

**Subclinical mastitis survey on milk composition in dairy sheep in
Kurdistan region of Iraq**

ABSTRACT:

Mastitis is an inflammatory condition of the mammary gland, characterized by the changes in the physical characteristics of the udder or milk. A cross sectional study was carried out in June, 2017 to December, 2018 to estimate the effect of subclinical mastitis (SCM) on milk composition in dairy sheeps in Kafri city of Kurdistan region of Iraq. Milk samples were gathered from residences of 295 sheeps with subclinical mastitis (California mastitis test (CMT) positive and somatic cell counts (SCC) >600,000 cells/ml in individual quarter foremilk), as well as from 50 healthy controls. Contrasted to the levels watched in milk from healthy quarters, milk from quarters with subclinical mastitis exhibited raised high chloride (>0.12 in contrast with <0.12 g/dl), pH (5.65 in comparison to 5.57), sodium (86.87 vs 47.81 mg/dl), albumin (4.52 in contrast with 1.75 g/dl), immunoglobulins (24.66% in comparison to 5.73%) and lactate dehydrogenase (LDH) activity (1344.14 vs 449.84 IU/L). In compare, reduced values were discovered for potassium (147.47 in comparison to 161.34 mg/dl), inorganic phosphorous (19.42 in comparison to 26.48 mg/dl), calcium (86.35 vs 121.12 mg/dl), β -lactoglobulin (30.22% in comparison to 52.18%) and α -lactalbumin (19.15% vs 24.52%). In this study, no changes were seen in blood serum LDH activity. Moreover, an increase in positive response to CMT was found to be accompanied by an almost proportionate increase in immunoglobulin values to 44.32% and reduce of α -lactalbumin levels in milk serum ($p < 0.01$). These alterations in LDH activity, pH, mineral concentrations and protein fractions in milk of quarters display the presence of tissue injury provoked by SCM. Therefore, these parameters can be used in the diagnosis of mastitis. The current study revealed that changes of the foremilk chemical composition are connected to

28 the subclinical mastitis; and that mastitis progression of quarters (CMT scores) influenced protein
29 fractions in milk.

30

31 **Keywords:** Composition , CMT, Kafri, Kurdistan, Iraq, Milk, Subclinical mastitis

32

33 **1. INTRODUCTION:**

34 Mastitis is the single most costly disease of dairy animals. Although large technological advances
35 in the prevention and treatment of mastitis have been made in recent years, mastitis continues to
36 cause major economic losses in dairy industry [1, 2]. This disease is usually connected with
37 physical and chemical abnormalities of milk and udder through which it can be grouped into
38 clinical or subclinical [3]. The gold standard diagnostic tool in both clinical and subclinical
39 mastitis is the identification of the causative agent by culture [4,5]. Anyway, California mastitis
40 test, somatic cells count (SCC), and changes in milk constituents are other important tools for
41 detection subclinical mastitis in bovine [6] and these tests may be used for ovine mastitis
42 detection. In addition, Mastitis is an important problem causing very large economic losses in
43 dairy industry throughout the world [7]. Many of the intramammary infections (IMI) originate
44 during the dry or non lactating period and result in clinical or subclinical mastitis during early
45 lactation [8,9]. Subclinically infected udder quarters can improve clinical mastitis and the rate of
46 new infections can be high [11]. Dairy sheep produce about 12.2 million metric tons (MT) of
47 milk, accounting for about 1.5% of the world total amount of milk produced by livestock species,
48 the largest amount of sheep milk is produced in India, followed by Iraq and Sudan [12]. The
49 dairy sheep industry is quickly gaining in importance throughout the world in new years. Among
50 the several problems hindering the livestock development in Iraq, sanitary problems constitute a
51 serious threat to the successful production of livestock and its industry. Hence, any factor that
52 adversely affects the quantity and quality of cattle and goat milk is of sheep financial interest.
53 Milk quality is mainly influenced by bacterial contamination of the mammary gland, which
54 causes clinical or subclinical mastitis [13]. Mastitis is described as an inflammation of the
55 mammary gland, affects lactating animals including sheep, goats, cattle, buffaloes and camels
56 and is almost always caused by bacterial infection. Mastitis in sheeps is mainly subclinical

