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NOTE

Clinical Pathology

The bacterial load in milk is associated with clinical severity in cases of bovine coliform mastitis

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ABSTRACT. We evaluated the relationship between the severity of coliform mastitis and bacterial load in 106 quarter milk samples. We found no significant relationship between somatic cell count and coliform bacterial load in milk in bovine clinical coliform mastitis. Results of the Cochran-Armitage test for trend in milk bacterial load proportions indicated a significant decreasing low group ($P<0.001$), increasing medium group ($P<0.002$) and increasing high group ($P<0.02$) with increasing clinical grade. The present study indicates that the coliform bacterial load in milk is significantly associated with clinical severity states in cases of bovine coliform mastitis, and can be a useful indicator for optimal management of this disease.

KEY WORDS: bacterial load, clinical severity states, coliform mastitis

Bovine mastitis, an inflammatory disease in dairy cows caused by bacterial infection of the mammary gland, has detrimental effects on milk quantity and quality [16, 33]. It is a complex disease responsible for serious economic loss in the dairy industry [16, 19]. Successful treatment of mastitis is dependent on early detection and proper diagnosis, including accurate identification of the pathogen involved, because the treatment varies depending on the causative pathogen [13]. Therefore, it is extremely important to identify causative bacteria for the treatment of bovine mastitis.

Coliform bacteria such as *Escherichia coli* (*E. coli*) and *Klebsiella* sp. are prevalent in the bovine environment and are among the most common mastitis-causing pathogens responsible for eliciting obvious clinical symptoms in cows [28]. The infection is initiated by the entry of the bacteria through the teat canal, and after a short infection period, is characterized by a strong inflammatory response, including influx of neutrophils into the udder [2, 11]. In dairy cows, coliform mastitis can range from a mild disease of short duration to a severe, peracute, life-threatening condition. The severity of coliform mastitis is associated with the degree of production loss and clinical outcome [40]. Therefore, evaluating the severity of coliform mastitis in dairy cows is important in determining appropriate treatment and making sound management decisions.

Various scoring systems have been developed to quantify the severity of clinical mastitis. In most of these scoring systems, disease severity is determined according to normal or abnormal physical characteristics of the mammary gland and milk, and the presence or absence of systemic signs of disease; thus, the severity of bovine mastitis is associated with the degree of mammary gland damage [1, 31, 40]. In a previous study of a case of bovine mastitis caused by *Staphylococcus aureus* (*S. aureus*), we found a significant correlation between the *S. aureus* load and the proportion of mammary epithelial cells in milk, indicating that the *S. aureus* load in milk reflects the exfoliation of mammary epithelial cells due to mammary damage [29]. Thus, the bacteria load in milk may be one of the factors that an indicator of the severity of bovine mastitis. In addition, the probability of a successful treatment outcome depends on both the cow's condition and various pathogenic factors related to the infected udder [43]. Evaluation criteria for determining the economic value of the cow and/or udders are often based on only the somatic cell count (SCC) of the milk [22]. However, although the bacterial load and SCC in milk are potential factors determining the severity of coliform mastitis, little is known about the relationship between the severity of coliform mastitis and these factors. Identification of

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good indicators of the severity of this disease would be helpful in determining the prognosis and optimal clinical management, and evaluating the treatment effect.

Therefore, in the present study, we evaluated the relationship between coliform bacteria load or SCC in mammary secreted milk and the severity of coliform mastitis according to systematic grading. The study results would provide information that would enable accurate diagnosis and optimal treatment of coliform mastitis.

Quarter milk samples from Holstein dairy cows with clinical mastitis were collected between 2016 and 2017 from dairy farms located in Ishikari district, Hokkaido, Japan. All cows examined in this study had no history of bovine mastitis before the present study. These cows had been examined for clinical mastitis by farm workers and/or veterinarians, and milk samples for bacteria isolation were collected aseptically prior to antimicrobial treatment during the initial farm visit by veterinarians.

Farm workers and/or veterinarians were trained to classify the severity of clinical mastitis by using a previously defined scoring system as follows [31]: mild (grade 1), when only the milk was abnormal (flakes, clots, or serous milk); moderate (grade 2), when abnormal milk was accompanied by swelling or redness of the mammary gland; or severe (grade 3), when the cow exhibited systemic signs of illness such as depression, anorexia, dehydration, or fever. These scores were added to the clinical records.

