Clinical, Microbiological and Hematological Findings in Ovine Subclinical Mastitis

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Abstract
Due to its richness in nutrients, milk represents a complete and balanced food that can be prepared for many other important products in human nutrition. But milk is also a medium of culture for numerous microorganisms. Although milk possesses antimicrobial mechanisms, in many cases it can be contaminated by endogenous or exogenous sources. This microorganism contamination of sheep milk and finished products depreciate its qualitative characteristics and produce severe food poisoning to consumers. In this study we aimed a bacteriological control of five samples of milk haphazardly collected from six particular flocks of sheep in the western part of Romania. In parallel a clinical examination of the animal was carried out, insisting on mammary gland and examination of blood samples collected from the same animals. In laboratory, a bacteriological examination of milk samples and leukogram on blood samples was performed. The identified bacterial species in milk samples were *Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus uberis, Enterococcus durans, Enterococcus faecium.* Although it started from the idea that the animals were healthy, subclinical mammary gland infections was still identified in more than half of the subjects examined.

Key words: *blood samples, clinical examination, mammary gland infection, microorganism.*

1. Introduction

The inflammation of mammary gland, named mastitis can be many times subclinical form of evolution, caused by physical or chemical agents, in majority of cases being caused by bacteria invasion. Mastitis in small ruminants in subclinical form is estimated with an annual prevalence of 5-30% [1,2]. Milk obtained from animals with subclinical mastitis is not altered and the taste is apparently normal. Subclinical mastitis occurs when a mastitis pathogen infects one or both udder halves but does not cause enough disruption of secretory tissue to result in visibly abnormal milk. In these circumstances, the immune system of the animal responds to the bacterial invasion by sending white blood cells to the inflamed mammary gland. The migration of inflammatory cells to the affected gland is in response to bacterial infection but because the inflammatory cells are part of the immune response and are active in engulfing and destroying bacteria, pathogens are not always present in the milk in detectable quantities. Mastitis causing bacteria are often categorized as "*contagious*" if the source is thought to be infected the milk that came from a gland infected with subclinical mastitis pathogens or "*environmental*" if the bacteria are considered as opportunistic pathogens that normally reside in the environment of the animals. Ewes that are affected with subclinical mastitis produce milk that appears visually identical to milk produced from healthy ewes, but the milk is produced from glands that

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have been damaged by bacteria and thus produce less quantities of lower quality milk. Outbreaks of mastitis can occur in ewes housed lambing, probably due to contamination of bedding from udder secretions. Almost any bacteria can theoretically cause mastitis but several groups of pathogens are commonly obtained from milk samples of affected ewes. The incidence of intramammary infection in dairy ewes is typically greatest in early lactation and ewes may be subclinically infected in the immediate postpartum period, but apparently healthy at later periods. The prevalence of mastitis increases with the age of ewe and with the number and weight of lambs. Also, the traumatic lesions caused by vigorous suckling lambs may conduce to mastitis. There are many factors that predispose to mastitis. Lactating females with more lambs or those who produce more milk are most exposed to infection of the udder. Like the elderly, as well as females with poor conformation of the udder were more likely to be affected by mastitis. Also milking procedures can have a large impact on the udder health and somatic cell number. There is also a genetic component to mastitis and udder conformation since it is a hereditary trait. However, females with poor conformation of mammary gland should be removed from the herd. Subclinical mastitis affects the quality and milk production, even lamb mortality [3]. Mastitis has a major impact both in animal welfare and in economy of sheep production. Contamination of the milk can be achieved with microorganisms from extremes, thus at the exit of the mammary gland is hardly a sterile milk. Almost always there are ordinary germs in mammary gland contaminating milk during milking. Microorganisms that come from healthy mammary gland are generally in small number, at milking: tens or hundreds, rarely thousands per ml. This is happen in the case when milking is due in aseptic conditions. When the number of germs in milk is very high during milking time, it comes to mammary gland infections consists of typical species that cause such diseases: staphylococci and streptococci, in most cases. There are perfectly healthy animals which still give milk from milking with large numbers of microorganisms: 10,000 germs/mililiter. This happens because of the broad and permanent apertures of the nipples, their sphincter being relaxed all the time. It's hard to know the measure of openness, but sphincters can easily appreciate by milking speed. As the jet milk is thicker the nipple has greater openness, and milk contains a greater number of germs. Due to anatomic constitution of nipples, microbial milk varies from ewe to ewe in the same farm, although they are all healthy and exploited under the same conditions of hygiene and feeding. The probability of subclinical mastitis occurrence and milk contamination with microorganisms outside the mammary gland is higher in the stables atmosphere where it roosts or cleaning animals contains many microorganisms involved in dust flux of straw bedding, feed, faeces and vehicles. Another way of contamination is the animal from which milk is obtained [4,5,6]. From this large amounts of microorganisms go into the milk especially during hand milking if vessels are wide open. This contamination can be reduced by keeping the animal body in a clean state and tying their tails during milking. The personnel that perform the milking, with dirty clothing and hands or naso-pharynx swelling, represent additional source of milk contamination. Contamination coming from employees milking the ewes, especially those with various diseases is much more dangerous because it often involves pathogens transmissible to humans through milk. Another way of milk contamination are the objects used for milking, including those used for mechanical milking, a major source of contamination. The importance of this contamination is the first in its massiveness, slack in hygiene, and secondly, the fact that it is done in most micro-organisms adapted in logarithmic phase reproduction, resume their activity as soon as they arrive in milk [7].

