Characteristics of mammary secretions from Holstein cows at approximately 10 days before parturition: with or without intramammary infection

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ABSTRACT
To evaluate the relationship between intramammary infection and basic characteristics of mammary secretion at late dry period, regarding mammary secretions, macroscopic observations, infection status, somatic cell counts (SCC), serum albumin concentrations, immunoglobulin (Ig) G1, and IgG2 levels were examined on 20 dairy cows at 9–12 days before calving. Intramammary infections were found in mammary secretions from 37 of the total 80 quarters. All of the mammary secretions with intramammary infection showed lower viscosity than that of normal colostrum. In four mammary secretions without intramammary infection, some macroscopic abnormalities were found. For mammary secretions without intramammary infection or macroscopic abnormality, viscosities were apparently higher than that in normal colostrum, indicating that viscosity is associated with macroscopic normality of the mammary secretion at approximately 10 days before calving. SCC and serum albumin concentrations were significantly higher in mammary secretions with intramammary infection or macroscopic abnormality. The SCC and serum albumin concentrations were correlated with viscosity of the mammary secretions, suggesting that most intramammary infections at approximately 10 days before calving may cause mastitis with increased permeability of the blood–milk barrier. No significant difference was observed in concentrations of IgG1 and IgG2, regardless of the presence of intramammary infections or macroscopic abnormalities.

1. Introduction
In dairy cows, incidence of intramammary infections increases during and around the dry period of a lactation cycle (Oliver and Sordillo 1988; Pyörälä 2008), and such infection may be a cause of mastitis in the early lactation stage. The incidence of clinical mastitis actually increases after calving (Bradley and Green 2004). To prevent mastitis early after parturition, diagnosis, management, and therapeutics during the dry period are important (Pyörälä 2008), resulting in preventing or decreasing milk production losses in the early stage of lactation.

Foremilk and milk contain proteins derived from plasma and tissue in addition to milk-specific proteins (Wall et al. 2015). Serum albumin is the most abundant plasma protein, and concentrations of serum albumin in mammary secretions are used for estimating the permeability of the blood–milk barrier (Wall et al. 2015; Tóthová et al. 2016). Immunoglobulins are important milk components for udder and calf health (Maunsell et al. 1999). A class of bovine immunoglobulin (Ig) G, IgG1, normally becomes incorporated into mammary secretions by the positive transport system during colostrogenesis (Larson et al. 1980; Castro et al. 2011; Moreno-Indias et al. 2012), in addition to the passive transport via a deficient blood–milk barrier (Nguyen and Neville 1998). Another class of IgG, IgG2, has also been shown to passively infiltrate into mammary secretions (Larson et al. 1980; Samarüüt et al. 2016). Determining the concentrations of serum albumin and IgGs can aid the estimation of the pathophysiological condition of the mammary gland in addition to examining intramammary infection status and somatic cell counts (SCC) for finding mastitis.

The objectives of the present study were to evaluate the relationship between intramammary infection, characteristics of mammary secretions and concentrations of serum albumin and IgGs in mammary secretions at approximately 10 days before parturition to determine patterns that may be useful for the diagnosis of intramammary infections.

