Genome-wide DNA methylation pattern of mastitic mice model reveals two novel genes associated with

*Staphylococcus aureus* mastitis

Di Wang¹,†, Yiyuan Wei¹,†, Liangyu Shi¹, Muhammad Zahoor Khan¹, Lijun Fan¹, Yachun Wang¹ and Ying Yu¹

*Corresponding Author: Ying Yu*

Tel: +86-010-62734611, Fax: 86-10-62732439, E-mail: yuying@cau.edu.cn

¹ Key Laboratory of Agricultural Animal Genetics and Breeding, National Engineering Laboratory for Animal Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, PR China;
Title of the manuscript: Genome-wide DNA methylation pattern of mastitic mice model reveals two novel genes associated with *Staphylococcus aureus* mastitis

ABSTRACT

Objective: The *Staphylococcus aureus* (*S. aureus*) is one of the major microorganisms responsible for subclinical mastitis in dairy cattle. The present study was designed with the aim to explore the DNA methylation patterns using the Fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) techniques in a *S. aureus*-infected mouse model.

Methods: A total of 12 out-bred ICR female mice range from 12 -13 week-old were selected for building a mastitis model. F-MSAP analysis was carried out to detect fluctuations of DNA methylation between control group and *S. aureus* mastitis group.

Results: The visible changes were observed in white cell count in milk, percentage of granulocytes (GRN %), percentage of lymphocytes (LYM %), CD4+/ CD8+ ratio (CD4+/ CD8+), and histopathology of mice pre and post-challenge with *S. aureus*. These findings showed the uniformity and suitability of the *S. aureus*-infected mouse model. The total of 369 fragments was amplified from udder tissue samples of the two groups (*S. aureus*-infected mastitis group and control group) using eight pairs of selective primers. Results indicated that the methylation level of mastitis mice group was higher than that in the healthy group. In addition, *NCK-associated protein 5* (*Nckap5*) and transposon *MTD* were identified to be differentially methylated through secondary PCR and sequencing in the mastitis mice group. These outcomes might play an important role in the development of *S. aureus* mastitis.

Conclusion: Collectively, our study suggested that the methylation modification in *Nckap5* and transposon *MTD* might be considered as epigenetic markers in resistance to *S. aureus*-infected mastitis and provided a new insight into *S. aureus* mastitis research in dairy industry and public health.

Keywords: *S. aureus*-infected mouse model; F-MSAP method; DNA methylation; udder tissue; differentially methylated genes
INTRODUCTION

Bovine mastitis is considered as a serious problem to the dairy industry [1, 2], which reduces the quantity and quality of milk as well as threatens public health [3]. It was responsible for more than $2 billion annual losses to the US dairy sector [4] and about 35$ billion losses to the world [5]. The incidence of subclinical mastitis in modern dairy farms is much higher than clinical mastitis (< 5%) and normally around 26%-68%.

Subclinical mastitis can be detected by increased milk somatic cell counts (SCC) [6], 200,000 to 500,000 of cells/mL in milk were considered as an indicator of subclinical mastitis in dairy cattle, while SCC in healthy mammary gland is less than 100,000 of cells/mL. The main reason of increased SCC in mammary gland is the invasion of pathogens, nowadays, the major pathogen responsible for bovine subclinical mastitis is Staphylococcus aureus (S. aureus) [7].

Inflammation progress of udder tissue is controlled by genetic and epigenetic factors as well as pathogens. DNA methylation, one of the main epigenetic modification mechanisms, plays an important role in gene expression [8, 9]. DNA methylation around the STAT5-binding enhancer in the CSN1S1 promoter was shown to be associated with shutdown of αS1-casein synthesis during acute mastitis [10]. Our previous research revealed that bovine mastitis enhances the level of methylation in the promoter of CD4 and alternatively decreases the expression of CD4 by blocking the transcription factor binding [11]. Chang et al., [12] suggested TLR4 promoter was linked to a recognized mechanism of epigenetic regulation of gene expression in Escherichia coli mastitis.

In 2016, Song et al., [13] found three genes (MST1, NRG1 and NAT9) with DNA methylation changes can serve as potential biomarkers in S. aureus subclinical mastitis. These studies showed that epigenetic has key role in bovine mastitis and should be evolved in the strategies of mastitis control. However bovine mammary gland tissue is difficult to obtain because of injury to animals and economic losses, thus animal model for such researches were carried out [14].

