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Original Article
Özgün Araştırma

Do CMT, SCC, and Bacteriological isolation overlap in subclinical mastitis cases of Anatolian buffaloes?

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Abstract: Subclinical mastitis is the most important and costly disease in the dairy sector. In this study, it was aimed to compare the results of bacteriological examination with those of California Mastitis Test (CMT) and Somatic Cell Count (SCC)in the milk samples collected from Anatolian buffaloes with no clinical signs of mastitis. For this purpose, 96 milk samples were collected from 24 Anatolian buffaloes of each quarter. All milk samples were examined for the presence of mastitic pathogens by bacteriology regardless of SCC values and CMT scores. A total of 103 isolates were recovered from the infected quarters. The first three frequently isolated mastitic pathogens were determined to be *E. coli*, *S. agalactiae*, and *S. aureus* with the rate of 31.07%, 22.33%, and 21.36%, respectively. According to the bacteriology results, threshold value for SCC was accepted as ≥78.000 cells/ml. The correlation value between CMT and SCC, CMT and bacteriology, and SCC and bacteriology was found as 0.737, 0.845 and 0.872, respectively, and the mean of inter-item correlation was determined 0.818. These results showed that the test results were highly correlated with each other. The results of the ROC analysis of the cut-of-value of the SCC test for this study chosen as 78.000 cells/ml supported the results obtained from the reliability analysis with sensitivity 85% and 1-specifity 100%. To sum up, a combination of CMT, SCC, and bacteriological investigation provides benefits in detecting mastitis early and avoiding misdiagnosis, allowing for timely action and treatment.

Keywords: Anatolian Buffalo, CMT, SCC, bacteriology

Anadolu mandalarının subklinik mastitis vakalarında CMT, SCC ve Bakteriyolojik izolasyon örtüşüyor mu?

Özet: Subklinik mastitis, süt sektöründe en önemli ve maliyetli hastalıktır. Bu çalışmada, klinik belirti göstermeyen Anadolu süt mandalarından toplanan süt örneklerinde bakteriyolojik inceleme sonuçları ile California Mastitis Testi (CMT) ve Somatik Hücre Sayısı (SCS) sonuçlarının karşılaştırılması amaçlandı. Bu amaçla, 24 Anadolu süt mandasınının her meme lobundan olmak üzere toplam 96 süt örneği alındı. SCC değerleri ve CMT skorlarına bakılmaksızın, tüm süt örnekleri mastitik patojenlerin varlığı açısından bakteriyolojik olarak incelendi. Enfekte loblardan toplam 103 izolat elde edildi. İlk üç en sık izole edilen mastitik patojenlerin sırasıyla %31.07, %22.33 ve %21.36 oranlarıyla *E. coli*, *S. agalactiae* ve *S. aureus* olduğu belirlendi. Bakteriyoloji sonuçlarına göre, SCC için eşik değer ≥78.000 hücre/ml olarak kabul edildi. CMT ve SCC, CMT ve bakteriyoloji, SCC ve bakteriyoloji arasındaki korelasyon değeri sırasıyla 0.737, 0.845 ve 0.872 olarak bulundu ve madde içi korelasyon ortalaması 0.818 olarak belirlendi. Bu sonuçlar, test sonuçlarının birbirleriyle yüksek derecede ilişkili olduğunu gösterdi. Bu çalışma için seçilen SCC testi kesim değeri için ROC analizi sonuçları; 78.000 hücre/ml olarak seçildi, duyarlılık %85 ve 1-özgüllük %100 ile güvenilirlik analizinden elde edilen sonuçları destekledi. Sonuç olarak, CMT, SCC ve bakteriyolojik inceleme kombinasyonu, mastitisin erken teşhis edilmesine ve yanlış tanıdan kaçınılarak zamanında müdahale ve tedavi sağlanmasına olanak tanır.

Anahtar kelimeler: Anadolu süt mandası, CMT, SCC, bakteriyoloji

Introduction

Water buffaloes provide the most important source of non-cattle milk with the rate of 13.2% worldwide. Buffalo milk production is a major animal husbandry activity in Asia, Italy, and Brazil, accounting for a significant portion of global milk production (FAO, 2015).

