The Journal of Animal & Plant Sciences, 29(4): 2019, Page: 1193-1197
ISSN: 1018-7081

Short Communication

THE RELATIONSHIP BETWEEN LACTOFERRIN GENE POLYMORPHISM AND SUBCLINICAL MASTITIS IN AWASSI EWES

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ABSTRACT

The objectives of this study were to determine lactoferrin (LF) gene polymorphism in Awassi ewes and milk LF concentration and their relationships with somatic cell count (SCC) and milk bacterial culture results in Awassi ewes affected with subclinical mastitis. Milk and whole blood samples from 130 adult lactating ewes were collected and LF gene polymorphism and sequence were determined using restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR). Milk LF concentration was determined using commercially available ELISA kits. Somatic cell count was determined manually and milk bacterial culture was performed as per routine laboratory procedures. One allele of LF gene was found (allele A) that controlled one genotype (AA) with a frequency of 100%. Digestion of the RFLP-PCR product using *EcoRI* enzyme confirmed the absence of allele B due to a single mutation at G to C site. There was a statistically significant correlation ($r = -0.80$, $p < 0.05$) between LF concentration and SCC (log^2) in milk of ewes with subclinical mastitis. In addition, a statistically significant correlation ($r = -0.60$, $p < 0.05$) was found between the mean LF concentration in milk and the outcome of bacterial culture (growth or no growth). In conclusion, lactoferrin genotype AA could be associated with increased resistance to intramammary infections in Awassi sheep.

Keywords: mastitis, lactoferrin, gene polymorphism, Awassi sheep.

INTRODUCTION

Lactoferrin (LF) is a transferrin family member of glycoproteins. LF binds and transfers Fe3 + ions into living tissues and body fluids like tears, saliva, sperm and milk (Titov *et al.*, 2019; Giansanti *et al.*, 2016). It is considered an important part of the innate immune response against many microbials that can invade tissues and mucous surfaces (Actor *et al*., 2009). In addition to its immune modulating and anti-inflammatory function, LF is found to promote cell proliferation and differentiation, and to affect the growth of many species of bacteria, viruses and parasites (Luna-Castro *et al.*, 2014; Jahani *et al.*, 2015; Musayeva *et al.*, 2016; Samaniego-Barron *et al.*, 2016; Karavn *et al.*, 2017; Samaniego-Barron *et al.*, 2017). In milk, the concentration of LF was dependent on the stage of lactation, milk production, somatic cell count (SCC), and udder health (Doust *et al.*, 2014; Sobczuk-Szu *et al.*, 2014; Alhussien, and Dang 2018). A rise in milk LF concentrations was detected in cows with subclinical mastitis which may indicate an important role of LF in the defense against mastitis in cows (Cheng *et al.*, 2008; Alnakip *et al*., 2014; Alhussien and Dang, 2017). The correlation between LF gene polymorphism (AA, BB and AB) and SCC has also been studied in cattle (Zielak-Steciwko *et al.*, 2014). The highest SCC was found in cow's milk of the AB genotype while the lowest was in the AA genotype (Zielak-Steciwko *et al.*, 2014). The role of LF in ewe's mastitis is still unknown. Therefore, the objectives of this research were to investigate LF gene polymorphism and to determine the concentration of LF in milk and its relationship with SCC and type of bacterial isolates obtained from Awassi ewes with subclinical mastitis.

MATERIALS AND METHODS

All ethical approvals were obtained from the institutional animal care and use committee of Jordan University of Science and Technology (JUST-ACUC) before the start of the study. A total of 130 local Awassi ewes ranged between 2-5 years of age reared at JUST farm and Khanasry station were used in this study. Milk samples were collected at mid lactation period in two sterile tubes. One tube was used within 24 hours to measure SCC and perform bacterial culture and the other tube was stored at -20°C for measuring LF concentration. Blood samples were also collected via jugular vein puncture and placed in EDTA-containing blood tubes and stored at -20°C until DNA isolation.

Total DNA was isolated from whole blood using tissue DNA kit (Omega Biotek Inc., USA) according to manufacturer's instructions. Extracted DNA was visualized using 1% agarose gel to ensure its quantity and quality. DNA was stored at −20°C until PCR analysis.

