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Microbiological and Cytological Investigation of Clinical Equine Mastitis in Türkiye

Türkiye'de Klinik At Mastitisinin Mikrobioyolojik ve Sitolojik Yönden Araştırılması

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ABSTRACT

Equine mastitis is an uncommon but may cause some serious clinical conditions including septicemia, arthritis, and pneumonia when transmission of the microbial pathogen to the nursing foal. Mares themselves also may show local and systemic clinical signs associated with mastitis. Little data are available evaluating microbial etiology of clinical equine mastitis associated with cytologic examination in Türkiye. Milk orudder secretion samples, which were admitted to the diagnostic laboratory from a total of 22 clinically mastitic mares, were examined by bacterial-fungal culture and cytological methods between 2016 and 2022. The most common bacterial isolate was found to be *Streptococcus equi* subsp. *zooepidemicus* (54.6%), followed by *Escherichia coli* (27.4%). No fungal pathogen was isolated. Cytologic examinations revealed the presence of strong neutrophilic infiltration (<80%) associated with degenerative changes and the presence of intracellular bacteria. In lactating mares (n = 9), *E. coli* and *S. zooepidemicus* were equally isolated from a total of 6 samples, followed by *S. aureus* (n = 1), *E. cloacae* (n = 1), and *S. maltophilia* (n = 1). On the other hand, in non-lactating mares, *S. zooepidemicus* was the most prevalent agent isolated from 9 samples, followed by *E. coli* from 3 samples and *S. epidermidis* from 1 sample. Further, more comprehensive studies should be conducted regarding subclinical cases and antimicrobial resistance profiles of the agents isolated from equine mastitis cases in horse populations in Türkiye.

Keywords: Bacterial infection, cytology, mare, mastitis

ÖZ

At mastitisi nadir görülen bir durumdur ancak mikrobiyal patojenin emziren taya bulaşması durumunda septisemi, artrit ve pnömoni gibi bazı ciddi klinik durumlara neden olabilir. Kısrakların kendileri de mastit ile ilişkili lokal ve sistemik klinik belirtiler gösterebilir. Türkiye'de klinik at mastitisinin mikrobiyal etiyolojisini sitolojik inceleme eşliğinde değerlendiren çok az veri mevcuttur. 2016-2022 yılları arasında klinik olarak mastitisli toplam 22 adet kısraktan tanı laboratuvarına kabul edilen süt/meme salgı örnekleri bakteriyel-fungal kültür ve sitolojik yöntemlerle incelendi. En yaygın bakteri izolatının *Streptococcus equi* subsp. *zooepidemicus* (%54,6), ardından *Escherichia coli* (%27,4) gelmektedir. Fungal patojen izole edilmedi. Sitolojik incelemeler, dejeneratif değişiklikler ve hücre içi bakteri varlığı ile birlikte güçlü nötrofilik infiltrasyonu varlığını (%80<) ortaya çıkardı. Laktasyondaki kısraklarda (n = 9), *E. coli* ve *S. zooepidemicus* toplam altı örnekten eşit olarak izole edilirken, bunu *S. aureus* (n = 1), *E. cloacae* (n = 1) ve *S. maltophilia* (n = 1). Laktasyonda olmayan kısraklarda ise dokuz örnekte en sık izole edilen etken *S. zooepidemicus* olurken, bunu 3 örnekle *E. coli* ve 1 örnekle *S. epidermidis* izledi. İleride Türkiye'deki at popülasyonlarında subklinik mastitis olguları ve etkenlerin antimikrobiyal direnç profilleri ile ilgili daha kapsamlı çalışmalar yapılmalıdır.

Anahtar Kelimeler: Bakteriyel enfeksiyon, kısrak, mastitis, sitoloji.

