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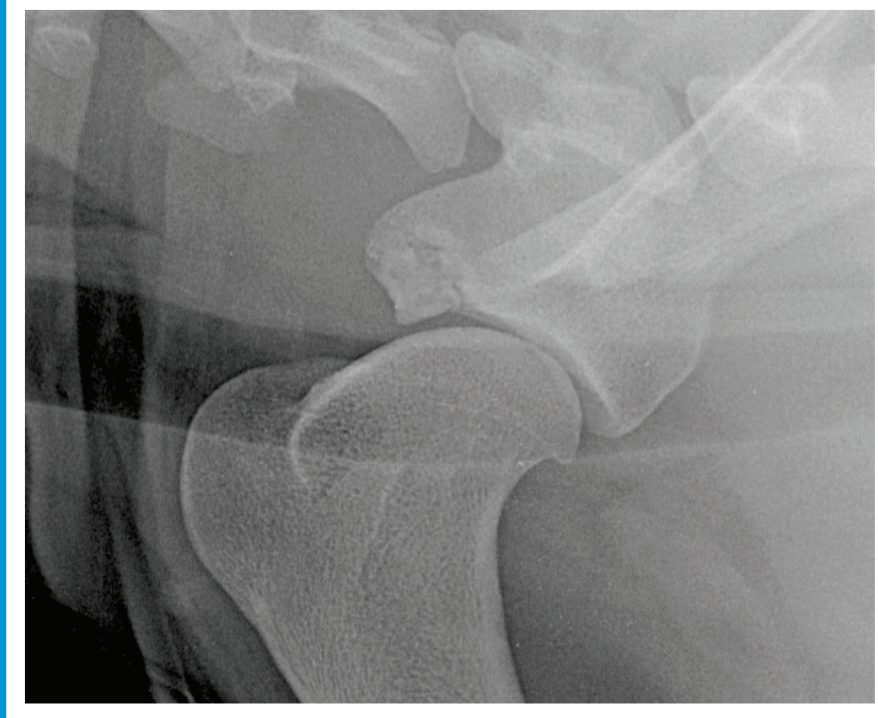

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Original Scientific Article

THE EFFECT OF SODIUM BENTONITE SUPPLEMENTATION IN THE DIET OF MINK (*Neovison vison*) ON THE MICROBIOLOGICAL QUALITY OF FEED AND ANIMAL HEALTH PARAMETERS

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Summary: The objective of the study was to assess the effect of mink feed supplementation at weaning on the hygiene and sanitary conditions of feed and selected parameters of animal health . The experiment was carried out in two stages. In the first stage of the study, as 0.5% addition of bentonite and a 1% supplement are incorporated into the daily feed intake mink experimental groups D1 and D2, respectively. In the second step, the increase in the proportion of bentonite in the two groups is up to 1% and 1.5%. K group (control group) was not supplemented. The microbiological examinations of the chosen indices of mink feed were performed twice in each feeding period. The mink health state was monitored and evaluated via hematological analyses and lysozyme activity. The research results enable a conclusion that the application of sodium bentonite in the analyzed amounts does not negatively influence the analyzed parameters of mink blood. In fact, it may be recommended to reduce fungus numbers in feed.

Key words: mink; bentonite; feed quality; haematological parameters; lysozyme

Introduction

Montmorillonite is commonly included into an animal diet due to its adsorptive properties. Bentonite is part of this group. Some authors tend to highlight bentonite therapeutic efficacy which is similar to antibiotics (1). Breeders feed fur carnivores with animal-origin by-products from other sectors of animal production. Under such conditions, saprophytic bacteria and opportunistic pathogens can quickly develop; and their occurrence favors the putrification of feed stuff ingredients. According to the study by Kopczewski et al. (2), poor hygiene feed control directly affects animal reproductive performance, which leads to infertility, reduced litter size and even miscarriages. Feeds administered to fur animals are relatively seldom microbiologically evaluated and, importantly, animal health is unequivocally impacted by feed hygiene and sanitary practices (3,4). Hematological examinations are used to indicate animal welfare, i.e. to determine the optimal physical and psychical standard against the environmental conditions where a feeding strategy and management systems are most important (5).

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Lysozyme is one of the key components of nonspecific defensive mechanisms in azurophilic granules, specific granules, neutrophil gel granules, as well as in grains of monocytes and macrophages (6). Its activity in blood is enhanced at lower airway disorders, renal diseases, and some diseases of the cardiovascular system (7,8,9). The objective of the study was to assess the effect of mink feed supplementation at weaning on the hygiene and sanitary conditions of feed and selected parameters of animal health.

Material and methods

The study concerned pastel-type minks at weaning, caged in the farm pavilion system. All of the animals had prophylactic treatments that are appropriate for the species. The animals received the same diet in terms of composition, nutritive value and as specified by feeding standards (10). The experiment proceeded in two stages. In the first research stage, a 0.5% bentonite additive and a 1% one were added as feed additive supplement into the daily feed intake into in the D1 and D2 experimental groups, respectively. In the second stage, the feed additive level was increased in both of the groups: D1 received 1% of bentonite in a daily feed intake, whereas 1.5% of bentonite was administered in D2. Feed for Group C (control group) was not supplemented. Each group consisted of 30 animals. Sodium bentonite was analyzed in the Polish Geodesic Institute in Warsaw (11) before it was used in the study. The microbiological examinations of the chosen indices of mink feed were performed twice in each feeding period. The sample analyses were made in two replications to determine total bacterial and fungal counts in feed according to feeding standards (12,13). The mink health state was monitored and evaluated cia hematological analyses of erythrocytes, hematocrit, hemoglobin, leucocyte level and lysozyme activity. The animal blood was collected from a clipped claw after previous local anesthetic administration (ointment with lidocaine) and from the heart at mink slaughter after previous stunning. The material was collected in test tubes with EDTA K2 PROFILAB and analyzed with an automatic blood analyzer MS4 VET. Lysozyme concentration was determined using a plate with the method modified by Hankiewicz (14). The experiment was conducted with the consent of the local ethical committee (No. 40/2009). The results were analyzed statistically with statistical program Statistica10.0 (StatSoft, Poland).

Results

The results of the microbiological evaluation of mink feed (*Neovison vison*) with the sodium bentonite supplement are summarized in Table 1. Total bacterial numbers in the first research stage reached the highest level in the experimental animal feed. The average concentration of mesophilic aerobic bacteria was $2.x10^6$ cfu/g/ feed in group D1 with a dietary 0.5% sodium bentonite additive, whereas in group D2 with a 1% bentonite supplement – $2.3x10^6$ cfu/g/feed. The lowest load of mesophilic aerobic bacteria, i.e. 1.5x10 cfu/g/feed was determined in the control group (C). The statistical analysis showed no significant differences in the numbers of studied microbes ($p>0.05$) between the groups in the first research stage. Different values, however, were noted for total fungal numbers. The highest level of fungal contamination of feed was recorded in the control group (C) as its average concentration was $2.1x10^4$ cfu/g/feed (Table 1). The experimental groups D1 and D2 had a similar but lower total fungal number. The fungal number obtained in the investigated mink feed did not exceed the statistical significance threshold between the groups ($p>0.05$), (Table 1). The studies on the microbiological quality of mink feed in the second research stage are illustrated in Table 1. The examination of total mesophilic aerobic bacteria counts showed their highest number in the feed of the experimental groups, i.e. D1 and D2. The mean bacteria concentration in this period reached 2.8x106 cfu/g/ feed and $2.7x10^6$ cfu/g/feed in group D2 and group D1, respectively, whereas $1.7x10^6$ cfu/g/feed in the control group (C). The statistical analysis in the second research stage did not exhibit significant differences between the groups in total mesophilic aerobic bacteria counts either. ($p>0.05$), (Table 1).

In the second study stage, just like in the first stage, the control group feed had the highest total fungal numbers which averaged $7.4x10^4$ cfu/g, while the lowest numbers were found in group D2: on average $3.1x10^3$ cfu/g/feed (Table 1).

Table 1: Total count of bacteria and fungi in animal feed in I and II research stage (cfu/g)

Group	I research stage				II research stage			
	Total bacteria count		Total fungal count		Total bacterial count		Total fungal count	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
K	$1.5x10^6$	$4.8x10^5$	$2.1x10^4$	$1.5x10^4$	$1.7x10^6$	$9,6x10^4$	$7,4x10^3$	$6,1x10^3$
D1	$2.4x10^6$	$4.8x10^5$	$1.1x10^4$	$2.8x10^3$	$2.8x10^6$	$1,7x10^5$	$4,7x10^3$	$2,3x10^3$
D2	$2.3x10^6$	$9.3x10^5$	$1.1x10^4$	$7.0x10^3$	$2.7x10^6$	$1,6x10^6$	$3,1x10^3$	$3,1x10^3$
Analysis	H=3.43; p=0.18		H=0.86; p=0.65		H=1.14; p=0.56		H=0.96; p=0.62	

Explanation: - the arithmetic mean, SD - standard deviation, Z-Mann Whitney U test, H-Kruscala-Wallis test

Table 2: Lysozyme activity in mink blood in I and II research stage

Group	I research stage				II research stage			
	I collection		II collection		I collection		II collection	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
K	3.27	0.51	3.29	0.43	2,86	0,35	3,75	0,65
D1	4.30	0.32	3.90	0.35	2,36	0,78	4,10	0,26
D2	4.06	0.59	4.23	1.53	1,36	0,73	4,41	2,68
Analysis	H=8.19; p=0.02*		H=2.86; p=0.24		H=0.57; p=0.75		H=8.58; p=0.01*	

Designation as in Table 1

Table 3: Level of chosen morphological parameters in research stage I

Parameter	Collection	Group						Statistical analysis, p
		K		D1		D2		
		$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
Erythrocyts [m/mm^3]	I	9.59	1.32	10.37	0.57	9.31	2.78	H=3.17 p=0.20
	II	9.65	0.71	10.42	0.48	10.01	0.56	H=6.53p=0.04*
Hematocrit [%]	I	55.07	8.17	56.94	3.23	52.08	15.08	H=1.09; p=0.58
	II	58.07	2.68	61.52	2.08	60.27	2.72	H=7.35;p=0.03*
Hemoglobin [g/dl]	I	17.64	2.63	19.86	1.53	16.82	5.17	H=6.47;p=0.04*
	II	17.53	0.85	18.56	0.77	17.84	0.85	H=7.06;p=0.03*
Leucocytes [m/m^3]	I	12.89	4.55	7.88	3.39	8.11	5.00	H=7.02 p=0.03*
	II	10.17	2.26	8.90	2.39	10.68	2.37	H=3.14 p=0.21

Designation as in Table 1

Table 4: Level of chosen morphological parameters in research stage II

Parameter	Collection	Group						Statistical analysis, p
		K		D1		D2		
			SD		SD		SD	
Erythrocyts [m/mm^3]	I	10.05	0.53	10.35	0.87	10.69	0.87	H=2.54;p=0.28
	II	9.82	0.60	9.69	1.55	10.12	0.28	H=1.54;p=0.46
Hematocrit [%]	I	57.53	2.23	58.29	4.12	59.22	5.33	H=1.07; p=0.58
	II	56.00	3.35	55.49	7.01	59.32	3.13	H=4.57; p=0.10
Hemoglobin [g/dl]	I	19.00	1.57	18.66	1.15	19.14	1.98	H=0.47; p=0.79
	II	18.80	2.21	18.94	2.20	19.14	1.62	H=0.47; p=0.79
Leucocytes [m/m^3]	I	7.60	2.97	7.68	3.45	8.71	4.10	H=0.40; p=0.81
	II	9.25	3.13	7.69	3.58	7.56	5.66	H=1.69; p=0.43

Designation as in Table 1

The examination indicated higher lysozyme activity in the blood plasma of experimental animals in both the first and second study stages. The analysis performed in the first research stage showed the increased lysozyme activity in the animals from the experimental groups D1 and D2 compared to control group C (p=0.02). The lowest lysozyme content was in control group C (3.27μg/ml), whereas it was the highest in the experimental group D2 (4.30 μg/ ml). Significant differences between the groups in the second study stage (p=0.01) were determined. The highest lysozyme activity was again observed in the animals from the D2 group (4.1μg/ml) and the lowest in control group C (2.86μg/ml). In the first collection in the second stage, the differences in this parameter level were statistically negligible (Table 2).

The hematological evaluation in the first blood collection showed significant differences in the evaluation of leucocytes (p=0.03) and hemoglobin (p=0.04), while the differences in other parameters were statistically insignificant (p>0.05). The leucocyte content in the first collection was the highest in the control group (C) (12.89 m/m3), whereas it was lower though still similar in the experimental groups (Table 3). Similarly, an increase in average WBC count in the control group (C), though statistically insignificant, was observed in the second stage. The obtained white blood cell count in all of the analyzed groups was within the reference range presented by Hunter (16) and in the upper limits given by Berestov et al. (17). One of the basic blood parameters conditioning its transportation function is hemoglobin (Hb). This parameter level is endorsed as a tool for diagnosing anemia in an organism. An Hb level in the first research stage was statistically significant in both of the analyzed blood samplings. The highest Hb level in the first stage was observed in the minks from the D1 group, while in control groups C and D2 its concentration had similar values (Table 3). The obtained hemoglobin content in the mink blood in all of the groups under study was found to slightly surpass the mean values reported by Hunter (16) and within the interval depicted by Berestov et al. (17). In the second blood collection, statistically significant differences were determined in the evaluation of erythrocytes (p=0.04), hematocrit (p=0.04), hemoglobin (p=0.03). In the first research stage, the erythrocyte level was highest in experimental group D1 (10.37 m/mm^3 in the first sampling and 10.42 m/mm^3 in the second one (Table 3). The values obtained in the second sampling were statistically significant (p=0.04). This parameter in all of the groups under investigation exceeded the values suggested by Berestov et al. (17) but fell within the upper limits presented by Hunter (1996). The hematocrit value in the second blood collection was higher in both of the experimental groups, i.e. D1, D2, compared to the control one. The statistical analysis showed that the differences were significant (p=0.04) (Table 3). In both of the analyzed samplings, the parameter was found in the upper limits given by Hunter (16).

Studying the results of the hematological assessment of mink blood, no statistically significant differences were determined in the evaluation of erythrocytes, hematocrit, and hemoglobin ($p>0.05$) in the second research stage in the first and second blood collection (Table 4). Similarly, the analysis of the white blood cells of mink blood in the second stage did not exhibit statistically significant differences between the groups ($p>0.05$).

Discussion

The research results available thus far have indicated that dietary bentonite included into a diet of animals that show varied degrees of diarrhea can cause the symptoms to regress. In the case of calf diarrhea, bentonite improves therapeutic efficacy better than antibiotics and chemotherapeuticals do. A bentonite-supplemented diet for pigs enhances growers' body conditions and increases weight gains. Dobrzański et al. (18) administered bentonite to chicken broilers' feed for 2 weeks and then observed a substantial reduction in fungal numbers and a decrease in mesophilic bacteria count, up to 70%. Grata et al. (19) studied the use of urea phosphate for disinfecting poultry liquid manure, and its strong bactericidal properties were observed as early as after 2-week studies. The examples of research on animals indicated that bentonite could improve the efficiency of treatment and relieve the symptoms of various cases of diarrhea (20, 21). In the studies by Kulok et al. (22) and Kołacz et al. (23), halloysite (aluminosilicate clay mineral) was used in fatteners' diet to decontaminate bacteria, fungi and mycotoxins in feed mixtures as well as reduce ammonia emission; the authors highlighted its high efficiency. The studies by Pasha et al. (24) also confirmed the strong adsorptive properties of this aluminosilicate towards aflatoxins. These authors indicate a beneficial effect of 0.5% of sodium bentonite used per 100 (mcg/kg) aflatoxins in feed regarding boosting the bird immune system measured by the antibody titers and phagocytosis process rate. Sodium bentonite has proven to be efficient in binding adverse aflatoxins in feed as it can prevent the "depression" of immune response by the elevation of antibody titers against hemagglutinin (HA) as well as improving feed conversion by 23.8% in birds whose diet included a sodium bentonite additive.

The obtained results of total mesophilic aerobic bacteria count in mink feed are lower than those given by Urlings et al. (25). The authors studying the possibility of using fermented poultry by-products in a mink diet determined a level of mesophilic aerobic bacteria in the range from 7 to 7.4×10^6 cfu/g. Similarly, the values reported by Powell et al. (26) for the total bacteria count exceed those obtained in this study. The authors assessed the impact of formalin as a preservation means for mink feed and established mesophilic aerobic bacteria count at the level of 7.7×10^6 cfu/g feed.

Lysozyme is known to have a bacteriolytic function and can degrade the mucin cell wall. The studies on ferrets by Wells et al. (27) showed the abundance of lysozyme in tracheal submucosal glands, which secrete proteins of antibacterial properties that in turn favor airway protection against bacterial infections. The significant increase of lysozyme activity proves the activation of innate immunity cellular mechanisms (28). In contrast, the maintenance of significantly higher lysozyme activity as compared to the initial values and the control group indicates the efficiency of phagocytic system cells as the main source of its production. Lysozyme activity grows in the presence of immunoglobulins. Interpreting the obtained research results can be challenging because there are no reference values for the lysozyme level in mink blood plasma. The vast majority of reports addressing mink blood parameters concerns studies on small populations and employs only a few parameters. The white blood cell count in each organism is frequently considered as an organism response to diverse environmental stressors (physical, chemical, biological). In carnivorous animals, leucocytosis is often recognized as soon as feed contaminated by bacteria is administered. In this research, the WBC count in the experimental groups of D1 and D2 was lower or similar, which may be attributed to the detoxificative operation of a sodium bentonite dietary additive. Grosicki and Kowalski (29), who analysed the health state of rats fed with preparations with a 2% bentonite supplement, showed no statistically significant differences in a level of erythrocytes, leucocytes, hemoglobin or hematocrit. Similar values of the hematological parameters of mink blood were reported by Douglas et al. (30), who aimed to establish a reference range. In this study, slightly

higher values for red blood cell components (RBC, Ht, Hb) were recorded. The results obtained in this research were consistent with these given by Fletch and Karstad (31). Analyzing the hematological parameters of mink blood, the authors focused on the influence of color on each blood parameter.