2. Materials and methods
The experiments conducted in this study were reviewed and approved by the Animal Care and Use Committee of the National Institute of Animal Health, National Agriculture and Food Research Organization of Japan. Approximate 1.0 ml of mammary secretion was collected from each mammary quarter from 20 Holstein cows raised in 4 local farms in Japan. Mammary secretions were collected at approximate 10 days (9–12 days) before the second parturition,
immediately after successive treatment for each teat as follows: the teat was dipped in 0.5% iodine solution (Theratec; GEA ORION Farm technologies, Suzaka, Japan) for 45 s, the solution was removed with a sterile paper towel, and the teat was scrubbed with a 70% ethanol-soaked cotton pad, and then dried. Following the collection of the mammary secretion, the teat was dipped in the iodine solution again. A part of the mammary secretion was subjected to somatic cell counts (SCC) by direct microscopic observation (National Mastitis Council: Subcommittee on screening tests 1968); in addition, microbiological examinations were conducted for causative pathogens of mastitis following a threefold dilution with phosphate-buffered saline (PBS). Procedures for microbiological cultivation tests were performed in accordance with the National Mastitis Council (1999). A total 120 μl from each mammary secretion sample was used for six blood agar plates (10–30 μl of mammary secretion was used for each plate). Identification of bacteria was performed using the analytical profile index (API) tests (API 20E, API 20Strep, API Staph and API Coryne; bioMérieux, Lyon, France). In accordance with the request of the dairy farmers, the quarters in which intramammary infections were observed were treated with antibiotics within one to two days of the diagnosis (eight to nine days before parturition). The remaining volume of each mammary secretion was gently stirred with an 18-G needle to find gelatinous fibrin, and then macroscopically examined for viscosity, colour (including contamination of the blood pigment, either haemorrhagic pigment or red blood cells), formation of milk clots, and flakes. A non-transparent, deep yellow colour of the mammary secretion (orangish to greenish) was judged as normal. Viscosity was divided into three groups: thick (seemingly thicker than normal colostrum and a glutinous starch syrup-like texture), intermediate (between thicker than normal milk and normal colostrum), and thin (between serous fluid and normal milk) (Maunsell et al. 1999). The somatic cells, fat, and fibrin were removed from the mammary secretion and colostrum by centrifugation at 3500×g for 15 min at 4°C following a threefold dilution with PBS and stored at −20°C until use for determinations of serum albumin, IgG1, and IgG2 concentrations.

Concentrations of IgG1 and IgG2 were determined by enzyme-linked immunosorbent assay (ELISA) with the Bovine IgG1 ELISA Quantitation Set and Bovine IgG2 ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA), according to the instruction manual with a modification as described by Samaritul et al. (2016). The concentration of serum albumin was determined by single radial immunodiffusion (Watanabe et al. 2000).

From 18 quarters treated with antibiotics and 22 quarters without intramammary infection of 10 cows, colostrum was also collected within 8 h of parturition. The colostrum was also subjected to macroscopic observations, microbiological cultivation tests, counting somatic cells, and determination of concentrations of serum albumin, IgG1, and IgG2, as described above.

Data were expressed as mean ± SD. Counts of somatic cells were logarhythmically transformed to be normally distributed before statistical analyses were performed. The InStat 3.0 (GraphPad Software, La Jolla, CA, USA) statistical software program was used for statistical analyses. Student’s t-tests were used to compare SCC and concentrations of serum albumin, IgG1, and IgG2 between mammary secretions with and without abnormal macroscopic observations, and between colostrum from quarters affected and unaffected by intramammary infection at approximately 10 days before parturition. Analysis of variance and Tukey–Kramer methods were used to compare SCC and concentrations of serum albumin, IgG1, and IgG2 in mammary secretions of different viscosities, and p < .05 was regarded as significant.

Table 1. Relationships between intramammary infections and characteristics of mammary secretions at 9–12 days before parturition of dairy cows:

| Intramammary infection† | Presence (number of sample) | Absence (number of sample) |
|-------------------------|----------------------------|---------------------------|
| Total                   | 37                         | 43                        |
| Mastitis-causative pathogen |                            |                           |
| S. uberis               | 11                         | NDp                       |
| S. dysgalactiae         | 7                          | ND                        |
| CNS†                    | 18                         | ND                        |
| S. aureus               | 2                          | ND                        |
| C. bovis                | 2                          | ND                        |
| E. coli                 | 1                          | ND                        |
| Colour†                 |                            |                           |
| Normal                  | 14                         | 40                        |
| Abnormal                | 23                         | 3                         |
| Blood pigment†          |                            |                           |
| Uncontained             | 28                         | 41                        |
| Contained               | 9                          | 2                         |
| Flakes or clots         |                            |                           |
| Uncontained             | 27                         | 40                        |
| Contained               | 10                         | 3                         |
| Gelatinous fibrin†      |                            |                           |
| Uncontained             | 33                         | 41                        |
| Contained               | 4                          | 2                         |
| Viscosity†              |                            |                           |
| Thick                   | 0                          | 41                        |
| Intermediate            | 26                         | 2                         |
| Thin                    | 11                         | 0                         |

†A total 120 μl of each mammary secretion sample was used to isolate mastitis-causative pathogen.

