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ANALYSIS OF FAECAL PH IN PIGLETS FROM BIRTH TO WEANING

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ABSTRACT

The health of the gastrointestinal system of pigs is still a topical issue. When focusing on the youngest categories of pigs, we routinely evaluate it on the basis of appetite assessment and physical examination of faeces. A piglet's gut health is also related to the development and changes of pH in the digestive system. Because there is little scientific work in this area, the aim of this study was to evaluate the physiological range of faecal pH in healthy suckling piglets from birth to weaning. Faecal pH measurements were performed in thirty-five suckling piglets at the time of the piglets' birth, on the 7th, 14th, 21st, and 28th day of their life. We found the mean pH values to be 6.38±0.46 within 24 hours after birth; 7.14±0.23 on the 7th day after birth; 7.23±0.23 on the 14th day after birth; 7.42±0.60 on the 21st day after birth; and 7.72±0.61 on the 28th day after birth. Statistical analysis showed significant differences between the pH of the faeces of new-born piglets and the samples taken in the following weeks of the experiment (P<0.05; P<0.01; P<0.001). Measuring faecal pH can be a simple, quick and inexpensive method used to determine the health status of piglets' intestines.

Key words: faeces; faecal pH; stool; suckling piglet

INTRODUCTION

The health status of the digestive system can be assessed and evaluated according to various parameters and examinations [13, 14, 23]. Faecal examination, which is a completely non-invasive approach, plays an important role here. The consistency and colour of the faeces, undigested feed ingredients, as well as mucus and blood admixtures are evaluated. In the case of a severe diarrhoea, bacteriological, parasitological or virological examination of the faecal samples is required [9].

According to various studies conducted on different species of animals and also in humans, pH measurement can be used to assess the health of the intestinal system [8, 17]. By testing stool pH in humans, a stool sample is tested for acidity to diagnose a medical condition. Acid stool may indicate a digestive problem such as: lactose intolerance, an infection such as rotavirus, or an overgrowth of acid-producing bacteria such as lactic acid bacteria. Faecal pH is also a commonly used response variable in equine nutrition studies focused on evaluating gastrointestinal health [2, 8]. Faecal pH and buffering capacity are indirect tests useful to detect hindgut changes of horses [1]. Hindgut acidosis can be problematic on many levels, as horses with this condition tend to have subclinical symptoms, such as: a take-it-or-leave attitude toward feed,

weight loss, recurrent mild colic, unusually soft manure, or behavioural changes [24].

However, to correctly read and understand faecal pH values, reference values from healthy animals without problems are needed. To date, there is very little data on faecal pH measurement in pigs [12]. There are several studies that examine pH in the intestinal tract, but not in faeces [4, 20, 21].

The aim of this study was to evaluate the physiological range of faecal pH in suckling piglets from birth to weaning.

MATERIALS AND METHODS

Animals

A range of faecal pH was determined based on the readings of 35 healthy piglets from 7 sow litters (a cross bred between Pietrain, Duroc and Landrace) at the pig clinic of the University of Veterinary Medicine and Pharmacy in Košice. Sows in the maternity ward were housed on a concrete floor with a separate and heated area for the piglets. From the first minutes after birth, the piglets had access to the sow's colostrum, later to the milk as well as the water. Due to the short time of mammary gland secretion in sows (approx. 30 seconds), colostrum and milk were collected after a previous intramuscular injection of 3 ml of oxytocin. Pelleted dry feed mixture (Creep feed 3, De Heus®) was provided from the seventh day after the birth of the piglets. The sows in the maternity ward were fed three times per day with the standard feed mixture for this category of pigs (KPK Vital, De Heus®), while its quantity depended on the number of piglets in the litter.

Experimental design

The faecal pH was determined at the birth of the piglets (within 24 hours), and also on the 7th, 14th, 21st, and 28th day of their life. After collection, the samples were immediately examined in the laboratory. The experiment also included the analysis of the pH of the fed feed mixtures and water. The respective legislative rules concerning animal welfare were observed during manipulation with animals.

Diagnostic testing

Upon arrival at the laboratory, the samples of faeces were diluted 1 : 3 with distilled water, according to the procedure described by Hydock, Nissley and Staniar [8]. The samples were stored at room temperature (20–23 °C) and stirred for one minute before measuring the pH. The measurements were performed using a calibrated Greisinger G 1500-GE pH meter (Greisinger®, Germany). The device was calibrated twice during the experiment and cleaned with a plastic brush and distilled water after each measurement. The measuring range of the instrument is from 0.00 to 14.00 pH, with an accuracy of ± 0.01 pH.

Statistical processing of results

The statistical processing of the results was performed by assessment of means (x) and standard deviations (SD). The significance (P) of differences in the means of corresponding variables were evaluated by One-way analysis of variance and by Tukey's Multiple Comparison Test. We used GraphPad Prism 9 for the statistical processing.

RESULTS

The pH value of piglet faeces showed an upward trend over a period of four weeks (Figure 1). We found the lowest values in the period up to 24 hours after birth. On the contrary, the highest values in the last week of the experiment (Table 1). During the entire experiment, the average pH ranged from 6.38 to 7.72. The lowest recorded value was 5.72, the highest 8.96. Tukey's multiple comparison test showed significant differences between the pH of new-born piglet faeces and samples taken in the following weeks of the experiment ($P < 0.05$; $P < 0.01$; $P < 0.001$).

Analysis of colostrum pH within 24 hours after farrowing the sows showed values close to 6.09, in the following weeks of lactation we recorded increased milk pH values. The highest pH values of milk were recorded in the 3rd week of the experiment (7.66 ± 0.02, Table 2.).

In the analyses of feed mixtures and water, the lowest values were recorded in the pelleted feed mixture for suckling piglets (Table 3).

DISCUSSION

Faeces are solid or semi-solid remains of food that has not been digested in the small intestine and has been broken down by bacteria in the large intestine. They are the

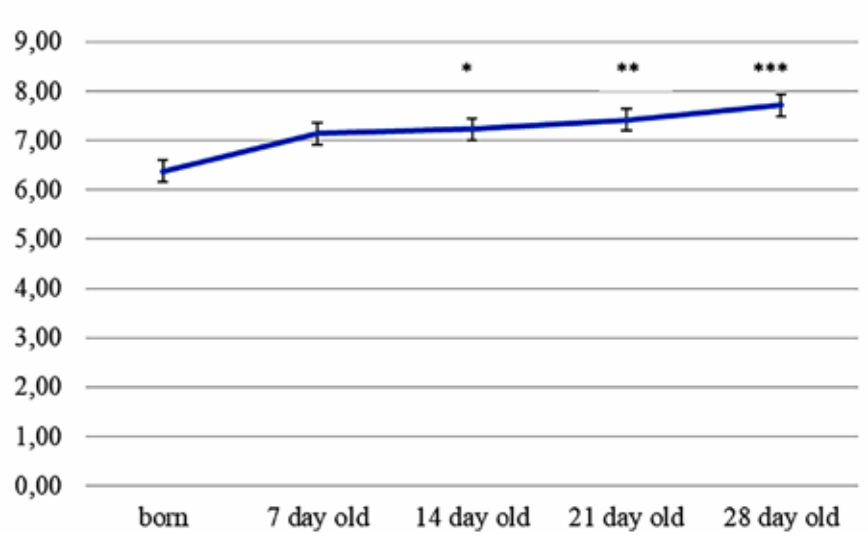

Fig. 1. Average pH of piglets' faeces

Table 1. pH values of piglet faeces (x ± SD)

	Born	7 days old	14 days old	21 days old	28 days old
Piglets/sow P15	5.72	7.04	7.10	7.55	7.60
Piglets/sow P16	6.22	7.08	7.16	8.02	8.96
Piglets/sow P17	6.29	7.55	7.23	7.53	7.91
Piglets/sow P18	7.01	7.32	7.15	8.03	7.76
Piglets/sow P19	6.79	6.89	6.98	6.73	7.42
Piglets/sow P20	5.99	6.97	7.30	7.60	7.21
Piglets/sow P09	6.65	7.16	7.70	6.47	7.20
x ± SD	**6.38 ± 0.46**	**7.14 ± 0.23***	**7.23 ± 0.23***	**7.42 ± 0.60****	**7.72 ± 0.61*****

Statistical significance: * – P < 0.05; ** – P < 0.01; *** – P < 0.001

Table 2. pH of colostrum (lactated within 24 hours) and milk of sows (x ± SD)

	Within 24 hours	7 days	14 days	21 days	28 days
Milk	6.09 ± 0.12	6.98 ± 0.04	7.08 ± 0.01	7.66 ± 0.02	7.01 ± 0.03

Table 3. pH of water and feed mixtures (x ± SD)

Feed	Water	Mixture for piglets	Mixture for sows
pH	7.19 ± 0.01	4.92 ± 0.18	6.07 ± 0.05

end products of the activity of the digestive tract, when intestinal fluid thickens due to water absorption [6, 22]. Daily production in healthy piglets is very low, only a few grams, which makes it difficult to obtain a sufficient amount of faeces samples and subsequent analysis. The small size of the anus and rectal ampoule also makes the collection difficult. For these reasons, in our experiment, we took fresh samples of faeces only during the spontaneous defecation of piglets.

Faeces examination can provide important information about diseases that affect the gastrointestinal system. Stool can be examined: macroscopically, microscopically, chemically, immunologically, and microbiologically [9]. Piglet diarrhoea is one of the most common pig diseases, leading to heavy mortality and thereby causing economic loss [25].

Assessment of diarrheal pH can be an initial step in clinical examination. Although researchers are not completely united on the best method of measuring pH, faecal pH is a suitable representation of what is happening in the gastrointestinal system. Accuracy depends on the number sampled because variation within a population of scouring pigs can be expected. Sufficient stool to determine pH can usually be obtained by gently squeezing the abdomen of diarrheic piglets. Stools of piglets with colibacillosis are rich in bicarbonate and tend to be alkaline, i.e. pH 8 or greater. Malabsorptive diarrhoeas due to atrophic enteritis commonly are pH 7 or less. Variations within groups of pigs are expected, and mixed infections are common [3].

Faecal pH values depends on the diet. Suckling piglets receive colostrum only within 24 hours after birth [18], and then the basis of their nutrition is milk. Milk secretion can be divided into two parts, transient milk and mature milk depending on composition in each period. The transitional milk is a secretion from 34 hours after birth to the 4th day of lactation, characterized by a high concentration of fat [19]. Mature milk is released from the following days of lactation until weaning, with the milk composition being relatively stable [15, 16]. The penetration of serum from blood vessels into the glandular follicles of active mammary glands has a fundamental effect on the pH of milk [5].

The pH of sow colostrum is more acidic than that of mature milk. Kent et al. [10] reported that the pH of colostrum immediately before and after parturition was 5.7, rising to 6.0 at day 1, and reaching 6.9 by day 9, consistent with other reports [5]. In our experiments, we found the lowest pH within 24 hours after the birth of piglets (pH of colostrum 6.09 ± 0.12), on the contrary, we found the highest pH of milk 21 days after the birth of suckling piglets (7.66 ± 0.02). One must not forget about the potential intake of water (if there is enough breast milk, the intake is limited) and the intake of piglets' feed mixture – pre-starter. In our experiments, this mixture was provided from the 7th day of life, but the actual intake did not start until a few days later, depending on the milk yield of the sows. The pH of feed depends on the feedstuffs used. The pH of our feed mixture was around 4.9. The suckling piglet has been use to a low stomach pH during lactation because lactic acid bacteria from the sow milk convert lactose to lactic acid [11]. Lactic acid helps to lower the pH of the sucklings' stomachs. In addition, acid production in the gastrointestinal tract has been shown to modify the composition of the gut microflora by lowering gut pH, thereby reducing the growth and proliferation of some gut pathogens [7].

CONCLUSIONS

Our experiment allowed us to come to the conclusion that in healthy suckling piglets, the lowest pH of faeces is during colostrum intake with a gradual increase in the values measured on the 7th, 14th, 21st and 28th days of the piglets' life. Measuring the pH of faeces can be a suitable examination in the evaluation of the health status of the digestive tract of piglets.

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A CASE REPORT IN A SYRIAN HAMSTER WITH HYPERADRENOCORTICISM DIAGNOSED AND TREATED WITH THERAPEUTIC TRIAL

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ABSTRACT

This case report describes a practical approach for diagnosing and treating a 17-month-old Syrian hamster with hyperadrenocorticism based on: history, systemic signs, dermatological lesions, and therapeutic trial. The patient was monitored for 16 weeks while he was treated with trilostane and achieved hair regrowth and the resolution of systemic and demeanour signs.

Keywords: alopecia; Cushing disease; hamster; hyperadrenocorticism; trilostane

INTRODUCTION

Hyperadrenocorticism (HAC), also known as Cushing disease, is a disease of the endocrine system resulting primarily from chronic excess production of glucocorticoids, particularly cortisol. Cortisol is an important hormone that plays an essential role in the regulation of the metabolism of carbohydrates, proteins and lipids. Of all pet animals, Cushing syndrome with similar clinical signs is most common in dogs (mostly in older patients), in cats it is rarely present [2, 4, 9].

Hyperadrenocorticism is rarely diagnosed in domestic hamsters in a clinical practice [7, 13], however, in laboratory hamsters the disease is found in higher numbers [1]. Primary HAC due to neoplasia of adrenal cortex is most common in males and older animals, with adrenocortical adenoma being one of the most commonly reported benign neoplasms in the Syrian hamster [12]. Secondary HAC can also develop due to pituitary neoplasia causing excess adrenocorticotropic hormone (ACTH) secretion [1]. Iatrogenic HAC may also occur when the patient receives excess glucocorticoid treatment [11]. All three types of HAC lead to hypercortisolemia and show similar systemic signs such as polydipsia (PD) and polyuria (PU), polyphagia and cutaneous lesions such as bilateral symmetrical flank alopecia and lateral thigh area, hyperpigmentation, skin thinning, lack of skin elasticity and comedones [5, 10, 11].

CASE PRESENTATION

Signalment and history

A 17-month-old male Syrian hamster (*Mesocricetus auratus*) was presented for a second opinion due to five months of non-pruritic progressive alopecia. The patient was previously treated with topical ivermectin, systemic

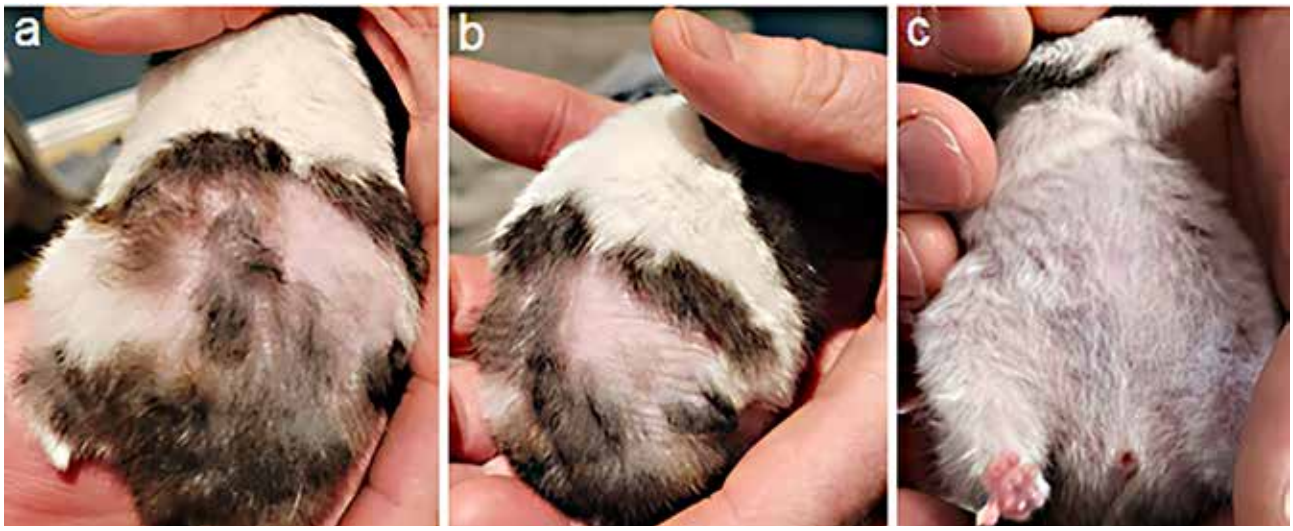

Fig. 1. Initial presentation
a – Bilateral, non-symmetrical, multifocal, regional alopecia on the flanks and lumbosacral area;
b – Diffuse hypotrichosis on the ventral abdomen and medial hind limbs; c – Generalised dermal thinning
(Figures courtesy of the owner)

dexamethasone (5 months prior to the second opinion and a month after the clinical signs appeared) and systemic enrofloxacin, which did not improve the clinical signs. The owner also described signs of PD and PU, changes in demeanour by hiding in his bedding, lethargy, weight loss and a strong urine odour of the bedding despite being replaced frequently.

CASE MANAGEMENT

General physical examination

The patient appeared bright, alert and responsive. The body weight was 0.150 kg which appeared to be slightly below the normal body condition. The mucous membranes were pink and moist. On abdominal palpation the patient was found to be comfortable and no masses were palpated.

Dermatological examination

There was bilateral, non-symmetrical, multifocal, regional alopecia on the flanks and lumbosacral area and diffuse hypotrichosis on the ventral abdomen and medial hind limbs (Figure 1). The remaining hair was not easily epilated. Additionally, there was dermal thinning and an area of comedones.

Differential diagnosis

Given the history, the clinical signs and the dermatological lesions, top on the list of differential diagnoses was hyperadrenocorticism (HAC). Other conditions were also considered including parasitic (demodicosis, sarcoptic mange, notoedrosis), bacterial (staphylococcal folliculitis), fungal (dermatophytosis), systemic (nephrosclerosis, amyloidosis), endocrinopathies (*diabetes mellitus*, hypothyroidism), neoplastic (epitheliotropic lymphoma), nutritional (pantothenic acid deficiency), and hypersensitivity (allergic or contact dermatitis) disorders, however these were not highly suggestive given the whole clinical presentation.

TREATMENT AND OUTCOME

Diagnostic approach

The author and the owner agreed to proceed with a therapeutic trial as the remaining differential diagnoses were unlikely and there was a high index of suspicion for HAC.

Treatment

Week 1: Trilostane (Trilostane 10 mg.ml^{-1}; Summit) at a dose of 2.5 mg.kg^{-1} once daily (sid) *per os* (p. o.) was prescribed. For the urine odour and the PU, the patient was prescribed enrofloxacin (Baytril 2.5 % oral solution; Elanco) 5 mg.kg^{-1} twice daily (bid) p. o. for seven days as a urinary tract infection (UTI) was suspected.

Fig. 2. Week 2: New hair started to grow on the alopecic areas

Follow up

Week 2: the patient was reviewed and some of the previously described clinical signs improved. The urine odour was resolved, the PD and PU improved. The patient's body weight increased 20 g during the two-week treatment period. The patient also showed signs of demeanour improvement where he was more active and playful. Lastly, new hair started to grow on the alopecic areas and there was no further hair loss (Figure 2). It was agreed to continue the treatment with trilostane at the same dose.

Week 6: the owner reported that the patient was better in himself, active and lively, and the PU reduced compared to when he was initially presented. The owner also reported that there was one incident where a creamy brown liquid was found in his housing and that was put down as diarrhoea as the trilostane is known to cause gastrointestinal signs as an adverse reaction [3]. It was also noted that the patient's weight had reduced by 35 g. It was advised to increase the trilostane dose to 3.75 mg. kg^{-1} sid/p. o. Lastly, more hair grew and the alopecic areas were reduced (Figure 3).

Week 7: the owner reported that the patient had erythematous genitalia, anorexia, more episodes of creamy brown liquid was found in his housing and was also PD. The owner ceased the treatment for seven days and the reported clinical signs were resolved.

Week 8: the patient was presented for a review and there was a further 10 g weight loss. The genitalia had crusting, however there was no erythema. PU and urine odour were also reported. More hair grew and there was no further hair loss. It was advised to reintroduce trilostane at the original dose, 2.5 mg.kg^{-1} sid/p. o., as the adverse reactions were suspected to be due to the higher dose. In view of the PU, enrofloxacin at the dose of 5 mg.kg^{-1} bid/p. o. for seven days was prescribed for a suspected UTI.

Week 11: the patient gained 10 g, the owner reported that there was significant improvement with the demeanour, the PU resolved and the urine odour reduced. The alopecic areas improved even more with the exception of some small areas on the right side of the flank and lumbosacral area (Figure 4). It was suspected that the hair follicles may have been irreversibly damaged.

Week 16: the patient was presented with altered demeanour and neurological signs with the suspicion of pituitary neoplasia. Although there was a further weight gain of 35 g and the patient was still having a good appetite, it was agreed to humanely euthanize as the condition was affecting the quality of life of the patient. No necropsy was performed.

Fig. 3. Week 6: a–c – More hair grew and the alopecic areas were reduced. The abdomen and medial hind limbs, previously described with hypotrichosis in week 1, in week 6 present a normal hair density

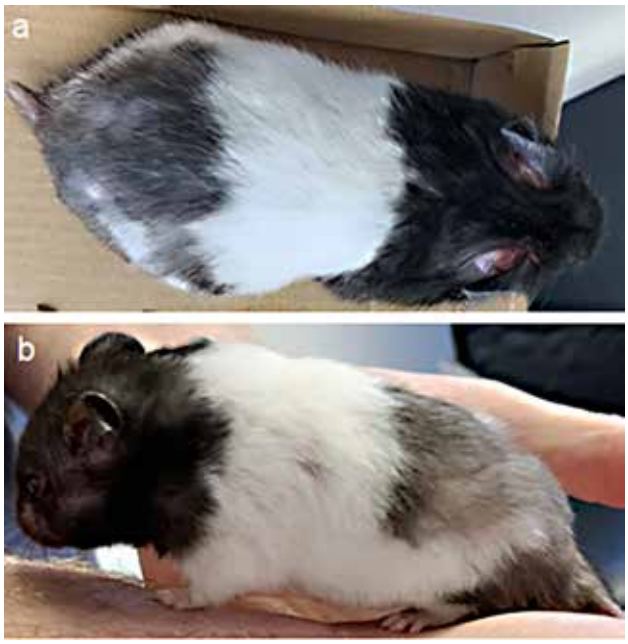

Fig. 4. Week 11: Alopecic areas improved even more with the exception of the lumbosacral area (a) and of some small areas on the right side of the flank (b)

Final diagnosis

Pituitary dependent HAC diagnosis was reached based on the history, systemic signs, dermatological lesions and therapeutic trial.

Ethical considerations

Consent for permission to publish this case report was obtained from the owner of the patient. The author declares no known conflicts of interest.

DISCUSSION

Although in dogs and cats the diagnostic process of HAC can be challenging in some cases, it is certainly much more practical to diagnose compared to hamsters [8]. In laboratory hamsters, the diagnosis of HAC is usually done using biochemistry for serum alkaline phosphatase and cortisol, and also urine cortisol creatinine ratio. Dynamic function tests such as ACTH stimulation test and dexamethasone suppression test are commonly used for the diagnosis of canine HAC, however in hamsters those tests are currency empiric [10]. Those tests were not considered, as the majority of them include high cost, lack of practicability and risks. Sampling may require several blood samples for the diagnosis and treatment monitoring which may need to be taken under general anaesthesis (GA). Furthermore, due to the size of the hamsters and blood volume restrictions, clinicians may fail to obtain the right amount in order to run the laboratory tests [6].

In terms of treatment, in literature searches, only a few case reports were found and no controlled clinical trials, involving treatment with metyrapone and 1,1-dichloro-2-2bis (p-chlorophenyl) ethane for HAC [1]. Mitotane is another drug of choice, with limited results [8, 10, 11]. An adapted canine HAC protocol with ketoconazole treatment was mentioned in a suspected case of HAC in a hamster with limited results too [7]. Trilostane was the only available drug to source in order to block the adrenal synthesis of glucocorticoids for this patient [3]. When the dose of trilostane was at 3.75 $mg.kg^{-1}$ sid/p. o. the patient lost some body weight, was anorexic and also developed diarrhoea. When the dose was reduced back to 2.5 $mg.kg^{-1}$ sid/p. o.the side effects were resolved. In the study that described treatment of two Syrian hamsters [13], a trilostane dose of 5 $mg.kg^{-1}$ sid/p. o. was used and anorexia and lack of activity was reported. Subsequently, the treatment was ceased for two days and then trilostane was introduced at a dose of 2.5 $mg.kg^{-1}$ sid/p. o. and the adverse reactions improved [13]. On the dermatological note, the alopecia and hypotrichosis improved significantly and the therapeutic trial also confirmed the diagnosis of HAC even though no diagnostic techniques were performed.

During the treatment and monitoring, the patient had two occasions of suspected UTI. The patient was treated with enrofloxacin with a good response resolving the clinical signs [3]. UTIs are commonly reported in canine patients with HAC [4, 8], however in the exotics literature UTIs are not reported as a secondary complication of HAC.

In conclusion, this case report presented a 16-week treatment with trilostane with minimal side effects and had significantly improved hair regrowth. Even though no laboratory tests were performed, the therapeutic trial confirmed the diagnosis of HAC due to the high index of suspicion. This practical clinical approach for diagnosing and treating HAC could be established in domestic hamsters in order to avoid the risks of a GA and sampling but also to minimise the costs involved during the process.

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YEASTS OF THE *MALASSEZIA* GENUS – RECENT FINDINGS

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ABSTRACT

The genus *Malassezia* is a medically important genus of yeasts that can colonize the skin of humans and other warm-blooded animals. The genus currently comprises 18 species of which four new species were identified recently. The most widely known species, *M. pachydermatis*, occurs in animals but was detected also in humans, namely at life endangering septicaemias and in prematurely born children. Proliferation of *Malassezia* occurs most frequently as a result of disturbances in the normal homeostasis of host immunity on the one hand and virulence of these yeasts on the other hand. The successful management of the disease depends on the therapeutic control of overgrowth of the yeasts and any concurrent bacterial infection by local or systemic antimicrobial treatment, as well as, on identification and potential correction of the predisposing factors.

Key words: cultivation; *Malassezia* spp.; occurrence; virulence

INTRODUCTION

Yeast infections are a relatively common problem in a small animal veterinary practice. In dogs and cats the genera *Malassezia* and *Candida* are the most frequently encountered opportunistic organisms. They can be a part of the normal microbiota of skin and mucous membranes of these animals, not acting as pathogens, but under certain conditions, particularly those with weakened local and total immunity of an individual, they may become pathogenic and cause mycosis, either as a primary disease or as a secondary infection [5].

Yeasts of the genus *Candida* are associated more likely with mucosal epithelium and in dogs they are the main aetiological agents of infections of the oral cavity, the gastrointestinal and the urogenital tracts. In comparison with *Candida*, *Malassezia* yeasts are more associated with skin and are the most frequent cause of otitis and dermatitis. The prevalence of inflammation of the external auditory canal in dogs in veterinary ambulance may reach 10 % and as many as 70 % of such cases may be related to the yeast of the *Malassezia* genus [18, 34].