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INTRODUCTION

Mastitis cases in horses is an uncommon condition unlike in dairy cows because of having been attributed to a short lactation period and the small size of the udder contributes to a more frequent expelling of the udder.[1] Anatomically hidden position of the udders protects them reducing exposure

to trauma and low contact probability to the contaminated ground surface. While mastitis is less prevalent in mares when compared with cows, serious outcomes regarding mastitis can occur in horses as well.[1] In the worst-case scenario, the transmission of the microbial pathogen to the sucking foal can cause septicemia, arthritis, and pneumonia.[1] Agalactia can also lead to subsequent foal malnutrition.[2] Mastitis can also trigger abortion in the mare in case of pregnancy and systemic compromisation, or less commonly, severe infection can cause permanent loss of function in the affected mammary gland due to fibrosis and obstruction.[3] Local swelling or heat in the affected udder, pain, udder asymmetry, firmness, ventral edema with or without concomitant lower limb edema, a congested mammary vein, rejection of the foal, and abnormal purulent and/or serosanguineous secretions are the clinical signs associated with mastitis.[3] Mares can also demonstrate systemic signs such as pyrexia (up to 41°C), anorexia, depression, and hindlimb lameness, but the most common clinical signs are a firm and swollen udder with purulent discharge. Blood analysis of the affected mares often yields unremarkable but may also show neutrophilia and hyperfibrinogenemia. Based on the clinical presentation, mastitis can be encountered as acute, chronic, and clinical or subclinical. Mastitis is mostly caused by bacteria, and less commonly by fungi, nematodes, or non-septic etiologies such as avocado tree poisoning.[4] Over 20 bacterial agents have been associated with mastitis in the equidae family including *Streptococcus* spp. which were the most common isolates reported by different authors.[3,5-7] In California, it was also reported that 42% of the mares suffering from clinical mastitis had Gram-negative bacteria isolated.[6]

In the diagnosis of mastitis, clinical inspection of the udder can be performed to observe typical signs. It is recommended to confirm bacterial isolation by performing a culture associated with the cytology of the milk/udder secretions.[8] The cytologic appearance of mare milk has a proteinaceous background and is either acellular or may contain scarce neutrophils.[9] Horses having mastitis associated with a bacterial agent usually have a high number of neutrophils with a degenerative appearance of the cells.[6]

Equine mastitis may be encountered during early lactation[1]. However, mastitis can be seen in mares at any period of lactation and also in post-lactational regression associated with weaning and is therefore commonly encountered during summer or autumn season.[9] Additionally, mastitis may occur in association with milk build-up relating to illness or loss of a foal and may also be seen in pregnant mares, non-pregnant mares, young fillies, and neonatal foals as well.[9] Different breeds can be affected by mastitis including thoroughbreds, standardbreds, quarter horses, and ponies. Most of the mares are likely to present with unilateral disease, and in some cases, only 1 ductal tree within a mammary can be affected.[9]

One report from Germany predicted that as much as 5% of breeding mares are affected by mastitis.[7] The incidence does not seem to be high in North America.[3,6] However, the true incidence across breeds and countries remains to be determined.[1]

According to the author's knowledge, little data are available evaluating the microbial etiology of equine clinical mastitis in Türkiye. Therefore, this study was designed to evaluate and provide information about the microbial etiology of equine clinical mastitis together with cytological findings.

MATERIALS AND METHODS

Animals

The analysis records of clinical mastitis cases were reviewed for the study. An official consent was taken from general management of Jockey Club of Turkey for publishing the results (Number: 21). A total of 22 mastitic case samples were admitted to Jockey Club of Turkey diagnostic laboratory of İstanbul between 2016 and 2022. Nineteen out of 22 (86.4%) of the mares were English thoroughbreds and 3/22 (13.6%) were Arabian thoroughbreds. The number of non-lactating and lactating mares was 13/22 (59.1%) and 9/22 (40.9%) respectively. The mean age (±SD) of the mastitic mares was 10.6 (±3.6) years old. Initial diagnosis of the cases was based on acute clinical signs associated with mastitis. The clinical signs consisted of 1 or multiple following signs: swollen udder, sensitivity of udder in palpation, and purulent or serosanguineous secretion from the udder. Milk or udder secretions of the mastitic cases were collected by the clinicians after cleaning and disinfection of the udder with standard protocols.[10] Following the discard of the first udder ejections, samples were collected into sterile plastic containers and were sent to the laboratory immediately for microbiological and cytologic analysis with ice packs. Nineteen of the 22 samples were like purulent discharge those had high viscosity with brownish appearance. Three of the samples analyzed were white in color but had a higher viscosity than normal consistency. The analysis of the samples was carried out within 24 hours of sample collection.