The coliform bacterial load and SCC of the milk samples were measured as follows. First, 50 μl of each sample was plated on a sheep blood agar plate (Nissui pharmaceutical, Tokyo, Japan). Then, after 24 hr of incubation at 37°C, each plate was inspected for bacterial growth, which was identified as coliform by colony characteristics. We considered milk samples to be contaminated if three or more kinds of different bacterial species were clearly observed on these bacterial cultures, and these samples were excluded from this study as described by Pinzón-Sánchez and Ruegg [31]. To measure the coliform bacteria load, we spread a 1-m*l* aliquot of each milk sample on a Petrifilm coliform count plate (3M, Minneapolis, MN, U.S.A.), which is known as suitable for identifying mastitis pathogens [14, 25, 26], incubated the plates at 37°C for 24 hr, and then counted the colonies. The counts were used to calculate the colony forming units (CFU)/m*l*. The milk samples were divided into three tertiles according to their coliform bacteria load: low (0< $\log_{10}$ CFU ≤3), medium (3< $\log_{10}$ CFU ≤6), and high (6< $\log_{10}$ CFU) coliform bacterial load groups. The SCC of the milk was measured using a DeLaval cell counter DCC (DeLaval, Tumba, Sweden) as described by Kawai *et al* [23].

A one-way ANOVA was conducted using SPSS software (IBM SPSS Statistics version 25, Tokyo, Japan). Prior to performing the statistical analysis, we converted the SCC and coliform bacteria load data to $\log_{10}$ to obtain a normal distribution. Statistical analyses were performed using one-way ANOVA followed by Scheffe's test to evaluate statistical differences among the coliform bacterial load groups. A test for trends in clinical severity states was performed using a Cochran-Armitage test for each bacterial load group. The Cochran-Armitage trend test was conducted using R software (R core team, 2016). *P*-values <0.05 were considered statistically significant.

A total of 206 quarter milk samples were initially identified as eligible for inclusion in this study. After performing bacterial cultures, nine samples were excluded because of contamination. Of the remaining samples, 106 yielded colonies of coliform bacteria on Petrifilm Coliform count plates, and these samples were used for the subsequent analysis. On the basis of the coliform bacteria load, we classified the 106 milk samples into low (n=36), medium (n=47), and high (n=23) coliform bacterial load groups. Moreover, on the basis of the severity scoring system, the 106 milk samples were divided into grade 1 (n=18), grade 2 (n=14), and grade 3 (n=74) clinical score groups.

First, we investigated the relationship between the SCC and severity score or coliform bacteria load. No significant differences in SCC could be detected among the severity score groups (average SCC in grade 1, grade 2, and grade 3 groups was 6.9, 6.6, and 6.5 counts/m*l* respectively) or the coliform bacterial load groups (average SCC in low, medium, and high groups was 6.5, 6.6, and 6.9 counts/m*l*, respectively; Fig. 1A and 1B). Next, we investigated the relationship between the severity score and coliform bacteria load in milk. Results of the Cochran-Armitage test for trend in proportions indicated a significant decreasing low group (P<0.001), increasing medium group (P<0.002) and increasing high group (P<0.02) with increasing clinical grade (Fig. 2).

Evaluating the severity of bovine mastitis caused by coliform bacteria in dairy cows is important in determining appropriate treatment and making milk production predictions. We focused on coliform bacterial load and SCC in milk as indicator of the severity of bovine mastitis, and investigated the relationship between coliform bacterial load, SCC in milk, and the severity of mastitis according to a clinical severity scoring system.

Disease severity is determined by interactions between the host, environment, and infectious agent. The SCC in milk is considered to be a marker for the onset of bovine mastitis [22, 35]. However, in this study, we investigated the relationship between the SCC and severity score or coliform bacterial load in cows with clinical coliform mastitis, but found no significant correlation (Fig. 1A and 1B). Previous studies have demonstrated that the SCC in milk is affected by many bovine factors such as cattle species, milk production level, and lactation stage [10, 34, 37]. Moreover, somatic cells, which include neutrophils, macrophages, and dendritic cells, play an important role in the defense mechanism of the mammary gland. Indeed, we have also investigated the relationship between the SCC and *S. aureus* load in the milk of an experimental model of *S. aureus*-induced mastitis, but found no significant correlations [29]. Although SCC itself is thought to be a useful marker for the onset of bovine mastitis, we concluded that it is unsuitable as criteria for directly evaluating the severity of coliform mastitis. However, the dynamics of bacterial growth and elimination from infected glands are determined by many factors. Burvenich *et al*. [9] concluded that the severity of *E. coli* mastitis is strongly correlated with bovine host factors rather than bacterial pathogenicity. In their report, they suggested that the number of bacteria in the mammary gland is likely dependent on the innate immunity of the cow, specifically on neutrophil function. Indeed, the severity of experimentally induced *E. coli* mastitis during early lactation was strongly correlated with the pre-infection innate capacity of isolated blood neutrophils to generate reactive oxygen species after zymosan and phorbol ester stimulation and on the chemotactic response of these cells [17, 24, 27]. Future studies should aim to elucidate the role of SCC,

