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Prevalence of subclinical streptococcal mastitis in dairy cows in Chaharmahal and Bakhtiari province

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Abstract

Mastitis is an inflammatory disease of the mammary gland, caused by many infectious agents such as bacteria, fungi, and viruses. Streptococci are reported to be among the major pathogens causing bovine mastitis around the world, which may cause clinical and subclinical forms of mastitis. Mastitis is one of the primary diseases of dairy cows and is responsible for remarkable economic losses due to reduction in quantity and quality of milk, cost of treatment, and the early culling of the cows. Considering the existence of substantial industrial and traditional dairy cattle farms in Chaharmahal and Bakhtiari province and the fact that mastitis is the most common disease in dairy industries, this study aimed to identify the role of streptococci in subclinical mastitis in dairy cows in Chaharmahal and Bakhtiari province. For this purpose,134 subclinical mastitis milk samples were collected from 8 dairy farms in Chaharmahal and Bakhtiari province, based on the california mastitis test (CMT) results and screened for the streptococcal cause of mastitis. DNAs were extracted from collected specimens and PCR was performed with specific primers for *Streptococcus agalactiae*, *Streptococcus uberis*, and *Streptococcus dysgalactiae*. Out of 134 mastitis milk samples 10 (7.5%), 14 (10.4%), and 5 (3.7%) samples were positive for *S. agalactiae*, *S. uberis*, and *S. dysgalactiae* respectively. The result of this research shows that 21.6 (29:134) percent of mastitis in dairy cattle farms in the studied region may be due to streptococci. The obtained data can be used in management and prevention strategies for cattle mastitis control in Chaharmahal and Bakhtiari province.

Key words: Mastitis, *Streptococcus*, Cattle, PCR, Chaharmahal and Bakhtiari

Introduction

Mastitis, characterized by the inflammation of the mammary gland, is a pervasive and economically significant disease affecting dairy cows worldwide (Libera et al, 2021). As the most common infectious ailment in the farm industry, it poses substantial challenges, necessitating a comprehensive understanding of its etiology, classification, symptoms, economic implications, and diagnostic methodologies. The mammary gland's vulnerability to infection is heightened

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during the post-milking period when the teat canal remains open for 1-2 hours. This path of susceptibility is crucial, as it correlates with a reduction in local antimicrobial protection, creating an opportune environment for pathogens to invade (Reshetka, 2013; Zverzhanovskiy et al, 2017; Serdyuchenko et al, 2018; Sobol et al, 2017; Zykova et al, 2018; Donnik et al, 2017; Ruegg, 2017; Phuektes et al, 2001).

Although viruses, fungi, and algae are acknowledged as potential mastitis causes, the predominant cause of bovine mastitis is the invasion of the udder by pathogenic bacteria. Among the lot of bacterial strains associated with mastitis, *Staphylococcus aureus, S. agalactiae, S. dysgalactiae,* and *S. uberis* stand out as the most common causes (Phuektes et al, 2001). Streptococci causing mastitis are classified into contagious and environmental groups (Cheng and Hen, 2020). Contagious pathogens are adept at surviving within the host and spreading among cows during the milking process, facilitating easy dissemination within the herd. On the other hand, environmental pathogens can survive outside the host and are part of the cow's normal microflora (Hogan and Smith, 2003). *S. agalactiae* is categorized as a contagious pathogen, *S. uberis* is considered environmental, and instances of contagion are observed. The classification of *S. dysgalactiae* as either environmental or contagious remains a subject of ongoing investigation, underscoring the complexity of mastitis pathogenesis (Oliver et al, 2003).

