Short communication

Cathelicidin Responded to *Streptococcus agalactiae* and Associated with the Severity of Subclinical Mastitis

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Abstract

Cathelicidin in milk was reported as a possible protein marker for the assessment of the severity of subclinical mastitis. We hypothesized that cathelicidin would be elevated in the mastitic milk of the *Streptococcus agalactiae*-subclinical mastitis and the elevation of cathelicidin may be related to somatic cell count (SCC). Western blot was used to determine the amount of cathelicidin to differentiate between normal milk and *S. agalactiae* subclinical mastitic milk. The correlation between amount of cathelicidin and the SCC was also determined. We found that *S. agalactiae* subclinical mastitis induced cathelicidin expression in milk. There was a significant correlation between the amount of cathelicidin and the SCC (n=56, r= 0.411). Our findings confirmed cathelicidin as a promising biomarker for subclinical mastitis and indicator of the severity of mammary infection.

Keywords: antimicrobial protein, bovine, innate immunity, mammary gland, somatic cell count

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Introduction

Cathelicidin is a neutrophil-derived antimicrobial protein, part of the innate immune system in mammals (Zanetti, 2004). Since neutrophils are the predominant white blood cells that infiltrate into mammary gland to protect from invading pathogens, cathelicidin may play an important role in inflammation. Cathelicidin was reported in naturally occurring clinical mastitis with several pathogens—including *Staphylococcus aureus*, *Streptococcus uberis*, *Enterococcus*, *Nocardia asteroides*, *Streptococcus dysgalactiae*, *Escherichia coli* and *Pseudomonas aeruginosa* (Smolenski et al., 2011).

In our previous study on the 2D-PAGE protein profiles—comparing between healthy and naturally occurring *S. agalactiae*-subclinical—differences were found in mastitic milk protein profiles: one of the prominent proteins was cathelicidin. A preliminary investigation on mastitis severity revealed that the 2D-PAGE image intensity of cathelicidin correlated with the somatic cell count (SCC). Further investigation, however, was needed into the presence of cathelicidin in the subclinical mastitis milk. The confirmation of cathelicidin's presence is imperative before proposing it as a possible biomarker for subclinical mastitis and as an indicator for the severity of mastitis.

The present study utilized western blot to validate cathelicidin in an attempt to investigate its expression in subclinical mastitis. Natural subclinical mastitis caused by *S. agalactiae* was the focus of this study—a well-known major contagious pathogen which elevates SCC and decreases milk quality and quantity (Keefe et al., 1997).

Materials and Methods

In order to classify samples as control and *S. agalactiae*-subclinical mastitic milk, the identification of the presence of the pathogen in milk samples and determination of SCC were conducted. Western blot was used to verify the presence of and quantify cathelicidin in milk samples. Finally, the correlation between SCC and the amount of cathelicidin was investigated.

Milk sample preparation: Quarter milk samples were aseptically collected according to the National Mastitis Council (NMC) guidelines (Hogan et al., 1999), from twelve lactating crossbred Holstein Friesian cows between August and September, 2012. They were raised at a dairy farm in Khon Kaen province, Thailand. The cows were 3 to 6 years old and not subjected to any antibiotics for at least three weeks prior to sampling. Clinical examinations and the California Mastitis Test (CMT) were performed as screening. The milk samples were processed for bacterial culture identification and total bacterial count (Hogan et al., 1999). The severity of mastitis was determined from the SCC using an automatic fluorescent-based cell counter (Fossomatic 5000, Foss Electric). Milk samples having an SCC < 1×10^5 cells/ml and a negative bacterial culture were classified as normal. By contrast, *S. agalactiae*-positive samples with an SCC > 1×10^5 cells/ml were classified as having *S. agalactiae*-subclinical mastitis (Smith and Harmon, 2001). Milk samples identified as *S. agalactiae*-subclinical mastitis were collected every 2 days 8 times in order to evaluate for any changes in SCC and cathelicidin.

Bacteriological test: Ten microliter milk samples were streaked on blood agar plates containing 5% bovine blood and then incubated aerobically from 24 to 48 h at 37°C. The identification of pathogens grown on the agar plate was done as per Hogan et al. (1999). *S. agalactiae* was identified by the individual hemolytic pattern (Christie, Atkins, Munch-Petersen; CAMP test) on the blood agar plate and α-hemolysis, pinpoint colony on blood agar, Gram's stain positive, negative catalase test, negative-oxidase test, negative inulin sugar utilizations, and the negative-bile esculin test (Hogan et al., 1999).

