The Detection of *Brucella* Bacteria with PCR and Bacteriological Method in Raw Milk and Some of the Dairy Products Which Are Consumed in Kars*

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**Abstract**

Because of the abort by the contamination to the humans via direct contact with infected animals or the consumption of contaminated raw milk and dairy products, brucellosis greatly causes to economic loss. For this reason, the annihilating of the effect of this disease is considerably important. Here, we aimed to detect the existence of *Brucella* bacteria in raw milk, cheese which is produced from raw milk and butter samples by using isolation, identification and PCR molecular techniques. *Brucella* type agents were detected in within a year by using 315 samples; 215 raw milk, 50 cheeses, 50 butter. Cheese and butter samples were obtained from raw milk. The grams coloring, oxidase, catalase, urease and H₂S analysis of 215 raw milk samples showed that only 4 (1.86%) samples was *Brucella* positive. As a result, we have determined that there are several types of *Brucellosis* bacteria in milk, cheese and butter samples. Milk and dairy products can create a risk in Kars because of brucellosis.

**Keywords:** *Brucella* spp, butter, cheese, PCR, raw milk

**INTRODUCTION**

Brucellosis is commonly known as a threat to public health because of the consumption of milk and dairy products that are produced in traditional ways in Turkey and many other countries in the world (Corbel, 1997; Pappas et al., 2006; Akpınar, 2016). Human brucellosis remains a prevalent zoonotic disease around the world with more than 500,000 new cases reported each year (Atluri et al., 2011). Brucellosis is a major zoonosis that is transmitted either by direct contact with animals or their secretions, or by consuming contaminated milk and dairy products (Aparicio, 2013, Corbel, 1997; Renukaradhya et al., 2002).

Brucellosis (also known as 'Mediterranean fever', 'fluctuating fever' 'Malta Fever' and 'Bang Disease') presents as an infectious, contagious, acute, sub-acute or chronic disease created by microorganisms and it is common throughout the world. This disease causes significant yield losses in animal production, making it a significant concern for human, domestic and wild animal diseases (Corbel, 1997; Garcell et al., 2016; Leong et al., 2016). The infection is caused by *B.abortus* in cattle, *B.melitensis* or *B.ovis* in goats and sheep, and these bacteria have been found in many animal species including sea mammals (Leal-Klevezas et al., 1995; Aparicio, 2013).

Brucellosis poses a major public health risk because human brucellosis is still a significant public health problem in Turkey (Yumuk and O’Callaghan, 2012). Scientists have made contributions to the overall picture of Brucellosis in Turkey with local studies (Çetinkaya et al., 2005; Arasoğlu et al., 2013; Çelebi et al., 2013; Kara and Akkaya, 2013). As it is well known that the Brucellosis generally appears in developing countries, several studies have been conducted...
to investigate Brucellosis rates in those countries (Karagiannis et al., 2012; Mailles et al., 2012; Havas et al., 2014; Shehu, 2014; Wareth et al., 2014; Mugizi et al., 2015; Rock et al., 2016). In Turkey, a study conducted by Iyisan et al., (2000) found that the rate was 3.56% in cattle and 1.26% in goats in 1989, and that this percentage was 1.2% in cattle, 2.08% in goats in 1990, and that it was 1.01% in cattle, 1.83% in sheep and goats in 1991. Kaynak et al., (2016) published a study reporting that they found Brucella spp. in 2% of raw milk samples.

In a study done by Charisis (1998) in Mediterranean countries, it was emphasized that the scale of human brucellosis cannot be determined and that there has been a significant increase in human cases in Turkey, from 3.03/100,000 in 1986 to 15.11/100,000 in 1996. This increase was probably seen as a result of improved observation and diagnosis techniques, more accurate results and better record keeping. Because cases of Brucella infection in animals can last as long as the animal’s life, it is common to observe continuous infection of mammary glands and lymph nodes of the mammary gland. In light of this, it has been reported that the Brucella agents are excreted in the milk during the ongoing lactation period. For this reason, it is recommended that the cows be isolated from the milk production process (Ataş et al., 2007).

Although cultures and serologic tests are widely used to identify Brucella agents in tissue, waste, blood and food, the PCR technique has also become more prevalent in recent years (Gupta et al., 2006; Ali et al., 2014; Wareth et al., 2014; Qasem et al., 2015; Kaynak et al., 2016). The polymerase chain reaction (PCR) technique is a rapid and sensitive method (Hamdy and Amin, 2002) used to determine the genus of Brucellosis and other pathogenic bacteria (E. coli) (Herman and Ridder, 1992; Allmann et al., 1995; Rudi et al., 2002). This technique can be used with specific enzymes to identify a specific genus (Tantillo et al., 2001; Funk et al., 2005). In addition, the PCR technique is useful for detecting Brucellosis not only in bovine milk and its products but also in ovine milk and its products.