The clinical presentation of mastitis in cows encompasses a spectrum of symptoms, including udder swelling, heat, pain, abnormal milk appearance, raised body temperature, lethargy, and anorexia (Heringstad et al, 2000; Kibebew, 2017). Mastitis is stratified into three classes - clinical, subclinical, and chronic (Cheng and Hen, 2020). Clinical mastitis is characterized by visible abnormalities in both the cow and the milk. Changes are limited to alterations in milk yield and somatic cell count, distinguishing it from subclinical mastitis. Subclinical mastitis is estimated to occur at rates 15–40 times higher than clinical mastitis. Lack of visible clinical symptoms is one of the challenges of timely diagnosis of this type of mastitis (Martin et al, 2018).

The economic ramifications of mastitis encompass the costs of treatment and veterinary interventions, reduced milk production, and the culling of livestock. In addition to economic costs, it affects the overall productivity and profitability of dairy products (Sordelli et al., 2000). Consequently, there is an urgent need for correct and timely treatment, necessitating fast and reliable diagnostic tools for mastitis (Godkin et al, 1993).

Traditional diagnostic methods such as microbial culture have historically been employed for mastitis diagnosis. However, their time-consuming nature prompted the adoption of molecular methods, including polymerase chain reaction (PCR), as valid alternatives with high sensitivity and specificity (Hassan et al, 2001). The PCR method offers a swift and reliable option for identifying bacterial pathogens. It enables the recognition of pathogens within hours, compared to the days required by traditional cultural methods. The heightened sensitivity of PCR allows for the detection of pathogens in the early stages of infection and in the carrier animals, even when bacterial concentrations in milk are minimal. However, the excessive sensitivity of PCR lies in its susceptibility to misdiagnosis due to minor contaminants in samples (Phuektes et al, 2001).

This study aims to investigate the prevalences of bovine streptococcal (*S. dysgalactiae, S. uberis,* and *S. agalactiae*) subclinical mastitis, in Chaharmahal and Bakhtiari province, by PCR method. By considering the prevalence of these streptococci, the research endeavors to provide valuable insights into the epidemiology and management of this prevalent dairy cattle ailment.

Materials and Methods

From June to September 2023, 134 subclinical mastitis milk samples were collected from eight distinct dairy farms located in Chaharmahal and Bakhtiari province. Mastitis suspected samples were collected based on CMT. Following the exclusion of the initial three milkings from each quarter, starting from the fourth milking, approximately 2-3 ml of milk was deposited into the corresponding compartment of the CMT container and the CMT procedure was conducted according to the company (Bovivet, Denmark) instructions.

The CMT positive samples were collected in sterilized test tubes and subsequently transported to the laboratory for further analysis. The samples underwent a centrifugation process at 6000 rpm for 30 minutes. The resulting sediments were then earmarked for molecular testing.

DNA was extracted from all 134 subclinical milk samples, and also positive controls including *S. agalactiae* (PTCC:1768), *S. uberis* (IBRC-M 10804), and *S. dysgalactiae* (PTCC:1236) using DNP Kit EX6071 (Sinaclon, Iran) following the specified instructions meticulously. Briefly, 1 ml of each milk sample was centrifuged at 3000 rpm for 10 minutes and then 100 µl protease buffer and 5 µl of protease were added to the precipitate and vortexed and incubated at 55° C for 30 minutes. After that, 400 µl of lysis solution was added to it and homogenized by vortexing for 15-20 seconds. In the next step, 300 µl of precipitation solution was added and mixed by vortexing for 5 seconds. The lysed sample was centrifuged at 12000 rpm for 10 minutes. The supernatant was decanted by gently inverting the tube and placing the tube on tissue paper for 2-3 seconds. Washing of precipitate was done with the addition of 1 ml of wash buffer and mix by 3-5 seconds vortexing. In the next step, the sample was centrifuged at 12000 rpm for 5 min and the supernatant was poured off and the pellet was dried at 65° C for 5 minutes. The pellet was suspended in 50µl of solvent by gentle shaking and placing at 65° C for 5 min. The purified DNA was harvested by centrifugation at 12000 rpm for 30 seconds and the supernatant was stored at -70 ℃ and used as a template in the PCR reaction.

