz and Şen 2007, Büyük et al. 2011, Erdenliğ Gürbilek et al 2014).

In this study, a small part of field *Brucella* strain collection of Kafkas University was evaluated by genus specific Real-Time PCR and species specific Bruce-Ladder PCR in addition of standardized phenotypical characterization.

Materials and Methods

Bacterial isolation and identification

In this study, field *Brucella* strains were evaluated by bacteriological and molecular methods. For this purpose a small part of culture collection of Microbiology Department of Veterinary Faculty, Kafkas University, Kars (Turkey) was used. The collection was developed from the samples taken from the cattle and sheep which had a history of abortion and human who were in a febrile disease in Kars region. The collection totally includes 259 *Brucella* strains that were composed 115 sheep, 139 cattle and 5 human isolates. Thirty field strains of *Brucella* sp. were randomly selected and included in the study as originated 16 isolates from sheep (all were from aborted foetus) and 14 from cattle (4 were from aborted foetus, 5 from milk and 5 from vaginal secret).

Bacterial isolation was made from the samples such as aborted foetus, milk and vaginal secret were duly taken and handed meticulously in accordance with the report of Büyük and Şahin (2011). In brief, milk and vaginal secret samples were first enriched in Farrel Broth for 5-7 days. Then the content was plated on Brucella Selective Agar (Oxoid, UK) plates containing Brucella selective supplement (Oxoid, UK). The fetal tissue samples were directly plated on selective agar plates. All plates were incubated at atmospheric condition with and without 5-10% CO_2 at 37°C for 5-7 days.

For the identification of the genus and/or species of *Brucella*, cultural and biochemical tests, including colony and microscopic morphology, catalase, oxidase, urease tests, H_2S production, Tbilisi phage susceptibility and CO_2 requirement were performed (Alton et al 1988).

Molecular typing

For molecular typing, genus-specific real-time PCR and species-specific BruceLadder-PCR were conducted. Genomic DNA was extracted from pure cultures of strains through the commercial extraction kit (Qiagen, Germany) in line with the manufacturer's recommendations.

Real-time was carried out with Freeze-Dried Reagent Kit specific for Brucella genus. The kit is specially adapted for amplification in glass capillaries using the Idaho Technoogy's Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) Instruments and TaqMan probes. The kit includes negative control (NC), unknown sample assays, positive control (PC) vials, 2X Reconstitution Buffer and Reagent Grade Water. Each reagent vial contains enough master mix for two reactions. 20 µL of 2X Reconstitution Buffer was added on to the NC, unknown and PC reagent vials, respectively. Twenty µL of Reagent Grade Water was added on to the NC and PC reagent vials. Twenty µL of sample (template DNA) was added on to the unknown reagent vial as well. After vortex and centrifugation of all tubes, 19 µL of the hydrated mixture was transferred into a capillary in duplicate. Once the samples were prepared the protocols R.A.P.I.D. 7200 was followed in LightCycler 2.0 Instrument.

For confirmation of Brucella species Bruce-Ladder PCR was carried out by modified from Garcia-Yoldi et al. (2006) (Table 1).

Brucella reference strains (*B. abortus* biotype 1 (544) and *B. melitensis* biotype 1 (16M)) and negative control (*E. coli* OP50) were used in both PCR. The amplified products were resolved by electrophoresis using a 1.5% agarose gel and followed by staining with ethidum bromide.

Results

The reaction content and *Brucella* species-specific Bruce-Ladder PCR conditions and sources and results of samples tested by conventional and molecular methods were displayed in Table 1 and Table 2. Results of *Brucella* genus-specific real-time PCR and *Brucella* species-specific PCR (Bruce-Ladder-PCR) in Figure 1A ad 1B. All 30 field strains were found as *Brucella* spp. by cultural and biochemical tests. Out of 16 sheep samples and 14 cattle originated samples 15 and 13 were identified as *B. melitensis* and *B. abortus*, respectively. Interestingly one strain (#3) was characterized as *B. abortus* isolated from aborted sheep foetus and one (#19) as *B. melitensis* from aborted cattle milk sample

All strains previously identified as *Brucella* spp. by conventionally were future confirmed as *Brucella* spp. with the genus-specific real-time PCR. The expression level of Target 1 region related to the all certain strains of *Brucella* genus was measured using an average crossing point (Cp) of each sample that was run in duplicate. And Cp values were found with a range of 13,69 and 14,44 when compared the Cp value of PC as 21,59. The NC and one strain (#28) didn't give any significant Cp values.

