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THE SIGNIFICANCE OF MONITORING SERUM AND FAECAL CALPROTECTIN IN HEALTHY DOGS

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ABSTRACT

The aim of this study was to monitor the concentrations of serum and faecal calprotectin in healthy dogs, determine confidence intervals, and observe its dependence on factors such as age. This study included 70 dogs representing 16 breeds, including samples collected from small and medium sized mixed breeds. All dogs were fed dry kibble. The concentrations of calprotectin in serum and faeces were measured using the Quantitative Sandwich ELISA kit from MyBioSource, specifically designed for canine samples. Faecal sample preparation followed the manufacturer's instructions for the specific canine ELISA test, and the obtained concentrations of serum and faecal calprotectin were divided into groups in relation to age. The first group consisted of individuals up to one year of age and one-year-old dogs. The following groups consisted of dogs of age 2–4 years, 5–7 years, 8–9 years, and 10–12 years. Monitoring the faecal calprotectin in healthy dogs showed a range of values from 2.63 to 28.81 ng.ml^{-1}, while serum calprotectin concentrations ranged from 3.23 to 13.51 ng.ml^{-1}. The statistical analysis confirmed that calprotectin levels in faeces and serum did not differ significantly across different ages. However, when the monitoring calprotectin concentrations in serum and faeces of healthy dogs was based on weight, the dogs were divided into groups according to size. The group with large dog breeds consisted of individuals over 20 kg (n = 15), the group with medium-sized dogs included individuals weighing between 12 kg and 20 kg (n = 16), and the largest group consisted of small dog breeds under 12 kg (n = 39). Statistical analysis confirmed a significant difference when comparing faecal calprotectin levels based on the size of dogs, but the serum calprotectin concentrations did not differ significantly with respect to dog size. Based on these calculations, confidence intervals for faecal calprotectin and serum calprotectin in healthy dogs ranged from 10.96 to 15.14 ng.ml^{-1} and 7.96 to 8.49 ng.ml^{-1}, respectively. Since this is the first study on calprotectin in serum and faeces of healthy dogs using the mentioned ELISA test, further studies on larger numbers of dogs will be needed in the future.

Key words: calprotectin; chronic inflammatory enteropathy; dog

INTRODUCTION

Chronic inflammatory diseases of the gastrointestinal tract constitute 10–20 % of all conditions seen in clinical practice, with their incidence on the rise [8, 33]. Chronic inflammatory enteropathies (CIE) in dogs and cats represent a diverse group of gastrointestinal disorders characterized by clinical signs lasting at least three weeks, following the exclusion of other intestinal conditions such as parasitism, intestinal obstructions or neoplasia [2, 21].

The most common clinical signs of CIE include diarrhoea, vomiting, stomach rumbling, bloating, weight alterations, anorexia and abdominal pain [8, 40]. However, these symptoms are frequently observed in extra-gastrointestinal conditions such as hepatic disease, hypoadrenocorticism, renal insufficiency, acute or chronic pancreatitis, exocrine pancreatic insufficiency (EPI), and, in cats, hyperthyroidism. Thus, diagnosing chronic gastrointestinal conditions necessitates an exclusionary approach, requiring a thorough diagnostic workup to eliminate all known causes of extra-gastrointestinal inflammation [2].

Chronic inflammatory bowel diseases (IBD) are also well-known as chronic idiopathic multifactorial conditions in humans, with Crohn's disease and ulcerative colitis being the most prevalent [6].

Dogs can also exhibit symptoms of inflammatory bowel disease (IBD), which is classified under chronic enteropathies when clinical signs are clearly defined and histological findings from endoscopic examination reveal characteristic infiltrations in the intestinal wall [6, 34]. When describing the histological changes, significant emphasis is placed on the type and severity of cellular infiltration in the lamina propria of the intestine, where various types of cell populations can be found, such as lymphocytic-plasmacytic, eosinophilic, or granulomatous infiltrations. The interpretation of the biopsies contributes to the assessment of the severity and distribution of the disease [2, 25].