The concentration of extracted DNA was calculated at a wavelength of 260/280 nm using a nanodrop spectrophotometer (Eppendorf, Germany). Pure DNA was defined as samples having 260/280 absorbance ratios of less than 1.8.

A pivotal aspect of this study accomplished the Polymerase Chain Reaction (PCR) method. In this process, a total volume of 20 µL was employed for DNA amplification. This comprised 10 µL of PCR 2x Master Mix (GeneDireX, Inc., Taiwan), 1µL (10 picomole) of each of forward and reverse primers supplied by Metabion in Germany, 3µL of template DNA, and 5µL of nuclease-free water. The sequence of selected primers, as outlined in Table 1, played a crucial role in targeting specific genetic sequences associated with *S. agalactiae*, *S. uberis* and *S. dysgalactiae*. In each PCR reaction along with samples, DNA from the above-mentioned strains was used as positive control and distilled water as a negative control.

The thermal cycling conditions for the PCR process were meticulously optimized for each streptococcal species. For *S. agalactiae* and *S. uberis*, the cycling involved an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. In the case of *S. dysgalactiae*, the cycling conditions were adapted, incorporating an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes.

The PCR products were analyzed through 1.5% agarose gel electrophoresis. This meticulous step, post-staining with a safe stain (Yekta Tajhiz Azma, Iran) and visualization under UV light (Uvitec, England), served as the cornerstone for confirming the success of DNA amplification and providing a visual representation of the molecular composition of the mastitis-causing streptococcal species in the examined samples.

Table 1: The sequence of primers used in the PCR

Species	Sequence (5′–3′)	PCR product size (bp)	Reference
S. agalactiae	F: AAGGAAACCTGCCATTTG R: TAACCTAGTTTCTTTAAAACTAGAA	270	(Phuektes et al., 2001)
S. dysgalactiae	F: GAACACGTTAGGGTCGTC R: AGTATATCTTAACTAGAAAAACTATTG	264	(Phuektes et al., 2019)
S. uberis	F: CGCATGACAATAGGGTACA R: GCCTTTAACTTCAGACTTATCA	445	(Momtaz et al., 2012)

The statistical analysis of the obtained data was conducted through a descriptive analysis utilizing Microsoft Excel (Microsoft Corporation, 2018).

Result

Based on the CMT performed on milk samples, all collected samples were positive. According to the PCR results, 10 (7.5%) samples were positive for *S. agalactiae*, 14 (10.4%) samples were positive for *S. uberis*, and 5 (3.7%) samples were positive for *S. dysgalactiae* (figure 1).

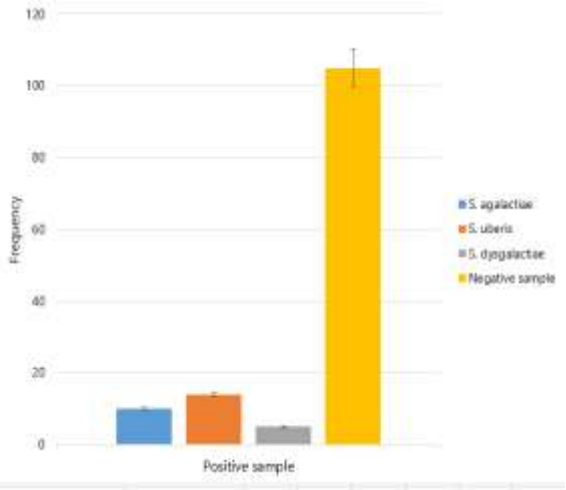

Figure 1: Frequency chart of bovine streptococcal mastitis in 134 individual mastitic millk samples from Chaharmahal and Bakhtiari province.

Figure 2: Agarose gel electrophoresis of the *S. agalactiae* PCR products. Lane 1: 100 bp DNA marker; lane 2: positive control (270 bp); lane 3: negative control and lane 4 is a positive sample.