Mastitis is an important infectious disease that may affect quantity and quality of milk in dairy ruminants. It is characterized by an inflammation of the

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mammary gland parenchyma due to different type of microorganisms, including bacteria and fungi (Baloch et al. 2011). The infection induces physical, chemical, bacteriological, and pathological changes in udder and milk since it is possible to identify a subclinical and clinical presentation (Guha et al., 2012). For the susceptibility of subclinical mastitis in water buffalo, milk quality, body weight, calving time, udder type, and milking hygiene conditions have all been established as factors (Hussain et al., 2013). Despite the fact that the incidence of mastitis in water buffalo was reported to be 10% lower than in cows, subclinical mastitis had a financial impact on buffalo breeders (Khan and Muhammad 2005; Ali et al., 2011). Therefore, this disease can negatively impact on the economy of milk industry other than on animal health and well-being.

Subclinical mastitis can be detected by both somatic cell count (SCC) and bacteriological culture (Moroni et al. 2006). The number of leukocyte and epithelial cells in the milk, which are defined as somatic cells, increase in milk during mastitis (Ruegg, 2017). Among the pathogens, the *Staphylococcus aureus* (*S. aureus)*, *Streptococcus agalactia* (*S. agalactiae*) and *Escherichia coli* (*E. coli*) are the most important bacteria in subclinical mastitis cases even if the 78% of mastitis cases are caused by coagulase negative bacteria (Moroni et al. 2006; Fagiola and Lai 2007). Quick and accurate diagnosis can help to control mastitis, which can cause serious problems in water buffaloes (Ruegg, 2017). The California Mastitis Test (CMT) is a fast, inexpensive, and easily applicable test that can be used to diagnose subclinical mastitis indirectly according to SCC level (Viguer et al., 2009).

The goal of this study was to compare the bacteriological examination and the CMT, and SCC of milk samples to diagnose subclinical mastitis in Anatolian buffaloes.

Materials and Methods

Collected samples

This study was carried out in the Karacabey district of Bursa province, Turkey. The study period was between April and August in 2020. A total of 96 milk samples were collected from each udder of half of 24 apparently healthy Anatolian buffaloes that showed no clinical signs of mastitis in order to perform CMT, SCC, and bacteriological examination. Twelve out of 24 apparently healthy adult Anatolian buffaloes were randomly selected from a farm (their ages ranged between 4 and 7 years), and the remaining 12 adult Anatolian buffaloes were randomly selected from five small-scale buffalo farms owned by families (their ages ranged between 4 and 6). All the farms had mechanical milking. The milking practice was applied twice a day. The detailed information about Anatolian buffaloes was emphasized in Table 1.

Table 1. Information about Anatolian buffaloes

Herds	Number of Anatolian buffaloes	Body weight	Milk yield (kg day-1)
H1	12	521.50 ± 5.50	5.39±0.16
H2	3	512.00±15.09	5.30±0.16
H3	2	465.50 ± 8.50	4.80±0.12
H4	2	498.50 ± 3.50	4.50±0.18
H5	2	492.00±4.63	5.20±0.12
H6	1	526.33 ± 6.71	4.48 ± 0.17

Sampling and CMT test

The milk samples from each udder half were collected aseptically. CMT was performed with approximately collected 5ml milk samples and the same volume of CMT solution. The results were scored as (-), (+), (++), (+++), and recorded.

Sampling for SCC and bacteriological examination

After the CMT test, teat ends were scrubbed with a gauze pad soaked with 70% alcohol, and the milk samples from each udder half were aseptically collected into two sterile tubes, one of the two for SCC, the other one for bacteriological examination. The milk samples collected for bacteriological examination were transferred to the laboratory in a cold chain immediately, and stored at 4°C for a maximum of 24 hours until cultured on standard bacteriological media.

SCC

Milk samples from each udder half collected in the other sterile test tube were analyzed to detect SCC on the sampling day at the farm. Milk aliquots were analyzed with DeLaval Cell Counter (DCC; DeLaval International AB, Tumba, Sweden) by using the kits of the same firm.

Bacteriological examination

The bacteriological examination was carried out according to standard procedures defined by Quin et al. (2011).

Statistic analysis

IBM SPSS 25 and MedCalc were used for data analysis. The pairwise comparison of results from CMT and bacteriology, and SCC and bacteriology were examined. In addition to this, Croostabs were obtained. Mc-Nemar test (α =0.05) was applied for assessing statistical association in terms of pairwise comparisons because of dependent data structure. Kappa statistic for assessing of agreement between the CMT, SCC, and bacteriology test results was also examined. At the end, reliability analysis and ROC analysis were also applied.

Results

CMT results

The CMT results of the quarter milk samples revealed that 28 quarter were negative. The remaining 68 quarter milk samples showed a CMT scores between (+) and (+++) levels. Of the examined 96 milk samples from the quarters belonging to 24 Anatolian buffaloes, 24 were found to be negative for CMT, CMT scores ranking (+) to (+++) were determined in 72 milk samples (Table 2).

