

Study for determining the presence of *Brucella spp*, *Salmonella spp*, *E. coli O157* and some other gram negative microorganisms in fresh cow's cheeses

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Abstract: This study was conducted to determine the presence of *Brucella spp*, *Salmonella spp*, *E. coli O157* and some other gram negative microorganisms in fresh cow's cheeses (n:100). *E. coli* was determined in all of the samples, *E. coli O157* was found in 8 samples and *Aeromonas hydrophila* was found in 12 samples. In the analyzed cheeses the prevalences of *Klebsiella pneumonia*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas auroginosa*, *Citrobacter freundii* and *Morgonella morganni spp. siboni*. were 16%, 16%, 7%, 16%, 12%, and 10% respectively. Neither *Salmonella spp.* nor *Brucella spp.* were determined in any of the samples. In conclusion, it is our opinion that the hygienic qualities of fresh cow cheeses are low and they may constitute a public health risk.

Keywords: *Brucella spp*, *E. coli O157*, Fresh Cheese, Microbiological Quality, Gram Negative Microorganism

1. Introduction

Brucellosis commonly seen in human beings and animals in the Mediterranean countries, Middle East, Central Asia and South America [1,2] is a zoonotic disease which is also called undulant fever, Mediterranean fever, Maltese fever and Bang's disease [3]. The disease causes abortions, loss of milk productivity and infertility in animals which are causes of economic losses. [4]. Brucellosis causes symptoms such as undulant fever, joint pain, muscle pain, abdominal pain, and headache [1,5] which also leads physical ailments and loss of labor in human beings[3]. The most dangerous strain for human beings is *Brucella melitensis*; however *B. suis*, *B. abortus*, *B. canis*, *B. pinnipedialis*, *B. ceti* and *B. innopinata* are also known to cause infection. [1]. Infection spreads via direct contact with infected animals, laboratory accidents, consumption of infected food or inhalation of infectious particles [6]. 63.6% of human cases are reported to be due to the consumption of fresh milk and fresh milk products. [5] Brucellosis has a high morbidity but low mortality [3]. The

prevalence of *Brucella* is reported between 0.6-22% in different regions of Turkey [4,7,8].

Fresh cheeses are produced with traditional methods in rural areas, either in small establishments or in households. The milk might be the potential source of the *Brucella spp.* and other pathogenic microorganisms due to lack of pasteurization and salt treatment. The presence of pathogenic organisms such as *Salmonella spp.*, *E.coli*, *E.coli O157* in food is a sign of direct or indirect fecal contamination and thusly poor hygienic conditions [9,10]. *Aeromonas spp.* are considered as an important food borne pathogenic organisms which can cause gastroenteritis [11] meningitis, wound infections and bacteremia in human beings [12]. *Klebsiella spp.* is an important opportunistic pathogenic organism. It can cause infections in the respiratory system, the nasal mucosa and the urinary tract [13]. Opportunistic pathogen bacteria such as *Morgonella morganni spp. siboni* and *Proteus mirabilis* can be dangerous for immunocompromised persons, the elderly

and children [14, 15, 16].

In this study the presence of *Brucella* spp, *Salmonella* spp, *E. coli* O157 and some other gram negative microorganisms was determined in fresh cow's cheeses to find out possible dangers in terms of public health and food quality.

2. Material and Methods

2.1. Collectiong the Samples

In this study, 100 fresh cow's cheeses which were on the market in Diyarbakir/Turkey were examined. The sampled cheeses were produced by adding commercial rennet to fresh cow's milk at the temperature of milking. Heat treatment or suppression in brine was not utilized. 250 grams of samples were obtained under aseptically conditions from the cheeses which were on sale with no packaging and were brought to our laboratory with cold containers immediately.