57 [14,15,16]. It is one of the serious problems of the dairy industry worldwide including Iraq.
58 Subclinical mastitis is 10 to 35 times more common than the clinical form, is of long duration and
59 difficult to discover [17, 18]. In Bangladesh, the prevalence of SCM is recorded from 20 to 44%
60 at cow level based on California Mastitis Test (CMT) [19, 20]. The efficacy of antibiotic therapy
61 for intramammary infections (IMIs) early in lactation is rare and Slight, with the ones carried out
62 reporting mixed results. The response to therapy with intramammary (IMM) cephalosporin sodium
63 on CMT positive quarters in lactating sheep on cure rates and somatic cell count [21]. It was
64 determined that by the 4-week post-calving evaluation, quarters treated with cephalosporin sodium
65 had significantly increased cure rates, and SCC were significantly decreased.
66 Lessening the exposure of the udder to potential pathogens and/or increasing the immune
67 response of dairy animals against infection remain some of the most effective mastitis control
68 measures today [18]. There have been some research studies that proved the effectiveness of
69 vaccination programs with a different combination of agents against mastitis in dairy sheep and
70 cattle [22, 23]. Unluckily, most of the mastitis vaccines are only labeled for dairy sheep. Early
71 identification of udder health problems is necessary for dairy farmers and veterinarians to ensure
72 not only the animal well-being but also the milk quality and dairying productivity. Economic
73 aspects interfere with the routine application of bacteriologic test of quarter milk samples. For
74 this reason, alternative parameters are used to identify trends in the development of the udder
75 health in a dairy herd, despite the fact that these parameters show inflammation. The aim of this
76 study was to investigate the effects of relationship between a set of chemical parameters
77 including pH, mineral concentrations, lactate dehydrogenase (LDH) activity and protein fractions
78 and subclinical mastitis occurred naturally on dairy sheep.

79

80 **2. MATERIALS AND METHODS**

81 **2.1. Study area**

82 The effects of subclinical mastitis on milk composition, was done at three dairy herds located in
83 some villages in Kafri city, Kurdistan region of Iraq. All the laboratory investigations were
84 conducted at the Biology Laboratory of College of Agriculture - Kifri, Garmian University,
85 Kalar, As Sulaymaniyah, KRG of Iraq. The study was conducted for the period from June, 2017
86 to December, 2018.

87 Sheeps were in the second to fifth lactation and were milked twice daily by hand milking. They
88 were fed ad libitum by a total mixed diet that had been formulated to meet the nutritional
89 requirements of a 350-kg sheep, yielding 10–18 kg of milk/d with about 1.2% protein and 1.1%
90 milk fat. All sheeps were subjected to post dipping , those were dried off nearly two months
91 before anticipated calving and all mammary glands of sheeps were infused with an antibiotic
92 preparation for use in non-lactating sheeps following the last milking of lactation.

93

94 **2.2.Milk sampling and milk component analysis**

95 Milk samples were collected from quarters of 295 sheeps with subclinical mastitis (SCM), as
96 well as from 50 healthy controls just before morning milking. Teats were scrubbed
97 comprehensively and dried with a single use paper towel. The first three flows of milk from each
98 teat were discarded. The teat end and aperture was disinfected with cotton swabs drenched in
99 90% ethyl alcohol and nearly 8 ml foremilk sample were gathered from each quarter of sheep in a
100 sterile tube held horizontally.

101

102 **2.3.California Mastitis Test (CMT)**

103 The experimental material was divided into four groups according to the California mastitis test
104 (CMT) results—0 = negative or trace, 1 = weak positive, 2 = distinct positive and 3 = strong
105 positive—obtained from the test performed directly in the herds, using the method described by
106 Schrick *et al.* [11]. Blood samples were also gathered from jugular vein for the LDH assay.
107 Samples were right away placed in crushed ice and submitted to the laboratory analysis within 3–
108 5 hrs. To diagnosis of SCM, the total somatic cell count of milk was decided, using Breed's
109 smudges with Newman's stain and leukocyte count more than 600,000 cells/ml of individual
110 quarter milk was taken as a positive index of mastitis [6]. In all other cases, the samples were
111 considered uninfected (healthy). All milk and blood samples were tested at midlactation and none
112 of the ewes were sampled twice in the study.