SCC results

The SCC values of the milk samples ranged from 50.000 cells/ml to 980.000 cells/ml. The results of SCC were emphasized in Table 2 in detail.

Bacteriology results

The bacteriological examination of 96 quarter milk samples identified 27 (28.12%) negative samples, 37 (38.54%) positive to one bacteria, and 32 (33.33%) positive to at least 2 bacteria. A total of 103 pathogens were isolated from the infected quarters. Out of those, 31.07%, 22.33%, 21.36%, 12.62%, 6.80%, 5.82% were found to be *E. coli*, *S. agalactiae*, *S. aureus*, *S. epidermidis*, *E. faecalis, S. uberis*, respectively (Fig1). *E. coli* and *S. aureus* were found to be the most common bacteria in the mixed cultures. Different mixed culture profiles and single cultures from each quarter were emphasized in Table 2.

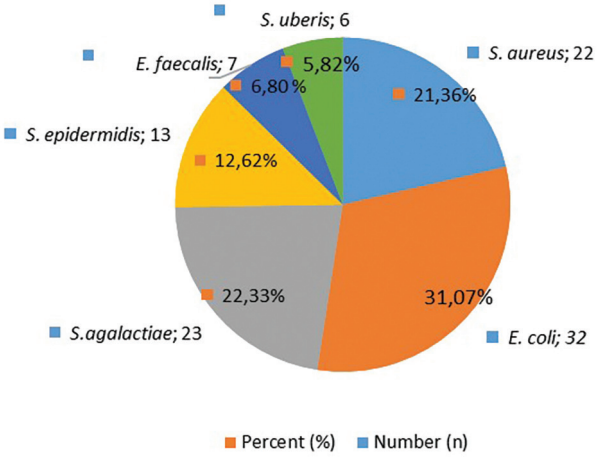

Fig 1. Distribution of isolated bacteria species

Statistical results for 200.000 cell/ml and above

SCC≥200.000 cells/ml was accepted as positive according to Tripladi et al. (2010). The Mc-Nemar test result (p-value=0.5>α=0.05) provided stronger evidence that CMT and bacteriology test results did not differ. The point estimates of the test for CMT and bacteriology examinations was 0.833. Significantly perfect agreement between these test was obtained. On the other hand, Mc-Nemar test result (p-value=0.031<α=0.05) provided that SCC and bacteriology results significantly differed. And also, the point estimates of the Kappa test for SCC and bacteriology was 0.437. The level of agreement between the CMT and SCC results was classified as slightly moderate and this agreement rate was supportive of the Mc-Nemar test result.

Table 2. The results of CMT scores, SCC and bacteriology in milk samples of Anatolian buffaloes (ABs)

Anatolian Buffalo ID	CMT Scores (+),(++),(+++)				SCC value	Bacteriological results			
	AML	PML	AML	PML		AML	PML	AML	PML
AB1	+++	+++	+++	++	980.000	*E.coli, S.aureus*	*E.coli, S. aureus*	*E.coli, S. aureus*	*S. aureus*
AB2	+++	++	+++	-	570.000	*S. agalactiae*	*E.coli, S.agalactiae*	*S. agalactiae*	*S.epidermidis*
AB3	+	-	+	+	135.000	*S.epidermidis*	-	*S. agalactiae*	*S. agalactiae*
AB4	-	-	-	-	66.000	-	-	-	-
AB5	++	+++	++	+	375.000	*S. agalactiae*	*S. agalactiae S. aureus*	*S. aureus*	*S. agalactiae*
AB6	+++	+++	+	++	880.000	*S. aureus, S.epidermidis*	*E.coli, S.agalactiae*	*S. agalactiae*	*E.coli E.faecalis*
AB7	-	-	-	-	50.000	-	-	-	-
AB8	+	-	+	-	78.000	*E.coli, E.faecalis*	-	*S. agalactiae*	-
AB9	-	+	-	-	55.000	-	*E.coli*	-	-
AB10	+	++	+	+	260.000	*S. agalactiae*	*E.coli, E.faecalis*	*E.coli*	*E.coli*
AB11	++	++	+	+	450.000	*S. agalactiae, S. aureus*	*S.epidermidis, S. uberis*	*E.coli*	*E.coli*
AB12	++	+++	++	++	400.000	*S. aureus*	*E.coli, S.agalactiae*	*E.coli*	*S.epidermidis, S. uberis*
AB13	+	+	+	++	250.000	*S. agalactiae*	*S. uberis*	*S.epidermidis*	*S.epidermidis S. uberis*
AB14	+	++	+	++	150.000	*E.faecalis S.epidermidis*	*S.epidermidis S. uberis*	-	-
AB15	++	++	++	+	375.000	*S.epidermidis, S. uberis*	*E.coli, S. aureus*	*S. aureus*	*E.coli*
AB16	+++	+++	+++	++	850.000	*E.coli, S.aureus*	*E.coli, E.faecalis*	*E.coli, S.aureus S.agalactiae*	*S.agalactiae S.aureus*
AB17	-	-	-	+	50.000	-	-	-	-
AB18	+	-	+	-	78.000	*E.faecalis*	-	*S. agalactiae*	-
AB19	-	-	-	-	50.000	-	-	-	-
AB20	+++	++	++	++	550.000	*E.coli, S.aureus S.agalactiae*	*E.coli, S.aureus*	*E.coli, S.aureus*	*S. aureus, S.epidermidis*
AB21	++	++	++	++	450.000	*E.coli S.agalactiae*	*S.aureus*	*S.aureus*	*E.coli, S.agalactiae*
AB22	++	+++	++	++	400.000	*S.agalactiae*	*E.coli, S.epidermidis*	*E.coli*	*E.coli*
AB23	+	++	+	++	250.000	*S.epidermidis*	*E.coli, S.aureus*	*E.coli*	*E.coli, S.aureus*
AB24	+	++	+	-	130.000	*E.faecalis*	*E.coli*	*S.agalactiae*	-

