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Determination of the presence and antibiotic resistance of *Listeria* species and aerobic mesophilic bacteria count of cow milks

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ABSTRACT:

Listeria species lead to mastitis infection in cows. The aerobic mesophilic bacteria count (total bacteria count) is one of the most important factors affecting udder health and determining the milk quality. The aim of this study was to determine the aerobic mesophilic bacteria count, one of the most important factors affecting cow's milk quality, and presence and the antibiotic resistance profiles of *Listeria* spp., one of the factors causing mastitis in cows. As a result of isolation and identification for *Listeria* spp., totally 3 *L. monocytogenes* (n: 68, 4.41%), 7 *L. innocua* (n: 68, 10.29%) and 3 *L. ivanovii* (n: 68, 4.41%) were isolated from cow milk samples. According to results of the disc diffusion method performed to determine antibiotic susceptibility, it was found that *L. monocytogenes*, *L. innocua*, and *L. ivanovii* isolates were susceptive against sulfamethoxazole/ trimethoprim, meropenem, vancomycin, streptomycin, oxacillin and erythromycin. The aerobic mesophilic bacteria in the cow milk samples were detected 1.1x107 cfu/ml as the highest and 2.3x102 cfu/ml as the lowest. The average aerobic mesophilic bacteria count of milk samples was calculated 256623.971 cfu/ml. The total bacteria (aerobic mesophilic bacteria) count (cfu/ml) of milk samples in the study was found to be high based on the criteria stated in the national and international standards. Also, *Listeria* species were isolated from these samples. Since intermediate and resistant *Listeria* species were determined against the antibiotics used as a treatment option in these isolates, it is thought that Listeria species should also be considered in mastitis infections in terms of etiology and treatment. It is considered that a national mastitis control program is needed for preventing the mastitis infections and antibiotic resistance development causing economic losses in dairy cattle enterprises in order to provide milking hygiene completely.

İnek sütlerinde Listeria türlerinin varlığı ve antibiyotik direnci ile aerobik mezofilik bakteri sayısının belirlenmesi

ÖZET:

Listeria türleri ineklerde mastitis infeksiyonuna da neden olmaktadır. Aerob mezofilik bakteri sayısı (toplam bakteri sayısı), meme sağlığını etkileyen ve süt kalitesini belirleyen en önemli parametrelerden biridir. Bu çalışmada inek sütü kalitesini etkileyen en önemli parametlerden olan aerob mezofilik bakteri sayısı ve ineklerde mastitise neden olan etkenlerden olan *Listeria* spp. varlığının ve antibiyotik direnç profillerinin belirlenmesi amaçlandı. *Listeria* spp. için izolasyon ve identifikasyon sonucunda toplam 3 *L. monocytogenes* (n: 68,% 4.41), 7 *L. innocua* (n: 68,% 10.29) ve 3 *L. ivanovii* (n: 68,% 4.41) inek sütü örneklerinden izole edildi. Antibiyotik duyarlılığını belirlemek için yapılan disk difüzyon yönteminin sonuçlarına göre *L.monocytogenes, L.innocua* ve *L.ivanovii* izolatlarının sülfametoksazol/ trimetoprim, meropenem, vankomisin, streptomisin, oksasilin ve eritromisine karşı duyarlı oldukları bulundu. İnek sütü örneklerinde aerob mezofilik bakteri sayısı en yüksek 1.1x107 cfu/ml; en düşük ise 2.3x102 cfu/ml bulundu. Süt örneklerinin ortalama aerob mezofilik bakteri sayısı 256623,971 cfu/ml olarak bulundu. Çalışmada süt örneklerinin toplam bakteri (aerob mezofilik bakteri) sayısının (cfu/ml) ulusal ve uluslararası standardlarda belirtilen kriterlere göre yüksek bulundu, aynı zamanda bu örneklerden *Listeria* türlerinin izole edildi. Ayrıca, bu izolatlarda tedavi seçeneği olarak kullanlan antibiyotiklere karşı orta (ilaçla artmış temeasta duyarlı-I-intermediate) ve dirençli *Listeria* türleri tespit edildiğinden, mastitis infeksiyonlarında etiyolojik ve tedavi açısından *Listeria* türlerinin de göz önünde bulundurulması gerektiği düşünüldü. Sağım hijyeni tam anlamıyla sağlanması için, süt ineği işletmelerinde ekonomik kayıplara neden olan mastitis infeksiyonlarınnı ve antibiyotik direnci gelişiminin önlenmesi amacıyla ulusal düzeyde mastitis kontrol programına ihtiyaç duyulduğu düşünüldü.