Not detectable.

Coagulase-negative Staphylococci including intramammary infections of Staphylococcus chromogenes, S. haemolyticus, and S. xylosus.

Includes mixed infection: two cases of infections of S. xylosus and S. uberis, one case of infections of S. aureus and S. dysgalactiae, one case of infections of S. aureus and S. chromogenes.

Classification was done as described in the ‘Materials and Methods’.

3. Results and discussion

None of the mammary glands exhibited oedema or abnormality in size, colour, consistency, or temperature or pain response following ocular inspection and palpation during the study (Maunsell et al. 1998). In 37 of the total 80 mammary secretions at approximately 10 days before parturition, causative pathogens of mastitis (Streptococcus uberis, S. dysgalactiae, coagulase-negative staphylococci (CNS) (including S. chromogenes, S. haemolyticus, and S. xylosus), Staphylococcus aureus, Corynebacterium bovis, and Escherichia coli) were isolated (Table 1). All of the infected mammary secretions showed intermediate or thin viscosities. The uninfected mammary secretions with intermediate viscosity showed at least one macroscopic abnormality intimating inflammation, including the presence of milk flakes,
glandular tissue, and blood pigmentation. In all the mammary secretions with thick viscosity, exhibiting a glutinous starch syrup-like texture, no intramammary infections of mastitis-causing pathogens and macroscopic abnormalities were found, except two mammary secretions that contained trace levels of milk flakes. These results suggest that thick viscosity indicates a macroscopic normality of mammary secretions from uninfected mammary glands at 9–12 days before parturition.

The intramammary infection caused significant increases in SCC and serum albumin concentration (Table 2), showing that the intramammary infections caused mastitis without palpable or macroscopically observable abnormalities of the mammary glands. Concentrations of IgG1 and IgG2 exhibited high variation and were not significantly different between infected and uninfected intramammary infection statuses. In comparison with mammary secretions without macroscopic abnormalities, mammary secretions with macroscopic abnormalities showed higher SCC, indicating that the macroscopic abnormalities were related with mastitis (Table 2). The serum albumin concentration was also significantly higher in mammary secretions with macroscopic abnormalities than in those without macroscopic abnormalities. In mammary secretions both with and without macroscopic abnormalities, high variations in IgG1 and IgG2 concentrations were found. Similar high variations in IgG1 and IgG2 concentrations were also observed in bovine colostrum (Baumrucker et al. 2014; Gross et al. 2016; Samarütel et al. 2016). In IgG1 and IgG2 concentrations, no significant difference was observed between mammary secretions with and without macroscopic abnormalities.

SCC concentrations of serum albumin, IgG1, and IgG2 were compared across thick, intermediate, and thin viscosities of the mammary secretions (Table 2). SCC were significantly higher in the mammary secretions of intermediate and thin viscosities in comparison with those of thick viscosities. The SCC in the mammary secretions of thin viscosities was higher than that in the intermediate viscosities. The SCC in mammary secretions of thick viscosities was similar to those in previous reports on uninfected mammary secretions from cows approximately 10 days before calving (Jensen and Eberhart 1981).

