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Original

Investigation of biochemical parameters and cytokine profile in sheep with contagious ecthyma

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ABSTRACT

Objective. This study investigated the serum biochemistry parameters and interleukins (IL-2, IL-4) in sheep naturally infected with infectious ecthyma, identified the immune types formed, and revealed the direction of the dominant cytokine response. **Materials and methods.** 28 unvaccinated sheep aged 1-4 months showing clinical symptoms of ecthyma and 10 healthy sheep in the same age range were used. Measurements were taken of albumin (ALB), alanine aminotransferase (ALT), urea nitrogen (BUN), cholesterol (CHOL), creatinine (CRE), gamma-glutamyl transpeptidase (GGT), glucose (GLU), total bilirubin (TBIL) and total protein (TP). Enzyme-linked immunosorbent assay (ELISA) was used to determine interleukins levels in the polymerase chain reaction (PCR) positive animal serum. **Results.** The biochemical analysis revealed that ALT, BUN, GGT, and CRE values in infected animals were significantly higher than in the control group (p=0.000 and p= 0.001) whereas TP and GLU values were significantly lower (p=0.000). There were no significant differences in ALB, CHOL, and TBIL values (p=0.1, p=0.05, p=0.08). Regarding the immune profile, infected animals had significantly higher IL-2 (%28) and IL-4 (%60) levels than the control group (p=0.008 and p=0.001). **Conclusions.** The findings indicate that Th1 (IL-2) and Th2 (IL-4) cytokines coexist while the dominant cytokine response in infected animals is Th2.

Keywords: Immunology; interleukin; zoonosis (*Source: AIMS, MeSH*).

RESUMEN

Objetivo. Este estudio investigó los parámetros bioquímicos del suero y las interleucinas (IL-2, IL-4) en ovejas naturalmente infectadas con ectima infeccioso; identificó los tipos inmunes formados y reveló la dirección de la respuesta de citocina dominante. **Materiales y métodos**. Se utilizaron 28 ovejas no vacunadas de 1 a 4 meses de edad que presentaban síntomas clínicos de ectima y 10 ovejas sanas del mismo rango de edad. Se tomaron medidas de albúmina (ALB), alanina

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aminotransferasa (ALT), nitrógeno ureico (BUN), colesterol (CHOL), creatinina (CRE), gamma-glutamil transpeptidasa (GGT), glucosa (GLU), bilirrubina total (TBIL) y proteína total (TP). Se utilizó un ensayo de inmunoabsorción ligado a enzimas (ELISA) para determinar los niveles de interleucinas en el suero de animal positivo para la reacción en cadena de la polimerasa (PCR). **Resultados**. El análisis bioquímico reveló que; los valores de ALT, BUN, GGT y CRE en los animales infectados fueron significativamente más altos que en el grupo de control (p = 0,000 y p = 0,001) mientras que los valores de TP y GLU fueron significativamente más bajos (p = 0,000). No hubo ninguna diferencia significativa en los valores de ALB, CHOL y TBIL (p = 0,1, p = 0,05, p = 0,08). En cuanto al perfil inmune; los animales infectados tenían niveles de IL-2 (% 28) e IL-4 (% 60) significativamente más altos que el grupo de control (p = 0,008 y p = 0,001). **Conclusiones**. Los hallazgos indican que las citocinas Th1 (IL-2) y Th2 (IL-4) coexisten mientras que la respuesta de citocina dominante en los animales infectados es Th2.

Palabras clave: Inmunología; interleucina; zoonosis (*Fuente: AIMS, MeSH*).

INTRODUCTION

The causative agent of the disease known under various names, such as contagious ecthyma, cutaneous pustular dermatitis, orf or scabby mouth is parapoxvirus from the family Poxviridae. The virus has a worldwide widespread and can survive for years in infected environments. The disease, which is usually transmitted by contact, infects the host through the oral mucosa and skin portantelles before multiplying in the epidermal cells. While it can occur in lambs and goat kids 10-12 days after birth, it is more generally seen in animals that are 3-6 months old in enzootic regions (1). It is characterized by gingival lesions adjacent to the tongue and palate, multiple papules, and ulceration covered with yellowish exudate in lambs (2).