Species of the genus *Malassezia*

Only three *Malassezia* species were known until 1990. With the development of molecular biological methods, new species were gradually identified. Currently, the following 18 species have been identified: *M. pachydermatis*, *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. dermatis*, *M. japonica*, *M. nana*, *M. yamatoensis*, *M. caprae*, *M. equina*, *M. cuniculi*,

Table 1. Occurrence of individual species of *Malassezia* in animals and man [30, 44]

Malassezia spp.	Principal host/other hosts	Year of 1st identification
M. pachydermatis	Dog, cat/ruminants, pig, cow	1925 (Dodge) [14]
M. furfur	Man/cow, pig	1989 (Baillon) [3]
M. sympodialis	Man/horse, pig, ruminants	1990 (Simmons, Guého) [39]
M. globosa	Man/cow	1996 (Guého et al.) [21]
M. obtusa	Man	1996 (Guého et al.) [21]
M. restricta	Man	1996 (Guého et al.) [21]
M. slooffiae	Man, pig/cat, ruminants	1996 (Guého et al.) [21]
M. dermatis	Man	2002 (Sugita et al.) [43]
M. japonica	Man	2003 (Sugita et al.) [42]
M. nana	Cat, cow	2003 (Hirai et al.) [23]
M. yamatoensis	Man	2004 (Sugita et al.) [41]
M. caprae	Goat/horse	2007 (Cabañes et al.) [7]
M. equina	Horse/cow	2007 (Cabañes et al.) [7]
M. cuniculi	Rabbit	2011 (Cabañes et al.) [8]
M. brasiliensis	Parrot	2016 (Cabañes et al.) [9]
M. psittaci	Parrot	2016 (Cabañes et al.) [9]
M. arunalokei	Man	2016 (Honnavar et al.) [24]
M. vespertilionis	Bat	2018 (Lorch et al.) [30]

M. brasiliensis, *M. psittaci*, *M. arunalokei* and *M. vespertilionis* (Table 1) [30, 44].

Some species from the genus *Malassezia* (particularly *M. pachydermatis*) have a broad spectrum of hosts while other are more specific and occur only in one animal species or in a group of related animals and in man. The use of genotype identification methods, e. g. PCR-RFLP, RAPD, sequencing and other, is inevitable for accurate species diagnosis of *Malassezia*. Phenotype methods developed for distinguishing of species of the genus *Malassezia* on the basis of characterization of cultural and biochemical properties differ which makes their definite identification more difficult. Moreover, individual *Malassezia* species include a wide range of genotypes with different phenotype properties even within particular species [11, 12, 16, 20, 26, 46].

The presence of *Malassezia* in pet animals, such as dogs or cats are more common than in farm animals. *Malassezia* were isolated from both healthy animals and those suffering from otitis and dermatitis. The yeast isolated most frequently from dogs is *M. pachydermatis*. *M. furfur* was identified in dogs, but very rarely. The *M. nana* and *M. slooffiae* species were diagnosed in cats. In comparison with dogs, *M. pachydermatis* occurs in cats far less than in dogs [2, 31, 32].

Information about the occurrence of *Malassezia* in farm animals is very scarce. The clinical role of *Malassezia* in farm animals is not clear, also with regard to the fact that the presence of *Malassezia* in these animals have been investigated very little. Their occurrence in farm animals was confirmed more frequently in healthy individuals and only sporadically in animals suffering from dermatitis or otitis; maybe due to the small number of studies concerned with the presence of *Malassezia* in sick animals. In cattle, the following species were identified by use of the genotyping methods: *M. furfur*, *M. sympodialis*, *M. nana*, *M. slooffiae*, *M. pachydermatis* and *M. equina* [7]. Of *Malassezia* species occurring in small ruminants there were identified as *M. caprae*, *M. slooffiae* a *M. sympodialis* [6, 7, 45]. The most typical *Malassezia* species

identified in horses is *M. equina* (most frequently isolated from skin around the anus). From healthy skin of horses there were sporadically isolated and genotypically determined also as *M. caprae*, *M. sympodialis* and *M. slooffiae* [6, 7, 47]. In pigs there were confirmed species, such as *M. furfur*, *M. sympodialis*, *M. slooffiae* and also *M. pachydermatis* [19, 33]. *M. cuniculi* was isolated from rabbits in New Zealand [8]. The mentioned studies originate from countries such as Spain, Italy, United Kingdom, Brazil and California with typical subtropical climates characterised by higher humidity and lower temperature differences and differing from that in the Czech and Slovak republics. It was observed that the occurrence of *Malassezia* in farm animals in Slovakia is very scarce (even none). The testing of 223 cattle detected *M. pachydermatis* only in two animals. Another species, *M. sympodialis*, was identified in one animal from 77 tested pigs. In the Slovak territory, no *Malassezia* were isolated from horses, small ruminants or rabbits [38].

New species of the genus *Malassezia*

The increasing availability of genomic and molecular tools and methods played a key role in the determination of new species of the genus *Malassezia*. Sequencing of the genome of various field isolates of *Malassezia* allowed scientists to uncover recently as many as four new species.

In 2016 two new species, *M. brasiliensis* and *M. psittaci*, were uncovered in five pet parrots in Brazil after sequencing which showed that they did not correspond to sequences of that time known species. *M. brasiliensis* and *M. psittaci* were isolated from lesions on the beak and oropharynx of the parrots and their properties were similar to those of other species of the genus *Malassezia* [9].

In 2016, *M. arunalokei* was recognized as a new species. It was isolated from scalp skin of a human patient with seborrheic dermatitis in Chandigarh in India in December 2012 and was confirmed as a new species four years later. It was identified in patients with moderate to medium serious seborrheic dermatitis but also in healthy individuals. Recently, it was determined that it occurs more frequently on the forehead and cheeks. It is interesting that phylogenetically *M. arunalokei* is very close to the species *M. restricta*, but it was determined that it deflected from this species 7.1 million years ago [13, 24].

In 2018, new species, *M. vespertilionis*, was isolated from bats in the eastern and western states of the USA. The isolates were obtained by swabbing the skin of the wings of hibernating bats in the subfamily Myotinae. The cultivation properties of this newly discovered species were distinct from the other *Malassezia*. The typical temperature of growth of *Malassezia* spp. is 32 °C (some species are able to grow even at 40 °C) but this new species grows best at 24 °C. It is able to grow over a broad temperature range – it can be cultured already at 7 °C up to the maximum of 40 °C. This thermal growth range confirms the adaptation by the yeast to survive on the skin of bats during both the host's hibernation and active seasons. The culturing period of *M. vespertilionis* can require as many as 50 days while with other species it usually lasts 2–14 days [30].

Lipid dependence of *M. pachydermatis*

M. pachydermatis was long considered a lipophilic but not lipid-dependent species, capable of growth without addition of lipids, namely on Sabouraud dextrose agar. This specific property was frequently used as a rapid diagnostic method to distinguish it from other lipid-dependent species. Recent genome sequencing determined that *M. pachydermatis* lacks the fatty acid synthase gene present in other species of this genus [48]; but is able to use for its growth the lipid fractions of the peptone component of the Sabouraud dextrose agar. The dependence of *M. pachydermatis* on lipids was confirmed by its failure to grow during culturing on a special lipid-free medium [36].

Complex evaluation of clinical cases

Persistent or recurrent *Malassezia* dermatitis or otitis in dogs is usually associated with insufficient identification and elimination of predisposing or unceasing factors. Clinical disease associated with the overgrowth of *Malassezia* frequently reflects proliferation of yeasts due to the disturbances of normal homeostatic balance of a host's immunity on the one hand and virulence of the yeasts on the other hand. Dog breeds with increased risk of *Malassezia* dermatitis include: West Highland White Terrier, English Setter, Shi Tzu, Basset Hound, American Cocker Spaniel, Boxer, Dachshund, Poodle and Australian Silky Terrier. Predisposed are also cat breeds Devon Rex and Sphynx [2]. The localization on the body of animals is derived from the lipophilic character of these yeasts. The most frequently inhabited sites are: the auditory canal, face, skin folds, interdigital space, axillary and inguinal region and anal region [22].

The successful management of *Malassezia* diseases frequently depends on the treatment of yeast overgrowth and concurrent bacterial infection by local or systemic antibiotics, as well as, identification and potential correction of predisposing factors.

The proliferation of yeasts is linked to factors such as: hypersensitivity (atopy, skin food allergies, allergy to flea bite and contact allergy), anatomic defects (skin folds or serous auditory canals), cornification disorders, ectoparasite infections, bacterial pyodermy, and endocrine disorders (hyperadrenocorticism, hypothyroidism, *diabetes mellitus*, in cats also viral leukaemia and viral leukopenia). Long-term corticoid or antibiotic treatment can also result in an increase of yeast populations. In addition, lesions can develop due to hypersensitive response to yeast allergens [5].

Virulence factors may play an important role in the pathogenicity of *Malassezia*. These factors include: enzymes esterase, lipase, acidic phosphatase, lipoxygenase, protease, phospholipase and proenzyme zymogen of the cellular wall of yeasts that can activate the complement system, initiate damage to integrity of keranocytes and result in epidermal spongiosis, inflammation and pruritus. Relevant studies have demonstrated that the expression of phospholipase in *M. pachydermatis* is modified by endogenous opioid peptide β-endorphin, which is facilitated by receptors present in the cell wall of *M. pachydermatis*. It was assumed that under the action of suitable agonists (β-endorphin) or antagonists (naloxone), these receptors affect the development of commensal or pathogenic phenotype in this species [10]. The pathogenic role of *Malassezia* may be related to the changes in chemical or immunological mechanisms of the skin that may modify the composition of the cell wall of *Malassezia*. The composition of the cell wall of the yeast *M. restricta* confirms that this is a specific type of yeast in the fungal kingdom. The cell wall of this species contains an average of 5 % chitin, 20 % chitosan, 5 % β-(1,3)-glucan and 70 % β-(1,6)-glucan that form a large alkali-insoluble complex [40].

Resistance

When dealing with a clinical case, the attending veterinarian does not need inevitably to know the species identity of *Malassezia* that caused the disease. The diagnosis on the level of yeast genus, i. e., genus *Malassezia* spp., suffices for the treatment. More important is the determination of antifungal susceptibility of the relevant clinical isolate (the agent). The cases of reduced susceptibility of *Malassezia* to commonly used antimycotics detected under field and laboratory conditions indicate the necessity of control and alertness with antimycotic treatment. In cases of treatment of recurrent or persistent mycosis, repeated testing of susceptibility of relevant isolates is recommended. It was observed that "resistant" cases were in the past 12 months treated on average by 4.4 cycles of antimycotics while dogs with "sensitive" isolates received on average only 0.8 cycles. This observation supported the view that this involved acquired resistance of yeast isolates. The latest research confirmed that increasing frequency of azole resistance in field strains, that can cause problems with the treatment, as azoles are among the most frequently used antimycotics and generally are considered as the most effective drugs for the therapy of *Malassezia* mycosis. Concrete genes, e. g. ERG11, ATM1, PDR10 and others, have been regarded as responsible for resistance of *Malassezia* [27, 29, 35, 37].

The resistance of *Malassezia* is closely related to formation of biofilms. The formation of biofilm is an important factor of virulence of yeasts of the genus *Malassezia* and a serious issue concerning future antimycotic therapy. Biofilm cells are protected against the action of antimycotics in several ways. The biofilm environment allows the cells to multiply and protects them against the immune system of the host and the effect of antifungal compounds. Investigations confirmed the ability of *M. pachydermatis* to form biofilms and much lower susceptibility of biofilms of all tested strains to ketoconazole and itraconazole in comparison to susceptibility of their planktonic forms. It was also observed that the cells persisting in biofilms exhibited increased expression of genes responsible for their resistance [25].

New therapeutic possibilities

The increasing antimycotic resistance of *Malassezia* has encouraged the search for new ways of therapy. Recently the role of plant essential oils as antimicrobial compounds became the subject of intensive research because of their documented action on fungal, viral and bacterial pathogens [1, 17]. Essential oils are mixtures of volatile oils obtained from plants, soluble in alcohol and aether but insoluble in water. Plant essential oils contain a broad range of chemical compounds capable of acting

by multiple mechanisms on various components of microbial cells. During the past five years, scientific studies described properties of a large number of tested essential oils obtained from plants in various genera: *Thymus* (thyme), *Artemisia* (wormwood), *Melaleuca* (tea tree), *Cinnamonum* (cinnamon), *Ocimum* (basil), *Rosmarinus* (rosemary), *Origanum* (oregano), *Syzygium* (clove), *Foeniculum* (fennel), *Trachyspermum* (ajwain), *Myrtus* (myrtle), and others. A number of studies confirmed that plant essential oils are promising effective components in fighting the *Malassezia* yeasts [4, 15, 17, 28].

CONCLUSIONS

Lipophilic *Malassezia* spp. are commensal fungi of the mammalian skin, responsible for dermatitis and otitis in dogs. It has been recognised that they may be the cause of primary mycosis, but as well as responsible for concurrent infection at bacterial diseases of skin and ears. They represent a risk factor at some dermatitis types, e. g., atopic or seborrheic. Proliferation of *Malassezia* occurs as a result of weakened immunity of an individual and is supported by virulence factor of *Malassezia* in the form of various enzymes and proenzymes. *Malassezia* mycoses are frequently characterised by relapses and successfulness of their treatment requires a complex approach.

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DETECTION OF SOME VIRULENCE FACTORS IN STAPHYLOCOCCI ISOLATED FROM MASTITIC COWS AND EWES

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ABSTRACT

About 150 million families around the world are engaged in milk production. However, inflammation of the mammary gland (mastitis) remains a major problem in dairy ruminants that affects the quality of milk worldwide. The aim of this study was the examination of udder health with detection of contagious and environmental pathogens causing mastitis in 960 and 940 dairy cows and ewes, respectively. The presence of selected virulence factors such as: the formation of haemolysis, gelatinase, biofilm, hydrolyse DNA, and resistance to antibiotics with detection of methicillin resistance gene (*mecA*), were determined in selected virulence factors associated with isolated staphylococci. These isolated staphylococci with selected virulence factors can have untoward effects on the severity of mastitis. The results of our study indicated that, in addition to the major udder pathogens (*S. aureus, S. uberis,* and *S. agalactiae*) causing mastitis, non-aureus staphylococci (NAS), is a major risk to dairy cows and ewes. NAS, such as *S. chromogenes*, *S. warneri*, and *S. xylosus* isolated from infected animals with clinical and chronic mastitis, had the highest representation of virulence factors in comparison to less virulent strains. In addition, the isolates of *S. aureus* and NAS demonstrated 77.0 % and 44.2 % resistance to one or more antimicrobial classes from mastitic milk samples obtained from dairy cows and ewes, respectively. Due to the high resistance to β-lactamantibiotics in two isolates of *S. aureus* and two species of NAS isolated from cows' mastitic milk samples, the presence of a methicillin-resistant gene *mecA* poses serious complications for the treatment and a serious health risk to milk consumers.

Key words: antibiotics; biofilm; dairy cows; ewes; mastitis; methicillin resistance gene; non-aureus staphylococci

INTRODUCTION

Milk and milk products are important global dietary products, consumed by more than 6 billion people worldwide. In 2019, the recorded milk consumption was 852 million tons, distinguishing the dairy industry as a very profitable market. The milk obtained is a traditional raw material for the production of a range of dairy products, which are unique in their composition, but EU rules emphasize that such products must come from healthy animals, which significantly limits their production and quality [6].

Despite the increasing animal hygiene level in dairy farming, the inflammation of the mammary gland (mastitis) is still one of the main health problems. This disease is associated with pain and adversely affects animal health, welfare, milk quality and the economics of milk production [28]. According to a study by H o g e v e e n et al. [11] the losses for the global dairy industry estimated at 16–26 billion euros per year; based on a global population of 271 million dairy cows, with a cost of 61–97 € per animal for farmers. In the United States, economically, bovine mastitis costs around $2 billion every year. It has also been identified as one of the most economically relevant diseases in Ireland by the Animal Health Ireland [19].

Due to their polyethological origin, infections of the mammary gland are most often caused by a complex of interactions among the host, environment, and infectious agents result in mastitis, one of the most frequent diseases of dairy cows and ewes (Fig. 1). In comparison with most other animal diseases, mastitis differs by the fact that several diverse kinds of bacteria can cause the infection. These pathogens are capable of invading the udder, multiplying there, and producing harmful, inflammation causing compounds [3].

Up to this date, more than 137 different organisms have been recognized as causative agents of ruminant intramammary infections (IMI). They include: bacteria, viruses, mycoplasma, yeasts, and algae, but bacteria have been identified as the principal causative agents of mastitis (95 % of all IMI). In general, each mastitis case is believed to be caused by one primary pathogen, as in milk samples from the affected udder usually only one bacterial species has been identified. However, it is not rare to detect simultaneous infections by two different pathogen species and even three pathogens have been found in a small proportion of samples [32].

The most common forms of mastitis have been caused by agents from two groups of bacteria. The group of con-

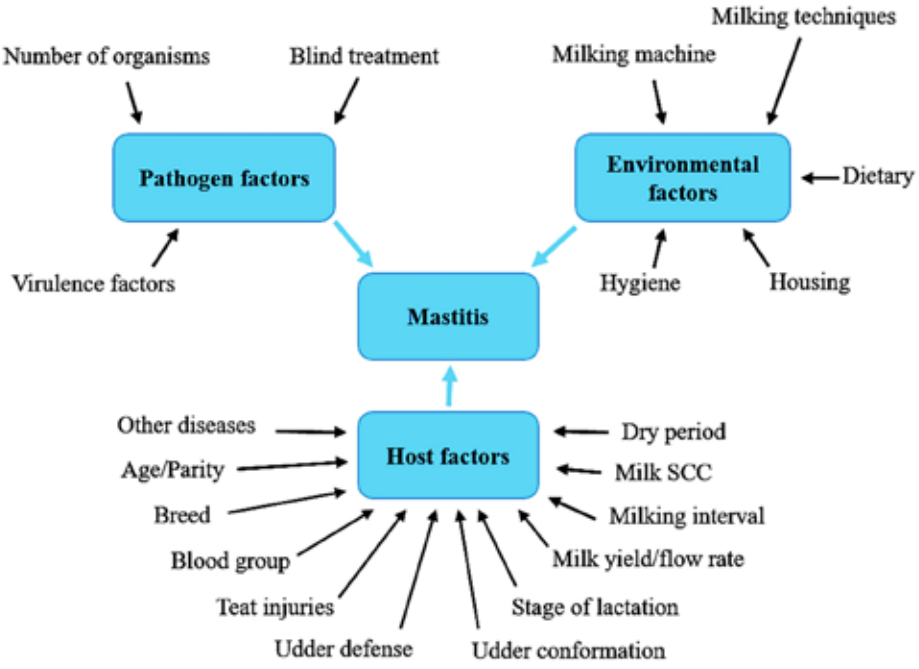

Fig. 1. Factors influencing the development of mastitis
Source: Zigo et al. [31]

tagious pathogens, (e. g., *Staphylococcus aureus, Streptococcus agalactiae*, or *Streptococcus dysgalactiale*) includes bacteria that survive and grow within the mammary gland (MG) and thus the infection can spread from infected to uninfected quarters and from cow to cow. This occurs most frequently during milking. The group of environmental pathogens varies by staphylococcal species. They prosper in the environment, especially in the presence of cow faeces [3].

Of this group, *E. coli* is the most important with multiple strains of varying pathogenicity for animals and humans. Others include: *Streptococcus uberis*, non-aureus staphylococci (NAS), *Corynebacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Proteus* spp., *Pasteurella* spp., *Listeria* spp., *Leptospira* spp., *Yersinia* spp., *Enterobacter* spp., *Brucella* spp. and *Mycobacterium* spp. [24, 26].

In recent years, *S. aureus* and NAS belong to the most common microorganisms causing mastitis in dairy cows and health disorders among consumers of milk and dairy products. According to the World Health Organization, 420,000 lives are lost due to food poisoning; and *Staphylococcus* spp. is characterized as an important agent that can cause foodborne diseases. Poisoning occurs due to the ingestion of preformed enterotoxins in food [23, 30].

Lately, NAS have become a concern among dairy producers, as their potential as mastitis-pathogens has been observed; and they have already been found in the majority amongst other pathogens. Their predominant isolation can be explained by the fact that NAS are pathogens adapted to survive in cows or ewes and may be in the mammary gland of sick or healthy animals. Among all the NAS found in dairy animals: *S. haemolyticus, S. chromogenes, S. epidermidis, S. warneri, S. cohnii, S. simulans, S. hominis, S. capitis,* and *S. xylosus* are the most prevalent species [5].

The main complications associated with the treatment of *S. aureus* and NAS infection include the fact that many strains can cause this disease and increasing number of them becomes resistant to increasing range of antibiotics (ATB) available for veterinary use. The increase in resistance occurs also because, in addition to treating clinical cases of IMI, the common routine on farms is to treat dry dairy cows across the board with ATBs [32].

The increase in the occurrence of NAS on dairy farms was observed after a decrease in the incidence of mastitis caused by the main pathogens; the causative NAS demonstrated an increased resistance to common ATBs and disinfectants [25]. Compared to *S. aureus*, NAS usually exhibits a lower number of virulence factors. The essential factor of pathogenicity of NAS is biofilm formation that allows them to survive application of disinfectants and other sanitation procedures. N a s c i m e n t o et al. [15] reported that the NAS (*S. epidermidis, S. saprophyticus, S. hominis,* and *S. aerletae*) isolated from mastitic ruminants, were resistant to the ATBs used to treat cows during lactation and were able to produce some of the staphylococcal enterotoxins.

Especially, multiresistant strains of staphylococci associated with resistance to more than one ATB are a serious risk to public health [25]. Recent studies also suggest that multi resistant staphylococci, especially to β-lactams ATBs indicates the presence of methicillin- resistant staphylococci (MRS) that have been identified in raw milk and dairy products, including cheeses [10, 15, 32].

In addition to the increased antibiotic resistance of staphylococci, the authors V a s i l' et al. [29] and H a v e r i et al. [8] confirmed biofilm formation and lysines in mastitic milk samples and considered them as important virulence factors involved in the development of clinical mastitis (CM).

Previous research has linked staphylococci and their virulence factors to the pathogenesis and clinical manifestations of mastitis. They stressed the importance of a thorough knowledge of their virulence factors, structures and products. It is crucial to understand how these microorganisms facilitate adhesion and colonization of the mammary gland epithelium which allows them to survive, successfully establish themselves and persist in the host tissue. Therefore, this study was aimed at the aetiology and determination of contagious and environmental udder pathogens in dairy cows' and ewes' herds. Particularly in isolated staphylococci, the presence of selected virulence factors such as: haemolysis, gelatinase, biofilm, hydrolyse DNA, and resistance to antimicrobials with detection of methicillin resistance gene (*mecA*), and their effect on the severity of mastitis were determined.

MATERIALS AND METHODS

Monitored dairy farms

The practical part of this study was carried out in four

different cows' and four sheeps' herds located in Slovakia with conventional (non-organic) farming. From dairy cows, each herd size ranged from 150 to 300 of Slovak spotted cattle breed between 1st and 4th lactation. The dairy cows investigated on all four farms were kept in a free housing system on straw litter, with *ad libitum* access to water. They were fed a total mixed feed based on silage, hay, and concentrate, in agreement with international standards [18]. The rations met the nutritional requirements of cows weighing 65 kg, with average milk yield 20–30 kg per day. In the first phase of lactation, the mean average dry matter intake per cow per day was 23.6 kg ± 3.7 kg. All cows were milked twice daily in parallel (Boumatic, USA) or fishing (DeLaval, Sveden) parlour. From all bovine dairy farms, we investigated 270 cows from first, 215 cows from the second, 250 cows from the third and 225 cows from the fourth herd.

The four sheep farms were in herd sizes ranging from 200 to 400 animals and consisted of Improved Valachian, Lacaune, and Tsigai breeds. In April, the ewes were on pasture during the day and received concentrates in amounts of 200 g per day during milking. After their lambs were weaned in early April, the ewes were milked twice a day on each farm. In the first two herds, machine milking was performed using a two-line milk parlour 2×14 Miele Melktechnik, (Hochreiter Landtechnik, Germany) and in two other herds the sheep were milked in two-line milk parlour 2×16 Alfa Laval Agri (Alfa Laval, Sweden). From all the sheep farms, during the first month of pasture (April), 220 ewes from first, 250 ewes from second, 270 ewes from third, and 200 ewes from the fourth herd were investigated.

Selection of dairy animals and examination of udder health

Dairy cows from the four farms were selected on the basis of the formation of production groups according to the stage of lactation (early lactation 14–100 days of lactation) and the phase nutrition, which were compiled by the animal husbandry technicians. The selected dairy cows of the same performance class (early lactation) were housed in individual husbandry groups, which included 45–90 animals on each farm.

Ewes were included in the study after lambing between the 1st–3rd lactation with a stay on pasture and milked twice a day. Complex examination of health status of the udder in ewes from the four farms was carried out at the start of the milking season (April). Each dairy cow and sheep were comprehensively examined on the basis of a clinical examination, with sensory examination and palpation of the udder, and milk from the fore-stripping of each udder quarter or halve was subjected to sensory examination and assessed by the California mastitis test (CMT) (Indirect Diagnostic Test, Krause, Denmark) with the collection of raw milk samples from positive cows and ewes [12]. CMT scores were 0, +, ++ and +++ for "negative", "weak positive", "positive" or "strong positive", respectively [27].

Subsequently, from the 960 cows, 689 cows had a negative CMT score, and 271 cows, based on clinical manifestations and with a CMT score indicating trace or positive (score of 1–3) were chosen for aseptic collection of 12 mm mixed quarter milk samples by discarding first squirts with the cleaning of the teat end with 70 % alcohol for laboratory analyses of bacterial pathogens, according to H o l k o et al. [12]. From 940 ewes, 756 animals had negative CMT scores and from 184 animals with CMT score trace or 1–3 were taken of 12 ml mixed halves milk samples for laboratory analyses. All milk samples from cows and ewes were cooled to 4 °C and immediately transported to the laboratory and were analysed on the following day.

Each mastitis case was assigned a corresponding mastitis grade according to the National Mastitis Council [17], with the mastitis grade categorized into severity levels. Subclinical mastitis (SM) was detected by a high somatic cell count (SCC) using CMT evaluation without any visible abnormalities of the milk or apparent signs of local inflammation or systemic involvement. Clinical mastitis (CM) was classified as mild mastitis, being characterized by visible changes in secretion, moderate mastitis, additionally showing local signs of inflammation of the mammary gland (Fig. 2), and severe mastitis; also showing general signs such as fever, low temperature, loss of appetite or inability to stand. A history of chronic mastitis or persistent mastitis was detected based on previous treatment of clinical examination of the udder with a positive CMT score and cultivation.