Amplification of 301 bp fragment of LF gene was performed using the following primers: Forward: 5'-GCC TCA TGA CAA CTC CCA CAC-3' and Reverse: 5'-CAG GTT GAC ACA TCG GTT GAC-3'. PCR reagents and profile were performed according to Chaneton *et al.*, 2008. The PCR reaction volume was 50 µl. It contained 3 µl of template, 10 µl of 5× Firepol master mix (Solis Biodyne, Tartu, Estonia) and 10 pmol of each primer. Non-template, negative control was performed with each PCR run. PCR thermal conditions were set to an initial denaturing step at 95°C for 5 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. The amplified DNA product was analyzed using 2% agarose gel electrophoresis and the final product was visualized under UV light.

RFLP-PCR was used to study LF gene polymorphism. *EcoRI* restriction enzyme was used to cut the amplified product of LF gene. Incubation time and temperature was in accordance to Zielak-Steciwko *et al.*, 2014. The digestion pattern was 301 bp for allele A and 201 bp, and 100 bp for allele B. The digested products were visualized on 2% agarose gel electrophoresis.

Amplified DNA was purified from agarose gel using PCR purification kit (Jena Bioscience GmbH, Germany) according to manufacturer's instructions and the purified product was stored at −20 °C until DNA sequencing.

Sequencing of the purified PCR amplicons was performed (Macrogen, Korea). Bio Edit software was used to view the sequences and aligned using the Meg Align software of the DNASTAR package. Identification of the nucleotide and phylogenetic analysis were performed using the Bio Edit software.

Lactoferrin concentration in milk samples was measured using sandwich enzyme immunoassay technique using sheep commercial ELISA kit (MyBiosource, Inc., San Diego, USA) according to manufacturer's instructions. The final absorbance was measured in a microtiter plate reader at 450 nm wavelength. The sensitivity was 10 µg/mL. The intra- and inter- assay coefficient of variation were less than 15%, and the detection range was 62.5-2000 µg/ml.

Somatic cell count was performed manually. Briefly, 0.01 ml of gently mixed milk sample was spread over 1 cm2 area on a glass slide. After drying, the slides were stained using Newman-Lampert stain. The stained slides were then examined microscopically and somatic cells were counted according to the previously published procedure (Halasa *et al.*, 2007). To make sure SCC were normally distributed; the raw data of SCC was transformed to a logarithmic (log2) scale using the following statistical model:

$Yijklm = \mu + ai + bj + dl + (ab)ij + eijklm$ where,

Yijklm: Somatic cell count (3 + log2 SCC)

µ: Mean somatic cell count for herd (3 + log2 SCC)

ai: Effect of genotype

bj: Effect of lactation number

dl: Effect of season

(ab)ij: Interaction effect between genotype and lactation number

eijklm: Erro

Bacterial culture was performed according to standard laboratory procedures (Lagier *et al.*, 2015). Briefly, samples were smeared on blood agar and MacConkey agar plates and incubated at 37°C for 24 hours. Preliminary bacterial identification was performed based on colony morphology, hemolytic features on blood agar and Gram's stain characteristics. Biochemical tests such catalase, coagulase and sugar fermentation test were used to distinguish between *Staphylococcus aureus* or coagulase negative Staphylococci (CNS). Microtube identification system (Microbact, Staph 12S STAPH-IDENT System MB1561, Oxiod, UK) were used to further identify species of CNS. *Escherichia coli* were identified based on growth MacConkey agar followed by gram staining and biochemical testing using catalase, oxidase, IMVIC test. Further classification of isolated Gram negative strains was performed using Microbacter GNB 24E (Oxiod, UK). *Streptococci* were initially identified using CAMP test, esculin hydrolysis, hemolysis, and no growth on MacConkey agar. Isolated *Streptococci* were further identified using Strepto system 9R (Lio Filchem S.KI, Italy).

The possible associations between LF gene polymorphism and milk SCC (log^2) and between LF concentration in milk and results of bacterial culture (growth vs no growth) were analyzed using GLM procedure of SAS program.

