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USING SHEEP FACIAL GRIMACE SCALE, INFRARED THERMOGRAPHY AND CORTISOL HORMONE TO MEASURE PAIN IN SHEEP INFECTED WITH MASTITIS DISEASE

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ABSTRACT:

The purpose of this study was to apply sheep pain facial expression scales (SPFES) to sheep infected with mastitis, measuring peripheral temperatures and serum cortisol hormone. Twenty-three healthy ewes and 23 infected ewes with clinical mastitis were included in this research from January 2022 to April 2023. Images were taken from each ewe to detect facial grimace scales of ewes. Nasal, eye and ear temperatures were collected using infrared thermal imaging camera. Moreover, serum cortisol hormone was collected from the blood. It was found that there was a significant decrease ($p<0.01$) in nasal, eye and ear temperatures of infected ewes in comparison to healthy ewes. Additionally, cortisol hormone was found significantly ($p<0.01$) higher in infected ewes at day 1 and day 7 compared to non-infected animals. The changes in facial expressions for all the determined five regions were significantly ($p<0.01$) different than healthy ewes. The total pain score was significantly ($p<0.05$) higher on day 1 and day 7, compared to healthy ewes. It was concluded that sheep facial grimace scale was a useful and practical tool for measuring pain in sheep. In addition, cortisol from the serum and peripheral temperatures were found to be useful pain indicators.

KEYWORDS: Facial Expressions, Pain Measurement, Peripheral Temperatures, Cortisol.

1. INTRODUCTION

Mastitis is an infection of the mammary glands. It causes considerable economic losses by reducing milk yield and milk quality, and milk rejection during the treatment period (Gelasakis *et al*., 2015). The most discussed animal disease in the world is probably mastitis, which is responsible for large economic losses to dairy, beef, sheep and goat producers (Freitas, 2005). Udder infection may produce an increase in temperature at this organ. Recently, there has been speculation regarding the application of infrared thermography (IRT) to obtain udder surface temperature and, consequently, use it as tool for mastitis diagnosis, thus, IRT was used to detect early subclinical mastitis as this type of mastitis happen with no obvious early symptoms (Berry *et al*., 2003). Mastitis can be clinical or subclinical. Diagnosing clinical mastitis is easier and requires direct observations. Its symptoms include abnormal milk, painful and swollen udder, increasing body temperature (Mavrogianni *et al*., 2011).

Animals infected with mastitis suffer from pain. Researchers use behavioral and physiological methods to measure pain in animals. In recent years, researchers use facial expressions to indicate pain levels in different animals, including sheep (McLennan *et al*., 2016). Sheep pain facial expression scales (SPFES) were used to indicate pain in sheep during mastitis. McLennan *et al*. (2016) presented a scale for facial expressions of sheep, which provides a reliable tool for pain assessment initiated with diseases such as foot rot and mastitis. This scale analysed 5 interested regions of the face or head to define the level of pain, including ears' position, nostril shape, cheeks tightening, orbital tightening and lip and jaw profile. The scale was highly accurate not only in pain identifications, but also in identifying illness signs. During disease states, sheep had tightened their eyes more than half, more backward ears, more tightened the masseter muscle of the cheek, and V-shaped nostril was performed by the sheep (McLennan *et al*., 2016). In addition to facial expressions, measuring surface temperature is frequently used.

Infrared thermography (IRT) is a non-invasive method to evaluate a body's surface temperature by an emitted electromagnetic radiation by an object when a temperature is only above absolute zero; that is zero Kalvin or -273 °C (Speakman & Ward, 1998; Travain & Valecchi, 2021). IRT measures changes in blood flow as well as heat transfer. The temperature is represented as a graphic on a body or object surface is called thermographic spectroscopy. The surface temperature is frequently affected by blood flow. For that reason, IRT is a familiar technique in medicine as a non-invasive method that detects the blood circulation through the emitted radiation (Turner, 2001).