Bacteriological culture and evaluation of growth on plates

In the laboratory, 0.2 ml of milk was inoculated from each sample onto blood agar plates (Oxoid LTD, Hamshire, UK) and incubated aerobically at 37 °C during

Fig. 2. Clinical mastitis with a change in the consistency of the milk secretion caused by *S. aureus* and confirmed on blood agar culture
Photos: F. Zigo

24 hours. The primocultivated colonies from the blood agar and identified as *Staphylococcus* spp. were sub-cultured onto different selective bacteriological media (No. 110, Baird-Parker agar, Brilliance UTI Clarity Agar, (Oxoid, Hampshire, UK) and incubated at 37 °C for the next 24 hours. The colonies were identified on the basis of: cell morphology, Gram staining, type of haemolysis, the activities of catalase (3 % H_2O_2, Merck, Darmstadt, Germany) aesculin hydrolysis, and cytochrome oxidase C (Bactident Oxidase, Merck). The presumptive *Staphylococcus aureus* were detected with the clumping factor test (DiaMondiaL Staph Plus Kit, Germany). Aesculin positive streptococci were cultivated on modified Rambach agar for the identification of *Streptococcus uberis* or *Enterococcus* sp. according to studies by V a s i l' et al. [29] and H o l k o et al. [12]. Lancefield serotyping (Dia MondiaL Strept Kit, Germany) was used to characterize aesculin negative streptococci. The species of gram-negative rods were identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) according to H i k o [10]. Contagious pathogens (*Staphylococcus aureus, Streptococcus agalactiae*) were classified as positive if one or more colony forming unit (CFU) were found. Other pathogens were classified as positive if at least five CFU were found. Samples were classified as contaminated if three and more pathogens were isolated from one milk samples and growth of contagious pathogens were not identified.

Detection of virulence factors in staphylococci

Staphylococci confirmed by MALDI-TOF analysis were exposed to deoxyribonuclease (DNase test) and tested to produce extracellular proteolytic enzymes (Gelatin hydrolysis test) according to H i k o [10]. The formation of biofilm was determined by the phenotypic method, and by the growth on Congo Red agar (CRA) according to V a s i l' et al. [30].

The ability of staphylococci to produce haemolysins was also determined according to M o r a v e j i et al. [14]. Types of haemolysis were phenotypically characterized based on the lysis zone of each staphylococcal isolate on the plates of blood agar base supplemented with 5 % sheep blood after 24 and 48 h incubation at 37 °C.

The susceptibility of staphylococci isolated from cows (n = 136) and sheep (n = 86) infected milk were tested *in vitro* against 14 antimicrobial agents. The susceptibility tests of isolates were carried out on Mueller Hinton agar using a standard disk diffusion procedure [1]. In our study, antibiotic discs containing penicillin (PEN;10 µg), ampicillin (AMP; 10 µg), amoxicillin (AMC; 10 µg), amoxicillin+clavulanic acid (AXC; 20/10 µg), ceftiofur (CEF; µg), oxacillin (OXA; 1 µg), cefoxitin (CFX; 30 µg), ciprofloxacin (CPR; 5 µg), lincomycin (LNC; 15 µg), neomycin (NMC; 10 µg), novobiocin (NVB; 5 µg), rifaximin (RFX; 15 µg), streptomycin (STR; 10 µg), and tetracycline (TET; 30 µg) were used. The zone of inhibition was recorded in millimetres, and the results were interpreted as previously described. The determined diameters of the respective inhibition zones were evaluated (susceptible, intermediate, resistant) according to CLSI breakpoints [2]. In the tests, the strains *S. aureus* CCM 4750 and *S. chromogenes* CCM 3386 (Czech Collection of Microorganisms, Brno, Czech Republic) were used as a control. The choice of antimicrobials reflects the range contained in a number of intramammary products to treat mastitis, which are available in Slovakia.

Detection of the *mecA* gene from the isolated staphylococci

Phenotypical positive staphylococci (45 and 26 isolates from cow and sheep mastitic milk samples) based on their antimicrobial resistance to β-lactam antimicrobials were subjected to the polymerase chain reaction (PCR) to test for methicillin resistance. The total genomic DNA was isolated according to H e i n et al. [9]. The DNA quality was checked using a BioSpec spectrophotometer (Shimadzu, Japan).

The source of DNA obtained as supernatant with centrifugation was used in the PCR reactions using primers *MecA*1 (GGGATCATAGCGTCATTATTC) and *MecA*2 (AACGATTGTGACACGATAGCC) (Amplia s.r.o, Bratislava, Slovakia) for detection of the *mecA* gene according to P o u l s e n et al. [22]. The confirmation of the identity of the PCR products (527 bp) with the selected primers was in accordance with the instructions specified by GATC Biotech (AG, Cologne, Germany) using Sanger sequencing. The similarity of the DNA sequences obtained from the isolates with those available from the GenBank–EMBL (the European Molecular Biology Laboratory) database were determined using the BLAST program (NCBI software package). *S. aureus* CCM 4750 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a reference strain for PCR in this study.

Statistical analysis

The data were entered into Microsoft Excel 2007® (Microsoft Corp., Redmond, USA) and analysed using Excel, State 11, and SPSS version 20 (IBM Corp., Armonk, USA). The dependence of the production of virulence factors on the most frequently isolated staphylococci from clinical, chronic and subclinical mastitis was statistically analysed using the chi-squared test with the significance level $\alpha = 0.05$; critical value $\chi^2 = 1.824$ for testing staphylococci isolated from mastitic ewes and $\chi^2 = 2.206$ for testing staphylococci isolated from mastitic cows; and the testing value G. Statistical independence between isolates with virulence factors and isolates without virulence factors within each species was confirmed when $G > \chi^2$; the independence was not statistically significant when $G < \chi^2$.

RESULTS

Based on the clinical examination of the MG, assessment of CMT, and laboratory diagnosis of milk samples, the occurrence of CM in the cow and sheep dairy farms was 9.1 % and 4.5 %, respectively. The most common form of IMI in cows and ewes was subclinical mastitis, with an incidence of 11.3 % and 10.2 %, respectively. The occurrence of chronic mastitis was 3.6 % and 1.8 % in dairy cows and ewes, respectively.

Investigation of four dairy herds demonstrated that of 960 dairy cows examined during the early lactation phase (14–100 days of lactation), 689 cows (71.8 %) had a negative CMT score and 271 cows (28.2 %) had a CMT score of trace or 1–3 for one or more quarters. The evaluation of udder health from the 940 ewes during their first month of the pasture season had 756 (80.4 %) animals with a negative CMT. One-hundred eighty-four ewes (19.6 %) had a positive CMT with a score of trace or 1–3 (Fig. 3).

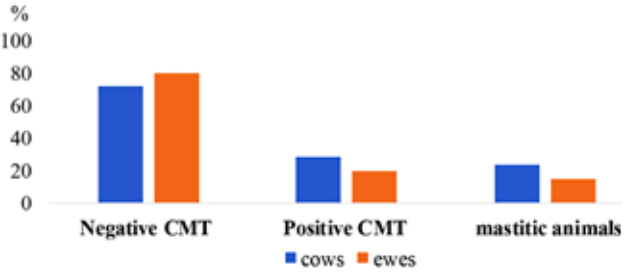

Fig. 3. Evaluation of CMT in monitored dairy herds
CMT – California mastitis test

Of the mixed milk samples taken from each cow and sheep based on the anamnesis and positive CMT score, bacterial agents causing clinical or subclinical mastitis were identified in 230 (84.8 %) and 155 (84.2 %), respectively. For the presence of udder pathogens, 41 (15.1 %) and 29 (15.7 %) samples from cows and ewes with a positive CMT score were identified as negative or contaminated.

Of the cows' and ewes' with positive samples, 136 and 86 cases (59.1 % and 55.4 % of the infected samples) contained the most commonly isolated staphylococci, respectively. The NAS represented the most commonly detected bacteria (42.6 % and 39.9 % of positive findings in cows and ewes) causing mainly subclinical mastitis. The *S. aureus* was the second most common pathogen (16.5 % and 18.2 % of positive findings in cows and ewes, respective-

Table 1. Udder pathogens isolated from milk samples of four monitored dairy cows and four sheep herds

Pathogens	Cows	Ewes	Clinical IMI[1] [n/%]		Subclinical [n/%]		Chronic [n/%]	
	[n/%]	[n/%]	Cows	Ewes	Cows	Ewes	Cows	Ewes
NAS	98/42.6	59/39.9	37/16	9/6.1	53/23.0	44/29.7	8/3.5	6/4.1
S. aureus	38/16.5	27/18.2	18/7.8	11/7.4	9/3.9	9/6.1	11/4.7	6/4.1
Escherichia coli	26/11.2	18/12.2	7/3.0	7/4.7	17/7.4	10/6.7	2/0.9	1/0.7
Str. uberis	21/9.1	0/0	9/3.9	0/0	5/2.2	0/0	7/3.0	0/0
Str. agalactiae	8/3.4	4/2.7	3/1.3	4/2.7	2/0.9	0/0	3/1.3	0/0
***Streptococcus* spp.**	10/4.3	9/6.0	4/1.7	1/0.7	6/2.6	6/4.1	0/0	2/1.4
***Enterococcus* spp.**	14/6.1	24/16.2	3/1.3	5/3.4	11/4.8	17/1.5	0/0	2/1.4
Mixed infection	15/6.5	7/4.7	6/2.6	5/3.4	5/2.2	10/6.7	4/1.7	0/0
Total	**230/100**	**155/100**	**87/37.8**	**42/27.1**	**108/46.9**	**96/62.0**	**35/15.2**	**17/11.0**

Clinical IMI[1] – clinical mastitis represented in mild, moderate, or severe forms of intramammary infection; n – number

Table 2. The role of *S. aureus* and NAS in the form of mastitis from infected cows and their virulence factors

***Staphylococcus* spp.** [number]	**IMI[1]** [number]	**Haemolysins[2]**	**DNAse[3]**	**Gelatinase**	**Biofilm**	***mecA* gene**	**Testing value**
S. aureus (38)	Clinical (22)	6α/4δ/1β	14	17	9	2	5.447*
	Chronic (8)	3α/2δ/2β	8	7	7	0	
	Subclinical (8)	3α/1β	6	7	5	0	
S. chromogenes (22)	Clinical (11)	4β/3δ	3	4	4	0	3.204*
	Chronic (4)	3β	1	1	2	1	
	Subclinical (7)	2β/2δ	1	1	2	0	
S. warneri (20)	Clinical (9)	4δ/2β	2	2	4	1	2.688*
	Chronic (3)	3β	0	0	1	0	
	Subclinical (8)	3β/1δ	2	0	2	0	
S. xylosus (18)	Clinical (7)	2δ/2β	2	0	3	0	2.255*
	Chronic (1)	0	0	0	0	0	
	Subclinical (10)	4β/1δ	0	0	2	0	
S. epidermidis (9)	Clinical (2)	1δ	0	0	1	0	1.012
	Subclinical (7)	2δ	0	0	2	0	
S. haemolyticus (7)	Clinical (4)	2β/1δ	1	0	2	0	0.742
	Subclinical (3)	0	0	0	0	0	
S. capitis (6)	Clinical (2)	2δ	0	0	0	0	0.401
	Subclinical (4)	0	0	0	0	0	
S. piscifermentans (6)	Clinical (2)	1β	0	0	1	0	0.851
	Subclinical (4)	2δ	0	0	0	0	
S. hyicus (10)	Clinical (0)	0	0	0	0	0	0.332
	Subclinical (10)	1δ	0	0	1	0	

IMI[1] – number of isolates and their influence on type of mastitis; haemolysins[2] – production of haemolysin type α, β or δ; DNAse[3] – ability of staphylococci to hydrolyse DNA; *– Chi-squared test significance level $\alpha = 0.05$; critical value $\chi^2 = 2.206$; Testing value (G) – statistical independence of virulence factors in isolated staphylococci was confirmed when $G > \chi^2$; the independence was not statistically significant when testing value was $G < \chi^2$

Table 3. The role of *S. aureus* and NAS in the form of mastitis from infected ewes and their virulence factors

***Staphylococcus* spp.** number	**IMI1** number	**Haemolysins[2]**	**DNAse[3]**	**Gelatinase**	**Biofilm**	***mecA* gene**	**Testing value**
S. aureus (27)	Clinical (11)	4α/2δ/2β	6	9	4	0	3.288*
	Chronic (6)	2α/1β	3	6	4	0	
	Subclinical (10)	4α/2β	4	8	3	0	
S. warneri (14)	Clinical (3)	1α/1β	1	2	2	0	2.305*
	Chronic (3)	2β	1	1	1	0	
	Subclinical (8)	2α/2β/	3	4	3	0	
S. chromogenes (11)	Clinical (2)	1β	0	0	1	0	1.824*
	Chronic (2)	1β	1	1	1	0	
	Subclinical (7)	3β/1δ	3	2	2	0	
S. xylosus (11)	Clinical (1)	1β	1	0	0	0	1.140
	Subclinical (10)	4α/2β	2	2	2	0	
S. haemolyticus (9)	Clinical (2)	1β	0	0	0	0	0.435
	Chronic (1)	0	0	0	1	0	
	Subclinical (6)	3β	0	0	0	0	
S. caprae (8)	Clinical (1)	1β	0	0	0	0	0.341
	Subclinical (6)	0	0	0	0	0	
S. epidermidis (6)	Subclinical (7)	0	0	0	1	0	0.215

IMI[1] – number of isolates and their influence on type of mastitis; haemolysins[2] – production of haemolysin type α, β or δ; DNAse[3] - ability of staphylococci to hydrolyse DNA; *– Chi-squared test significance level α = 0.05; critical value χ^2 = 1.808; Testing value (G) – statistical independence of virulence factors in isolated staphylococci was confirmed when G > χ^2; the independence was not statistically significant when the testing value was G < χ^2

ly), primarily causing clinical or chronic mastitis, followed by *E. coli*, streptococci, and enterococci (Table 1).

Table 2 and 3 summarize, in descending frequency, the isolated strains of *Staphylococcus* spp., and indicated their role in the type of mastitis and the occurrence of selected virulence factors. The *S. aureus* isolated from clinical, chronic or subclinical cases of mastitis had the highest ability to possess virulence factors compared to NAS and revealed: haemolysis in the blood plates, production of gelatinase, biofilm, and the ability to hydrolyse DNA. The *mecA* gene was detected in two isolates of *S. aureus* from a cows' clinical mastitis. Eight species of NAS were isolated from mastitic cows, with the following recorded: *S. chromogenes* (22.4%), *S. warneri* (20.4%), *S. xylosus* (18.4%), *S. epidermidis* (9.1%), *S. haemolyticus* (7.1%), *S. hyicus* (10.2%), *S. capitis* (4.4%), and *S. piscifermentans* (4.4%). From the mastitic ewes there were isolated six species of NAS with the following recorded: *S. warneri* (23.7%), *S. chromogenes* (18.6%), *S. xylosus* (18.6%), *S. haemolyticus* (15.2%), *S. caprae* (13.6%), and *S. epidermidis* (10.2%). From all cow and ewe mastitic samples caused by NAS, 48 and 26 (48.9% and 44.1%) cases involved production of: haemolysins, 12 and 11 (12.2% and 18.6%), hydrolysis of DNA, 8 and 12 (8.1% and 20.3%), production of gelatinase, and 27 and 14 (27.5% and 23.7%) were involved in biofilm formation.

In Table 2, the significance level of α=0.05 was confirmed in the isolated staphylococci *S. aureus, S. chromogenes, S. warneri* and *S. xylosus* from CM and chronic cows' mastitis, which had the most numerous representations of virulence factors (production of haemolysins, gelatinase, the ability to hydrolyse DNA and the formation of biofilm) in comparison to less virulent strains. In addition, the *mecA* gene was confirmed from one chronic

Table 4. Phenotypic resistance profile in isolates of *Staphylococcus* spp. from mastitic cows and ewes

Number of groups of antimicrobials	Phenotypic resistance profile	Cows (n = 136)		Ewes (n = 86)	
		Number of isolates	% of isolates	Number of isolates	% of isolates
0		41	30.1	48	55.9
1	PEN	7	5.1	4	4.7
1	STR	7	5.1	2	2.3
1	NMC	8	5.9	2	2.3
1	AMX	7	5.1	4	4.7
1	NVB	6	4.4	2	2.3
1	AMP	6	4.4	3	3.5
1	LNC	4	2.9	2	2.3
1	OXA	5	3.7	3	3.5
2	NMC, STR	8	5.9	2	2.3
2	OXA, NVB	0	0	2	2.3
2	OXA, TET	4	2.9	0	0
2	CPR, NVB	2	1.5	0	0
2	LNC, NVB	2	1.5	4	4.7
3	PEN, AMX, OXA	4	2.9	3	3.5
3	PEN, LNC, NVB	2	1.5	0	0
3	AMP, OXA, NMC	3	2.2	3	3.5
3	CPR, NMC, STR	4	2.9	0	0
3*	NVB, LNC, STR	4	2.9	2	2.3
4*	RFX, CPR, STR, TET	2	1.5	0	0
4*	CPR, LNC, NMC, NVB	3	2.2	1	1.2
4*	NVB, CPR, NMC, STR	2	1.5	0	0
4*	AMP, CEP, FOX, PEN	3	2.2	1	1.2
5*	OXA, AMP, LNC, NMC, STR	2	1.5	0	0
Total multi drug resistant isolates		**16**	**11.8**	**4**	**4.7**
Total antimicrobial-resistant isolates		**95**	**70.0**	**38**	**44.2**

*MDR – multi drug isolates resistant to three or more antimicrobial classes; AMX – amoxicillin, AMC – amoxicillin + clavulanic acid, AMP – ampicillin, CEP – cephalexin, CPR – ciprofloxacin, FOX – cefoxitin, LNC – lincomycin, NMC – neomycin, NVB – novobiocin, OXA – oxacillin, PEN – penicillin, RFX – rifaximin, STR – streptomycin, TET – tetracycline

case of mastitis in *S. chromogenes* and one CM case in *S. warneri*. In isolated staphylococci from mastitic ewes demonstrated in Table 3, the significance level in *S. aureus, S. warneri*, *S. chromogenes,* and *S. xylosus* was confirmed. The presence of the *mecA* gene had not been confirmed in the tested *S. aureus* and NAS.

In 136 and 86 isolates of staphylococci from mastitic cow and ewe milk samples, *in vitro* resistance to 14 antimicrobials were tested by the standard disk diffusion method (Table 4). Generally, low resistance was shown to tetracycline, amoxicillin reinforced with clavulanic acid, rifaximin and cephalexin. Of the tested staphylococci, 95 and

38 isolates (70.0% and 44.2%) from mastitic cows and ewes showed resistance to one or more antimicrobials. To one antimicrobial, 50 and 22 isolates (36.7% and 25.6%) from mastitic cows and ewes were resistant. Mastitic cows and ewes produced 55 and 16 (39.7% and 18.6%) resistant staphylococci isolates to two or more antimicrobials, respectively. Multi-drug resistance to three or more antimicrobial classes were recorded in 16 and 4 isolates (11.7% and 4.7%) from cows' and ewes' samples. The tested staphylococci revealed multi-resistance to a combination of antimicrobial classes, such as: aminoglycosides, β-lactams, macrolides and cephalosporins.

The 45 and 22 isolates (33.1% and 25.6% of all isolated staphylococci) from mastitic cows and ewes in which phenotypic resistance was confirmed to β-lactam antimicrobials were tested by PCR for methicillin resistance with the detection of the *mecA* gene. Only from positive cows' milk samples, the presence of *mecA* gene was confirmed in four isolates of staphylococci (two isolates of *S. aureus* and one isolate each of *S. chromogenes* and *S. warneri*), which at the same time demonstrated resistance to both cefoxitin and oxacillin (Table 2). Based on the results of our study, these isolates were considered as methicillin-resistant staphylococci (MRS).

DISCUSSION

The incidence of mastitis is, of course, highly dependent on the lactation stage and health status of dairy animals [31]. In our study, we monitored the prevalence and aetiology of mastitis in four cow and four ewe dairy farms during the early lactation phase. In particular, cows in this lactation stage (14–100 days after calving) represent the largest group in farms because milk production depends on them. The dairy cow produces a quantity of milk representing 42–45% of the total milk produced during first 100 days of lactation. With such an enormous milk production burden, cows are exposed to stress factors, such as: hormonal changes associated with lactogenesis, reduced dry matter intake (which is in contrast to the desired increasing milk yield), increased lipomobilisation of body reserves with a negative energy balance, and a change in body score [16].

All of the risk factors affect the non-specific and specific immune system; in particular, the MG, through which pathogenic microorganisms penetrate more easily from the external environment. With the onset of intramammary infection, one of the indicators is an increased Somatic Cell Count (SCC) [27] which was confirmed in our study based on the evaluation of the CMT. Of the 960 dairy cows, 689 (71.7%) cows, based on anamnesis, clinical examination and evaluation of CMT, were negative and 271 cows (28.2%) showed a trace or positive CMT, with a score of 1–3. Cows with a high SCC were in 84.9% cases (230 positive cows from 271 cows) positive for the presence of an udder pathogen, which poses a significant risk to the health of the individual and the spread of infection to the environment. On the sheep farms during the first month of pasture season 756 sheep (80.4%) had a negative CMT and 184 animals (19.6%) had increased SCC on the basis of the CMT score. The laboratory examination revealed that 136 samples (14.5%) were positive for the presence of an udder pathogen (Fig. 3).

Generally, IMI begins when pathogens pass through the teat canal, interact with the mammary tissue cells, multiply and disseminate in the cisterns and throughout the duct system. The manifestation of mastitis depends mainly on the degree of reaction of the udder tissue to injury or infection [3].

Of the 230 and 136 infected cows and ewes, 46.9% and 62.0% had subclinical, 37.8% and 27.1% clinical and 15.2% and 11.0% had chronic mastitis (Table 1). According to S i n g h a et al. [26], CM represents a serious health problem that can result in: the reduction of milk yield, milk quality deterioration, treatment costs, involuntary culling, death, increased risk of antimicrobial resistance, and reduced animal welfare. Therefore, the prevalence of CM should be at the lowest level in lactating ruminants. Our results indicate that the prevalence of CM in a cows' dairy farm is in contrast with the study of R a h m a n et al. [24], who reported the prevalence of clinical forms from 2.3% to 4.1% in lactating cows.

The incidence of mastitis in sheep farms are extremely variable. F t h e n a k i s [7] found the occurrence of mastitis in sheep was between 4–50%. In our study, the incidence of mastitis at the beginning of the pasture season was 16.4% in sheep herds with the most frequently of subclinical form (11.5%). The occurrence of CM was 4.9% which is considered an acceptable value. On the contrary, a study from British slaughterhouses reported a very high prevalence of CM ranging from 13–50%. This suggest

that CM or chronic mastitis is a major cause of culling of ewes in the UK [4].

A Finnish study focused on the detection and aetiology of mastitis; P y ö r ä l ä and T a p o n e n [23] pointed to a much higher risk of CM caused by *S. aureus* and NAS, which was also confirmed in all of dairy herds across our study. Of the 230 and 155 infected cows and sheep samples, NAS (42.6% and 39.9% of positive findings) and *S. aureus* (16.5% and 18.2%) were the most frequently detected, in 136 and 86 cases (59.1% and 55.5%). The isolates of *S. aureus* and NAS accounted for 7.8% and 7.4%, and 16.0% and 6.1% of CM from mastitic cows and ewes milk samples; however, *S. aureus, S. chromogenes, S. warneri* and *S. xylosus* often caused chronic mastitis due to persistent IMI. As the results of our study revealed, the incidence of clinical and chronic mastitis in the cows and sheep farms caused by *S. aureus* and some NAS is higher compared to other udder pathogens (Table 1).

Consistent with the results of P e r s s o n et al. [21], we assume that the chronic IMIs caused by *S. aureus* and NAS in our study are predominantly persistent rather than new infections. It has been shown that many cows and ewes with *S. aureus* IMI in early lactation were already positive during the previous lactation or at drying off, which can precipitate a persistent, subclinical infection into CM in immunocompromised animals after calving or lambing.

The high detected incidence of staphylococci in our results was consistent with the study of H o l k o et al. [12], who recorded a high incidence of NAS and *S. aureus* isolated from infected milk samples during the examination of 42 dairy farms in the west of Slovakia. The NAS represented 35.9% of the positive findings and were the most commonly detected bacteria. On the other hand, the authors confirmed a high resistance to aminoglycosides and β-lactam antimicrobials but without the presence of methicillin resistance genes, which contradicted our study.

The increased incidence of staphylococcal infection in dairy ruminants also encourages the highest degree of pathogenicity in the production of more virulence factors, which are of crucial importance in persistent and CM cases [20]. These include, among others: cell wall–associated factors, different enzymes, and exotoxins that facilitate the infection pathway. For the individual virulence factors, co-production of hemolysins, hydrolysis of DNase was detected in *S. aureus, S. chromogenes, S. warneri, S. xylosus,* and *S. haemolyticus* as well as the production of gelatinase. The isolated staphylococci *S. aureus, S. chromogenes* and *S. warneri* from mastitic cows and sheeps had the most numerous representations of virulence factors resulting in the increasing incidence of CM and persistent cases in comparison to strains with no virulence factors (Table 2 and 3).

In their study of staphylococci isolated from mastitis milk in cows, M e l c h i o r et al. [13], reported that biofilm formation and resistance to antimicrobials were the most frequent virulence factors in strains isolated from CM. Increasing biofilm formation was evident in strains from CM and repeat cases of mastitis after previous unsuccessful treatment. The IMI caused by *S. aureus* or NAS is difficult to treat, even with intramammary antimicrobials, so proper consideration should be given to the infections produced by biofilm-producing bacteria.

The resistance to one or more antimicrobials in our study was detected in 95 and 38 isolates (77.0% and 44.2%) of staphylococci isolated from infected cows and ewes. Multi-resistant isolates for three or more groups of antimicrobial classes represented 16 and 4 isolates (11.8% and 4.7%). The tested staphylococci phenotypically showed multi-resistance to a combination of antimicrobial classes, such as: aminoglycosides, β-lactams, macrolides and cephalosporins (Table 4). In addition, the presence of β-lactam-resistant strains confirmed in 45 and 22 isolates (33.1% and 25.6% of all isolated staphylococci) from mastitic cows and ewes in our results indicated the presence of methicillin-resistant staphylococci (MRS). By PCR was confirmed the presence of *mecA* gene in two isolates of *S. aureus* and one isolate each of *S. chromogenes* and *S. warneri* only from mastitic cows. All positive staphylococci (n=4; 2.9%) with the *mecA* gene showed resistance to oxacillin and cefoxitin and were considered as MRS.

CONCLUSIONS

Our study confirmed that more than half of the IMIs were caused by staphylococci, especially NAS, followed by *S. aureus* in cows' and ewes' dairy farms. In addition to *S. aureus, S. chromogenes, S. warneri* and *S. xylosus* isolated from CM and chronic mastitis indicated a high degree of pathogenicity in the production of more virulence factors in comparison to other strains of NAS. The

resistance to aminoglycoside and β-lactam antimicrobials was frequently found in staphylococci, possibly because these are the antimicrobials most commonly used in dairy ruminant drying and mastitis treatments.