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1. Introduction

Mastitis is defined as an inflammatory response in udder glands leading to economic losses due to non-use of milk in dairy cattle enterprises and treatment expenses, impairing milk quality, and being caused by the bacterial, chemical, and traumatic reasons or heat (6,7,15,24,38). Mastitis are classified as clinical and sub-clinical mastitis. The ones not causing clinical symptoms and visible change in udder tissue and milk are classified as "subclinical" and those causing more or less swelling, pain, temperature increase and color change in sick udders by causing visible symptoms are classified as "clinical". Subclinical mastitis infects easily among cows and these kinds of mastitis cause economic loss in the enterprises. In the clinical mastitis, disorders such as decrease in milk yield, agalactia, presence of watery secretions and clotting may develop (6,11).

The aerobic mesophilic bacteria count (total bacteria count) is one of the most important parameters determining the udder health and milk quality (16,24,28). The total bacteria count in raw cow milk must be <100.000 (piece) in milliliter (ml) at 30°C according to raw and heat-treated drinking milk communique (2) in the Turkish Food Codex numbered 2000/6 published in the Official Gazette dated 14/02/2000 and numbered 23964 and the EU regulation dated 30/04/2004 and numbered 853/2004 (3,4).

There are National Mastitis Councils in the developed countries in order to eliminate mastitis. Nationwide struggle with mastitis is performed with the National Mastitis Control Programs prepared by these councils (6,7,38) The targets in the mastitis control programs are stated to be the elimination of the current infections, protecting from new infections and following udders constantly in terms of mastitis. It has been reported that all cows in a herd is enabled to be protected simultaneously by the udder health control program. The protection from the udder infections has been achieved at the rate of 80-90% in the herds in which the udder health control programs are applied for a long time (6,31).

Listeria genus is composed of totally 17 species, identified within the 2 groups, which are gram-positive, small rod-shaped bacteria surviving in the environments containing low pH and temperature and high salt (5,29,36). One of these groups is *Listeria* sensu stricto including *Listeria monocytogenes*, *Listeria seeligeri*, *Listeria marthii*, *Listeria ivanovii*, *Listeria welshimeri*, and *Listeria innocua* and the other is *Listeria* sensu lato including *Listeria grayi* and 10 *Listeria* species recent identified as of 2009 (29). Among these species, only *L. monocytogenes* and *L. ivanovii* identified to be pathogenic species (29,36). The species in *Listeria* sensu stricto group exhibit high adaption in soil and water (26). *Listeria* species are also observed in farms. Especially, they are frequently found in fertilizer and fermented silage (8,36). For this reason, animals may be infected through bad quality silage, pathogenic inhalation, direct contact, water, forage components, litter mat, soil and dung (5). *Listeria* species (12), which are ubiquiter and intracellular pathogen, cause abortion, stillbirth, encephalitis and neonatal septicemia in human beings and animals (5,23,30). Also, *Listeria* species cause mastitis infection in cows (5,23). *L. monocytogenes* causes encephalitis, abortion, septicemia, ocular form and mastitis infections in cows (27). Also, *L. monocytogenes* is an opportunistic pathogen and causes especially important food-borne infections in human beings as well as the infections it causes in animals (10). Particularly elderly people, pregnant women, newborns and the people with a suppressed immune system are quite susceptible to these infections (25,36). *L. ivanovii* is a predominant pathogenic species in animals (8) and causes clinical infections in cows and sheep causing abortion and stillbirth (26,27). In their study, Alexander et al. (2) declared *L. ivanovii* is the potential abortion agent in cows. Rocha et al. (34) isolated *L. innocua* from fatal meningoencephalitis case in fattening bull and this was the first. *Listeria* species, mainly *L. monocytogenes*, have been isolated from cow milk and identified to be clinical mastitis cause in cows in numerous studies. Haggag et al. (19) was isolated and identified *L. monocytogenes* (2.4%), *L. ivanovii* (2%), *L. innocua* (1.2%), and *L. grayi* (2.8%) in totally 250 milk samples including 150 cows, 50 buffaloes, and 50 sheep. Jamali et al. (24) was isolated 17 L. monocytogenes, *3 L. innocua*, and 1 *L. ivanovii* from 201 milk samples obtained from clinical mastitis of cows.

