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RESEARCH ARTICLE

An Enzyme-Linked Immunosorbent Assay for Brucella Specific Antibody and Real-Time PCR for Detecting *Brucella Spp*. in Milk and Cheese in Şanlıurfa, Turkey

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A B S T R A C T

The objective of this study was to investigate the presence of anti-Brucella antibody and *Brucella spp*. DNA in cow, sheep and goat milk and in Urfa cheese collected from markets and bazaars in Şanlıurfa, located in southeast of Turkey. A total of 258 samples consisting of 178 raw milk (48 cow milk, 65 sheep milk and 65 goat milk) samples and 80 Urfa cheese samples were investigated. Anti-Brucella antibody was detected by indirect ELISA (i-ELISA), and the presence of *Brucella spp*. DNA was screened by real time Polymerase Chain Reaction (RT-PCR). 16.6% of the cow, 6.1% of the goat and 6.1% of the sheep milk and 16.25% of the cheese samples were found as positive for brucella antibodies by i-ELISA. The RT-PCR assay amplified Brucella DNA from 18.75, 7.6 and 6.1% cow, goat and sheep milk samples respectively. Brucella DNA was amplified from 22.5% cheese samples. The 11.2% and 13.9% of the samples were found as positive by i-ELISA and RT-PCR respectively. This study indicates that milk and milk products consumed in Şanlıurfa poses a risk to public health in terms of brucellosis. The combining usage of both i-ELISA and RT-PCR methods could lead to more reliable results to detect anti-Brucella antibody and *Brucella spp.* DNA from milk and cheese samples.

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INTRODUCTION

Brucellosis is a zoonotic disease, which represent a serious threat to public health and has a significant impact on the economy of the food industry. Brucellosis is seen in many regions in the world particularly in the Eastern Europe, Mediterranean Basin, Africa, Asia, the Middle East and in central and southern America (WHO, 2006; Guerra *et al*., 2015; Lor and Chukwu, 2015; Gul *et al*., 2014; 2015; Zhang *et al*., 2015; Abu-Seida *et al*., 2015).

Brucellosis is endemic in Turkey, and the disease is most commonly seen in people living in the central, east and south-east Anatolia. In human, 18 264 cases (24 cases/100 000) were reported in 2004. The human cases were 7703 in 2010 including 1268 cases from south-east Anatolia (Erbaydar *et al.*, 2012). The number of cases declined to 4403 in the year 2014 as the consequence of the disease control efforts (MoH, 2015). In spite of the decrease in the incidence of the disease, Turkey is still among the endemic countries (Erbaydar *et al*., 2012).

Several studies have shown that the consumption of raw milk and dairy products is the main source of the human Brucellosis in Turkey (Tekin *et al*., 2012; Güler *et al*., 2014). Although, there are some studies conducted in different provinces (Türütoğlu *et al*., 2003; Keskin and Tel, 2003; Alper and Nesrin, 2013; Arasoğlu *et al*., 2013; Kara *et al*., 2014; Kaynak-Onurdag *et al*., 2016), a comprehensive survey on the frequency of *Brucella spp*. in raw milk and dairy products in Turkey is lacking. In spite of the high livestock potential and very common consumption of cheese produced from raw milk in Şanlıurfa, to the best of the authors' knowledge, no study investigating the existence of the *Brucella spp*. in milk and cheese in Şanlıurfa is available. Therefore, the present

study was performed to determine the presence of *Brucella spp.* in milk of cow, sheep, and goat and in Urfa cheese, which is produced from raw milk, in order to evaluate the risk for both animal and human health.

MATERIALS AND METHODS

Milk and cheese samples: A total of 258 samples consisting of 178 raw (unpasteurized) milk and 80 home made fresh white (traditional, unpasteurized) cheese samples were collected between March 2015 and October 2015 from markets and bazaars in Şanlıurfa province. All samples were subjected to i-ELISA and RT-PCR.

Antigen preparation: A hot saline extract antigen (HSE) from a local *Brucella abortus* biotype 3 isolate (A53) was used following the method described by Barrouin-Melo *et al*. (2007), with minor modifications. Briefly, bacterial cells were harvested with 20 ml sterile PBS and inactivated by heat, washed three times, pellets were re-suspended in PBS and autoclaved. Then centrifuged and the supernatants stored at -20ºC until analysis.