Based on the phenotypic manifestation of antimicrobial resistance, detection of the presence of the *mecA* gene was confirmed in MRS (2.9 %) in two isolates of *S. aureus* and two isolates of NAS (one isolate each of *S. chromogenes* and *S. warneri*) from mastitic cows. We can state that *S. aureus* still comes on top in the number of chronic or severe mastitis cases, as well as the number of virulence factors but some NAS species could have the same aggressive potential based on their production of gelatinase, haemolysis, biofilm, hydrolysed DNA and multi-drug resistance.

The knowledge gained regarding the virulence of both *S. aureus* and NAS species associated with mastitis, especially in combination with resistance patterns and the presence of MRS isolates, is important for designing efficient prophylaxis and treatment guidelines to minimize the negative effects on milk yield and culling hazards in dairy animals.

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THE EFFECT OF THE CRYOTHERAPY ON THE HEALTH AND WELFARE OF DOGS: PRELIMINARY STUDY

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ABSTRACT

This study is focused on determining the cryotherapeutic effects in the treatment and welfare of dogs. We characterized the basic principles of cryotherapy as well as summarized and statistically processed the current state of the application of this form of therapy in a veterinary practice. Recent scientific studies have shown that cryotherapy is mainly effective in treating skin diseases and problems with the musculoskeletal system including minor injuries caused by excessive muscle strain. It can also be used in combination with anti-inflammatory drugs to treat the respiratory system. Moreover, cryotherapy can be an effective form of treatment for ageing. Our study involved a survey evaluation given to veterinarians in Slovakia, Czechia, and Hungary with a key question regarding their experiences with cryotherapy and cryosurgery in dog therapy. Statistical results demonstrated that Slovak veterinarians do not utilize cryo-methods satisfactorily. A summarization of the reasons could start a change in this unfavourable aspect in Slovak veterinary medicine and contribute to better promotion of cryotherapy application in the therapy of animals.

Keywords: cryosurgery; cryotherapy; cynology; dogs; questionnaire

INTRODUCTION

Cryotherapy (the word cryos is of Greek origin and means frost or cold) has been a therapeutic method used in human and veterinary medicine to treat certain diseases, mainly injuries and inflammations since ancient history. It is documented that the ancient Egyptians had utilized the cold for the injuries treatment, even before 2500 BC. Simple tools like ice or cold compress were substituted with liquid carbon dioxide (CO_2) in the early 1920s to treat skin lesions and gynecological problems in humans. The deficiencies of therapy with liquid CO_2 led to the application of liquid nitrogen in cryotherapy [13].

The effect of low temperature on the cellular level manifests mostly by necrotisation. Rapid cooling of the tissue to a temperature below –25°C followed by slow thawing results in the damage of the tissue cells by the necrosis process which is influenced by several factors acting synergistically on the cells.

The most important consequence caused by rapid

freezing is the formation of intracellular crystals inducing irreversible lesions and electrolyte concentration changes within the cells. Then the phospholipid cell membranes are broken, and in turn, extracellular crystals are formed. These phenomena lead to cellular dehydration caused by osmosis across the cell membrane. Finally, the concentrations of intracellular and extracellular electrolytes reach toxic levels.

At the same time, mechanical damage to the cell membrane occurs, and changes in the vascular system happen to result in the formation of so-called cryothrombi in the capillaries. We speak about cryothrombosis if the low temperatures cause alteration of vasomotor function and interstitial oedema promotes a slowing of circulation and an increase in vascular permeability. The decrease in temperature destroys blood elements on the endothelium of the vessels and allows thrombus fixation on the vessel walls. Thrombosis occurs several hours after application of cold to tissue, but usually within 48 hours at the most [28].

Characteristic features of cryotherapy include: reliable necrotisation of soft tissues, thrombotisation of capillaries, reversible anaesthesia of the peripheral nerves, larger vessels remaining intact, and blood circulation being fully restored in them after thawing without negative consequences, and the mineral envelope of the bones remains unchanged. The cold reduces blood flow by increasing its viscosity, vasoconstriction, and metabolic activity reducing, which reduces the oedema formation at the site of the injury [19]. Analgesia results from an alteration in the cellular metabolism and slowing of nerve conduction velocity in the motor and local sensory neurons [9, 15, 18, 26]. An additional benefit is that it decreases the concentration of inflammatory mediators, including tumour necrosis factor-α and nitric oxide [31].

Cryotherapy uses freezing temperatures to destroy cells in target tissues. Most cells are made up of about 70 % water. Inside the cells, ice crystals begin to form after exposure to freezing temperatures, which tear them apart. Subsequently, further damage to cells and tissues occurs when the blood vessels supplying the diseased tissue freeze. Cryosurgery (a special part of cryotherapy) is usually performed using liquid nitrogen. Supercooled liquid nitrogen (–196 °C) is sprayed onto the affected tissue with a cryoprobe or by using a swab. More modern applications include ultrathin needles that are capable of using argon to form ice. This procedure provides good control over the amount of frozen tissue and helps to minimize complications [14, 29, 30]. Cryotherapy in conscious dogs appears to be well-tolerated [2].

The treatment progress can be schematically divided into several stages. After the actual freezing and subsequent thawing, necrosis formation and debridement occur. The necrosis of the cryopreserved tissue usually takes 48 hours. Then, the necrotic masses begin to separate and are eliminated from the organism. The stage of necrosis debridement usually lasts about one week, then it is replaced by the stage of granulation when the lesion formed by cryallisation is filled with granulation tissue. The granulation stage can last up to three weeks, possibly longer depending on the size of the defect. The whole procedure concludes with the epithelization process, which is a smooth continuation of the previous stage. The treatment can be accelerated, especially at the necrosis removal stage, with the help of enzyme therapy. The granulation can be promoted by massaging the healing tissue, e. g. under a stream of running lukewarm water. For this reason, animals are not prevented from licking the healing defect at this stage [28].

The advantages of cryotherapy include the possibility of using it even in critical areas where the application of a conventional procedure would pose too high a risk if the immune system responds favourably to the therapy. This method is almost painless, it can be used without general anaesthesia, only in local anaesthesia of frozen tissue, or with the use of tranquilization of the patient. It presents minimal risks of postoperative bleeding and complications [2, 5, 7]. If necessary, the procedure can be repeated or the cryotherapy can be combined with other treatment methods. After cicatrisation, a very good cosmetic effect is achieved without adverse side effects [2, 7].

The positive effects of cryotherapy in the treatment of individual health problems in dogs are: reduced stress and anxiety [10], improved coat quality [27], elimination of skin disorders (atopic dermatitis, parasites, folliculitis, pustular dermatitis, dermatophytosis, yeast infections, and seborrhoea), improved mobility in patients with chronic inflammation of the joints and neurological diseases. For joint inflammation and pain, it shortens the healing phase and also relieves pain in hip and elbow dysplasia [21].

This type of therapy is also used in the treatment of other diseases such as: tumours, glaucoma, oral surgery, epulis, cysts, itchy lesions, distichiasis (removal of aberrant or extra eyelashes), warts, eczema, and other skin

disorders, such as: joint inflammation, musculoskeletal problems, gynecological diseases, and cryoextraction of the lens in cataract surgery [3, 6, 11, 16, 23].

We consider all contraindications to cryotherapy to be relative. Cryosurgery is believed to have more effective and a safer use in benign or less active malignant tumours. Melanomas are considered unsuitable for cryosurgery, but recent research in this field has been very intensive [28].

Cryotherapy like the other therapeutic methods based on physical phenomena has been predominantly used in human medicine. Step by step it expands into veterinary practice including dog therapy as can be seen from the above-mentioned applications. Our study was aimed at bringing some information about therapeutic advantages linked to the use of low temperatures in veterinary medicine. The main goal of this paper was a preliminary exploration of a fact how frequently cryotherapeutic methods are utilized by veterinarians in our close area.

Application of cryotherapy in practice

The simplest method of cryotherapy in practice is the use of an ice pack or bandage. Cryobandages enable the patient to begin using the limb earlier after surgery, reduce recovery time, and improve tolerance to other rehabilitative techniques [4]. The recommended length of time for ice pack application per session is 10–20 minutes to reach deeper tissues (to a depth of 1.5 cm) and provide longer-lasting effects [1]. The ideal frequency of administration of cryotherapy in dogs is unknown, but protocols using cryotherapy once daily up to every 4–6 hours have been advocated. Compared to control dogs following surgery, using an ice pack secured around the stifle with an elastic bandage for 20 minutes provided continuous cooling and allowed passive range of motion exercises to be tolerated [22]. Cryotherapy can also be administered using a compression system that circulates ice water in a bladder wrapped around the limb which massages the limb at the same time as reducing tissue temperature [17]. This system improved weight-bearing when used every 4–6 hours in the first 24–72 hours following surgery, and improved range of motion, reduced pain scores, and reduced lameness for up to 42 days postoperatively compared to dogs not receiving the therapy [12, 15].

Technical equipment working with liquid nitrogen, so-called cryocautery machines, are commonly available, also in the Czechia. The new generation devices are controlled by microprocessors, they are autonomous, i. e. not connected to the nitrogen bomb, which makes it possible to control the cooling rate. Cryoprobes, by which wave freezing is carried out, have interchangeable endings depending on the shape and nature of the lesion being treated. For flat lesions and smaller tumours, passive tips are suitable, which are only applied by the doctor to the part of the patient's body that is being treated with freezing. They are usually made of aluminum. To ensure a better contact with the tissue, it is recommended to coat the passive tips with an intact gel before cryallisation. When treating larger tumours, it is necessary to use active tips, which are equipped with tiny channels leading the liquid nitrogen to the frozen area to be treated. The freezing surface of these tips can also be tipped with spikes that penetrate directly into the tumour, thus achieving significantly lower temperatures not only on the surface but also inside the tumour. The active tips are made of copper and their surface is gold-plated [28].

The most commonly used cryotherapy devices include cryospray and cryocautery machines. Cryospray is an effective way to remove pathological skin processes as gently as possible and without the need for surgery. Warts, skin tumours, and ectopic eyelashes on the eyelids are eliminated precisely with liquid nitrogen. The temperature of –196 °C causes the destruction of pathological tissue under local anaesthesia [3]. A cryocautery is a device designed for cryosurgery. It provides fast and efficient cooling of tissue to a temperature of –89 °C. A wide range of interchangeable instruments and spray nozzles are used for the operations, designed by the needs of the different medical fields where cryocautery is used (gynecology, urology, laryngology, ophthalmology, proctology, and phlebology). The nozzles allow cooling of very small areas of affected tissue (several mm) but also large pathogenic areas (a few cm) [25].

MATERIAL AND METHODS

A survey was selected as a method for our research. We have chosen two forms of the questionnaire to monitor the current state of using cryotherapy on dogs in veterinary practice. The first form was addressed to the dog owners in Slovakia and it was published on the internet. The second one was assigned to veterinarians, it was sent via

e-mail, and the doctors were contacted by phone. We also monitored the application of cryotherapy abroad, namely in the Czech Republic and Hungary, by sending the questionnaires to veterinarians. The questionnaire dedicated to Czech and Hungarian owners has been published on the internet.

Questionnaire for dog owners

The questionnaire for dog owners consisted of nine questions. It contained seven structured questions (the owner had the choice of several answers), one open question (the respondent was allowed to answer individually), and one semi-closed question (the possibility to choose an answer or answer individually). General information such as their age or sex was considered in the questionnaire. The questionnaire was preferentially focused on the owners' experiences with cryotherapy.

Questionnaire for veterinarians

The questionnaire addressed to the veterinarians consisted of nine questions, containing seven structured, one open, and one semi-closed question. Most of the questions that were proposed have led to the collection of information about veterinarians' knowledge, use, and experiences with cryotherapy applications on dogs' patients. Only two initial questions were devoted to general information about the respondents (age, and gender).

RESULTS

Evaluation of the questionnaire done by dog owners

The study involved 87 dog owners, 42 Slovaks, 25 Czechs, and 20 Hungarians. The average age of the respondents was 21–30 years, 73 were women and 14 were men. The majority of dog owners had completed secondary school.

At the beginning of the questionnaire, we were interested in whether dog owners know what cryotherapy means. 41% of respondents answered positively and up to 59% did not know about cryotherapy. However, of the owners who responded positively, only 9% had visited a veterinarian's ambulance for cryotherapy. A comparison of the results obtained from the different countries shows that the dog owners in Hungary have the most experience with cryotherapy. On the question of which disease the owners visited the cryocentre and what was the progression of treatment, they answered as follows. Approximately 30% of respondents stated treatment of tumours, 20% removal of cysts, and 10% respondents each for diabetes, itchy lesions, elimination of warts, and extraction of the lens during cataract surgery. Ten percent of the dog owners listed that they had not yet visited the cryocentre (Fig. 1).

We further investigated the owners' level of knowledge about the effects of cryotherapy in the treatment of skin defects and musculoskeletal disorders. Surprisingly, up to 68% of respondents did not have this knowledge, and only 32% answered positively (Fig. 2).

One of the questions in our questionnaire focused on the awareness of dog owners about the benefits of cryotherapy. The majority of responses (93%) were negative. Participating dog owners were unaware of the benefits of cryotherapy. Only 7% of the respondents were aware of the positives of the therapy we studied (Fig. 3).

The next question followed the previous one. In this open question, respondents could indicate their own opinion, and the benefits they detected after receiving cryotherapy for their dogs. The most frequent answers included: restoration and relaxation of muscles, an increase of energy and positive mood, reduction of inflammation, relief of pain in muscles, and joints, alleviation of arthritis symptoms, as well as the elimination of skin eczema. As an advantage of cryotherapy, the owners also mentioned the method of treatment, since the patient was treated standing in the cryo-box, its head was out of reach of liquid nitrogen, thus there was no risk of inhaling it, which is very dangerous.

Evaluation of the questionnaire by veterinarians

Fifteen veterinarians from Slovakia, ten from Czechia, and also ten from Hungary responded to the questionnaire intended for them. A total of only 35 veterinarians participated in the anonymous questionnaire survey. To obtain more relevant results, we sent the questionnaire to the Chamber of Veterinarians in Slovakia and the Czech Chamber of Veterinarians. To follow the trend in other countries of the European Union, we distributed the questionnaire to the European Board of Veterinary Specialisation. Unfortunately, concerning the validity of the General Data Protection Regulation, the organizations in question were not able to contact their members on such matters. For comparison, we present the results of a questionnaire de-

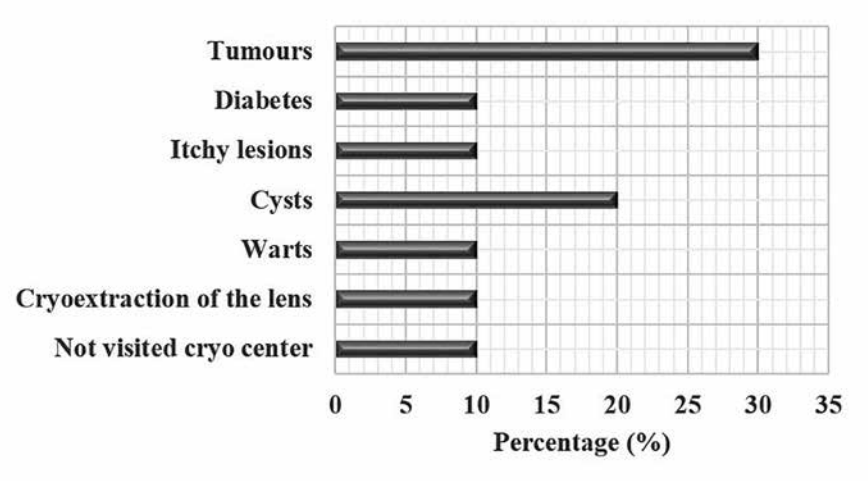

Fig. 1. Types of diseases that dog owners visited the cryocentre with

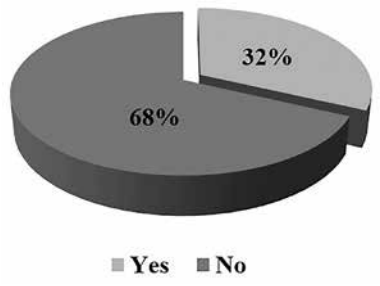

Fig. 2. The owners' level of knowledge about the effects of cryotherapy in the treatment of skin defects and musculoskeletal disorders

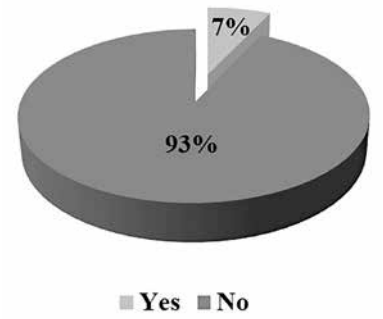

Fig. 3. The awareness of dog owners about the benefits of cryotherapy

signed by a veterinarian together with Slovakia, Czechia, and Hungary. For a better explanation, we discuss selected issues separately on a country-by-country basis. The average age of the respondents was 31–40 years, of which 18 were women and 17 were men.

In the first question, we were interested in whether veterinarians have enough information about the possibilities of applying cryotherapy in the treatment of dogs. Almost a third (29%) of veterinarians surveyed are familiar with cryotherapy. However, 71% of practitioners have insufficient information about the use of this therapy in their practice. In the Czech Republic (20%) and Hungary (11%), the situation is worse compared to Slovakia. In Slovakia, up to 47% of respondents answered that they had enough information about the possibilities of applying cryotherapy to their patients (Fig. 4).

When we asked how many veterinarians use this method in their practice, only 29% responded positively. The rest (71%) do not use this therapy at all. The results allow us to conclude that the therapeutic method studied by us is comparably used in practice in Slovakia (33%), the Czech Republic (30%), and Hungary (22%) (Fig. 5).

The following question was asked to find out the number of veterinarians who have experience with cryosurgery. Cryosurgery is a special field of cryotherapy that is used in their practice by 40% of respondents, and 60% of veterinarians have no experience with this therapeutic method. However, it is clear from our survey that cryosurgery is most often practiced by Czech doctors, compared to Slovak and Hungarian ones (Fig. 6).

The next question followed the previous one. With this open-ended question, we monitored, what experience veterinarians have with cryosurgery. The most frequent answers were the positives of the therapy, such as faster recovery after injury, painlessness, and shorter convalescence. As the main advantage, doctors mentioned the unnecessity of general anesthesia during treating patients.

From the results of the questionnaire survey, we can conclude that veterinarians use cryotherapy most often in the treatment of cysts (55%), warts (42%), eczema, and

other skin disorders (21 %) and inflammation of joints (21 %). Diseases such as tumours, epulis, and musculoskeletal disorders each account for a consistent 11 % of total applications (Fig. 7).

12.5 % of respondents do not use this form of therapy in their practice. Slovak and Czech veterinarians do not use cryotherapy in the treatment of cancer. This trend is clearly visible in Hungary, where up to 25 % of respondents routinely apply cryotherapy for tumour removal. In contrast, in Slovakia and the Czech Republic, this method is used for inflammation control, while in Hungary it is not.

The last question of our questionnaire was devoted to the age of patients who received cryotherapy. Up to 84 % of the patients were elderly; younger individuals accounted for only 16 % of the total (Fig. 8).

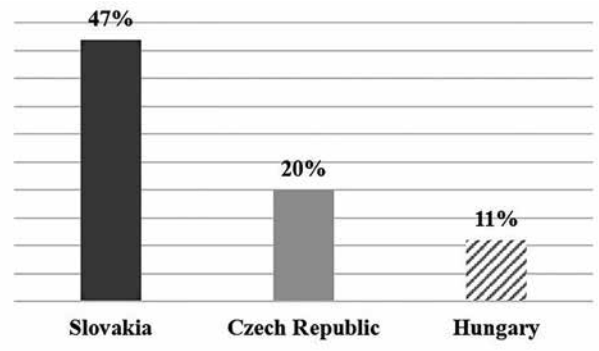

Fig. 4. Awareness trends in Slovakia and neighbouring countries

DISCUSSION

Cryotherapy is one of the new therapeutic methods. Together with laser and ultrasound therapy, it should be an integral part of every modern veterinary practice. This

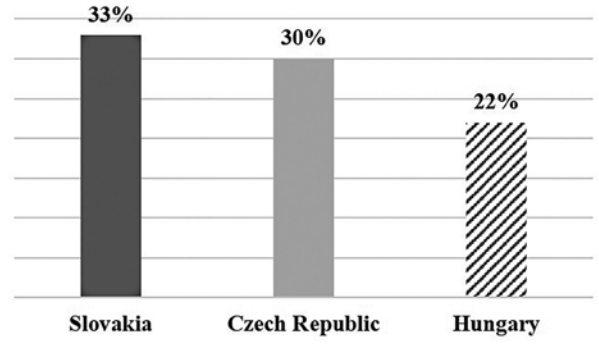

Fig. 5. Usability of cryotherapy in veterinary clinics in Slovakia and abroad

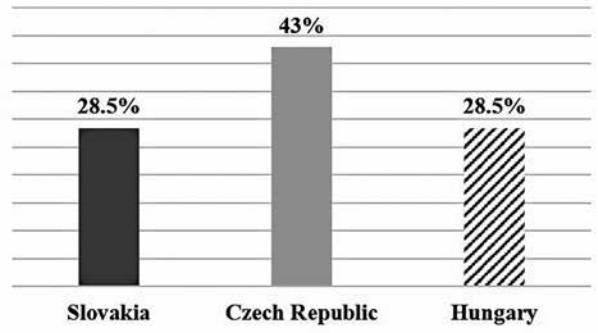

Fig. 6. Cryosurgery in Slovakia and abroad

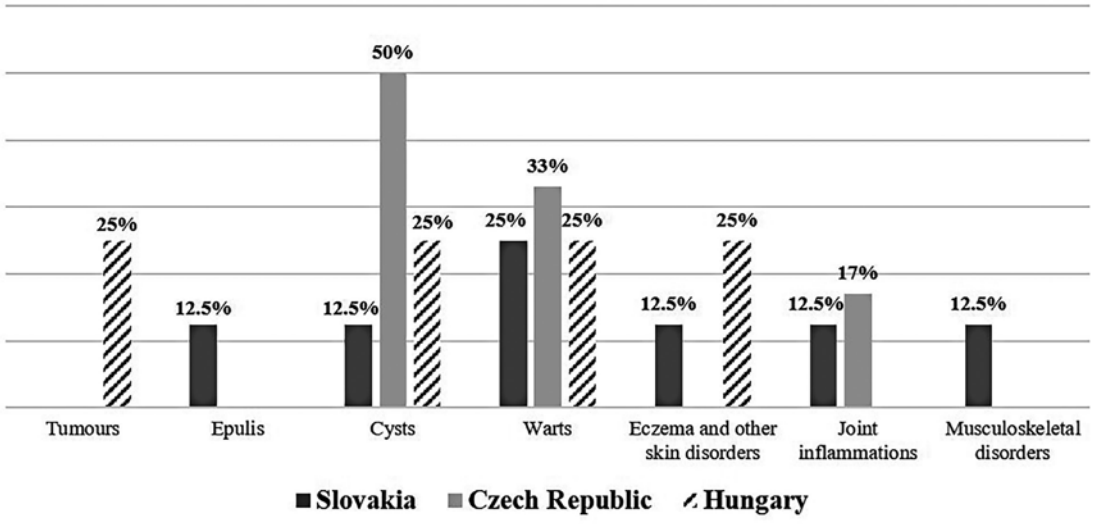

Fig. 7. Diseases treated by cryotherapy

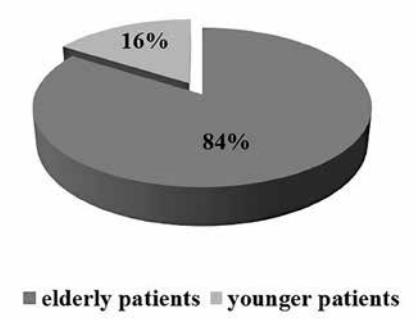

Fig. 8. Use of cryotherapy to the age of the patient

work aimed to determine whether veterinarians and dog owners have sufficient knowledge and experience with this therapy. The results obtained from the questionnaires prepared for veterinarians and dog owners allow us to state that almost half of the doctors have knowledge about cryotherapy, but only one-third of them use this therapeutic method in their practice. Cryotherapy is mainly used by doctors in elderly patients for the treatment of arthritis, epulis, eczema, itchy lesions, and other skin defects, removal of warts, cysts, musculoskeletal problems, and lens extraction during cataract surgery. In Slovakia and the Czech Republic, the method we have studied is not used in the treatment of tumours, unlike in Hungary, where up to 25 % of cryotherapy applications are for tumours.

Surprisingly, Slovak doctors have more information about the principles and benefits of cryotherapy than their Czech and Hungarian colleagues, but the number of doctors who use this technique in their practices is almost the same in each country. This may be due to the concern of Slovak veterinarians about the high initial financial costs. However, these fears are unfounded, as the profitability of the initial costs is good considering the price of cryo-therapeutic treatment of dogs. We estimate the return on initial financial costs within 2 to 3 years.

The technique we have studied can also be used in surgery. Cryosurgery is used by only one-third of doctors in their practice in Slovakia as well as in Hungary. In the Czech Republic, cryosurgery is practiced by almost 40% of veterinarians. We assume that this is a consequence of the fact that the first cryosurgical autonomous nitrogen system for tumour treatment (KCH-3) was developed in the Czech Republic in the years 1977–1983. Czech producers are still innovating their cryocautery machines used in surgery. Promotion, interpretation of the advantages of using these cryocautery devices among veterinarians, and, last but not least, the producers have adapted the price of cryosurgical devices to the financial possibilities of veterinarians in the Czech Republic. If we compare the use of cryosurgery in our geographic area (Slovakia, Czechia, Hungary) with worldwide cryosurgery exploration, it is clear that this method is known and applied more frequently in other countries [8, 20, 24]. Thirty dog patients underwent only one cryosurgery procedure – the removal of eyelid masses under local anaesthesia. Compared with surgical removal and blepharoplasty, the procedure by cryocauter was time- and cost-effective with the additional benefit of not requiring general anaesthesia [20]. Even 200 dogs participated in the cryosurgery treatment of eyelid masses differentiated by benign and malignant tumours in the study by American researchers [24]. The mean recurrence time after cryosurgery was 7.4 months, whereas it was 28.3 months after surgical excision. Moreover, the overall cosmetic appearance was observed to be better with cryosurgery [24].

Another clinical study of cryosurgery efficacy deals with the treatment of skin and subcutaneous tumours in dogs and cats [8]. Cryosurgical tumour ablation was performed by liquid nitrogen cryosurgical spray and the authors declare effective results, especially, in older animals [8].

Dog owners have very little information and knowledge about the therapeutic technique. Only 9% of them have visited a cryocentre with their dog. However, it is pleasing to note that both practitioners and owners who are familiar with cryotherapy rate its use very positively. They consider the main benefits to be faster muscle recovery, reduced inflammation, relief from muscle and joint pain and help with eczema and warts. A benefit that owners have noticed in their dogs is an increase in positive energy and mood after the application of cryotherapy.