Statistical results for 78.000 cell/ml and above

Considering the results obtained the cut-of-value from analysis, the SCC test was taken into account again. In the first analysis run, the SCC positive threshold value was 200,000 and above. When re-evaluated with all data and analysis results, the cut-of-value for this study was updated as 78.000 cells/ml due to bacteriological isolation of *S. agalactiae* in one of the quarters from AB3, AB8, AB18, and AB24. The analysis was repeated, disregarding that the SCC values were below 200,000 cells/ml in the samples with *S. agalactiae* isolation. To put it more clearly, when we accepted SCC ≥ 200.000 cells/ml as positive, we realized we were missing one of the

major mastitis pathogen, *S. agalactiae*. In the light of this update, in the cut-of-value of the SCC test, samples AB4, AB7, AB9, AB17 and AB19 were considered negative for SCC and the analysis were repeated. The results obtained were given in Table 3, 4.

Table 3. Pairwise comparison of results: (a) from CMT and bacteriology, (b) from SCC and bacteriology

		Bacteriology		
		Negative	**Positive**	**Total**
CMT	Negative	3	0	3
	Positive	1	20	21
Total		**4**	**20**	**24**

		Bacteriology		
		Negative	**Positive**	**Total**
SCC	Negative	4	1	5
	Positive	0	19	19
Total		**4**	**20**	**24**

From Table 3(a), it was clear that CMT and bacteriology test results seemed to be quite compatible. They gave different results in only 1 sample (sample 17), that was, the bacteriology was negative while the CMT was positive. This difference was not statistically significant, see Table 3(a) analysis results. From Table 3(b), it was clear that SCC (with new cut-of-value 78.000) and bacteriology test results seemed to be quite compatible. They gave different results in only 1 sample (sample 9), that was, the bacteriology test was positive while the SCC was negative. This difference was not statistically significant, see Table 3(b) analysis results.

Mc-Nemar test was applied for CMT and bacteriology and also SCC and bacteriology. Mc-Nemar test result (p-value=0.5>α=0.05) provided stronger evidence that was CMT and bacteriology results do not differ In addition, Kappa statistic for assesing of agreement between the CMT and bacteriology results was also examined. The point estimates of the test for CMT and bacteriology was 0.833 (Data not shown). Significantly perfect agreement between these test were obtained. Mc-Nemar test result (p-value=0.5>α=0.05) provided stronger evidence that was SCC and bacteriologyresults did not differ statistically significant. In addition, the point estimates of the Kappa test for SCC and bacteriology was 0.864 (Data not shown). Significantly perfect agreement between these test were obtained.