Microbiological Analysis

All samples were inoculated into 5% sheep blood agar, Mac Conkey agar for bacterial isolation, and inhibitory mold agar (IMA) for fungal isolation. Bacterial culture plates were incubated at 37°C in both aerobic and microaerophilic conditions for 72 hours. On the other hand, IMA plates for fungal isolation were inoculated and incubated at 25°C in an aerobic atmosphere for 10 days. After incubation, isolated microbial colonies were initially examined based on Gram staining and catalase and oxidase activity. Further identification of the suspected colonies was made by biochemical methods using a commercial bacterial identification system as described by the manufacturer (Diagnostics SK Inc., Galanta, Slovenia).

Cytology

Cytological examinations were carried out by following the standard protocol described previously with some minor changes.[11] Briefly, 50 µL of milk or secretions was radiated on a microscope slide and left to dry at 37°C for 15 minutes. After drying off, prepared smears were stained with May Grunwald–Giemsa quick stain according to the protocol described by the manufacturer (GBL, İstanbul, Türkiye). The stained smears were then examined by visualization of at least 10 different microscopic fields at 100× magnification by using immersion oil. One slide per mare was prepared for the cytologic examination. The cytology results were expressed as the relative number (%) of neutrophils, macrophages, and lymphocytes in the smear. Briefly, it was calculated according to the formula below.

$$\text{Relative number}(\%) = \frac{\text{Number of the individual cell(NEU or MAC or LYM)}}{\text{Number of the total counted cells(NEU} + \text{MAC} + \text{LYM)}} \times 100$$

RESULTS

According to the bacterial isolation results, the most commonly isolated bacterial agent was determined to be *Streptococcus equi* subsp. *zooepidemicus* 54.6% (n=12) followed by *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter cloacae*, and *Stenotrophomonas maltophilia* with the isolation rates of 27.4% (n=6), 4.5% (n=1), 4.5% (n=1), 4.5% (n=1), and 4.5% (n=1), respectively. No fungal agent was isolated from the samples analyzed in the study. In lactating mares (n=9), *E. coli* and *S. zooepidemicus* were equally isolated from a total of 6 samples, followed by *S. aureus* (n=1), *E. cloacae* (n=1), and *S. maltophilia* (n=1). On the other hand, in non-lactating mares *S. zooepidemicus* was the most prevalent agent isolated from 9 samples, followed by *E. coli* from 3 samples and *S. epidermidis* from 1 sample.

Cytological examinations revealed all of the samples had <80% neutrophil with a mean value of 93.5% (± 3,8), which demonstrated degenerative changes and the presence of intracellular bacteria (Figure 1). Degenerative changes were seen as swollen nuclei that partially lose their lobulation (karyolysis) and/or rupture of the nuclear membrane (karyohexis) of the cells probably caused by bacterial endotoxins. On the other hand, mean macrophage and lymphocyte values were found to be 6.0% (±3.3) and 0.6% (±0.9), respectively (Table 1). The distribution of clinical mastitis cases according to the months included in the study is given in Figure 2.

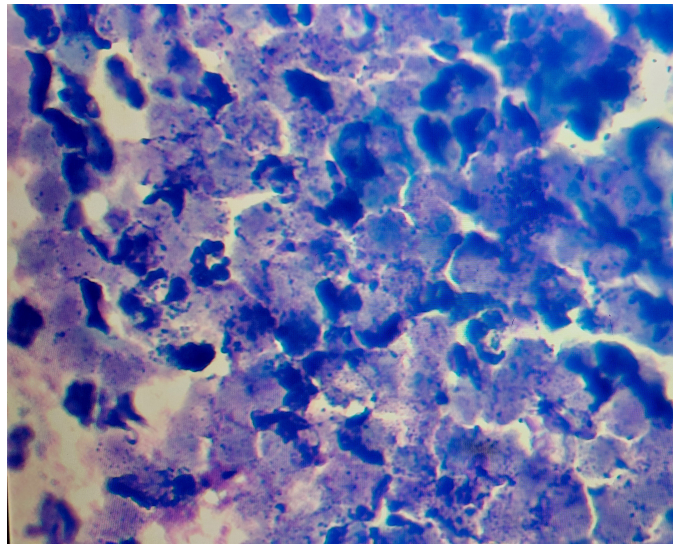

Figure 1. Cytological evoluation of mastitic sample from a mare. Note the NEU cells with degenerative changes and intracellular cocci microorganisms (MGG quick stain, 100x objective).