2.2. Isolation and Identification of *Brucella* spp

10 gr of cheese sample were added in 90 ml Farrell's broth and homogenized with a stomacher. Two 0.5 ml dilute material obtaining from the homogenized samples were inoculated onto Farrel Broth in which *Brucella* Selective Supplement (Oxoid SR83) was added. One of the inoculated tubes was incubated in aerobic oven and another one in 10% CO₂ for 5 days at 37°C. The tubes were vortexed everyday throughout the incubation period. At the end of the incubation periods, 0.1 ml of enriched samples were obtained from each tube and inoculated onto *Brucella* Medium Base (Oxoid CM169). The aerobically inoculated samples were incubated in aerobic conditions and microaerophilically inoculated samples were incubated in microaerophilic conditions for 5 days at 37°C. At the end of this period, 1-2 mm in diameter, pale yellow colored, transparent, round colonies on the *Brucella* Selective Agar were considered suspicious [17]. *Brucella* suspicious colonies were purified with Tryptic Soy Agar (Merck-1.05458). The identification of colonies was carried out with a VITEK II automatic system device.

2.3. Isolation and Identification of *Aeromonas* spp

Preliminary enrichment process of 25 gram cheese samples was performed in sterile bags with 225 ml of buffered peptone water (BPW, LAB204). After incubation at 37°C for 24 h, one loop of the enriched fluid was inoculated with the streak method onto *Aeromonas* Agar (Oxoid CM 83) containing 5 mg/l Ampicilin (Oxoid SR 136). The plates were then incubated at 30°C for 24 h. At the end of the incubation period, green opaque colonies with dark green centres on the *Aeromonas* Agar were considered suspicious. Suspicious colonies were purified with Tryptic Soy Agar (Merck-1.05458) and identification was carried out with a VITEK II automatic system device [18].

2.4. Isolation and Identification of *Pseudomonas* spp

The samples were inoculated on a *Pseudomonas* Agar Base (Merck 1.10989) and the petri dishes were incubated at 35°C for 5-7 days. The resulting colonies were purified with Tryptic Soy Agar (Merck-1.05458) and identification was carried out with a VITEK II automatic system device [19].

2.5. Isolation and Identification of *Salmonella* spp

Preliminary enrichment process was applied to 25 grams of cheese samples in sterile bags added with 225 ml of buffered peptone water (BPW, LAB204). 0.1 ml of enriched fluid was obtained after incubation at 37°C for 24 h and selective enrichment was carried out in 10 ml Rappaport-Vassiliadis Broth (RVS, LAB086). RVS tubes were incubated at 37°C for 24 h. At the end of the incubation period, samples from these tubes were inoculated onto Xylose Lysine Deoxycholate Agar (XLD, LAB032) and incubated at 37°C for 24 h. Pink colored and black centered colonies on the XLD agar were identified after the purification using Tryptic Soy Agar (Merck-1.05458) with a VITEK II automatic system device [20].

2.6. Isolation and Identification of *E. coli* and *E. coli* O157

For the isolation of *E. coli*, 10 grams of cheese samples were homogenized with 90 ml of 0.09% sodium chloride and inoculated onto TBX (Tryptone Bile X-glucuronide) Agar (Merck-1.16122-0500). Colonies with the typical green color were processed with the Vitek 2 compact 15 device for identification. For the identification of *E. coli* O157, suspicious colonies were also verified with the latex agglutination test (oxoid *E. coli* DR 0620M) [21].

2.7. Isolation and Identification of *Enterobacteriaceae*

VRBG (Violet Red Bile Glucose) Agar (Merck 1.10275) was used for the analysis of enterobacteriaceae and the typical colonies obtained were passaged with EMB (Eosin Methylene-blue) Agar (Merck 1.01347). Positive samples were purified with Tryptic Soy Agar (Merck-1.05458) and identification was carried out with a VITEK II automatic system device [22].

2.8. Determination of Antimicrobial Susceptibility

Antibiogram testing was planned for pathogens (*Brucella* spp., *Salmonella* spp. and *E. coli* O157) which have the potential to cause serious disease in human beings. Since only *E. coli* O157 was isolated, antibiogram testing was done only for this bacterium.

Antimicrobial susceptibility testing of the isolates was done by the Kirby-Bauer disc diffusion method, according to Clinical Laboratory Standard Institute (CLSI M100-S20, 2012) protocol. All isolates were cultured on Muller Hinton agar added with 5% defibrinated sheep blood, and then all of them incubated microaerobically at 30°C for 48 h.