Table 4. The results of reliability analysis

Reliability Statistics		
Cronbach's Alpha	Cronbach's Alpha Based on Standardized Items	N of items
0,928	0,931	3

Item Statistics			
	Mean	Std. Deviation	N
CMT	0,88	0,338	24
SCC	0,79	0,415	24
Bacteriology	0,83	0,381	24

Inter-Item Correlation Matrix			
	CMT	SCC	Bacteriology
CMT	1,000	0,737	0,845
SCC	0,737	1,000	0,872
Bacteriology	0,845	0,872	1,000

Summary Item Statistics							
	Mean	Minimum	Maximum	Range	Max/Min	Variance	N of Items
Inter-Item Correlations	0,818	0,737	0,872	0,135	1,183	0,004	3

The reliability analysis procedure was applied to obtain the reliability measure of the CMT, SCC and bacteriology results and also to obtain information about the relationship between individual tests. In addition, inter-test reliability estimates were calculated with the obtained intra-class correlation coefficients. Table4 represented the reliability statistics of this study. In this table, we had the Cronbach's Alpha (0.928) and the number of items (N of items) measuring Qu(3). The reliability (internal consistency) of Qu was 0.928 which was well above the recommended threshold of 0.70, this value assigned good reliability. The items (CMT, SCC, bacteriology) had relatively high internal consistency, thus, an internal consistency problem was not the case. Also, Table 4 showed the Item Statistics (Mean, standard

deviation and sample size, N) for each of the type of tests. Considering three types of test, averages of giving positive test result of three types were very close to each other. Also, the standard deviation for all the test types do not differ each other significantly. In addition, Inter-Item correlation matrix gave the correlations between CMT, SCC and bacteriology results. While the correlation value between CMT and SCC was 0.737, correlation between CMT and bacteriology, and, SCC and bacteriology scaled as 0.845 and 0.872, respectively. Additionally, mean of inter-item correlation was 0.818. These results showed that the test results are highly correlated with each other, an encouraging result (Table 4).

In addition to the reliability analysis, ROC analysis was also applied to highlight the validity of the new cut-of-value of SCC used in this study. ROC analysis was used to determine the diagnostic success (highest specificity and sensitivity values) of a test, and to compare the diagnostic success of the tests by comparing the sensitivity and specificity values obtained at different cut-off points. Thus,"1-speciticity" showed the false positive rate (Figure 2). Figure 2 illustrated the ROC curve, results of the data using MedCalc statistical software program, respectively.

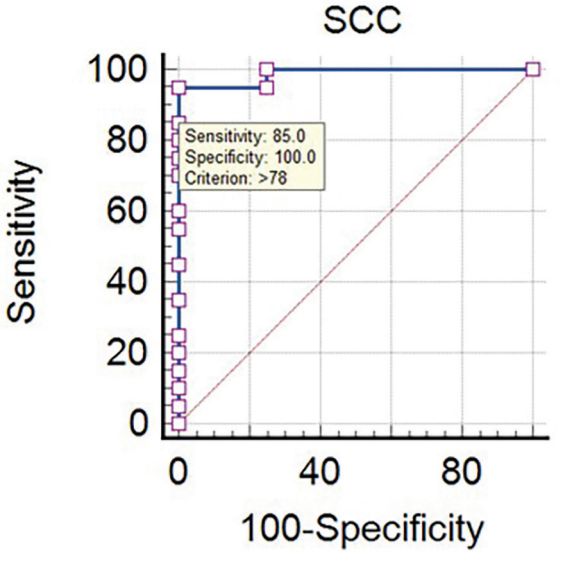

Figure 2. ROC Curve

From Fig 2, we could see that the ROC curve (the blue line) in this example huged the top left corner of the plot. The area under ROC curve (AUC) was obtained 0.988 which was extremely high (data not shown). Thus, it was concluded that SCC test did a good job of predicting of bacteriological results. Additionally, p-value < 0.0001 showed that the area under the ROC curve was significantly different from 0.5 and that therefore there is evidence that the SCC test does have an ability to distinguish between the two groups (positive and negative outcomes) (Figure 2). According to the general concept of ROC analysis, the position of the cut-off determined the number of true positives and false positives. The cut-of-value of SCC test for this study was chosen as 78.000 cells/ml with sensitivity %85 and 1-Specificity %0.00 (p-value < 0.0001)(Figure 2) (data not shown) The results of ROC analysis supported the results obtained from the reliability analysis, it showed that there is no problem in the use of the SCC cut-of-value as 78.000 cells/ml (Figure 2).

Discussion and Conclusion

Mastitis shows an important economic impact due to the decrease in milk yield, increased treatment costs and discarded milk, and high rate of animals replacement in dairy livestock (El-Kohedry and Osman 2008). Early and accurate diagnosis is known to be important for effective treatment, and control of mastitis (Charaya et al., 2015). Subclinical mastitis was declared as a type of mastitis that affected the milk composition and cellular structure without affecting the mammary glands (Fagiola and Lai, 2007).