The response to stress is characterized by the activation of the hypothalamus–pituitary–adrenal (HPA) axis, both adrenal catecholamines and steroids are known to be involved in the stress response, immune function, blood pressure and energy homeostasis (Weaver *et al*., 2021). Thus, an animal can loss heat (Schaefer *et al*., 2002; McManus *et al*., 2016). This heat loss can be detected by decreasing peripheral temperatures such as the nasal (Kuraoka & Nakamura, 2011; Proctor & Carder, 2015, 2016). Measuring surface temperature of an object using IRT needs suitable tools, frequently infrared thermal camera (Travain & Valecchi, 2021). There is a limited number of studies undertaken on measuring facial expressions of sheep during diseases; therefore, the main purpose of this study was to apply SPFES to sheep infected with mastitis in addition to measure peripheral temperatures and serum cortisol hormone.

2. MATERIALS AND METHODS

2.1 Subjects and study site

The present study was undertaken at Batifa District, Zakho, Kurdistan Region of Iraq from January 2022 to April 2023. Twenty-three healthy ewes and 23 infected ewes with clinical mastitis were included in this research. Diagnosis of clinical mastitis was based on the appearance of abnormally appearing milk and swollen udder. When milk was off colour, watery, bloody or had the appearance of serum, abnormal milk night also

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contained different amounts of pus and clots. The diagnosis was undertaken before any datum was collected, then the infected ewes were separated from the flock. The sheep understudy were treated by a specialized veterinarian.

2.2 Ethical Approval

Animal Ethics Committee of University of Zakho approved all process of the present study with the code of approval: AEC-020. All ewes were normally infected with mastitis and were carefully treated by a veterinary specialist.

2.3 Data Collection

2.3.1 *Sheep Facial Grimace* Scale:

A Sony camera (SONY, Cyber-Shot, DSC-H20, Japan) with a high resolution was used in the present study to capture pictures. Two pictures (one from the front and the other from the side) were captured for each ewe's head; that is 2 pictures for each healthy ewe and 4 pictures for each infected ewe with mastitis at days 1 and 7 with the total of 138 pictures. The pictures were scored in May 2023 to avoid any bias that might have happened by the researchers. Five regions of the face were scored (for details, see McLennan *et al*., 2016). If two or more areas were not identified by the authors, the image was removed for pain scoring, thus, only 114 images were included. To score the images, a three-scale was used, i.e., a score 2 for severe pain level, 1 for moderate pain and 0 for no pain in sheep (for details, see: McLennan *et al*., 2016).

2.3.2 *Temperature*:

Infrared thermal camera (FLIR E4, FLIR Systems, OU, Estonia) was used to capture thermal pictures from the eye, ear and nasal of ewes (Figure 1). Images were taken for 23 healthy ewes, and 23 infected ewes at days 1, 7, 21 and 28. A total of 345 images were collected from selected peripheral areas. Images were taken from half to 1 meter distance away from each animal.

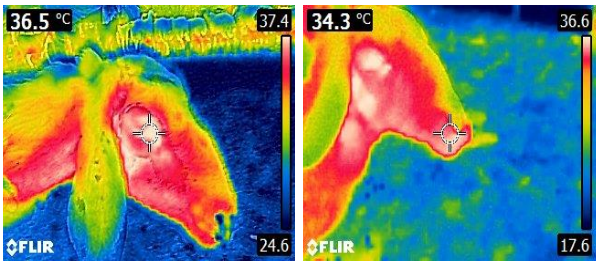


Figure 1: Infrared thermal images show the temperature of both eye (left side) and nasal (right side) on the top-left corner of each image.

2.3.3 *Serum Cortisol*:

Five mL of the blood was collected from the jugular vein from healthy ewes and infected ewes at days 1 and 7 of infection. Directly after collecting the blood samples, they were centrifuged at a lab in Batifa for five minutes at 2000 rpm. The collected serum was then placed in a freezer for later analysis. Cortisol hormone was determined using Cobas Elecsys Analyzer (Roche Diagnostics, Germany).