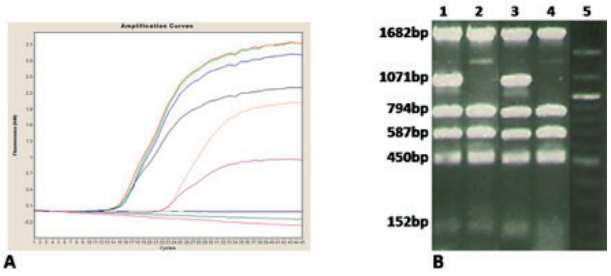

Figure 1 (A-B): Results of *Brucella* genus and species-specific PCR (Bruce-Ladder PCR). (A) Results of Brucella genus-specific real-time PCR, blue and green curves; field *B. abortus* (#22), red and black curves; field *B. melitensis* (#173), pink and dark green; negative control (NC), dark blue and grey; uncharacterized strain (#312); pale red and purple; positive control (PC). (B) Results of *Brucella* species-specific PCR (Bruce-Ladder-PCR), lane 1; positive control (*B. melitensis* 16M), lane 2; positive control (B. abortus 544), lane 3; field *B. melitensis* isolate, lane 4; field B. abortus isolate, lane5; molecular weight stand (100 bp DNA ladder, New England BoiLab).

Fifteen isolates of *B. abortus* and 13 of *B. melitensis* presented a common Bruce-Ladder PCR profile expected for field strains, excluding the vaccine strains, with the several band sizes of 1682, 794, 587, 450 and 152bp, except the size of 1071bp that is specific for only *B. melitensis* (Figure 1B). Interestingly one strain (#19) of cattle and one strain (#3) of sheep presented unusual Bruce-Ladder PCR band profiles specific for *B. melitensis* and *B. abortus*, respectively.

Furthermore one strain (#28) from aborted sheep foetus was unable to be identified as *Brucella* spp. by genotypically (neither by real-time PCR nor Bruce-Ladder PCR) though all phenotypic features are very similar to *Brucella* genus. This strain was suspected to be one of *Ochrobactrum* species and kept under strict conditions for future characterization.

Discussion

In comparison with the broad host range of *Brucella* species exist, the infection has very strict host specificity. Thus, goats are the natural hosts of *B. melitensis* and sheep are preferred hosts of the pathogen, and cattle are the natural hosts of *B. abortus*. In some cases, the certain *Brucella* species cross the border from its natural host, transmit to the others and lead to exceptional abortion in unfavorable hosts (Ocholi et al 2005). Contrary to this, the isolation of *B. melitensis* from cattle has been reported constantly during a decade (Refai 2002) and it substituted for *B. abortus* in cattle in South Europe (Godfroid and Kasbohrer 2002). Similar habit was reported for *B. abortus* as a causative agent of sheep brucellosis in both natural and experimental conditions in some regions (Shaw 1976, Zowghi and Ebadi 1988, Ocholi et al 2005, Shareef 2006).

Though it was scarce of cross-infection in interspecies it is possible due to the common use of shelters and pastures by different animal like in the reported study. This kind of mismatched isolation was first reported virtually by Büyükcangaz and Şen (2007) in cattle in Turkey and the causative agent was typed as *B. melitensis* biotip 3. A different study

Table 1. Brucella species-specific BruceLadder-PCR conditions.

PCR reagents		PCR reaction thermal conditions and cycles			
Reagent	Vol.(µL)	Cycle element	Temp (°C)	Time (sec)	# Cycles
PCR buffer (x10)	2.5	Initial denaturation	95	35	1
dNTP mix (2.5 mM)	2.5	Denaturation	95	35	
MgCl2 (50 mM)	1.5	Annealing	63	45	35
Bruceladder Primer mix (10 pmol)	2	Elongation	72	180	
Taq DNA polymerase (5u/ul)	0.2				
Water	15.3	Final elongation	72	360	1
Templete DNA (10 ng/ul)	1				

Table 2. Sources and results of samples tested by conventionally and molecular methods.