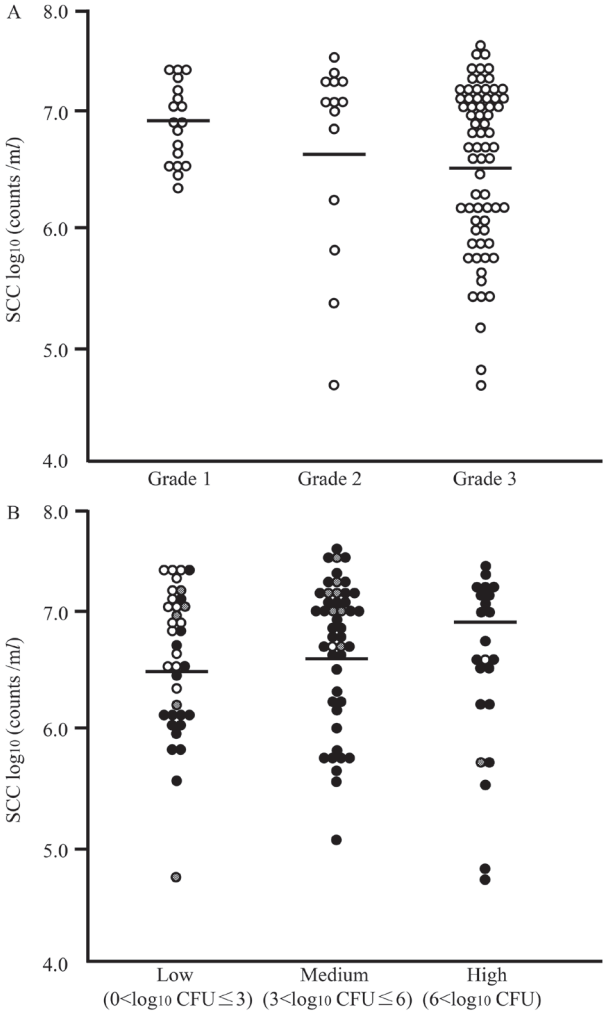
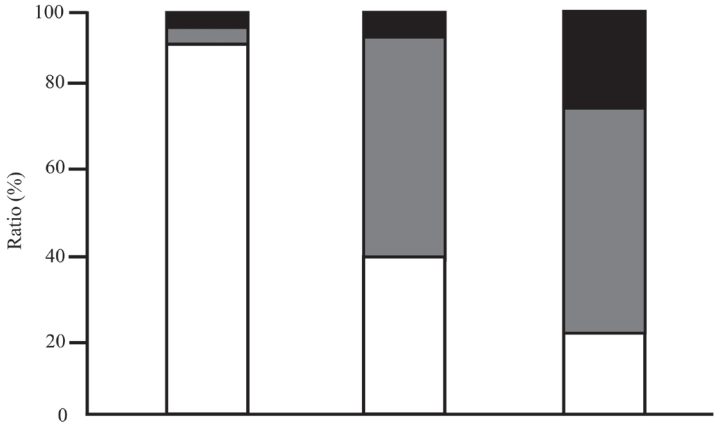


Fig. 1. Comparison of somatic cell counts among clinical severity score and coliform bacterial load groups. (A) On the basis of clinical severity scores, we divided 106 milk samples into three tertiles: grade 1 (n=18), grade 2 (n=14), and grade 3 (n=74) clinical score groups. Each data point represents one quarter milk sample (White circle, n=106), and the bar represents the mean. (B) On the basis of coliform bacterial loads, we divided 106 milk samples into three tertiles: low (n=36), medium (n=47), and high (n=23) coliform bacterial load groups. Each data point represents one quarter milk sample (n=106), and the bar represents the mean. White circles, grade 1; gray circles, grade 2; black circles; grade 3. SCC=somatic cell count. CFU=colony forming units.



	Grade 1	Grade 2	Grade 3
Total number of udders	18	14	74
Low (%)	16(88)	5(36)	16(22)
Medium (%)	1(6)	8(57)	37(50)
High (%)	1(6)	1(7)	21(28)

Fig. 2. Distribution of cows among the severity score and coliform bacteria load groups. On the basis of coliform bacteria loads, we divided 106 milk samples into three tertiles: grade 1 (n=18), grade 2 (n=14), and grade 3 (n=74) clinical score groups. The stacked bar graph shows the distribution of cows in each coliform bacterial load group between the three different clinical severity grades: low (white), medium (gray), and high (black). Values are given as number of cows (%). CFU=colony forming units.

including the individual cell types comprising the SCC, and the relationship of the number of each of these cell types with the severity of coliform mastitis.