CMT and SCC could be considered as reliable markers in the detection of subclinical mastitis (Chandra et al. 2019). In buffalo, the number of somatic cells is a critical parameter for detecting udder health and milk quality, as well as it is in other dairy animals (Costa et al., 2020). In addition to this, the scores of CMT are directly related to the average SCC. A higher CMT score is indicative of a higher SCC. In other words, CMT is like an indirect measure of SCC in milk (Harmon, 2001).

CMT score ≥1+ was detected in 21/24 (87.5%) animals while bacteriology was found to be positive at the rate of 83.33 %(20/24). The point estimates of the test for CMT and bacteriology was found to be 0.833 meaning a significantly perfect agreement between these tests. Birhanu et al. (2017) found that bacteriology and CMT results were nearly identical, with 105/262 (40.1%) cows being CMT positive and 101 (38.5%) out of the 105 CMT positive cows were culture positive. Coinciding with the results of our study, it has been reported that there was a qualitative correlation between the bacteriological culture and the CMT (Middlettonet al., 2004). It was reported that the number of somatic cells used as an important marker of udder inflammation in dairy cattle could be used in the early diagnosis of subclinical

mastitis in buffaloes, and the SCC threshold was determined as 200.000 cells/ml (Tripaldi et al., 2010). On the other hand, when the threshold value was accepted as 200.000 cells/ml, the point estimates of the Kappa test for SCC and bacteriology were 0.437, significantly different (data not shown). In our study, CMT scores ≥1+ were discovered in milk samples from AB3, AB8, AB9, AB14, AB18, and AB24 with somatic cell counts of 135.000, 78.000, 55.000, 150.000, 78.000 and 130.000 cells/ml, respectively, Morover, in contrast to the results of Tripladi et al. (2010), bacteria were isolated from at least one or more milk samples from at least one of the quarters. Inflammation in the udder tissue could be detected with CMT sensitivity without an increase in the number somatic cells, as seen in cows (Middleton et al., 2004). In particularly, isolation of *S. agalactiae*, a well-known contagious mastitic pathogen, from the milk samples obtained AB3, AB8, AB18 and AB24 with CMT score ≥1+, we re-evaluated with all data and analysis results, the cut-of-value for this study was updated as 78.000 cells/ml so as not to miss *S. agalactiae*. In support of our decision, Sharma et al. (2011), demonstrated that 15% of infected cows had SCCs<200.000 cells/ml (12.5-200x10^3 cells/ml). This time, the point estimates of the Kappa test for SCC and bacteriology were found to be 0.864. Significantly perfect agreement between these test was obtained. While the correlation value between CMT and SCC, CMT and bacteriology, and SCC and bacteriology were scaled as 0.737, 0.845, and 0.872, respectively. Additionally, mean of the inter-item correlation was found to be 0.818. These results showed that the test results were highly correlated with each other, which was an encouraging result. A ROC analysis was performed to emphasize the statistical validity of the obtained test results. When the SCC positive threshold value was taken as 78.000 cells/ml due to bacteriologic isolation of *S. agalactiae* in one of the quarters from AB3, AB8, AB18, and AB24, the specificity was obtained at 100.0%. So, the the rate of false positives was indicated as 0.00%. Moreover, for 78.000 cells/ml, the sensitivity for SCC was reached as 85.0%. These high values showed that this value could be used for identifying bacteriology in milk samples of Anatolian buffaloes for this study. According to our findings, this SCC threshold may be a useful detection criterion in Turkey field conditions. Parallel to our results, Sharma et al. (2010) also revealed the Kappa value of SCC was higher than that of CMT and CMT was concluded to be the most accurate test after bacteriology and SCC. Milk samples from one of the quarters of AB14 and AB17 with 150.000 cells/ml SCC and 50.000 SCC, respectively, in the current study showed that CMT (+) score without bacteriologic isolation was not enough to decide whether the quarters are infected or not in keeping with Guccione's claim of a significant difference between CMT score and SCC (Guccione, 2013). Similiarly to our findings, the author found 10-20% CMT positivity despite a negative bacterial culture (Guccione, 2013). This could be explained by short-term infections or intermittent bacterial shedding from the udder tissue (Sanford et al., 2006). In Stetca et al.'s (2010) study, the SCC was declared as normal up to 100.000 cells/ml, the somatic cell count was evaluated as an increasing value of 100.000-300.000 cells/ml, and the number of somatic cells 300.000 cells/ml and above was reported as a suspicion of infection. Unlike Stetca et al (2010), in milk samples of AB10, AB13 and AB23 with CMT sores (++), the SCC were determined as 260.000, 250.00 and 250.000 cells/ml, respectively and bacteria such as *S. aureus and S. agalactia* were isolated from all three (AB10, AB13, AB23), in line with the claim of a high probability to have a positive bacteriological culture if SCC was higher than 200.000 cell/ml by Sgorboni et al. (2014). CMT (+) to CMT (+++) scores, SCC of 300,000 cell/ml and above were observed in most of the milk samples belonging to AB1, AB2, AB5, AB6, AB11, AB12, AB15, AB16, AB20, AB21, AB22 with bacteriologic isolation of major mastitic pathogens such as *S. aureus*, *S. agalactia, E. coli* in line with Tripladi et al. (2010) and Stetca et al. (2010).