Table 1. Distribution of Neutrophil (NEU), Macrophage (MAC), and Lymphocyte (LYM) Cells (%) Against Different Bacterial Isolates

Isolate	NEU (Mean ±SD)	MAC (Mean ±SD)	LYM (Mean ±SD)
Gram positive			
Streptococcus. zooepidemicus (n=12)	96.1 (±2.4)	3.9 (±2.1)	0.07 (±0.1)
Staphylococcus aureus (n=1)	92.8	4.6	2.6
Staphylococcus epidermidis (n=1)	84.7	8.2	7.1
Gram negative			
Escherichia coli (n=6)	91.4 (±2.5)	7.8 (±1.5)	1.1 (±1)
Enterobacter cloacae (n=1)	90.2	8.6	1.2
Stenotrophomonas maltophilia (n=1)	88.4	11.4	0.2

DISCUSSION

Mastitis appears to be less prevalent in horses when compared especially with other body site infections. During a 6-year period, only 22 mastitic milk/secretion samples were admitted to our laboratory. On the other hand, 2804 bacterial culture analyses were carried out, which mostly consisted of respiratory system samples during the same time period. The seemingly reduced cases of mastitis in horses can be explained by smaller size and relatively concealed location of the udder, coupled with a smaller storage capacity contributes decreased probability of infection than cows and goats.[12]

A previous study from California demonstrated that 42% of mares were affected by mastitis during the lactation period, another 28% displayed signs within the first 8 weeks of postweaning, and the remaining 30% of the mares were in the non-lactating period.[6] According to the study, in the period of 8 weeks postweaning, drying-off mares were considered to be more likely to suffer from mastitis, which coincides with summer and early fall in the Northern Hemisphere, when insect populations are peaking.[6] Moreover,

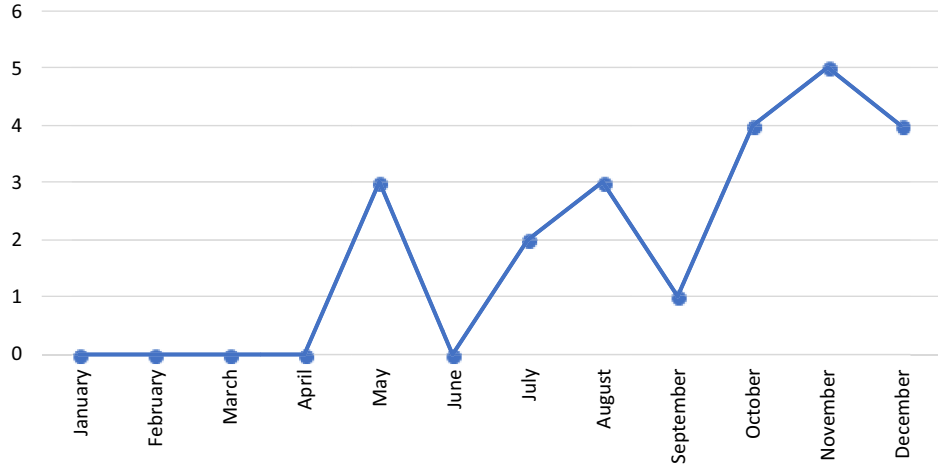

Figure 2. Distribution of clinical mastitis cases according to the months recorded in the study

following the weaning period, udder secretions will accumulate and potentially drip, facilitating the entrance of pathogens to the teat canal.[12] Mares those were presenting mastitis right after parturition usually have a history of dripping milk prefoaling, or may have lost a foal at, or immediately after, parturition.[3] The report from California[6] claimed that 70% of the mares had signs of mastitis from May to September when most mares in reproduction are lactating or have been weaned. In the present study, 40.1% (n = 9) of the mastitis cases were encountered between May and September during lactation period, but 59.1% (n = 13) of the mares were in non-lactating period that coincides October to December. The results of the present study showed to a degree of similarity but mostly revealing that mastitis cases occurred in non-lactating mares. Dry mares may present irregular idiopathic lactation, particularly in autumn, and also improper lactation is encountered in mares with Cushing's disease, possibly due to the secondary increase in blood prolactin level.[13]