In conclusion, cryotherapy is not sufficiently used in clinical practice, despite the benefits it provides. This is probably related to the lack of awareness and promotion of this therapeutic method among physicians as well as in general public society. The solution could be to organize popular lectures for dog owners and subscription seminars, interactive workshops with the opportunity to try out the therapeutic method for veterinarians, to raise awareness of cryotherapy and cryosurgery in society. A deeper knowledge of the positives by the general public could contribute to an increased interest of veterinarians in cryotherapy

and help them to overcome possible doubts (profitability and amount of initial financial investment) about the application of this therapeutic method in their professional practice.

CONCLUSIONS

Cryotherapy is a non-invasive, painless, innovative method that can be used even without general anaesthesia and in places where the application of conventional treatment methods would pose a great risk. It involves treatment with cold or frost. It can be effective alone or in combination with anti-inflammatory drugs. It is a suitable alternative for older and younger patients with movement problems caused by excessive physical stress, skin defects, or tumours.

The subject of cryotherapy in our study was to monitor the trend in Slovakia and compare it with the situation in neighbouring countries. We processed the current state of cryotherapy use in practice through a questionnaire. From the results, it is clear that cryosurgery is most used in the Czech Republic in comparison with Hungary and Slovakia. Cryotherapy is used very little, despite the benefits that this therapeutic method provides. This may be due to the lack of promotion of cryotherapy among professionals and the general public.

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EVALUATION OF URINE NITROGEN EXCRETION AS THE MEASURE OF THE ENVIRONMENTAL LOAD AND THE EFFICIENCY OF NITROGEN UTILIZATION

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ABSTRACT

The aim of this study was to evaluate the relationship between nutrition, the concentration of milk urea nitrogen (MUN) and the urinary nitrogen excretion in farm conditions and to use the MUN concentration as a tool to control protein nutrition and environmental load in dairy farming. Urinary N excretion was evaluated by an empirical model according to the intake and metabolic transformation of N to milk protein in selected farms was on average 208.8 ± 34.8 g with a range of values from 127.7 to 277.8 g N.day^{-1}. The evaluated proportion of excreted N in relation to crude protein (CP) intake in the total mix ration (TMR) was statistically significant ($R^2 = 0.504$; $P < 0.0001$). Urinary N excretion, evaluated according to the analysis of the MUN content, using selected regression equations, was on average 211.8 ± 24.3 g.day^{-1} with an individual variation of 157.2–274.7 g.day^{-1} with a significantly higher positive correlation to the received CP in the TMR ($R^2 = 0.693$; $P < 0.0001$). The evaluated effect of CP concentration in the TMR on urine N excretion confirmed the higher nitrogen excretion in the urine by 25.6 g per day with an increased CP in the TMR by 1 %. The proportion of urea nitrogen in the total N excreted in the urine was on average 80.5 %. The validation of the models for the prediction of nitrogen excretion, according to the MUN for the practical application on farms, was determined the best equation by Kaufmann a St-Pierre, which used available data from routine analysis of milk composition by the Breeding service of Slovakia. The MUN analysis offered a simple and non-invasive approach to the evaluation of the urinary N excretion, as well as, the efficiency of N utilization from feed to milk.

Key words: dairy cows, milk urea nitrogen; nitrogen excretion; urinary urea nitrogen

INTRODUCTION

Urea as the final metabolite of nitrogen transformation, includes ammoniacal nitrogen absorbed from the rumen originating from the degradation of feed proteins, as well as the deaminated part of the absorbed amino acids (aAA) that are not used in proteosynthesis due to aAA imbalances or deficiency of energy [34]. Nitrogen compounds transformed in this way make up the proportion of urea in the blood, which in dairy cows has three metabolic excretion routes:

1) by recycling urea in the saliva and across the rumen wall from the blood back into the rumen, which is an

important source of nitrogen for microbial protein synthesis;
2) by the excreting of urea into milk;
3) excretion of urea by the kidneys is the main route of excretion of excess nitrogen from metabolism into urine [22].

The estimated amount of urea in milk (MU) represents less than 1% of the urea circulating in the blood; however, the concentration of milk urea is highly correlated to the protein content of the feed ration [2]. The results of measurements of the MU [respectively, urea nitrogen in milk (MUN)] provides valuable information on the nutritional status and enables the evaluation of the efficiency of nitrogen utilization (ENU) from feed to milk protein [35]. The analysis and evaluation of MU as well as ENU reflect the degree of nitrogen transformation from feed to milk [24] and serve as a tool for the management of protein nutrition on the farm [30, 35].

Increasing the intake of nitrogen generally leads to an increase in the concentration of MU or MUN, but also significantly increases the urinary N excretion (UN in g $N.d^{-1}$) as well as faecal N excretion [19, 33]. Rumen degradable proteins from the feed ration, which is not transformed into milk protein is largely excreted in the urine as urinary urea nitrogen (UUN). The UUN is the most unstable N compound in urine, and its estimated amount is an average of 77% of the total amount of N in the urine of lactating dairy cows and is closely correlated with the concentration of N excreted in urine [5, 9]. B u r g o s et al. [6] have confirmed a direct relationship between urea nitrogen in milk (MUN) and N excretion in the urine. Therefore, the concentration of urea nitrogen in milk (MUN; mg $N.dl^{-1}$) is a suitable tool for assessing the environmental load for the prediction of N excretion in urine (UN; g $N.d^{-1}$) on the farm. The confirmed relationships between the concentration of MUN, UN, and UUN have enabled the verification of predictive models for estimating UUN from MUN [7, 30]. Factors that influence the relationship between milk urea nitrogen and urinary urea nitrogen include: the body weight and genetics of cows, time of sampling, the quantity of urine produced, and significantly the content of protein in the feed ration [1, 18, 32].

The aim of this study was to analyse the relationship of nutrition (concentration of nutrients and daily intake of nutrients in the TMR) to the concentration of milk urea nitrogen to estimate the quantity of excretion nitrogen in urine and validate models for the prediction of nitrogen excretion in urine under farm conditions.

MATERIALS AND METHODS

The evaluations were carried out in 30 herds with an average annual production of milk of between 8,500 and 9,500 kg per cow. The evaluations involved the dairy cows of the first lactation phase, according to days in milk (DIM; 21–130 DIM), or by the daily milk production. Feed rations of dairy cows were predominantly based on corn and alfalfa silage, supplemented with different carbohydrate feeds (cereal grains and cereal grain by-products) and protein supplements (soybean meal and rapeseed meal) provided as the TMR *ad libitum*. At the farms, the nutritional composition of the TMR and daily feed intake was intensively monitored and the dairy cows on all farms were kept in a free-stall housing system.

Analysis of total mix rations

Samples of the TMR prepared on the farms were taken from the feeding trough on the control day and were analysed for dry matter (DM), crude protein (CP), acid and neutral detergent fibre (ADF, NDF), starch and ether extract (EE) contents according to conventional methods according to the Commission Regulation (EC) No. 691/2013 [10]. DM was determined by weight upon drying the sample at 105 °C under prescribed conditions. The CP content was determined according to the Kjeldahl method using a 2300 Kjeltec Analyzer Unit (Foss Tecator AB, Hoganas, Sweden). The fat (as aether extract) was determined by the device Det-Gras (JP SELECTA, Spain). The ADF, NDF were determined using Dosi-Fiber Analyser (JP SELECTA, Spain) and the content of starch was determined polarimetrically. The netto energy for lactation (NEL) was calculated using regression equations according to the nutrient requirements of dairy cattle (NRC) [25].

Analysis of production parameters

All farms were evaluated by the Breeding Services of the Slovak Republic in: the control day of milk collection, and in the framework of checking the performance of milk yield. Milk samples were analysed for: the total protein content, fat, lactose, and urea concentration by spectrophotometric tests in the near-infrared region using

MilkoScan FT+ and BENTLEY FTS in the Central Analytical Laboratory for milk with accreditation under registration number 096/5878/2015/2. The analysed content of urea in milk (MU) was converted to urea nitrogen in milk (MUN) according to O u d a h [26].

Statistical analysis

The average values, descriptive statistics, and variability of the examined markers, as well as the influence of factors on these properties, were studied using software XLSTAT2018. The evaluation of the regression equations to determine the excretion of nitrogen in urine have consisted in the validation of models to the correctness and accuracy based on the mean bias [20] and the root mean square prediction error (RMSPE) [3] or residual error [20].

RESULTS

Nutrient content of ration, feed intake and milk production in groups of dairy cows

The mean concentration of nutrients in the TMR, daily intake of nutrients and the production parameters of dairy cows on the farms are listed in Table 1.

The mean milk production was 35.4 ± 6.4 kg.day^{-1} (range 23.2 to 47.9), the content of milk protein was 3.15 ± 0.2 % and milk fat was 3.62 ± 0.4 %. The content of milk urea was 27.4 ± 3.6 mg.dl^{-1} and milk urea nitrogen 12.79 ± 1.67 mg.dl^{-1} (with ranges of 19.74 to 35.7 and 9.21 to 16.64, resp.). The fluctuations of production parameters reflected: the different content of nutrients, the composition of rations, genetic production potential, and the order

Table 1. Nutritional composition of the TMR, production and composition of milk on the farms

	Mean	SD	Minimum	Maximum
Nutrients in TMR [% of Dry Matter]				
Crude protein	16.20	0.8	14.42	17.85
NEL	6.76	0.2	6.38	7.13
NDF	34.44	2.6	28.04	38.75
ADF	20.69	1.6	16.07	24.68
Starch	25.01	3.3	16.93	29.78
NFC	37.99	3.4	31.65	48.95
NEL/CP	0.42	0.03	0.37	0.48
Starch/CP	1.55	0.3	1.02	2.07
NFC/CP	2.35	0.3	1.89	3.17
Daily intake of nutrients [kg.day^{-1}]				
Dry matter	22.35	1.3	20.76	24.90
CP intake	3.62	0.2	3.20	4.02
NEL intake	150.89	10.6	134.09	171.83
NDF intake	7.68	0.7	6.07	8.69
ADF intake	4.62	0.5	3.42	5.40
Starch intake	5.60	0.9	3.66	7.03
Production and composition of milk				
Milk yield [kg.d^{-1}]	35.40	6.5	23.20	47.90
Milk protein [%]	3.15	0.2	2.77	3.46
Milk fat [%]	3.62	0.4	2.95	4.55
Yield of milk protein [kg.d^{-1}]	1.11	0.2	0.77	1.46
Yield of milk fat [kg.d^{-1}]	1.26	0.20	1.00	1.67
Milk urea [mg.dl^{-1}]	27.40	3.58	19.74	35.66
Milk urea nitrogen [mg.dl^{-1}]	12.79	1.67	9.21	16.64

SD – standard deviation; TMR – total mix ration; CP – crude protein; NDF – neutral detergent fibre; ADF – acid detergent fibre; NFC – non-fibre carbohydrates; NEL – netto energy for lactation

of lactation. The parameters of milk production and composition of the TMR showed significant differences in the chemical composition of nutrients in relation to the actual production and composition of the milk on the farms.

Evaluation of urinary nitrogen excretion (UN)

The excretion of nitrogen in the urine for the set of dairy cows was determined by two methods:

a) **The empirical principle** of the evaluation was based on the metabolic transformation, i. e., the difference between the analysed nitrogen intake in the TMR and that excreted in the milk. The urinary N excretion was determined by the calculation according to the equation, UN (g N.day^{-1}) = (N intake × 0.83) – N in milk – 97 [16] where 0.83 was the value of the proportion of nitrogen digestibility and the value 97 representing the endogenous losses.
b) **The amount of nitrogen excreted in the urine** was determined by the calculation according to the analysed MUN content using the regression equation model of the meta-analysis of balance trials for monitoring urinary nitrogen excretion, which were separately described in studies by Kauffman and St-Pierre [18]; Nousiainen et al. [24]; Wattiaux, Karg [35].

The amount of urinary urea nitrogen (UUN) was determined by the calculation, according to the analysed content of urea in milk, using the regression equations of the meta-analytic evaluation separately proposed by Burgos et al. [7]; Powell et al. [31]; Wattiaux , Ranathunga [36]. The results of our investigations are summarized in Table 2.

The total amount of nitrogen excreted in the urine (UN) according to intake and metabolic transformation of nitrogen into milk in the observed farms (Tab. 2) was on average 208.8 ± 34.8 g N per day with a range of values from 127.7 to 277.8 g N.day^{-1}. The estimated values of UN according to the intake and metabolic transformation of N in relation to the analysed crude protein (CP) content in the TMR (Fig. 1) was statistically significant ($R^2 = 0.504$; $r = 0.710$; $P < 0.0001$).

The amount of nitrogen excreted in the urine, estimated according to the analysed urea content in milk, in the total average of the three selected models was 211.8 ± 24.3 g N.day^{-1}. Fluctuations in the values in individual farms with a minimum of 157.2 g N.day^{-1} at the lowest level of CP (14.4 %) in DM of the TMR and a maximum of 274.7 g N.day^{-1} with an upper limit of CP (17.9 %) in DM of the TMR. The daily amount of nitrogen excreted in the urine, calculated according to the analysed milk urea nitrogen, in relation to nitrogen intake in the TMR (Fig. 1) showed a statistical significance ($R^2 = 0.693$; $r = 0.832$; $P < 0.0001$). The dependence of CP content to excretions of N in urine was confirmed by regression and each increase of CP by 1 % in the range of 14.5–17.9 % CP in the TMR increased nitrogen excretion by 25.6 g.day^{-1}.

The estimated level of urinary urea nitrogen (UUN) from three verified models was on average 170.7 ± 24.6 g.day^{-1}with fluctuations of values (115.4–231.8). The proportion of urea nitrogen from the total excreted nitrogen in the urine formed 85.5 % on average. With a low content of CP in the TMR, UUN represented 73.4 % and increased to 85.2 % with higher contents of CP (Table 2).

Validation of models for determining excretion of nitrogen in the urine

The selected models of regression equations for the determination of UN based on the analysed milk urea nitrogen content were validated for accuracy and precision compared to the empirical model of UN according to intake and metabolic transformation of nitrogen [(N intake × 0.83) – N milk – 97]. The evaluation results are summarized in Table 3.

Accuracy and precision were evaluated in our tracking from calculated values of selected parameters as, mean bias, residual error (RE), and root means square prediction error (RMSE). By evaluation of the regression of the individual values of bias in the analysed models there was confirmed, a negative linear relationship with the coefficients of slope at the statistical significance level $P < 0.0001$ (Table 2).

From the evaluation parameters determining accuracy and precision, the model D was the most accurate to predict the excretion of urine N (UN) under breeding conditions compared to the models E and F, but the precision in the monitored models D and E in the evaluated parameters (RE, RMSPE) was very similar. A higher rate of dependence in the evaluated models was confirmed by the E model. Under farm conditions it is appropriate to use the model D that uses the regression relationship [18] to estimate the UN, despite a lower coefficient of determina-

Table 2. Predicted UN and UUN calculated according to analysed MUN and the metabolic transformation of nitrogen

	Average	SD	Range
Excretion of N in urine [g.day^{-1}] (UN)			
***N Intake × 0.83 – N milk – 97**	208.9	34.8	127.7–277.8
1. Kaufmann, StPierre [18]	206.1	24.7	150.8–267.3
2. Nousianen et al. [24]	224.3	26.8	156.8–257.9
3. Wattiaux, Karg [35]	204.8	21.4	164.1–299.0
****Average (1-2-3)**	211.8	24.3	157.2–274.7
Excretion of urea N in urine [g.day^{-1}] (UUN)			
1. Burgos et al. [7]	165.7	24.3	111.2–226.0
2. Powell et al. [31]	174.1	25.2	117.6–236.6
3.Wattiuax, Ranathunga [36]	172.2	24.4	117.4–232.8
Average (1-2-3)	170.7	24.6	–
% Proportion of UUN from UN	80.5 %	–	73.4 % – 85.2 %

SD – standard deviation; * – $R^2 = 0.504$; $P < 0.0001$; ** – $R^2 = 0.693$; $P < 0.0001$

Excretion of N in urine [g.day^{-1}] according to the regression equations of the authors:

Kaufmann, StPierre [18] (2001)	UN = 0.0259 × BW × MUN
Wattiaux, Karg [35] (2004)	UN = 0.0283 × BW × MUN
Nousianen et al. [24] (2004)	UN = 14.1 × MUN + 26

Excretion of urinary urea nitrogen [g.day^{-1}] according to the regression equations of the authors:

Burgos et al. [7] (2007)	UUN = –37.33 + 16.01 × MUN
Powell et al. [31] (2014)	UUN = –36.4 + 16.6 × MUN
Wattiuax, Ranathunga [36] (2016)	UUN = –32 + 16.1 × MUN

tion ($R^2 = 0.503$), since the model E took advantage of the evaluations [18].

Relationship between nitrogen intake in the TMR, efficiency of utilization into milk and urinary excretion

The efficiency of nitrogen utilization (ENU) was calculated by two ways:

a) By the empiric model, ENU was calculated as a percentage of the nitrogen intake that is transformed into milk protein. The calculation was made according to the actual production of milk protein (milk yield and % milk protein) and intake of CP in the ration by the equation ENU (%) = [100 x N milk/N intake] [11, 25]. The estimated efficiency of N utilization on the farms was determined on average at 30.1 ± 5.0 %, with significant fluctuations of average values on farms within the range from 21.1 % to 39.5 % [23].

b) The calculation of efficiency of nitrogen utilization (ENU) based on analysed MUN content for the production group in combination with the amount of milk produced by using the regression equation by Huhtanen et al. [15] from meta-analytical assessments of the experiments. The estimated efficiency of N utilization according to the analysed milk urea nitrogen was on average lower (29.2 ± 1.4 %), with a smaller range (26.0–32.1 %) of the fluctuation of average values according to breeds. The analysed values of ENU in relation to the CP content in the TMR, expressed by equation ENU % = 53.22–1.48 × CP % confirmed the higher ($P < 0.0001$, $R^2 = 0.729$) negative correlation [23].

Table 3. Validation of models for the predicted UN according to MUN content compared to the calculation using the empirical model

[g.day^{-1}]	Model D Kaufmann, StPierre [18]	Model E Nousiainen et al. [24]	Model F Wattiuax, Karg [35]
UN g.day^{-1} by Empiric model	208.88 ± 34.8	208.88 ± 34.8	208.88 ± 34.8
UN g.day^{-1} by Equation by MUN	206.11 ± 24.7	224.54 ± 21.4	204.84 ±26.8
Mean bias	–2.77	–4.04	15.46
Residual error	26.57	26.47	26.86
RMSPE	26.71	26.77	30.99
Regression of bias against empirical model for the UN evaluation			
R^2	0.503	0.623	0.424
Slope (probability)[a]	–0.5504 (P < 0.0001)	–0.609 (P < 0.0001)	–0.5106 (P < 0.0001)

[a] – coefficient of determination and statistical probability of existence of slope coefficient (F test);
RMSPE – root mean square prediction error; MUN – milk urea nitrogen; UN – excretion of nitrogen in urine

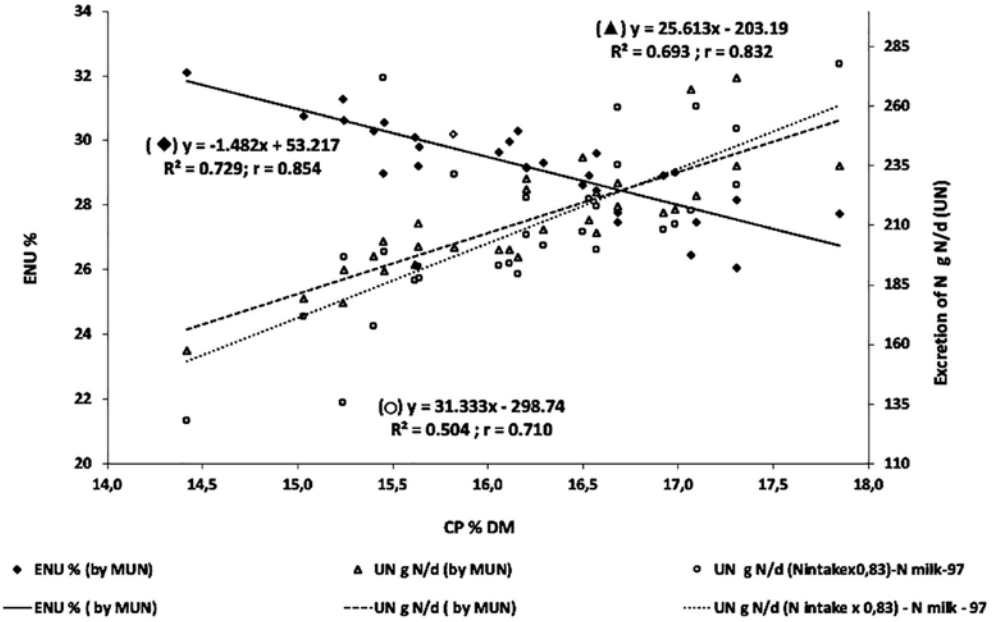

Fig. 1. Relationship between the CP content and the efficiency of N utilization (ENU) and excretion of N in urine

DISCUSSION

The high producing dairy cows on the first lactation phase with average milk production of 35.4 ± 6.6 kg per day in feed trials when feeding the TMR containing corn and alfalfa silage were analysed for milk urea nitrogen (MUN) values with an average of 12.8 ± 1.7 mg.dl^{-1} in a significant number of farms in the evaluated region (30/3150). At comparable nutritional and production parameters, the published values from the meta-analysis [33] confirmed the MUN content of 13.1 ± 3.6 mg.dl^{-1} for North America. In North-Western Europe, the MUN content achieved an average value of 12.5 ± 5.1 mg.dl^{-1}, at lower production (25.5 ± 4.5 kg) and at half the starch content in the TMR (13.2 ± 8.4 %). The MUN level ranging from 8 to 12 mg/dl, indicated the optimal intake and utilization of

nitrogen from the feed rations [24, 33]. The levels of milk urea nitrogen above 12 mg.dl^{-1}indicated an increased loss of ingested nitrogen that was excreted in the urine in the form of urinary urea nitrogen (UUN). For high producing dairy cows with an annual milk yield of 12,000 kg, the recommended milk urea nitrogen was on average above 14.5 mg.dl^{-1} [22] or more precisely from 10 to 16 mg.dl^{-1} [36].

The kidneys together with the liver, play an important role in reducing the excretion of nitrogen [21]. In the kidneys, the urea can be retained or excreted depending on the level of CP in the feed ration and the metabolic needs of the dairy cows. The content of CP in the feed ration affects the expression of transport-proteins in the inner collecting duct of the kidneys, as well as, the influence on the renal reabsorption of urea with an impact on the ratios between blood urea nitrogen and urinary urea nitrogen (UUN), respectively, milk urea nitrogen and UUN [33]. Reducing the intake of nitrogen in the ration of ruminants did not change the rumen synthesis of microbial protein (14.6 g microbial N per kg digested organic matter in the rumen), but there was confirmed a decreasing of UUN by 84 %. The reason for such a decrease in UUN was a decreasing in the filtration load of urea due to a decrease in renal plasma flow (by 64 %), glomerular filtration rate (by 71 %), and plasma urea level (by 55 %).

A higher intake of nitrogen increased the concentrations of milk urea nitrogen (MUN) and urinary urea nitrogen (UUN), and thus contributed to a decrease in the efficiency of the nitrogen use [16, 24, 33]. The regression analysis by C a s t i l l o et al. [8] confirmed a positive linear correlation between the intake of nitrogen up to the level of 400 g N per day and proportionate level of excreted nitrogen in the faeces, urine and milk. When reducing the content of crude protein in the TMR, reduced excretion of urea nitrogen in urine and improvement in the efficiency of nitrogen utilization from feed to milk were confirmed [8]. However, it is necessary to ensure that improving the efficiency of N utilization does not affect the yield of milk production [28]. Increasing the nitrogen intake over 400 g N per day exponentially increased the excretion of nitrogen in the urine and the excretion of nitrogen in the faeces and milk decreased linearly.

The regressive effect of CP content on UN excretions confirmed for each increase of CP by 1 % in the range of 14.5–17.9 % CP in the TMR increased nitrogen excretion by 25.6 g.day^{-1}. According to the values presented in the study by W a t t i a u x, R a n a t h u n g a [36] the authors assumed that each decrease in CP in feed rations by one percentage point in the range of the CP content 19–13 % (in DM) reduced the urinary urea nitrogen by 28.1 g. day^{-1}. When reducing MUN by 1 mg.dl^{-1} (MUN range 10–16 mg.dl^{-1}), P o w e l l et al. [31] observed a daily decrease of UUN to 16.6 g.day^{-1}. Although, there is a narrow correlation between the concentration of MUN and excretion of N in urine, the concentrations of urea in urine were 32–38 times higher [4] than in the milk.

The efficiency of N conversion from the feed into the milk protein ranges from 25 to 35 % in dairy cows and is mainly influenced by the content of the crude protein in the feed and the level of milk production [13, 14]. The observed efficiency of N utilization (ENU) in our study farms expressed a similarity according to the evaluation method at the level of values of the mean, but a different range of fluctuations of individual values for those farms. Studies of some authors [12, 17, 29] found that ENU under commercial conditions reached the level of 28 %, with a fluctuation between 18 % and 35 %. P a c h e c o [27] reported the average value of ENU at the level of 29 % with a fluctuation from 19 to 40 % between farms, which are values comparable to our results obtained by calculation according to the metabolic conversion. The regression relationship overestimates ENU and at the values over 17 % of CP underestimates EUN, compared to the biologically accepted efficiency of nitrogen transformation from feed to milk [14].

CONCLUSIONS

Evaluation of urea in milk at the farm level provides an analytical tool for adjusting the content and relative representation of proteins and carbohydrates in the rations for optimizing the protein needs of lactating dairy cows. Such treatment increases the efficiency of nitrogen transformation from feed to milk and the reduction of nitrogen excretion in urine as a tool for reducing the ecological load. The validation of the models for the estimation of urinary excretion of N according to the analysed MUN content from our evaluations confirmed that the best equation for the use under our breeding conditions was the one based on meta-analytic experiments by Kauffman and St-Pierre [18] which is expressed as UN (g N.day^{-1}) = 0.0259 × body weight × MUN.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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BACTERIAL ETIOLOGIES OF SUBCLINICAL MASTITIS IN COWS JOS METROPOLIS, NIGERIA

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ABSTRACT

This study was conducted to investigate the prevalence of subclinical mastitis and its contagious and environmental bacterial causes in dairy cows in Jos Metropolis of Plateau State. A total of 208 milk samples were collected aseptically from 52 lactating cows and subjected to the California Mastitis Tests (CMT). Milk samples were transferred into peptone water broth for enrichment, followed with bacteriological assays and biochemical identification of bacteria. Using R Commander version 3.6.2, data such as: breed, age, parity, lactation, and management system of cows were collected, and analysed to determine their relationship with bovine subclinical mastitis. Out of 208 quarter milk samples collected from 52 cows, 35.09 % showed subclinical mastitis. While the Cross-breed cows had 15 % subclinical mastitis recorded, the White Fulani had 78.13 %. Cows of the age 3 to 5 years had the highest prevalence of mastitis, while cows with 4 to 6 calves showed the highest prevalence in the parity category. The early lactation stage of these cows showed the highest prevalence. The environmental bacteria isolated where *E. coli, K. pneumoniae, K. oxytoca, C. freundii, K. aerogenes,* and *Proteus* sp. with *E. coli* having the highest frequency of 40.86 %, followed by *K. Pneumoniae* with 17.79 %. The contagious bacteria isolated were *S. aureus, S. agalactiae,* and *Corynebacterium* sp. with *S. aureus* occurring most with 69.71 %. This study concluded that the prevalence of subclinical mastitis is high in cows in the study area. It was also discovered that the breed of cow, age of cow, parity number, stage of lactation and husbandry systems have an impact on subclinical mastitis. *S. aureus* with the highest frequency, indicated that contagious microbes have more effect on subclinical mastitis than environmental microbes.