The clinical stages of ecthyma lesions are followed by the development erythematous macules, papules, vesicles and pustules finally crusts. Locally found virus scatters with the crust. Although its spread to all organs was clear, similar lesions were noted in the respiratory and gastrointestinal tracts of severely affected lambs and goats (3). The acute and contagious disease, which affects sheep, goats, some domestic and wild ruminants, reduces productivity. It is thus an economically important zoonotic disease that harms national and international trade of animal products by reducing feed intake, fleece, and meat quality (4,5). Morbidity can reach 100% while mortality from secondary bacterial infections can reach 15% (6). The disease can become fatal in lambs if the lesions hinder suckling while accompanied by fungal or bacterial lesions (7). In adult animals, udder lesions make breastfeeding painful, which may cause the to be abandoned (8).

The most important reason for interest in immunologically infectious ecthyma is the infection can sometimes occur repeatedly in the same region despite a normal immune response at the first infection (9). Several studies have established the condition of immune cells in the skin of first-time infected or reinfected sheep. The main cells in infected keratinocytes are neutrophils, B-cells, dendritic cells and T-cells (10,11).

Ecthyma lesions contain abundant dendritic cells. These cells are vital for antigens, stimulation, and maintenance of immunity (12). Despite the host's defense system, these infections are smaller in scale and shorter lasting when the virus re-infects the host. Thus, first-time lesions usually resolve within 4-6 weeks while recurrent infections are less severe and heal in an average of 2-3 weeks. Cytokines secreted from immune cells are important in both primary and reinfection. In particular, Interleukin 2 (IL-2) and IFN-ɣ cytokines play a preventive role during reinfection (3,13). Interleukin 4 (IL-4) production is carried out by T lymphocytes and mast cells. This arranges the growth the development and involution of cells like B and T lymphocytes, and plays a role in immunity against extracellular pathogens and helminths by increasing antibody output and inflammatory responses (14).

The aim of this study was to investigate the serum biochemistry profile, and IL-2 and IL-4

levels in sheep naturally infected with infectious ecthyma, to reveal which responses develop in the immune system and identify the direction of the dominant cytokine response. Although there are few in vivo studies in this area, our study is the first in animals infected with native ecthyma. We think that the findings will contribute to the pathobiology of the disease.

MATERIALS AND METHODS

Animals and study design. The study material consisted of sheep aged between 1-4 months with clinical ecthyma symptoms that had been brought to the Internal Medicine Clinic of the Faculty of Veterinary Medicine of Harran University, Turkey. The study group comprised 28 sheep with macroscopic lesions around the mouth and gingiva while the control group had 10 clinically healthy sheep. Both groups were in the same age range. In the control group, no loss of appetite, lethargy, weakening, sores in the mouth, or other symptoms were detected in any animals in the control group and normal values were found in respiratory rate, heart rate, and body temperature. No animals in either group had been vaccinated against ecthyma.

Collection of samples and biochemistry. Five ml blood was collected from V. jugularis of each sheep in yellow-capped (BD Vacutainer® SST™II Advance) tubes. After the blood samples were centrifuged (Nuve, NF 800R, Turkey) at 3000 rpm/10 minutes, the serum samples were stored at -20°C until analysis. Fujı Drı-Chem NX500i instrument (Fujifilm Corporation®, Tokyo, Japan) was used for serum biochemistry analysis. A comprehensive panel of measurements was made of albumin (ALB), alanine aminotransferase (ALT), urea nitrogen (BUN), cholesterol (CHOL), creatinine (CRE), gamma-glutamyl transpeptidase (GGT), glucose (GLU), total bilirubin (TBIL) and total protein (TP).