Sample number	Origin	Sample type	Time originally isolated	Phenotypic characterization						Genotyping c haracterization		Final result
				CO_2 req.	H_2S	Catalase	Oxidase	Urease	Tbilisi Phage	Real-time PCR	Bruce-Ladder PCR	
#1	Sheep	Foetus	2005	-	--	+	+	+	-	+	+	*B. melitensis*
#2	Cattle	Foetus	2005	+	+	+	+	+	+	+	+	*B. abortus*
#3	Sheep	Foetus	2005	+	+	+	+	+	+	+	+	*B. abortus*
#4	Sheep	Foetus	2006	-	-	+	+	+	-	+	+	*B. melitensis*
#5	Sheep	Foetus	2007	-	-	+	+	+	-	+	+	*B. melitensis*
#6	Cattle	Foetus	2007	+	+	+	+	+	+	+	+	*B. abortus*
#7	Sheep	Foetus	2007	-	-	+	+	+	-	+	+	*B. melitensis*
#8	Sheep	Foetus	2008	-	-	+	+	+	-	+	+	*B. melitensis*
#9	Sheep	Foetus	2009	-	-	+	+	+	-	+	+	*B. melitensis*
#10	Sheep	Foetus	2009	-	-	+	+	+	-	+	+	*B. melitensis*
#11	Sheep	Foetus	2009	-	-	+	+	+	-	+	+	*B. melitensis*
#12	Cattle	Milk	2009	+	+	+	+	+	+	+	+	*B. abortus*
#13	Cattle	Milk	2009	+	+	+	+	+	+	+	+	*B. abortus*
#14	Cattle	V. secret	2009	+	+	+	+	+	+	+	+	*B. abortus*
#15	Cattle	V. secret	2009	+	+	+	+	+	+	+	+	*B. abortus*
#16	Cattle	Milk	2008	+	+	+	+	+	+	+	+	*B. abortus*
#17	Cattle	V. secret	2008	+	+	+	+	+	+	+	+	*B. abortus*
#18	Cattle	Milk	2010	+	+	+	+	+	+	+	+	*B. abortus*
#19	Cattle	Milk	2010	-	-	+	+	+	-	+	+	*B. melitensis*
#20	Sheep	Foetus	2010	-	-	+	+	+	-	+	+	*B. melitensis*
#21	Cattle	V. secret	2010	+	+	+	+	+	+	+	+	*B. abortus*
#22	Cattle	V. secret	2010	+	+	+	+	+	+	+	+	*B. abortus*
#23	Sheep	Foetus	2011	-	-	+	+	+	-	+	+	*B. melitensis*
#24	Cattle	Foetus	2011	+	+	+	+	+	+	+	+	*B. abortus*
#25	Sheep	Foetus	2011	-	-	+	+	+	-	+	+	*B. melitensis*
#26	Sheep	Foetus	2012	-	-	+	+	+	-	+	+	*B. melitensis*
#27	Cattle	Foetus	2013	+	+	+	+	+	+	+	+	*B. abortus*
#28	Sheep	Foetus	2013	-	-	+	+	+	-	-	-	*Uncharacterized*
#29	Sheep	Foetus	2013	-	-	+	+	+	-	+	+	*B. melitensis*
#30	Sheep	Foetus	2014	-	-	+	+	+	-	+	+	*B. melitensis*

DİP: Deneme intraperitoneal, KİP: Kontrol intraperitoneal, DİM: Deneme immersiyon, KİM: Kontrol immersiyon

was from Kars region followed this with a report of case more than half *Brucella* isolates originated from cattle samples were representing as if PCR profile that was very similar to *B. melitensis* vaccine strain-Rev 1 (Unver et al 2006). Another exceptional case was reported by Büyük and Şahin (2011) for Kars strains of which one strain was isolated from aborted cattle foetus and identified as *B. melitensis* subsequently. This kind of reports are leaving away especially for B. melitensis as unusual causative agent of cattle brucellosis in Turkey (Sarisayin et al 1969, Erdenliğ Gürbilek et al 2014). Conversely sheep cases arising from B. abortus are more rarely in Turkey (Erdenliğ Gürbilek et al 2014) even it was reported many countries heretofore (Zowghi and Ebadi 1988, Ocholi et al 2005, Shareef 2006).

In this study one strain was characterized as *B. abortus* isolated from aborted sheep foetus and one as *B. melitensis* from aborted cattle milk sample. The results are close similarity and the explanation of this kind of unusual case can illustrated as in the studies reported before; having common habitats of animals, lack of an effective vaccine for cross-protection and fully optimized precaution method of disease and limited knowledge on the epidemiology in these host species (Pishva and Salehi 2008, Alvarez et al 2011, Büyük and Şahin 2011). But the important that it should not be ignored of reporting of *Brucella* cross-infection among certain hosts ceased to be extraordinary and gained an usual face over time.

An other interesting result is an unusual strain (#28) isolation from aborted sheep foetus that was unable to be confirmed as *Brucella* spp. by genotypically though all phenotypic features (aerobic, motile, oxidase and urease positive, Gram-negative rods) are very similar to *Brucella* genus. This strain was suspected to be one of Ochrobactrum species based on its features. This kind of close relationship has been reported between *B. melitensis* and *Ochrobactrum* spp. and has also led to misidentification (Elsaghir and James 2003). Besides this phenotypic features O. anthropi is one of the closest *Brucella* relatives based on DNA, rRNA, and protein analyses (Yanagi and Yamasato 1993, Velasco et al 1998, Cloeckaert et al 1999). It is known that some of *Ochrobactrum* strains can cause severe systemic infections in healthy human opportunistically and mimics *Brucella* infection (Kettaneh et al 2003, Ozdemir et al 2006). Despite all, *Ochrobactrum* species are unable to establish a chronic infection and have never associated with abortive animal infections. But it is still kept under strict conditions for future characterization and on probation as an unusual inductive agent for abortive animal infection.

Conclusion

In conclusion, owing to the ability of *Brucellae* to adapt to different conditions that leads to changes in epidemiologic features of *Brucella* species it would be advantageous to reveal strain diversity and illuminate complexity of interactions between the organism and animals. No doubt that we will continue to expand our strain pool and to full characterization of our remaining *Brucella* strains and thus will be able to make out these questions. These laborious approaches should help in further understanding the evolution, host specificity and pathogenicity of the genus *Brucella*. Thus the preliminary results would be beneficial and will attract attention of authorized persons to improve control measurements and even encourage them to contribute of production of novel polyvalent vaccine that is able to protect both hosts.

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