Laboratory mouse is widely used for scientific research in epigenetics and disease progress. There is little information in the cited literatures about the whole genome DNA methylation changes in S. aureus-induced mastitis mice model. Methylation-sensitive amplified polymorphism (MSAP) analysis depends on two different DNA methylation-sensitive restriction isoschizomers (Hpa II and Msp I) for the same restriction site (CCGG) and has been extensively used to explore genomic DNA methylation levels and patterns because of its reliability, sensitivity and convenient operation [15]. Fluorescence-labeled MSAP (F-MSAP) which is based on
fluorescently labeled primers is more sensitive, safer and effective than the original MSAP [16]. To determine the DNA methylation changeability in udder tissue of S. aureus mastitis, genomic DNA methylation variation and related genes were investigated in a mouse model using the F-MSAP method.

MATERIALS AND METHODS

Care and Use of Animals

All protocols and procedures for the experimental mice were reviewed and approved by the Institutional Animal Care and Use Committee at China Agricultural University, China (Permission number: DK996). All the experiments were performed in strict accordance with the regulations and guidelines established by this committee. Before tissue sampling, the mice were euthanized by cervical dislocation. All efforts were made to minimize their suffering.

Sample selection and size

A total of 12 out-bred ICR female mice range from 12-13 week-old were selected for building a mastitis model. S. aureus was collected from fresh milk of mastitis dairy cattle. Bacteriological culture of milk samples was performed according to National Mastitis Council standards [17]. A volume of 3 mL milk was mixed into trypticase soy broth containing 7.5% NaCl and cultured at 37° C for 18-24 h. The S. aureus was confirmed by specific halo and transparent ring around the colony. nuc (thermonuclease) gene has been used for rapid identification of S. aureus, thus PCR for amplification of nuc gene was performed to identify S. aureus for double check [18, 19]. The primer of nuc gene is shown as follows: F: 5’-GCGATTGATGGTGATACGGTT-3’, R: 5’-AGCCAAGCCTTGACGAACACTAAAGC-3’. A 50μL of the S. aureus culture (5x10⁶ CFU) was carefully inoculated into the fourth abdominal pair teats (left and right) of the mammary gland of six mice with a blunt head capillary glass tube to induce S. aureus mastitis. Simultaneously, six healthy control mice (C1-C6) were inoculated with sterile, pyrogen-free saline.

Physiological and biochemical indicators detection

Body temperature test

The temperature of the rectum of the mice was measured at 5 time points per day, i.e., 0 h, 6 h, 12 h, 18 h and 24 h using an electronic thermometer.
Paraffin section and HE section

The 4th pair of udder tissue was collected after the mice were softly killed through cervical dislocation. Then tissues were paraffin embedded and sectioned, and finally HE stain was performed.

Milk white cell count

A total of 10 μL of milk was evenly spread onto the slides. Then after NEWMANS staining, the number of white cells in 10 fields was counted through microscope, and the average value was taken as the result.

Complete blood count (CBC)

A total of 1 mL blood was collected from orbital sinus and CBC (including granulocytes (GRN) and percentage of granulocytes (GRN %), lymphocytes (LYM) and percentage of lymphocytes (LYM %)) was conducted by Xiyuan Hospital CACMS, Haidian, Beijing, China.

CD4+/CD8+ ratio

A total 200 μL of fresh peripheral blood was collected from orbital sinus, and the cell counts of T helper/inducer lymphocytes (CD4+) and T suppressor/cytotoxic lymphocytes (CD8+) were measured by flow cytometry (conducted by Xiyuan Hospital CACMS, Haidian, Beijing, China; antibody information: anti-CD4+CD8 antibody [EDU-2+733], Abcam, Shanghai, China). The CD4+/CD8+ ratio is the ratio of T helper cells (with the surface marker CD4) to cytotoxic T cells (with the surface marker CD8).

Genomic DNA isolation from udder tissue

Related physiological and Biochemical indicators and udder tissue samples were obtained at 24 h after intra-mammary infection (IMI) with S. aureus. The mice were gently euthanized using cervical dislocation method. The skin surface of the fourth pair of mammary gland was washed with 75% ethanol and dried; consequently, the udder tissues were sampled quickly and carefully. The collected udder tissues were used for DNA extraction with Wizard® Genomic DNA Purification Kit (Promega, USA) and the genomic DNA was run in 1×TBE on 1% agarose gel electrophoresis to check its integrity.