The severity of IBD as well as CIE can clinically be assessed by using a scoring system based on the symptoms presented, such as the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) and the Canine Chronic Enteropathy Clinical Activity Index (CCECAI) [1, 26]. The first index evaluates six parameters, including activity level, appetite, vomiting, stool consistency, stool frequency, and weight loss. Each variable is rated from 0 (normal) to 3 (severe change). Based on the cumulative score, the disease is classified as mild (0–3), moderate (4–5), intermediate (6–8), or severe (≥ 9) [26]. The above-mentioned clinical signs are also considered in the CCECAI, along with albumin concentration, the presence of ascites, peripheral oedema, and pruritus. Using a similar scoring pattern, CCECAI diseases are classified as insignificant (0–3), mild (4–5), intermediate (6–8), severe (9–11), and very severe (≥ 12) [1]. These scoring systems are largely based on the subjective evaluation of the patient, which may not always reflect the degree of intestinal inflammation or the severity of histological lesions [19]. Therefore, most veterinarians rely on the dog's response to treatment in cases of CIE [15].

Given the fact that most dogs do not require immediate immunosuppressive therapy, veterinarians often prefer to use a therapeutic trial for treatment. Based on the response to therapy, CIEs are classified as food-responsive enteropathies (FRE), antibiotic-responsive enteropathies (ARE), or those that respond only to immunosuppressive drugs (IRE). IRE can also be referred to as steroid-responsive enteropathy (SRE), in which the CCECAI activity index is higher than 12, indicating the most severe clinical symptoms which often represents a form of IBD [7, 21]. Histological examinations are required to confirm the most severe form of CIE, and such examinations have shown that changes in mucosal architecture better reflect the clinical severity of the disease (i.e., epithelial lesions, villus morphology, lymphatic dilation, fibrosis, and crypt changes) rather than the number of infiltrating lymphocytes and plasma cells in the intestinal wall [4].

However, many authors stated that histological and endoscopic diagnostic procedures are invasive for the patient and relatively expensive [9]. Due to this, it is more appropriate to use adequate biomarkers for diagnosing CIE, which could reflect the degree of intestinal mucosal damage through serum or stool examination [5, 10]. For a biomarker to be clinically useful, it must effectively assist in evaluating intestinal function and assessing the risk of disease development [36].

One example of such a biomarker is calprotectin. Calprotectin is a complex of the S100A8/A9 proteins and is a member of the S100/calgranulin family of damage-associ-

ated molecular pattern (DAMP) molecules that accumulate in the areas of inflammation. Calprotectin is expressed and released by activated macrophages and neutrophils, and its expression can also be induced in epithelial cells [21].

Serum calprotectin concentrations are elevated in dogs with CIE, however, this increase is not specific to the gastrointestinal tract [18]. Calprotectin is a stable protein complex that can be extracted and measured in faecal samples. Faecal calprotectin concentrations have been assessed in dogs with chronic gastrointestinal inflammation, demonstrating their utility as a biomarker for intestinal inaflammation in dogs. Additionally, faecal calprotectin concentrations serve as a reliable surrogate marker for disease severity in dogs with CIE [20, 34]. Although faecal calprotectin has been shown to reflect an overall gastrointestinal health, its concentration may also be elevated in acute gastrointestinal inflammatory conditions. Hence, the patient population for faecal calprotectin testing must be carefully selected to gain relevant information from this marker [15].

In order for calprotectin to be used in the diagnosis of digestive disorders, it is essential to monitor its concentrations in both serum and faeces of healthy dogs.

The aim of this study was to monitor the concentrations of serum and faecal calprotectin in healthy dogs, develop confidence intervals, and observe its dependence on factors such as age and breed size.