Penicillin and/or its derivatives are an effective treatment option in the infections in animals caused by *Listeria*. Gentamicin provides an effective treatment in the genital system listeriosis infections of cows. Additionally, erythromycin and trimethoprim/sulfamethoxazole may be useful in septicemic form treatment (27). Also, ampicillin, chloramphenicol, rifampicin, tetracycline and aminoglycoside antibiotics may be used in treatment (5).

In this study looked at the aerobic mesophilic bacteria count, one of the most important parameters affecting cow milk quality, and presence and the antibiotic resistance profiles of *Listeria* spp., one of the factors causing mastitis in cows.

2. Material and Methods

In the study, 68 cow milk samples, which were taken from Holstein and Simental cows and sent to laboratory for analysis by veterinarians, were examined in terms of the prevalence of *Listeria* spp. and the aerobic mesophilic bacteria count. The milk samples were collected aseptically from each cow to sterile container during milking and sent them to laboratory under aseptic and cold chain conditions (+2-+8°C) and their analyses were performed immediately.

Detection of *Listeria* species:

The cow milk samples were analyzed for *Listeria* spp. isolation based on ISO 11290-1 method (20).

25 milliliters from each sample were put into the sterile sample bags and homogenized in stomacher (Bigmixer, Intersience) by 225 ml sterile Half-Fraser Broth (Merck, Germany) for 1 minute. The homogenized samples were incubated at 30 °C for 24±2 hours for pre-enrichment. And then, 0.1 ml of the samples was taken and these samples were inoculated to Fraser Broth (Merck, Germany), a pre-enrichment, and incubated at 37 °C for 24-48 hours. And then, Fraser Broth's of each sample was inoculated in selective agars Ottoviani and Agosti (Merck, Germany), PALCAM (Oxoid, UK) and Rapıd' L. mono agars (Bio-Rad, USA) and incubated at 37 °C for 24-48 hours (20). In Rapid' L. mono agar (Bio-Rad, USA), *L. monocytogenes* colonies in green color, *L. ivanovii* colonies reproduce in blue color by forming a yellow zone around them. *L. innocua* colonies reproduce in white color without forming a zone around them (12).

After incubation, the colonies were confirmed by gram staining, catalase, oxidase and mobility tests based on Bergey's Manuel of Systematic Bacteriology (35). Afterwards, he isolates were verified in automatic identification system (Vitek 2 Compact). *Listeria* spp. suspected colonies were arranged in tubes with 3 ml sterile salty water based on McFarland 0.5-0.63 turbidity and identified with Gram positive identification card in Vitek 2 Compact (Biomerieux, France) device. Afterwards, verified strains were kept by being taken into bead storage tubes and kept at (-20) °C.