Western blot analysis: The concentration of milk protein was determined using Lowry's method (1951). The optical density (OD) of sample solution was measured at 750 nm (OD_{750}). Bovine serum albumin (BSA) was used as the standard protein. Western blotting was conducted as described in Kurien and Scofield (2006). Briefly, 50 µg of separated protein from the SDS-PAGE was transferred to the Polyvinylidene Fluoride (PVDF) membrane in accordance with the standard western blot semi-dry blotting method. The PVDF membrane was blocked by incubation in 2% (w/v) advanced ECL blocking (Amersham Life Science Inc.) at room temperature for 60 min. The membrane was then rinsed with super Tris-buffered saline (STBST) pH 7.4. The PVDF membrane was stained with 1:10,000 (v/v) Anti-Cathelicidin antibody (Rabbit polyclonal to cathelicidin, Abcam, UK) in 2% advanced ECL blocking at room temperature for 60 min followed by rinsing with STBST. The membrane was incubated with a secondary antibody following 1:20,000 (v/v) anti-rabbit IgG in 2% advanced ECL blocking, horseradish peroxidase-linked species specific whole antibody (from sheep) (Amersham Life Science Inc.) at room temperature, 60 min). The ECL detection reagents (Amersham Life Science Inc.) were used for visualizing the band, and the band intensities quantified by images capture ChemiDoc XRS (Bio-Rad) chemiluminescence and ChemiDoc™ XRS Plus system Image Lab™ software compared by using the cathelicidin (Abcam, UK) as the standard protein.

Statistical analysis: The correlation coefficient (r) between the SCC and quantity of proteins was calculated according to Kaps and Lamberson (2009) and declared significant if $p < 0.05$.

Results and Discussion

Of all the milk samples collected, 7 had an SCC between 631 and $9{,}101\times10^3$ cells/ml and a single infection of *S. agalactiae*: these were classified as *S. agalactiae*-infected subclinical mastitis. The 2 milk samples having an SCC < 1×10^5 cells/ml (range, 44–54 × 10^3) and being negative for bacterial culture were grouped as the controls.

The *S. agalactiae* subclinical milk samples and the controls were subjected to protein studies. The two main findings were (a) cathelicidin was found only in infected milk samples and (b) the quantity of cathelicidin was significantly correlated with SCC.

Cathelicidin was reported in naturally occurring clinical mastitis with several pathogens; including *Staphylococcus aureus*, *Streptococcus uberis*, *Enterococcus*, *Nocardia asteroides*, *Streptococcus dysgalactiae*, *Escherichia coli* and *Pseudomonas aeruginosa* (Smolenski et al., 2011). The present study revealed that in *S. agalactiae*-subclinical mastitis, cathelicidin was also found in the milk.

Verification of the presence of cathelicidin in *S. agalactiae*-subclinical mastitis in the present study employed the specific of antigen-antibody reaction of the western blot technique. The affinity of the cathelicidin-antibody to the cathelicidin in the milk sample was illustrated in the western blotting result (Fig. 1). The presence of cathelicidin in infected milk samples while absent in the control samples confirms cathelicidin as a promising biomarker for the detection of subclinical mastitic milk.

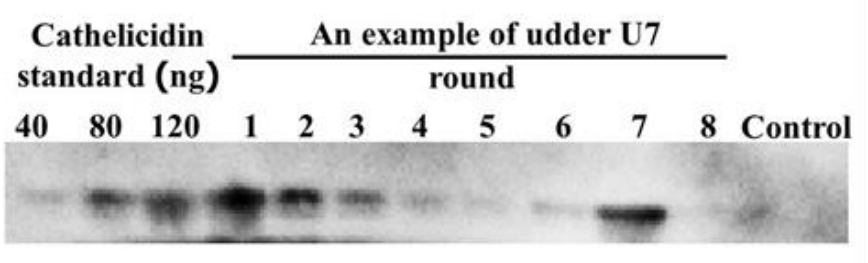

Figure 1 Cathelicidin in milk samples of a cow with different levels of SCC

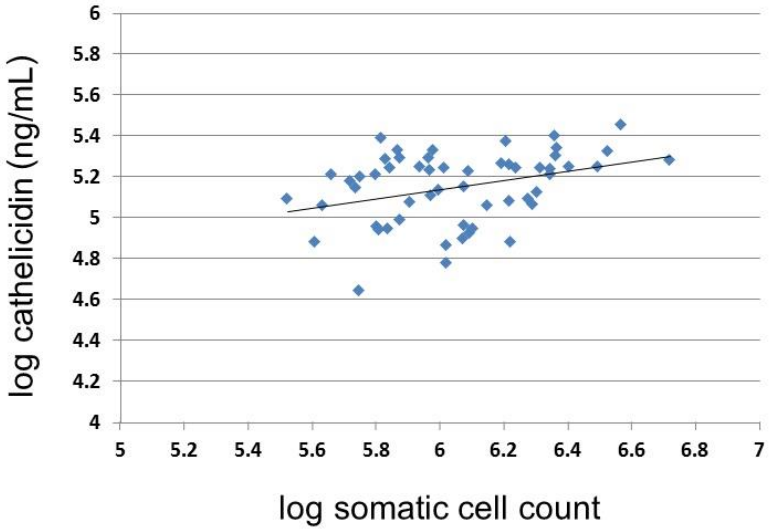

Figure 2. A linear association between the amount of cathelicidin and somatic cell count. Data were transformed with logarithm.