**MATERIALS AND METHODS**

Our aim was to use isolation, identification and PCR molecular techniques to investigate Brucella agents in samples of raw milk and in cheese and butter produced from raw milk sold in Kars province. This study employed traditional analysis to determine that 1.86% of the raw milk samples contained the brucellosis agent, and these results were supported with PCR measurements, which is a molecular technique.

**Milk samples**

The samples used in the analysis were taken from markets, dairy farms and grocery stores which sell these products in sterilized bags under aseptic circumstances. Approximately 100 ml/gr amounts of the samples were brought to the laboratory at Kafkas University in the Faculty of Veterinary Food Hygiene and Technology, and they were immediately analyzed without waiting. The existence of the Brucella type bacteria were investigated over one year and evaluated 315 samples: 215 of cow milk, 50 of cheese and 50 of butter.

**Microbiological analysis**

In this study, 10 ml milk, 10 gr cheese and 10 gr butter samples were homogenized for 2-3 minutes in a stomacher, by putting them in a bag which contained 90 ml Brucella Broth. After that, we took 1 ml from each sample and put them to the different two tubes which contained 9 ml Brucella Broth, at next stage the samples were vortexed. One of the tubes was incubated in aerobic environment and the other tube was incubated in an atmosphere of 10% CO₂ at 37 ± 2°C for 5-7 days. At the end of this 5-7 day period, a tube which contained Brucella Agar was cultivated twice by the streaking method. One of them was incubated in a 37 °C incubator and the other in 37 °C incubator in 10% CO₂. Gram coloring, oxidase, catalase, urease and H₂S tests were applied to the samples that exhibited reproduction (Farrell and Robertson, 1972).

**PCR analysis**

*Extraction of genomic DNA from the samples:* The protocol that was developed for DNA extraction was carried out as follows: The PCR process was applied to Brucella-positive samples. For the PCR process, the DNA was obtained by using a DNA extraction kit (FERMENTASE). Samples were taken from colonies in Brucella agar, placed in 1.5 ml FTS and centrifuged at +4 °C for 10 min. After the supernatant on top of the pellet was poured off, 180 µl digestion solution and 20 µl proteinase K were added, and then it was incubated at 56°C for 30 minutes. Afterward, 200 µl lysis and 400 µl...
50% ethanol were added to the samples. Lysate GeneJet was prepared and centrifuged at 6000 g for 1 min. The upper portion was removed, 500 µl ethanol washing solution I was added and then it was centrifuged at 8000 g for 1 min. The upper portion was removed again, 500 µl ethanol washing solution II was added and then it was centrifuged at a minimum of 12000 g for 3 min. 200 µl Elution buffer was added to the resulting pellet, which was held at room temperature for 2 min. and then centrifuged at 8000 g for 1 min. The resulting DNA pellet was stored at -20 °C until it was analyzed.

Implementation of PCR protocol: Amplification reaction mixtures were prepared in volumes of 50 µl containing PCR buffer, 1.5 mM MgCl₂, 200 µM deoxynucleoside triphosphate, 1µM primer, 200 ng of genomic DNA and 2.5 U of DNA polymerase. The temperature cycling for the amplification was performed in a thermocycler (BIO RAD T100) as follows: the first cycle was 94 °C for 2 min and the next 40 cycles were 94 °C for 2 min, followed by 58 °C for 45 seconds, 72 °C for 45 seconds and a final cycle of 58 °C for 45 seconds and 72 °C for 10 min/1 cycle. The size of the amplified DNA was determined by electrophoresis on 0.8% agarose gels and compared with DNA molecular weight standards. A DNA-free control (distilled water) was also used to monitor contamination. The sample was examined in a UV transilluminator emitting light at a wavelength of 312 nm, and the results were recorded in a gel imaging system. Each sample was tested at least twice.

Statistical analysis: Chi-square analysis showed no differences between the bacteriological culture and PCR methods for detecting the B. abortus antigen.

RESULTS AND DISCUSSION
At the end of the laboratory analysis, which included gram coloring, oxidase, catalase, urease and H₂S of 215 raw milk samples, it was determined that only 4 (1.86%) samples were Brucella positive.

According to the above data, it was seen that there was reproduction in 17 of the 215 milk samples. In gram coloring done in the colonies which had reproduction, 8 samples were gram positive and 9 samples were gram negative. As a result of the oxidase, catalase and urease experiments that were applied to gram negative samples, only 4 (1.86%) samples were found positive for Brucella. After applying the PCR process on the Brucella positive samples, DNA extraction revealed that these 4 Brucella isolates were 100% B.abortus.