Key words: bovine; CMT; contagious bacteria; environmental bacteria; subclinical mastitis

INTRODUCTION

Mastitis is a multi-etiologic disease of the mammary gland, characterized by reduction in milk production and considered as an economically important disease in the diary subsector in developed and developing nations [19]. Mastitis is the most economically significant disease of

dairy cattle, accounting for 38 % of the total direct costs of the common production diseases [3]. Mastitis is a global problem as it adversely affects animal health, quality of milk, and economics of milk production, and every country, including developed ones, suffer substantial financial losses [15]. It is the most critical deadly disease of dairy animals. It is responsible for heavy economic losses due to reduced milk yield (up to 70 %), milk discard after treatment (9 %), cost of veterinary services (7 %), and premature culling (14 %) [16].

Any microbe that can opportunistically invade tissues and cause infection can cause mastitis. Bacteria are the most common culprits of microbial mastitis. They are classified distinctly into two groups, viz., contagious and environmental. Contagious mastitis involves introducing pathogens during the milking processes via milking equipment (malfunctioning pulsation and vacuum controllers) or milkers' hands. Some of the more commonly listed species that exploit this transmission mode include *Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis,* and *Mycoplasma* spp. Environmental mastitis is caused by pathogens found in the habitat of the cow, such as: soil, plant material, manure, bedding, or a contaminated water source. Frequently, isolated causative pathogens contributing to environmental bovine mastitis include streptococci and gram-negative bacteria, such as coliforms (*Escherichia coli, Klebsiella* spp., and *Enterobacter*) [5]. Studies have shown that coliform mastitis, apart from resulting in agalactia, can cause substantial loses to producers [12]. Cows affected with coliform causing mastitis can show clinical signs and progress to a per-acute form that can lead to the animal's death. These cases have a sudden onset, with swollen and hot quarters and yellowish, watery milk that contains clots and flakes. Severe cases occur in early lactation, particularly around calving [8]. Both contagious and environmental mastitis can result in subclinical and clinical forms with profound economic implications for the dairy industry in developing countries.

Most important predisposing factor increasing the chance of mastitis is poor hygienic management and unhygienic housing systems. The more the presence of bacteria in areas where cows are kept, the higher the chances of infection. Just after milking it takes about 20 minutes before the teat canal is fully closed, so lack of clean floors and resting places increases the chance of contracting mastitis. Another risk factor is milk yield; cows with a high milk yield will develop mastitis more easily [6]. Their udder tissue is more active and has longer milking time. This makes the teat canal open for a longer period of time. High milk production is also connected with more energy demand which possibly decreases the resistance against infections. Poor milking technique and unhygienic milking procedures are risk factors [6]; force-milking can cause injury to the teat and teat canal making the entrance of bacteria to the udder easier. Lack of cleaning utensils, the hand of the milker and the udder, as well as, incomplete milking are other factors which may cause the multiplication of bacteria.

Therapy is given on the premise that productivity gains will outweigh treatment costs after the elimination of infections. In the case of contagious pathogens, elimination may also decrease the reservoir of infection for previously noninfected cows. By intra-mammary administration, antibacterial agents are used in the treatment of mastitis [10]. For instance, in *Streptococcus agalactiae*, all four quarters of infected cows should be treated to eliminate the pathogen and prevent possible cross-infection of a noninfected quarter. Cure rates can often be 75–90 %. The labelled use of commercial intramammary products that contain amoxicillin, penicillin, and erythromycin are as efficacious as procaine penicillin G infusions derived from multiple-dose vials [10]. Most other streptococci also display *in vitro* susceptibility to numerous antibacterial agents, especially β-lactam drugs.

The objective of this study was to determine the prevalence of subclinical mastitis and its contagious and environmental causes in dairy cows in Jos Metropolis.

MATERIALS AND METHODS

Study area

The research was carried out in Jos Metropolis covering three Local Government Areas in Plateau State viz., Jos East, Jos North, and Jos South from June 2019 to May 2020. It has a total area of 1,821 km^2 and a population of 821,618 people. Its headquarters is located in the city of Jos having coordinates of 09°55′00″N 08°53′25″E. At an altitude of 1,217 m (3,993 ft) above sea level, Jos' climate is closer to temperate than that of the vast majority of Nigeria. Jos receives about 1400 millimetres of rainfall annually. Due to security issues bordering on herders/farmers

clashes, the dairy farms involved in this study were selected by convenience.

Study population and sample collection

The three farms that were selected for this study from Jos Metropolis had 54, 63 and 68 dairy cows respectively. Two types of dairy cows were sampled, namely White Fulani and cross-bred cows (Fig. 1 and 2). White Fulani cows are medium sized animal with long horns. They are easily characterized by their long and lyre-shaped horns. They weigh about 250–380 kg while the cross-bred weigh 450 kg on the average. A White Fulani cow produces about 6 litres of milk per day while the cross-bred cows can produce 30 litres per day. At each farm, cows included in this study were selected by a blind random sampling technique. A total of 208 milk samples were collected aseptically from 52 lactating cows based on the minimum sample size according to A n u e y i a g u et. al. [1]. During the farm visits for sample collection, 52 structured questionnaires were administered to collect information concerning the herds such as: breed, age, parity, lactation stage, blind-udder, previous history of mastitis, and husbandry system.

Ethical clearance

In line with the guidelines of the Animal Use and Care Committee (AUCC) of National Veterinary Research Institute (NVRI), Vom, Nigeria, the procedure involved in the milk sampling was non-invasive and does not inflict pain or discomfort to the animals, Therefore, no ethical approval was required.

Fig. 1. White Fulani cow

Detection of subclinical mastitis

To eliminate debris and bacteria from the environment on the udder and teat, cows were restrained and each udder was rinsed clean with water and swabbed with 70 % ethanol. The presence of subclinical mastitis was determined by the California Mastitis Test (CMT) and the severity of the infection by the nature of coagulation and viscosity of the mixture (milk and CMT reagent) [14]. Briefly, a squirt of milk sample from each quarter of the udder was deposited in each of the cups on the CMT paddle, and an equal amount of 3 % CMT reagent was added to each cup and thoroughly mixed. Negative reactions were reported as 0 and trace, whereas positive reactions were graded as +1, +2 [2].

Bacteriological isolation and identification

The milk positive for the California Mastitis Test (CMT) samples were bacteriologically examined according to the procedures given by A n u e y i a g u et al. [2]. One ml of each milk sample was inoculated into 9 ml peptone water broth for enrichment and incubated at 37 °C

Fig. 2. Cross-bred cow

overnight. Thereafter, a loopful of the broth culture was streaked on blood agar (BA), mannitol salt agar (MSA), MacConkey agar (MCA) and eosin methylene blue agar (EMB).

The isolates were identified using, colony morphology, Gram staining and biochemical tests. The confirmatory screening was conducted on presumptive Gram negative bacteria using Oxiod™ Microbact™ GNB 24E. Coagulase test and CAMP tests were used on Gram positive bacteria.

Statistical analysis

Data collected were analysed using R Commander Software (Version 3.6.2) to conduct Chi-squared test (χ^2), and p values $P < 0.05$ were considered statistically significant.

RESULTS

Out of the total of 208 milk samples collected aseptically from lactating cows, 35.09 % showed subclinical mastitis (Table 1). Clinical mastitis was not observed in this work. The back left and back right quarters of cows sampled showed higher prevalence than the front quarters (Table 2). There was statistical significance in the quarter-wise prevalence of the subclinical mastitis, with p values less than 0.05. If not appropriately managed, the cows with these positive results might develop into either stronger CMT positive or clinical mastitis.

Data from: breed, age, parity, lactation, floor type, and husbandry system were studied from the questionnaires collected. While the cross-breed cows had 15 % subclinical mastitis recorded, the White Fulani had 78.13 % (Table 3). The age 3 to 5 had the highest prevalence of mastitis, while cows with 4 to 6 lactations showed the highest prevalence in the parity category (Table 3). The early lactation stage of these cows showed the highest prevalence. The nomadic system of rearing showed a lesser prevalence of mastitis than the intensive system (Table 3).

The environmental bacteria isolated were: *E. coli, K. pneumoniae, K. oxytoca, C. freundii, K. aerogenes*, and *Proteus* sp. with *E. coli* having the highest frequency of 40.86 %, followed by *K. Pneumoniae* with 17.79 % (Table 4). The contagious bacteria isolated were *S. aureus, S. agalactiae*, and *Corynebacterium* sp. with *S. aureus* occurring the most. There was statistical significance in subclinical mastitis in both groups of bacteria with P values less than 0.05 (Table 4).

Table 1. Prevalence of clinical and subclinical mastitis of cows

Forms of mastitis	No. of positive Cow level	Prevalence [%] (n = 52)	No. of positive Quarter level	Prevalence [%] (n = 208)
Clinical	0	0	0	0
Subclinical	28	53.84	73	35.09
Total	**28**	**53.84**	**73**	**35.09**

DISCUSSION

In this study, the overall prevalence of subclinical mastitis was 35.09 % and was corroborated by the results pre-

Table 2. Quarter-wise prevalence of subclinical mastitis in lactating cows

Quarters	Number tested	CMT positive [%]			χ^2	P-value
		Trace	1+	2+		
FR	52	12(23.08)	2(3.85)	6(11.54)	29.443	0.000***
FL	52	9(17.31)	4(7.69)	6(11.54)		
BR	52	13(25)	18(34.62)	11(21.15)		
BL	52	9(17.31)	20(38.46)	6(11.54)		
Total	**208**	**43(20.67)**	**44(21.15)**	**29(13.94)**	–	–

FR – Front right quarter; FL – Front left quarter; BR – Back right quarter; BL – Back left quarter;
Trace – 200,000–400,000 SCC; 1+ – 400,000–1,200,000 SCC; 2+ – 1,200,000–5,000,000 SCC

Table 3. Risk factors associated with bovine mastitis in Jos

Risk factors	Category	No. of cows examined	No. of cows affected	Prevalence [%]	χ^2	P-value
Breed	White Fulani	32	25	78.13	19.734	0.000***
	Cross	20	3	15.00		
Age	3–5 years	7	5	71.43	2.079	0.354
	5–9 years	19	8	42.11		
	10–14 years	26	15	57.69		
Parity	1–3 calves	23	10	43.48	3.079	0.214
	4–6 calves	20	11	55.00		
	7–12 calves	9	7	77.78		
Lactation stage	1–3 months	25	14	56.00	3.166	0.205
	4–7 months	21	9	42.86		
	8–12 months	6	5	83.33		
Husbandry system	Nomadic	37	19	51.35	0.321	0.571
	Intensive	15	9	60.00		

Table 4. Frequency of environmental and contagious aetiologies

Bacterial species	No positive [%] (n = 208)	χ2	P-Value
Environmental bacteria			
E. coli	85 (40.86)	253.41	0.000***
K. pnuemonie	37 (17.79)		
K. oxytoca	15 (7.21)		
C. freundii	2 (0.96)		
K. aerogenes	3 (1.44)		
***Proteus* sp.**	2 (0.96)		
Total	**144**	–	–
Contagious bacteria			
S. aureus	145 (69.71)	364.81	0.000***
S. agalactiae	2 (0.96)		
***Corynebacterium* sp.**	1 (0.48)		
Total	**148**	–	–

sented in a systematic review of 14 articles by A n u e y i-a g u et al. [1] which recorded a prevalence range between 26.9 and 57.8 % for subclinical mastitis. This high prevalence in this work necessitates prompt action by policy makers and farmers to reduce economic downturn in the dairy industry.

Statistically, the prevalence of mastitis among the breed types of ruminants was significant. This may be due to a better adaptive ability of the White Fulani breeds to environmental factors compared to cross breeds that may have traits of foreign breeds imported into the nation from temperate environments. The findings in this study agreed with reports by other authors that stated that exotic breeds like Friesian breeds were more susceptible to bovine mastitis than the indigenous breeds [7].

Cows between the age of 3 and 5 years showed a higher

prevalence of subclinical mastitis than older ones. The report of C o m p t o n et al. [7] disagreed with the results of this study. This may be due to the low immunity in younger animals compared to the older ones. However, the result of this study was in agreement with the report of S h i t t u et al. [17], who claimed to have younger cows more susceptible to mastitis. Cows that have had more calves, in this study, showed a higher prevalence than those with lesser number of calves [13]. Early lactation stages and nomadic husbandry system showed higher prevalence as well [2, 18].

This study showed that from 208 samples, environmental udder pathogens in 144 cases of Sub-clinical Mastitis (SCM) were identified as: *E. coli, K. pneumoniae, K. oxytoca, C. fruendii, K. aerogenes*, and *Proteus* sp. This was in close agreement with K a l l a et al. [11], who reported: *Escherichia coli, Klebsiella spp., Enterobacter* spp., *Citrobacter, Serratia* and *Proteus* as the major mastitogens [9]. These bacteria are generally found in high concentrations in organic matter, such as bedding and manure (environment). Therefore, from an epidemiologic standpoint, the primary source of infection for some pathogens in this study was environmental. These bacteria invade the udder through the teat sphincter when teat ends come in contact with coliform bacteria. Once they have entered the mammary gland, they either multiply rapidly or remain dormant. However, 148 contagious bacteria such as *S. aureus, S. agalactiae*, and *Corynebacterium* sp. were identified with *S. aureus* having the highest prevalence (69.71 %). This was in line with the work of B i r h a n u et al. [4] where *S. aureus* had the highest prevalence of 44.9 % followed by *Streptococcus* sp. with 25.3 %.

CONCLUSIONS

This study concluded that the prevalence of subclinical mastitis was high in cows in the study area. It was also discovered that: the breed of cow, age of cow, parity number, stage of lactation and husbandry systems have impact on subclinical mastitis. *S. aureus* with the highest frequency indicated that contagious microbes had more effect on subclinical mastitis than environmental microbes.

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TOXIC EFFECTS OF CADMIUM ON THE FEMALE REPRODUCTIVE ORGANS A REVIEW

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ABSTRACT

Cadmium (Cd) is a common environmental pollutant present in soil and associated with many modern industrial processes. Cadmium may adversely influence the health of experimental animals and humans and exert significant effects on the reproductive tract morphology and physiology. During embryonic development, cadmium suppresses the normal growth and development of the ovaries, and in adults it disrupts the morphology and function of the ovaries and uterus. The exposure to cadmium has adverse effects on the oocyte meiotic maturation affecting the structure of ovarian tissue. The distribution of follicles and corpus luteum in the ovarian tissues has been shown to be disrupted, affecting the normal growth and development of the follicles. In the ovarian cortex, the number of follicles at different stages of maturation decreased, and the number of atretic follicles increased. In the medulla, oedema and ovarian haemorrhage and necrosis appears at higher doses. Granulosa cells exposed to cadmium exhibited morphological alterations. Oocyte development was inhibited and the amount of oocyte apoptosis was higher. Cadmium exposure also caused changes in the structure of the ovarian blood vessels with reduction in the vascular area. Cadmium effects included increased uterine weight, hyperplasia and hypertrophy of the endometrial lining. Exposure to cadmium had specific effects on gonadal steroidogenesis by suppressing steroid biosynthesis of the ovarian granulosa cells and luteal cells. Progesterone, follicle stimulating hormone, and luteinizing hormone decreased significantly after $CdCl_2$ administration. Cadmium can suppress the female's ovulation process and cause temporary infertility.

Key words: cadmium; female; mammals; reproductive organs

INTRODUCTION

Cadmium toxicity is associated with several clinical complications, hepatic dysfunction and bone diseases. Cadmium can also cause various forms of diseases: osteomalacia and osteoporosis, hypertension, arteriosclerosis, and anaemia [25, 85, 92]. Chronic exposure leads to: obstructive airway diseases, emphysema, end-stage renal failures, diabetic and renal complications, deregulated blood pressure, bone disorders, and immune-suppression

[24, 26, 30]. Cadmium accumulates over time in blood, kidney, and liver as well as, in the reproductive organs, including the placenta, testis, and ovaries [20, 21, 29, 57].

At the cellular level, Cd stimulates: cell proliferation, inhibit apoptosis, inhibit DNA repair, and promote cancer in a number of tissues [63, 76]. Several authors have outlined the genotoxic effects of Cd on animal cells and tissues [15, 87]. Cadmium acts as a mitogen by promoting cancer cell proliferation and is associated with increased risk of carcinogenesis in multiple organs, most notably: kidney, prostate, liver, pancreas, lung, stomach, and testis [9, 23, 44, 86, 87]. Cadmium is known as an endocrine disruptor compound, interfering with the synthesis and regulation of some hormones in both females and males [75]. Exposure of rodents to the metal resulted in a down-regulation of pituitary hormones; including: gonadotropins, prolactin, ACTH, growth hormone, and thyroid-stimulating hormone [33, 65].

Cadmium exposure is associated with reproductive toxicity, resulting in both animal and human male and female. The detrimental effects of Cd on morphology, physiological and biochemical dysfunctions of male reproductive organs have been documented [4, 25, 41, 45, 70]. Experimental animal studies have shown *in vivo* that Cd was also able to temporarily induce sterility in female reproductive systems [80]. Long-term exposure to low doses of Cd had adverse effects on the health of the next generation. Some harmful effects showed gender differences in offspring [100]. In this article, we review the findings of the effect of Cd on the female reproductive organs of mammals in relation to hormonal disturbances obtained in experimental animals.

Mechanism of toxicity

At the cellular level, cadmium affects: cell proliferation, differentiation, apoptosis and other cellular activities. Current evidence suggests that the exposure to Cd induces genomic instability through complex and multifactorial mechanisms. Cadmium may cause the DNA single chain breaks, and harmed the DNA repaired system, inducing apoptosis in many kinds of cells [94].

Cadmium has the potential to affect reproduction and development in many different ways, and at every stage of the reproductive system. Gestational Cd treatment induces ovarian toxicity and reproductive dysfunction through increased oxidative stress [6, 14, 38, 56, 68]. Furthermore, the deregulation of cell growth, the resistance to apoptosis, as well as epigenetic alterations have been demonstrated in diverse experimental systems. This had been reported to cause apoptosis, necrosis and cell proliferation [7, 18]. Epigenetic changes in DNA expression [90], stimulates the synthesis of Cd binding proteins metallothioneins and heat proteins [32], inhibit or upregulate transport pathways [78, 81, 89].

Cadmium-induced oxidative stress has been associated with production of reactive oxygen species comprising mainly superoxide radical anion, hydrogen peroxide and hydroxyl radicals which lead to lipid peroxidation, membrane protein and DNA damage [72, 73]. Recent studies have described lipid peroxidation to be an early and sensitive consequence of cadmium exposure, and free radical scavengers and antioxidants have been reported to attenuate cadmium-induced toxicity. Toxicity could result from Cd interacting with cellular components even without entering the cell, but by interaction with receptors on their surface [8].

The overall effect of Cd on any cell or tissue is likely due to the synergy of several mechanisms. A large proportion of the cellular reactions affected by ionic Cd2+ are mediated by cellular signalling cascades [77, 79]. The combination of the multiple mechanisms can give rise to a high degree of genomic instability in cadmium-adapted cells, relevant not only for tumour initiation but also for later steps in tumour development [19]. Other mechanisms of Cd toxicity have been suggested, including ionic and molecular mimicry, interference with cell adhesion and signalling, genotoxicity and cell cycle disturbance [80].

Distribution of Cd in female reproductive organs

In female reproductive organs, Cd was found mainly in the ovaries and uterus [52]. In the ovary, the presence of Cd has been identified in follicular fluid and oocytes [95] which may accumulate in ovaries, eventually affecting the quality of developing oocyte and embryo [22]. The incorporation of Cd into the chromatin of the developing gamete has been demonstrated [80]. In the mouse, Cd was deposited in the ovary after 8 days of its injection [91]. Cadmium chloride in a dose of 0.25 $mg.kg^{-1}$ per week resulted in almost the same Cd content in ovaries, adrenals, and the pituitary gland [53, 82]. Several studies have demonstrated increased accumulation of Cd in the ovary with an increase in age [102], thus leading to failure of progres-

sion of the oocyte development from primary to secondary stage, and failure to ovulate [80, 97, 102]. A significant increase in Cd concentration was also found in the uterine tissue. After 30 days of administration, the mean Cd concentration increased in uterine tissues depended on the dose. Cadmium displays the ability to accumulate in human endometrium, and the lining of the uterus [66]. There was a significant correlation between the Cd concentration in the blood and in the uterine tissues just after 30 days of Cd administration, as well as, 3 months after the exposure termination [48].

Effects of cadmium on the structure of the ovary

The deleterious effects of Cd reflected in the structure of the ovary and its toxicity was seen in all zones of the ovary. In the ovary cortex, a decreased number of follicles in different stages of maturation have been noticed. In the interfollicular zone areas of disorganization, oedema and necrosis has been identified, whereas in the medulla, congestion, hyperaemia and large sanguineous vasodilatation tending to haemorrhage was observed [42]. The distribution of follicles and corpus luteum became highly confused in ovarian tissues in the Cd group. The average size of the corpus luteum seemed to be larger [42]. The histopathology of the ovaries revealed a significant decrease in follicle number of follicles [52]. These changes resulted in the increased constituent ratio of the original follicles and a decreased diameter of all levels of follicular oocytes [99].

Cadmium affected the maturation of follicles, and increased the number of atretic follicles. In mouse foetal gonads, Cd increased female germ cell apoptosis [3]. Cadmium, at concentrations as low as 1 μM, significantly decreased the germ cell density in human foetal ovaries. The developing or mature follicles are sensitive to cadmium treatment. Cadmium is able to block the growth of follicles; and developing follicles transform into atretic follicles [80], and increases the apoptotic follicular cells [52]. The follicle growth and differentiation were significantly disturbed by ≥1.2 $\mu g.ml^{-1}$. The exposure to $CdCl_2$ with concentrations of 1.6 $\mu g.ml^{-1}$ on day 2, had caused a significantly reduced survival rate and rate of antral follicles, and increased abnormal follicle rate [81].

A decrease in the granulosa cell numbers and changes in their morphology with Cd exposure was found [5, 27, 50, 55, 71]. Cadmium has a catalytic role on mouse ovarian granulosa cell apoptosis [16, 40]. Apoptosis of the granulosa cells surrounded the oocyte as well as, the apoptosis due to the oocyte may cause follicular atresia [46, 83, 93]. Cadmium caused a significant reduction in gonadotropin binding, which altered the steroidogenic enzyme activity of granulosa cells. These changes exhibited a positive correlation with membrane changes of the granulosa cells [49]. The oocyte nucleus damage and the morphological changes of granulosa cells, and the cells of the internal and external theca layers of the ovarian follicle caused by Cd were found in the ovary of the $CdCl_2$ group [91]. Cd causes damage to the folliculogenesis which results in the decline in the number and quality of ovulated oocytes and the failure in the fertilization [62].

The exposure to Cd of cultured human ovarian granulosa cells caused morphological alterations in a time- and dose-dependent manner [80]. *In vitro* study revealed that the suppression of steroid forming was due to the direct influence to the granulosa cells and luteal cells function [54]. In ovarian granulosa cells isolated from 21-day-old female Sprague Dawley rats cultured *in vitro* for 36 h and exposed to $CdCl_2$ (0, 5, 10, and 20 μM) was found that mRNA and protein levels of all factors were upregulated in each cadmium-dose group [40]. Cadmium decreased: antioxidants like superoxide dismutase, catalase, glutathione peroxidase and glutathione, and raised the concentrations of malondialdehyde and hydrogen peroxide in the uterus and ovaries of rats [52].

Cadmium and oocyte development

Oocyte development and associated events were seen to be disrupted by Cd administration in several species, although variations have been observed. Cadmium had affected the development of mice ovaries, it induced the oocytes into the status of apoptosis, resulting in the death of a large number of oocytes [36]. The amount of oocyte apoptosis in mouse treatment groups was remarkably higher. Cd induced apoptosis in meiotic cells as it also decreased the number of mouse oocytes after meiotic initiation [3].

Cadmium caused a reduction in the maturation of oocytes. Cadmium chloride was toxic to the bovine oocyte maturation process *in vitro* in a concentration-dependent manner [43]. This was related to the failure of the progression of oocyte development from primary to secondary stage [80] and had direct detrimental effects on the bovine oocyte maturation and developmental ability [1]. The high concentrations of Cd completely suppressed the oocyte

maturation rate and also, suppressed an integral component of expanded cumulus cells in porcine oocytes [84]. When oocytes were exposed to different concentrations of Cd (0.4, 2, 10 or 50 μM) for 44 h during *in vitro* maturation Cd exposure, prominently resulted in the failure of cumulus cell expansion and arrested meiotic progression [102]. It was found that maturation rate of the bovine oocyte was significantly affected at 2 and 20 M $CdCl_2$ and also reduced the percentage of oocytes reaching metaphase II stage. Both Cd concentrations also decreased numbers of oocytes resting in MII, reduced numbers of fertilized ovine oocytes in culture, and increased the rate of oocyte degeneration in both groups [34]. At the lowest dose tested Cd *in vitro* affects the physiological function of ovine gametes, but at higher dose tested can compromise cell viability [34]. A significant decline in viability of buffalo oocytes was observed at 1.0 $mg.ml^{-1}$ Cd [1]. The concentration of cadmium causing 100 % oocyte death (1-day culture) was 18 $\mu g.ml^{-1}$ [51]. The germinal vesicle breakdown of oocyte was disturbed dose-dependently after the culture follicles were exposed to ≥1.6 $\mu g.ml^{-1}$ $CdCl_2$ [88, 89].

Cadmium exposure decreased the number of ovulated oocytes and impaired oocyte meiotic maturation rate both *in vivo* and *in vitro*. In female mice, Cd impaired meiotic maturation of oocytes by affecting the cytoskeletal organization, mitochondrial function, and histone modifications. Cadmium disrupted meiotic spindle morphology and actin filament, which are responsible for successful chromosome segregation and the polar body extrusion during oocyte maturation and fertilization [17, 103]. Cadmium exposure impeded the mouse oocyte meiotic progression by disrupting the normal spindle assembly, chromosome alignment and actin cap formation [11]. Cadmium exposure perturbed the assembly of spindle structure by weakening the microtubule stability, accompanied by the misaligned chromosomes at the equatorial plate. Failure of meiotic progression results from the dysfunctional dynamics of cytoskeleton and nuclear maturation in Cd-exposed oocytes [102]. Exposure to Cd-induced oxidative stress with increased reactive oxygen species leads to abnormal mitochondrial distribution, insufficient energy supply and DNA damage, ultimately leads to oocyte deterioration [11]. Its exposure led to faulty nuclear maturation of oocytes through damage to the cytoskeleton assembly, resulting in: aberrant spindle organization, chromosome alignment, and actin polymerization [11, 103]. The subchronic cadmium poisoning may destroy or suppresses the oocyte fission process, as well as reduce the activity of ATP enzyme in the ovary.

