

# Development of a Monoclonal Antibody to Detect $\alpha$ 1-casein in the Milk of Healthy and Mastitis-Affected Goats

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Article ID: KVFD-2019-22287 Received: 17.03.2018 Accepted: 26.08.2019 Published Online: 29.08.2019

## How to Cite This Article

Pang M, Guan X, Zuo CX, Liu MJ, Rehman S, Fan QL, Lu P, Chen DK, Ma WT: Development of a monoclonal antibody to detect  $\alpha$ 1-casein in the milk of healthy and mastitis-affected goats. *Kafkas Univ Vet Fak Derg*, 26 (1): 71-74, 2020. DOI: 10.9775/kvfd.2019.22287

## Abstract

This study aimed to evaluate the expression level of caseins during mastitis of goats. Whole goat caseins were used as primary antigens for mouse immunization. A monoclonal antibody (mAb) named 5B with high specificity to goat  $\alpha$ 1-casein was developed. Further results showed that mAb 5B can successfully be applied to western blot and ELISA. In addition, immunofluorescence analysis using this mAb showed increased milk  $\alpha$ 1-casein level in mastitis-affected goats. In conclusion, this study has established an effective tool to evaluate the expression level of  $\alpha$ 1-casein during mastitis development.

**Keywords:**  *$\alpha$ 1-casein, Monoclonal antibody, ELISA, Mastitis, Immunofluorescence, Goat*

## Sağlıklı ve Mastitisli Keçi Sütünde $\alpha$ 1-kazeini Belirleyen Monoklonal Antikor Geliştirilmesi

### Öz

Bu çalışma keçilerde mastitis sırasında kazein ekspresyon seviyesini değerlendirmeyi amaçlamıştır. Farelerde immünizasyon için birincil antijen olarak keçi kazeinleri kullanıldı. Keçi  $\alpha$ 1-kazeine yüksek spesifite gösteren ve 5B olarak adlandırılan bir monoklonal antikor (mAb) geliştirildi. Sonuçlar mAb 5B'nin western blot ve ELISA'ya başarıyla uygulanabileceğini gösterdi. Ek olarak, geliştirilen mAb kullanılarak yapılan immünofloresan analizi, mastitisten etkilenen keçilerde süt  $\alpha$ 1-kazein seviyesinin arttığını gösterdi. Sonuç olarak, bu çalışma ile mastitis gelişimi sırasında  $\alpha$ 1-kazeinin ekspresyon seviyesini değerlendirmek için etkili bir faktör belirlenmiştir.

**Anahtar sözcükler:**  *$\alpha$ 1-kazein, Monoklonal antikor, ELISA, Mastitis, İmmünofloresans, Keçi*

## INTRODUCTION

Goat milk and its productions begin gaining attention because of their easy digestibility and lower allergenic properties compared to the cow milk<sup>[1]</sup>. An important role for goat milk is to provide the necessary nutrients for infants affected by cow milk allergy<sup>[2]</sup>. In addition, the nutritional value of goat milk has been widely accepted considering the total protein, fat, vitamin and mineral contents<sup>[3,4]</sup>. Caseins in milk account for more than 80% of total proteins and consist of  $\alpha$ 1-,  $\alpha$ 2-,  $\beta$ -, and  $\kappa$ -caseins and  $\alpha$ 1-casein is one of the most important highly-phosphorylated proteins among caseins<sup>[3]</sup>. Goat milk

contains less  $\alpha$ 1-casein (5%-20%) compared to (30%-35%) cow milk, making goat milk more similar to human milk and less likely to cause allergy<sup>[3]</sup>. In addition,  $\alpha$ 1-casein influences the coagulation properties of milk<sup>[3]</sup>. With the development of hybridoma technology, monoclonal antibodies (mAbs) against caseins were used to determine the content of  $\beta$ -casein in bovine milk<sup>[5]</sup>. However, up to the present, mAbs against goat caseins have not yet been developed.

In this paper, we used purified goat caseins for mouse immunization. Two strains of hybridoma cells targeting goat caseins (named 5B and 7H) were screened by indirect



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ELISA. To explore the effect of mastitis on as1-casein, we analyzed the level of as1-casein of goat milk samples collected from healthy goats, goats with sub-clinical mastitis and clinical mastitis by indirect competitive ELISA. Immunofluorescence of goat mammary epithelial cells was carried out to further examine as1-casein expression profile in lipopolysaccharide (LPS)-induced mastitis *in vitro*.

