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Short Communication

Live Bacteria in Clots from Bovine Clinical Mastitis Milk with No Growth in Conventional Culturing

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Abstract

Background and Objective: Although screening of clinical mastitic milk for bacteria is necessary for evidence-based antibiotic treatment and prevention of mastitis, these milk samples are frequently culture negative. The purpose of the present study was to evaluate clots from culture-negative mastitic milk for the presence of viable bacteria in an attempt to unravel the reason for the no-growth (NG) status of mastitic milk. **Methodology:** A total of 449 clinical mastitic milk samples were enrolled, visual inspections were performed to confirm the presence of clots in milk samples and specimens were subjected to bacterial culturing by conventional methods. Milk samples that were NG by primary culture were re-cultured. Furthermore, clots in NG milk samples were recovered aseptically and cultured. Bacteria isolated from milk or clots were identified using biological methods. **Results:** In primary culture, NG was detected in 96 out of the 449 samples tested (21.4%). The proportion of mastitic milk with clots was significantly higher in NG milk (45.1%, 78/173) than in growth (G) milk (6.5%, 18/276). In the secondary culture of NG milk and its clots, the culture-positive rate of clots from NG milk (53.8%, 42/78) was significantly higher than that from NG milk (34.4%, 33/96). Although no differences were found in other pathogens, the isolation rate of coliforms from clots (20.8%, 20/78) was more than two times that from milk (8.3%, 8/96). **Conclusion:** These findings suggested that elimination of mastitic bacteria by the host immune system may be a cause of culture-negative mastitic milk and that this pattern is often observed upon infection with coliforms.

Key words: Clots, culture negative, mastitic milk, mastitis, coliform

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Clinical mastitis affects milk yield and quality can lead to major economic losses for the dairy industry worldwide¹. Given that the primary causative agents of mastitis are bacteria, the use of antibiotics is considered to be the standard of care for treatment and prevention of the disease in dairy herds². Hence, reliable identification of the causal bacteria is important not only for treatment but also for developing mastitis control strategies for dairy herds. Recent studies have shown that no bacterial growth is detected in at least 20% to over 40% of milk samples obtained from cows that present with clinical mastitis^{3,4}. The no-growth (NG) samples pose problems for veterinarians and farmers owing to a lack of objective evidence for treatment. Possible reasons for the NG status of mastitic milk samples may include the following: clearing of the infection by the cow's immune system, the presence of organisms at numbers below the limit of detection, selection of improper test(s) for the organism(s) of interest, failure of the cow to shed the pathogen and non-infection⁵. Nonetheless, the precise reason for the NG status remains unknown at present⁶.

In clinical mastitis, several significant changes occur in the tissue and in the milk in response to infection. These effects include not only local inflammation signs but also gross changes in the milk such as the presence of flakes, clots, or serous milk, where increases in somatic cell counts (SCCs) may be observed. The large increase in SCC reflects an influx of polymorphonuclear neutrophils (PMNs) flowing from blood vessels into milk resulting in the formation of clots in mastitic milk through an aggregation of leukocytes and phagocytes. However, it remains unclear whether bacteria confronted by PMNs remain viable⁷.

The purpose of the present study was to investigate clots of culture-negative mastitic milk for the presence of viable bacteria, an evaluation that was expected to reveal the underlying cause for the NG status of mastitic milk.

MATERIALS AND METHODS

Milk collection and primary bacterial culture: Milk samples from animals who presented with clinical mastitis between December, 2016 and March, 2017 were enrolled in the present study. These cases occurred at a commercial dairy of 2,000 Holstein cows, a facility located in Tochigi Prefecture, Japan (just North of Tokyo). All cows had been examined for mastitis by farm personnel and milk samples for bacterial isolation were collected aseptically prior to antimicrobial treatment. After visual inspection for the presence of clots in the milk,

10 μ L of each milk sample were directly cultured onto a sheep blood agar plate (Nissui pharmaceutical, Tokyo, Japan). After 24 h of aerobic incubation at 37°C, each plate was inspected for bacterial growth and the number of colony-forming units was counted. Samples from quarters with clinical mastitis that had no colony growth after 24 h were identified as NG, samples that yielded colony growth were classified as G. The NG milk samples were stored at -20°C pending the secondary bacteriological culturing. The experiment was performed in compliance with the ethical code for animal welfare of the Azabu University.

Secondary bacterial cultures: All NG milk samples (n = 96) were thawed at room temperature before secondary bacterial culturing. To confirm the NG status obtained in the first cultures, 10 μ L of each milk sample were plated as described above. In addition, NG milk samples with clots (n = 78) were used for clot cultivation. Specifically, clots observed in NG milk samples were recovered aseptically using an inoculating loop and spread onto sheep blood agar as described above. After 48 h of aerobic incubation at 37°C, the plates were inspected for bacterial growth. All operations were performed aseptically on a clean bench. Bacteria isolated from milk and/or clot samples were identified and confirmed using biological methods as described in a previous report⁸.

Statistical analysis: Differences in culturing results between each sample type were tested using the chi-square test. The $p < 0.05$ were considered significant. All statistical analyses were performed with EZR (The R foundation for Statistical Computing, Vienna, Austria). This program is a modified version of R commander that enables the application of statistical functions frequently used in biostatistics⁹.