The divergences observed are likely to arise from a method of blood collection for analyses. An increase in erythrocyte numbers in many animal species results from spleen contraction due to animal's stress and excitement at blood sampling. In minks, however, this effect has not been studied in detail.

The research results enable a conclusion that the application of sodium bentonite in the analyzed amounts does not influence negatively the analyzed parameters of mink blood. Reducing fungus numbers may be recommended in feed as it lowers mycotoxin concentration and improves the feed's sanitary state.

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VPLIV DODATKA NATRIJEVEGA BENTONITA V HRANI KANADSKIH KUN ZLATIC (*Neovision vision*) NA MIKROBIOLOŠKO KAKOVOST KRME IN ZDRAVSTVENE PARAMETRE HRANJENIH ŽIVALI

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Povzetek: Cilj raziskave je bil oceniti učinek dodatka natrijevega bentonita v krmi kun na higieno in sanitarne lastnosti krme ter izbrane parametre zdravja živali ob njihovi odstavitvi. Poskus je bil opravljen v dveh fazah. V prvem delu je bilo v dnevno količino krme vključenih 0,5 odstotka oziroma 1 odstotek bentonita pri poskusnih skupinah kun D1 in D2. V drugem delu poskusa je bil delež bentonita v obeh skupinah povečan na 1 oziroma 1,5 odstotka. Skupina K (kontrolna skupina) v krmi ni imela dodatka. Mikrobiološke preiskave krme kun so bile opravljene po dvakrat v vsakem obdobju hranjenja. Zdravstveno stanje kun smo spremljali in ocenjevali preko hematoloških analiz in meritve aktivnosti encima lizocima. Rezultati raziskave omogočajo sklep, da uporaba natrijevega bentonita v analiziranih količinah ne vpliva negativno na analizirane parametre krvi kun. V resnici je dodatek priporočljiv za zmanjšanje števila glivic v krmi.

Ključne besede: kanadska kuna zlatica; bentonit; kakovost krme; hematološki parametri; lizocim

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Original Scientific Article

THE DETECTION OF RELATIVE mRNA EXPRESSION OF CYTOKINE IN CHICKENS AFTER *Enterococcus faecium* EF55 ADMINISTRATION AND *Salmonella enterica* Enteritidis INFECTION

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Summary: Quantitative RT-PCR was used to determine the mRNA expression of pro-inflammatory cytokines IL-15, IL-17, IL-18, LITAF, iNOS, and LyTact chemokine at the caecum and spleen of experimentally infected chicks with *S. enterica* serovar Enteritidis SE147 preventively treated with *Enterococcus faecium* EF55. One-day-old ROSS 308 female chicks (100) were randomly divided into 4 groups (n=25). The chicks of the probiotic (EF) and *Salmonella*+ probiotic (EFSE) groups received per os *E. faecium* EF55 (10^9 CFU/day) from 1 to 7 days of the experiment. The birds of the *Salmonella* (SE) and EFSE groups were perorally infected with *Salmonella* Enteritidis SE147 in a single dose (10^8 CFU) on Day 4 of the experiment.
The preventive administration of *E. faecium* EF55 showed higher, however no significant, mRNA levels of studied pro-inflammatory cytokines and LyTact chemokine except of iNOS in caecum of EFSE experimental group of chicks mainly at Day 1 after *S.* Enteritidis SE147 infection compared to other groups. In contrast, the dynamics of cytokine/chemokine gene specific responses in caecum are not correlated with responses in the spleen.

Key words: cytokines; chemokines; qRT-PCR; chickens; *E. faecium* EF55; *S.* Enteritidis SE147

Introduction

Salmonella enterica is one of the major causes of human food-borne gastroenteritis worldwide. Poultry is considered to be the most significant source of *S. Enteritidis* for humans (6). Therefore, knowledge of the host immune response against *Salmonella* is essential for the understanding of its pathophysiology, prevention and treatment.

Aviansystemic salmonellosis has three distinct phases characterised by significant interaction withthe immune system. In the first phase, the invasion via the gastrointestinal tract occurs (11). *Salmonella* spp.penetrates the intestinal epithelium and enters the Peyer's patches. From the Peyer's patches, *Salmonella* spp. moves to the mesenteric lymph nodes, spreads to the circulatory system, and leads to transient bacteraemia (27). In this phase, there is massive chemotaxis of chemokines (IL-8, CXC, MIP-1β) together with IL-1 and IL-6 into intestinal mucosa. Bacteria are rapidly cleared from the blood by phagocytes in spleen and liver, and a large fraction of bacteria are killed by these cells (4). The second phase includes the establishment of systemic infection, mainly as an intracellular infection of macrophages (11). Activation of macrophages leads to the secretion of pro-inflammatory Th1 cytokines, such as IL-1, TNFα, IFNγ, IL-12, IL-15 and IL-18. Finally, infection may be cleared by the immune response, the birds may succumb to the infection or a subclinical carrier state may develop.

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Manipulationof the gut microbiota of chickens by the administration of probiotic bacteria may help to control enteric bacterial infections, including *Salmonella* invasion (20). The mechanisms of probioticeffects are, however, poorly understood, especially at the molecular level. Several mechanisms might be involved in mediating the effects, including the competitive exclusion, production/presence of antibacterial substances (e.g. bacteriocins or colicins), and the modulation of immune responses (22, 9). These activities include the ability to induce cytokine production leading to the regulation of innate and acquired immune responses. Finally, it has been shown that certain species of lactobacilli strains induce the production of cytokine-promoting Th1 effector functions, such as IL-12 (13, 10), while the other strains of probiotic bacteria induce the production of regulatory inflammatory cytokines, such as IL-10 and TGF-β (23).

Therefore, the current study was undertaken to determine the influence of probiotic bacteria on the pro-inflammatory cytokine and chemokine mRNA profiles during *S.* Enteritidis SE147 infection in chickens to study their potential role in pathogenesis and activation of the avian immune system.

Materials and methods

Experimental animals

Atotal of 100 one-day-old hybrid ROSS 308female chicks were included in the experiment. One day-old chicks were placed in large pens with cellulose cotton (Pehazell, Slovakia) and reared with a lighting regimen of 23^h light and 1^h dark. The initial room temperature of 32–33°C was reduced weekly by 1°C to a final temperature of 28°C. Relative humidity was within a range of 50–6 0%. Birds had free acces to feed (BR1 – starter diet) and water. Application of cleaning and feeding regimens prevented them from cross-contamination effectively throughout the experiment. Chicks were randomly divided into 4 groups (n=25): control (C), *E. faecium* EF55 (EF), combined *E. faecium*EF55+*S.* Enteritidis SE147 (EFSE) and *S.* EnteritidisSE147 (SE).The probiotic strain *Enterococcus faecium* EF55 (provided by Lauková, IAP SAS, Košice, Slovakia) was individually *per os* administered to EF and EFSE groups from 1 to 7 days (1.10^9 CFU/0.2 ml PBS). Experimental infection of SE and EFSE groups was also done individually *per os* using *Salmonella enterica* serovar Enteritidis SE 147 (provided by Rychlík, VRI, Brno, Czech Republic) in a single dose (1.10^8 CFU/0.2 ml PBS) on Day 4. Five chicks from each group were euthanized on Days 1, 2, 3, 4 and 7 post infection (p.i.) with *salmonella*. Samples of spleen and the caudal part of both caeca were taken from each animal during necropsy.

Homogenization of tissue and isolation of total RNA

Tissue samples (spleen and caecum) were cut into 20 mg pieces, immediately placed into RNALater solution (Qiagen, UK) and stored at -70 °C prior to RNA purification. After storage, a single tissue fragment was transferred into 1 ml of TRI Reagent (Molecular Research Center, USA) and homogenized using zirconium silica beads (BioSpec Products, USA) and a vortex mixer (Labnet, USA). To separate the phases, 50 µl of 4-bromanisole (Molecular Research Center, USA) was added. The entire content of the tube was centrifuged and the upper aqueous phase was collected for total RNA purification by using the RNAeasy mini-kit (Qiagen, UK) following the manufacturer's instructions. A TurboDNA-free kit (Ambion, USA) was used for treatment of RNA samples to remove genomic DNA. The purity and concentration of RNA was determined spectrophotometricallywith NanoDrop 200c, (Thermo Scientific, USA) and 1 µg of total RNA was immediately reverse transcribed by using iScriptcDNA Synthesis Kit (Bio-Rad, USA). The resulting cDNA was 10× diluted in UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and used as atemplate in real-time PCR (RT-PCR) or stored at -20 °C until used.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The mRNA levels of IL-15, IL-17, IL-18, lipopoly-saccharide-induced TNF-α factor (LITAF), iNOS and chemokine Lymphotactin (LyTact) were determined. In addition, mRNA expression of two references genes, coding for GAPDH (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin (UB), was determined and used for data normalization. The primer sequences used for qPCR are listed in Table 1. All primer sets allowed DNA amplification efficiencies between 94% and 100%.

The amplification and detection of specific products were performed using CFX 96 RT system

(Bio-Rad, USA) with the following temperature-time profile: initial denaturation 15 min 95 °C and 45 cycles: denaturation 95 °C for 20 sec, annealing 60 °C for 30 sec, and final elongation 72° C for 30 sec. A melting curve from 50 °C to 95 °C with a reading at every 0.5 °C was performed for each individual RT-PCR plate. Each sample was subjected to RT-PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. We also confirmed that the efficiency of amplification of each target gene (including GAPDH, UB) was essentially 100% in the exponential phase of the reaction, where the cycle quantification (Cq) was calculated. The Cq values of interest genes were normalised to an average Cq value of the reference genes (ΔCq), and the relative expression of each representative value was calculated as $2^{-\Delta Cq}$. These expression levels were then used for comparative data analysis. Relative mRNA expression of cytokine in the spleen and caecum were determined in five independent animals, and we present the results combined from all of these animalsin the figures.

Statistical analysis

Statistical analysis of the results was performed using one-way ANOVA with Tukey post-test by Minitab 16 software (SC&C Partner, Brno, Czech Republic). In the results, the average values are expressed as mean ± SEM. The values of $P < 0.05$ were considered significant.

Results

All studied genes for cytokines (IL-15, IL-17, IL-18, LITAF,iNOS, as well as chemokine LyTact) were detected during whole experiment in allsamplings and groups; however, no statistically significant differences were determined.

Interleukin-15 achieved the maximal levels in the spleen (Fig.1a),and picked up the highest levels of expression in the SE group from 1-4 day p.i. whencomparedto controls, followed with the EFSE group on days 1 and 2 p.i. In three days p.i., the values of EFSE group were higher than SE and the maximal improvement showed EF group. In contrast, the IL-15 levels of EFSE group in the caecum exceeded the level of C and SE groups in all samplings with exception on Day 4 p.i. (Fig.1b).

Interleukin-17 showed in spleen thehighest expression in SE group on Day 2 p.i. butin EF group on Day 1 p.i.(Fig. 2a). In the caecum,the expression of EFSE group at Day 1 p.i. reached the maximum level, similarly to IL-15 (Fig. 2b).

Interleukin-18 in the spleen achieved the highest expression on Day 1p.i. in SE group followed with EFSE group (Fig.3a), butin the caecum this combined group over-exceeded the values of SE infected group at this day (Fig. 3b).

Table 1: List of primers used for chicken cytokine mRNA quantification

Primer	**Sequence 5'–3'**	**References**
GAPDH For	CCTGCATCTGCCCATTT	(De Boever *et al.*,2008)
GAPDH Rev	GGCACGCCATCACTATC	
UB For	GGGATGCAGATCTTCGTGAAA	(De Boever *et al.*,2008))
UB Rev	CTTGCCAGCAAAGATCAACCTT	
IL-15 For	TGGAGCTGATCAAGACATCTG	(Kolesárová *et al.*, 2011)
IL-15 Rev	CATTACAGGTTCCTGGCATTC	
IL-17 For	TATCAGCAAACGCTCACTGG	(Crhánová *et al.*,2011)
IL-17 Rev	AGTTCACGCACCTGGAATG	
IL-18 For	ACGTGGCAGCTTTTGAAGAT	(Rýchlik *et al.*, 2009)
IL-18 Rev	GCGGTGGTTTTGTAACAGTG	
LITAF For	AATTTGCAGGCTGTTTCTGC	(Kolesárová *et al.*, 2011)
LITAF Rev	TATGAAGGTGGTGCAGATGG	
LymphotactinFor	CATAGTCTGGCTTGGCGTCTT	(Withanage *et al.*, 2004)
LymphotactinRev	GCGCATTGACTGACTTGCA	
iNOS For	GAACAGCCAGCTCATCCGATA	(Berndt *et al.*, 2007)
iNOS Rev	CCCAAGCTCAATGCACAACTT	

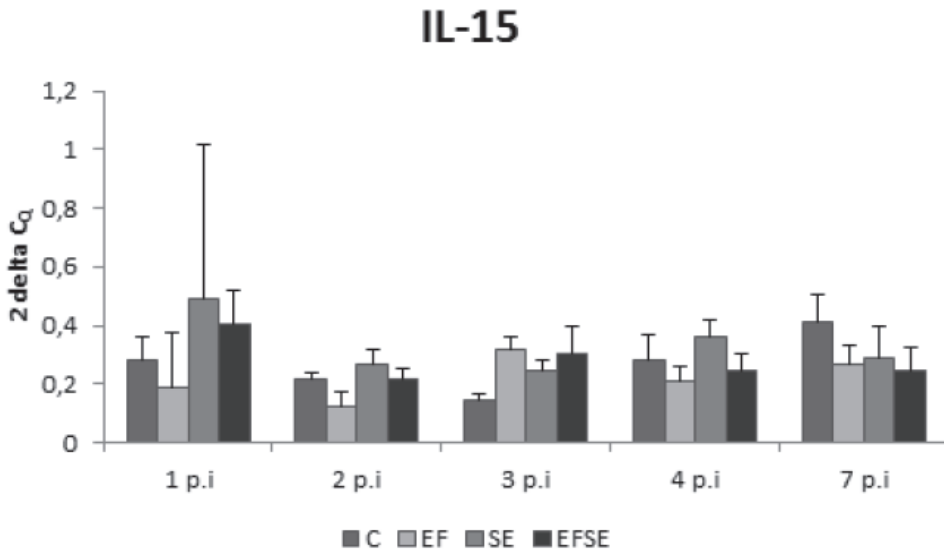

Figure 1a: Relative expression of IL-15 mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147, EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

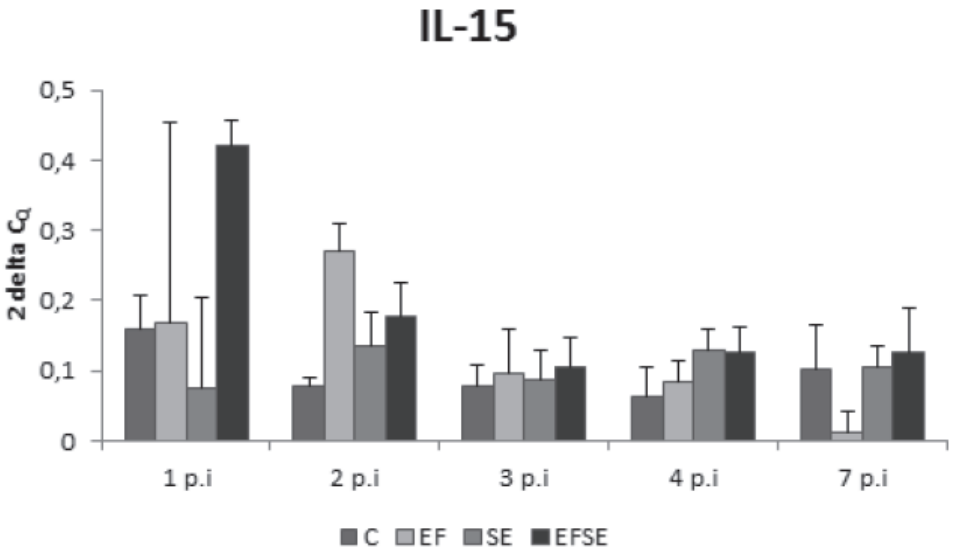

Figure 1b: Relative expression of IL-15 mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

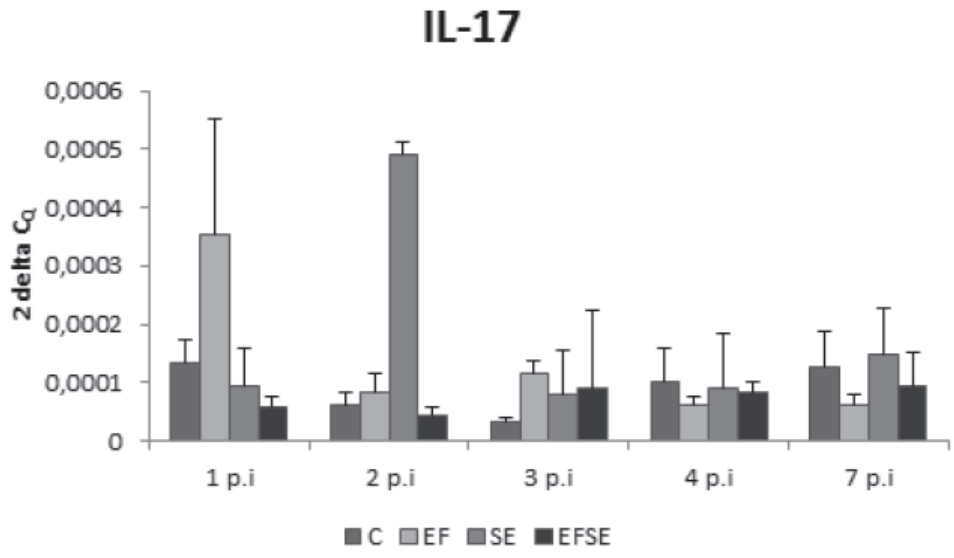

Figure 2a: Relative expression of IL-17 mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.*Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