In our study, our aim was to investigate quickly and accurately the prevalence of Brucella agents in milk and dairy products using the PCR molecular technique. Therefore, this study investigated 215 raw milk samples, 50 cheese samples and 50 butter samples (a total of 315 samples) that were sold in Kars province. B.abortus was identified in 4 of 215 raw milk samples that were analyzed in this study. While this value was found to be lower than those reported in other studies, Zowghi et al. (2008) found a rate of 25.2% in raw cow milk, Leal-Klevezas et al. (1995) found a rate of 64% in raw goat milk, Turutoglu et al. (2003) found a rate of 3% in cow milk samples using MRT and a rate of 17.7% in sheep milk, and Gulluce and Leloglu (1996) found a rate of 56% using MRT and 65.6% using ELISA. Our results were similar to the result that Celebi and Otlu (2011) reported, which was 4.4% at after a serological survey of cow milk samples. Kara and Akkaya (2013) investigated 100 fresh cheese samples produced in Afyonkarahisar and found 2% B.abortus and 7% B.melitensis. Romero et al. (1995) conducted a study using ELISA and PCR techniques to detect Brucella antibodies and Brucella DNA, respectively. They performed their study on milk samples from 56 cows and found that milk from 37 of the cows was culture-positive for Brucella. They determined that 87.5% of the

### Tab. 1. Brucellosis isolation results of the studied samples

<table>
<thead>
<tr>
<th>Investigated Material</th>
<th>Number of Samples</th>
<th>Reproduction Number</th>
<th>Gram Staining</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Urease</th>
<th>H₂S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>215</td>
<td>17</td>
<td>+</td>
<td>9</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cheese</td>
<td>50</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Butter</td>
<td>50</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Bulletin UASVM Veterinary Medicine 73 (1) / 2016
milk samples were positive for *Brucella* DNA and and 98.2% were positive for *Brucella* antibodies.

With regard to other studies that used PCR, Ali *et al.* (2014) identified *Brucella* spp. at a rate of 9.5% in raw milk in Pakistan, Mugizi *et al.* (2015) identified it in cow milk at a rate of 5.31%, and Kaynak *et al.* (2016) identified it in raw milk at a rate of 2%.

Out of 54 milk samples collected from goats having a history of abortions, Funk *et al.*, found that only 32 (59%) of the samples tested positive using serology. However, 48 (88.8%) of the samples were found to be positive with PCR. This number includes all 32 samples that tested positive with serology. After a single PCR, sensitivities of $2.2 \times 10^6$ and $2.8 \times 10^7$ CFU/ml were obtained for detection using agarose gel electrophoresis. In a controlled experiment, the sensitivity and specificity of this PCR was found to be 90% and 100%, respectively. The specificity and high sensitivity of the PCR assay may provide a valuable tool for the diagnosis of brucellosis in goats (Funk *et al.*, 2005). Hamdy and Amin (2002) conducted a study to examine milk samples using culture and PCR techniques to detect *Brucella* species. They isolated *Brucella* strains from milk samples of cattle (n.24), sheep (n.12) and goats (n.11) but did not isolate any *Brucella* strains from camel's milk.

*Brucella* was not isolated from the cheese and butter samples, which were investigated separately from the raw milk. Similar to some studies (Gulluce *et al.*, 2003, Atas *et al.*, 2007), we did not find any *Brucella* bacteria in the cheese and butter samples. On the other hand, several studies reported a wide range of percentages of positive values. For example, Atas *et al.* (2007) found a rate of 5.9% in fresh and pickled cheese samples, Gulluce *et al.*, (Gulluce *et al.*, 2003) reported 21.66% in white cheese, civil cheese and lor cheese, (Alim and Tomul, 2005) identified a rate of

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**Fig. 1.** The image of *Brucella* DNA which exists in 1.5% of agarose gel, obtained from the PCR

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**Fig. 2.** The image of *Brucella* DNA which exists in 1.5% of agarose gel, the determined on the basis of the species with help of applied Multiplex PCR kit.
7.1-8.5% in fresh cheese samples, and Barrow et al. (1968) reported a rate of 1.64% in cream and cream products.

There are a number of significant studies conducted on Brucellosis with different aims in the literature. For instance, Santiago-Rodriguez et al., studied the survival of the Brucella abortus aqpx mutant gene during the elaboration and conversion of fresh and ripened cheeses at and below room temperature (Santiago-Rodriguez et al., 2015). In another study, Ebrahimi et al. (2014) investigated the rate of B. melitensis seropositives and its probable shedding in lactating goat flocks in Iran’s district zone. Mugizi et al. (2015) studied the epidemiology of Brucellosis by using phenotypic and molecular approaches to identify the Brucella species, biovars and genotypes occurring in cattle milk.

CONCLUSION
This study showed that milk samples pose a risk for B. abortus. The results of this research along with that of prior studies indicate that there has been a decrease in brucellosis infections thanks to positive results gained from increased efforts to fight brucellosis and reducing non-isolated Brucella agents in cheese and butter. Moreover, especially in recent years, the mentality of the producers has changed. They know that they need to heat or pasteurize raw milk before using it and this is a factor leading to the apparent decrease in the disease. Apart from these factors, in recent years large milk companies have been replacing family companies. Therefore, animal products have been produced in more hygienic conditions and from healthier animals. In addition, there has been an increase in programs that fight against contagious diseases. All of these factors can be considered to be reasons that the incidence rate of the brucellosis disease has decreased.

REFERENCES