Figure 3: Agarose gel electrophoresis of the *S. uberis* PCR products. Lane 1: 100 bp DNA marker; lane 2: positive control (445 bp); lane 3: negative control and lane 4 a positive sample.

Figure 4: Figure 2. Agarose gel electrophoresis of the *S. dysgalactiae* PCR products. Lane 1: 100 bp DNA marker; lane 2: positive control (264 bp); lane 3 and 4 two positive sample and lane 4: negative control.

Discussion

Mastitis is the most prevalent disease affecting dairy cows worldwide (Ziv, 1992). Subclinical mastitis is one of the most important diseases causing substantial economic loss to the farmers and the dairy industries due to the long-term effects of chronic infections (Abdella, 1996) and also through decrease in milk quality and quantity. Subclinical mastitis in dairy cattle is a chief and silent problem whose early detection is critical to prevent its associated economic losses and also to make decisions for its rapid and effective treatment (Chagunda et al, 2006). Laboratory methods should be used to identify it because mostly remains unnoticed by the farmer (Singh et al, 2008). Streptococci are among important cow subclinical as well as clinical mastitis bacterial agents (Kibebew, 2017); so, the present research was conducted to detect the importance of streptococcal subclinical dairy cows mastitis in Chaharmahal and Bakhtiari province. In line with the findings of various researchers, our study underscores the role of streptococci as causative agents in subclinical cases of cattle mastitis.

The role of specific streptococcal species in subclinical mastitis has been explored in several studies, providing valuable insights into the diverse microbial landscape associated with this bovine ailment. In agreement with our results, Phuektes et al, (2001), in their research for developing a multiplex PCR assay for the simultaneous detection of the four major bacterial causes of bovine mastitis including *S. agalactiae*, *S. aureus S. dysgalactiae* and *S. uberis* reported that these agents play respective roles in 13.7%, 3.4%, 1.7% and 0.85% of bovine subclinical mastitis. Moatemedi et al, (2007) revealed the role of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* respectively in 20%, 12.5% and 0.83% of subclinical mastitis in dairy cattle in Ahvaz. Ehsani et al, (2024) reported that the contamination rates of bulk tank milk with *S. uberis* in Isfahan were 16% and 20% as determined by culture and RT-PCR methods, respectively. Comparing their results with the present study, the role of *S. uberis* was more prominent than those of the two streptococci. This discrepancy may be due to the difference in time, region, sampling season and the type of milk (bulk tank and individual sample) in the two studies (Song et al, 2020). In accordance with our results, Emadi et al, (2013) also reported that *S. uberis* is involved in 6.6% of subclinical mastitic cow milk samples in Tehran province.

Molecular methods have created a fundamental revolution in the detection and identification of microorganisms; these methods are simple, low-cost and highly sensitive (Rezazadeh Zarandi et al, 2017). Nithin Prabhu et al, (2013) reported that the PCR method can be successfully used for the identification of the major mastitis caused streptococci, especially *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*. Vojgani et al, (2006) investigated the contamination of the bulk milk tanks of livestock farms with mastitis ascociated pathogens by using universal primers and reported that the use of this primer was able to identify the main cause of mastitis. Soltao et al, (2017) highlighted the utility of PCR assays for identifying mastitis pathogens in bulk tank milk. While our study focused on

individual cow samples, the insight from bulk tank milk examinations emphasizes the potential of repeated testing as a valuable monitoring tool, particularly when considering *S. dysgalactiae, S. agalactiae*, and *S. uberis*. Wang et al, (2016) investigated the frequency of mastitis-causing pathogens in bulk tank milk and reported that *S. aureus*, *S. agalactiae, S. dysgalactiae* and *Trueperella pyogenes* are the most frequently detected pathogens in bulk tank milk samples.