RESULTS

Among 130 dairy ewes studied in this work, one allele of LF gene was found, which limited the occurrence of one genotype with a frequency of 100% (Figure 1). Based on the results of the *EcoR1* enzyme digestion, the revealed allele gene (301 bp) was designated as A, and the related genotype was identified as AA (Figure 2). Digestion of the RFLP-PCR product using *EcoRI* enzyme revealed lack of digestion site due to a single mutation (G to C). Therefore, neither BB homozygous nor AB heterozygous genotypes were observed in the studied population.

There was a statistically significant and strong correlation ($r = -0.80$, $p < 0.05$) between the mean LF concentration and SCC (log^2) in milk of ewes with subclinical mastitis. The mean LF concentration in mastitic milk was 136.89±49.6 µg/ml.

There was a statistically significant and strong correlation ($r = -0.60$, $p < 0.05$) between the mean LF concentration in the milk and the outcome of bacterial culture (growth or no growth). The mean LF

concentration in culture positive samples was 140.69±44.89µg/ml, while in the negative culture samples; LF concentration was 115.68±68.98 µg/ml.

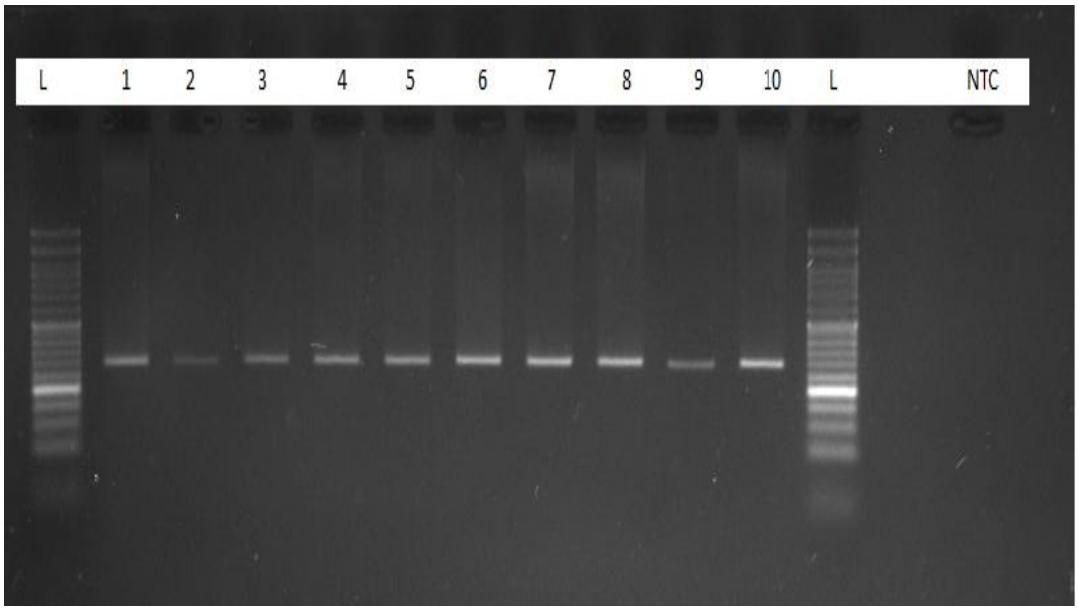

Figure 1. Agarose gel of amplified lactoferrin gene. L: 50bp leader; 1-10: 301bp amplified lactoferrin gene; NTC: non template control. The size of intense band is 200bp.