Next, we investigated the relationship between the severity scores and coliform bacteria load. Results of the Cochran-Armitage test for trend in proportions indicated a significant decreasing low group, increasing medium group and increasing high group with increasing clinical grade (Fig. 2). Previous studies in cows with experimentally induced coliform mastitis have shown significant positive correlations between the number of *E. coli* in milk, milk endotoxin concentration, and severity of systemic clinical signs [21]. Endotoxin, also known as lipopolysaccharide, is a structural component of the outer membrane of all gram-negative bacteria, including *E. coli* and *Klebsiella* spp. [38, 42]. Endotoxin is a potent stimulator of the immune system of animals, and clinical disease occurs either when an excessive amount is released within the body or when the host responds in an overly sensitive manner [32]. Most of the clinical disease signs associated with acute coliform mastitis are attributed to the endotoxin-mediated induction of endogenous inflammatory mediators, especially the cytokine tumor necrosis factor-α [20, 21]. Thus, the severity of coliform mastitis could be dependent on endotoxin-induced immune mediator responses. Indeed, Wenz *et al*. demonstrated a positive relationship between systemic severity signs and the number of bacteria in the infected mammary glands of dairy cows with acute coliform mastitis [39]. Taken together, these reports and our present study results indicate that the number of bacteria in the mammary gland is directly associated with clinical disease severity.

In the present study, we did not evaluate the differences in the species of coliform bacteria. The most common species, isolated in more than 80% of cases of coliform mastitis, is *E. coli* [7]. In addition, this bacterial species is the most common cause of clinical mastitis in well-managed dairy herds with low milk somatic cell counts [3, 8]. However, *Klebsiella* spp. may also cause either individual clinical mastitis cases or outbreaks in dairy herds [28, 30]. Economic losses due to *Klebsiella* spp. are much greater than those due to *E. coli* because of reduced survival and milk production [12, 15]. In future studies, it is necessary to further investigate the relationship between severity score in coliform mastitis and bacterial load of each of the different coliform bacterial species.

Interestingly, the present study indicated that few cases were categorized with low bacterial loads, yet classified as grade 3 with systemic symptoms, and cases categorized with high bacterial loads, were yet classified grade 1 with only local symptoms (Fig. 2). This fact indicates that severity state based on coliform bacteria load does not necessarily depend on bacteria count. In some cases, in the low bacterial load group with systemic symptoms, one plausible explanation is that excess endotoxin remains in the mammary gland, and indicates that the infection caused by endotoxin of coliform pathogens is continuing. Indeed, direct injection of lipopolysaccharide into the mammary gland reveals signs such as coliform mastitis, despite no living bacterial pathogens and no control associated with bacterial growth [36, 41]. On the other hand, in the case of high bacterial load with only local symptoms, it is necessary to consider that multiple factors such as strain differences and bovine sensitive host factors. Recently, Blum *et al*. indicated that differences in the intensity and duration of SCC, milk yield, bacterial count, and other mammary immune responses were observed in a challenge study comparing a strain of *E. coli* isolated from the environment and peracute, recurrent, and acute mastitis. In particular, the K71 strain of *E. coli* from the environment did not elicit inflammation in bovine mammary glands [5]. In addition, one recent report showed that the pathogenicity of *E. coli* causing mastitis in cows is dependent on the *fec* locus of the ferric citrate uptake system [4]. These results indicate that the severity of coliform mastitis may be dependent on variations within single bacteria species. Another reason is that milk yield may affect the concentration of bacterial load. In rare cases, there are long term detrimental effects on mammary gland health and milk quality following an *E. coli* mastitis infection, and in some cases the mammary gland does not fully recover, resulting in reduced milk yield [6]. As we do not have data on milk yield in this study, future research needs to evaluate changes in concentration of bacterial load and the decreasing milk yield caused by coliform mastitis. Additionally, persistent *E. coli* infections in the mammary gland causing recurrent episodes of mastitis have long been documented [18]. Generally, cases with recurrent infections are more difficult to treat, and treatment is different from treatment for the initial infection. Thus, the medical history of the cow also needs to be considered for the prognosis and optimal clinical management; however, the present study focused on initial coliform mastitis infections. It is important that future research also focuses on the endotoxins of the coliform pathogen, strain differences, and bovine sensitive host factor, to further elucidated the precise relationship between bacterial load and severity state.

In conclusion, the present study revealed increasing bacterial loads with increasing severity grade, and thus demonstrated that coliform bacterial load in milk is associated with clinical severity score of bovine mastitis. In future studies of cows with coliform mastitis, measurement of bacteria load could help reduce confounding associated with disease severity and increase the accuracy of result comparisons among studies. The present study results suggest that measurement of bacteria load could be important for determining appropriate treatment and making sound management decisions in coliform mastitis.

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