The serum albumin concentration was higher in mammary secretions of thin viscosities than of intermediate and thick viscosities. In secretions of intermediate viscosities, the serum albumin concentrations were apparently higher than those of thick viscosities. Increased serum albumin concentrations in mammary secretions may occur through the influx of the protein through leaky tight junctions, which are caused by increased permeability of capillary vessels during mastitis (Stelwagen et al. 2009; Lehmann et al. 2013). The serum albumin concentrations in the mammary secretions were highly correlated with viscosity of the mammary secretions. The viscosities of the mammary secretions might relate with influx of plasma constituent into the mammary secretions.

Regarding the concentration of IgG1, no significant difference was observed between the different viscosities of the mammary secretions. Increased infiltration of IgG1 passively transferred in the mastitic mammary secretions via leaky tight junctions (Stelwagen et al. 2009; Lehmann et al. 2013), in addition to the inherent high variation in the IgG1 concentration, could be the likely causes.

For the IgG2 concentration, a significant difference was found, but only between thick and thin viscosities of mammary secretions. Although IgG2 is presumed to enter mammary secretions via leaky tight junctions similar to serum albumin (Nguyen and Neville 1998), a higher variation of IgG2 concentrations than serum albumin concentrations would result in a lower correlation between IgG2 concentrations and viscosities of the mammary secretions. The concentration of IgG2 (which is lower than serum albumin in the blood plasma) was higher than serum albumin in the mammary secretion, although both serum albumin and IgG2 are considered to enter mammary secretion in a similar manner. Samarütel et al. (2016) stated that these patterns in concentrations of serum albumin and IgG2 in colostrum are currently unexplained and deserve further study.

Increased SCC and serum albumin concentrations were observed in the mammary secretions with macroscopic abnormalities, and mastitis-causing pathogens were isolated from most of the mammary secretions with macroscopic abnormalities. Macroscopic observation of mammary secretions could be proposed as a method to detect mastitis at approximately 10 days before calving.

During the first two weeks of the non-lactation period in dairy cows, SCC in the mammary secretions increase and then remain at high levels until the last week of the dry period (Jensen and Eberhart 1981, Sordillo et al. 1987). In contrast, synthesis and secretion of milk-specific proteins such as caseins, α-lactalbumin, and β-lactoglobulin are suppressed until a few days before parturition (Schanbacher et al. 1993). In the last week of pregnancy, the colostral formation (including IgG1 transfer) is considered to be largely developed in the bovine mammary gland (Jensen and Eberhart 1981; Baumrucker and Bruckmaier 2014). At most, barely 1.0 ml of each of the glutinous starch syrup-like thick mammary secretion was collected in this study, suggesting that the colostrogenesis had not yet begun at 9–12 days before parturition. Approximately 10 days before parturition may be a suitable time point for checking intramammary infection, since the mammary secretion constituents do not change largely.

| Table 2. SCC and serum albumin concentrations, IgG1 and IgG2 levels in mammary secretions with and without intramammary infections, macroscopic normality, and different viscosities at 9–12 days before parturition. |
|---------------------------------|--------------|--------------|--------------|--------------|
| **SCC** (counts/ml) | **Serum albumin** (mg/ml) | **IgG1** (mg/ml) | **IgG2** (mg/ml) |
| Intramammary infection | | | |
| Uninfected (n = 43) | 6.06 a ± 0.34 | 1.81 a ± 0.56 | 19.3 ± 8.8 | 4.84 ± 3.23 |
| Infected (n = 37) | 7.18 b ± 0.37 | 3.59 b ± 1.29 | 18.4 ± 10.5 | 5.88 ± 3.91 |
| Macroscopic normality* | | | |
| Normal (n = 39) | 5.98 ± 0.21 | 1.79 ± 0.58 | 19.1 ± 8.5 | 4.75 ± 3.29 |
| Abnormal (n = 41) | 7.14 ± 0.39 | 3.43 ± 1.31 | 18.7 ± 10.5 | 5.86 ± 3.79 |
| Viscosity** | | | |
| Thick (n = 41) | 6.02 ± 0.29 | 1.81 ± 0.57 | 17.4 ± 7.7 | 4.77 ± 3.28 |
| Intermediate (n = 28) | 7.00 ± 0.38 | 2.98 ± 1.10 | 20.7 ± 10.8 | 5.37 ± 3.94 |
| Thin (n = 11) | 7.34 ± 0.26 | 4.08 ± 0.79 | 14.8 ± 9.3 | 7.23 ± 3.27 |

*Means with different superscripts differ significantly (p < .05) among each measurement item in each classificatory criterion.