Economically, bovine mastitis incurs significant disadvantages in livestock production. The compromised milk quality and quantity directly impact the profitability of dairy operations. Timely diagnosis and intervention are essential to curtail economic losses associated with decreased milk yield, veterinary expenses, and potential culling of infected animals. Implementing preventive measures based on accurate diagnostic tools can contribute to substantial economic benefits by minimizing treatment costs, preserving milk production, and maintaining the overall health of the dairy herd (Wang et al, 2016; Kibebew, 2017).

In conclusion, our research reinforces the significant contribution of streptococci, particularly *S. uberis.* and *S. agalactiae* to subclinical cases of bovine mastitis in Chaharmahal-Bakhtiari. Regular testing, coupled with preventive measures, is crucial for controlling the incidence of mastitis in this region. The PCR-based test demonstrated specificity in identifying and determining the prevalence of these bacterial pathogens. The economic advantages of timely diagnosis and intervention cannot be overstated, as they play a pivotal role in sustaining the economic viability of dairy operations.

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Conflict of interest

The authors declare no conflict of interest.

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اهمیت ورم پستان‌های استرپتوکوکی در گاوهای شیری در استان چهارمحال و بختیاری

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چکیده

ورم‌پستان بیماری التهابی غده پستان است که توسط عوامل عفونی متعددی همچون باکتری‌ها، قارچ‌ها و ویروس‌ها ایجاد می‌شود. استرپتوکوک‌ها جز پاتوژن‌های اصلی ایجادکننده ورم پستان در سراسر جهان هستند که می‌توانند اشکال درمانگاهی و تحت درمانگاهی ورم پستان را ایجاد کنند. ورم پستان یکی از بیماری‌های اولیه گاوهای شیری است که به دلیل کاهش کمیت و کیفیت شیر، هزینه درمان و حذف زود هنگام گاوها، زیان‌های اقتصادی قابل توجهی را به همراه دارد. با توجه به وجود گاوداری‌های صنعتی و سنتی فراوان در استان چهارمحال و بختیاری و این مهم که ورم پستان شایع‌ترین بیماری در گاوداری‌های شیری است، این مطالعه با هدف شناسایی نقش استرپتوکوک‌ها در ورم‌پستان‌های تحت درمانگاهی در گاوهای شیری استان چهارمحال و بختیاری انجام شد. برای این منظور، ۱۳۴ نمونه شیر ورم‌پستان تحت درمانگاهی از ۸ گاوداری در استان چهارمحال و بختیاری بر اساس نتایج آزمایش کالیفرنیایی ورم‌پستان (CMT) جمع‌آوری و از نظر علل استرپتوکوکی ورم‌پستان غربال شدند. DNA نمونه‌های جمع‌آوری شده استخراج شد و با پرایمرهای اختصاصی *استرپتوکوکوس آگالاکتیه*، *استرپتوکوکوس یوبریس* و *استرپتوکوکوس دیسگالاکتیه* واکنش زنجیره‌ای پلیمراز انجام شد. از ۱۳۴ نمونه شیر ورم‌پستانی، ۱۰ نمونه (۷/۵درصد)، ۱۴ نمونه (۱۰/۴ درصد) و ۵ نمونه (۳/۷درصد) به ترتیب از نظر *استرپتوکوکوس آگالاکتیه*، *استرپتوکوکوس یوبریس* و *استرپتوکوکوس دیسگالاکتیه* مثبت بودند. نتایج این مطالعه نشان می‌دهد که ۲۱/۶ (۲۹:۱۳۴) درصد از ورم پستان‌های تحت درمانگاهی گاوهای شیری در منطقه مورد مطالعه قرار گرفته، می‌تواند ناشی از استرپتوکوک‌ها باشد. نتایج به دست آمده می‌تواند در راهبردهای مربوط به مدیریت و پیشگیری ورم پستان گاو در استان چهارمحال و بختیاری مورد استفاده قرار گیرد.

کلمات کلیدی: ورم پستان، *استرپتوکوکوس*، گاو، PCR، چهارمحال و بختیاری

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