MATERIALS AND METHODS

In 2022 and 2023, blood and faecal samples were collected from healthy dogs (n = 70) that visited the Small Animal Clinic at the University of Veterinary Medicine and Pharmacy in Košice for preventive examinations or preoperative evaluations prior to castration. In order for the dog to be included as a healthy individual into this study, the dogs had to be clinically healthy and not be receiving any medications. Additionally, owner consent was obtained for each dog before their participation in this study.

In this group of healthy dogs, a single blood draw was performed during the clinical examination in order to obtain a serum sample. Moreover, faecal samples were obtained from natural defecation. The haematological and biochemical tests showed no elevated values for any of the laboratory parameters. Animals in this study were carefully selected to meet the breed diversity criteria as well as age diversity. All dogs were fed dry kibble, which was a set criterion for the selection of healthy dogs taking part in this study.

Various dog breeds were included in this study, and the most represented breeds were Yorkshire Terrier (n = 12), German Shepherd (n = 7) and Maltese (n = 6). The following breeds were: Dachshund (n = 4), Poodle (n = 4), English Cocker Spaniel (n = 4), and Staffordshire Terrier (n = 4), Hungarian Vizsla (n = 3), Chihuahua (n = 3), Miniature Pinscher (n = 2), Belgian Shepherd (n = 2), Boxer (n = 2), and West Highland White Terrier (WHWT) (n = 2). The study also included mixed breeds up to 12 kg (n = 4) and medium mix-breeds up to 20 kg (n = 8). Dog breeds represented by only one individual included: Golden Retriever, Shih Tzu, and French Bulldog. The youngest individual was a 6-month-old Golden Retriever, and the oldest one was a 12-year-old small mixed breed dog. When monitoring the concentrations of serum and faecal calprotectin based on the age of the dog, the individuals were divided into groups. The first group consisted of individuals up to one year of age and one-year-old dogs. The folllwing groups consisted of 2 to 4 year-old dogs, 5 to 7 year-old dogs, 8 to 9 year-old dogs, and the final group consisted of individualas aged 10–12 years. When monitoring the concentrations of serum and faecal calprotectin based on size, the individuals were divided by weight into a large dog breed group, which included dogs over 20 kg (n =15), a medium-sized dog breed group, which included dogs weighing between 12 and 20 kg (n = 16), and the largest group consisted of small breed dogs under 12 kg (n = 39).

To determine calprotectin concentrations in serum and faeces, the Quantitative Sandwich ELISA kit from MyBioSource, designed specifically for canine samples, was used. The resulting concentrations were expressed in $ng.ml^{-1}$ units. Faecal sample preparation followed the manufacturer's recommendations for the specific canine ELISA test. Furthermore, the owners consented to the measurement of calprotectin during routine check-ups or preoperative examinations of their dogs.

Statistical analysis

Statistical evaluation of the obtained results of calprotectin concentrations in healthy dogs was performed using a one-way analysis of variance. The results were assessed at a significance level of 0.05.

Ethical considerations

The study was approved by the Ethics committee for the approval of research involving animals in accordance with the legislative requirements applicable at the UVMP in Košice, permit No. EKVP/22-17.

RESULTS

The monitoring of calprotectin in faeces of healthy dogs indicated a range of values from 2.63 ng.ml^{-1} to 28.81 ng.ml^{-1}. The concentration of calprotectin in serum of healthy dogs ranged from 3.23 ng.ml^{-1} to 13.51 ng.ml^{-1}.

The largest group of dogs observed were small breeds (n = 39), where the concentration of calprotectin in faeces ranged from 2.63 ng.ml^{-1} to 18.29 ng.ml^{-1}. The average faecal calprotectin level for healthy small dog breeds was 10.06 ng.ml^{-1}. For medium-sized dog breeds (n = 16), the range of faecal calprotectin was from 3.98 ng.ml^{-1} to 24.67 ng.ml^{-1}, with the average concentration being 12.96 ng.ml^{-1}. The widest range of calprotectin concentrations in healthy dogs was observed in large dog breeds (n = 15), ranging from 2.78 ng.ml^{-1} to 28.81 ng.ml^{-1}, with the average concentration being 20.9 ng.ml^{-1} (Fig. 1).