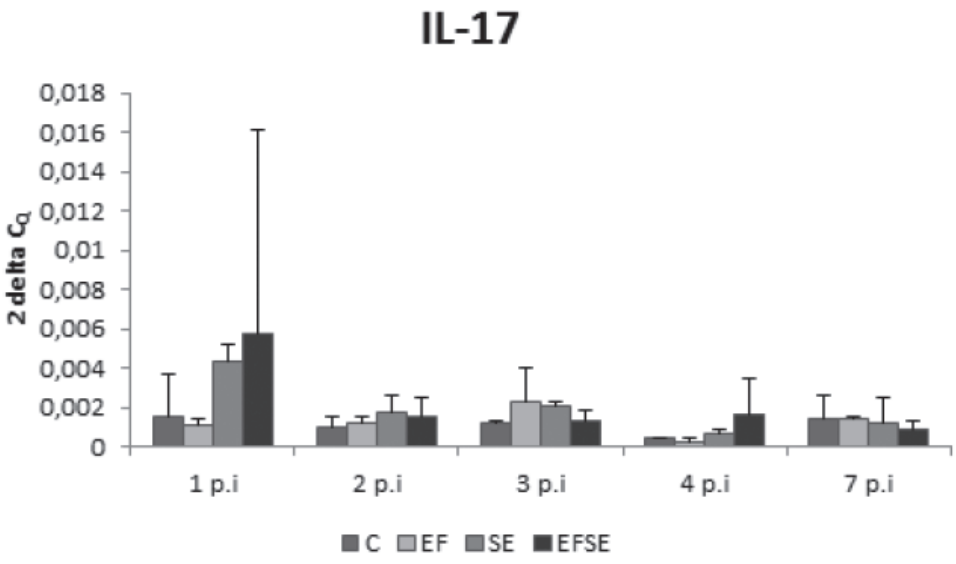

Figure 2b: Relative expression of IL-17 mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.*Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

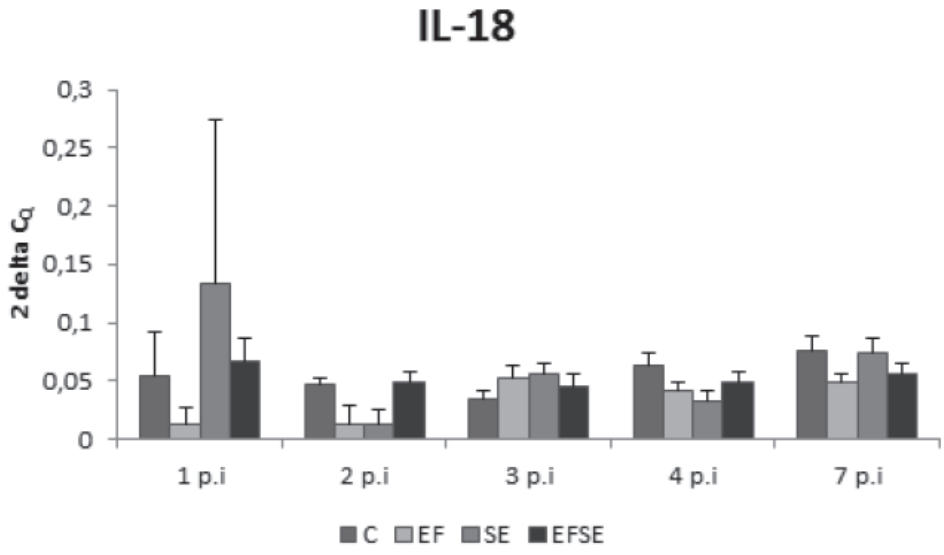

Figure 3a: Relative expression of IL-18 mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.*Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

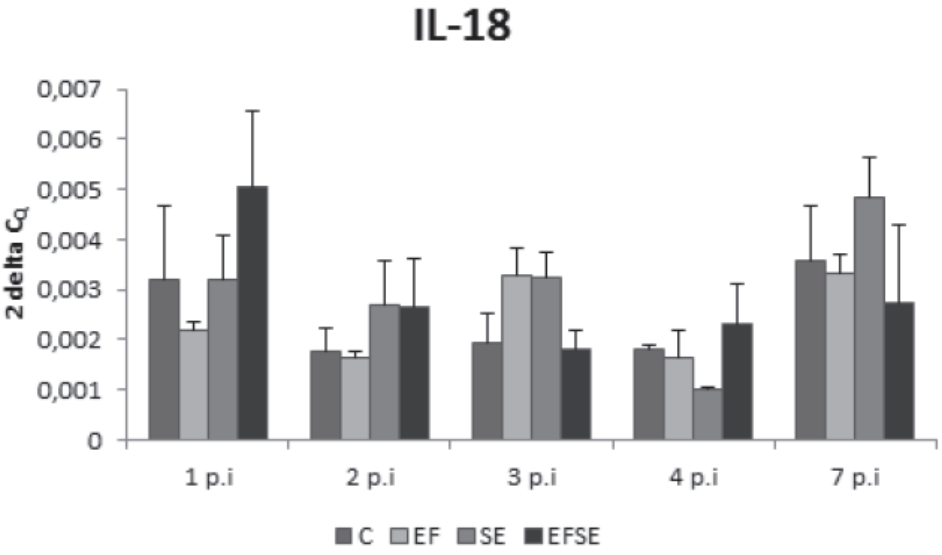

Figure 3b: Relative expression of IL-18 mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

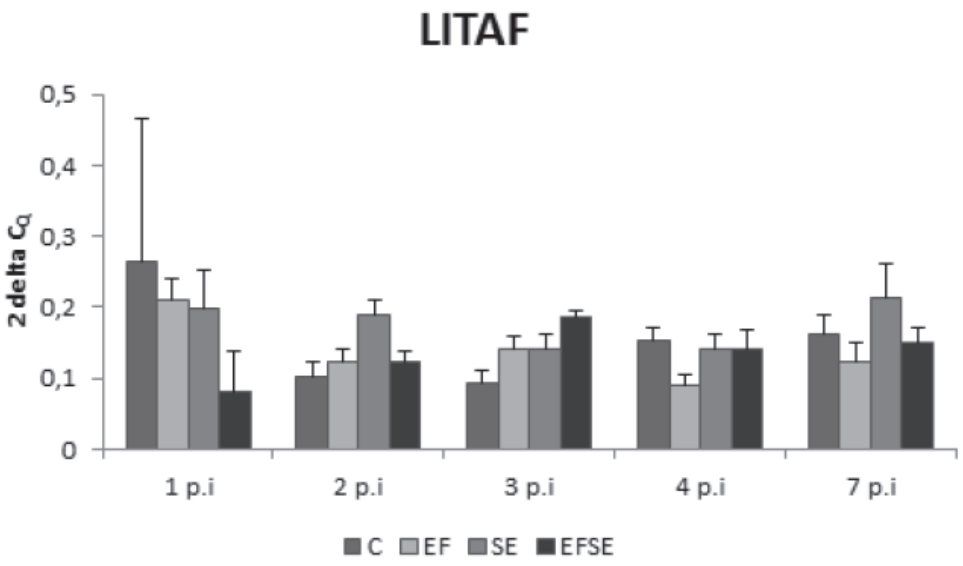

Figure 4a: Relative expression of LITAF mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

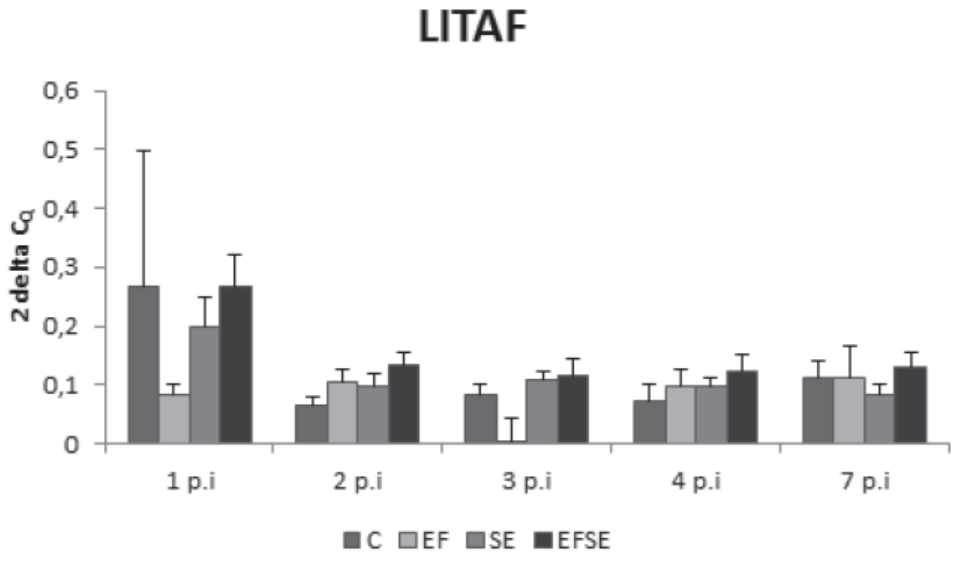

Figure 4b: Relative expression of LITAF mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

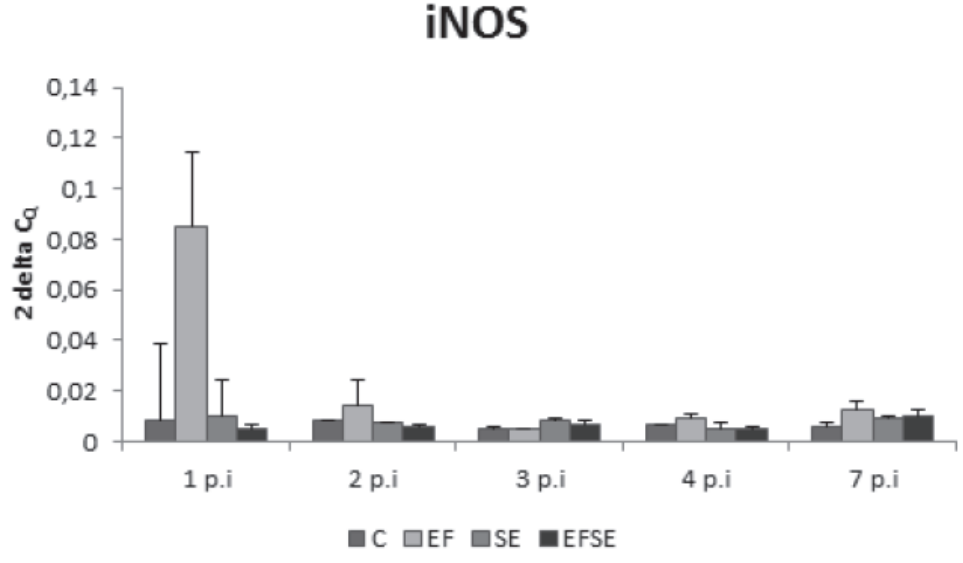

Figure 5a: Relative expression ofiNOS mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

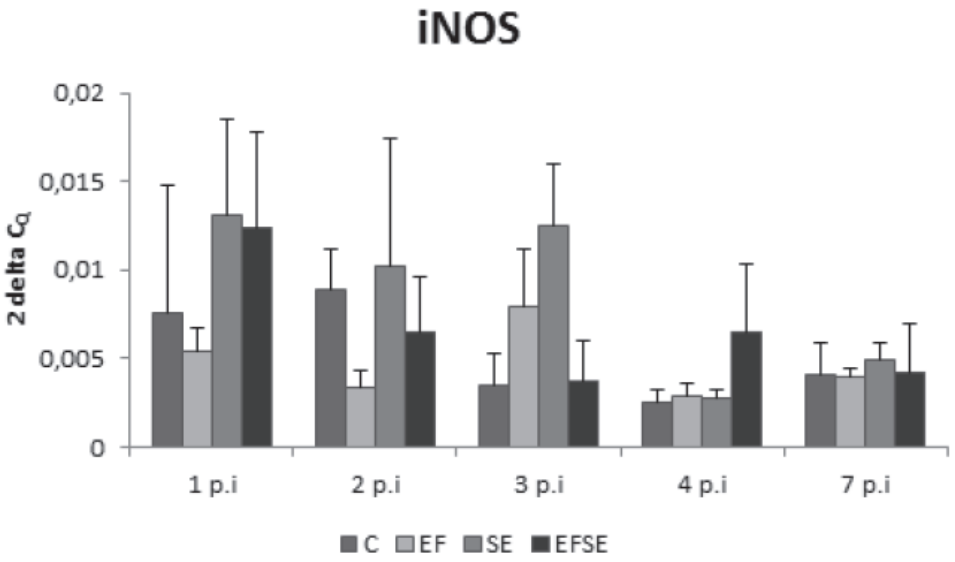

Figure 5b: Relative expression of iNOS mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

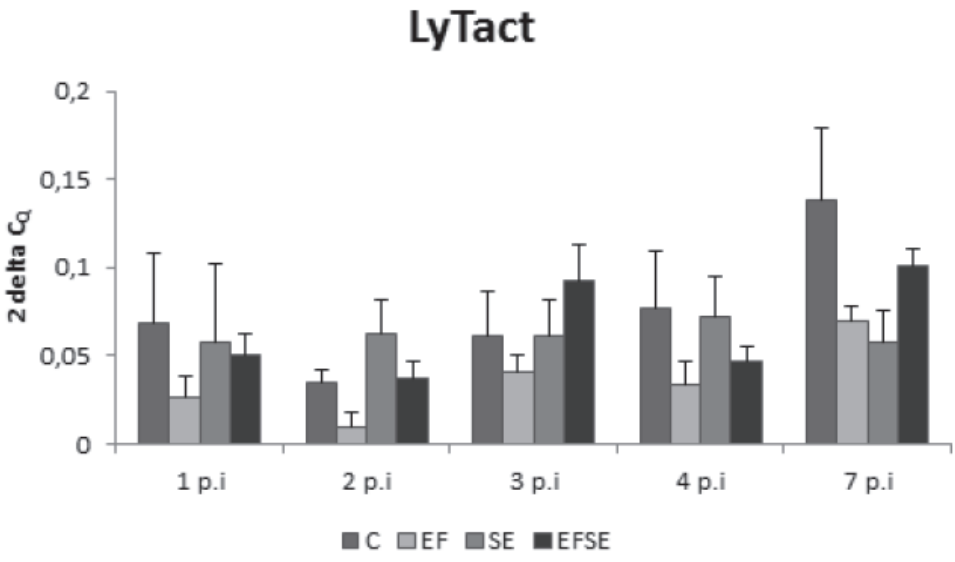

Figure 6a: Relative expression of LyTact mRNA in the spleen.
Legend**:** C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

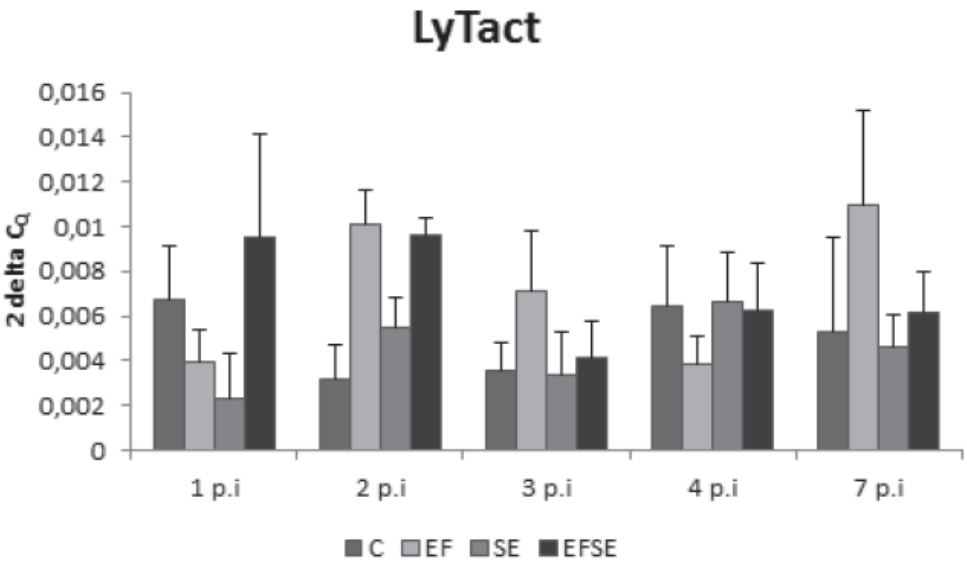

Figure 6b: Relative expression of LyTact mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

LITAF expression in higher levels was found in the spleen on Days 2 and 7 p.i. in SE group, as well as in EFSE group on Day 3 p.i. (Fig 4a).In contrast, in the caecum, the expression of LITAF was the highest in the EFSE group compared with another groups from Days 1 to 7 p.i. (Fig.4b).

iNOS detection in the spleen showed the highestlevel of expression in EF group during experiment on Day 1 p.i. in comparison with other groups(Fig.5a). However, in the caecum the maximum levels reached SE groups except for Day 4 p.i. when values of EFSE group were the highest (Fig. 5b).

LyTactchemokine expression in the spleen increased on Day 2 p.i. in SE, and on day 3 p.i. inEFSE groups. (Fig. 6a).Caecum of chickens treated with *E. faecium* EF55 showed the highest levels of expressionon Days 2, 3 and 7 p.i. together with SE+EF group on Days 1, 2, 3, and 7 p.i. (Fig. 6b).

Discussion

Cytokines are essential effector molecules, which initiate and coordinate cellular and humoral immune response against pathogens.Specifically, the increased mRNA expression and protein secretion of chemokines, proinflammatory and Th1 cytokines such as IFN-γ, IL-1, IL-6, IL-8, IL-12, and MIP-1β are observed following infection with various *Salmonella* species (12).

The current results demonstrate the modulation of other kinds ofcytokine mRNA expression in chickens after administration of *E. faecium* EF55 and challenged with *S.* Enteritidis SE147. The early increased expression of IL-17 mRNA in the intestinal mucosa of chickens in EFSE group suggests that enhanced expression of cytokine can be consistent with the moving of phagocytic cells into infected intestine. Earlier *in vivo* study indicated that IL-17 is a potent activator of neutrophils, both through expansion of the lineage via the regulation of G-CSF receptor as well as recruitment through regulation of chemokine expression. Ectopic expression of IL-17 stimulated a strong neutrophilic response (8). Furthermore, our earlier work with *S.* EnteritidisPT4 (17) demonstrated the increased density ofheterophilsin the blood of EFSE group on Day 3 p.i. In the current experiment the increased of IL-17as well as chemokine lymphotactin in the caecum of combined treatment EFSE group on the Day 1p.i confirms the assumption of mutual cytokine interactions at the local level. This result demonstrates the early activation of the natural immunity components in the caecum after preventive *E. faecium* EF55 administration. Moreover, increased mRNA expression of IL-17 only in the caecum may suggestthat cytokine is involved mainly in mucosal immunity.Coccia (2012) showed that IL-17 enhances production of antimicrobial peptides by intestinal epithelial cells. This finding is consistent with our previous report about decreased numbers of *S.* Enteritidis in the caecum of chickens after administration of *E. faecium* EF55 and challenged with *S.* Enteritidis (16). Similarly, our laboratory recently demonstrated the increased number of IgA+ caecal intraepithelial lymphocytes in the EFSE group (18), which suggests an important role of IL-17 in local immune response.