Preparation of test samples

For milk: 20 ml of milk was chilled and centrifuged. The fatty layer was removed and 100 µl of the defatted milk or supernatant was used in ELISA as milk test samples.

For cheese: 2 g of triturated cheese and 8 ml dichloromethane were incubated at 50ºC for 30 min and centrifuged. 4 ml of the extract was evaporated, the oily residue was re-dissolved in 0.5 ml methanol, and 0.5 ml distilled water and 2 ml hexane. Centrifuged and 100 µl of lower methanolic-aqueous phase was used in ELISA as cheese test sample.

Positive and negative test samples: *Brucella* positive and negative sera were diluted 1:100 in milk and cheese test samples and used as positive and negative controls in the test.

Indirect ELISA (I-ELISA): Each well of the microplate (Nunc 269620, Denmark) was coated with 100 µl of diluted antigen. Then washed in PBS with Tween 20 (PBS/T) following the incubation for overnight at 4ºC. 100 µl of control and test samples were added and incubated for 1h at room temperature (RT). After washing, 100 µl of conjugate was added and incubated for 1h at RT. after washing again, 100 µl of substrate was added and incubated for 15 min before reading at OD_{450} nm with a microplate reader (VERSAmax 3.13/B2573).

Data analysis: Optical densities (OD_{450}) of the cheese and milk samples were determined three times and the OD value of the test sample was subtracted from the mean OD of negative test sample. The results were expressed as a percent positivity value (%P). When the result was more than 50, the test sample was considered as positive.

DNA isolation: DNA was extracted by using a 20 mg of cheese and 500 µl aliquot of milk. They were centrifuged at 13.000×g for 5 min after homogenization with 300 µl of lysing buffer and boiled for 15 min. The supernatants were collected into tubes and 40 µl PK and 200 µl of lysing buffer were added then kept overnight at 56ºC. Centrifuged at 13.000 × g for 15 min and 3 min with 500 µl isopropanol and 300 µl ethanol respectively, the supernatants were discarded, after drying 35 µl ddH_2O was added to each sample. The isolated DNA specimens were kept at -20°C until analysis.

The identification of *Brucella spp.* by Real-Time PCR: For the *Brucella spp.* DNA obtained by the nucleic acid isolation, *BCSP31* gene was examined and all samples were analyzed in duplicates by RT-PCR. Light Cycler FastStart DNA Master SYBR Green I kit (03003230001, Roche Diagnostics, Germany) was used according to manufacturer's instructions. The nucleic acid sequence indicated below (Table 1) was targeted for the identification (Queipo-Ortuño *et al*., 2005**)**. 2 µl (50 ng/µl) DNA templates were used for detection.

The thermal cycling was carried out in Rotor-Gene Q (Qiagen, Germany) instrument. *Brucella spp.* was obtained from Harran University Veterinary Faculty Microbiology Department as positive control and *ddH_2O* has also been added as negative control to the RT- PCR analysis.

RESULTS

Of the 48 cow's milk, 8 (16.6%) were found positive with i-ELISA whereas 9 (18.75%) samples were found positive with RT-PCR. Of the 65 goat's milk 4 (6.1%) were detected as positive with i-ELISA whereas 5 (7.6%) were found positive with RT-PCR. The same samples were found positive with both i-ELISA and RT-PCR. On the other hand, *Brucella spp*. DNA was detected with RT-PCR in one milk sample of cow and goat, which were found negative with i-ELISA. Anti-brucella antibodies were determined with ELISA and *Brucella spp*. DNAs were amplified with RT-PCR in the same 4 (6.1%) sheep milk samples. Out of 80 cheese samples, anti-brucella antibodies were determined with i-ELISA in 13 (11.2%) samples but *Brucella spp.* DNAs were detected with RT-PCR in 18 (13.9%) samples. Five cheese samples were found negative with i-ELISA but they were found positive with RT-PCR (Table 2). The analysis of melting peaks and curves can differentiate the unspecific products from the specific products. In this study, the target melting temperature (T_m) was about 86.5ºC. To confirm of *Brucella spp*. positivity, T_m of positive samples were compared with T_m of positive control (Fig.1).