## MATERIAL and METHODS

### Ethical Approval

Animal-related experiments in the present study were approved by the Research Ethics Committee of Northwest A&F University according to the guidelines of the Ministry of Health in China for the care and use of laboratory animals.

### Antigen (Whole Goat Caseins) Preparation

Goat caseins were extracted using isoelectric precipitation. Briefly, 10 g of whole goat milk powder (purchased from Kabtrita, Holland) was dissolved in 10 mL of 2M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O buffer. The pH was adjusted to 4.2 using 1M CH<sub>3</sub>COOH solution (preheated to 40°C). After complete precipitation at 37°C, the solution was centrifuged at 4000g for 10 min, and the precipitate was washed with 20 mL of 95% (v/v) ethanol 3 times to remove lipid. Next, 1:1 (v/v) ethanol-ether mixture was added to wash the precipitate 3 times. Final precipitation was washed twice with ether and was placed in a fume hood until all ether volatilized. Casein powder was preserved at -80°C and its purity and molecular weight were analyzed by SDS-PAGE [6].

### Mouse Immunization, Cell Fusion, Hybridoma Cell Screening and Ascites Preparation

Mouse immunization, cell fusion, hybridoma cell screening and ascites preparation were conducted as previously reported by our group [6].

### Competitive Indirect ELISA

Competitive indirect ELISA was conducted as previously reported [6]. Specifically, the dilution of goat milk was 15 fold with PBST and the dilution of mAb 5B was 7 fold with PBST.

### Milk Sample Collection and Processing

For clinical mastitis samples, milk with the detection of flakes and clots with gland swelling or systemic illness such as fever, depression, weakness and dehydration were selected. For sub-clinical mastitis diagnosis, the California Mastitis Test was used as previously reported [7]. All the milk was stored at 4°C after collection and was used for further assays within 1 h.

### Isolation and Culture of Goat Mammary Epithelial Cells (MECs)

Goat MEC isolation was performed as previously reported [6].

After isolation, the cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, New York, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 1% GlutaMAX Supplement (Thermo Fisher Scientific) and 100 units mL<sup>-1</sup> of penicillin-streptomycin (Sigma Aldrich, Missouri, USA). The culture medium was refreshed every 24 h. The cells were cultured at 37°C in 5% CO<sub>2</sub> and were passaged when they were 80% confluent.

### LPS Stimulation and Immunofluorescence Staining

The *in vitro* mastitis model was established by adding LPS (Sigma Aldrich) to the culture medium at a final concentration of 10 µg mL<sup>-1</sup>. Meanwhile, the control group was treated with culture medium of the same volume. All cells were treated for 12 h before immunofluorescence analysis. Procedures of immunofluorescence were the same as previously reported [8].

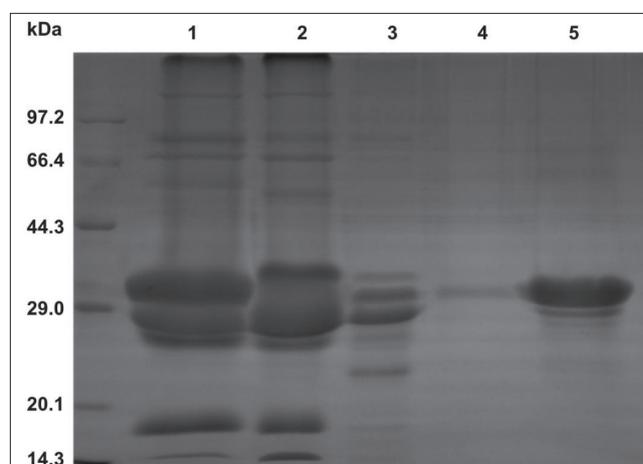
### Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Differences were assessed using t-tests or one-way ANOVA with Dunnett test. P<0.05 was considered significant.

## RESULTS

Isoelectric point precipitation method was used for the purification of the goat casein and was identified by SDS-PAGE. After precipitation, caseins were purified and contained as1-casein because lane 3 and lane 4 has an identical band at 23.9 kDa (Fig. 1). Consistent with a previous study, purified as1-casein showed a higher molecular weight when analyzed by SDS-PAGE than expected [9].