DNA digestion, ligation and amplification

DNA digestion

DNA samples of both the mice groups were digested with two different isoschizomer systems (EcoR I / Hpa II and EcoR I / Msp I) respectively in a water bath at 37°C for overnight. The EcoR I / Msp I digestion system was performed in a reaction including 500-800 ng genomic DNA, 1μL EcoR I, 1μL Msp I, 4μL 10×B4...
buffer and 13μL ddH2O. *EcoR I* / *Hpa II* digestion was performed in a reaction containing 1μL genomic DNA, 1μL *EcoR I*, 2μL *Msp I*, 4μL 10×B1 buffer and 12μL deionized water.

### Ligation

The ligation was then performed in a final volume of 30 μL including 12.5 μL enzyme-digested products, 1 μL (10 pmol) *EcoR I* adapter, 1 μL (10 pmol) *Hpa III* / *Msp I* adapters, 0.5 μL T4 ligase, 4 μL 10×T4 buffer and 9 μL ddH2O and incubated at 16° C overnight and then inactivated at 65° C for 10 minutes.

### Pre-amplification PCR

Four μL of the above ligation product was pre-amplified as a template in a final volume of 20 μL contained a 42 ng H-M+1 primer, 41 ng E+1 primer, 0.1 μL Ex Taq polymerase, 1.6 μL dNTP, 1.2 μL MgCl2, 2 μL 10×Ex buffer and 9.5 μL deionized water. The PCR conditions were as follows: 94° C for 5 min; 30 cycles of 94° C for the 30 s, 56° C for 1 min, and 72° C for 1 min; and a final extension at 72° C for 7 min.

### Selective amplification PCR

Selective amplification PCR was performed in a final volume of 20 μL including 4 μL pre-amplified products, 41 ng H-M+3 primer, 12 ng E+3 primer, 0.1 μL Ex Taq polymerase, 1.6 μL dNTP mixture, 1.2 μL MgCl2, 2 μL 10×Ex Taq buffer, and ddH2O. The PCR amplification reactions were performed using touch-down cycles under the following conditions: 94° C for 5 min; 13 touch-down cycles of 94° C for 30 s, 65° C (subsequently reduced each cycle by 0.7° C) for 30 s and 72° C for 15 s; 23 continued cycles of 94° C for 30 s, 56° C for 30 s and 72° C for 15 s; and extension at 72° C for 7 min. The adapters and primers used in the present study are summarized in Table 1.

### Silver staining

After the selective amplification PCR, the products were loaded into polyacrylamide gel electrophoresis (PAGE) and then silver stain was performed. The silver staining steps are as following.

Firstly, the PAGE gel was fixed in a solution of 75% ethanol for 15-30 min with gentle shaking. Then washed the gel three times using deionized water. Later, a total of 200 mL 0.5% AgNO3 solution was added, incubating the gel 30 min at room temperature with gentle shaking (100-120 rpm). After incubation, the AgNO3 solution was discarded and both sides of the gel were washed for 20 s using deionized water. Next, a total of 200 mL freshly made aqueous solution of 7.5% sodium carbonate was added with gentle shaking for 5 min. Afterwards, a total of 1.25 mL solution of 40% formaldehyde was added, then the gel was incubated at room temperature with gentle shaking. Washing the gel carefully and the stained bands should be appeared within a
Continuing the incubation until all desired bands appeared. Finally, quenched the reaction by washing the gel in 1% acetic acid for a few minutes and wash the gel several times with deionized water (10 min per wash).

**Fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) analysis**

Based on the silver staining results, four pairs of selective-primers with more different bands were selected to be fluorescent labeled (FAM-labeled) to perform F-MSAP analysis.