Interleukin-18 is the most important growth factor for avian CD4+ cells and enhances the cytotoxic activity of NK cells (14). IL-15 is indispensable for long-term survival of memory CD8+ T cells and up-regulates the proliferation of cytotoxic and helper T-cells for the direction of heterophils and NK cells to the site of inflammation (19). Chickens infected with *S.*Enteritidis SE147 in the current trial presented the highest increase of IL-18 and IL-15 mRNA expression on the first day p.i. to confirm the activation of specific immune components. However, further sampling showed declined level of IL-18 mRNA expression.

In the EFSE group, the IL-15 and IL-18 expression was maintained approximately at the same levels for all the samplings. The infection of chickens with *S.*Enteritidis showed a significant increase of CD4+ and CD8+ lymphocytes. However, chickens treated with EF55 and infected with *S.* Enteritidis revealed increases of CD4+ and CD8+ cells (25), whichsuggests enhancing the role of IL-15 and IL-18 also in our current trial. Modulation of these cytokines suggests the stimulatory action of *E. faecium* EF55 in the direction of Th1 immune responses.

Several authors demonstrated suppressive effect of some probiotic strains on TNF-α production by host immune cells (13, 24, 29). We observed similar effect of *E. faecium* EF55 on LITAF cytokine production in caecum at the first day p.i. in EF group.

Macrophages and other effector cells express inducible nitric oxide synthase (iNOS) that is an integral part of the host defence mechanisms. The synthases activate the nitric oxide (NO), which is

typical of their strong bacteriostatic effect against intracellular bacteria (28). Similarly, *in vitro* cultivated chickens' macrophages infected with *Salmonella* accounted for the important role of the iNOS enzyme in the defence against *Salmonella* (30). In the current experiment, the highest although not significant iNOSexpression observed in the spleen of EF group at first day p.i.suggests the action of *E. faecium* EF55 in the direction of Th1 polarization. The highest iNOS expression found in the spleen of the SE and EFSE groups at the first day p.i. suggests that *E. faecium* EF55 could also stimulate splenic macrophages.

Chemokines can stimulate the chemotaxis of leukocytes adhering to the activated endothelium via adhesion molecules (selectins, integrins, and members of the large family of immunoglobulin) (3). Our current results showed that *S.* Enteritidis SE147 could affect the expression of immune system components, including chemokines. Lymphotactin acts as a potential chemoatractant for T and NK cells (2). However, Zhang et al. (32) found a decreased level of lymphotactin expression in chickens infected with *S.* Enteritidis. Similarly, we also observed a decreased level of LyTactexpression in the spleen of SE group in comparison with the control group at first day p.i. In contrast, Meyer et al. (21) showed that *in vitro* some probiotic enhanced strains stimulated production of chemokines. In the current study, we found the increased of LyTactexpression in ceaca of EFSE group at Days 1, 2, and 7 p.i. This finding suggests that an increased expression of lymphotactin in the caeca during intestinal infection may play a key role in the migration of cells into site of inflammation.

In conclusion, the preventive administrationof *E. faecium* EF55 to *S.* Enteritidis SE147 infected chicks showed some immunomodulatory effect in the caeca, mainly at the first day p.i. presented by no significant higher levels of pro-inflammatory gene expression cytokines IL-15, IL-17, IL-18, LITAF, and LyTact chemokine. However, the dynamics of cytokine/chemokine gene specific responses in caecum are not correlated with responses in the spleen.

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RAZLIKE V IZRAŽENOSTI GENOV ZA CITOKINE PRI PIŠČANCIH PO DODAJANJU *Enterococcus faecium* EF55 IN OKUŽBI S *Salmonella enterica* enteritidis

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Povzetek: Kvantitativna metoda RT-PCR je bila uporabljena za določanje izražanja mRNK vnetnih citokinov IL-15, IL-17, IL-18, LITAF, iNOS in kemokinov LyTact v slepem črevesu in vranici pri piščancih, poskusno okuženih s *S. enterica* serovar enteritidis SE147 ter nato preventivno zdravljenih z *Enterococcus faecium* EF55. En dan stari piščanci ROSS 308 ženskega spola (100) so bili naključno razdeljeni v 4 skupine (n=25). Piščanci iz probiotične (EF) in salmonele+ probiotične (EFSE) skupine so prejeli per os *E. faecium* EF55 (109 CFU/dan) od 1. do 7. dneva poskusa. Piščanci iz skupin salmonele (SE) in skupin EFSE so bili peroralno okuženi s *Salmonella* enteritidis SE147 v enkratnem odmerku (108 CFU) 4. dan poskusa.
Preventivno dodajanje *E. faecium* EF55 je povzročilo statistično neznačilno povišanje, izraženosti m RNK raziskanih vnetnih citokinov in LyTact kemokina, razen pri izraženosti mRNK za iNOS v slepem črevesu poskusne skupine piščancev EFSE 1. dan po okužbi s *S.* enteritidis SE147. Prav tako niso bili ugotovljeni specifični odzivi v izraženosti citokinov/kemokinskih genov v vranici.

Ključne besede: citokini; kemokini; QRT-PCR; kokoši; *E. faecium* EF55; *S.* enteritidis SE147

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Original Scientific Article

EFFECTS OF *DGAT1* AND *GH1* POLYMORPHISM ON MILK YIELD IN HOLSTEIN COWS REARED IN TURKEY

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Summary: The aim of this study was to analyse the associations among the K232A polymorphism in the diacylglycerol acyltransferase1 (*DGAT1*) gene and L127V polymorphism in the bovine growth hormone (*GH1*) gene and milk yield in Holstein cows. A total of 281 Holstein cows from three different dairy cattle farms in the Burdur and Kayseri provinces of Turkey were included in this study. The PCR-RFLP method was used for *DGAT1* and *GH1* genotyping. The frequencies of genotypes and alleles of *GH1* gene were found to be 0.78 for LL, 0.18 for LV and 0.04 for VV; 0.87 for the L allele and 0.13 for the V allele. The frequencies of genotypes and alleles of the *DGAT1* gene were to be 0.61 for AA, 0.30 for KA and 0.09 for KK; 0.76 for the A allele and 0.24 for the K allele. No relations were found between *DGAT1*-K232A genotypes and the average milk yield in the first three lactations. However, an association between *GH1*-L127V polymorphism and the average milk yield of Holstein cows was found in the first three lactations. Cows with LL genotype had higher milk yield compared to LV and VV cows ($P<0.05$).

Key words: *DGAT1*; *GH1*; Holstein; milk yield; PCR-RFLP

Introduction

Estimation of the future performance of livestock is a crucial subject in farm animal breeding. Male and female animals with superior features should be selected to accelerate genetic improvement (1). The genetic potential of breeder candidates can be directly determined using the available molecular genetic methods (2). For this purpose, quantitative genetic methods have been used. Recent developments in the field of molecular biology have been used in livestock breeding and selection methods as an additional tool (3).

Selection programmes in dairy cattle breeding are basically aimed at milk production traits, milk components and fertility. These traits are known as polygenic traits, and they are controlled by numerous genetic loci and influenced by many environmental factors. Therefore, it is thought that candidate genes with close linkage of the encoding loci can be used to estimate milk production performance (4). Several polymorphisms in different gene loci have been noted to affect production traits such as milk yield and milk composition (2). For instance, it has been reported that there are

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strong association between acylCoA-diacylglycerol acyltransferase1 (*DGAT1*) genotypes and milk traits (5); bovine growth hormone (*GH1*) genotypes and milk yield (6). Many genome scans have shown that QTLs on *Bos taurus* autosomal (BTA) chromosomes 6, 14, 20, and 26 have significant effects on milk yield traits (7). It is thought that the selection of animals with favourable genotypes of genetic markers may be possible, thus allowing rapid genetic progress in dairy cattle breeding. Particularly in the last few decades, with regard to this topic, several potential candidate genes have been recognised in cattle.

Triglyceride synthesis is catalysed by *DGAT1*. This enzyme plays a major role in intestinal fat absorption, lipoprotein synthesis, the development of adipose tissue and lactation in higher eukaryotes (8). The bovine *DGAT1* gene, which is located on the centromeric end of BTA14, has been reported as a candidate gene for QTLs associated with fat content and milk yield (5, 9, 10,11).

Bovine growth hormone directly or indirectly affects many physiological processes such as lactation, growth and reproduction (12). Therefore, it is thought that *GH1* can be considered as a genetic marker of milk productivity in cattle (13). Previous studies have shown that there might be an association between allelic variants of the *GH1* gene and milk yield traits (14). Several polymorphisms were found in the *GH1* gene (1, 4). The best known of these polymorphisms is the leucine (L allele) to valine (V allele) substitution at position 127 in exon 5 of the *GH1* gene. The effects of L127V polymorphism on milk production in cattle have been studied to some extent but the results obtained by various researchers have been found to be contradictory (1, 15).

The Holstein is the most commonly reared dairy cattle breed (16) and 92% of annual milk production is obtained from imported breeds such as Holstein, Simmental and Brown Swiss and their crossbreeds in Turkey. The association among *GH1* and *DGAT1* gene polymorphisms and milk yield has been studied in different countries (1, 11, 12). However, according to the authors' knowledge, no study has as yet been conducted on the association among *GH1* and *DGAT1* polymorphisms and milk yield in the Holstein breed in Turkey.

The aim of this study was to analyse the association of *GH1* and *DGAT1* gene polymorphisms with average (1., 2. and 3. lactations) milk yield in a Turkish Holstein-Friesian cow population.

Materials and methods

PCR-RFLP assay for DGAT1 and GH1 genotypes

A total of 281 Holstein cows, in the third lactation, were used in this study. The DNA was extracted from whole blood using phenol-chloroform method (17).

For detection of *DGAT1* genotypes, a 411 bp DNA fragment was amplified by PCR. PCR was carried out in a 25μL volume containing 5 pmol of each primer (forward: 5'-GCACCATCCTCTTCCTCAAG-3'; reverse: 5'-GGAAGCGCTTTCGGATG-3') (Genbank no. AJ318490.1) 1.5 mM $MgCl_2$, 200 μM dNTP mix, 1 X PCR buffer, 1U Taq polymerase and 100 ng of genomic DNA template. PCR included the following steps: pre-denaturation of 95 °C/5 min followed by 35 cycles at 94 °C/1 min, 60 °C/1 min, 72 °C/1 min and final extension at 72 °C/10 min. Within the *DGAT1* gene the non-conservative polymorphisms K232A at position 10433 and 10434 in exon 8 giving rise to a lysine by alanine amino acid substitution. The amplified PCR products were digested using the *Cfr*I (MBI Fermentas) enzyme. The PCR mixture for the *GH1* gene was prepared in the same way as for *DGAT1*, and a 223 bp DNA fragment was amplified. PCR products were amplified using forward 5'-GCTGCTCCTGAGGGCCCTTCG-3' and reverse 5'-GCGGCGGCACTTCATGACCCT-3' (Genbank no. JQ711182.1) primers. The cycles applied were as follows; pre-denaturation at 95 °C/4 min, followed by 35 cycles at 94°C/40sec, 60 °C/40 sec, 72°C/40 sec and the final extension at 72 °C/5 min. Amplified PCR products were digested with the *Alu*I (MBI Fermentas). Polymorphism is the leucine (L allele) to valine (V allele) substitution at position 127 in exon 5 of the *GH1* gene.

Data sets and statistical analysis

Data for daily milk production in the first three lactations were obtained from the Cattle Breeders' Association of Burdur and Kayseri. Direct counting was used to estimate the genotype and allele frequencies of the *DGAT1* gene *Cfr*I and *GH1* gene *Alu*I genetic variants. The chi-square test (x^2) was used to check whether the populations were in Hardy-Weinberg equilibrium using PopGene32 software (18). Mean differences of milk yield among genotype groups were assessed by analysis

Table 1: Allelic and genotypic frequencies of *DGAT1* and *GH1* genes

Loci	Frequency	Genotype			Allele Frequency		Statistical Significant (Chi-squared HWE)
		AA	KA	KK	A allele	K allele	
DGAT1	Observed	172	83	26	0.76	0.24	X^2 = 10.23 P<0.01 (df=1)
	Expected	162.2	102.6	16.2			
		LL	LV	VV	L allele	V allele	
GH1	Observed	219	51	11	0.87	0.13	X^2 = 10.91 P<0.001 (df=1)
	Expected	212.7	63.5	4.7			

HWE: Hardy-Weinberg Equilibrium; df: degree of freedom

Table 2: Means and standard error of mean (SEM), coefficient of variation of milk yields (average daily milk yield in the first three lactations) in Holstein cows with different *DGAT1* and *GH1* genotypes

Loci	Genotype	N	Mean ± SEM (liter)	%V	Statistical Significance (ANOVA)
	AA	172	26.55± 0.39	19.2	
DGAT1	KA	83	25.64± 0.55	19.6	F: 1.73 P>0.05
	KK	26	27.60± 0.93	17.2	
β= 0.20	P= 0.966	R^2 = 0.001			
	LL	219	26.79± 0.33[a]	18.4	
GH1	LV	51	24.60± 0.69[b]	20.0	F: 3.95 **P<0.05**
	VV	11	26.43± 2.03[ab]	25.4	
β= -1.18	**P= 0.042**	R^2 = 0.015			

[a,b,c] : Different superscripts within the same column demonstrate significant differences; %V: Coefficient of variation; β: Regression coefficient; R^2: Determination coefficient

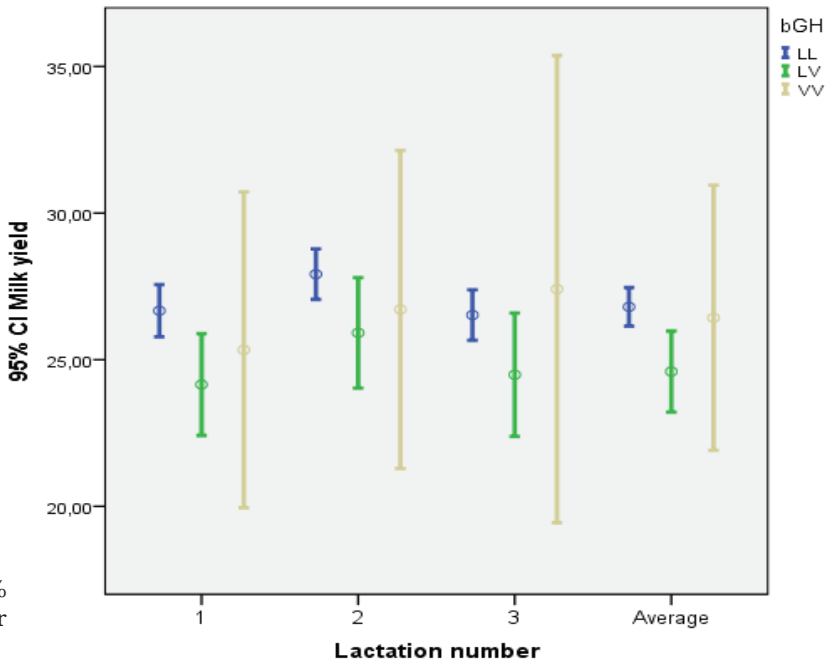

Figure 1: Error Bar graph with 95% Confidence interval (CI) of milk yields for *GH1* genotypes in Holstein cows

of variance and Sidak multiple comparisons test as post hoc test. The effects on the average milk yield of *GH1* and *DGAT1* genotypes were estimated using regression analysis. The software SPSS for Windows Version 14.01 (License number: 986964) was used in statistical analysis data.

Results

PCR amplification yielded a 411 bp long *DGAT1* gene fragment. Restriction digestion of 208 and 203 bp PCR products with the *Cfr*I enzyme revealed three genotypes of AA (208 and 203 bp), KK (411 bp) and KA (411, 208 and 203 bp). PCR amplification yielded a 223 bp long *GH1* gene fragment. Restriction digestion of 171 and 52 bp PCR products with the *Alu*I enzyme revealed three genotypes of LL (171 and 52 bp), VV (223bp) and LV (223, 171 and 52 bp). The allelic and genotypic frequencies of the *DGAT1* and *GH1* genes, and the polymorphisms for the Holstein cows are given in Table 1. Significant deviation was observed from HWE in the Holstein breeds on *DGAT1* ($P<0.01$) and *GH1* ($P<0.001$) genes.

Our findings revealed that no-significant difference was found among cows with different *DGAT1* genotypes (AA, AK, KK) in terms of average milk yield per day ($P>0.05$). The LL and VV genotypes of the *GH1* gene were better than the LV genotype for average daily milk yield (Table 2) ($P<0.05$). Additionally it was found that animals who carry the LL genotype had a higher, and more homogeneous milk yield than those with the other two genotypes (Figure 1). On the other hand, it was found that VV genotype cows show a similar feature together with LL and LV genotypes in terms of daily milk yield, but VV genotype cows showed a higher variation coefficient (25%) (Table 2).

In this study, regression analysis was also performed and the regression coefficient values of genotypes were calculated. The effect of *DGAT1* genotypes on average milk yield was not found significant ($p=0.966$). The regression coefficient (R^2) value of the *DGAT1* gene was found to be very low (<0.001) in the Holstein cows studied. On the other hand, the effects of *GH1* genotypes on milk yield were found to be significant, and the regression coefficient (R^2) value was found as 0.015.

Discussion

In this study, due to their potential roles in milk yield, the *GH1* and *DGAT1* genes were studied to evaluate their effects on milk yield in Holstein cows.