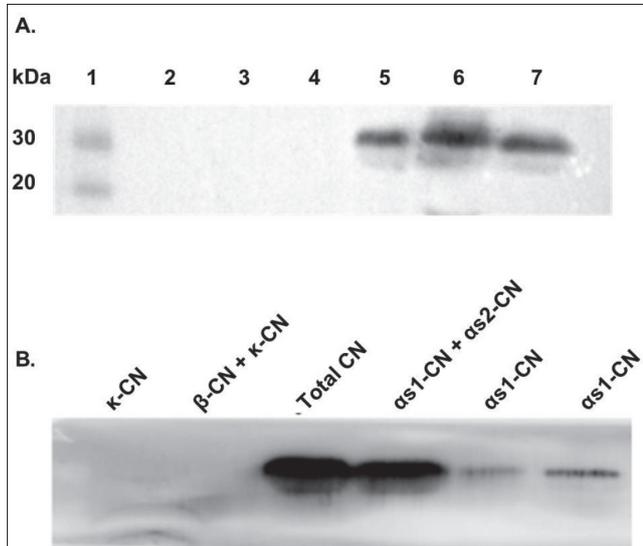
The titers of sera collected from the immunized mice 7-10 days after the forth immunization were determined by indirect ELISA. All immunized mice produced anti-goat



**Fig 1.** SDS-PAGE analysis of caseins. Lane 1, bovine milk powder; lane 2, goat milk powder; lane 3, total goat caseins; lane 4, goat as1-casein; lane 5, bovine  $\alpha$ -casein

casein antibodies. Several mice were selected for further experiment and were injected with 100  $\mu$ g of immunogen intravenously 3 days before cell fusion. Then, hybridoma secreting antibodies specific to goat caseins but unrecognizable to bovine milk powder were selected by

indirect ELISA, and were subcloned by limiting dilution method. Two stable hybridoma cell lines were established and named as 5B and 7H. These two hybridoma cell lines showed strong proliferation ability and high specificity to goat caseins and was chosen for further studies.



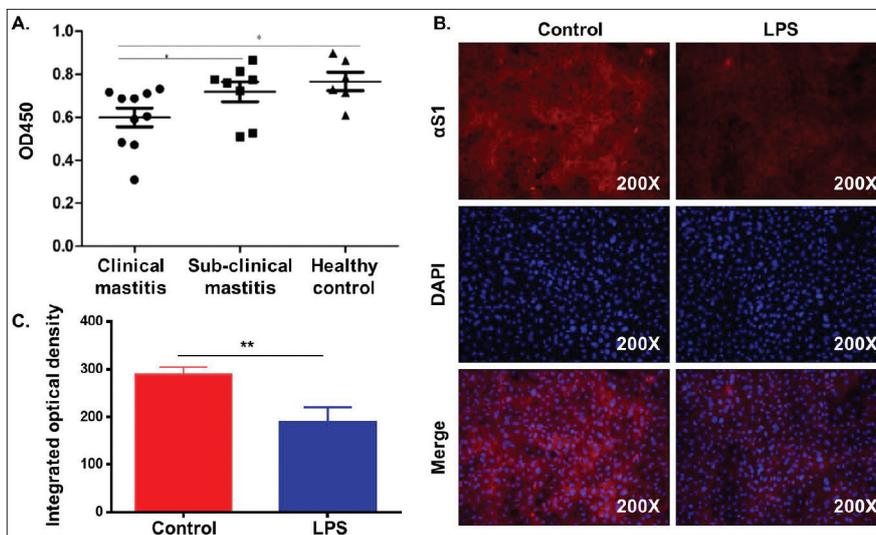
**Fig 2.** mAb specificity tests by western blot analysis. (A) Goat casein was loaded as a positive control, the content of goat casein and bovine casein was 1.7  $\mu$ g, milk powder was at the concentration of 0.1 g/mL, the milk was 20-fold diluted. Lane 1, marker; lane 2, bovine  $\alpha$ -caseins; lane 3, cow milk; lane 4, cow milk powder; lane 5, total goat casein; lane 6, goat milk; lane 7, goat milk powder. (B) Isolation of goat caseins and recognition of 5B to  $\alpha$ 1-casein as determined by western blot. Goat caseins are purified by cation-exchange chromatography. Each lane is loaded with the correspondingly labeled proteins

Cross-reactivity of mAbs 5B and 7H was analyzed by indirect ELISA using goat casein, bovine milk, goat milk, goat milk powder and bovine milk powder as antigens. The results showed that these two mAbs both specifically recognized goat caseins and had no cross-reactivity with bovine caseins (Table 1), which was supported by western blot analysis using mAb 5B (Fig. 2A). In addition, the specificity of mAb 5B was further evaluated using different subtypes of goat caseins. The result showed that this mAb only reacted with goat  $\alpha$ 1-casein, while no other reactions existed between mAb 5B and purified  $\beta$ - and  $\kappa$ -caseins (Fig. 2B).