Bacterial colonies from fresh pure culture were mixed with Muller Hinton broth to prepare the turbidity of each inoculum was adjusted to McFarland 0.5 standards. Bacteria from each suspension were inoculated onto Muller Hinton agar using a sterile cotton-tipped swab. The plates were kept at 37°C for 1–2 min, to get them dry, before antibiotic discs were dispensed. Incubation of the plates took place in a microaerobic atmosphere at 30°C for 48 h and the diameter of the inhibition zones was measured with calipers. The susceptibility patterns (resistance/sensitivity) of the strains were determined according to the National Committee for Clinical Laboratory Standards [23].

3. Results and Discussion

The presence of *Brucella* spp., *Salmonella* spp., *E. coli* and *E. coli* O157 was given in Table 1, the gram negative microorganisms detected were given in Table 2 and the antimicrobial susceptibility results of *E. coli* O157 were given in Table 3. *E. coli* was found in every sample (100%) whereas *E. coli* O157 was found in 8% of the samples. No cases of *Brucella* spp or *Salmonella* spp. were detected. *A. hydrophila* was found in 5 samples.

All of the *E. coli* O157 isolates obtained from fresh cheeses were resistant to ampicillin, erythromycin, penicillin G and spiramycin, on the other hand they were susceptible to ceftiofur, cefoperazone, gentamicin+amoxicillin, amoxicillin/clavulanic acid, enrofloxacin, nalidixic acid and streptomycin.

Table 1. Presence of *Brucella* Spp., *Salmonella* Spp., *E. coli* and *E. coli* O157 in Fresh Cheese Samples

Organism	Presence
<i>Brucella</i> spp.	not detected
<i>Salmonella</i> spp.	not detected
<i>E. coli</i>	100%
<i>E. coli</i> O157	8%

Table 2. Gram Negative Microorganisms Detected in Fresh Cheese Samples

Microorganism	Number of Samples
<i>Klebsiella pneumonia</i>	16
<i>Proteus mirabilis</i>	16
<i>Proteus vulgaris</i>	7
<i>Pseudomonas aeruginosa</i>	16
<i>Citrobacter freundii</i>	12
<i>Morganella morganii</i> spp. <i>siboni</i>	10
<i>Aeromonas hydrophila</i>	12

Table 3. Susceptibility of *E. coli* O157 Spp. to Antimicrobial Agents Isolated from Fresh Cheese

Antimicrobial Agent, concentration (µg/disk)	<i>E. coli</i> O157 (n=8)		
	R	I	S
Penicillin/Novobiocin (40)	6	2	-
Ceftiofur (30)	-	-	8
Cefoperazone (30)	6	2	-
Cefoperazone (75)	-	-	8
Tetracycline (30)	2	-	6
Gentamicin+Amoxicillin (35)	-	-	8
Ampicillin (10)	8	-	-
Erythromycin (15)	8	-	-
Amoxicillin/clavulanic acid (30)	-	-	8

Antimicrobial Agent, concentration (µg/disk)	<i>E. coli</i> O157 (n=8)		
	R	I	S
Enrofloxacin (5)	-	-	8
Penicillin G (10)	8	-	-
Spiramycin (100)	8	-	-
Doxycycline(30)	-	2	6
Nalidixic acid (30)	-	-	8
Streptomycin (25)	-	-	8
Trimethoprim/sulfamethoxazole (25)	2	-	6

In scientific studies the presence of a wide variety of bacteria such as *Brucella* spp., *Salmonella* spp., *E. coli*, *E. coli* O157, *H7*, *Klebsiella* spp., *Proteus* spp. in cheeses was reported. [24, 25, 26, 27, 28]. The presence of these bacteria indicates that cheese can be contaminated with human or animal sourced enteric bacteria. [10].