2.4 Statistical Analysis

All the collected data were analysed using GenStat software program (17th edition, VSN international). All findings were written as mean ± standard error of mean (SEM) unless otherwise stated. According to the normality test of Shapiro-Wilk test, cortisol and temperature data were normally distributed, thus they were analyzed using a one-way analysis of variance (ANOVA). Repeated measures with Fisher's Unprotected LSD test were used for post hoc comparison. Whereas the data of face regions were non-parametric. Therefore, they were analyzed by Kruskal-Wallis test with Mann Whitney U test for post-hoc comparison. Correlations were indicated using a nonparametric correlation test of Spearman's test. Differences are reported as significant at $p<0.05$ and tendencies are reported when p is between 0.09 and 0.05. Both specificity and sensitivity were scored as follows:

Sensitivity = TP / TP+FN

1 - Specificity = TN / TN+FP

Where: TP = True Positives (sick ewes recognized as sick)

TN = True Negatives (healthy ewes recognized as healthy)

FP = False Positives (healthy ewes recognized as sick)

FN = False Negatives (sick ewes recognized as healthy)

3. RESULTS

3.1 Facial Grimace Scale

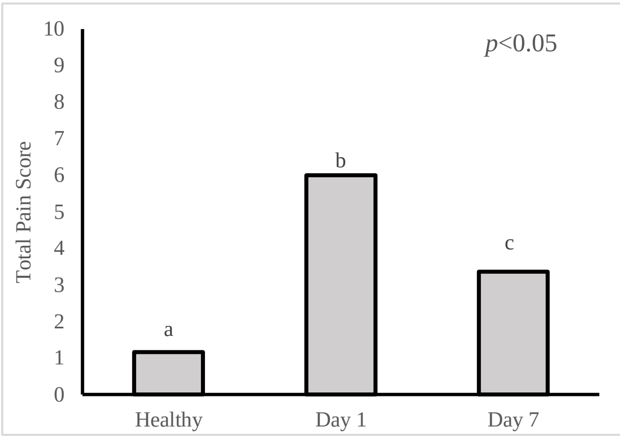
There were significant differences ($p<0.01$) between healthy and infected ewes with mastitis in all the five-measured facial regions (Table 1). However, lip and jaw profile, and ear position were not significantly differed between healthy and infected ewes at day 7.

Table 1: The difference in SPFES between healthy and infected ewes at days 1, and 7.

Facial Regions	Animal State			*p*-value
	Healthy	Foot rot Day 1	Foot rot Day 7	
Orbital Tightening	0.16 ± 0.08 a	1.42 ± 0.11 b	0.84 ± 0.11 c	0.01
Lip and Jaw Profile	0.21 ± 0.09 a	1.16 ± 0.13 b	0.47 ± 0.12 a	0.01
Nostril Shape	0.11 ± 0.07 a	1.00 ± 0.17 b	0.79 ± 0.12 b	0.01
Ear Position	0.53 ± 0.12 a	1.47 ± 0.12 b	0.58 ± 0.16 a	0.01
Cheek Masseter Muscle	0.15 ± 0.08 a	0.95 ± 0.12 b	0.68 ± 0.13 b	0.01

Note: different letters in the same row means significant difference.

Figure 2 shows the difference in total pain scores recorded by facial units. There was a significant difference ($p<0.05$) between healthy, infected ewes at day 1 and day 7. The total score



was with the range of 0-10 based on the five regions recorded and each was out of 2. The total scores were 1.16, 6.00 and 3.36 for healthy, infected at day 1 and day 7, respectively.

Figure 2: The total pain score recorded from facial expressions of ewes.

The sensitivity of obtaining true positives was 0.77, while 1-specificity was 0.24. Lower 1-specificity means that higher percentage of ewes were correctly identified as healthy whereas higher specificity values means that higher percentage of ewes were correctly identified as infected.

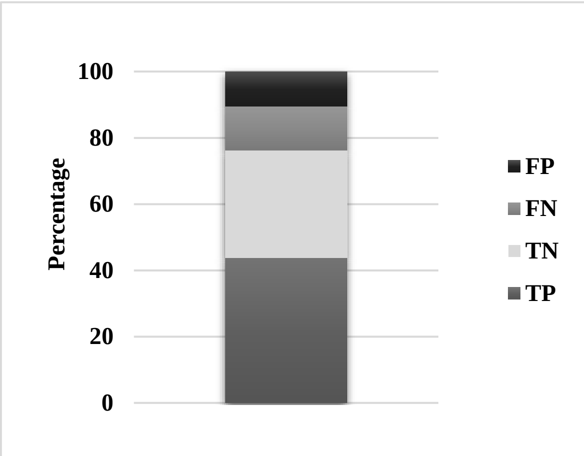


Figure 3: The ration of sensitivity and 1-specificity.

The results of Spearman's test for indicating correlations are shown in Table 2. Strong correlations were found between some face areas such as orbital tightening and nostrils in infected animals at day 1.