DNA extraction and PCR amplification. Samples were taken by scraping from lesions located on the gingiva and lip margins. The crusts were mechanically homogenized with 200 µl phosphate buffer. The homogenized shells were used for extraction. Polymerase chain reaction (PCR) was performed using the primers PPP-1 and PPP-4 targeting the partial region (594 base pairs) of the virus's B2L gene (15). For each sample, the amplification volume was 30 µL while the PCR mix comprised 3 µL of DNA, 0.25 µLTaq DNA polymerase (5 U/1µL, Thermo Scientific, USA), 3 µL10xbuffer (20 mM, Thermo scientific, USA), 2 µL $MgCl_2$ (25 mM, Thermo Scientific, USA), 1 µL of each primer (10 pmol), 1 µL of dNTP (10 mM) solution (Thermo Scientific, USA), and 18.75 µL of ddH_2O. A Biometra TOne Thermal cycler (Analytik Jena, German) device was used for amplification. The obtained PCR products were evaluated by running on a 1% agarose gel stained with EtBr (ethidium bromide).

Evaluation of immune profile. After serum of ecthyma positive animals was thawed at room temperature, interleukin levels (IL-2 and IL-4) were measured using sheep IL-2 ELISA kit (E0029Sh, Bioassay Technology Laboratory, China) and sheep IL-4 ELISA kit. (E0079Sh, Bioassay Technology Laboratory, China).

Statistical analysis. Statistical analyzes were performed using the SPSS 22 program. First, normality test was performed to determine whether the variables were normally distributed. The coefficient of variation, Shapiro-Wilk value and histogram were examined during the evaluation. Student's t tests were used for normally distributed biochemical parameters. Mann-Whitney U tests were used to compare IL-2 and IL-4 levels between non-normally distributed and ordered variable groups. A statistically significant result was considered when the p value was less than 0.05.

Ethical note. Before starting to collect samples for the study, ethical confirmation was obtained from the Animal Experiments Local Ethics Committee of Harran University, dated 07.05.2021 and decision number 2021/004/06.

RESULTS

Animals with clinical symptoms of ecthyma (especially gingiva and lip margins) were included in the study (Figure 1).