The exposure of Cd changed the ultrastructure of cells in the ovary tissues. Remarkable changes have been observed on the fine structure of oocytes. Under the electron microscope, chromatin margination, karopyknosis, swelling of mature cisternae of Golgi apparatus, mitochondrial cristae disappearance, and swelling of the rough endoplasmic reticulum can be observed in the $CdCl_2$ group mice [91]. At the same time, the numbers of DNA damage of mouse oocytes in treatment groups were higher. The ultrastructure of oocyte in periods of apoptosis showed the phenomena such as mitochondrion vacuole, and karyotheca invagination [36]. Cd exposure caused the impaired cytoplasmic maturation by showing the disrupted dynamics of mitochondrial integrity and cortical granules, and thereby resulting in the compromised sperm binding ability and fertilization capacity of porcine oocytes [102].

Effect of Cd on ovulation and the oestrous cycle

Cadmium can suppress the female ovulation process and cause temporary infertility [36]. A minimum dose of 5 $mg.kg^{-1}$ of $CdCl_2$ was needed to inhibit ovulation. Ovulation was suppressed when the dose of 10.0 $mg.kg^{-1}$of $CdCl_2$ was administered on the day before ovulation. Cadmium is able to decrease preovulatory luteinizing hormone levels in the blood and inhibit ovulation in rats [55]. When $CdCl_2$ was given closer to the time of the luteinizing hormone surge on the day of proestrus, a more pronounced effect on ovulation was recorded. An administration of Cd to female rats during oestrus and diestrus resulted in increased serum progesterone levels [10].

The circulation sex hormone disturbances in Cd-exposed rats caused irregular oestrous cycle, persisting for 3 months after exposure termination [48]. The ovarian Cd concentration increased with age, and has been associated with failure of progression of oocyte development from primary to secondary stage, and failure to ovulate. The incidence of the failure of ovulation was associated with the decreased progesterone levels in serum and inflammation, haemorrhages and necrosis in the ovary. The mechanism by which ovulation could be rendered ineffective is by the failure of pick-up of the oocyte by the tubal cilia due to suboptimal expansion of the oocyte-cumulus complex and mis-expression of cell adhesion molecules [80].

Effects on steroidogenesis

Many experiments have revealed that the Cd administration profoundly alters ovarian steroidogenesis [55, 59, 98]. Several well-characterized estrogenic responses were induced in rodents after intraperitoneal injection of low doses of Cd [28]. Exposure to even low concentrations of Cd is sufficient to significantly affect the steroidogenic pathway [20]. Cadmium at nanomolar concentrations displayed xenoestrogenic activities by affecting lactotroph proliferation and hormone release from anterior pituitary cells [65].

Depending on the identity of the steroidogenic tissue involved and the dosage used enhance or inhibit the biosynthesis of progesterone, a hormone that is inexorably linked to both normal ovarian cyclicity and the maintenance of pregnancy. *In vitro* and *in vivo* studies showed that Cd can both increase and inhibit the secretion of the major classes of steroid hormones: oestradiol (E2) and progesterone (P). Oral Cd exposure induced changes in the plasma levels of steroid hormones: decrease in E2 and increase in P after the highest dose of Cd in female rats [48]. On the other hand, extremely low doses have been reported to stimulate luteal progesterone biosynthesis in the ovaries [20]. The exposure of cultured rat ovarian granulosa cells to Cd causes a decrease in progesterone production [96]. *In vitro*, there were significant differences in P and E between different concentrations of Cd and also between different stages of the oestrous cycle. When Cd was administered i. p. for 5 days per week for 6 weeks at doses of 1.0, 0.5, 0.25 and 0 mg.kg^{-1}, this suggested that Cd may inhibit progesterone and release oestradiol in the ovaries [98].

Acute Cd effects on steroidogenesis are most severe in rats evaluated in proestrus or in early pregnancy, while in late pregnancy steroidogenesis is relatively unaffected [58]. It was found that after Cd exposure, the most affected were productions of progesterone and testosterone in proestrus rats and less in pregnant dams, whereas oestradiol was not affected at all. Cadmium appears to interfere with the ovarian steroidogenic pathway in rats at more than one site [58] and was able to reduce the level of progesterone, by disrupting the function of two steroidogenic enzymes, cytochrome P450 and 3β-hydroxysteroid dehydrogenase, which are essential for the synthesis of progesterone [20]. Once the corpus luteum is formed, the effect of $CdCl_2$ is reduced. Several *in vitro* studies suggested that these effects are mediated via oestrogen receptors [2]. At lower concentrations, Cd competes with oestradiol for binding to oestrogen receptors and activates the receptors, leading to changes in gene expression [74].

Cadmium displays the ability to mimic oestrogens. Cd exposure induces a limited spectrum of estrogenic responses *in vivo* and that, in certain targets, effects of Cd might not be mediated via classical oestrogen receptors signalling through oestrogen responsive element-regulated gene [2]. It seems that Cd inhibit ovarian steroidogenesis by downregulating StAR gene expression along with inhibiting activities of steroidogenic enzymes and antioxidant system [60].

Toxic effects of Cd on the vascular system of the ovary

An important route of Cd exposure is the circulatory system whereas blood vessels are considered to be the main stream organs of Cd toxicity [61]. Light microscopy revealed that small arteries in developing follicles and the interstitial stroma were particularly susceptible to damage. In rats, haemorrhagic necrosis and endothelial damage in the vessels has been found [12]. Exposure to $CdCl_2$ resulted in the coagulation of blood around the ovarian follicles of the female Golden hamster, which follicles within 24 h showed signs of degeneration [67]. In Syrian hamsters the small arteries of the developing follicles and interstitial stroma seemed selectively susceptible to $CdCl_2$ toxicity while the corpora lutea, mesothelium, primordial oocytes, and the rete ovarii appeared resistant [64]. In the ovarian medulla, hyperaemia and large sanguineous vasodilatation tending to haemorrhage have been found [42].

Cd toxicity on uterus

Cadmium effects included, increased uterine weight, hyperplasia, and hypertrophy of the endometrial lining [101]. The high levels of steroid hormones led to the proliferation of the endometrium. In the ovariectomized rats treated with cadmium by the dose of 0.1–2.5 mg.kg^{-1}, uterine weight was increased and a dose-effect manner was found [69]. The increase in wet weight was accompanied by proliferation of the endometrium and induction of progesterone receptor and complement component C3 [28, 39]. Both 3-day and 4-week oral Cd administration resulted in a dose-dependent stimulation of C3 expression in the uterus, significant at and above 0.5 mg.kg^{-1} b.w. [22].

Differences in Cd tissue levels and hormonal potency for the two routes of administration was found. A single

i.p. injection of Cd increased dose-dependently uterine wet weight. Uterine wet weight increased 1.7 times when ovariectomized rats were administered a single intraperitoneal injection of 5 μg $CdCl_2$ kg^{-1} b.w. [28] and 1.2 and 1.4 times after a single intraperitoneal injection of 500 or 2,000 μg $CdCl_2.kg^{-1}$ b.w. [22]. The thickness of the endometrium and weight of the uterus were also increased in F1 female offspring. A marked increase in the endometrial thickness during oestrus after 30-day exposure to Cd was found [48]. Histological evaluation of uterine tissues showed that the height of the luminal epithelium was significantly increased [2, 22]. The increase of uterine weight and by the increase of the thickness of luminal epithelium cell and endometrium was accompanied by the decrease of nuclear/cytoplasm of luminal epithelium cell and endometrium [96].

Cadmium caused morphologic alterations and dysfunction in blood vessels. The effects of Cd are a multisystem phenomenon involving inflammation, hypertrophy, apoptosis and angiogenesis [31]. Exposure to Cd increase the thickness of the basal lamina in the blood vessels in a dose-related manner in the uterus of female rats subcutaneously injected with 0.36 and 0.18 $CdCl_2.kg^{-1}$ for 8 to 60 weeks. Uterine and cervical stromal haemorrhages were seen in immature hamsters at doses of ≥30 μmol $CdCl_2.kg^{-1}$ [12].

In the vasculature of the uterus, Cd interacts with the vessels, leading to an inflammatory response, that finally causes the formation of high endothelial venules, blood vessel dilatation and oedematisation [13, 47]. A dose- and time-related increase of the thickness of the media in arteries with signs of perivascular inflammatory reaction could be observed [37]. Cadmium might affect endometrial angiogenesis and as a consequence cause endometrial dysfunction with an increased risk for fertility problem. Long-term exposure was reported to give rise to changes in the blood vessels of the myometrium of the uterus in female rats and may cause small vessel wall thickening in the rat uterus [35]. After Cd exposure, the endometrial endothelial cells of blood vessels have a key role in the regulation of endometrial angiogenesis.

CONCLUSIONS

Female reproductive organs are sensitive to Cd treatment. Cadmium has been shown to exert significant effects on ovarian and reproductive tract morphology. It has the potential to affect reproduction in many different ways, and at every stage of the reproductive process. Experiments in animals showed dose- and age-dependent Cd-toxicity in the ovaries, uterus, and cervix. Oral exposure of rats to Cd induced damage of the ovaries. Cadmium caused the reduction in the ovary size and inhibited folliculogenesis which resulted in the diminution of the numbers of primordial, growing, and tertiary follicles. After Cd exposure, oocyte development is inhibited, and ovarian haemorrhage and necrosis supervene at higher Cd doses. The cadmium may suppress ovarian granulosa cells. Exposure was linked to a decline in the number of corpora lutea, as well as a reduction in their vascular area. Cadmium exposure has the adverse effects on the oocyte meiotic maturation and can suppress the female animal's ovulation process and cause temporary infertility. Morphometric changes in the epithelium of uterus have been presented. An increase of stroma was a sign of uterus oedamatisation caused by damage in the wall of the blood vessels. The effects of Cd, are a multisystem phenomenon involving: inflammation, hypertrophy, apoptosis, angiogenesis and important processes involved in vascular remodelling systems. Cadmium has been proposed to be an endocrine disruptor by inducing estrogenic responses and luteal cells steroid biosynthesis and had the ability to alter the levels of circulating hormones and sexual cycle phases.

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THE EFFICIENCY OF THE NATURAL COLLAGEN COATING IN THE TREATMENT OF EXTENSIVE CHRONIC DEFECTS OF THE SKIN AND SURROUNDING TISSUES IN DOGS

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ABSTRACT

The occurrence of extensive non-healing and chronic skin defects with loss of tissue substance in companion animals are a frequently solved problem. The management of the therapy of large-area defects and absent tissues is a challenge for setting up successful therapeutic management and achieving wound closure, satisfactory cosmetic effect, and restoration of the functionality of the damaged area. In veterinary medicine, we often encounter the failure of wound therapy methods commonly used in closing defects, as a result of which the defect closure time is prolonged. The longer the time required to close the defect, the greater the risk of microbial infection and complications associated with healing in the case of extensive damage to the surrounding soft tissues. Direct influence of the individual phases of healing with supportive alternative therapy appears to be a very suitable solution for the treatment of chronic wounds. To overcome the shortcomings related to partially efficient conventional wound dressings, efforts are oriented toward developing new and effective platforms for wound healing applications. Five patients referred to the Small Animal Clinic of the University Veterinary Hospital were included in this clinical study. Patients were referred to the clinic with extensive long-term non-healing wounds, necrosis and secernation, or ongoing infection, with loss of tissue substance, which showed signs of chronicity. After assessing the patient's state of health and subsequent cleaning of the wound bed from contaminants and damaged tissues, collagen sponge Suprasorb® C was applied to the surface of the cleaned wound bed. The average time for the complete closure of the defect was 24.6 days since the introduction of the primary treatment. In all patients, we observed the complete closure of the defect, restoration of functionality of the damaged tissues, and achievement of a cosmetic effect without complications in the recovery process, which points to the excellent effectiveness of the collagen covering in the wound healing process.

Key words: collagen sponge; conservative management; chronic skin defect; non-healing wound; wound healing

INTRODUCTION

In companion animals, the healing of large skin defects resulting from traumatic accidents can be challenging

to treat since they need a complex management consisting of accurate debridement of the damaged and necrotic tissues, local and/or systemic infection control, protection of the underlying tissues, and induction of cutaneous tissue regeneration. To this aim, surgical tissue reconstruction (i. e., skin graft) and medical treatments are often associated to reach complete anatomical and functional recovery of the damaged area, reducing debilitating side effects, and pain [18, 29].

Wounds have a variety of causes, from surgery, injuries, extrinsic factors (e. g., pressure, burns and cuts), to pathologic conditions such as diabetes or vascular diseases. These types of damage are classified into acute or chronic wounds depending on their underlying causes and consequences [11]. Acute wounds usually proceed through an organized and appropriate repair process, resulting in the sustained restoration of anatomical and functional integrity. On the contrary, chronic wounds are not able to achieve optimal anatomical and functional integrity. Healing is related to, and determined by, both pathological processes nature, degree, and status of host and environment. Systemic factors such as patient age, the presence of vascular, metabolic and autoimmune diseases, as well as ongoing drug therapy, may affect the wound healing process [12]. In the case of chronic lesion, characterized by the failure of normal phases of wound healing in an orderly and timely manner, with a recovery time that usually exceeds eight weeks [10, 14]. An ideally healed wound is an area returned to normal anatomical structure, function and appearance after an injury; a minimally healed wound is characterized by the restoration of anatomical continuity, but without sustained functional results; the wound can recur [12].

The wound management goal is to complete the healing in a rapid manner, with functional and aesthetic outcomes [17]. For many years, wound management was based on covering the wound and the materials used for that had a passive action in encouraging the healing process. However, in recent years, the wound management has been updated due to a greater understanding of the molecular and cellular processes involved in wound healing and preventing the wound from healing. Accordingly, the design and functionality of wound dressings has evolved in the direction of multi-functionality. The critical necessities of modern wound dressings include: biocompatibility, no cytotoxic effect, no antigenic or inflammatory stimulation, a rate of biodegradability directly proportional with the rate of formation of new tissue, a release of incorporated bioactive constituents (drugs), and the control of possible infections [9, 26].

The multidisciplinary association between tissue engineering and regenerative medicine includes aspects from engineering, biology, and material science and ultimately results in the progress of viable alternatives for organs and tissue regeneration/replacement. As an alternative treatment of wounds, tissue engineering has recently suggested a range of solutions, such as the introduction of scaffolds, to manage the treatment process. Scaffolds, 3D structures, not only offer sustenance for tissue formation, but also can shield a wound, becoming an efficient “fence” against external contamination [21]. Biodegradable polymeric materials are ideal carrier systems for biomedical applications. Features like controlled and sustained delivery, improved drug pharmacokinetics, reduced side effects and safe degradation make the use of these materials very attractive in a lot of medical fields [19]. They can be loaded with growth or angiogenic factors to favour tissue regrowth or antimicrobials to avoid wound infection [7, 8].

Collagen is one of the most frequently used materials in protein-based scaffolds for skin wound healing; this is not only because of its sheer abundance in the body, but also because it is the main component of the dermal extracellular matrix (ECM). Specifically, collagen type I constitutes 80–85 % of the dermal ECM, while collagen III constitutes 8–11 % [22]. Collagen sponges are especially useful in wound healing because their wet-strength allows their suturing to soft tissue and provides a template for new tissue growth [4, 13]. Their porosity is controlled by varying the collagen content and freezing rate. These sponges are capable of absorbing large amounts of tissue exudate, adhere smoothly to a wet wound bed, and maintain a moist environment, while shielding against mechanical trauma and bacterial infection [30]. Collagen is part of the ECM and interacts with macromolecules and cells promoting cellular attachment and spreading [2]. It provides directional information for cell attachment, migration, and proliferation through integrins which are expressed on the surface of most cells of the ECM [20, 25] and its breakdown products become integrated into the developing physiological ECM. Although there has been much discussion about the potential immunogenicity and adverse reactions to the application of collagen within biomedical

devices, collagen is considered to contain very weak antigenic components and the incidence of such adverse reactions to the presence of collagen are extremely rare [15, 16]. Indeed, studies have demonstrated that the presence of collagen within biological implants and scaffolds do not elicit immune responses [24]. The treatment of extensive chronic defects of the skin and surrounding tissues in dogs presents challenges in the form of ending the chronic condition, the inflammatory phase and starting the closure of the skin defect. Collagen as a biodegradable external component brings significant progress in the process of closing a skin defect, as well as a carrier of supporting substances that help the process of granulation and epithelization. Therefore, the clinical study was aimed to the monitoring of the effectiveness of wound treatment by straight bovine collagen covering attached on the surface of defect.

MATERIALS AND METHODS

In this clinical study, five patients from the Small Animals Clinic, Section of surgery, orthopaedics, roentgenology and reproduction at the University of Veterinary Medicine and Pharmacy in Košice were included. Patients were with long-term non-healing wounds of various aetiologies with loss of tissue substance that showed signs of chronicity (persistent inflammatory phase of the healing process). The group of patients consisted of five dogs: crossbreed (n = 4) and a Rhodesian Ridgeback (n = 1), with a mean age of patients of 5.6 years. There were three males and two females. The clinical study included wounds of various aetiologies, such as stab wounds (n = 2), wound dehiscence (n = 2) and laceration (n = 1) located on different parts of the body.

After the primary clinical examination of the patient with an assessment of the patient's state of health, the wound was macroscopically evaluated with regard to: the extent and localization of the wound, hyperaemia, oedema, secernation, granulation, necrotization, and contraction of the wound edges. Macroscopic evaluation of the wound changes and the healing process was performed at each wound check, during the entire period of therapy. The regularity of the wound check, the replacement of the wound covering, and the length of the therapy were different for individual patients depending upon the individual stages of healing in which the current injury was located.

The patients were sedated at the initial clinical examination and prepared for surgery and the mechanical removal of contaminants from the surface of the wound bed by an intravenous application of butorphanol (Butomidor, RP Richter Farma, Austria) at a dose of 0.2 $mg.kg^{-1}$ b.w. and medetomidine (Cepetor, CP pharma, Germany) at a dose of 0.020 $mg.kg^{-1}$ b.w. The introduction of the patient into general injection anaesthesia was performed by propofol (Propofol, Fresenius Kabi, Germany) at a dose of 3 $mg.kg^{-1}$ b.w., with intravenous (IV) prolongation of anaesthesia as needed. A combination of amoxicillin and clavulanic acid (Synulox RTU, Zoetis, Czech Republic) at a dose of 15 $mg.kg^{-1}$ b.w. was used as the initial antibiotic therapy administered subcutaneously. In each patient, a sample was taken from the wound using a sterile microbial swab for bacteriological cultivation and determination of the antibiogram in a certified laboratory. The microbial culture indicated the need to use a systematic antibiotic therapy. Based on the results of the microbial culture and antibiogram, it was not necessary to change the set antibiotic therapy in all five patients.

After the primary examination, the wound was flushed with a bacteriostatic and bactericidal lavage solution (Braunol, B. Braun Melsungen AG, Germany) diluted in an isotonic solution (0.9 % NaCl, B Braun Melsugen AG, Germany). The wound bed and edges of the skin defect were refreshed by excochleation, while total removal of the changed structures was not possible based on the state of the surrounding tissues. Enzymatic preparations containing collagenase (Irulox Mono, TJ Smith & Nephew Ltd., United Kingdom) or containing pancreatin, trypsin, chemotrypsin or papain (Pana-Veyxal Salbe, Veyx-Pharma, Germany), were applied in places where it was necessary to gently separate the changed structures, necrotic and damaged tissue without iatrogenic damage. In the case of painful inflammation of the skin, a dermal gel containing ketoprofen (Fastum gel, A. Menarini Industrie Farmaceutiche Riunite Srl, Italy) was applied to the site of inflammation. Collagen sponge (Suprasorb® C, Lohmann & Rauscher, Germany) was applied to the surface of the cleaned wound bed.

In places where the wound exceeded the coverage of the collagen sponge, preparations based on ointment and cream were used – cream containing the silver salt of sulfadiazine and sodium hyaluronate (Ialugen plus, IBSA, Slovakia s.r.o., Slovakia), or ointment containing neomy-

cin and bacitracin (Baneocin, Sandoz GmbH, Austria), or ointment containing iodine (Betadine, EGIS Pharmaceuticals PLC, Hungary).

A three-layer bandage with non-sterile gauze squares (Lohmann&Rauscher, Germany), a secondary layer of Cellona® synthetic cotton wool (Lohmann&Rauscher, Germany), and an elastic CoPoly wrap (CoPoly, Czech Republic) placed circularly on the surface to the site of damage was used as a surface covering.

RESULTS

The average healing time for patients from the primary treatment and application of collagen covering in combination with bactericidal preparations, to closure of the defect was 24.6 days. The shortest duration of therapy from the primary treatment was 13 days. The longest period of therapy from the primary treatment was 45 days. These results pointed out and confirmed the excellent effectiveness of the collagen covering in the wound healing process of extensive non-healing chronic wounds with loss of tissue substance. The expected effect of collagen as a natural material was not to create toxic intermediates and not force the body to react to the material; effectively support the healing process and not causing any complications in the patient's recovery process. During the entire time of the regeneration process, from the beginning of the application of the collagen sponge until the closure of the defect, the point of contact of the collagen with the surrounding soft structures was evaluated using macroscopic indicators of the state of healing, which were: secernation, hyperaemia, oedematization, and necrotization of the defect surface. The progress of the healing process was evaluated by every re-bandaging of the wound. The state of the aforementioned indicators of macroscopic assessment of wound healing was different in each patient at the beginning of the treatment, but after the start of tissue regeneration at the place of application of the collagen coating, they were very similar and almost not observed.

The first patient that was referred to the clinic with the dehiscence of a surgically treated bite wound, localised on the cranial surface of the chest, extending laterally and dorsally along the scapula and neck, irregular in shape (Fig. 1). The closure of the skin defect occurred on the 45th day after the onset of therapy.

The second patient was referred to the clinic with a devastating injury to the right thoracic limb (Fig. 2). The patient had symptoms of grade four lameness with massive swelling in the metacarpal region. After 28 days from the start of the therapy, there was complete healing of the wound with first-degree lameness.

In the third patient with a dehiscence of a surgically treated bite wound with necrosis and secretion on the right side of the abdominal wall, extending from the right flank to the lateral side of the thigh, complete closure of the wound occurred on the 23rd day from the start of the therapy.

In the fourth patient with a puncture wound on the palmar surface of the left thoracic limb with an ongoing infection, the defect was closed on the 13th day from the start of therapy (Fig. 3).

The fifth patient was referred to the clinic with a stab wound of a circular shape on the foot pad of the fifth toe of the left hind limb. The wound was closed after 11 days from the start of the therapy and re-epithelialization of the wound surface occurred on the 14th day from the start of therapy.

All five patients point to the difference in the condition and location of the skin defects and thus the impossibility of a uniform assessment of the wound and individual setting of therapy. In all patients, it was damage to the skin and surrounding structures after the failure of previous therapy of a different provoking aetiology. Swelling, hyperaemia, and secretion of varying extent were noted in all patients after the primary examination. Necrotization around the defect was noted in two patients. The initiation of therapy with the application of enzymatic preparations resulted in a calming of the inflammation around the defect and significant progress in the wound healing process. Detachment of the changed structures and the formation of a granulation bed were recorded as early as three to five days (Fig. 4). The disappearance of swelling and secretion was observed in the following days. The subsequent application of a collagen cover, together with an ointment containing the silver salt of sulfadiazine and sodium hyaluronate, brought progress in the steps of closing the skin defect with new epithelization and tissue remodelling (Fig. 5). The difference in the closure time of the skin defect was directly proportional to its localization with regard to the lateral pull, size and extent of the damaged structures.

Fig. 1. Dehiscence of a surgically treated bite wound, localised on the cranial surface of the chest

Fig. 2. Devastating injury to the right thoracic limb

Fig. 3. Puncture wound on the palmar surface of the thoracic limb with an ongoing infection

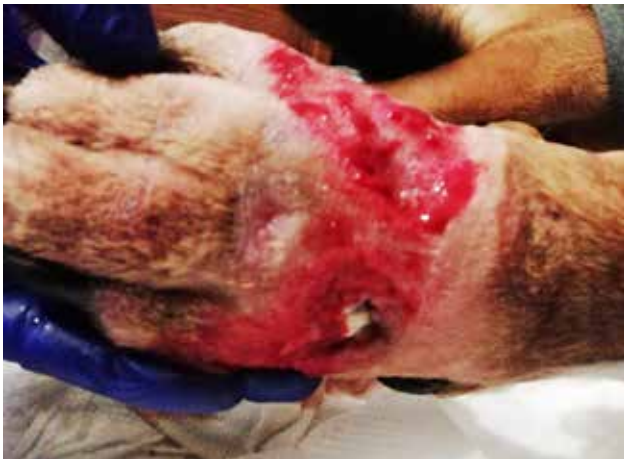

Fig. 4. The massive formation of a granulation bed

Fig. 5. Closing the skin defect with new epithelization and tissue remodelling

DISCUSSION

The wound healing process unites several overlapping phases: i. e., homeostasis, inflammation, proliferation/granulation, and remodelling. It involved a cascade of cells, matrix components and other biological factors to act together in order to facilitate the healing and restore the tissue integrity. Still, when the healing course deviates from the normal path, the healing does not advance past the inflammatory phase [28].

During our study, we focused on the treatment of five patients which were referred to the clinic with chronic wounds without progress in the healing process. All patients had a desirable outcome and closure of the defect in the fastest possible time. The results of our work points to the effectiveness of collagen covering in the therapy of

wounds that cannot pass through the inflammatory phase of the healing process and show signs of chronicity.

Collagen offers structural support to the body and regulates cellular functions such as: cell shape, proliferation, differentiation and cell migration [5]. It stimulated cellular and molecular cascades of wound healing and enabled the development of new tissue and wound debridement. An abrupt change could be noticed with the application of the collagen matrix in the wound bed within 10–14 days and an improved wound would be seen with the chronic wounds. When such dressings come into contact with wound exudate, they absorb wound fluid and thereby create a moist environment around the wound. These dressings are semipermeable to water and oxygen. Because of the hydrophilic nature of collagen, the fluid is retained and cells having phagocytosed microorganisms are picked up. It has an important function in the natural wound healing system by inducing clotting and forming matured scar in the area of the wound. In the event of wound healing as collagen combines with dermal cells, growth factors and cytokines derived from the patient, it accelerates the re-epithelization of the skin. The collagen dressings are also designed as vehicles to deliver therapeutic drug on the wound surface [23].

In our case, we observed a significant improvement after the application of the collagen sponge within the first few days. It created a moist environment for closing the wound in the defect site and the beginning of the formation of new granulation tissue occurred on average three days after application. In wounds with massive secernation, a large amount of exudate was absorbed and secernation stopped on average on the fifth day after the application of the collagen. The collagen, which we applied as a natural material to the site of damage, did not create toxic intermediates after application and did not cause an organism's reaction to the material. It protected the wound site from further external mechanical and microbial stress; thanks to the fact that it created a mechanical barrier against the external environment.