In the F-MSAP technique, the digestion of DNA was performed with different methylation sensitive isoschizomer (*Hpa II* and *Msp I*) as well as an internal control restriction enzyme (*EcoR I*) as shown in (Figure 1A and Figure 1B). The enzyme-digestion products were then ligated to adapters and pre-amplification and selective amplification with FAM-labeled primers was performed [16]. The amplified products with FAM-labeled primers were detected through the ABI3730 platform. Finally, the F-MSAP fragments were analyzed using GeneMarker V1.65 by detecting fluorescent signals of different intensity and locations. Four types of bands were detected. *t*-test and *chi*-square test were used in significance analysis of methylation levels between the two experimental groups. The level of statistical significance was set at *p* < 0.05.

Ultimately, a total of 20 different bands were excised from polyacrylamide gels after silver staining, followed secondary PCR and sequencing to identify the differentially methylated genes.

**RESULTS**

**Physiological differences between *S. aureus* infected mice and healthy controls**

The *S. aureus* strain used to instruct mastitis mice model was confirmed by specific PCR amplification of the *nuc* gene (Figure S1). In order to get uniformity and standard *S. aureus*-infected mice model, several parameters were checked pre and post-challenge with *S. aureus* (Figure 2A).

Before the *S. aureus* attack, there was no difference for the body temperatures between C group and SM group; however, a significant rise of body temperature was observed in SM group compared to the controls after 24 h IMI (Figure 2B). Furthermore, white cell counts in milk and GRN % were also seen to be increased (Figure 2C and 2E), while the LYM %, as well as CD4+/ CD8+, were observed to be significantly decreased in SM group after IMI (Figure 2D and 2F). For further confirmation, paraffin section and HE stain was carried out to check whether there was any abnormality in tissue, which showed inflammatory cells infiltration, the space
between the cells become wider and epithelial cell sloughing off in SM group (Figure 2G). These findings indicated that the mouse model was launched successfully and provided a foundation for the current research (Figure 2).

Four cleavage patterns of DNA methylation

The cleavage patterns were defined and shown into four types according to the methylation sensitive of the isoschizomers as mentioned in Figure 1C and 1D. The PAGE gel electrophoresis indicated four methylation types shown in Figure 1E. Type I bands (un-methylated) appeared in both EcoR I / Msp I and EcoR I / Hpa II digestion. Type II bands (inner methylated of double stranded sequence) appeared only in EcoR I / Msp I digestion but not digested in the EcoR I / Msp I. Type III bands (semi-methylated, outer methylated of single stranded sequence) obtained in EcoR I / Hpa II digestion but not cannot be digested in the EcoR I / Msp I. Type IV (outer methylated of double stranded sequence) represents the absence of band in both enzyme combinations.

Genome-wide DNA methylation profiles of the S. aureus infected mice and healthy controls

Genome-wide DNA methylation profiles of the two groups were generated using the F-MSAP method. Eight pairs of selected primers were labeled with fluorescent dyes to detect DNA methylation patterns in udder tissue of the two groups. The fragments between 100 bp and 500 bp were highly intense. C1 (the first sample of C group) showed inner methylated of double stranded DNA and SM1 (the first sample of SM group) showed outer methylated of double stranded DNA (Figure 3A). The results were consistent with a signal peak (Figure 3B) and data (Figure 3C) output from GeneMarker V1.65 software. A total of 369 clear and reproducible bands were amplified from udder tissue samples of the two groups are shown in Figure 3D, and the bands in the red frame were the differentially methylated fragments.

DNA methylation levels of the two groups

Methylation levels of the two groups were detected according to the bands of four FAM-labeled primers. The analysis of variance and Duncan's multiple range tests were performed to evaluate the different methylation levels of the two groups. Variances of four DNA methylation patterns between two groups are shown in Table S1. In S. aureus-infected mice group, the type I bands (un-methylated) are extremely lower than those in the healthy control group ($p < 0.01$). As for whole methylation bands (II+III+IV (%)) and full methylation bands (II+IV (%)), there were also significant differences between the two groups with $p < 0.01$ and $p < 0.05$, respectively.
respectively (Figure 3E). These findings indicated that DNA methylation level in S. aureus-infected mice was increased.

**S. aureus-induced changes in the differentially methylated bands of udder tissue of mouse**

All possible banding patterns between S. aureus-infected mastitis mice and healthy controls were explored to identify the changes in cytosine methylation patterns. Differentially methylated bands refer to the differences between the bands of M and H lane in two experimental groups. The different DNA methylation patterns of the four FAM-labeled primers in the control group and SM group were shown in Table S2. Out of 29 bands, three were the highly significant difference, 25 bands showed significant differences and only one band remained unchanged under S. aureus-induced events between the two groups. These findings indicated the frequently DNA methylation events when S. aureus attack udder tissue. The analysis of significantly differential methylated bands between two groups was carried out using t-test in EXCEL.