113 Milk serum (whey) was readied at a two-step centrifugation procedure. At first, milk samples
114 were centrifuged at 5000 rpm for 15 min to remove their creams and cells. Samples were then
115 treated with 0.2 M hydrochloric acid at the controlled pH of 3.5 for casein precipitation. Treated
116 samples were recentrifuged and the supernatants (whey) were gathered. The pH of milk samples
117 was determined electrometrically. Total calcium and phosphorous concentrations were

118 determined using by colorimetric method, a hand-held spectrophotometer by commercial kits
119 based on cresolphthalein complexation and phosphomolybdic acid complex formation, at
120 wavelengths of 500 and 310 nm, respectively. Albumin was determined by bromocresol green
121 method, using commercial kit at wavelength of 546 nm; chloride based on rapid spot test using K
122 chromate and sodium and potassium by flame photometer; and silver nitrate (observation of
123 yellow colour, >0.15 g/dl and brownish colour less than that amount) [9]. LDH activity was
124 determined by spectrophotometer, using commercial kit by the method of Siddiquee et al. [10] at
125 wavelength of 320 nm. Protein fractionation of milk was segregated according to molecular mass
126 by cellulose acetate membrane electrophoresis (Sebia preference, France) at 90 V for 20 min and
127 barbital buffer; pH = 6.8. After fractionation, membranes were stained with fixative dye solution
128 (4.5% trichloroacetic acid, 0.4% Ponceau red, 97.5% double distilled water) at 10 min and then
129 decolorized and purified. After drying, the relative levels of proteins were determined using
130 densitometry at wavelength of 430 nm.

131

132 **2.4. Somatic Cell Count (SCC) determination**

133 Milk samples for SCC determination were gathered before vaccine administration (T0) and on
134 days 30 and 32 of the experiment. SCC was determined using spreading 0.03 ml of gently blend
135 milk from each sample over 2 cm² area of a glass slide and staining by Newman-Lampert stain.
136 The stained slides were then tested by the same technician every time by light microscope
137 according to previously published procedure [24]. SCC was expressed in log₃.

138

139 **2.5. Statistical analysis of the experimental data**

140 The software of SPSS [25] was used of data analysis. Student's t-test was carried out to find the
141 differences between the results of mastitic, non-mastitic milk and serum. The changes in the
142 content of protein fractions in milk with different positive CMT scores were appraised by one-
143 way analysis of variance (ANOVA) followed by Duncan's multiple range test. The results were
144 given as mean \pm SEM. A repeated measures ANOVA test was used to estimation milk
145 composition variables over different sampling points in vaccinated and non-vaccinated normal
146 ewes. $p < 0.05$ was measured statistically significant.

147

148 **3. RESULTS**

149 Present study was done in order to investigate the effects of relationship between a set of
150 chemical parameters including pH, mineral concentrations, lactate dehydrogenase (LDH)
151 activity, protein fractions and subclinical mastitis occurred naturally on dairy sheep. The results
152 of Table 1 showed that the concentrations of potassium, phosphorous and calcium were
153 significantly lower in the milk of inflamed (SCM) mammary glands than those of normal glands
154 ($p<0.01$).

155 The concentrations of albumin, chloride and sodium were significantly higher in the milk of
156 inflamed mammary glands than those in normal ones ($p<0.01$). The pH was considerably higher
157 in the subclinical mastitic milk than in the normal ones ($p<0.01$)

158

159 **Emplacement of Table 1**

160

161 The LDH activities of milk and blood serum samples of normal animals and animals affected by
162 subclinical mastitic were presented in Table 2. The mean LDH activity was considerably higher
163 in milk from inflamed (SCM) quarters than in normal milk ($p<0.01$). No significant difference
164 was observed in LDH serum values.

165

166 **Emplacement of Table 2**

167

168 The contents of protein fractions were contingent upon the CMT progression. Statistically
169 significant ($P<0.01$) influence of high mastitis progression on the increase in milk
170 immunoglobulin values to 35.20% was detected. Milks obtained from highly inflamed glands
171 (milk samples with high score in CMT) had significantly ($p<0.01$) lower albumin and pre-
172 albumin and α -lactalbumin, but the content of β -lactoglobulin in milk was comparable between
173 quarters with different CMT scores (Table 3).