Table 2: The correlations between the five regions of spfes of healthy and infected ewes with mastitis. The upper row of each facial unit is for healthy ewes, the middle row is for day 1 and the bottom row is for day 7 of infection.

Facial parts	Orbital tightening	Nostril shape	Ear position	Cheek muscle	lip and jaw profile
Orbital tightening	-	0.53	0.48	0.43	0.34
	-	1.00	0.86	0.76	0.20
	-	0.78	0.50	0.36	0.65
Nostril shape	0.53	-	0.94	0.53	0.45
	1.00	-	0.55	0.57	0.28
	0.78	-	0.57	0.88	1.00
Ear position	0.48	0.94	-	0.60	0.91
	0.86	0.55	-	0.21	0.85
	0.50	0.57	-	0.90	0.35
Cheek muscle	0.43	0.53	0.60	-	0.34
	0.76	0.57	0.21	-	0.37
	0.36	0.88	0.90	-	0.59
lip and jaw profile	0.34	0.45	0.91	0.34	-
	0.20	0.28	0.85	0.37	-
	0.65	1.00	0.35	0.59	-

3.2 Temperature

The temperature of the eyes, ears and nasal over time is shown in Table 3. A significant (p<0.01) drop in the temperatures of the eye, ear and nasal were observed during the infection of days 1, 7 and 21, in comparison to the healthy ewes and day 28. In addition, the eye temperature was significantly (p<0.01) lower on day 1 than on days 7 and 21. Similarly, the ear temperature n days 1 and 21 were lower significantly (p<0.01) than day 7.

Table 3: The temperature of the eyes, ear and nasal of healthy and infected ewes with mastitis over time.

Body Region	Healthy animals	Infected animals				*p*-value
		Day 1	Day 7	Day 21	Day 28	
Eye	37.3 ± 0.2a	29.1 ± 0.6b	32.3 ± 0.8c	33.6 ± 0.7c	35.9 ± 0.3a	0.01
Ear	30.8 ± 0.4 a	21.4 ± 1.3bc	24.0 ± 1.3b	18.9 ± 1.0c	30.7 ± 1.0a	0.01
Nasal	33.2 ± 0.6a	23.6 ± 0.7b	24.5 ± 1.1b	25.2 ± 1.2b	33.0 ± 0.9a	0.01

Note: different letters in the same row means significant difference.

3.3 Serum Cortisol

The serum cortisol level was significantly (p<0.01) increased in infected ewes at day 1 (44.17 ± 7.9 nmol/l) in comparison to the healthy ewes (14.68 ± 2.9 nmol/l). In day 7 (22.01 ± 3.8 nmol/l), the level of cortisol was decreased, however it was significantly higher than the healthy and lower than the infected ewes in day 1 (Figure 4).

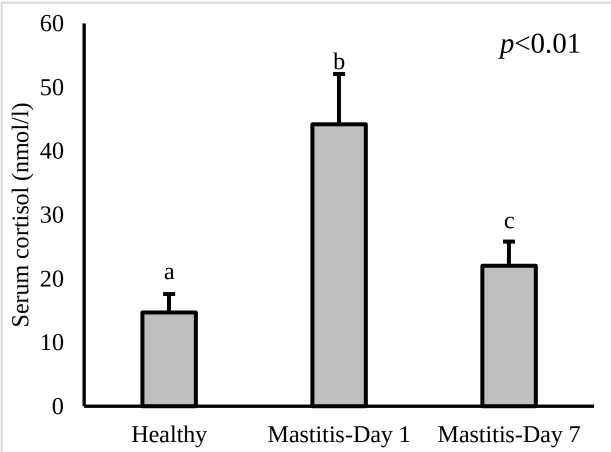


Figure 4: The level of serum cortisol in healthy with infected ewes at days 1 and 7.

4. DISCUSSION

The purpose of the present study was to determine the effects of mastitis disease on ewes. The findings revealed that total pain scores increased significantly. In addition, the peripheral temperatures decreased significantly. Mastitis had caused an elevation in cortisol levels in the serum.