Aerobic mesophilic bacteria count:

In order to determine the aerobic mesophilic bacteria count, 10 ml milk sample was diluted with 90 ml Maximum Recovery Diluent (MRD, Merck, Germany). Then, each milk sample was diluted 10 times up to 10^9. One ml was taken from each dilution and they were inoculated in two petri dishes by pour plate inoculation technique in Plate Count Skimmilk Agar (Merck, Germany) and incubated at 30°C for 72 hours. And then, the aerobic mesophilic bacteria count of the milk samples was calculated in colony forming unit (cfu)/ml (21,22).

Investigation of antibiotic susceptibility of isolated *Listeria* isolates:

Antibiotic susceptibility was investigated according to disc diffusion method based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) (9,13,14,18). For this aim, in first step, the isolates were taken from the bead storage tubes and inoculated to Nutrient Broth (Merck, Germany) and incubated at 37 °C for 24 hours. And then, they were inoculated in Rapıd' L. mono (Bio-Rad, UK) agar and incubated at 37 °C for 24-48 hours. All pure *Listeria* spp. isolates were diluted in tubes containing 3 ml sterile salty water with McFarland 0.5 turbidity. The sterile swabs were immersed to the bottom of the tubes. Then, the remaining liquid was removed by applying pressure inside tubes and it was inverted into the tubes. The inoculum was spread evenly over the entire surface by swabbing in three directions to Mueller-Hinton F agar (Merck, Germany). It was incubated at 35+/-1 °C for 16-20 hours. Gentamicin (Oxoid, 10µg), meropenem (Oxoid, 10µg), amoxicillin clavulanic acid (Oxoid, 30µg), streptomycin (Oxoid, 10µg), chloramphenicol (Oxoid, 30µg), ciprofloxacin (Oxoid, 5µg), amikacin (Oxoid, 30µg), ampicillin (Oxoid, 10µg), erythromycin (Oxoid, 15µg), trimethoprim-

sulfamethoxazole (Oxoid, 25µg), penicillin G (Oxoid, 10U), rifampicin (Liofilchem, 5µg) oxacillin (Oxoid, 1µg) and tetracycline (Oxoid, 30µg), vancomycin (Oxoid, 30µg) were used.

After incubation, the inhibition zones were measured on Mueller-Hingon F agar plates and evaluated as resistant (R), intermediate (I) and susceptible (S) based on the breakpoints suggested by EUCAST (18) for *L. monocytogenes* on ampicillin, penicillin G, oxacillin, erythromycin, trimethoprim-sulfamethoxazole, meropenem and for the other antibiotic discs commented based on the breakpoints suggested by CLSI (14) for other Gram-positive bacteria (1,13,14,18). *Escherichia coli* (ATCC ® 25922™) were used as reference on antibitoic susceptability tests.

3. Results

Three *L. monocytogenes* (n:68, 4.41%), 7 *L. innocua* (n:68, 10.29%) and 3 *L. ivanovii* (n:68, 4.41%) were isolated from cow milk samples (Table 1).

Table 1: Isolation and identification results of *Listeria* spp. from cow milk samples

Tablo 1: *İnek sütlerinden Listeria spp. izolasyon ve identifikasyon sonuçları*

***Listeria* species**	**Number of positive samples**
L. monocytogenes	3 (4.41%)
L. innocua	7 (10.29%)
L. ivanovii	3 (4.41%)
Total	13 (19.11%)

In antibiogram tests, *L. monocytogenes*, *L. innocua,* and *L. ivanovii* isolates were susceptible against sulfamethoxazole/trimethoprim, meropenem, vancomycin, streptomycin, oxacillin, and erythromycin. Table 2 shows the disc diffusion test results of *Listeria* spp. isolates.