A one-way analysis of the variance of faecal calprotectin concentrations of small, medium and large breeds in healthy dogs confirmed their statistically significant difference, as the P-value = 2.86.10^{-6} was lower than the chosen significance level of 0.05.

When monitoring the concentrations of calprotectin in the serum of healthy dogs, the widest range of values was observed in small breeds, ranging from 3.23 ng.ml^{-1} to 13.51 ng.ml^{-1}, with an average value of 7.84 ng.ml^{-1}. Furthermore, in medium and large dog breeds, the serum calprotectin values were similar and ranged from 4.29 ng.ml^{-1} to 11.32 ng.ml^{-1} and from 4.49 ng.ml^{-1} to 11.37 ng.ml^{-1}, respectively. The average serum calprotectin concentration in medium-sized dogs, with a value of 7.75 ng.ml^{-1}, was very similar to the average concentration in large breed dogs, which was 8.27 ng.ml^{-1}. No statistical significance was found when comparing serum calprotectin values among large, medium, and small dog breeds. The average values of serum and faecal calprotectin in healthy dogs based on breed size are shown in Fig. 1.

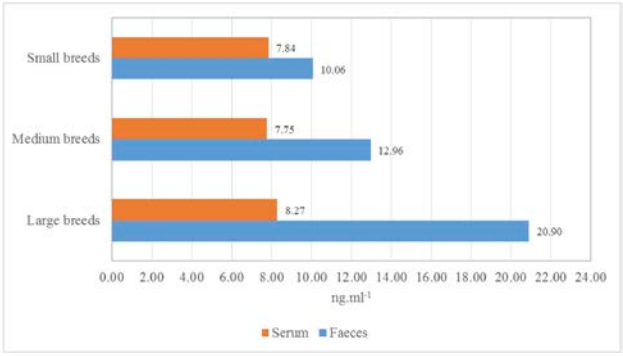

Fig. 1. Average concentrations of serum and faecal calprotectin in healthy dogs according to breed size

Further monitoring of calprotectin levels in healthy dog faeces and serum included tracking their concentrations based on age.

In each age category, dogs of various sizes were included. The youngest dogs in the first group of under one year and one-year-old dogs, formed a group of 8 individuals with the lowest average faecal calprotectin concentration of 4.5 ng.ml^{-1}, compared to the age group 2 to 4 years old, consisting of 20 dogs, where the average faecal calprotectin concentration was much higher at 11.5 ng.ml^{-1}. The 5–7 years age group included 25 dogs, with an average faecal calprotectin concentration of 7.2 ng.ml^{-1}, compared to the 8–9 years age group, consisting of 13 dogs, with an average level of 11.7 ng.ml^{-1}. The highest average faecal calprotectin concentration was observed in the oldest age group, 10–12 years, consisting of 4 individuals, with an average value of 13.2 ng.ml^{-1}.

The monitoring of calprotectin levels in the serum of healthy dogs revealed stable concentrations across the different age groups. In the youngest group, the average serum calprotectin concentration was 8.39 ng.ml^{-1}. In the 2–4 year age group, as well as in the 5–7 and 8–9 years age groups, very similar average values were observed: 7.35 ng.ml^{-1}, 7.95 ng.ml^{-1}, and 8.02 ng.ml^{-1}, respectively. The oldest individuals, aged 10–12 years, had the highest average serum calprotectin concentration at 9.16 ng.ml^{-1}. The average values of serum and faecal calprotectin in healthy dogs based on age are shown in Fig 2.