In the previous study,[6] the mean age of the affected mares was 13.2 ±6.2 years, ranging from 3 to 24 years old. In the present study, the mean age of the mares was determined to be 10.6 ±3.6 years old that seems ages around 10 years old mares were more likely to suffer from mastitis. But contrary to this, another study demonstrated a broader range of age including a 2-month-old foal and 3 young fillies those were 2-3 years old.[3] The present and previous studies show that mastitis can occur in a broad range of age in mares.

In a previous study, *S. zooepidemicus* was reported to be the most common isolates species (36.8%) in 28 mastitic samples.[6] In the same study, the second most common isolate was determined as *Staphylococcus* spp. and *E. coli* was reported only in 1 case (5.3%), but in the present study, *E. coli* was found to be the second most common agent (27.4%) isolated from clinical mastitis cases. In a different study conducted in Brazil, revealed the most common isolated species were reported as *Streptococcus* spp. (20%) and *S. aureus* (12.73%).[10] *E. cloacae* and *E. coli* were also isolated in 7/55 and 2/55 of the lactating mares, respectively.[10] Böhm et al[7] determined that most bacteria isolated from mastitic samples were also found on the skin of the udder and isolated in the milk of healthy post-partum mares. The present study was in concordance with most of the studies published related to *Streptococcus* spp., especially *S. zooepidemicus* was found to be the most commonly isolated pathogen (54.6%) in the present study. The other agents isolated in the present study were mostly originated from environmental and skin-related bacterial agents. Interestingly, *S. maltophilia* was isolated in 1 mastitic sample in the current study. There were studies reporting *S. maltophilia* isolated from mastitic samples in cows.[14] But no published study or case report could be encountered in mares up to date.

Diagnostic tools widely used in bovine mastitis such as California mastitis test (CMT) have conflicting results when used in mares.[1] CMT is based on somatic cells reacting to a detergent solution a producing a gradable agglutination to the degree of gel formation[15-18]. Waldridge[19] studied CMT and no association was found between CMT results and the presence of clinical or subclinical mastitis and aerobic culture results in mares. Similarly, no association was found between somatic cell count (SCC) and clinical disease.[7,20] In the diagnosis of equine mastitis, it is mostly utilized from clinical signs, culture, and cytology as well.[1] Since the data obtained in previous studies were taken into account, a different diagnostic method like CMT or SCC was not used in the diagnosis of mastitis, other than culture and cytological examination in the current study. Cytology results revealed a strong neutrophil response in clinical mastitic milk samples with bacterial etiology in the present study. Mc Cue and Wilson[6] determined large numbers of neutrophils in the cytologic evaluation of 18 milk samples indicating acute inflammation in 13 (72.2%) and bacteria in 6 (33.3%). Domańska et al[11] also revealed that values of neutrophil, macrophage, and lymphocyte were higher initially than in consecutive examined days after parturition until weaning in non-clinical mastitic mares. After weaning, neutrophil, macrophage, lymphocyte, and bacteriological index in milk increased and did not differ from the mean values in clinical mastitic mares. The results of the present and previous studies indicated that an inflammatory cell response mostly indicating an increase in neutrophil reaching and passing 80% occurs in clinical and non-clinical mare mastitis cases.

In conclusion, to the best of the author's knowledge, the present study is the first that presented a microbiological and cytological evaluation of clinical mastitis cases in mares up to the date in Türkiye. *S. zooepidemicus* was found to be the most common agent followed by *E. coli*. Cytology results yielded strong neutrophilic inflammation and intracellular bacteria with degenerative changes morphologically. More comprehensive studies should be conducted to determine the prevalence of subclinical cases besides clinical cases and also detect antimicrobial resistance profiles of the agents isolated from equine mastitis cases further.

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