174

175

176 **Emplacement of Table 3**

177 The Table 4 shows that the concentrations of protein fractions were significantly different
178 between normal and SCM milk ($p<0.01$). SCM caused increment in the immunoglobulin and

179 albumin content in milk. While, β - lactoglobulin, α -lactalbumin and pre-albumin content in SCM
180 milk was reduced relationship normal milk.

181

182 **Emplacement of Table 4**

183

184 **4. DISCUSSION**

185 Mastitis is an important problem causing very large economic losses in dairy industry throughout
186 the world [1, 2]. Many of the intramammary infections (IMI) are created during the dry or non
187 lactating period and result in clinical or subclinical mastitis during early lactation. Swelling of the
188 mammary gland leads to a diversity of compositional changes in milk either because of local
189 results or because of serum components entering the milk and the movement of some normal
190 milk components out of the alveolar lumen into the perivascular space [4]. Hypothetically, all
191 changes in mammary discharge during swelling might be used to measure the effects of mastitis,
192 but problems of instrumentation and standardisation have hampered farm application of most
193 examinations. Albumin content of milk in subclinical mastitis was meaningfully increased
194 compared to the healthy ones. The increase of albumin content in milk during mastitis has been
195 reported in goats [26, 27], sheep [28] and goats [2]. Although, it be usual think that the main site
196 of albumin synthesis is in the liver, and that the albumin enters the milk by leaking through the
197 epithelial tight junction from the blood stream [18], the extrahepatic synthesis of albumin has
198 been exhibited in mammary gland epithelial cells, albeit lesser than the liver [8]. The noticeable
199 increases of albumin in mastitic animals propose that a great source of the increase in the content
200 of albumin in milk under inflammatory situations is the inflamed gland itself. Our findings shows
201 that tissue disturbances of the mammary gland in subclinical mastitis were accompanied by
202 significant increase of LDH activity in the milk , but without obvious influence on enzyme levels
203 in blood serum. Higher LDH activity in milk serum of inflamed udders has been previously
204 reported in goats [10, 7] and cows [11]. The higher level of LDH in mastitic milks than in the
205 blood serum reveals that blood serum was not the sole source of this enzyme during mastitis
206 cases and that it is probably also liberated from disintegrated leukocytes and the parenchymal
207 cells of the udder [27]. The pH of SCM milk was higher than that of normal milk, which is
208 agreement with the results of earlier reports [2]. The circuitous pH testing can be measured as a
209 guide to detect the subclinical mastitis as this is economical, comfortably and rapid. It can be

210 done in the field at the time of milk collection. Later determining pH, the positive samples can be
211 checked to isolate the causative organism for further confirmation of SCM. Mastitis also
212 noticeably changed the ionic environment. Chloride and sodium are increased while potassium,
213 normally the predominant mineral in milk, is decreased. These increases in chloride and sodium
214 and reduce in potassium levels have been verified by other authors as methods of monitoring
215 udder health [8, 27]. Intramammary infection results in injury to the ductal and secretory
216 epithelium, an opening of the “tight junctions” between secretory cells, and the increased
217 permeability of the blood capillaries. Thus, chloride and sodium pour into the lumen of the
218 alveolus and, in order to keep osmolarity, potassium levels reduce relatively. The levels of
219 phosphorous and calcium is also influenced by mastitis. The reduction in phosphorous and
220 calcium levels in the case of intramammary infections have been previously reported [16, 18].
221 The current study showed that the types of proteins present in all of the milking fractions from
222 quarters with subclinical mastitis undergo dramatic changes. Quarters with SCM revealed higher
223 immunoglobulins and lower lactalbumin than did the corresponding milking fractions taken from
224 healthy ones. The increased proportion of immunoglobulins connect to inflammatory responses
225 of the udder compensated for the significantly lower proportion of lactalbumin. Actually, there is
226 a near balance between this reduce and increase. Changes in protein fractions of milk acquired
227 from mastitic sheep have been documented in previously studies [1, 22]. Immunoglobulins in
228 mammary discharges are serum-derived or produced in the udder and pass into the milk through
229 the mammary epithelium. The concentrations of immunoglobulins in normal milk are low and
230 depend on the degree of vascular permeability of the udder tissues. When this penetrability
231 barrier is broken during inflammation, immunoglobulin concentrations increase in discharges
232 from infected glands. The immunoglobulin has several important functions. They are believed to
233 prevent bacterial adherence to inhibit multiplication in epithelial membranes, agglutinate bacteria
234 and neutralize toxins. Also, an important function of immunoglobulins is opsonization of
235 microorganisms for phagocytosis. The increase in milk immunoglobulins may be effective in
236 decreasing severity of mastitis [4]. Specific proteins are greatly synthesized in the mammary
237 gland. This reduce in α - lactalbumin connect to SCM could be due to the decreased synthetic
238 activity of mammary gland. Some studies propose that α -lactalbumin may leak out of the
239 alveolus between epithelial cells; this component has been calculated in urine or blood of sheeps
240 with mastitis [28]. β -lactoglobulin and α - lactalbumin have physiological properties of whey