**Includes classification with viscosity: thick viscosities are included in normal macroscopic observations while intermediate and thin viscosities are included in abnormal macroscopic observations.
proteins involved in the host defence system, would assist in yield-
contains a complicated mixture of proteins including various
analysis in prepartum mammary secretion or colostrum, which
untreated (uninfected) or treated with antibiotics. Proteomic
gen was isolated from the colostrum from quarters that were
mary infections. It was not possible to determine how the intra-
quarters that were treated with antibiotics to eliminate intramam-
were observed in the colostrum, including colostrum from the
Disclosure statement
The authors would like to thank Mr Yukio Chikayama for his technical
assistance.

No macroscopic abnormalities or intramammary infections were observed in the colostrum, including colostrum from the quarters that were treated with antibiotics to eliminate intramammary infections. It was not possible to determine how the intramammary infections would progress if the infected quarters had not been treated with antibiotics, but no mastitis-causative patho-
gen was isolated from the colostrum from quarters that were untreated (uninfected) or treated with antibiotics. Proteomic analysis in prepartum mammary secretion or colostrum, which contains a complicated mixture of proteins including various proteins involved in the host defence system, would assist in yield-
ing an understanding of the immunological and health conditions of the mammary gland during colostrogenesis (Hernández-Castellano et al. 2014). The SCC, serum albumin concentrations, and IgG1 and IgG2 levels in the colostrum in this study were within previously reported ranges (Guidry et al. 1980; Olejník 1994; Baumrucker et al. 2014; Gross et al. 2016; Samarütel et al. 2016) (Table 3). No significant differences were observed in SCC, serum albumin, IgG1, and IgG2 between quarters that were uninfected and untreated versus infected and treated with antibiotics. Although a detailed discussion on the appropriate care of mammary glands of dairy cattle is needed, the results of the present study suggest that elimination of the causative bacteria of mastitis is possible with antibiotic treatment before calving. Diagnosing mastitis before calving may be useful to lower milk production losses in the early stage of lactation.

4. Conclusion
At approximately 10 days before calving of dairy cows, the observation of macroscopic characteristics of mammary secretions would be valuable for diagnosing mastitis, since most intramammary infections caused mastitis with macro-
scopic abnormalities of the mammary secretions. Treating mas-
titis of the level that did not show palpable or macroscopically observable abnormalities with antibiotics at 8 or 9 days before calving resulted in normal uninfected colostrum.

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No potential conflict of interest was reported by the authors.

Table 3. SCC and serum albumin concentrations, IgG1 and IgG2 levels in colostrum from quarters uninfected and infected at approximately 10 days before calving.

| Quarters                      | SCC (log10(counts/ml)) | Serum albumin (mg/ml) | IgG1 (mg/ml) | IgG2 (mg/ml) |
|-------------------------------|------------------------|-----------------------|--------------|--------------|
| Total (n = 40)                | 5.55 ± 0.16            | 1.40 ± 0.50           | 32.2 ± 9.9   | 2.39 ± 0.83  |
| Uninfected and untreated (n = 22) | 5.51 ± 0.16            | 1.38 ± 0.52           | 33.9 ± 10.8  | 2.33 ± 0.95  |
| Infected and treated (n = 18) | 5.60 ± 0.14            | 1.43 ± 0.49           | 30.2 ± 8.5   | 2.46 ± 0.66  |

Note: In all categories, no significant difference was detected between quarters of ‘Uninfected and untreated’ and ‘Infected and treated’.

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