A one-way analysis of the variance of calprotectin values in faeces and serum of healthy dogs divided into five age groups did not confirm their statistically significant difference when the calculated P-value = 0.09 was greater than the selected significance level of 0.05.

From the presented statistical calculations, confidence intervals for faecal and serum calprotectin values could

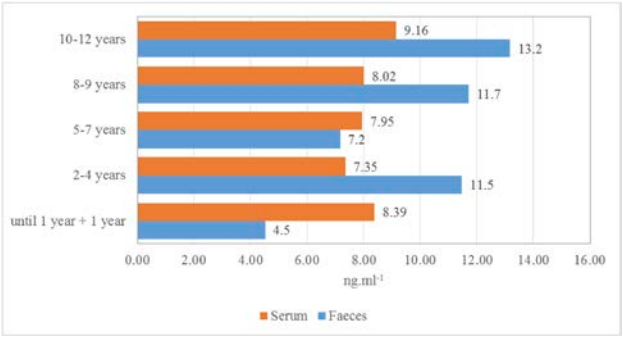

Fig. 2. Average values of serum and faecal calprotectin in healthy dogs in relation to age

also be derived. The confidence intervals for calprotectin in faeces and serum calprotectin levels ranged from 10.96 to 15.14 ng.ml^{-1}, and from 7.96 to 8.49 ng.ml^{-1}, respectively.

The confidence intervals indicate the range within which the calprotectin concentrations of reliably healthy dogs, as measured by the specified ELISA method, are most likely to be found.

DISCUSSION

Chronic enteropathies in clinical veterinary practice have shown a steadily increasing trend over the past decades, which has led to significant scientific attention aimed at understanding their etiopathogenesis, discovering suitable diagnostic parameters, and determining effective therapies for this group of diseases [3, 11, 13]. The diagnosis and monitoring of chronic inflammatory enteropathy (CIE) commonly involve a combination of clinical signs, laboratory findings, and endoscopic and histological results [27, 24]. Laboratory markers represent a group of non-invasive tests that are useful for assessing the inflammatory status of the intestines [17, 37].

The presence of active intestinal inflammation in patients with chronic inflammatory enteropathy (CIE) is associated with the migration of leukocytes into the intestine, which leads to the production of several proteins that can be detected in the serum or faeces. Calprotectin is an abundant protein found in infiltrating leukocytes, primarily granulocytes, but also lymphocytes, at the sites of inflammation, where it is released into the extracellular space as a result of cell degradation. Therefore, it can effectively reflect the intensity of the inflammatory process in the intestines [31, 32]. Due to this, we also chose to monitor this biomarker in healthy dogs to determine its relationship to age and breed size.

There are many publications that studied calprotectin in serum and faeces, describing increased concentrations in dogs affected by chronic enteropathies, but only a few studies focused on calprotectin in healthy dogs [12, 22]. Several authors noted that the levels of many biomarkers in healthy dogs can depend on various factors, such as age, sex, body condition, or external factors like diet [21, 16]. Therefore, to properly evaluate calprotectin levels in sick dogs, it is essential to monitor this parameter in as many healthy dogs as possible, including various breeds and considering their internal and external conditions.

In the study of 564 sick dogs, K a t h r a n i et al. [29] observed that certain dog breeds had a significantly higher risk of developing chronic inflammatory enteropathy (CIE). These breeds include Weimaraner, Rottweiler, German Shepherd, Border Collie, and Boxer. For practical purposes, it would be highly relevant to monitor calprotectin levels especially in healthy dogs of these high-risk breeds. In our study of healthy dogs (n = 70), we included German Shepherds (n = 7), Belgian Shepherds (n = 2), and two Boxers.