Brucellosis is a widespread [6] zoonotic infection which leads to economic losses due to abortion, loss of milk productivity and infertility [4]. Unpasteurized milk and milk products are important reasons in its spread [5, 9, 29]. There are differing results in studies which investigated the presence of *Brucella* spp. in cheeses. These differences in studies may depend on many factors such as regional differences, type of the cheese, production method or the utilization of heat treatment. In studies undertaken in Turkey, the presence of *Brucella* spp. was found to be 3.33-19.33% [28, 29, 30, 32] for feta cheese, 1.81-20.5% for tulum cheese [28, 29, 31] and 17.5% [9] for herbed cheese. In our study, *Brucella* spp. was not isolated in any of the cheese samples.

It is thought that the source of the milk, the vaccination status of the animals, the geographic region, heat treatment and the usage of salt in the production might influence the presence of *Brucella* spp. in cheeses. In the present study, the absence of *Brucella* spp. might be explained by the use of cow's milk, the vaccinated animals and the low incidence of *Brucella* spp. by cows in the region from where the cheese samples were obtained.

In this study, *E. coli* was detected in every sample investigated (100%). These results are in accordance with the results obtained by Ordiales et al. [24] and Baz et al. [26]. On the other hand Keskin et al. [32] notified the presence of *E. coli* in feta cheese samples as 86%. In our study the mean number of *E. coli* was 1.6×10^6 cfu/g (1.2×10^2 - 3.7×10^7); whereas Ordiales et al. [24] detected the *E. coli* number in cheeses which were produced with fresh milk, on the second day as 1.1×10^5 - 4.6×10^6 cfu/g. Akkaya and Alişarlı [34] detected *E. coli* O157:H7 in 1% of feta cheese samples obtained from street markets in Afyonkarahisar city centre. Gümüşsoy, Gönülalan [35] and Baz et al. [26] did not detect any *E. coli* O157:H7 in fresh feta cheeses which they sampled. In our study, the presence of *E. coli* O157 was determined at rate of 8%. The presence of *E. coli* and *E. coli* O157:H7 is thought to be directly related to the hygienic conditions during milking, storage, processing of the milk and the production of the cheese. The cheeses examined in this study are considered to be exposed to fecal contamination in various stages of production.

Salmonella spp. was not found in any of the sampled fresh cheeses. This Result is in accordance with the findings of Keskin *et al.* [33], Gülmez and Güven [35]. Akkaya and Alişarlı [26] found a 2% prevalence of *Salmonella spp.* in feta cheese samples in their study. Uraz *et al.* [36] reported an 8.13% incidence of *Salmonella spp.* out of 81 samples of feta cheeses. Primary and secondary contaminations might have played a role in the results of *Salmonella spp.*

In the present study, in fresh cheese samples, gram negative bacteria which could be opportunistic pathogens were also isolated. It was reported in the study of Dülger and Gücin [25] that out of 264 isolates of fresh white cheese samples were found *Klebsiella pneumoniae* and *Citrobacter spp.* at the rates of 5.69% and 11.75% respectively. In their study, Uraz *et al.* [36] reported, out of 81 isolates that were obtained from fresh white cheese samples, were determined *Klebsiella pneumoniae* at the rates of 1.16% , *Proteus mirabilis* 2.32% , *Proteus vulgaris* 1.16% , *Pseudomonas aeruginosa* 1.16% , *Citrobacter freundii* 3.49% and *Aeromonas hydrophila* 5.81% . Massa *et al.* [37] reported in their study that was performed on mozzarella cheeses out of 41 isolates, 37 cases of *Klebsiella pneumoniae* and 1 case of *E. coli*. In the present study the rates of gram negative bacteria were found to be higher. The variety and numbers of gram negative bacteria in cheeses could be related to hygienic conditions during production. These microorganisms have the potential of negatively influencing the quality and shelf life of cheeses.

4. Conclusion

In conclusion, it was detected that fresh cheeses did not contain *Brucella spp.* and *Salmonella spp.* but they were the sources for *E. coli O157* and other opportunistic gram negative bacteria. The presence of gram negative microorganisms indicates poor hygienic quality. The necessity of complying with hygiene rules at all stages from production to sales must be supported with training, legislation and inspection.

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