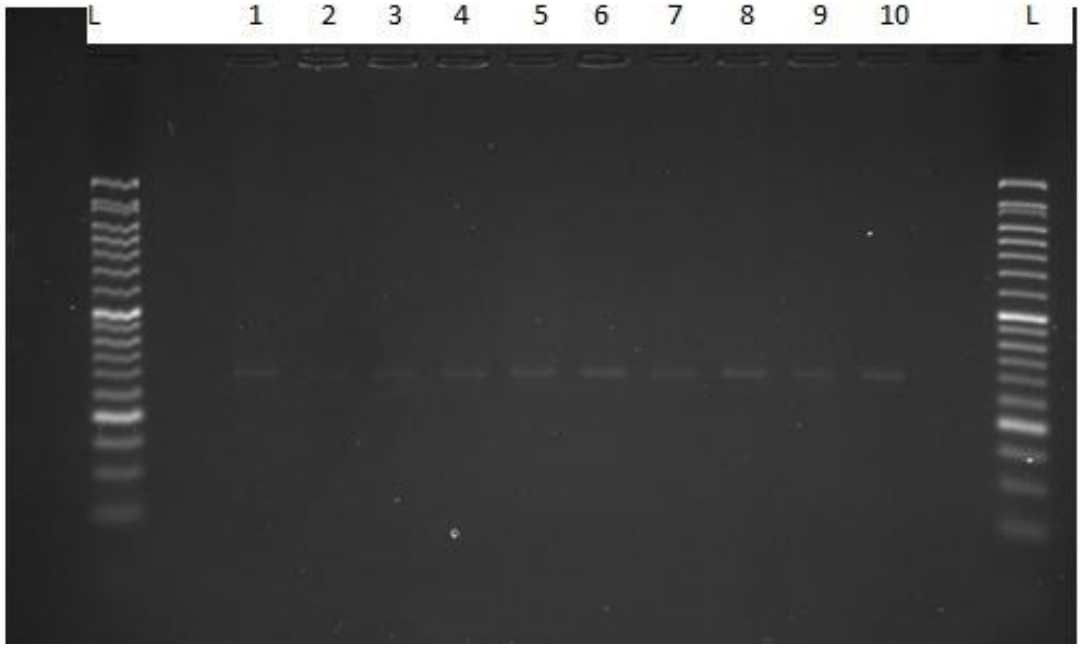

Figure 2. RFLP – PCR agarose gel electrophoresis image. L: 50bp leader; 1-10; no restriction pattern of Lactoferrin with *EcoR1*. The size of intense bands are 200bp and 500bp respectively.

DISCUSSION

In cow's milk, there are 2 alleles of LF gene namely A and B with 3 different possible genotype combinations; AA, AB, BB. Allele A does not have site for *EcoRI* digestion (Zielak-Steciwko*et al.*, 2014). However, allele B produces two bands after *EcoRI* digestion (Zielak-Steciwko *et al.*, 2014). In this study, only one allele (allele A) was found with 100% frequency of one genotype (AA). Similar to that in cattle, allele A in Awassi sheep was not found to have a site for *EcoR1* enzyme digestion. To our knowledge, this is the first time

in recent literature that LF gene polymorphism, LF concentration in milk in Awassi ewes with subclinical mastitis has been revealed.

Mastitis is still a substantial cause of concern in sheep enterprises worldwide. In dairy cows, somatic cell count (SCC) is an important diagnostic tool to identify cows with subclinical mastitis. A threshold of 250,000 cells/ml has been widely accepted to indicate intramammary infection in cows, however this threshold is poorly defined in ewes. In fact, in sheep, there has been large variations in the number of SCC in normal milk that could reach up to 1,600,000 cells/ml (Bytyqi, *et al.*, 2013). However, recent research have suggested the threshold SCC in normal sheep's milk should not exceed 250,000 cells/ml (Bytyqi, *et al.*, 2013; Alhussien and Dang 2018). These variations SCC content of milk are due to different udder pathogens, lactation stage and season (Alhussien and Dang 2018). This lack of consensus in the scientific community regarding acceptable SCC level in normal sheep's milk warranted the development of additional laboratory tests to determine the health of the udder in ewes. The significance of using LF concentrations and polymorphism as a diagnostic aid of subclinical mastitis has been therefore suggested in cows. This was based on the findings of strong associations between milk SCC and LF genotype with highest SCC in AB LF genotype and lowest in AA LF genotype (Zielak-Steciwko*et al.*, 2014). In this study, only one genotype (AA) was found in ewes and this could determine high resistance to certain mastitis pathogens in sheep. Similar relationships were also reported concerning LF concentrations in milk in various animal species (Al-Majali *et al.*, 2007).

In conclusion, this study determined for the first time the gene polymorphism and genotype of lactoferrin in Awassi sheep. One allele (A) controlling one genotype (AA) was found. This LF genotype is known to be associated with increased resistance to mastitis in dairy cows and goats. Detection of this allele in Awassi sheep could be used to indicate increased resistance to certain important udder pathogens.

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