Table I: The primer sets used for *Brucella spp.*

Brucella spp.	Primers	Ref.
(BCSP31) (Forward primer)	B4 5'-TGGCTCGGTTGCCAATATCAA-3'	Queipo-Ortun et al. (2005)
(BCSP31) (Reverse primer)	B5 5'-CGCGCTTGCCTTTCAGGTCTG-3'	Queipo-Ortun et al. (2005).

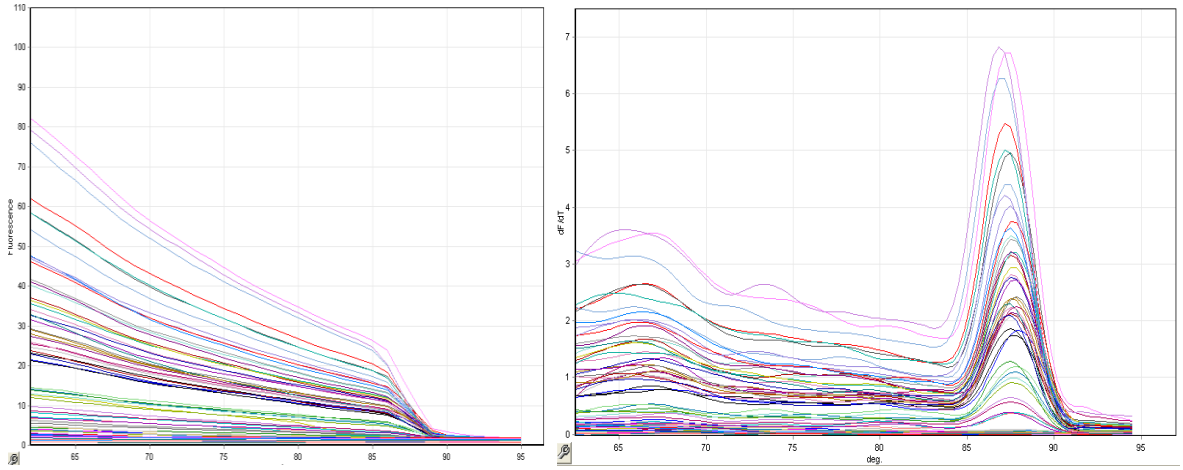

Fig. 1: Melting peaks (left) and curves (right) of *Brucella spp*. positive samples in milk and cheese samples and positive controls, Target T_m of about 86.5±2.5°C (right)

Table 2: *Brucella spp*. in Milk and Cheese Samples by ELISA and RT-PCR

		Number of positive samples	
Samples	n	ELISA	*Brucella spp*. by RT-PCR
Cow Milk	48	8 (16.6)	9 (18.75)
Goat Milk	65	4 (6.1)	5 (7.6)
Sheep Milk	65	4 (6.1)	4 (6.1)
Cheese	80	13 (16.25)	18 (22.5)
TOTAL	258	29(11.2)	36 (13.9)

Values in parenthesis indicate percentage.

DISCUSSION

The presence of *Brucella spp*. in bovine milk samples have been reported from different parts of Turkey (Türütoğlu *et al*., 2003; Kara *et al*., 2014; Kaynak-Onurdağ *et al*., 2016). In the present study, i-ELISA detected Brucella antibodies in 16.6% of the samples and the RT-PCR amplified *Brucella spp.* DNAs from 18.75% of the samples which is very close to the results of Terzi (2006) who had reported that 20% positivity in Samsun province. On the other hand, the presence of *Brucella spp*. in bovine milk samples in Şanlıurfa was rather higher than the results obtained in Burdur (Türütoğlu *et al*., 2003), Afyon (Kara *et al*., 2014) and Edirne (Kaynak-Onurdağ *et al*., 2016) provinces. The variability between results might be due to the differences in the specificity and sensitivity of the test methods used.

Similar results have been reported from Egypt (Wareth *et al*., 2014), India (Patel *et al*., 2008, Nigeria (Lor and Chukwu, 2015) and Pakistan (Shafee *et al*., 2011) in respect to the presence of the *Brucella spp*. in bovine milk, whereas higher prevalence was reported from Iran (Haghi *et al*., 2015), Egypt (Hamdy and Amin, 2002) and India (Patel *et al*., 2014). In contrast, lower prevalence was reported in Tanzania by Assenga *et al.* (2015). The differences between the countries may result from the control measurements implemented in the countries as well as the health status of the livestock in the countries, in which the studies were performed.