2. Materials and methods

Bacteriological and haematological analyses were performed on milk and blood samples haphazardly collected from five Merinos of Transylvania and Ţurcană ewes, 2-5 years of age, from six particular flocks of the western part of Romania, in April-June 2015. The animals were kept under extensive conditions, with at least 8 hours per day of grazing in natural pastures, with diet supplementations with alfalfa hay and commercial concentrates. The sheep had no contact with other animal species except male donkeys and shepherd

dogs. The ewes were housed in barns during the night, and the hygiene conditions of the barns and the welfare of the animals were considered up to standard in all six studied flocks. The ewes were hand milked twice daily by the same shepherds. The hygiene conditions for milking were not adequate. The shepherds never used gloves for milking but wash their hands before milking. No specific control measures against mastitis were taken. Milk from the studied ewes was collected from the mammary glands in sterile vials after disinfecting the teat and kept under refrigeration during transportation to the laboratory for microbiological analysis. For microbiological determination, ten millilitres from each sample was mixed with 90 ml of saline water to prepare the initial dilution which was used like mother dilution for further serial dilutions for standard plate counting (SPC). Serial dilutions (10^{-1} to 10^{-3}) the samples in saline water were plated on WL-nutrient agar (Merck Germany). The plates were then incubated at 30˚C for 48 hours [8]. After 48 hours the bacterial colonies or colony-forming units (CFU) were counted using the Nitech LKB 2002. From each milk sample a sub-sample was inoculated on the surface of blood agar containing 5% of washed sheep red blood cells and MacConkey agar plates. All plates were incubated aerobically at 37°C and examined for growth after 24 h. The presence of six or more bacterial colonies of the same type on the medium was considered to be significant and the samples were recorded as positive. Bacteria were identified by using colony morphology, haemolytic pattern on blood agar media and further microscopic examination (Gram staining), standard biochemical methods (catalase, haemolysis, coagulase test [8]. Also milk samples were cultured in special culture media to identify bacterial species: Manitol salt agar (MSA) medium, eosin methylene blue (EMB) also named Levine, enterococcus selective agar (Enterococcosel Agar), agar SS and MRS agar.

Blood samples were taken from the jugular vein and collected into ethylene-diamine-tetraacetic-acid (EDTA) and heparinised tubes (Vacutainer®, Becton Dickson). These samples were used for haematology. Total white blood cell (WBC) counts were carried out directly in the haemocytometer (haemocytometric method) and leukocytic formula on smears coloured through May-Grünwald-Giemsa method. Each animal was clinical examined on each half-udder within 1 h after milking, and included a general inspection, palpation, evaluation of half-udder consistency and supramammary lymph nodes. Presence or absence of lacteal cysts was observed. During clinical examination, presence of inflammations on known three phases (*rubor*, *calor*, *dolor*) have been also evaluated [9]. Nodules, abscesses, and lacteal cysts were differentiated as follows: a nodule was defined as a relatively hard, roughly spherical, often painless, abnormal structure; an abscess was defined as a collection of pus and infected material in or on the skin, that has accumulated in a cavity formed by the tissue as a result of an infectious process; a lacteal cyst was defined as a retented subcutaneous cyst in the mammary gland resulting from closure of a lactiferous duct. Consistency of udder after palpation was classified as: normal, oedematous, sclerotic, or atrophic [10].

3. Results and discussion

In Table 1 are presented the results of colony-forming units (CFU) or aerobic plate counting from the all 30 milk samples collected from six sheep flocks. In manitol salt agar (MSA) medium *Staphylococcus aureus* produced yellow colonies with yellow zones, whereas *Staphylococcus epidermidis* produced pink colonies. In MRS agar and Levin no bacteria colonies developed, while in enterococcus selective agar (Enterococcosel Agar) *Enterococcus durans* and *Enterococcus faecium* was developed.

In Table 2 the results of clinical examination are shown. From a number of 30 examined ewes, 11 were detected with clinical signs of mastitis. These clinical signs were observed after attentively examination and consisted in mild inflammation of supramammary lymph nodes and disseminated small nodules in mammary gland. A slight increase of temperature and sensitivity of mammary gland without affecting the general condition of examined animals was observed. These symptoms have been observed in primary stage of udder inflammation, following in short time to become clinically visible, with general condition affected, when ewes refuse to be milked.

In Table 3 the results of leukocyte formula on blood samples collected from studied animals are shown. A decrease of leukocytes, neutrophils,

eosinophils, lymphocytes and monocytes values was observed while basophiles remained approximately to appropriate values.