The frequency of the *GH1*-V allele was found to be lower than that of the L allele; this has been generally reported in all Holstein populations (12, 15). Similarly, in this study, the frequency of the *GH1*-V allele was found to be lower than that of the L allele. It was reported that the highest frequency of the L allele was found in larger sized dairy breeds such as Holstein, and this allele was also correlated with higher milk production (19). In addition, it was reported that selection for milk yield in the German Holstein cattle population has been indirectly effective in the spreading of the *GH1*-L allele. Hence, the frequency of the V allele was found to be very low in German Holstein sires (12). Similarly, the *GH1*-L allele frequency was also found to be higher than that of the V allele in our study. The high *GH1*-L allele frequency in the examined Holstein cattle may have caused the deviation from HWE. On the other hand, it is aimed to increase the milk yield in Holstein breeding in Turkey for many years. Hence, it may lead to HWE in terms of *GH1*-L allele in the Turkish Holstein population. It is thought that this selection in *GH1* gene may cause deviation from HWE in *DGAT1* gene as well. In addition, Thaller et al. (11) reported that *DGAT1*-A allele frequency was found to be higher than that of the K allele and was fixed in German Holstein cattle population. This information may be an explanation of the deviation from HWE for *GH1* and *DGAT1* genes in the Holstein cows examined in our study, because an important part of the Turkish Holstein cattle population originated from Germany and the US (20). Similarly, *DGAT1*-A allele frequency was also found to be considerably higher than that of the K allele in our study. On the other hand, the deviation from HWE for *GH1* and *DGAT1* genes may be due to the number of animal population used in this study.

It was reported that there was a significant association between the *GH1*-L127V genotypes and milk production traits in different cattle breeds (13, 21). Nevertheless, this information is still controversial. For example, Zwierzchowski et al. (22) reported that the presence of the V allele

may be indicative of better performance in daily milk yield and milk composition in Holstein cows. Similarly, V allele frequency was reported to be very common in best Canadian Holstein AI bulls by Sabour and Lin (23). Kovacs et al. (1) found that the V allele was preferred for increased milk production traits in a Hungarian Holstein cattle population. Khatami et al. (13) investigated the relationship between *GH1* genotypes and milk production in a Russian Holstein cattle population and the Yaroslavl cattle breed, and they found that the rate of cows with high milk production (more than 6000 kg per lactation) was higher in cows with the VV genotype than in those with other genotypes. This rate was 1.75 times higher in cows with the VV genotype than in cows with LL and LV genotypes, respectively. The V allele was reported by Khatami et al. (13) to be superior commercially to the L allele, especially when present in homozygote form. On the other hand, there are studies indicating that the LV genotype is superior to other genotypes for milk yield. For instance, in Polish Holstein bulls and heifers, significant differences were reported between *GH1* genotypes by Grochowska et al. (24), and the highest values for milk and protein yields were observed for cows with the LV genotype. Similarly, Kovács et al. (1) reported that the *GH1*-LV genotype compared to other genotypes was shown to have a positive effect on 305 days lactation yield. Furthermore, there have also been studies in which the L allele was associated with high milk yield. For instance, the LL genotype of the *GH1* gene was significantly associated with better milk production traits, mainly with fat content in the Brown Swiss breed (6). Furthermore, cows with the LL genotype were shown to have a higher milk yield than *GH1*-LV cows in the first lactation. No significant differences between the genotypes and milk production traits in the second and third lactations in Polish Holstein cows were found by Dybus (15). In this study, the LL genotype of the *GH1* gene was significantly better for milk yield than the other two genotypes in Turkish Holstein cows.

DGAT1 is another gene which is thought to be associated with milk yield in cattle, and this gene has two alleles. The *DGAT1*-K allele has been associated with high milk fat yield (25), the *DGAT1*-A allele has been associated with high milk yield (5, 11).

The milk fat yield has become a desired characteristic in dairy cattle breeding in recent years. This situation may have been caused by an increase in *DGAT1*-K allele frequency in some populations. As a result of selection to increase milk yield, the frequency of the *DGAT1*-K allele decrease from 15 to 5% between 1981 and 1990 in Israel Holstein cows (26).

The milk yield of daughters of bulls with *DGAT1*-AA genotypes was found on average to be 548 kg higher than that of bulls with *DGAT1*-KK genotypes in the Holstein breed (12). Similarly, the *DGAT1*-A allele was found to have economically beneficial effects in German Holstein across all lactations. However, it was reported that this allele showed better performance in Fleckvieh cattle in only the first lactation (11).

On the other hand, there have been studies in which no relationship between *DGAT1* genotypes and milk yield existed. For instance, Näslund et al. (27) found no differences among the three *DGAT1* genotypes and milk yield in Swedish Holsteins. Similarly, we did not find any relationship between *DGAT1* alleles and milk yield in Holsteins in our study. To estimate the possible effects of *DGAT1* alleles on milk yield traits more studies should be conducted in dairy cattle breeds.

Milk yield is a multifactorial trait, and milk yield traits have been shown to be primarily and considerably influenced by environmental factors such as farm environment, management and feeding. Therefore, the observed differences between the *GH1* and *DGAT1* genotypes and milk production characteristics could have resulted from another source of variation such as the effects of herd and sire. The effects of the *GH1* and *DGAT1* genotypes on the milk yield, milk fat and milk protein content should be confirmed in further studies. However, it is not yet possible to say which genotypes of *GH1* and *DGAT1* should be recommended for the improvement of milk production traits.

In terms of herd management in farms, high and uniform milk yield is very important. It was found in this study that LL genotype cows show uniformity in terms of daily milk yield, whereas VV genotype cows have a higher variation coefficient. The study showed that *GH1* may be used in selection programmes in dairy cattle breeding. The crucial role of growth hormone in lactation initiation and maintenance is well known, thus *GH1* polymorphisms and interaction with other yield traits should be the subject of further research. Furthermore, *DGAT1* polymorphism was not found to have a significant effect on milk yield.

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VPLIVI POLIMORFIZMA *DGAT1* IN *GH1* NA MLEČNOST KRAV PASME HOLSTEIN, GOJENIH V TURČIJI

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Povzetek: Namen raziskave je bil proučiti povezave med polimorfizmom K232A v genu za diacilglicerol acyltransferaso 1 (*DGAT1*) in polimorfizmom L127V v genu govejega rastnega hormona (*GH1*) ter mlečnostjo krav holstein. Skupno je bilo v raziskavo vključenih 281 prvesnic in krav pasme holstein v drugi laktaciji s treh različnih mlečnih govedorejskih kmetij v provincah Burdur in Kayseri v Turčiji. Za genotipizacijo *DGAT1* in *GH1* je bila uporabljena metoda PCR-RFLP. Frekvence genotipov in alelov gena GH1 so bile 0,78 za LL, 0,18 za LV, 0,04 za VV, 0,87 za alel L in 0,13 za alel V. Frekvence genotipov in alelov gena *DGAT1* so bile 0,61 za AA, 0,30 za KA, 0,09 za KK, 0,76 za alel A in 0,24 za alel K. Med genotipi *DGAT1*-K232A in povprečno mlečnostjo v prvih treh laktacijah niso bile ugotovljene povezave. Ugotovljena pa je bila povezava med polimorfizmom *GH1*-L127V in povprečno mlečnostjo krav pasme Holstein v prvih treh laktacijah. Pri kravah z genotipom LL je namreč mlečnost višja kot pri kravah z genotipoma NN in VV ($p < 0,05$).

Ključne besede: *DGAT1*; *GH1*; holstein; proizvodnja mleka; PCR

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Original Scientific Article

DISTRIBUTION OF *Salmonella* Enteritidis GENOTYPES AMONG SELECTED BROILER FLOCKS IN BOSNIA AND HERZEGOVINA

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Summary: Cases of human salmonellosis continued decreasing at the EU level in 2012; a total of 92,916 cases were reported by 27 EU Member States. With 91,034 confirmed cases, this represented a 4.7% decrease in comparison to 2011. In the EU, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the serovars most frequently associated with human illness. Human *S.* Enteritidis cases are most commonly connected with the consumption of contaminated eggs and poultry. In 2012, the prevalence of *Salmonella* spp. in broiler flocks was 3.1%. Serovar Enteritidis was isolated in 0.2% of broiler flocks at the EU level. According to the first post-war national monitoring program conducted during 2012 in Bosnia and Herzegovina, the prevalence of *Salmonella* spp. and *S.* Enteritidis in broiler flocks was 10.0% and 8.7%, respectively. To obtain better insight into the epidemiology of the dominant serovar *S.* Enteritidis within the selected broiler flocks, genotyping with pulsed-field gel electrophoresis (PFGE) was performed using *Xba*I with isolates obtained in 2010–2011 from broiler farms located in seven geographical regions with the highest density of them. Due to the apparent similarity of the genotypes found in several broiler flocks, our findings suggest a homogenous population of *S.* Enteritidis circulating among the vast majority of broiler flocks. Secondly, since identical or very similar genotypes were also found in faecal samples from broiler flocks and dust samples from hatcheries, a common source of infection can be indicated.

Key words: *Salmonella* Enteritidis; epidemiology; genotyping; PFGE; poultry; broiler flocks; Bosnia and Herzegovina

Introduction

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) continues to be one of the leading causes of bacterial foodborne diseases throughout the world (1–3). This is mainly attributed to the consumption of contaminated poultry products (4). In addition to health concerns, *Salmonella* spp. affecting animals and humans represents a recognized economic burden due to the chronic effects of infections. A common reservoir of salmonellas remains in the intestinal tract of predominantly domestic poultry. Animal-to-human transmission occurs when the contaminated poultry and meat products are introduced into the food-production chain. In humans, the symptoms are often mild and self-limiting, although a severe disease with a fatal outcome is possible (5).

The transmission routes of salmonellas are various. Human salmonellosis is mostly associated with the consumption of faecally contaminated foodstuffs, such as eggs and poultry for *S.* Enteritidis, and pork, poultry and beef for the second most common serovar in humans in

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the EU, *Salmonella* Typhimurium (3). In children, contact with the infected puppies or turtles (6, 7) and the consumption of raw milk (8) have also been reported to cause salmonellosis. In 2011 and 2012, some unusual sources for human salmonelloses were observed, including contaminated mung bean sprouts in Germany (9), smoked salmon in the Netherlands (10), and watermelons in the UK (11). Such findings indicate the variety of hosts and transmission routes in *Salmonella* epidemiology. Nevertheless, contaminated poultry eggs and meat remain the most common source of human salmonellosis (5, 12).

Knowledge of the dissemination of certain *Salmonella* serovars through the food chain, including primary poultry production, is crucial for the understanding of how food animals and/or food-processing procedures contribute to the contamination of products and subsequent human infections. Traditionally, phenotypic methods such as serotyping and phage typing have been used for the identification of *Salmonella* isolates in outbreak investigations. However, these methods have limited applicability for studies on the transmission of salmonellas because of their poor discriminatory power observed for closely related isolates (13, 14). For the latter, epidemiological studies, based on pulsed-field gel electrophoresis (PFGE), for example, are of great value (15–17). In many laboratories, PFGE has been accepted as the gold standard for the molecular typing of salmonellas during outbreaks of human salmonellosis (18). It was proven useful during the EU multistate outbreak of *Salmonella* Stanley infections in 2011–2012 (19), the *Salmonella* Brandenburg infections in patients hospitalized in a French clinic in 2010–2012 (20), the human *Salmonella* Dublin infection due to contaminated raw milk cheese in France in 2012 (21), and the human *S.* Enteritidis infection caused by contaminated eggs in Slovenia in 2009 (12). According to Uzunović-Kamberović (22) and Hadžiabdić (25), there is an evident lack of information on PFGE typing of *Salmonella* serovars isolated in Bosnia and Herzegovina (BiH). Although a retrospective study on human salmonellosis in the central part of BiH was previously described and provides some data on the prevalence and serovar distribution, their genetic diversity remains unknown (22).

The aim of our study was to perform a preliminary molecular typing of the selected *S.* Enteritidis isolates by PFGE, isolated in 2010–2011 from farms located in the major broiler-producing regions in BiH, in order to obtain an overview of their genetic relatedness and diversity, in addition to discovering the possible sources of infection. The obtained data would enable the preparation of the most appropriate *Salmonella* prevention model in broiler flocks.

Materials and methods

S. *Enteritidis isolates*

In 2010–2011, *S.* Enteritidis strains were isolated from broiler flocks (18 positive samples/isolates from 1,894 inspected samples) and hatcheries (two positive from 20 inspected) within the scope of a *Salmonella* research program in the primary poultry production in BiH. Broiler flocks and hatcheries originated from the geographical regions (GA 1–7) with the highest density of poultry population (Figure 1); see Table 1 for the distribution of samples, both the inspected and positive, according to their geographical origin. Sampling obtained faecal material from broiler flocks during the last three weeks of breeding (n = 1,894) and dust samples from hatcheries (n = 20). All the obtained *S.* Enteritidis isolates (n = 20) were subjected to PFGE typing.

Isolation and identification

The isolation and identification of *S.* Enteritidis from the faecal material of broilers and the dust samples from hatcheries was performed according to the standardized method (23). Briefly, samples were inoculated at the ratio of 1 : 9 into the non-selective pre-warmed BPW (Buffered Peptone Water; Himedia, India) and enriched for 18 ± 2 h at 37°C. The following day, selective enrichment in MSRV (Modified Semi-solid Rappaport Vassiliadis medium; Himedia, India) was performed and, in the case of swarming, followed by inoculation onto XLD (Xylose-Lysine-Deoxycholate agar; Himedia, India), BG (Brilliant Green agar; Himedia, India) and *Salmonella*-chromogenic agar (Oxoid, UK). *Salmonella*-suspected colonies were further identified by biochemical tests (API Rapid 20E; BioMerieux, France) and serotyped by commercial *Salmonella* antisera. All *S.* Enteritidis isolates were stored at -76°C in a cryo-protective medium for further genotyping by PFGE.

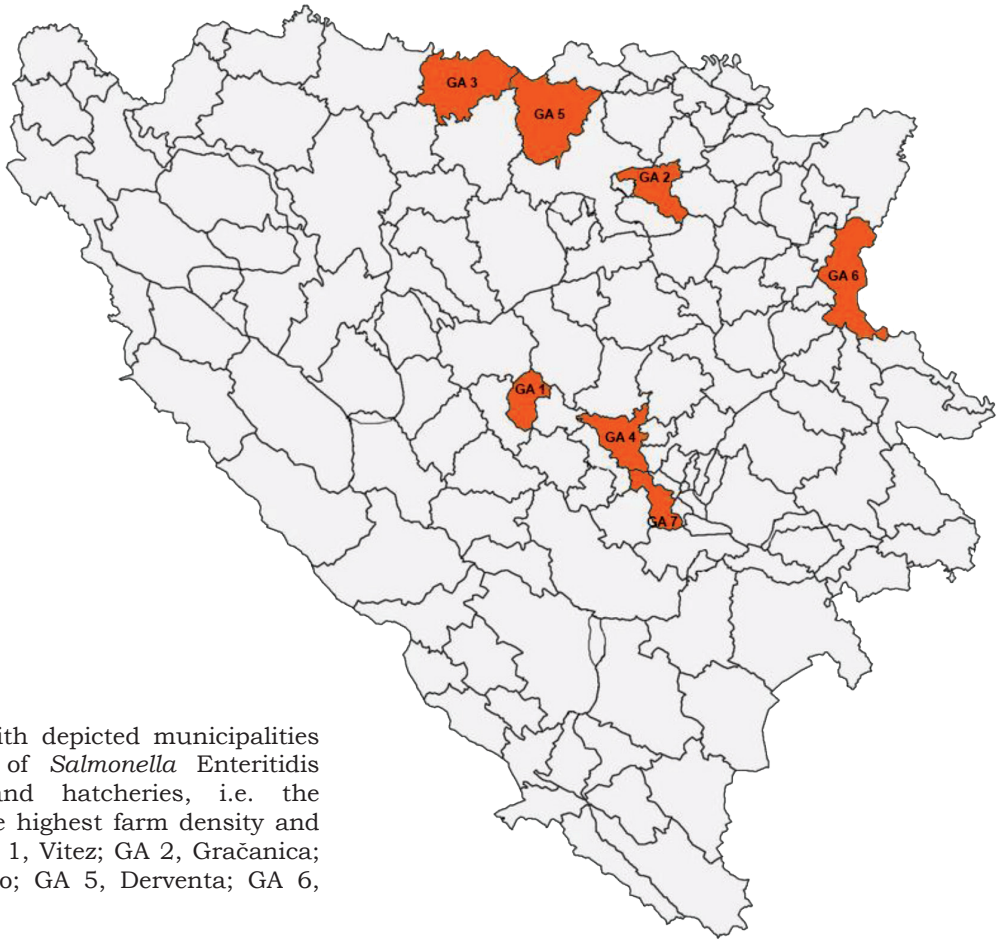

Figure 1: A map of BiH with depicted municipalities showing the distribution of *Salmonella* Enteritidis positive broiler farms and hatcheries, i.e. the geographical areas with the highest farm density and abbreviated as GA 1–7 (GA 1, Vitez; GA 2, Gračanica; GA 3, Srbac; GA 4, Visoko; GA 5, Derventa; GA 6, Zvornik; GA 7, Sarajevo)

Table 1: Distribution of samples according to the selected geographical regions

Geographical region	Broiler flocks (faecal samples)		Hatcheries (dust samples)	
	Number of samples			
	All	Positive	All	Positive
GA 1	100	**1** (SE 13)	-	-
GA 2	280	**3** (SE 15, SE 17, SE 20)	-	-
GA 3	574	**6** (SE 07, SE 10, SE 12, SE 14, SE 16, SE 19)	-	-
GA 4	494	**4** (SE 03, SE 06, SE 08, SE 18)	10	**1** (SE 01)
GA 5	88	**1** (SE 02)	-	-
GA 6	86	**1** (SE 05)	-	-
GA 7	272	**2** (SE 09, SE 11)	10	**1** (SE 04)
Total	*1,894*	***18***	*20*	***2***

Note: For details on geographical regions, see Figure 1. The names of the positive samples (*S.* Enteritidis isolates) are given in parentheses and were assigned according to the results of genotyping (see Figure 2).