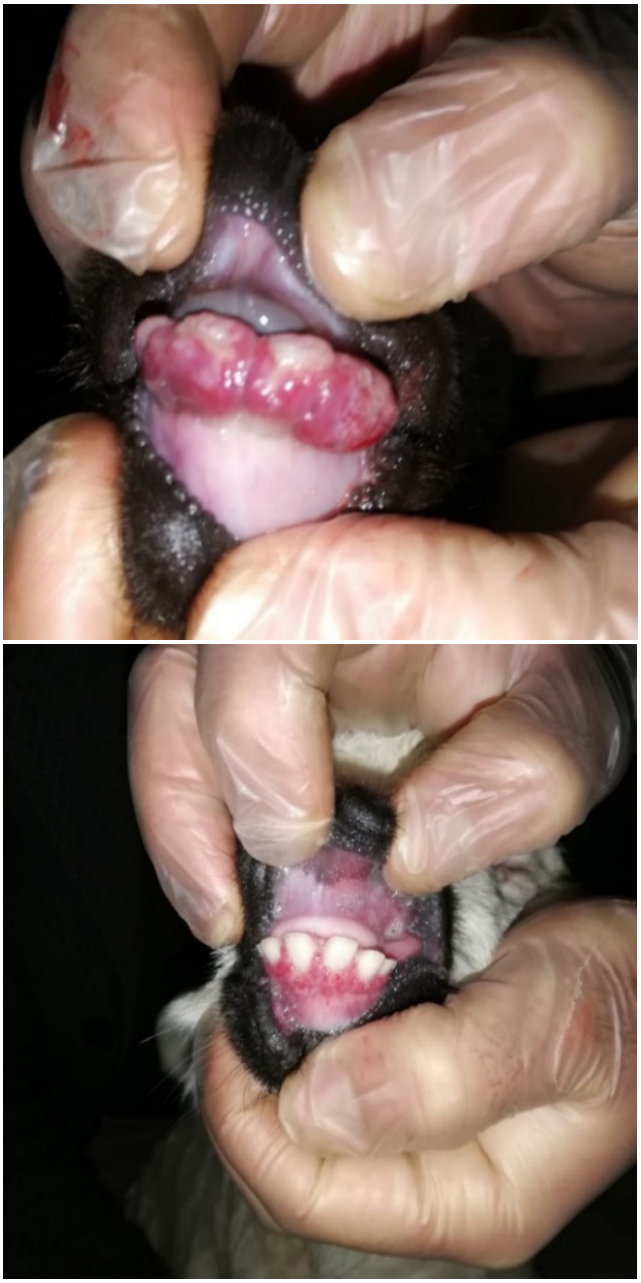

Figure 1. Papular macroscopic lesions on the gingiva and labium in sheep.

Of the 28 animals that showed symptoms, 25 were diagnosed as positive by PCR (Figure 2).

Serum biochemistry analyses were performed on all the samples both groups. For the samples from contagious ecthyma-positive animals, ALT, BUN, GGT and CRE levels were higher ($p<0.001$ and $p<0.01$) than the control group while TP and GLU values were significantly lower ($p<0.001$). There were no significant differences between the groups in ALB, CHOL, and TBIL values ($p>0.05$) (Table 1).

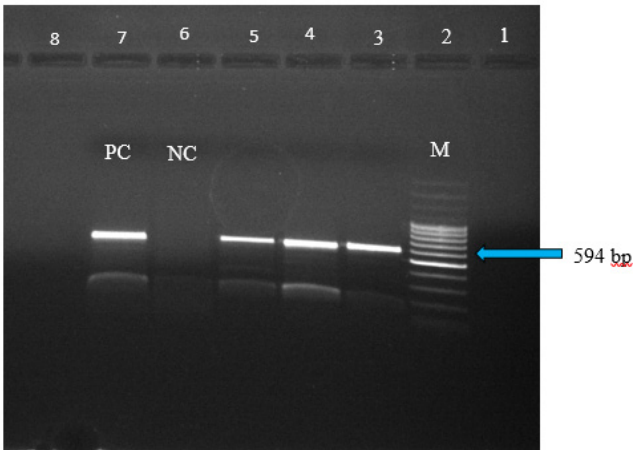

Figure 2. PCR amplicons of ORF virus (594 bp), line M. 100bp DNA ladder (Thermoscientific, United States), lines 3–5, positive samples. Line 6, negative control. Line7, positive control.

Table 1. Biochemical parameter values in sheep serum of control and naturally infected ecthyma groups.

Parameters	CG (n=10)	IG (n=25)	RI	P
ALB (g/dL)	2.6 ±0.18	2.8 ±0.31	2.4 - 3.0	0.1
ALT (U/l)	31.1 ±1.15	36.7 ±1.41***	26 - 34	0.000
BUN (mg/dL)	18.7 ±2.1	22.8 ±0.7***	8 - 20	0.000
CHOL (mg/dL)	57.4 ±1.26	57.3 ±1.49	52 - 76	0.05
CRE (mg/dl)	0.90 ±0.03	1.0 ±0.08**	0.5 - 2.2	0.001
GGT (U/l)	13.20 ±1.3	20.52 ±2.1***	6 - 17	0.000
GLU (mg/dl)	75.76 ±2.25	56.80 ±1.47***	50 - 80	.000
TBIL (mg/dl)	0.18 ±0.78	0.24 ±0.95	0.0 - 1.6	0.08
TP (g/dL)	6.15 ±0.15	5.30 ±0.11***	6.0 - 7.9	0.000

CG=Control group x±SD; IG=Infected group x±SD; RI=Reference intervals; **(p<0.01), ***(p<0.001), n = number of samples; x- means; SD – standard deviation, Student's t-test.

Serum cytokine levels and percentage increases were determined for all animals. Both cytokines levels were significantly higher in the infected animals than the control group ($p<0.01$). The IL-2 cytokine response was 28% higher in the

infected animals while the IL-4 response was 60% higher (Table 2).

Table 2. Cytokine levels and percent cytokine increases in ecthyma-infected sheep.