RESULTS

The results of primary and secondary bacterial culture are summarized in Table 1. A total of 449 milk samples were enrolled. Under standard growth conditions of primary bacterial culture, pathogens were detected in 353 of 449 clinical mastitis samples (78.6%), while 96 samples (21.4%) were classified as NG. The proportion of milk samples with clots was 38.5% (n = 173/449). The NG percentage of mastitic milk with clots (45.1%, n = 78/173) was nearly seven times higher than that of mastitic milk without clots (6.5%, n = 18/276) ($p < 0.01$). In the secondary bacterial culture of the 96 NG milk samples, no significant differences were observed in the isolation rate of bacteria between milk with clots (32.1%, n = 25/78) and milk without clots

Table 1: Results of bacterial growth by primary/secondary culture

Sample	n	Bacteria		p-value*
		Growth	No growth	
Primary culture				
Milk without clots	276	258	18	<0.01
Milk with clots	173	95	78	
Secondary culture				
Milk	96	33	63	0.0153
Clots	78	42	36	

*Chi-square test

Table 2: Percentage of isolated pathogen from milk and clots samples after freezing

Pathogen	Cumulative total (%)		
	Milk (n = 96)	Clots (n = 78)	p-value*
Coliforms	8 (8.3)	20 (20.8)	0.00395
Coagulase-negative staphylococci	6 (6.3)	7 (7.3)	0.697
<i>Staphylococcus aureus</i>	1 (1.0)		
Other streptococci	9 (9.4)	8 (8.3)	1
<i>Corynebacterium bovis</i>	18 (18.8)	12 (12.5)	
<i>Pseudomonas</i> spp.		4 (4.2)	0.702
Bacillus		5 (5.2)	
Other pathogens		2 (2.1)	0.0153
Culture-negative	63 (65.6)	36 (46.2)	

*Chi-square test

(44.4%, n = 8/18). On the other hand, in the cultivation of clots, bacteria were isolated from 42 of 78 (53.8%) samples, a frequency significantly higher than that obtained from the cultivation of milk itself (34.4%, n = 33/96) ($p = 0.0153$).

A comparison of the distribution of bacteria isolated from NG milk samples and from clots revealed that only coliforms were isolated at significantly different rates from the two matrices ($p = 0.00395$), with the rate being higher from clots. There were no differences in the isolation ratio of other classes of pathogens (Table 2).

DISCUSSION

Though the proportion of mastitic milk with NG status in the present study was similar to those in previous reports, it was apparent that NG milk is significantly more likely to contain clots compared to growth (G) milk. The clots, which result from an aggregation of leukocytes and phagocytes, may have been formed towards the end of the infection. After the secondary culture of NG milk, bacteria were isolated from 34.4% (33/96) of these samples, yet no relation between the bacteria and the presence of clots was observed. A possible explanation for this result may be the low concentration of bacteria in the milk sample. Meanwhile, bacteria were isolated

at significantly higher rates from the clots than from NG milk. It has been reported that substances in milk may hamper the viability of bacteria in culture, which suggests that it may be easier for the causative bacteria to survive in the clots than in milk¹⁰. This observation suggests in turn that NG milk arises from the clearance of infection by the cow's immune system. Comparisons of the distribution of isolated pathogens from NG milk and clots, revealed that only coliforms were isolated at a significantly different frequency, with coliforms isolated more often from clots than from NG milk. Previous reports have observed that the viable counts of a certain variety of pathogens in milk decreased after collection and during preservation at room temperature, reflecting the action of leukocytes and antimicrobial components present in milk¹¹. In the present study, coliforms in NG milk may also have been affected by this phenomenon prior to the initiation of plate culturing. It is suggested that the milk-mediated host immune response to coliform infection is among the causes of NG status in clinical mastitic milk.

The present study has several potential limitations that should be acknowledged. Firstly, secondary bacterial culture was carried out after freezing of the milk samples. Previous reports^{12,13} have noted that the post-freezing viability of pathogens may vary with species, so this issue will need to be addressed in subsequent experiments on the clot carriage of bacteria. Secondly, samples were cultivated under aerobic conditions on sheep blood agar in the work reported here. The recovery of bacteria (e.g., anaerobes or *Mycoplasma*) that do not grow aerobically or on conventional media was not tested here, but should be examined in future work. However, it is worth noting that *Mycoplasma* mastitis has not previously been reported on the farm that supplied the samples characterized here.

At present, it is uncertain whether the bacteria isolated from clots in NG milk can serve as causative agents of mastitis, although the recovery of these microbes can be interpreted as an evidence of past infection. Hence, this result could provide useful information for prevention as opposed to treatment, of this disease.

CONCLUSION

Spontaneous healing of clinical mastitis by the host immune system is thought to be one of the reasons of culture-negative mastitic milk. The recovery of coliforms from clots in these samples suggests that these microbes may be the causative agents in these infections.

SIGNIFICANCE STATEMENT

This study discovers the possibility of the causes for culture-negative mastitic milk that can provide beneficial information to prevent mastitis. This study will help the researcher to uncover the critical areas of high incidence rate of culture-negative mastitic milk that many researchers were not able to explore. Thus, a new theory on live bacteria in clots and the cause of culture-negative mastitic milk may be arrived at.

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