In Turkey, there have been limited studies detecting *Brucella spp*. in goat's and ewes' milk. Terzi (2006) investigated 50 goat's milk samples for *B. melitensis* in Samsun province and determined 12% positivity with Milk Ring Test whereas Whey-AT detected 6% positivity, which is very close to the positivity detected in the goat's milk in the present study. However, Keskin *et al*. (2009) have reported that PCR amplified *B. melitensis* in 22.5% of the goat's milk in Aydın province.

In a study performed in Van province by İlhan *et al*., (2008), PCR amplified *B. melitensis* DNA in 23.5% of sheep milk samples collected after abortion. In another study conducted in Afyon province by Kara *et al*. (2014), positivity rate was found as 5.40%. In Burdur province, Türütoglu *et al*. (2003) detected brucella antibodies in 17.7% of the sheep milk samples by Milk Ring Test and in 13.7% of the samples with Whey-AT. Their results were higher than those of this study.

Gupta *et al*. (2006) have found 59% positivity by serology and detected Brucella DNA in 88.8% of the milk samples by PCR. Hamdy and Amin (2002) have also detected high positivity (72.2%) with PCR, but in their study *Brucella spp.* was isolated in 10 of 18 milk samples by direct culture technique. Haghi *et al*. (2015) found that the most prevalent pathogen in milk samples is *Brucella spp*. (53.3%) in Zanjan, Iran. In a study by Hamdy and Amin (2002), PCR amplified Brucella DNA from 10 of 21 ewes' milk whereas the direct culture method detected *Brucella spp*. from 12 milk samples which was higher than our study. In this study, positivity detected in bovine milk was higher than the positivity in goat and the sheep milk but RT-PCR amplified Brucella DNAs from the same percentage of the goat and sheep milk samples which may result from the breeding of the goats and sheep in the same herd.

Studies performed in different provinces of Turkey (Güllüce *et al*., 2003; Pamuk and Gürler, 2014) revealed the existence of Brucella organisms in cheese except a study performed by Alper and Nesrin (2013) in Çanakkale province. In this study, detection of Brucella antibodies in 11.2% of the cheese samples by i-ELISA and amplification of Brucella DNAs in 13.9% of the 80 samples were very close to the results obtained by Pamuk and Gürler (2014) whereas our results were higher than the positivity of the Brucella in cheese tested by Öngör *et al*. (2006) and Ataş *et al*. (2007). On the other hand, our positivity rates were lower than the findings of Güllüce *et al*. (2003) who detected Brucella antigen in 21.66% of white cheese samples collected in Erzurum province, and Keskin and Tel (2003) who detected Brucella antibodies

in 30% of bovine milk samples in Şanlıurfa province and its surroundings. In this study, positivity rates determined in the cows' milk and cheese samples were similar. This similarity may be due to the production of the cheese mainly from bovine milk.

In this study, RT-PCR amplified *Brucella spp.* DNAs from one bovine and one goat milk in which no Brucella antibodies were detected by i-ELISA consistent with the findings of Wareth *et al.* (2014). On the other hand, Patel *et al*. (2008) could not detect Brucella DNAs in 9 bovine milk samples which were positive for Brucella antibodies. However, these authors detected Brucella DNAs by PCR from three of the 38 bovine milk samples that are negative for antibodies. Gupta *et al*. (2006) also found higher sensitivity and specificity of the PCR than serological methods in a study conducted for diagnosis of Brucellosis in goat. In the present study, detection of Brucella DNAs in some samples that were lacking antibodies might show a recent infection before antibodies are formed.

Conclusions: This study revealed the presence of Brucella antibodies and Brucella organisms in the bovine, goat and sheep milk as well as in the cheese in Şanlıurfa. Because, homemade cheese from raw milk is widely consumed particularly in this region, proper measures should be taken in terms of animal health as well as public health.

Author's contribution: SKA, OK, SEG conceived and designed the study. SKA, SEG, AY, OYT, MD performed the experiments; SG analyzed the data and wrote the manuscript, all authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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