The present study confirmed a positive correlation between cathelicidin and SCC (coefficient r=0.411; $p<0.01$; n=56). Since SCC is associated with the level of severity of mastitis, the relationship between SCC and cathelicidin can be used as an indicator of the severity of mastitis. Milk proteins have been used as indicators of the severity of mastitis in *S. uberis*-induced subclinical mastitis and *S. aureus*-natural clinical mastitis (cathelicidin (Smolenski et al. 2011) and HMGB-1 (Furukawa et al., 2011), respectively).

The correlation coefficient reported in the current experiment (r=0.411) suggests that naturally occurring *S. agalactiae*-mastitic milk has a moderate correlation between cathelicidin and SCC. This moderate correlation may indicate the limitation of having collected milk samples from natural mastitis. Samples might have been collected at any time (early, middle, late or recovery from infection), so the stage of infection was not defined. The varying stages of infection resulted in varying numbers of neutrophils—the main source of cathelicidin production. During the late stages of infection, cathelicidin would be diminished due to degeneration of neutrophils, and the macrophage in SCC predominate (Sladek et al., 2005). Reduction in the neutrophils/SCC ratio would result in the absence of cathelicidin despite being high in SCCs and being pathogen-positive. Late stages of infection would therefore limit cathelicidin detection.

On the other hand, Smolenski et al., (2011) illustrated the presence of cathelicidin in early infection and reported a strong linear relationship between SCC and cathelicidin (R^2= 0.784). They employed bacterial infusion into bovine mammary glands as being representative of early infection, and found SCC and cathelicidin were both immediately elevated. The current results and those of Smolenski et al., (2011) indicate that cathelicidin may help in the detection of subclinical mastitis and also in the

assessment of the severity of mastitis. Besides cathelicidin, there was another report showing that a high correlation of the levels of milk protein and SCC (r=0.975). Furukawa et al. (2011) determined both SCC and the protein HMGB-1—a proinflammatory cytokine. They conducted this experiment in natural mastitis, in which most of mammary glands were infected with *S. aureus*. Although the experiment was conducted in natural mastitis (in which the stage of infection could not be defined), the *S. aureus*, a high virulence pathogen, caused HMGB-1 to be produced and secreted at all stages of infection (Riollet et al., 2001; Lahouassa et al., 2007). HMGB-1 was highly correlated with SCC and therefore also proposed as an indicator for the detection of mastitis.

In conclusion, the present study verified and quantified the presence of cathelicidin in *S. agalactiae*-subclinical mastitic milk. It was also found that an increase of cathelicidin in milk was correlated with the severity of mastitis. These findings support cathelicidin as a promising biomarker for subclinical mastitis and as an indicator of the severity of mastitis.

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บทคัดย่อ

โปรตีน Cathelicidin ที่ตอบสนองต่อเชื้อ *Streptococcus agalactiae* และความสัมพันธ์กับความรุนแรงของเต้านมอักเสบแบบไม่แสดงอาการ

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โปรตีน cathelicidin ในน้ำนมเคยถูกรายงานว่าอาจเป็นโปรตีนทีสามารถใช้ประเมินระดับความรุนแรงของเต้านมอักเสบแบบไม่แสดงอาการได้ คณะนักวิจัยจึงมีสมมติฐานว่าโปรตีน cathelicidin จะมีปริมาณที่เพิ่มขึ้นในน้ำนมของโคที่เป็นเต้านมอักเสบแบบไม่แสดงอาการจากการติดเชื้อ *Streptococcus agalactiae* รวมทั้งการเพิ่มขึ้นของโปรตีน cathelicidin อาจมีความสัมพันธ์กับจำนวนเซลล์โซมาติก ในการศึกษาครั้งนี้จึงใช้เทคนิค western blot ในการวัดปริมาณของ cathelicidin เพื่อเปรียบเทียบในตัวอย่างน้ำนมปกติและน้ำนมจากเต้านมอักเสบแบบไม่แสดงอาการจากการติดเชื้อ *S. agalactiae* ในการศึกษาครั้งนี้มีการประเมินค่าสหสัมพันธ์ระหว่างปริมาณ cathelicidin และจำนวนเซลล์โซมาติก ผลการศึกษาพบว่าเมื่อเกิดเต้านมอักเสบแบบไม่แสดงอาการจากการติดเชื้อ *S. agalactiae* จะเหนี่ยวนำให้มีการแสดงออกของโปรตีน cathelicidin ในน้ำนม โดยมีค่าสหสัมพันธ์ระหว่างปริมาณ cathelicidin และจำนวนเซลล์โซมาติกเท่ากับ 0.411 (n=56) การศึกษาในครั้งนี้ยืนยันได้ว่าโปรตีน cathelicidin อาจถูกนำมาใช้เป็นตัวบ่งชี้การเกิดเต้านมอักเสบแบบไม่แสดงอาการและเป็นตัวบ่งบอกระดับความรุนแรงของเต้านมอักเสบได้

คำสำคัญ: โปรตีนต้านจุลชีพ โค ภูมิคุ้มกันที่มีมาแต่กำเนิด เต้านม จำนวนเซลล์โซมาติก

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