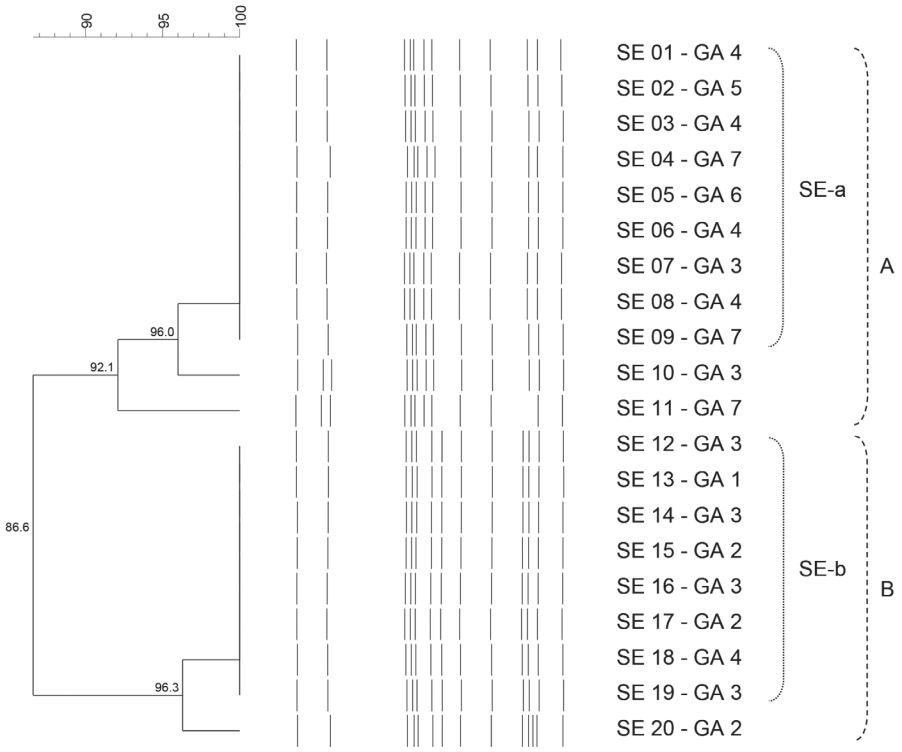

Figure 2: Dendrogram of 20 *Salmonella* Enteritidis pulsotypes showing the genetic relatedness of isolates (SE 01–20) obtained in 2010–2011 from broiler farms (n = 18) and hatcheries (n = 2; isolates SE 01 and SE 04) located in seven geographical areas of BiH (GA 1–7) with the highest farm density. A and B denote two clusters obtained according to the arbitrary 90% cut-off value for grouping by genotype similarity; Cluster A is subdivided into the SE-a group and two less similar isolates and cluster B into SE-b and one isolate

PFGE typing

Prior to PFGE, *S.* Enteritidis isolates were recovered on blood agar medium and subjected to PFGE typing according to the PulseNet standardized one-day protocol, using the restriction endonuclease *Xba*I (24). The obtained fragments were electrophoretically separated under the following conditions: 20 h at 6 V/cm and 14°C, with pulse times from 2 s to 64 s employing the CHEF-DR II system (BioRad, USA). PFGE profiles were subjected to computer-assisted analysis with BioNumerics software (version 6.6; Applied Maths, Belgium). For normalization, a molecular-sized standard *Salmonella* Braenderup strain H9812 (ATCC BAA-664) was used. For the construction of a similarity matrix, the band-based Dice coefficient with optimization and band-matching tolerance set to 1% was employed. A dendrogram was created using the UPGMA algorithm to enable the estimation of epidemiological relatedness among *S.* Enteritidis isolates.

Results

The prevalence of *Salmonella* spp. in broiler flocks during 2010–2011 was 2.9%, with a 1.0%. prevalence of *S.* Enteritidis Respecting the arbitrary 90% cut-off value for clustering by genotype similarity, a PFGE dendrogram of 20 selected *S.* Enteritidis isolates revealed two major clusters: A and B (Figure 2).

Cluster A (isolates SE 01–11) could be subdivided into SE-a group (isolates SE 01–09) with identical genotypes and two other isolates (SE 10 and SE 11; SE 10 was more similar to SE-a than to SE 11) differing in one or two bands from SE-a. Similarly, cluster B (isolates SE 12–20) could be subdivided into an SE-b group (isolates SE 12–19) with identical genotypes and isolate SE 20 differing in one band from SE-b. Regarding the origin of isolates, *S.* Enteritidis from GA 3 (n = 6) and GA 4 (n = 5) were found in both clusters A and B, but isolates from GA 1 (n = 1) or GA 2 (n = 3) in B, and isolates from GA 5 (n = 1), GA 6 (n = 1) or GA 7 (n = 3) in A.

Interestingly, an *S.* Enteritidis isolate from one of the hatchery dust samples (SE 04) and a faecal isolate from broiler flock (SE 09), both from GA 7, showed identical genotypes (the third isolate, SE 11, from GA 7 showed a two-band difference to SE 04 and SE 09). An identical banding pattern was also observed for the second dust isolate (SE 01) when compared to faecal isolates from broiler flocks (SE 03, SE 06 and SE 08) from GA 4 in cluster A, but one isolate from GA 4 (SE 18) clustered in B. In general, no marked dissimilarities could be observed among all the 20 *S.* Enteritidis isolates.

Discussion

The occurrence of numerous human salmonellosis cases indicates the need for related epidemiological studies and effective control measures, which should not be diminished by the reports on human campylobacteriosis as the most common zoonosis in 2012 with 214,268 confirmed cases in the EU (3, 9–12). An effective control program for *Salmonella* spp. in the primary production very often seems to be the only possibility for a reduction of the bacterial load in food animals. In Bosnia and Herzegovina, the prevalence of *Salmonella* spp. in broiler flocks was 2.9% in 2010–2011, determined in the present preliminary study dated with sampling to a two-year period prior to 2012 when the first official sampling in BiH was initiated. At the EU level, the prevalence was 3.1% in 2012 and 3.2% in 2011, which showed a decrease in comparison to the 4.1% prevalence in 2010 (1, 3).

The two most common serovars found in broiler and layer flocks are *S.* Enteritidis and *S.* Typhimurium (2, 3). The reported average prevalence of the two target serovars in broiler flocks at the EU level continued to decline from 0.7% in 2009 and 0.4% in 2010 to 0.3% in 2011 (1, 5). At the EU level, the prevalence of *S.* Enteritidis in broiler flocks was 0.2% in 2011 and 2012 (1, 3). In 2010–2011, the prevalence of *S.* Enteritidis in broiler flocks in BiH was 1.0% (*this study*). In 2012, the first official monitoring program for salmonellas in BiH was launched, yielding insight into the prevalence of *Salmonella* spp. (10.0%) and *S.* Enteritidis (8.7%) in broiler flocks (25). Such an evident disproportion in the prevalence data (2.9% vs. 10.0% of *Salmonella* spp. and 1.0% vs. 8.7% of *S.* Enteritidis obtained in the preliminary study in 2010–2011 vs. official monitoring in 2012, respectively) could have several reasons. Specifically, a different sampling strategy (sampling performed by the farmers themselves in 2010–2011 vs. expert sampling in 2012) and a different sampling method employed (sampling of fresh faeces into collection containers in 2010–2011 vs. sampling by boot socks according to the adopted sampling legislation).

In BiH, the prescribed method for the detection of *Salmonella* spp. in faecal material is the horizontal standardized microbiological method (23). This method, followed by serotyping, enables only limited options for a wider epidemiological research on the circulation of selected serovars. Therefore, the source-infection relations and the possibilities for intervention could only be discovered by the use of molecular typing methods, e.g. MLST, MLVA and PFGE that have been the most promoted in recent years (18). A standardized PFGE protocol has been routinely used in several laboratories (12, 26, 27) as promoted by EFSA and ECDC at the EU level. The initiative for protocol standardization and routine use of PFGE in epidemiological studies will lead toward the construction of a large database of pulsotypes, enabling prompt reactivity during outbreaks and adding markedly to the ensuring of public health (28).

Knowing the importance of *S.* Enteritidis for the poultry industry and public health, with 44.4% of all the reported serovars in human-confirmed cases (1, 3), the research on genetic diversity among circulating *S.* Enteritidis isolates in selected broiler flocks of BiH was initiated. It was shown by using PFGE that their genetic diversity was limited (86.6% similarity between the two discovered genetic clusters A and B); thus, they are closely related. According to the criteria by Tenover (29), an isolate is considered to be closely related to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with a single genetic event, i.e. point mutation or insertion/deletion. Such changes typically result in two or three band differences. Clustering of the pulsotypes could, therefore, indicate a dominant genotype circulating within geographical regions of BiH and a common source of infection. Since the vast majority of broiler farms are located in the regions that were selected for our study, intervention by taking the proper biosecurity measures could reduce *Salmonella* prevalence in these flocks and possibly lower the number of human salmonellosis cases. In several other studies, a continuous circulation of certain *Salmonella* serovars within poultry flocks has also been observed (26, 30). Taking into account the identical genotypes of *S.* Enteritidis isolates obtained in GA 4 and GA 7 from the hatchery (dust samples; SE 01 and SE 04, respectively) and broiler flocks (faecal samples; SE 03/06/08 and SE 09, respectively), vertical transmission of *S.* Enteritidis seems to be a reasonable explanation, consistent with other studies (26, 31, 32). Since the sampled parent flocks were negative for *S.* Enteritidis in

2010–2011, a more detailed sampling strategy would better explain, i.e. confirm or exclude, the vertical transmission of *S.* Enteritidis from parent flocks to progeny. This is strongly supported by the fact that the corresponding hatcheries are delivering one-day-old chickens to the majority of *S.* Enteritidis-positive broiler farms.

Regarding the considerable number of isolates and a limited value of PFGE in the epidemiological analysis of *S.* Enteritidis, due to high homogeneity among strains, this preliminary research is a significant innovation in the molecular typing of *S.* Enteritidis by PFGE in BiH. Therefore, it is a valuable contribution to future research on the epidemiology of salmonellas. This is strongly supported by our recent research in which an 85.0% similarity was observed among *S.* Enteritidis isolates obtained from broiler and laying-hen flocks (25), confirming the high homogeneity among *S.* Enteritidis isolates. It is also clear that a multiple approach that involves strict biosecurity measures, the education of poultry producers, consistent hygiene measures in the food-processing plants, and coordinated research by veterinary and public health experts is the only model for the prevention of *Salmonella* spp. contamination. In this regard, more attention should be given to establishing a national genotype database, enabling prompt reactions to food-related outbreaks, since such collaboration at the national level has already yielded promising scientifically verified results in Slovenia (12). The presented preliminary study calls for a wider coordinated research on *Salmonella* spp. epidemiology in BiH, indicating the emerging need for inclusion of molecular typing protocols in microbiological laboratories for the prevalent food-born pathogens, with the main goal of minimizing the incidence of human infections.

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RAZŠIRJENOST GENOTIPOV *Salmonelle* enteritidis V IZBRANIH REJAH PITOVNIH PIŠČANCEV V BOSNI IN HERCEGOVINI

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Povzetek: Leta 2012, ko je 27 držav članic prijavilo skupaj 92.916 primerov salmoneloz, se je v EU nadaljevalo upadanje števila primerov salmoneloz pri ljudeh. V primerjavi z letom 2011 je bil v letu 2012, ko je bilo 91.034 primerov tudi potrjenih, opazen 4.7% upad. *Salmonella* enteritidis in *Salmonella* typhimurium sta serovara, ki sta v EU najpogosteje povezana z obolenji pri ljudeh. Primeri *S.* enteritidis pri ljudeh so najpogosteje posledica uživanja okuženih jajc in perutninskega mesa. V rejah pitovnih piščancev je bila leta 2012 prevalenca *Salmonella* spp. 3.1 %. Serovar enteritidis je bil v EU izoliran pri 0.2 % rej pitovnih piščancev. Glede na rezultate prvega povojnega programa monitoringa, ki se je izvajal leta 2012, sta bili v Bosni in Hercegovini prevalenci *Salmonella* spp. in *S.* enteritidis v rejah pitovnih piščancev 10.0 % in 8.7 %. Namen našega dela je bil pridobiti boljši vpogled v epidemiologijo dominantnega serovara *S.* enteritidis v izbranih rejah pitovnih piščancev. Izolate, ki so bili pridobljeni v letih 2010–2011 na farmah pitovnih piščancev iz sedmih geografskih območij z največjo gostoto farm, smo tipizirali z metodo pulzne gelske elektroforeze (PFGE) ob uporabi encima *Xba*I. Zaradi očitne podobnosti genotipov, ki smo jih našli v različnih rejah pitovnih piščancev, rezultati kažejo na homogeno populacijo *S.* enteritidis, ki kroži med večino rej. Ker smo enake ali zelo podobne genotipe našli tudi v vzorcih fecesa iz rej pitovnih piščancev in v vzorcih prahu iz valilnic, lahko sklepamo tudi o skupnem viru okužbe.

Ključne besede: *Salmonella* enteritidis; epidemiologija; genotipizacija; PFGE; perutnina; reje pitovnih piščancev; Bosna in Hercegovina

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Case Report

MALIGNANT CATARRHAL FEVER IN AMERICAN BISON (*Bison bison*) IN SLOVENIA

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Summary: Malignant catarrhal fever (MCF) is a ubiquitous disease of cattle and other ruminants caused by ovine herpesvirus-2 (OvHV-2) in Europe and other continents and alcelaphine herpesvirus-1 (AlHV-1) in Africa. In March 2010 MCF was recognized in small private zoological garden in three American bison (*Bison bison*) imported from Vienna. They were housed in an enclosure next to llamas, goats and domestic sheep, with direct contact between all mentioned species. Typical clinical signs of the acute head and eye form of MCF were observed, with especially inflammation of conjunctives, oral and nasal mucosa and marked depression. Mortality was 100%. Necropsy findings in one bison were consistent with MCF. Acute ulcerative abomasitis and omasitis, acute hemorrhagic enterotyflitis, acute purulent bronchitis and moderate emaciation were found. The histopathology revealed mixed lymphohistiocytic and neutrophilic vasculitis in the brain, meninges, liver, spleen, heart, lungs and mixed lymphohistiocytic and neutrophilic hepatitis and adrenalitis. OvHV-2 DNA was detected post-mortem in tissue samples by PCR. Direct sequencing of the PCR product confirmed 100% nucleotide identity to OvHV-2 strain BJI035 and closely related to two OvHV-2 positive sheep samples collected in 2007. These are the first case of MCF confirmed by laboratory diagnostic methods in Slovenia. Furthermore, these are also the first cases of MCF in American bison in Slovenia.

Key words: *coryza gangraenosa bovum*; Europe; ovine herpesvirus-2; sheep

Introduction

Malignant catarrhal fever (MCF) is a ubiquitous and usually fatal lymphoproliferative disease of over 30 species of wild and domestic ruminants including cattle, deer, bison, water buffalo, moose and pigs. The most susceptible species are the American bison (*Bison bison*) and many deer species with rapidly progressing acute clinical signs followed by death in a few days (1, 2).

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Based on the reservoir hosts in which the virus was originally detected, the MCF-causing viruses are named as alcelaphine herpesvirus type 1 (AlHV-1) from wildebeest (*Connochaetes taurinus*), ovine herpesvirus type 2 (OvHV-2) from domestic sheep and caprine herpesvirus type 2 (CpHV-2) detected in domestic goats and a pig in Germany (3, 4). MCF-causing viruses are members of genus *Macavirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae* (5). They all induce disease with similar clinical signs and pathology in susceptible species. Death can occur within a few days or up to several weeks after the onset of clinical signs (6).

A common form of the disease is sheep-associated MCF (SA-MCF), which was initially observed in Europe, but is found worldwide wherever sheep and other MCF-susceptible species are kept together. Domestic and wild sheep are natural reservoir species of OvHV-2 and do not exhibit any clinical signs of infection, whereas the disease is dramatic and usually fatal in MCF-susceptible species. Another form of the disease is wildebeest-associated MCF (WA-MCF), which is a particular problem in Eastern and Southern Africa and in zoological gardens (2).

The virus is spread by nasal secretions. Adult sheep shed OvHV-2 sporadically with a short-lived episode usually lasting less than 24 hours, while new-born lambs are not the source of infection. Contrary to this observation, new-born wildebeest calves are infected and shed AlHV-1 continuously until 3-4 months of age (2). Respiratory tract of sheep is the primary target for OvHV-2 during natural infection (2, 7). OvHV-2 predominantly replicates in cells of nasal turbinates when naturally infected sheep experience intensive shedding episodes (8). The virus establishes a latent infection in the lymphocytes. In cattle herds, outbreaks are sporadic with few animals being affected, but outbreaks in American bison herds can affect up to 100% of animals (2, 9). A risk factor for cattle and bison infection is usually close cohabitation with domestic sheep (2, 7).

Laboratory confirmation of a clinical diagnosis of MCF is important and necessary, because clinical signs of other enteric or diseases with oral lesions are similar (2, 13). Such differential diagnoses are blue tongue, vesicular stomatitis, BVD (mucosal disease), severe form of IBR, foot and mouth disease, rinderpest and encephalitis of other aetiology. Virus isolation test for MCF caused by OvHV-2 is not useful, because OvHV-2 has never been propagated in monolayer cell culture, while AlHV-1 replicates in bovine turbinate cells (14, 15). Specific anti- AlHV-1 or anti-OvHV-2 monoclonal antibodies or conjugates are not available commercially. A direct ELISA has been developed for detection of specific antibodies, but in the acute form of the disease, the animals usually do not manage to develop antibodies before death (2). Diagnosis in MCF-susceptible species has benefited from recent development in molecular virology (16). PCR assays have been developed for the detection of OvHV-2 and AlHV-1 viral DNA (13, 17).

In Slovenia MCF has been diagnosed by clinicians and pathologists, but it had never been confirmed by a specific diagnostic method. This paper describes three field cases of MCF in American bison *(Bison bison)*.