Table 2: Disc diffusion test results of *Listeria* spp. isolates

Tablo 2: *Listeria spp.'lerin disk difüzyon test sonuçları*

Antibitotic discs	***Listeria monocytogenes strains* (n:3)**			***Listeria innocua* strains (n:7)**			***Listeria ivanovii strains (n:3)***		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Amikacin (Oxoid, 30µg)	1	1	1	7	-	-	2	1	-
Amoxicillin-clavulanic acid (Oxoid, 30µg)	3	-	-	7	-	-	3	-	-
Ampicillin (Oxoid, 10µg)	2	1	-	6	1	1	2	-	1
Erithromycin (Oxoid, 15µg)	3	-	-	7	-	-	3	-	-
Gentamicin (Oxoid, 10µg)	2	1	-	4	2	1	2	1	-
Chloramphenicol (Oxoid, 30µg)	3	-	-	6	-	1	3	-	-
Meropenem (Oxoid, 10µg)	3	-	-	7	-	-	3	-	-
Penicillin G (Oxoid, 10U)	2	1	-	4	-	3	1	1	1
Ciprofloxacin (Oxoid, 5µg)	3	-	-	5	2	-	3	-	-
Oxacillin (Oxoid, 1 µg)	3	-	-	7	-	-	3	-	-
Rifampicin (Liofilchem, 5µg)	2	1	-	6	1	-	2	1	-
Streptomycin (Oxoid, 10µg)	3	-	-	7	-	-	3	-	-
Tetracyclin (Oxoid, 30µg)	2	1	-	4	2	1	1	1	1
Trimethoprim-Sulfametoxazole (Oxoid, 25µg)	3	-	-	7	-	-	3	-	-
Vancomycin (Oxoid, 30 µg)	3	-	-	7	-	-	3	-	-

Table 3 shows the average aerobic mesophilic bacteria count of each milk sample. The aerobic mesophilic bacteria count in the cow milk samples were found to be 1.1x107 cfu/ml as the highest and 2.3x102 cfu/ml as the lowest. Average aerobic mesophilic bacteria count of the milk samples was calculated 256623.971 cfu/ml.

Table 3: Avarage Aerob mesophilic bacteria count results of cow milk samples

Tablo 3: *İnek sütü örneklerinin ortalam aerob mezofilik bakteri sayımı sonuçları*

Cow milks	Avarage aerob mesophilic bacteria count (cfu/ml)
68	256623.971

4. Discussion and Conclusion

For quality milk, the total bacteria count is quite important. The total bacteria count in the milk obtained in dairy cow enterprises is requested to be low. The most important factor affecting the bacteria count in milk is mastitis infection. Mastitis infections cause inflammation in udder and injury in udder tissue and increases in the total bacteria count and, thus, in the number of somatic cells and give harm to both cows and milk in dairy cow enterprises and therefore cause economic losses. Throughout the world, national mastitis control programs are formed in many countries. Within the frame of these programs, the applications on barn and environment planning, milking management and hygiene, the maintenance and cleaning of milking equipment, teat cleaning and applying antiseptic (teat dipping) on teats before and after milking, monitoring the udders of dairy cows in terms of clinical and subclinical mastitis infections in farms, and dry period management are determined as the standards (6,10).

Dhuol and Osman (17) said total bacteria count in the 30-morning milking and 30 evening milking milk samples was 650.000 cfu/ml on average. Tosun and Baki Acar (6) in Tekirdağ province, they determined that the total bacteria count was 1.796.718.36±156.573.31 cfu/mL in bulk tank samples. Darbaz et al. (16) on cow milk in the Turkish Republic of Northern Cyprus, they reported that the total bacteria count in bulk tank changed based on seasons and the highest average total bacteria count was 182.000 cfu/ml in fall. The aerobic mesophilic bacteria count in this study was found to be higher than limit values determined for raw cow milk in national and international standards. In the studies of the other researchers, this data was higher compared to the criteria. But, the average values of the aerobic mesophilic bacteria count were calculated lower compared to the results of the other researchers. This was considered to be associated with that fact that the milk samples taken from animals were individuals assessed instead of the bulk tank.