When we compared our findings to those of previous researches in terms of SCC, we came to the conclusion that using a combination of CMT, SCC, and bacterial isolation at the same time would prevent misleading diagnoses of subclinical mastitis. Since, evaluating the number of somatic cells below 200.000 cells/ml in milk samples alone without information about the microbiological status of the breast could be misleading. On the other hand, milk culture determines mastitic pathogens but does not reveal the severity of infection-related inflammation (Sharma et al., 2011). There are different results on threshold of SCC on either breast with infection or without infection in the world as well in Turkey (Dhakal etal. 2008; Sahin et al., 2017). Ozenc et al. (2008) reported that the number of somatic cells in the initial period of lactation was 130.000 cells/ml in buffalo milk samples with subclinical mastitis in Turkey which was lower than 200.000 cell/ml. Contrary to our results, in another study conducted in Turkey, Sekerden (2011) reported that the number of somatic cells was below 100.000 cells/ml in wa-

ter buffalo milks without breast infection. The average SCC was recorded at 90,701±6,372 cells/ml in Anatolian buffaloes in Turkey. (Sekerden, 2011). Comprehensive studies that will contribute to the determination of the standard SCC are required in order to develop the threshold values suitable for Turkey's conditions. SCC was affected by various factors such as stage of lactation, age/breed, parity/season/stress, diurnal variation, milking as stated by Sharma et al. (2011). The substantial difference in SCC values between udders infected by specific pathogens and healthy udders was described by Tripaldi et al (2010). It has been known that bacterial infections in buffaloes due to poor environmental hygiene and growing conditions increase udder damage and cellular reaction (Tripaldi et al., 2010).

Information about the prevalence and distribution of mastitis-causing bacteria is crucial in order to control and/or prevent the disease (Ozenc et al., 2008). Common mastitic bacterial pathogens in water buffaloes were declared contagious as *S. agalactiae*, *S. aureus, A. pyogenes, Mycoplasma* spp.; environmental as *S. uberis, S. dysgalactiae, E. coli, Enterobactericeae*, yeast and moulds and opportunist as coagulase negative staphylococci (Fagiola and Lai 2007). In our study, the range of the bacteria was found to be similiar to Fagiola and Lai (2007). In a study carried out in Afyon province, Turkey, *Candida* spp., CNS *Staphylococcus* spp. were determined to be mastitic agents in Anatolian buffaloes (Ozenc et al., 2008). To the best of our knowledge, the frequent microorganisms and their isolation rates in subclinical and clinical mastitis cases of buffaloes varied between countries, in the different regions of the same country, and even on different farms in the same region (Patel et al., 2019). For instance, in the studies conducted in Brazil, the most frequent agents isolated from mastitic buffalo milks were declared to be *Corynebacterium* spp. (19.76%-59.25%), *Staphylococcus* spp. (17.65%-37%), *Streptococcus* spp. (16.94%-39%) (Pardo et al. 2007). However, in another study conducted in Brazil, *Corynebacterium* spp. (5%) was reported to be observed with the low incidence (Vasquez-Garcia et al., 2017). Up to 50% *S. aureus* predominance followed by *S. agalactiae* and *S. dysgalactiae* were reported in mastitic buffaloe milks in Pakistan (Ali et al., 2008; Hussain et al., 2007). The variety of mastitis pathogens was attributed to the diversity of ambient microflora at the farms, as well as the hygiene and sanitation conditions of the farms. The distribution and impact of mastitis pathogens are affected by differences of countries legislation, veterinarian and laboratory practices, and farmer management methods (Zadok and Fitzpatrick, 2009).