Case description

History

The disease outbreak was suspected in a herd of three American bison *(Bison bison)* (age 3, 4 and 5 years) in a small private zoo located about 30 km east of Ljubljana. All three animals were bought from a foreign Zoo two years before the outbreak. They were housed in a pen of about 200 m^2, next to a pen with llamas *(Lama glama)*, domestic crossbreed goats *(Capra aegagrus hircus)* (buck and doe) and Istrian Pramenka sheep *(Ovis aries aries)* (ram, ewe and their four month old lamb) and an enclosure of wild boars *(Sus scrofa)*. A new pair of sheep was introduced to the zoo one year after the arrival of the bison, and had a lamb after their arrival. The bison had no contact with sheep before this introduction. All pens were constructed in a manner that allowed nose to nose contact between animals from neighbouring pens. The zoo also housed two roe deer *(Capreolus capreolus)*, three ibex *(Capra ibex)* and rabbits *(Oryctolagus cuniculus)*, but these animals were in another part of the zoo that had no direct contact with the bison, sheep and goats. Before the outbreak of MCF, the three bison were healthy and in good physical condition.

Clinical findings

In March 2010 all three American bison sickened one after another with 5 to 7 day intervals between death of one and occurrence of disease in another. The first animal to get sick was the youngest (3y) and the last was the 5 year old bison. The disease followed a very similar course in all three animals. In the first stage (2 days), the bison ate and drank less than usual and ruminated less. They were also presenting signs of mild colic, as they started to kick at the belly and were less agile. In the second stage (2 to 5 days) increased mucosal discharge appeared on the nares and excessive salivation was noted. The animals became progressively more depressed and showed

little interest in food. According to the owner's observations the affected animals also drank and consequently urinated more often than usually. Their corneas became progressively more opaque, blepharospasm and photophobia were noted and in 2 to 7 days corneas were completely cloudy and the animals became apparently blind, which was the case in the two animals that died naturally. At this stage the animals were completely anorexic; often dipped their mouths in water but did not drink. They were hyperaesthetic, but most of the day they were lying down, salivating excessively and had copious mucosal nasal and ocular discharge. Breathing was laboured and grunting notable. Obvious reluctance to walk was noted, as well as lethargy, ataxia and hypermetria. In the following two days the 3 and 4 year old bison that died naturally stood up and lay down often (almost every hour), kicked at their pectoral region with front feet and had seizures until finally they hardly stood up, swayed while standing and when they attempted to walk, fell down and did not attempt to stand up again. Before death, the 4 year old bison threw its head vigorously on the flooring and calmed down. The three year old animal was found dead in the morning. During the last week of its life the 4 year old bison did not eat at all, did not pass any manure or urinate. There was also much less salivation and nasal mucosal discharge during the last few days of the animals' life. Nasal erosions were also noted in the 4 year old bison at this time.

The youngest bison was treated with yeast and probiotics for indigestion with no success. The 4 year old bison was treated by a local vet, who sedated the animal and recorded 40.5°C body temperature and treated the animal for pneumonia with antibiotics and anti-inflammatory drugs. The antibiotic therapy was repeated 2 days after the first treatment. The therapy empirically improved the clinical status for a day (improved demeanour and appetite). Afterwards, the disease progressed rapidly; the bison appeared completely blind, it started to run into the fence and died within 24 hours. The last bison, which started to exhibit signs of disease just 5 days after the death of the 4 year old animal, was euthanized when it started to present corneal opacity and severe clinical disease 5 days after the onset. Time from the onset of the disease until death was 7 and 14 days in the 3 and 4 year bison, respectively.

Laboratory diagnosis

Five days after detection of clinical disease, a five-year old American bison was euthanized and immediately submitted for a post-mortem examination.

Necropsy

Specimens of the brain, spleen, liver, lungs, myocardium, adrenal gland and kidneys were fixed in 10% neutral buffered formalin for 24 hours, routinely embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (HE).

At the necropsy of the five-year old bison that was euthanized, moderate emaciation, mild corneal opacity, acute ulcerative end erosive omasitis and abomasitis, moderate haemorrhagic enterotyphlitis and cystitis and acute purulent bronchitis were observed. The liver was moderately congested; meninges were severely congested and oedematous. Numerous petechial haemorrhages were scattered through the renal cortex and spleen capsule. No lesions in the upper alimentary and respiratory tracts were noticed and lymph nodes were apparently normal.

Histologically, a mild to moderate mixed lymphohistiocytic and neutrophilic infiltrate was observed in the adventitia and media of numerous veins and arteries in the brain and meninges, liver, spleen, myocardium and lungs. In the liver, a severe mixed lymphohistiocytic and neutrophilic periportal hepatitis and small multifocal necroses of liver parenchyma were observed. Small dense multifocal accumulations of lymphocytes and macrophages and a severe parenchymatous degeneration were seen in the kidneys. Meninges were moderately oedematous and mildly to moderately infiltrated with lymphocytes, macrophages, neutrophils and fibrin, compatible with meningitis. Small lymphocytic aggregates and numerous small haemorrhages were noticed in the brain. A severe diffuse mixed lymphohistiocytic, neutrophilic and eosinophilic infiltrate was found in the adrenal cortex. The spleen was moderately atrophic and a large amount of haemosiderin was seen in the red pulp. Acute interstitial pneumonia and mild purulent bronchiolitis with severe diffuse pulmonary emphysema were also diagnosed.

Molecular diagnostics

Tissue samples of lymph nodes, spleen and kidney were collected for molecular diagnosis and genetic characterisation of the suspected MCF virus.

Blood samples of three sheep that were in contact with bison and showed no clinical signs of disease, were collected two weeks after the death of the five-year old bison.

Blood, lymph node, spleen and kidneys were kept frozen at -60 °C prior to DNA extraction. Total DNA was extracted from positive controls and unknown tissue samples using QIAamp® DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

PCR and primers for detection of SA-MCF (OvHV-2)

Oligonucleotide primers 556 and 775 (2) for the detection 422-bp fragment in ORF 75 gene of OvHV-2 were used. Positive control DNA extracts were prepared form archived blood samples of healthy sheep containing OvHV-2, negative control contained sterile distilled water. Two µl of the total DNA extract were used as the target in subsequent PCR amplifications. PCR was carried out in the final volume of 25 µl using 0.5 µl Platinum Taq DNA polymerase (Invitrogen, Germany), 2.5 µl 10 x PCR buffer, 1.0 µl 50mM $MgCl_2$, 20 pmol of each primer and 1 µl of dNTP mix (containing 10 mM of each dNTP). After denaturation in a T1 thermal cycler (Biometra, Germany) at 95°C for 5 min, the reaction mixtures were subjected to thermal cycling for 40 ramp cycles each of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. A time delay of 7 min at 72°C for was included prior to a 4°C soak. The amplified PCR products were detected by agarose gel electrophoresis. Total reaction volume of 15 µl was loaded onto a 2% (w/v) agarose gel containing ethidium bromide (0.5 µl/ml), and the PCR products were visualized under UV transilluminator.

Direct sequencing of PCR products

Four PCR products were used as templates in cycle sequencing reactions primed with the 556 and 775 primers, using the Macrogen sequencing service (Macrogen, South Korea). Individual nucleotide (nt) sequences were assembled and proofread using the SeqMan and EditSeq programs in the DNASTAR program package (DNASTAR Inc., Madison, WI, USA). For each sample, 360 nucleotide long sequences were aligned with the published data using BLAST (available at http://www.ncbi.nlm.-nih.gov/) at the National Centre for Biotechnology Information (NCBI). Multiple sequence alignment was carried out using the sequence analysis software (DNASTAR Inc., Madison, WI, USA).

Results

DNA extracted from the bison tissue samples (lymph node, spleen and kidneys) and from the three sheep blood samples was used in PCR amplification. The PCR product of predicted size (422 bp) was detected in lymph node, spleen and kidneys of the euthanized bison, while all three samples collected from sheep in contact with the bison were negative. The PCR products of appropriate size, including two OvHV-2 positive sheep samples collected in 2007, which were used as positive controls, were directly sequenced. The nucleotide sequence for three samples (one bison and two sheep) was determined, confirming the specificity of the amplification for OvHV-2. The three sequences of 360 nucleotides of partial ORF 75 were compared to each other and to the published sequences of OvHV-2 (Figure 1). The observed homology in nucleotide sequences between Bison/2010 and the strain BJ1035 in GenBank was 100% and closely related (99.7%) to strains 1905ZIEGEKY and Sheep 5/2007, which was detected in a sample of asymptomatic infected sheep collected in 2007 in Slovenia. Another sequence, Sheep 1/2007 was closely related (99.7%) to strains 27846ELCH and 25034REH in GenBank and revealed three and four nucleotide differences to strains Sheep 5/2007 and Bison/2010, respectively. On amino acid level the 120 aa sequences were 100% identical between Bison/2010, BJ1035, 1905ZIEGEKY and Sheep 5/2007, while the sequence Sheep 1/2007 was 100% identical with 27846ELCH, 25034REH, 25843HIRSCH, 15821BISON, 25058ZIEGEH and 21114HIRSCH (data not shown). This data confirmed that the bison was infected with OvHV-2 and died of MCF. A sequence detected in sheep in 2007 was closely related to the strain detected in the bison.

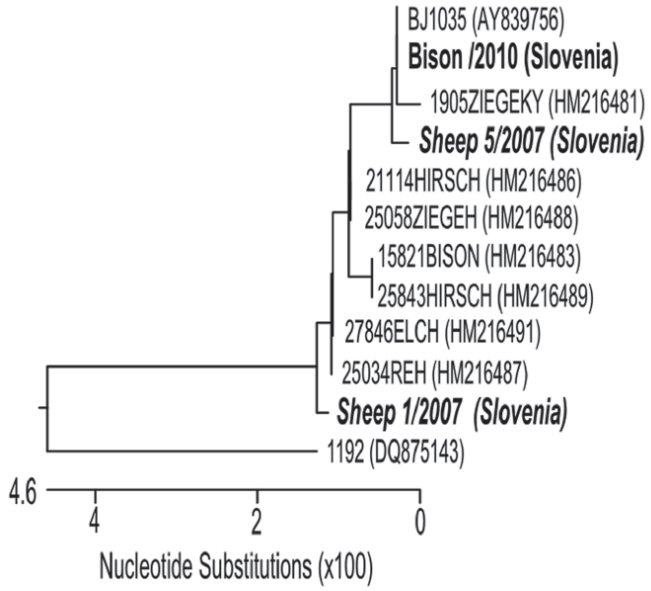

Figure 1: Phylogenetic analysis of partial tegument protein gene (360 nucleotides of ORF 75: genome positions of nucleotides 121137-121496, numbering according to OvHV-2 strain BJ1035, AY839756)

The tree was constructed from aligned nucleic acid sequences of three Slovenian OvHV-2 (one American bison sample and two sheep samples in bold-italic from this study) and nine OvHV-2 sequences of closely related strains found in GenBank. The original names and GenBank accession numbers are presented for each sample. The dendrogram represents a tree constructed using MegAlign program of sequence analysis software Lasergene®.

Discussion

The American bison in this study exhibited predominantly the clinical signs of the head and eye form of MCF. After the 5th day of the disease nasal discharge was more intensive, similarly as reported previously (11). Unlike some other investigators (6, 11, 12, 18), we did not note diarrhoea, dysentery or melena. The disease was acute, ending with death in a week and two weeks in the 3 and 4 year old bison, respectively. According to literature, clinical signs appear most often about a month and half to two months (but could also be much latter) after natural infection in the American bison (6, 7). Since the ewe and ram in the next pen were there for more than a year, we speculate that viral shedding appeared either in the periparturient period in the ewe or during the first 3 months of the lamb's life.

Gross lesions described in this case were consistent with other reports of acute MCF in bison, although no alteration of the upper alimentary tract were observed, which was reported in most cases of MCF in bison (7, 11, 12, 18, 19). A reason for this could be that the disease had not yet progressed to that point at the time of euthanasia, which was the fifth day after the first clinical sign appeared. The most prominent histological change – vasculitis, present in the majority of the examined organs, was of a mixed lymphohistiocytic and neutrophilic form, reported before only in bison experimentally infected with blood from a calf with MCF (19). Vasculitides described in other cases of acute MCF in bison were lymphocytic (11, 12, 18). Meningitis and periportal hepatitis reported by two authors (12, 19) were lymphocytic, whereas in our case they were mixed lymphohistiocytic and neutrophilic with an additional fibrinous component, found only in the meninges. Multifocal lymphocytic nephritis was also reported (19), whereas multifocal lymphocytic encephalitis and adrenalitis diagnosed in our bison, was not reported by other authors (7, 11, 12, 18, 19).

For the first time in Slovenia, the application of a specific PCR assay has enabled the detection of OvHV-2 DNA in clinical samples of SA-MCF in American bison. The usefulness of PCR method for the confirmation of virus after the suspicion of MCF in American bison was proved. The direct sequencing confirmed the specificity of PCR products and nucleotide comparison with previously published strains in GenBank determined nucleotide identity (2). Detection of similar strain in bison and sheep collected in Slovenia in 2007 is confirming that this strain

has been circulating in Slovenia for several years. Another detected positive sample from sheep is very similar but not identical to the strain Bison/2010, confirming the circulation of different strains of OvHV-2 in the region.

Considering the ability of herpes viruses to cause latent infections, it is not surprising that OvHV-2 DNA was not detected in the blood of the tree sheep in contact with the bison, since the samples were collected two weeks after the last bison died and these animals were probably no longer viremic, or the quantity of the virus was below the limit of detection.

Conclusion

This was the first case of acute, 100 % fatal MCF in American bison in Slovenia confirmed by laboratory methods, and also one of the few reported in Europe. High susceptibility and mortality of American bison to MCF was confirmed. The source of infection was probably a group of sheep housed in a pen next to the bison in a small zoo. We firmly advise not to keep sheep in the same facility as American bison in zoos, breeding operations, fairs or sales.

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MALIGNA KATARALNA MRZLICA PRI AMERIŠKEM BIZONU (*Bison bison*) V SLOVENIJI

J. Starič, J. Ježek, V. Cociancich, M. Gombač, P. Hostnik, I. Toplak

Povzetek: Maligna kataralna mrzlica (MCF) je vseprisotna bolezen goveda in drugih prežvekovalcev, ki jo povzroča ovčji herpesvirus-2 (OvHV-2) v Evropi in na drugih celinah ter alcelafini herpesvirus-1 (AlHV-1) v Afriki. V marcu 2010 je bila MCF ugotovljena v majhnem zasebnem živalskem vrtu pri treh ameriških bizonih (*Bison bison*), uvoženih z Dunaja. Bili so nastanjeni v ogradi poleg lam, koz in domačih ovc, z neposrednim stikom z vsemi omenjenimi vrstami. Opazili so tipične klinične znake akutne oblike MCF glave in oči, še posebej vnetje vek, ustne in nosne sluznice ter prepoznavne depresije. Umrljivost je bila 100-odstotna. Ugotovitve obdukcije enega od bizonov so bile skladne z MCF. Ugotovljen je bil akutni ulcerozni abomasitis in omasitis, akutni hemoragični enterotifilitis, akutni gnojni bronhitis in zmerno hujšanje. Histopatološka preiskava je razkrila mešani limfohistiocitični in nevtrofilni vaskulitis v možganih, možganskih ovojnicah, jetrih, vranici, srcu, pljučih in mešani limfohistiocitični in nevtrofilni hepatitis ter adrenalitis. DNK OvHV-2 je bila odkrita *post mortem* z uporabo metode PCR v tkivnih vzorcih. Neposredno določanje zaporedja baznih parov produkta PCR je potrdilo 100-odstotno nukleotidno identiteto OvHV-2 seva BJI035 in tesno povezanost z dvema OvHV-2 pozitivnima vzorcema ovc, zbranima v letu 2007. Gre za prvi primer MCF, potrjen s pomočjo laboratorijskih diagnostičnih metod, v Sloveniji. Poleg tega gre tudi za prve primeri MCF pri ameriških bizonih v Sloveniji.

Ključne besede: maligna kataralna mrzlica; ovčji herpes virus 2; Evropa; ovca

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Case Report

ARTHROSCOPIC TREATMENT OF AN INCOMPLETE AVULSION FRACTURE OF THE SUPRAGLENOID TUBEROSITY IN A DOG

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Summary: A thirteen month-old, female English Setter was referred for a non-weight-bearing lameness of the left thoracic limb. Clinical and radiographic findings were consistent with a left supraglenoid tubercle incomplete fracture. The anamnesis reported a lameness arising 5 months earlier after a traumatic injury. After a period of conservative treatment, lameness had not resolved. Clinical examination revealed grade 3/5 lameness and pain on manipulation of the shoulder joint. Medio-lateral radiographic projection revealed a distinct irregular radiolucent line between the greater tubercle and the glenoid.

An arthroscopic examination was performed and a careful and complete debridement and revitalisation of the subchondral bone was performed. As reported in the literature, a rigid compressive internal fixation is usually indicated when a joint is involved, but, in the case presented herein, the dog's history and young age led us to believe that a minimally-invasive treatment should be satisfactory.

Radiographic controls were carried out at 3, 6, 16 and 19 months following the surgical procedure, revealing a complete fusion and regular margins of the supraglenoid tubercle. Clinical signs of lameness resolved definitively at 3 months after surgical treatment.

To the best of the Authors' knowledge, cases of incomplete avulsion fracture of the supraglenoid tuberosity managed with arthroscopic surgery have not been described.

Key words: supraglenoid tubercle; shoulder; fracture; arthroscopic treatment; dog

Introduction

Traumatic injury of the shoulder joint is a common cause of foreleg lameness in dogs. In domestic carnivores, the supraglenoid tuberosity has its own centre of ossification and fuses with the remainder of the scapula towards five to six months of age (1).

In most cases (2), ligamentous or tendinous injuries, fractures or luxations are implicated. Avulsion fractures of the supraglenoid tuberosity usually occur in juvenile animals, as a result of a faulty landing following a jump (3). Typically, this involves a marked hyperflexion of the shoulder, which considerably increases the traction exerted by the *biceps brachii* muscle (4).

To the best of the Authors' knowledge, cases of incomplete avulsion fracture of the supraglenoid tuberosity managed with arthroscopic surgery have not been described.

Case description

A thirteen month-old, female English Setter weighing 15.5 kg was referred to the Veterinary Teaching Hospital of the University of Perugia with a non-weight-bearing lameness of the left forelimb.