There is a limited number of studies on *Listeria* spp. isolated from mastitis cases and antibiotic resistance profiles. Rahimi et al. (32) was examined 85 milk samples isolated 3 (3.5%) *L. monocytogenes*, 5 (5.9%) *L. innocua* and 1 (1.2%) *L. ivanovii* as a result of the identification of cultures. Rawool et al. (33) isolated *L. monocytogenes* from the milk of 1 of the 3 cows with mastitis and from the dung of the other 2 cows and *L. ivanovii* from the dung of 1 buffalo with mastitis, among the milk of 650 cows and buffaloes. Vilar et al. (37) stated that they isolated 6 (6.1%) *L. monocytogenes* and 7 (7.1%) *L. innocua* in 98 bulk tank samples they obtained from cow farms. Konosonoka et al. (25) isolated 3 (1.4 %) *L. monocytogenes* in 221 bulk tank cow milk samples. Yadav et al. (39) stated that they isolated 3 *L. monocytogenes* in 85 milk samples of the cows and buffaloes with clinical mastitis infection. Aksoy et al. (1) stated that they isolated 8 *L. monocytogenes*, 4 *L. seeligeri*, 5 *L. ivanovii*, and 1 *L. welshimeri* in 100 raw cow milk samples after isolation, identification, and polymerase chain reaction (PCR). The isolation findings and isolates rates obtained in this study had shown similarity with the results of other studies. For this reason, it was thought that the *Listeria* species causing mastitis in cows. So, these bacteria should also be considered in the causes of mastitis and treatment stages in mastitis infections.

Today, antibiotic resistance is a wide spreading issue addressed by the World Health Organization (WHO). Aksoy et al. (1) stated that they found intermediate and resistant to amikacin, meropenem, vancomycin and penicillin G, and Trimethoprim-Sulfamethoxazole in 15 *L. monocytogenes* isolates they isolated from raw cow milk, cheese and

butter. Jamali and Redmehr (23) stated resistance against penicillin G, amoxicillin-clavulanic acid, chloramphenicol, erythromycin, and tetracycline at the rates of 66.7%, 23.8%, 19%, 4.8% and 52.4%, respectively, in 17 *L. monocytogenes* isolates they have isolated from the clinical mastitis infections of cows. Also, they reported that the resistance against 1,2 and more than 2 antibiotic active substances was 33.3%, 38.1%, and 14.3%, respectively. They stated resistance against Penicillin G and tetracycline at the rate of 33.3% in 3 *L. innocua* isolates and identified that there were 2 isolates against 1 antibiotic active ingredient. They reported that 1 *L. ivanovii* strain they isolated was resistant against Penicillin G. As there were intermediate and resistant isolates in *Listeria* species isolated in this study against amoxicillin-clavulanic acid, gentamicin, chloramphenicol, rifampicin, tetracycline and Penicillin G, which are used in treatment option especially in animals, it was considered that the antibiotic resistance developed in *Listeria* species, may become common and, therefore, there may be decrease in the number of antibiotics, which are used in treatment option in the future.

The total bacteria (aerobic mesophilic bacteria) count (cfu/ml) was calculated high based on the criteria stated in the national and international standards in milk samples. Also, *Listeria* species were isolated from these samples. Since intermediate and resistant *Listeria* species were determined against the antibiotics used as a treatment option in these isolates, it is thought that *Listeria* species should also be considered in mastitis infections in terms of etiology and treatment. It is considered that a national mastitis control program is needed for preventing the mastitis infections and antibiotic resistance development causing economic losses in dairy cattle enterprises in order to provide milking hygiene completely.

Conflict of Interest

The author declared no conflict of interest.

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Authors' Contributions

Idea / concept: Orkun BABACAN
Experiment design: Orkun BABACAN
Supervision / Consultancy: Orkun BABACAN
Data collecting: Orkun BABACAN
Data analysis and interpretation: Orkun BABACAN
Literature search: Orkun BABACAN
Writing the article: Orkun BABACAN
Critical review: Orkun BABACAN

Ethical Approval

An ethical statement was received from the authors that the data, information and documents presented in this article were obtained within the framework of academic and ethical rules, and that all information, documents, evaluations and results were presented in accordance with scientific ethics and moral rules.

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