Table 1. The results of CFU/ml counting

Sample	Flock I	Flock II	Flock III	Flock IV	Flock V	Flock VI
1	500-2000	500-2000	100-500	50-100	100-500	500-2000
2	500-2000	500-2000	100-500	100-500	500-2000	100-500
3	100-500	100-500	100-500	100-500	500-2000	100-500
4	100-500	500-2000	100-500	100-500	500-2000	500-2000
5	100-500	100-500	100-500	100-500	500-2000	500-2000

Table 2. The results of clinical examination

Sample	Flock I	Flock II	Flock III	Flock IV	Flock V	Flock VI
1	+	+	-	-	-	+
2	+	+	-	-	+	-
3	-	-	-	-	+	-
4	-	+	-	-	+	+
5	-	-	-	-	+	+

Table 3. The results of leucocytic formula

Item	Flock I (n=5)	Flock II (n=5)	Flock III (n=5)	Flock IV (n=5)	Flock V (n=5)	Flock VI (n=5)	Normal [9] (Falcă,2000)
L(thousands/mm^3)	4.6±0.3	4.3±1.2	7.9±4.7	7.8±3.9	2.1±1.1	5±0.5	8±4
Neutrophils (%)	25.2±0.6	17.9±6	30.2±13	29.9±13.9	14.4±6	23.8±9.8	31±15
Eosinophils (%)	4.2±1.1	3.1±0.3	4.7±6.1	5.1±3.9	1.7±0.7	2.9±6	5±5
Basophils (%)	1.6±0.1	1.4±1.6	1.6±0.08	1.6±0.9	1.4±1.7	1.4±1.8	1.5±1.5
Lymphocyte s(%)	49±3.8	44.8±5	57.7±9,8	58±11.8	23.3±1.3	41.7±5	60±10
Monocytes (%)	2.4±1.7	2.1±1.1	2.9±2.3	2.7±1.3	1.2±0.7	2.2±0.1	3±3

L=Leucocytes

Considering that samples of milk from the sheep in six flocks were collected direct from mammary glands in sterile conditions, after disinfecting the teat and kept under refrigeration during transportation to the laboratory, don't enter into the discussion the subsequent contamination, to the ways it were presented in the introduction of this paper. The maximal values of colony-forming units (CFU) were obtained in flock V, with 500-2000 colonies/millilitre, followed by flocks II, VI and I. Sanitary veterinary actual norm [11] for raw milk of goats, sheep and buffalos must meet the following standards: if it is intended for the manufacture of heat-treated milk for human consumption or for the manufacture of milk-based products heat treated total bacteria count in plates at 30˚C /ml must be ≤ 1.500.000[1]. If it is intended for the manufacture of products derived from raw milk "through a process which does not involve any heat treatment "the standard total bacteria count in plates at 30˚C /ml should be ≤ 500.000[1]. Milk provided by flocks I, II, V and VI does not fit within the limits stipulated by the sanitary veterinary norm, so it can be considered mastitic milk. The isolation from studied milk samples of *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus durans* and *Enterococcus faecium* represents a serious problem because in short time these infected animals will register a decreasing milk production, with local inflammations, alteration of general state of health, and if will not be affected all implicated ewes can die. This subclinical mastitis becomes a serious economic problem for sheep breeders and this milk will represent for consumers a serious health risk [12]. European research in Mediterranean countries has indicated that most of the variation in mastitis is associated with differences in herd management. On the other hand, higher producing breeds were at greater risk of mastitis and the use of dry off treatment resulted in less mastitis. Mastitis in milking sheep is usually caused by bacteria that live on skin, and it is sensible to conclude that practices that reduce exposure of teat ends to bacteria should result in reduced prevalence of mastitis [13]. Pastures and shelters for ewes, control of milking equipment, the disinfection of teats before and after milking with commercially products and an improvement of nutritional

management must become measures applied in every sheep farm for avoiding the appearance of mastitis. An interesting study made in Spain emphasized the connection between clinical mastitis and to selenium deficiency. These researchers observed that a deficient diet in selenium evolved an incidence of 15.4% clinical mastitis, while treated ewes with barium selenite evolved an incidence of mastitis of 3.8% [13]. In U.S.A. clinical mastitis typically occurs in less 5% of lactating ewes but subclinical mastitis may occur in up to 15-30% of animals [14].

4. Conclusions

Subclinical sheep mastitis is very important for breeders because reduces production efficiency and farm profitability, attracting significant economic loss. In the condition of the fact that Romania is on the fifth place in Europe in terms of the number of sheep, understanding and preventing mastitis is essential to achieving successful management of sheep farms.

From a number of 30 samples of milk collected from six sheep farms, 12 were contaminated with a number of bacteria beyond the limits permitted by sanitary veterinary actual norm, being considered mastitic milk, with the isolation of *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus durans* and *Enterococcus faecium.* Clinical examination emphasized symptoms of primary stage of udder inflammation. The identified subclinical mastitis was correlated with changes of leukocytic formula, with decreasing of leukocytes, neutrophils, eosinophils, lymphocytes and monocytes values while basophils remained approximately to appropriate values.

The milk obtained from animals with subclinical mastitis represents a serious health risk for consumers. In these conditions the microclimate, the hygiene of milking and as well as nutritional management must be improved.

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