G r e l l e t et al. [15] also focused on monitoring of healthy dogs, including those from high-risk breeds. In their study they evaluated faecal calprotectin levels using a comparative control group of 69 healthy dogs. The majority of these were German Shepherds (n = 11), Rottweilers (n = 5), and Golden Retrievers (n = 3), while other breeds of varying sizes were represented in smaller numbers. Given that calprotectin concentrations may differ based on factors such as breed size, age, sex, body condition, and external factors such as diet [21], this group of individuals represented a suitable and valuable sample of the healthy canine population. Obtaining faecal calprotectin concentrations from healthy dogs of specific breeds can be particularly valuable, as some authors indicate a breed predisposition to chronic intestinal diseases in German Shepherds and Rottweilers [29].

In our study, we included the results of monitoring faecal and serum calprotectin levels in 70 healthy dogs of various breeds, focusing on comparing their levels based on age and breed size. Our findings confirmed the highest concentrations of faecal calprotectin in large healthy dogs. An interesting finding from our monitoring was the statistical evaluation of calprotectin concentrations in faeces

among large, medium, and small breed dogs, which were determined to be statistically significant. This statistical comparison confirmed significant differences in faecal calprotectin concentrations based on breed size. However, this was not observed in the serum concentrations, as no significant differences were confirmed depending on breed size. In the future, further studies would be needed to monitor this parameter in faeces from a larger number of healthy dogs of various breeds and to establish the reference ranges. G e f f r é et al. [14] acknowledged that establishing the lower and upper limits of the reference interval requires a large reference sample size (ideally n > 120).

G r e l l e t at al. [16] also suggested that calprotectin concentrations may depend on external factors, such as diet. In our study, all dogs were fed a dry kibble diet, which served as a criterion for selecting healthy dogs for the study. We made an effort to minimize the influence of varying diets on calprotectin concentrations, as diet is a potential factor that could affect the levels found in faeces.

The influence of age is also described in the study performed by G r e l l e t et al. [16], who investigated faecal calprotectin in 19 pregnant bitches from the fourth week of gestation to the eighth week of lactation, along with 30 puppies from nine litters, aged four to nine weeks. This study compared the faecal concentrations to the reference values obtained from 69 healthy dogs in a previous study [15]. Puppies aged four to six weeks had a significantly higher calprotectin levels in their faeces compared to puppies aged seven to nine weeks. The concentrations in the control group of healthy dogs were significantly lower than those in both groups of puppies. These elevated faecal calprotectin levels in young puppies were similar to those found in adult dogs with IBD. However, these high concentrations may be explained by other factors than intestinal inflammation. In this case, maternal milk and increased intestinal permeability in developing puppies could be contributing factors [41]. This study also emphasizes the importance of monitoring calprotectin in relation to different stages of a dog's life [16].

Many authors described faecal calprotectin concentration as a useful biomarker for intestinal inflammation in sick dogs, and therefore, it is essential to monitor the physiological levels of this parameter in healthy dogs, considering various internal and external factors [15, 20, 34].

The calprotectin protein complex (S100A8/A9) belongs to a group of molecules known as alarmins, which are associated with tissue damage and act as damage-associated molecular pattern molecules. These molecules accumulate in areas of inflammation [23] leading to disruption of intestinal permeability [30] and alterations in the composition and function of the gut microbiota [28]. This condition is referred to as dysbiosis [35, 38, 39].

When monitoring faecal and serum calprotectin, careful selection of dogs for testing is essential to obtain relevant information about this biomarker [27].

This study, using the specified canine ELISA test, was one of the first to monitor calprotectin in serum and faeces in 70 healthy dogs. In the future, it will be important to study these parameters in a larger number of healthy dogs, considering various factors, which would allow for the development of reference ranges for both biomarkers.

CONCLUSIONS

Given the rising prevalence of chronic enteropathies in clinical practice among dogs, the importance of monitoring calprotectin in affected individuals is also increasing. However, to accurately assess the severity and type of disease, it is essential to monitor the concentrations of this biomarker in healthy dogs, taking into account various factors such as age and breed. Our study, which involved measuring 70 healthy individuals using the ELISA method, contributed to the evaluation of these significant factors.

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