**Differentially methylated genes between S. aureus-induced mastitis mice and healthy controls**

To identify the differentially methylated genes, a total of 20 bands were excised from polyacrylamide gels after silver stain, followed secondary PCR and sequencing. Two differentially methylated fragments (100-200bp) with different methylation patterns were confirmed as gene Nckap5 (150bp) and Transposon MTD (116bp) through sequencing alignment (http://genome.ucsc.edu/cgi-bin/hgBlat) (Sequencing alignment results were shown in Figure S2 and Figure S3). Nckap5 is related to the promotion of the cell death, which should be methylated under normal conditions. In current results, Nckap5 is hypo-methylated in S. aureus mastitis mice (Figure 4A). In SM group, except for SM1 showing type III (semi-methylated), other samples all showed type I (un-methylated); while in C group, except for C3 showed type I, others showed type II (inner methylated of double stranded sequence). Transposon MTD showed lower methylation level in S. aureus mastitis group compared with control group. In SM group, SM1, SM5 and SM6 showed type I (un-methylated) and SM2, SM3 and SM4 showed type IV (outer methylated of double stranded); while in C group, C1, C4 and C5 showed type IV (outer methylated of double stranded); C2 and C6 showed type I (un-methylated) and C3 showed type III (semi-methylated) (Figure 4B). Since Nckap5 gene and transposon MTD were found to be hypo-methylated in S. aureus infected mice udder tissue, we assumed that these two genes might trigger inflammatory changes in udder tissue (Figure 4C).
DISCUSSION

The current study used a *S. aureus* mastitis mouse model to evaluate the changes of genome-wide DNA methylation patterns and levels in udder tissues post *S. aureus* infection. Two differentially methylated genes (*Nckap5* and transposon *MTD*) were discovered which could be indicators of *S. aureus* mastitis.

Mice can serve as an appropriate research model for bovine mastitis study due to its low cost and environmental control [20]. Here, a *S. aureus*-infected mouse model was established and optimized, which is the pre-requisite for obtaining the reliable results. Fan et al., [21] had evaluated the body temperature, udder tissue slices, milk white cell count, CBC and CD4⁺/CD8⁺ ratio pre and post-challenge in order to establish the *S. aureus* mice model, which is consistent with the present study. These observations suggest that our established *S. aureus* mice model showed consistency and reliability for the further experimental procedure.

In the current study, we used F-MSAP method to detect genomic DNA methylation variance of udder tissues between *S. aureus*-induced mastitis mice and healthy mice. F-MSAP assay is modified from MSAP, which using fluorescently labeled primers and capillary gel electrophoresis instead of traditional denaturing acrylamide gel electrophoresis and silver staining [16, 22, 23]. Compared with MASP, F-MASP method has been proven to be safer, more efficient and higher resolution because it can automatically detect genome-wide DNA methylation patterns with DNA sequencer. Based on F-MASP analysis, our findings revealed that the genomic DNA methylation level of mastitis mice was significantly higher than those in healthy mice. Similarly, higher methylation level was found in the lymphocytes of *S. aureus* mastitis cows compared with healthy cows [13]. Thus, the data suggest that the methylation level of *S. aureus* mastitis-related genes may be changed because of DNA methylation fluctuations in *S. aureus* infected mice.