241 proteins involving immunoenhancing effects. The possible role of α -lactalbumin as an antitumour
242 agent is being investigated [10].

243

244 **Conclusion**

245 The results of study showed that these alterations in LDH activity, pH, mineral concentrations
246 and protein fractions in milk of quarters display the presence of tissue injury provoked by SCM.
247 Therefore, these parameters can be used in the diagnosis of mastitis. The current study revealed
248 that changes of the foremilk chemical composition are connected to the subclinical mastitis; and
249 that mastitis progression of quarters (CMT scores) influenced protein fractions in milk.

250

251 **COMPETING INTERESTS**

252 Author has declared that no competing interests exist

253

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332 Table 1: Comparisons of pH, albumin and minerals in milk of normal and milk of mammary
333 glands with subclinical mastitis

Parameters	SCM milk	Normal milk
pH	5.65 ± 0.08	5.57 ± 0.01*
Albumin (mg/dl)	4.52 ± 0.1	1.75 ± 0.02*
Chloride(mg/dl)	<0.12*	>0.12
Potassium(mg/dl)	147.47 ± 201	449.84 ± 1.1*
Sodium(mg/dl)	86.87 ± 4.1	47.81 ± 1.1*
Calcium(mg/dl)	86.35 ± 1.1	121.12 ± 0.6*
Phosphorous(mg/dl)	19.42 ± 0.2	26.48 ± 0.2*

334

335

336 Table 2: : Comparisons of LDH in milk and blood sera of normal and infect animals (SCM)

	SCM milk	Normal milk	SCM serum	Normal serum
LDH (IU/L)	1340 ± 110.1	280.1 ± 11.3*	601.1 ± 18.14	640.2 ± 25.1

337

338

339 Table 3: Comparisons of milk albumin, pre-albumin, immunoglobulin, β-lactoglobulin and α-
340 lactalbumin according different scores in positive CMT

	+++	++	+
Albumin	5.1 ± 1.2 ^c	15.2 ± 1.3 ^b	25.1.3 ^a
Pre-albumin	0.1 ± 0.12 ^a	0.05 ± 0.2 ^b	0.1 ± 0.04 ^a
Immunoglobulin	45.1 ± 1.1 ^c	17.12 ± 0.32 ^b	9.1 ± 0.21 ^a
β-Lactoglobulin	32.1 ± 1.2 ^a	33 ± 1.01 ^a	28.1 ± 1.01 ^a
α-Lactalbumin	10.3 ± 1.03 ^c	22.1 ± 1.1 ^b	26.1 ± 0.6 ^a

341

342

343 Table 4: Comparisons of albumin, pre-albumin, immunoglobulin, β -lactoglobulin and α -

344 lactalbumin in milk of normal or mastitic mammary glands (SCM milk)

345

	SCM milk	Normal milk
Albumin	15.2 \pm 1.1	5.3 \pm 43 *
α -Lactalbumin	19.15 \pm 0.54	24.52 \pm 0.4*
β -Lactoglobulin	30.22 \pm 1.1	52.18 \pm 0.5*
Pre-albumin	0.06 \pm 0.3	0.15 \pm 0.02*
Immunoglobulin	24.66 \pm 0.52	5.73 \pm 0.22*

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UNDER PEER REVIEW