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The owner reported a lameness arising 5 months earlier, probably as a result of a traumatic event. For three months, the dog was kept at rest and showed an acceptable improvement. However, after a few weeks of normal activity, the lameness recurred. Clinical examination findings pointed out reluctance to walk, grade 3/5 lameness based on the Quinn scale (5). At rest, the dog maintained the shoulder and the carpus in a flexed position. Inspection revealed a little swollen area over the cranio–lateral aspect of the left scapulo–humeral joint, which was painful on palpation. There was some mild muscle reduction of the left forelimb that was judged to have been caused by disuse. Moreover, extension and flexion of the shoulder also caused the dog pain and movements of abduction. Neurological examination was within normal limits.

Findings of the orthopaedic examination were compatible with a traumatic injury of the proximal part of the bicipital tendon and/or its insertion. Other articular disease like an extra-articular scapular or humeral injury could not be clinically excluded.

After sedation with dexmetedomidine 4 µg/kg IV (Dexdomitor®: Orion Corporation Orion Pharma, Espoo, Finland), a radiographic study (mediolateral and caudocranial projections) was carried out. Mediolateral radiographic views of the affected limb showed an abnormal shape of the distal part of the supraglenoid tuberosity; this area showed also a radiolucent and irregular linear defect surrounded by small irregular radiolucent flaws and mild sclerosis. The trabecular pattern of the bone was not detectable (Figure 1). Since a traumatic event was implicated, a suspect of incomplete avulsion fracture was made.

To complete the diagnostic procedure and, if necessary, with the potential of following-up with minimally invasive treatment, an arthroscopic examination was performed to assess the fracture site as well as the other intra-articular structures (6). After premedication with acepromazine 10 µg/kg (Prequillan®, Fatro Spa, Bologna, Italy) and metadone 0.2 mg/kg IV (Eptadone®, Molteni Spa, Firenze, Italy), general anaesthesia was induced with 3 mg/kg of propofol (Proposure®, Merial Italia Spa, Milano, Italy.), and maintained after intubation with a mixture of isoflurane (Isoflo; Esteve Spa, Milano, Italy) and oxygen (50-100 ml/kg/min) via a circle breathing circuit with spontaneous ventilation.

The left forelimb was prepared for aseptic surgery: trichotomy and aseptic disinfection was performed at wide margin around the supraglenoid tubercle. The dog had an arthroscopic examination of shoulder using an arthroscope (2.7mm, 30° oblique arthroscope, Small Joint Arthroscope: Karl Storz GmbH & Co. KG, Tuttlingen, Germany) connected to a video camera and image-recording device. A standard lateral shoulder portal (7) allowed evaluation of all major structures. Inspection of the middle and caudal compartment of the joint revealed severe synovitis. Overall, arthroscopy ruled out any other associated injury, particularly capsular tears. In the cranial area, an intra–articular fracture of the supraglenoid process was found. The avulsion fracture had an extension from the gleno-humeral ligament to the biceps brachii insertion site. A circular fissure was clearly visible with partial cracks in the central portion of the fissure. Using an hook probe and also through the manipulation of the scapular joint during direct visualization of the fracture line it was possible to evaluate the degree of the mobility of the tuberosity. This portion did not showed to be mobile during these procedures. At the point of fracture, two fragments of non-vital subchondral bone and of yellow colour (diameter 2x1mm) were removed. In the fissure line, deep debridement and revitalisation of the subchondral bone was performed using bone graspers, double-spoon forceps, hand burr and mastoid curette (Karl Storz GmbH & Co. KG, Tuttlingen, Germany). alternately (Figure 2).

At the end of surgery, the arthroscope and surgical instruments were removed and the joint was flushed copiously with lactated Ringer's solution(8). After recovery from anaesthesia, 0.01 mg/kg of buprenorphine (Temgesic®, RB Pharmaceuticals Limited, Milano, Italy) and 0.2 mg/kg of meloxicam (Metacam®, Boehringer Ingelheim, Milano, Italy) were administered subcutaneously to provide postoperative analgesia.

The two fragments of non-vital subchondral bone and of yellow colour removed during arthroscopy were immediately fixed in 10 % neutral buffered formalin and routinely embedded in paraffin. The samples were sectioned and stained with Haematoxylin & Eosin for histopathological assessment that revealed cartilaginous tissue with regular cartilaginous lacunae and chondrocytes in different stages of maturation in irregular arrangement, associate with diffuse and small foci of fibrous tissue. Several and marginal areas of the samples showed focal activation of

normal endochondral ossification and focal mild hemorrhagies. Due to the histopathological findings, characterized mostly by cartilagineous activation process, and the clinical data, the diagnosis of suspect reparative process was done (Figure 3).

One day later, the dog was discharged and the owner was directed to follow a medical therapy based on the administration of FANS for 7 days and to keep the animal at rest for 3 months.

Radiographic controls were carried out at 3, 6, 16 and 19 months following the surgical procedure (Figure 4). At 3 months after surgery, at the physical examination, there were no abnormalities such as lameness or pain after manipulation of the affected limb. At the last control (19 months after arthroscopic surgery), clinical examination showed similar results to the previous controls. Indeed, a significant improvement of the bone shape and radiopacity of the bone was noted; however, a small radiolucent defect of the surface of the cranial glenoid cavity was identified (Figure 5).

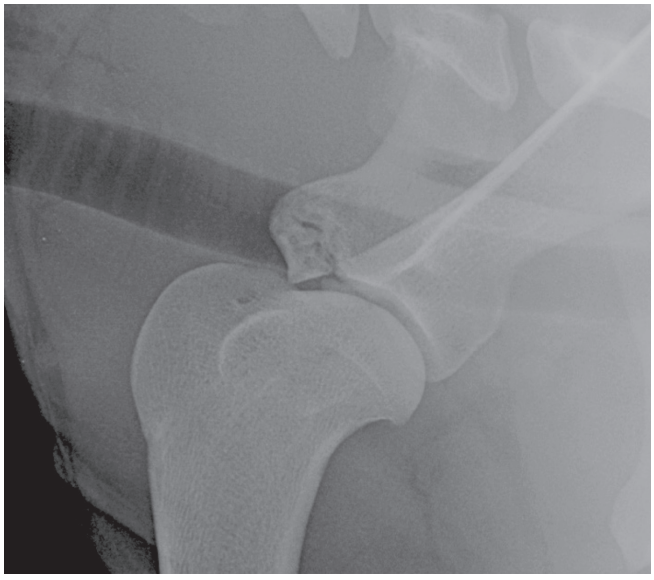

Figure 1: Mediolateral radiographic projection of the left shoulder

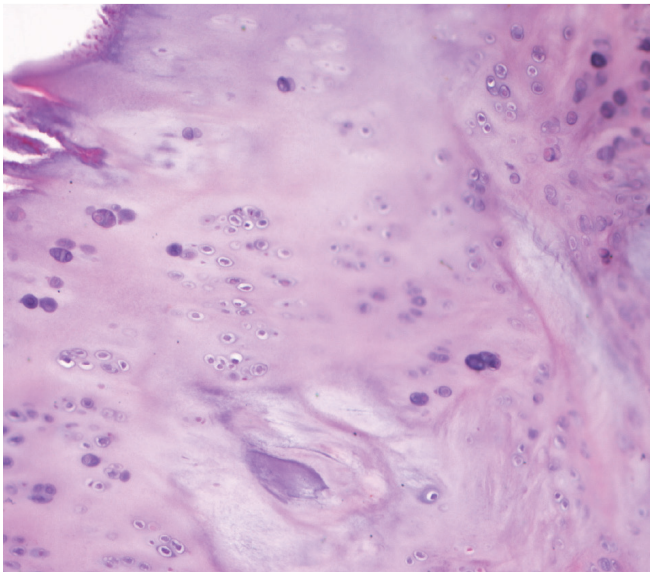

Figure 3: Histological section of subchondral bone, 10 x, Haematoxylin & Eosin. Cartilaginous tissue with regular cartilaginous lacunae and chondrocytes in different stages of maturation associated to area of normal endochondral ossification

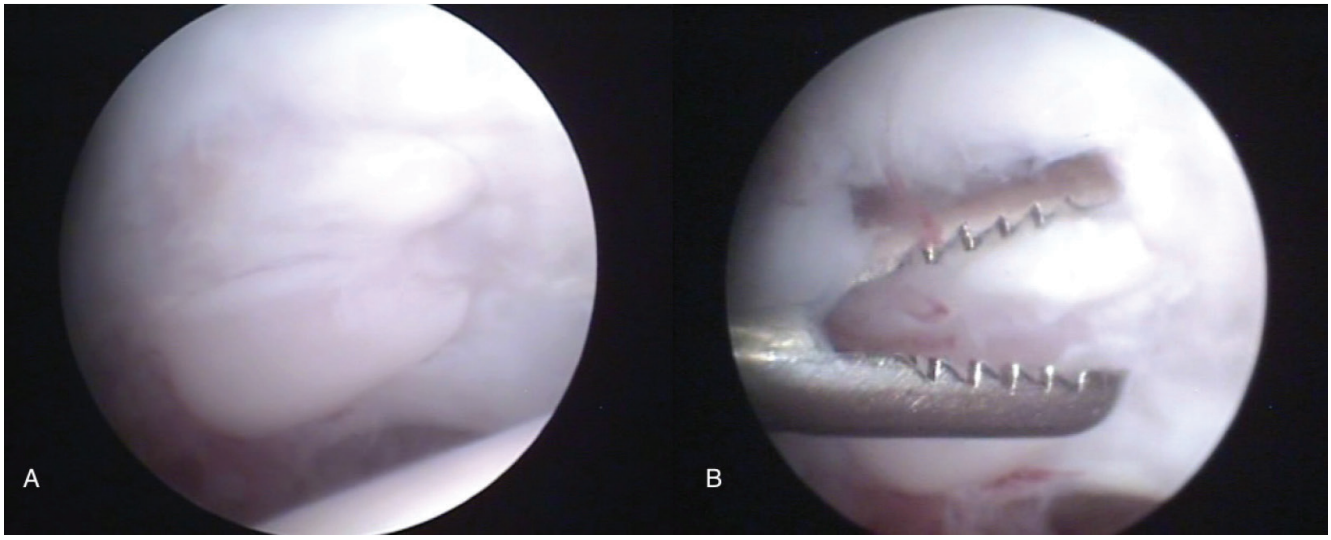

Figure 2: (A) Arthroscopic view of the avascular bone fragment. (B) Removal of the bone fragment by grasping forceps

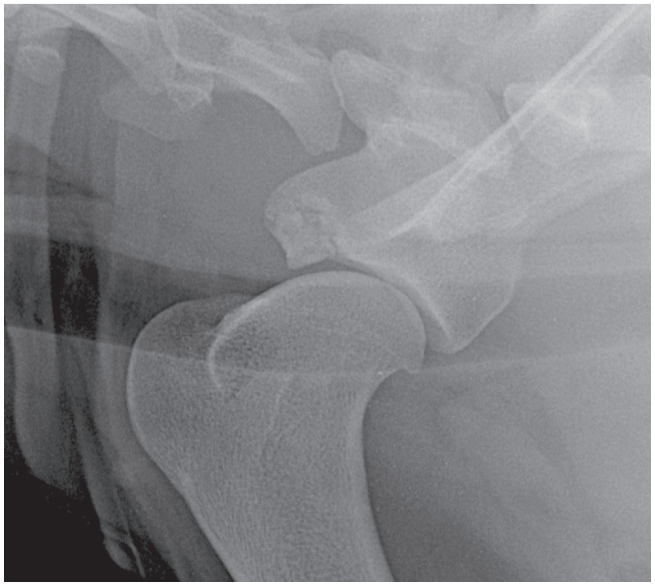

Figure 4: Mediolateral radiographic projection of the left shoulder 16month after arthroscopic treatment

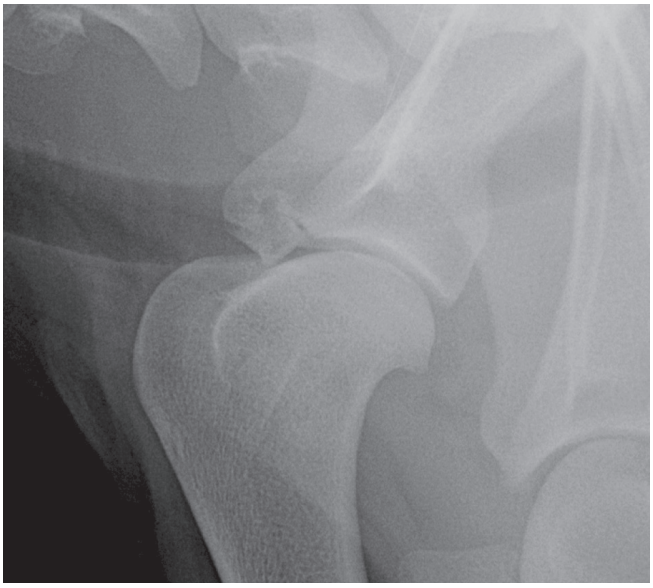

Figure 5: Mediolateral radiographic projection of the left shoulder at last control 19 month after arthroscopic treatment

Discussion

Supraglenoid tuberosity is the site of origin of the *biceps brachii* muscle and develops as a separate centre of ossification, normally fusing on to the scapula by 5 months of age. Although fracture is uncommon (9), it mostly occurs as a result of direct force exerted on the shoulder region; on the contrary, the avulsion fracture of supraglenoid tuberosity appears to be due to an indirect force (10). In this report, the aetiopathogenesis of the described lesion was unknown. Non-weight-bearing lameness and an abnormal limb posture, as observed in this dog, are classically described as an avulsion fracture of the supraglenoid tuberosity (11). As described in literature (4) the lameness is caused by pain due to instability of the suvraglenoid tuberosity that caused micro-movements at the level of the fracture line. Moreover, the pull due to the biceps brachii tendon during flexion of the shoulder has created continue micro-fractures that could have affect the healing process. This kind of injury is detectable by shoulder radiograph. In this case, the radiographic study showed an incomplete avulsion fracture of the supraglenoid tuberosity of left forelimb. Furthermore, it is necessary to address that at the first visit, x-ray exams were not carried out by the first Practitioner, which does not exclude the remote possibility that the fracture was originally complete. However, the Authors assume that the fracture was probably incomplete from the beginning because the physiologic pull of the *biceps brachii* tendon would create too large a gap that would not allow bony fusion. Moreover, the first radiographic exams showed a bone profile that was quite linear and homogeneous, which suggests an originally incomplete injury. When a joint is involved, surgery is indicated and the prognosis is more guarded, as described by Johnston (12). The prognosis for articular fracture is related to the alignment and rigidity of internal fixation (1). Some authors recommend a rigid compressive internal fixation, although this technique may compromise growth in the case of physeal injuries (13, 14). In the case presented here, the conservative treatment did not lead to a complete resolution of the injury. A minimally invasive arthroscopic treatment, instead of an internal fixation technique, was chosen despite the articular involvement. Management of articular cartilage lesions is based on the idea that healing, through the formation of fibrocartilage, is promoted when the lesion is exposed to blood that contains mesenchymal stem cell precursors (7, 15). Several methods are used. Abrasion arthroplasty involves the uniform removal of subchondral bone until bleeding is achieved. Here, we created several microcracks in the subchondral bone plate to allow bleeding at the lesion surface (7, 15, 16).

Another aetiopathogenetic hypothesis to consider is chondrodysplasia. In the literature, a

case in which an English setter showed bilateral abnormal ossification of the supraglenoid tubercles has been described (17). The ossification disorder involved the proximal and distal segments of the supraglenoid tubercle, as well as the cranial articular surface of the glenoid cavity. In that report, diagnosis was consistent with osteochondrosis or focal chondrodysplasia. In this case, the Authors did not consider this lesion as an abnormal ossification of the supraglenoid tubercle, since the lesion was monolateral and secondary to an injury (18). Moreover, radiographic aspect of the observed lesion showed a substantial difference compared to those showed by De Simone (17). A good conservative therapeutic strategy to obtain an immobilization of the forelimb, is the application of a Velpeau bandage. In the Authors' opinion , the complete immobilization of the limb for a long period could cause a certain degree of muscle atrophy that might reduce or slow down the full functional recovery of the limb. Therefore for this case, in the postoperative period, we considered more appropriate keeping the dog at rest in a limited cage both in height and in width without the application of any bandage, in order to maintain a physiological loading of the limb and also to avoid a severe amyotrophy. Accordingly to our clinical experience, the previous conservative treatment followed by the dog did not bring any significant improvement, while arthroscopic surgery was effective to ameliorate the clinical condition for the incomplete avulsion fracture of the supraglenoid tuberosity in this dog.

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ARTROSKOPSKO ZDRAVLJENJE NEPOPOLNEGA AVULZIJSKEGA ZLOMA NADPONVIČNE GRBICE PRI PSU

A. Bufalari, G. Moretti, A. Proni, V. De Monte, A. Di Meo

Povzetek: Trinajstmesečna samička angleškega setra je bila napotena na pregled zaradi nenosilnega šepanja leve sprednje okončine. Ugotovitve kliničnega pregleda in rentgenskega slikanja so kazale na nepopolni zlom leve nadponvične grbice. Anamneza je poročala o šepanju, ki izhaja iz travmatske poškodbe, povzročene 5 mesecev pred pregledom. Po obdobju konservativnega zdravljenja šepanje ni prenehalo. Klinični pregled je pokazal oceno šepanja 3/5 in bolečino pri manipulaciji ramenskega sklepa. Mediolateralna rentgenska projekcija je pokazala izrazito radiolucentno linijo med večjo grbico in sklepom. Opravljena je bila artroskopska preiskava, skrbno in popolno odstranjevanje odmrlega tkiva ter revitalizacija subhondralnega dela kosti. Kot poročajo v literaturi, je po navadi v takšnih primerih potrebna toga čvrsta notranja fiksacija, če je v poškodbo vključen sklep. Vendar bi v tem primeru glede na zgodovino psičke in njeno mladost moralo zadostovati minimalno invazivno zdravljenje. Rentgenske kontrole so bile opravljene 3, 6, 16 in 19 mesecev po kirurškem posegu in so razkrile popolno zlitje in ravne robove nadponvične grbice. Klinični znaki šepanja so dokončno izveneli v 3 mesecih po kirurškem zdravljenju. Po vednosti avtorjev v literaturi še niso bili opisani primeri nepopolnega avulzijskega zloma nadponvične grbice, ki bi bili odpravljeni z artroskopsko operacijo.

Ključne besede: nadponvična grbica; pleče; zlom; artroskopsko zdravljenje; pes

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