Subsequently, two differentially methylated genes (*Nckap5* and transposon *MTD*) in mice udder tissue were obtained, which may have important roles in the development or resistance of *S. aureus* mastitis. Our findings revealed hypo-methylation of *Nckap5* in *S. aureus*-induced mastitis mice. In a previous study, it was cited that *Nckap5* encodes Nck-associated protein 5 and the Nck protein reportedly plays a role in the process of the Fas ligand (FasL) factor-induced cell death [24]. These indicated that the *Nckap5* gene might have a role in promoting the death of immune cells. A genome-wide association study revealed that a polymorphism near *NCKAP5* showed significant link with symptoms of depression in human [25]. A recent study reported that structural variations in cell line MHH-CALL-2 include homozygous inversion (*NCKAP5*) are responsible for disturbance of epigenetic gene regulation in childhood acute lymphoblastic leukemia by using next generation
sequencing [26]. Similarly, in our results, we noted a higher level of methylation of transposon \textit{MTD} in the healthy control group compared to the mastitis group, which are in consistency with recently reported study showed that the transposon is under a state of hyper-methylation in healthy status [27]. Transposon \textit{MTD} belongs to Family \textit{ERVL-MaLR} (mammalian apparent long terminal repeat (LTR) retrotransposons), which is mammalian apparent LTR-retrotransposons, showed a lower expression in early embryos than other retrotransposons [28]. Studies suggested that DNA methylation status of the CACTA transposon can explaining the incomplete penetrance and poor expressivity of the maize (\textit{Zea mays}) mutant \textit{Unstable factor for orange1} \textit{(Ufo1)} [29]. Hypomethylation of \textit{Karma} transposon is associated with the mantled phenotype in oil palm [30]. In aggregate, the data obtained in current research and the previously reported studies infer the importance of \textit{Nckap5} and transposon \textit{MTD} as indicators and might be a target in remedies of mastitis. Further biological investigation is needed to validate the reliability of the current findings through pyro-sequencing and the gene expression via qRT-PCR; in addition, the regulatory mechanisms of methylation changes could be study in mammary gland of dairy cow infected by \textit{S. aureus}.

In conclusion, these findings offered a base line understanding of the investigation of dairy cow mastitis infected by \textit{S. aureus} and also provided a comprehensive picture of DNA methylation in \textit{S. aureus} infected udder tissue. In addition, we suggested that the DNA methylation variation of \textit{Nckap5} and transposon \textit{MTD} might be considered as indicators and methylation markers in control strategy of \textit{S. aureus} mastitis, and provided a provided a new insight into \textit{S. aureus} mastitis research in dairy industry and public health.

\textbf{CONFLICT OF INTEREST}

The authors have declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

\textbf{ACKNOWLEDGMENTS}

This research was financially supported by the Beijing Natural Science Foundation (6182021), Beijing Dairy Industry Innovation Team (BAIC06), National Natural Science Foundation of China (31272420), Modern
Agro-industry Technology Research System (CARS-36) and the Program for Changjiang Scholar and Innovation Research Team in University (IRT-15R62).

REFERENCES


Table 1. The adapters and primers used in F-MSAP

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<tr>
<th>Name</th>
<th>5′→3′sequence</th>
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<td>Adapters</td>
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<td>HpaII/MspI adapters</td>
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</tr>
<tr>
<td></td>
<td>CGTTCTAGACTCATC</td>
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<tr>
<td>EcoRI adapters</td>
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<tr>
<td></td>
<td>AATTGGTACGCAGTCTAC</td>
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<td>Pre-selective primers</td>
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<td>HpaII/MspI+T</td>
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<tr>
<td>EcoRI+A</td>
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<td>EcoRI+ATC</td>
<td>GACTGCGTACCAATTC-ATC</td>
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</table>

Note: There are eight pairs of combined selective-primers as below:

Primer 1: F: GATGAGTCTAGAAACGG-TAC; R: GACTGCGTACCAATTC-AAC
Primer 2: F: GATGAGTCTAGAAACGG-TAC; R: GACTGCGTACCAATTC-ATG
Primer 3: F: GATGAGTCTAGAAACGG-TAC; R: GACTGCGTACCAATTC-AAG
Primer 4: F: GATGAGTCTAGAAACGG-TAC; R: GACTGCGTACCAATTC-ATC
Primer 5: F: GATGAGTCTAGAAACGG-TAG; R: GACTGCGTACCAATTC-AAC
Primer 6: F: GATGAGTCTAGAAACGG-TAG; R: GACTGCGTACCAATTC-ATG
Primer 7: F: GATGAGTCTAGAAACGG-TAG; R: GACTGCGTACCAATTC-AAG
Primer 8: F: GATGAGTCTAGAAACGG-TAG; R: GACTGCGTACCAATTC-ATC
Figure legends