B o h l i n g et al. [3] in their study described the course of wound healing in dogs and cats. In secondary wound healing, the wound defect in cats was filled with granulation tissue more slowly than in dogs. The formation of granulation tissue in the wound bed began in dogs at an average of 4.5 days and in cats at 6.3 days. The complete granulation of the tissue occurred in 11.7 days in dogs, and in cats the granulation time was observed to be more than 19 days.

In a study published by A r a v a t h i n et al. [1], the effectiveness of the collagen cover was observed against the control group in a mammalian model for a period of 12 days. On the 6th day after the application of the collagen cover, a layer of granulation tissue was formed and the inflammation decreased. The secretion was present on average until the 6th day. The wounds were filled with compact granulation tissue on day 12 without the presence of exudate. A wound size reduction of 90 % was observed in the collagen-based sponge treated group, whereas it was 75 % in wound control group.

In a study conducted by V e v e s and S h e h a n [27], in human medicine performed on 276 patients of diabetic foot ulcer divided equally into two groups, one group was treated with collagen and the second with other dressing materials. They found no significant difference in the completeness of healing of wounds when old wounds (>six months old) were compared. But the healing was better with the rapid start of granulation tissue formation and wound closure in wounds of less than six months' duration treated with collagen dressings. In addition, patients with collagen covering experienced a reduction in pain and a reduction in bacterial colonization in the wound.

The results of the study by C o l a k et al. [6] demonstrated that collagen dressings are better than conventional dressings with regard to early granulation tissue and shorter hospital stay. The aim of this study was to compare the results of diabetic foot ulcer patients treated with and without collagen. Of the total 64 patients included in the study, 30 were treated with physiological serum (PS) and 34 with collagen. Complete closure was achieved in 17 (56.6%) of the PS group patients after 12 weeks of treatment. The rate was 25 (73.5%) in the collagen group. The mean duration of treatment was 9.2 weeks in the PS group and 8.08 weeks in the collagen group. The recovery time and recovery rates were determined to be better in the collagen group than in the PS group.

In our study of collagen wound therapy in dogs, the wound began to fill with granulation tissue on average on the 3rd day and was filled with compact granulation tissue on average on the 12th day from the start of the therapy. The average time for the complete closure of the defect since the primary treatment was 24.6 days. The shortest duration of therapy from the primary treatment to the closure of the defect was 13 days. The longest period of therapy from the primary treatment to the closure of the defect

was 45 days. The length of therapy in this patient with the dehiscence of a surgically treated bite wound was directly proportional to the large extent of the injury, bacterial contamination, the presence of necrotic tissue and massive secernation from the wound. The application of collagen covering to chronic types of wounds supports the healing process and the closure of the skin defect, depending on the size and location, in the shortest possible time with the restoration of the functionality of the damaged structures.

CONCLUSIONS

A big challenge in therapeutic management has been chronic large-area, deep wounds with a lack of loose skin for their closure. Macroscopic results after the targeted application of a collagen cover in combination with bactericidal and supportive preparations on the surface of wounds of various aetiologies, which remained in the inflammatory phase of healing without progress in the healing process, indicates the success of the chosen therapeutic method. Since the wounds were of different aetiology and extent, the collagen cover is effective for different types of wounds regardless of other factors such as breed predisposition, condition, body weight, age, etc. Collagen cover opens up the possibility of using collagen as a carrier of other therapeutic substances that support the wound healing process, such as growth factors, stem cells, antibiotics, or natural substances such as cinnamon and clove extracts and others that have an anti-inflammatory effect, in the therapy of chronic large-area non-healing defects with loss of tissue substance and impaired function of the damaged area for faster wound closure, restoring the functionality of damaged tissues and achieving the maximum cosmetic effect.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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PCR DETECTION OF AN EYE ANOMALY IN A FAMILY OF LONGHAIRED COLLIES

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ABSTRACT

Inherited eye diseases have been the subject of genetic research for many years. This paper focuses on the optimisation of the DNA test based on the polymerase chain reaction (PCR) for the detection of Collie Eye Anomaly (CEA) in dogs. A small family of four longhaired Collies (parents and their daughters) with a confirmed positive clinical ophthalmologic examination of CEA served as the source of affected animals. Both PCR reaction conditions examined were suitable for detecting canine *NHEJ1* gene mutation associated with CEA. One carrier was found in a small group of eleven randomly selected control healthy dogs. The PCR test confirmed the previous CEA-positive ophthalmological examination in Collies. The results indicated that all four family members of the examined longhaired Collies had a homozygous intronic deletion of 7799 bases in the canine *NHEJ1* gene. The affected female Collies may potentially transmit this CEA-associated mutation to their puppies.

Key words: Collie eye anomaly; dogs; hereditary eye diseases; PCR

INTRODUCTION

Hereditary eye diseases are becoming a serious problem in some breeds of dogs. In diagnosing these conditions, the first step is usually a clinical ophthalmological examination. However, in many cases with a genetic basis, specific genetic tests are also welcome to complement a comprehensive clinical examination. This is especially valuable in diseases with similar symptoms where molecular genetic methods help to refine the diagnosis. In addition, genetic tests are important in detecting asymptomatic carriers (heterozygotes) of recessive alleles in the case of autosomal recessive inheritance modes. When crossing with another carrier, heterozygotes may pass these alleles on to their offspring (Fig. 1). A congenital condition, Collie Eye Anomaly (CEA), was an example of that transfer in multiple dog breeds for many years [10, 12, 13]. However, there are doubts about the monogenic autosomal recessive inheritance of this disease at this time [3], based on research of Nova Scotia Duck Tolling Retrievers.

The CEA anomaly is not simple but complex in nature with the prevalent phenotype of choroidal hypoplasia in mild cases. More severe cases may also have optic nerve colobomas, retinal detachment and intraocular haemor-

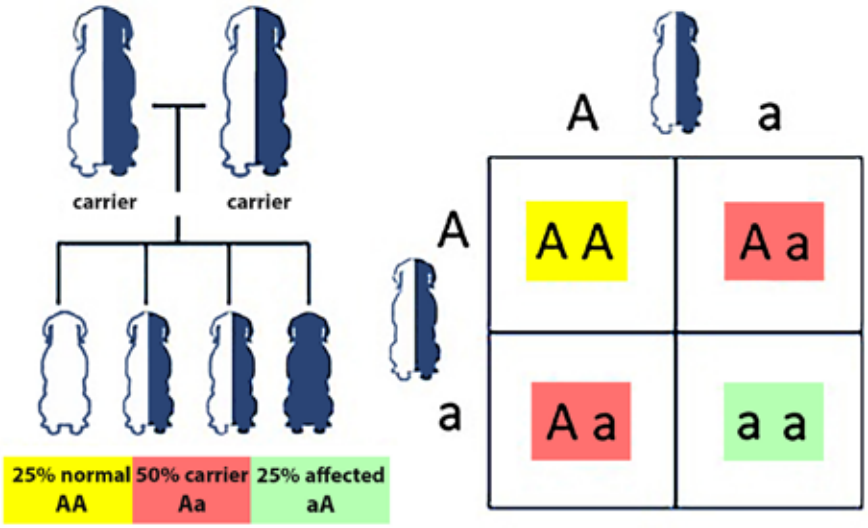

Fig. 1. Crossing of two asymptomatic carriers (Aa): 25 % of puppies are affected (aa)
Source: https://www.pawprintgenetics.com/blog/2018/11/13/who-test-autosomal-recessive-genetic-diseases/

rhage [7]. The current paper of Kucharczyk et al. [10] describes CEA as a congenital canine pleomorphic ocular disorder characterized by two main lesions: choroidal hypoplasia/chorioretinal dysplasia (CH/CRD) and papillary/peripapillary colobomata. The CH/CRD is bilaterally present, with varying degrees within the same individual and between affected dogs. As concerns colobomata, their size varies; the larger defects may be involved in post-natal retinal detachment. Similarly, Mizukami et al. [11] pointed out that CEA is a syndrome with variability in manifestation and severity of clinical and ophthalmological lesions. The most severely affected dogs may develop retinal detachment and intraocular bleeding leading to blindness [2, 6, 14]; however, this is usually with a low incidence.

CEA was first described in the Collie and Shetland sheepdogs and later in other breeds [1, 3, 11]. The eye examination is usually advisable at six weeks of age.

In 2007, Parker et al. [13] developed the scientific basis for DNA testing of CEA. The authors revealed that a large homozygous deletion of 7.8 kb within an intron 4 in the *NHEJ1* gene (non-homologous-end-joining factor 1; located on chromosome 37) was present in all CEA-affected dogs and absent in unaffected dogs of multiple breeds.

The aim of our study was to optimize the polymerase chain reaction (PCR) conditions for the detection of hereditary Collie Eye Anomaly (CEA) in groups of both the controlled clinically healthy dogs, and the affected animals. The affected dogs were members of a small family of longhaired collies with a confirmed positive clinical ophthalmologic examination of CEA.

MATERIALS AND METHODS

Sample collection

Blood and/or buccal (cheek) cells of dogs served as a source of DNA. The total number of dogs tested was 15. Of these 15 dogs, four came from the same family of longhaired Collies. The family consisted of mother (age 6.5 years), father (age 9.5 years), daughter 1 (age 4 years) and daughter 2 (age 11 months). In all of these Collies, CEA had been confirmed by ophthalmologic examination in previous years (as stated by the owner), but this finding was not supported by genetic verification. Eleven (11) ophthalmological healthy dogs of different breeds and ages (1 year to 14 years) served as phenotypically unaffected controls.

The whole blood samples were collected at the University Veterinary Hospital (Small Animal Clinics) into sterile EDTA tubes (0.5 M EDTA, pH 8.0) from seven dogs of the following breeds: Yorkshire terrier, Golden retriever, Rhodesian ridgeback, Bulldog, Bernese mountain dog, Bavarian painter and Hanoverian painter.

As for the buccal swabs of the dogs' mucosal cells, their owners mostly took the swabs. They used sterile cytology brushes according to the instructions of the Genomia laboratory (https://www.genomia.cz). These samples came from eight dogs of the following breeds: Labrador retriever (one dog), Jack Russell terrier (one dog), Border collie (one dog), Shetland sheepdog (one dog) and four above-mentioned longhaired Collies.

ETHICAL CONSIDERATIONS

All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (Protocol No. 3323/16-221/3) approved by the State Veterinary and Food Administration of the Slovak Republic and by Ethics Commission of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia.

Isolation of DNA

Genomic DNA was extracted using binding membrane-based systems (binding columns in microcentrifuge tubes) of two kits: Whole Blood Genomic DNA Fast Extraction kit (BioTeke, China) and ReliaPrepTM Blood gDNA Miniprep System (Promega, Madison, USA). Both kits were designed to extract DNA from whole blood, so the procedure for isolating DNA from buccal swabs was slightly modified. The main modifications included extending the lysis time (30 minutes instead of 10) and gentle thorough mixing of the cells before and after lysis (20 seconds instead of 10). The concentration and purity of DNA were determined at 260 and 280 nm using NanoPhotometer (Implen GmbH, Germany).

Primers and PCR reactions

Detection of CEA-associated deletion in the *NHEJ1* intron 4 was performed by PCR amplification with primers described in P a r k e r et al. [13]. Two sets of primers were used: *NHEJ1*-F17/*NHEJ1*-R17 to amplify within the deletion and *NHEJ1*-F20/*NHEJ1*-R23 to amplify across the deletion. The sequences of the primers were as follows:
NHEJ1-F17 5'-TCTCACAGGCAGAAAGCTCA-3'
NHEJ1-R17 5'-CCATTCATTCCTTTGCCAGT-3'and
NHEJ1-F20 5'-TGGGCTGGTGAACATTTGTA-3' /
NHEJ1-R23 5'-CCTTTTTGTTTGCCCTCAGA-3'.

Two final modifications of the PCR reaction were used, as indicated in Table 1.

Tab. 1. Final modifications of PCR conditions for CEA detection

Step	Reaction conditions 1		Reaction conditions 2	
	Temp [°C]	Time [s]	Temp [°C]	Time [s]
Initial denaturation	95	120	95	120
Denaturation	95	40	95	30
Annealing	50	50	55	30
Synthesis	72	90	72	60
Final synthesis	72	300	72	300

PCR reaction mixture (25 ml) consisted of 14.5 ml water, 5 ml buffer (5× Go TaqÒ Flexi Buffer, Promega), 2.5 ml $MgCl_2$ (25 mM), 0.5 ml dNTP (10 mM each), 0.625 ml of each primer (10 mM), 0.25 ml DNA polymerase (GoTaqÒG2Hot Start Polymerase, Promega) and 1 ml of genomic DNA.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to visualise the PCR results (amplicons) using 1.5 % agarose in 1×TAE buffer (1 h, 75 V, Gel Haus 300). Gels were stained by GelRed® (Biotinum, CA) and documented via UV light using the Digimage system (Major Science, USA).

RESULTS

The conditions of the PCR reaction 1 (Tab. 1) were shown to be suitable for amplification and detection of the resulting amplicon on a 1.5 % agarose gel. Using the first set of primers (*NHEJ1*-F17/*NHEJ1*-R17) that flank a sequence within a site of potential deletion, it is possible to determine whether the subject is healthy (normal, clear) (AA), i. e., no deletion is present in intron 4 of the *NHEJ1* gene (Fig. 2). Regarding the carrier status, the PCR reaction of the same individual with the second set of primers should be performed (Fig. 3, Fig. 4). This allows distin-

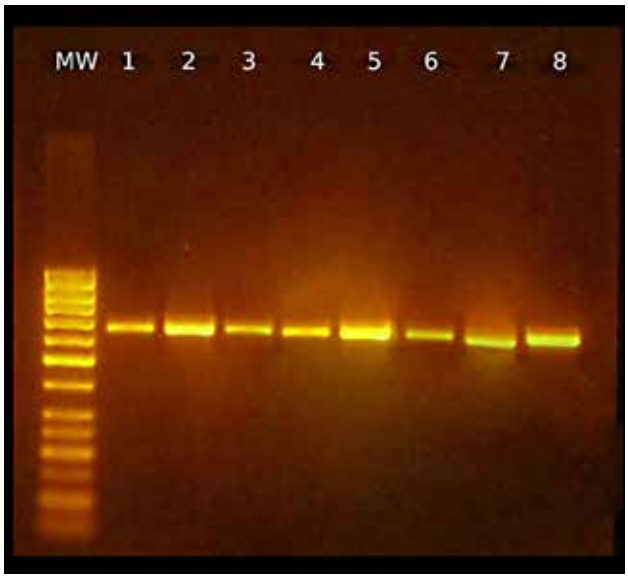

Fig. 2. Agarose gel with resulting PCR amplicons (636 bp) in healthy dogs
MW – Standard of molecular weight 50 bp; lanes 1, 2 – Labrador retriever; lanes 3, 4 – Jack Russell terrier; lanes 5, 6 – Yorkshire terrier; 7, 8 – Golden retriever

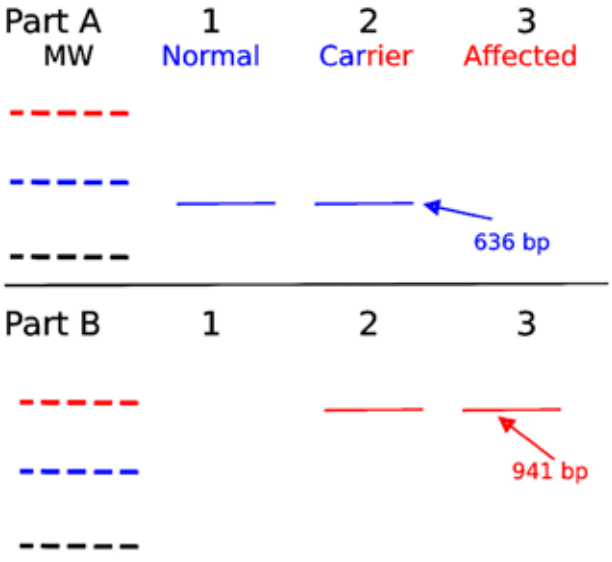

Fig. 3. Schematic representation of PCR results on agarose gel using two pairs of primers for each of the tested individuals 1, 2 and 3
Part A: the PCR results on agarose gel using the first set of primers (*NHEJ1*-F17/*NHEJ1*-R 17) for individuals 1, 2 and 3. **Part B:** the PCR results on the same agarose gel using the second pair of primers (*NHEJ1*-F20/*NHEJ1*-R23) in the same individuals. Considering simultaneously parts A and B of the same gel the individual 1 is normal (clear; healthy) (AA), individual 2 is a carrier (Aa) and individual 3 is genetically CEA-affected (aa)

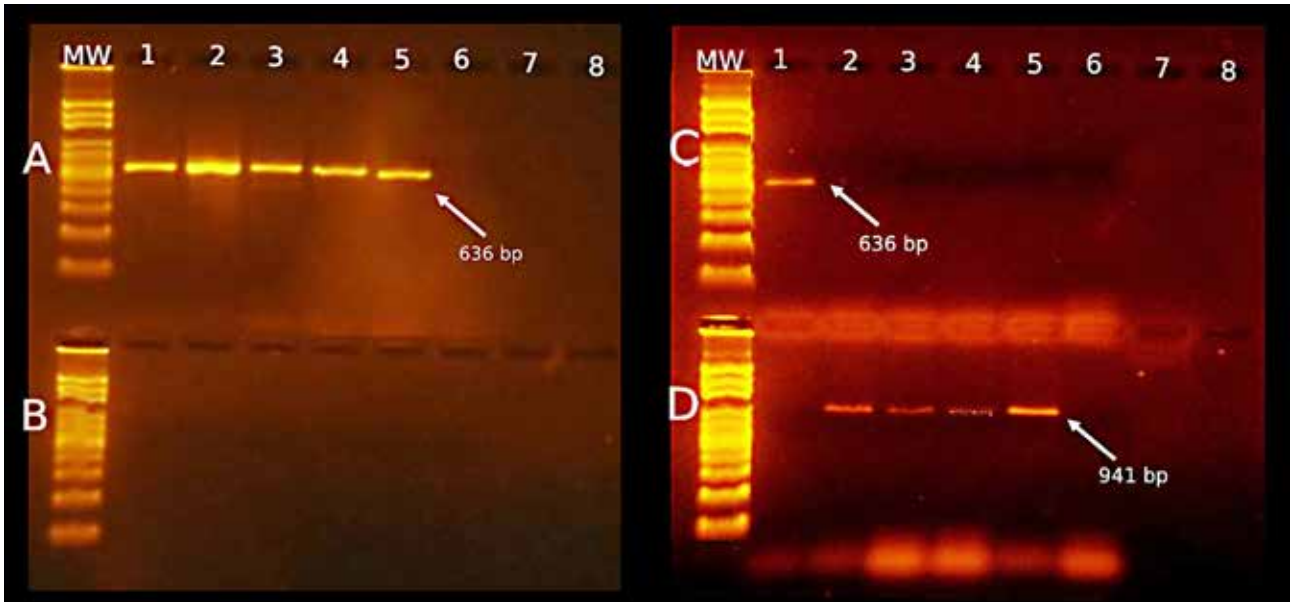

Fig. 4. PCR detection of normal (clear) and genetically CEA-affected dogs
Agarose gel 1 – normal (clear) dogs: MW – standard of molecular weight 100 bp; line 1 Rhodesian ridgeback; line 2 Bulldog; line 3 Bernese mountain dog; line 4 Bavarian painter; line 5 Hanoverian painter; line 6 negative control (water instead of DNA). Agarose gel 2 – normal (clear) (line 1) and CEA-affected dogs (lines 2–5): line 1 Border collie; line 2 Longhaired collie – father; line 3 Longhaired collie – mother; line 4 Longhaired collie – daughter one; line 5 Longhaired collie – daughter two; line 6 – negative control (water instead of DNA)

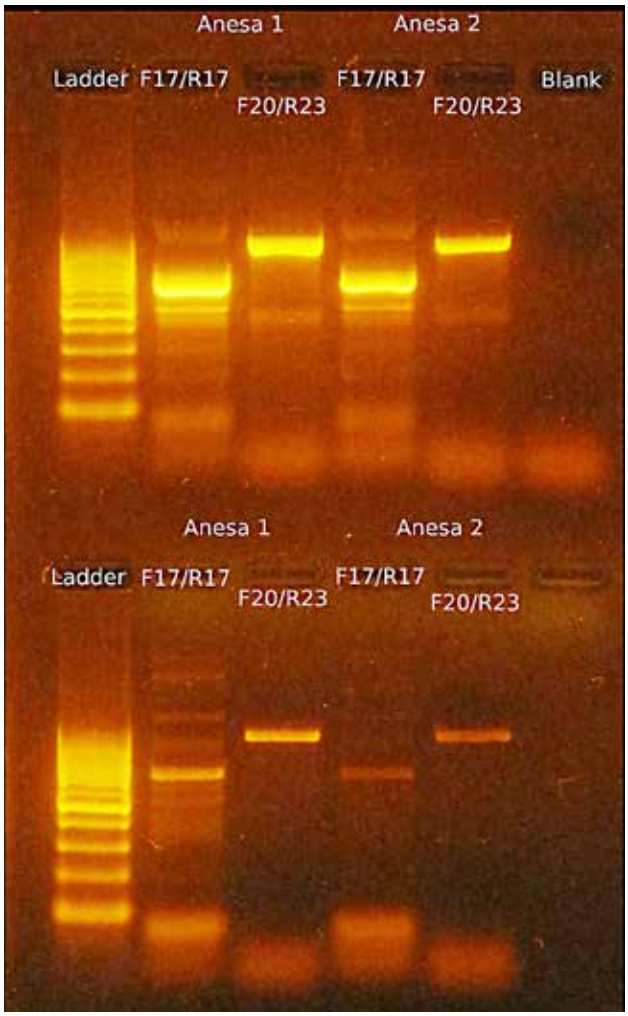

Fig. 5. PCR detection of amplicons in a Shetland sheep dog carrier of CEA
Agarose gel: line 1 ladder 100 bp; lines 3–5 positive PCR reactions with both sets of primers (*NHEJ1*-F17/*NHEJ1*-R 17 and *NHEJ1*-F20/*NHEJ1*-R23) indicate a carrier dog Anesa (636 bp and 941 bp); line 6 – negative control (water instead of DNA). At the bottom of the picture is the PCR result with tenfold diluted DNA

guishing healthy individuals (dominant homozygotes AA) from phenotypically normal carriers of recessive allele (heterozygotes Aa) (Fig. 3; Fig. 4/agarose gel 1) as well as to detect genetically affected animals (aa) (Fig. 4/agarose gel 2). Despite slightly less visible PCR amplicons, the second PCR reaction conditions tested (Reaction conditions 2, Table 1) with increased annealing temperature (from 50 °C to 55 °C) and shortening times of denaturation, annealing and synthesis showed to be sufficient for the detection of resulting fragments on an agarose gel. The results are on Fig. 4 (agarose gel 2), where is visible one amplicon of healthy Border collie and four amplicons of longhaired Collie family members (mother, father and two daughters) that were affected by CEA disease.

The result of the PCR testing of a CEA carrier dog (Shetland sheepdog) is in Fig. 5.

DISCUSSION

Although the genetic test for CEA is commercially available nowadays (e. g., Laboklin s. r. o.), we attempted to test the PCR reaction conditions for CEA detection using two sets of *NHEJ1* primers described in the scientific literature. Both PCR reaction conditions tested showed to be suitable for the visualisation of specific PCR amplicons (indicated the presence or absence of *NHEJ1* gene mutation – deletion) on an agarose gel (Table 1, Fig. 2, Fig. 4, Fig. 5). Similar results were obtained by D o s t á l et al. [6] using the same primers as in our study, but more extreme temperature of denaturation (98 °C), very short times and different chemicals. The authors did not isolate DNA but used a direct PCR amplification using the 20-times diluted unclotted blood samples. On the contrary, in our study, we used DNA isolated from the blood and buccal cells; we slightly modified mainly isolation method from buccal cells that are a good and non-invasive source of DNA. In addition, saliva specimens can serve for DNA analysis using the PCR, especially the real-time PCR with a melting curve analysis, as shown by C h a n g et al. [9] and confirmed by M i z u k a m i et al. [11] in the case study in Hokkaido dogs affected by CEA.

As indicated by G o u g h et al. [7], Collie Eye Anomaly is best diagnosed at 6–7 weeks. Similarly, C r i s p i n and M e l l e r s h [5] emphasised that CEA is a congenital condition, which can be diagnosed as soon as eye examination is possible (at five to six weeks of age), and it is diagnosed clinically with greatest accuracy in such young dogs. On the contrary, a genetically affected dog may be clinically unaffected at the time it has an eye examination, especially if it is very young [8].

According to G r o s å s et al. [8] who examined choroidal hypoplasia in 103 Norwegian Border Collie puppies in the age from 5 to 8 weeks, choroidal hypoplasia/chorioretinal dysplasia (CH/CRD) is consistently visible on ophtalmological examination only until the age of 10 weeks. Later development of the tapetal area may mask the lesions resulting in so-called “go-normal” phenomena [2]. For this reason, the authors recommend the clinical eye examination prior to the age of 10 weeks [2, 8]. In gen-

eral, it is recommended to perform a preventive clinical examination of the eyes before placing the dog in the breed [15]. To eliminate CEA from a breed, complex examination such as litter screening combined with DNA testing is proposed as the best approach [3, 5].

There is currently much debate about the type of inheritance in relation to CEA. The polygenic type of inheritance or single-gene coded (autosomal recessive) inheritance with incomplete penetrance was supposed for this disorder [12]. Moreover, ophthalmic phenotypes such as choroidal hypoplasia and coloboma are currently thought to be inherited separately. The collection of optic nerve head (ONH) coloboma affected Nova Scotia Duck Tolling Retrievers showed lack of concordance of the *NHEJ1* intronic deletion with ONH coloboma. Due to the complex genetic aetiology of ONH coloboma, the *NHEJ1* intronic deletion test results should be carefully considered when making breeding decisions [3].

Dog genetics is an interesting and very useful area of research. Some canine diseases are clinically similar to human diseases with the same gene involved in dog and humans [4]. Mainly, for this reason, a biological resource centre of canine samples Cani-DNA CRB (http://dog-genetics.genouest.org) now serves as a biobank of blood and tissue samples from affected and unaffected dogs for genetic studies.

CONCLUSIONS

Both PCR reaction conditions tested were suitable for detecting canine *NHEJ1* gene mutation associated with CEA using two different sets of primers in each individual. One carrier was observed in a small group of eleven randomly selected healthy dogs. Moreover, the PCR test confirmed the previous CEA-positive ophthalmological examination in Collie breeding. This test indicated that all four family members (mother, father and two daughters) of the examined longhaired Collies were genetically affected by homozygous intronic deletion of 7799 bases in the canine *NHEJ1* gene. The two young female Collies (daughters) may potentially transmit this CEA-associated mutation to the next generation of offspring. For this reason, it is advisable to remove affected dogs from a breeding programme in most cases.

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