E. coli, an environmental pathogen, was the predominant mastitic pathogen followed by contagious pathogens, *S. agalactiae* and *S. aureus*. Among the remaining 3 bacteria, *S. uberis, E. faecalis* were also environmental pathogens, except an opportunist pathogen, *S. epidermidis*. In our study, the single culture of *E. coli* isolation was determined to be low, commonly *E. coli* was observed to accompany with *S. aureus, S. agalactiae, E. faecalis* in mixed cultures (Table 2). Owing to the fact that environmental pathogens existed in the faeces, soil, litter and milking equipments, and do not colonize the milk duct, during bedding after milking or interval between two milking was thought to be the possible entrance for them. In particularly, teat lesions caused by contaminated milking equipment allows the penetration and invasion of *E. coli* in the udder during bedding after milking or during interval between two milkings (Fagiola and Lai 2007). Morover, pendulous udder and longer teat declared contributing a greater risk to mastitis (Chavoshie and Husaini 2012). Hence, high isolation rate of *E. coli* in particularly in mixed cultures mostly from posterior quarters accompanying with high SCC was attributed to fecal contamination due to pure hygenic conditions, but not for infection (Table 2). Morover, *E. coli* was reported to be dominant in particularly clinical mastitis accompanied with high SCC, but not for subclinical mastitis (Zadok and Fitzpatrick, 2009). The remaining environmental pathogens, *S. uberis* and *E. faecalis* observed with low incidence in the study was the same situation as stated above. The majority of the mastitic infections were known to be caused by contagiouse pathogens; *S. aureus* and *S. agalactiae* (Ruegg, 2017). *S. agalactiae* was determined to be the second frequently isolated mastitic pathogen in this study. *S. agalactiae* is known as an obligate pathogen of udder being transmitted during milking. Remaining in the milk ducts as superficial, it was reported to cause subclinical mastitis inducing significant increase in SCC (Fagiola and Lai, 2007) in line with SCCs≥200.000 cells/ml in our study (Table 2), however, in milk samples with SCCs<200.000 cells/ml, we also isolated *S. agalactiae* as stated above. It has been reported that *Staphylococcus* spp. was predominant in subclinical mastitis in milk samples of buffaloes(Ruegg, 2017; Pisanu et al., 2019; Coimbra-e-Souza et al., 2017). The most common cause of mastitis in buffaloes in particularly observed at the beginning of lactation was reported to be *S. aureus*, contagious agent, which is prevalent

in the skin and the surroundings and spreads to the udder tissue by abrasion of the teat tip and poor milking practices (Anderson et al., 2012). *S. aureus* was once thought to be a common cause of mastitis in cattle and water buffaloes in Asia. *S. aureus* prevalence in milk from cattle and buffaloes have been observed in Asia, ranging from 29.0 percent in China to 54.87 percent in India (Badua et al., 2020). After colonization to the nipple channel, *S. aureus* adheres to the epithelium of the ducts and alveoli in the gland and begin to produce toxins. After the adhesion of bacteria, macrophage stimulation and the migration of neutrophils from blood to milk are stimulated, resulting in an increase in SCC and swelling of the mammary gland (Jia et al., 2020). In this study, the milk samples with high SCC between 250.000-980.00 cells/ml were observed to harbour *S. aureus* as seen in Table 2. While some studies claimed*S. aureus* isolation rates of more than 50% in buffalo milk (Khan et al., 2013; Ali et al., 2008), others claimed rates as low as 2.5 percent (Chavoski and Husaini 2012). Due to remaining in the farms for along time *Staphylococcus* spp. was reported to be the causative agent of subclinical mastitis (Fagiola and Lai 2007). The fourth frequently isolated microorganism, *S. epidermidis* is well-known resident of the normal udder skin. When the predisposing factors such as skin lesions, unproper milking, inadequate hygenic conditions exists in the farm, it can cause mastitis as an opportunistic pathogen in subclinical mastitis cases. In accordance with our study, Dhakal et al (2018) reported predominance of CNS *Staphylococcus* spp. such as *S. albus* and *S. epidermidis* associated with subclinical mastitis.

Consequently, bacteriological examination based on CMT and SCC results might be deceiving when determining the subclinical mastitis is prevalent in a flock. Therefore combination of CMT, SCC, and bacteriological examination simultaneously-provides correct diagnose as well as the opportunity to take immediate action and initiate treatment. Given the satisfactory correlation between the tests when threshold value for SCC can be accepted below 200.000 cells/ml such as 78.000 cells/ml in this study, it was assumed that variations affecting SCC and differences at environmental hygiene and growing conditions varying between countries were the cause. Major mastitic pathogens, *S. aureus* and *S. agalactiae*, circulating among the Anatolian buffaloes should be taken in consideration in Turkey.

Ethics committee for the use of experimental animals and other ethics committee decisions and permissions: Pursuant to paragraph k of article 8 of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" published in the Official Gazette dated 15.02.2014 and numbered 28914; milking is not subject to the permission of the Animal Experiments Local Ethics Committee (HAYDEK).

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