Figure 1. **Schematic representation of the two isoschizomers and four DNA methylation patterns.** (A) *Hpa II* is digestible in inner and outer methylated of single stranded CCGG sequence; *Msp I* is digestible in inner methylated of single and double stranded sequence; *EcoR I* is an internal control restriction enzyme which recognizes the GAATTC sequence. (B) An example DNA sequence is digested with isoschizomers (*Hpa II* and *Msp I*) and divided into different fragments. (C) Activity of the two isoschizomers. *Msp I* can recognize inner methylation of single and double stranded CCGG sequence, but cannot recognize outer methylation of single and double stranded CCGG sequence. *Hpa II* can recognize inner and outer methylation of single stranded CCGG sequence, but cannot recognize inner and outer methylation of double stranded CCGG sequence. The digested sites were shown in red circle and undigested sites are shown in black circle. (D) Illustration of four DNA methylation pattern types. (E) The agarose gel electrophoreses (silver stain) indicate four methylation types. Line M: a system of *EcoR I*/*Msp I*, line H: a system of *EcoR I*/*Hpa II*; H+: *Hpa II* digested; H-: *Hpa II* undigested; M+: *Msp I* digested; M-: *Msp I* undigested.

Figure 2. **Differences of the physiological and biochemical indexes between the two groups.** (A) Schematic sketch of establishing *S. aureus*-induced mastitis mouse model. (B) Temperature difference between healthy control and *S. aureus* mastitis; *: p < 0.05. (C) Milk white cell counts; **: p < 0.01. (D) LYM %: percentage of lymphocytes; **: p < 0.01. (E) GRN %: percentage of granulocytes; **: p < 0.01. (F) CD4+/CD8+ ratio; **: p < 0.01. (G) Udder tissue slices: ① Alveolus are complete in HC group, ② Inflammatory cells infiltration, ③ The space between the cells becoming wider, ④ Epithelial cell sloughing off can be found in SM group.

Figure 3. **The electrophoresis and illustration of selective amplification.** (A-C) An example showing how to name the signals in F-MSAP; (A) C: control group; SM: *S. aureus* induced mastitis group; Line M: a system of *EcoR I*/*Msp I*, line H: a system of *EcoR I*/*Hpa II*; (B) The signal peak represents methylation-sensitive amplified polymorphic fragments labeled with FAM fluorescent dye; the height of the peak represents the molecular weight of the fragments; (C) 1 = band, 0 = no band. (D) The fluorescent electrophoresis of the fourth primer of the 12 samples. C1-C6 are six replicates of control group; SM1-SM6 are six replicates of *S. aureus* induced mastitis group; Line M: a system of *EcoR I*/*Msp I*, line H: a system of *EcoR I*/*Hpa II*; red arrow indicated the different band pattern between the two groups. (E) DNA methylation levels of the four primers of
the two groups. Whole methylation represents the sum of type II, type III and type IV; full methylation represents the sum of type II and type IV.

Figure 4. Silver staining and sequencing results of two differentially methylated fragments between SM mice group and controls. (A) Silver staining and partial sequencing result of gene Nckap5; C1-C6 are six replicates of control group; SM1-SM6 are six replicates of S. aureus induced mastitis group; Line M: a system of EcoRI/MspI, line H: a system of EcoRI/HpaII. Different color of the frames represents different DNA methylation pattern: light blue = Type I; wine red = Type II. (B) Silver staining and partial sequencing results of transposon MTD; C1-C6 are six replicates of control group; SM1-SM6 are six replicates of S. aureus induced mastitis group; Line M: a system of EcoRI/MspI, line H: a system of EcoRI/HpaII. Different color of the frames represents different DNA methylation pattern: light blue = Type I; orange = Type III; light green = Type IV. (C) The genome-wide DNA methylation level of S. aureus-induced mastitis mice is higher than that in healthy controls, gene Nckap5 and transposon MTD showed hypo-methylation in S. aureus mastitis group. The circles with letter M inside means methylation, and the number of circles represents level of methylation.
Figure 1
Figure 2

A. *S. aureus*-induced mastitis vs. Healthy control

- Temperature, milk white cell count, LYM%, GRN%, CD4+CD8+ and histopathology of udder tissue

To establish the *S. aureus* mouse model

B. Temperature

C. Milk white cell count

D. LYM %

E. GRN %

F. CD4+CD8+

G. Control (C) vs. *S. aureus* mastitis (SM)

Figure 2
Figure 4