Infected sheep in the current study had high total pain score and this score was decreased at day 7; however, both were higher than the control (healthy sheep) (see Figure 2). This elevation in pain score may be due to the involuntary movement of facial muscles of animals (Langford *et al*., 2010). Similar results were found in a study of McLennan *et al*. (2016) who found that sheep infected with foot rot disease had high total pain score and McLennan *et al*. (2016) also found that the pain score decreased when sheep were recovered. It was also shown that there were significant changes in the facial expressions of sheep in day 7 in comparison to control, but the changes were not observed at day 90 of treatment (McLennan *et al*., 2016). In another study, the total pain score was significantly higher in days 1 to 17 than the baseline (control) in sheep (Häger *et al*., 2017). The results of the present study are in agreement with the previous studies' findings of McLennan *et al*. (2016) and Häger *et al*. (2017). This means that using SPFES is an effective method in detecting pain level in infected sheep and in line with other studies on different animal species (Langford *et al*., 2010; Sotocinal *et al*., 2011; Keating *et al*., 2012; Holden *et al*., 2014; Reijgwart *et al*., 2017; Müller *et al*., 2019; Evangelista *et al*., 2019).The sensitivity was found to be high in this study, which was 0.77 and the result of the present study was similar to the results of the previous study conducted by McLennan *et al*. (2016) who found the sensitivity of facial expressions in detecting pain was 0.80 and 0.81 for mastitis and foot rot, respectively. In another study conducted by Häger *et al*. (2017), who found the accuracy of 68%; and for horses was slightly higher than 70% (Dalla Costa *et al*., 2014); whereas higher accuracy or sensitivity was found in mice, which was 97% (Langford *et al*., 2010).

Serum cortisol in the present study was found to be significantly higher in infected sheep with mastitis on both day 1 and 7 in comparison to healthy sheep. In addition, the day 1 of infection cortisol levels were significantly higher than day 7 (Figure 4). In a study conducted by Häger *et al*. (2017), it was found that a significant elevation in saliva cortisol in sheep post-surgery on day 1 and the cortisol levels were decreased on day 7. Similar results were found in a recent study undertaken to measure the pain levels in infected goats with foot rot disease, in which serum cortisol levels were increased on day 1 and day 7 post-infection (Hussein and Al-Naqshabendy, 2023).

Cortisol levels were also found to be increased in animals such as of calves after normal husbandry procedures such as disbudding (Gaab *et al*., 2022); and sheep after shearing (Arfuso *et al*., 2022). In another study undertaken by Weaver *et al*. (2021) on Merino ewes was to measure cortisol levels when administrating animals with saline or hydrocortisone acetate injection. Weaver *et al*. (2021) found elevations in serum cortisol concentrations during the experimental injection of these chronic-induced stressors.

The vasoconstriction of peripheral regions like the eye, ear and nose of animal during a fight or flight response, pain or emotional fever, the blood diversion from these areas to the internal body occurs and causes a drop in surface temperature (Proctor & Carder, 2015). Both arteriolar and venous vasoconstriction in the abdominal viscera and skin occurs when heart rate and contractibility of the cardiac is increased when hormones such as epinephrine and norepinephrine are released by adrenal medulla (Vieira *et al*., 2018). This initial vasoconstriction will drop the surface skin temperature; after a period of time, the heat built-up in the core body is released and causing an elevation in skin temperature (Loughmiller *et al*., 2001; Soerensen & Pedersen, 2015).

The peripheral temperatures dropped significantly in the present study after the infection, and increased as in healthy animals on day 28 (Table 3). In a study of Hussein and Al-Naqshabendy (2023), it was also found that the peripheral temperatures of the eye, ear and nasal dropped in infected goats. Similarly, Zebari *et al*. (2021) found a drop in the eye and ear temperatures after ear tagging of kid goats. Thus, the results of the present study are in line with the previous studies. However, Arfuso *et al*. (2022) found different results as the total eye temperature measured from three regions of the sheep eyes was increased post-shearing.

CONCLUSIONS

It was concluded that spfes was a useful and practical tool for measuring pain levels in sheep. In addition, cortisol from the serum as well as peripheral temperatures from the eye, nasal and ear temperatures were found to be useful indicators in detecting pain experience in sheep infected with diseases such as mastitis. More studies are required to apply the SPFES to the sheep with different painful and stressful states such as shearing, disbudding and ear tagging. In addition, future studies are essential to study sheep grimace scale using positive emotional states.

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