Cytokines (ng/L)	Healthy controls (n=10) x±SD	Sheep with ecthyma (n=25) x±SD	P value	% cytokine increase
IL-2	9.76 ±1.60	12.47 ±3.46**	0.008	%28
IL-4	17.18 ±6.21	27.48 ±7.35**	0.001	%60

** : p<0.01, n = number of samples; x- means; SD – standard deviation, Mann-Whitney U test.

Finally, serum cytokine concentrations ranged from 5 to 40 ng/L, Th1 and Th2 cytokines were found together, and the predominant cytokine response in infected animals was in the direction of Th2 (Figure 3).

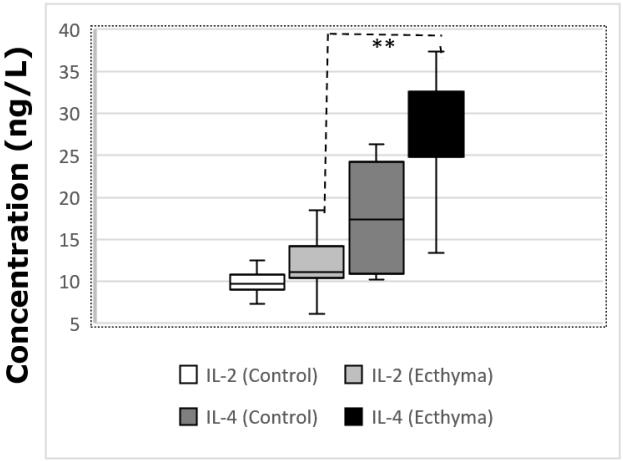

Figure 3. Interleukin concentrations and predominant cytokine response.

DISCUSSION

Although contagious ecthyma progresses with low mortality, its high morbidity rate causes serious economic losses, especially in developing countries. The failure to eradicate this disease is probably due to the lack of appropriate observation, adequate epidemiological knowledge and control strategies. Recognition of the disease, control measures, correct care, and feeding and transport conditions are critical for preventing epidemics and reduced yields. Compared to other poxviruses, this pathogen needs to be extensively investigated given its wide host range, potential for zoonosis, and ability to create immune-avoiding reinfection (1). While there have been several immunological studies (1,3,7,13,16), ours is the first study to investigate biochemical parameters together with the dominant cytokine response in contagious ecthyma.

This study compared the serum biochemistry parameters of ecthyma-positive and disease-free control animals. In the infected animals, ALT, BUN, CRE, and GGT were higher while GLU and TP were lower. ALB, CHOL, and TBIL values did not differ between the groups. Asl et al. reported similar findings for serum biochemistry parameters in goats with infectious ecthyma. In domestic animals, serum GGT activity is derived only from the liver. Elevated levels may indicate damage to biliary epithelial cells. Unlike our study findings, Asl et al. reported higher GLU values in infected than control animals but no significant difference in TP. The raised GLU value may have been related to stress (17). The lower GLU level in infected animals in our study may be related to insufficient food intake once symptoms appeared in these animals. In addition, the high serum creatine and BUN levels in infected animals caused protein loss, which is consistent with lower TP levels in infected animals in our study.

Kataria et al (18) reported similar findings to the present study from blood biochemistry analysis of healthy and infected animals with Peste des petits ruminants, which progresses with mouth sores and causes a higher mortality rate than ecthyma (18). Similarly, Narnaware et al (19) evaluated serum biochemical parameters in camels infected with contagious ecthyma and found increased ALT but decreased TP and GLU (19) while Gameel et al (20) detected hypoproteinemia and increased ALT in lambs infected with infectious ecthyma. They concluded that raised ALT in the blood may be directly related to the extent of tissue damage, levels of inflammatory mediators in the blood, and severe skin lesions (20). Similarly, Asl et al (17) suggested that changes in biochemical parameters may be associated with malnutrition, weight loss, oxidative stress, inflammation, and secondary bacterial infection (17).