In order to evaluate  $\alpha$ 1-casein content in milk of goat with mastitis, OD<sub>450</sub> values of different goat milk samples were compared by indirect competitive ELISA. As illustrated in Fig. 3A, milk from goats suffering from clinical mastitis had the lowest OD<sub>450</sub> value, representing the highest levels of  $\alpha$ 1-casein in these samples. In comparison, milk from goats with sub-clinical mastitis and healthy controls had relatively lower levels of  $\alpha$ 1-casein compared to the goats with clinical mastitis ( $P < 0.05$ ). We further built an *in vitro* mastitis model by challenging goat MECs with LPS. Interestingly, LPS treatment resulted in significantly decreased cytoplasmic  $\alpha$ 1-casein levels in MECs (Fig. 3B-C).

**Table 1.** Testing specificity of monoclonal antibodies 5b and 7b against goat casein by ELISA

Antibody	Protein					
	Goat Casein	Goat Milk	Goat Milk Powder	Bovine Casein	Bovine Milk	Bovine Milk Powder
MAb 5B	1.762	1.531	1.824	0.080	0.075	0.082
MAb 7H	1.634	1.520	1.627	0.092	0.065	0.087



**Fig 3.** Analysis of  $\alpha$ 1-casein level in goat milk samples and goat MECs. (A) Indirect competitive ELISA analysis of  $\alpha$ 1-casein content in milk samples from goats with clinical mastitis ( $n=10$ ), sub-clinical mastitis ( $n=8$ ) or healthy goats ( $n=6$ ). (B) Immunofluorescence analysis of  $\alpha$ 1-casein expression levels in goat MECs with (LPS,  $n=4$ ) or without LPS (Control,  $n=4$ ) treatment. (C) Integrated optical density analysis of the immunofluorescence results in (B)

## DISCUSSION

Casein expression level is an important index when evaluating the lactation function of the mammary gland. While mAbs against  $\beta$ -casein<sup>[10]</sup> and  $\kappa$ -casein<sup>[11]</sup> have been developed, no studies have reported the successful development of mAbs against  $\alpha$ s1-casein, the major part (38%) of total caseins in milk<sup>[12]</sup>. In addition, considering that  $\alpha$ s1-casein is the most important protein causing milk allergy<sup>[13]</sup>, mAbs with high specificity to goat  $\alpha$ s1-casein are therefore of critical importance for people to avoid milk allergy.

Mammary epithelial cells constitute an important part of the mammary gland and are the structural basis for the lactation of the mammary gland. The secretion of caseins is affected by the condition of MECs. By using mAbs against  $\alpha$ s1-casein, we can measure its expression level in MECs under different conditions. Intriguingly, our data suggest that the concentration of  $\alpha$ s1-casein was significantly increased in milk samples of clinical mastitis-suffered goats, indicating that the milk of these goats may be problematic and chances of allergy are higher. However, we found the expression of  $\alpha$ s1-casein in the cytoplasm of MECs was decreased after stimulation of LPS *in vitro*, consistent with a previous study<sup>[14]</sup>. The severely milk yield loss in goats with clinical mastitis may be the main reason for the higher concentration of  $\alpha$ s1-casein in milk of clinical mastitis-affected goats. However, we could not rule out the possibility that whether an enhanced  $\alpha$ s1-casein secretion ability accounted for less  $\alpha$ s1-caseins retention. Thus, future studies are needed to further explore the underlying mechanisms.

In conclusion, a mAb named 5B that specifically reacted with goat  $\alpha$ s1-casein was developed in the present study. This mAb can successfully be applied to western blot, ELISA and immunofluorescence and served as a convenient tool to assess the dynamics of  $\alpha$ s1-casein during mastitis development. More future studies investigating the biological function of  $\alpha$ s1-casein and its relationship with certain diseases are encouraged. Specially, studies concerning the involvement of  $\alpha$ s1-casein in milk allergy will lay important foundation for elucidating the underlying mechanisms and for effective therapy development.

## CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

## FUNDING

This work was supported by National Natural Science Foundation of China (31902282), Qinghai province Major

R&D and Transformation Project (2018-NK-125) and Key industrial innovation chains of Shaanxi province (2018ZDCXL-NY-01-06).

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