T lymphocytes, which are an important part of immunity, include CD4+ (Th) and CD8+ (Tc) helper molecules. What is important here is that antigen-presenting cells must available the antigen to CD4+ Th cells to form both cellular and humoral immune responses. Immunocompetent Th cells activated by the presented antigen begin differentiating and proliferating. Th cells differentiate into Th1, Th2, or Th17 cells. Many cytokine genes are activated in this process. These Th subgroups perform different functions with the cytokines they secrete. Th1 cells fight intracellular bacteria and viruses by producing IL-2, TNF-α and IFN-γ (inflammatory effect) (21,22,23). IL-2 secreted by Th1 cells is required to activate Tc cells, which play a key role in killing virus- and bacteria-infected cells. This indirect effect further increases the importance of Th1 activation in the fight against infections. Th2 cells generate the cytokines IL-4, IL-5, IL-10, IL-13. Thus, it stimulates B cell proliferation and immunoglobulin secretion from plasma cells (24). Th2 cells have no effect on cell-mediated reactions (anti-inflammatory effect) (25). Haig et al (26) investigated the cytokine reaction of the afferent lymph following the injection of inactivated virus into sheep pre-infected with infectious ecthyma. CD4+ T cells increased in both groups compared to other cell types in the lymph while there were also differences in individual cytokine increases in lymph following reinfection, although the ecthyma lesions had similar sizes and healing times (26). Anziliero et al (27) investigated the effects of inactivated parapoxvirus ovis (iPPOV) in mice with intraperitoneal vaccination on cytokine expression. A complex cytokine response occurred in which the Th1 cytokine response was followed by the Th2 response (27). Avcı et al (28) administered iPPOV to rats intraperitoneally to investigate its effects on anti-inflammatory and proinflammatory cytokine levels. Serum samples were taken at hourly intervals for TNF-α (16 and 24 hours) and IL-6 (12, 16, and 24 hours). The syntheses were stimulated causing undulations in IL-10 and IL-12 levels. Increased cytokine levels were associated with iPPOV immunomodulatory activity (28). Wang et al (29) studied the answer to target cells after orf virus infection in goat skin fibroblast cells, which the main host target. Viral infection increased decorin, IL-8, and TNF-α levels in the early stage, which provided important data about the disease's pathogenesis and its interaction with the host (29). Pacheco et al (30) investigated the Th1/Th2 balance in three groups of 7-month-old sheep: Fasciola hepatica-infected, vaccinated and control. They found higher Th2 regulation and lower Th1 response in 9-18 days after infection in vaccinated and unvaccinated sheep with Fasciola hepatica (30).

Th1/Th2 balance is important because it can change the onset and course of acute and chronic infections, autoimmune or inflammatory, allergic, and neoplastic diseases (24,31,32). Glucocorticoids, which are the end products of HPA axis activity, increase IL-4, IL-10, IL-13 production by Th2 cells while inhibiting lymphocyte proliferation, cytotoxicity, and secretion of IL-2, TNF-α, IFN-γ (33). The use of glucocorticoids in veterinary medicine is quite common, particularly in the field to treat chronic inflammation, pain, and accelerate healing. However, it is very important to measure the immune response balance when using glucocorticoids to support the treatment.

Our study sampled sheep brought to the hospital for routine clinical examination while naturally infected sheep of the same age group and with lesions in the same region were used for uniformity and determination of cytokine response. However, the immune response can be affected by many different factors. Therefore, a future study with greater financial resources can compare cytokine responses while controlling for care and feeding conditions, age, and time since the onset of infection. Different studies can be conducted to understand the similarities and differences in cytokine response by performing in vivo studies in ecthyma or different diseases.

Our study analyzed serum biochemistry profiles and cytokine levels of unvaccinated, first-time, naturally infected sheep. Especially cytokine findings were remarkable. The findings show that both cellular and humoral responses develop in the immune response (anti-inflammatory effect) where the Th2 response is dominant. The results obtained may provide us with more information about the pathogenesis, prognosis and treatment options of the disease.

Conflict of interest

There is no conflict of interest between the authors.

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