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Original Article - Veterinary Medicine

Ultrasound and histopathological analysis of the gallbladder in dogs

[*Análise ultrassonográfica e histopatológica da vesícula biliar de cães*]

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ABSTRACT

In Veterinary Medicine, diseases restricted to the gallbladder (GB) are uncommon in dogs. The histopathological examination of GB is considered the gold standard. However, abdominal ultrasound examination is increasingly included in the veterinary clinical routine, as it allows changes in previously underdiagnosed GB to have a more accurate diagnosis. The aim of this study was to identify, quantify and correlate the different aspects of GB in dogs through ultrasound and histopathological examinations. The GB of 60 dog cadavers were evaluated, without predilection for sex, age, and breed. The results demonstrated that the ultrasound examination, when compared to the histopathological examination, presented 83% agreement in the analysis of general data for the presence or absence of GB changes. The level of agreement in the Kappa test was considerably high (K=0.63). There was a high agreement in the diagnosis of changes observed by ultrasound and histopathological examinations, when compared "affected" or "non affected". However, when compared the findings of mucocele, cystic mucinous hyperplasia and cholecystitis, there is a low Kappa agreement. Thus, ultrasound examination is an excellent tool for identifying changes related to the GB. However, more studies must be carried out to deepen the understanding of the correlation between imaging and pathological findings to lead diagnostic conclusions.

Keywords: canine, bile ducts, ultrasound, pathology

RESUMO

Na Medicina Veterinária, doenças restritas à vesícula biliar (VB) são incomuns em cães. O exame histopatológico da VB é considerado padrão-ouro, porém o exame ultrassonográfico abdominal está cada vez mais incluído na rotina clínica veterinária, pois permite que alterações da VB, antes subdiagnosticadas, tenham um diagnóstico mais preciso. O objetivo deste estudo foi identificar, quantificar e correlacionar os diferentes aspectos da VB em cães, por meio de exames ultrassonográfico e histopatológico. Foram avaliadas VB de 60 cadáveres de cães, sem predileção por sexo, idade e raça. Os resultados demonstraram que o exame ultrassonográfico, quando comparado ao exame histopatológico, apresentou concordância de 83% na análise dos dados gerais para presença ou ausência de alterações na VB. O nível de concordância no teste Kappa foi consideravelmente elevado (K=0,63). Houve alta concordância no diagnóstico das alterações observadas pelos exames ultrassonográfico e histopatológico, quando comparados "afetados" ou "não afetados". Entretanto, quando comparados os achados de mucocele, hiperplasia mucinosa cística e colecistite, observou-se baixa concordância Kappa. Assim, o exame ultrassonográfico é uma excelente ferramenta para identificar ou excluir alterações relacionadas à VB. No entanto, mais estudos devem ser realizados para aprofundar a compreensão da correlação entre exames de imagem e achados patológicos para conclusões diagnósticas.

Palavras-chave: canino, ductos biliares, ultrassom, patologia

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INTRODUCTION

The gallbladder (GB) is an organ attached to the liver and an important component of the digestive system. The group formed by the liver, organic ducts and the GB is called bile ducts. (Gomes *et al*., 2018). The GB connects with the liver through these organic ducts, which drain and transport bile. GB is responsible for reserving and concentrating bile produced by the liver, which is later released into the small intestine (Prolo *et al*., 2017). Absorption of terminal fat products through the intestinal mucosa and contribution to the excretion of other substances are highlighted functions, such as bilirubin and excess cholesterol, are some of the highlighted functions of the bile (Guyton *et al*., 2006).

The main risk factors related to bile duct disorders reported in human medicine include age, sex, level of physical activity, diet, lifestyle, and rapid weight loss (Prolo *et al*., 2017). Bile tract diseases result in high morbidity and mortality rates, which can range from 10% to 60%. However, recent improvements in the diagnosis, treatment, and prognosis of GB conditions have had a considerable impact on the lifespan of affected animals (Claesson *et al* 1984; Loh *et al*., 2019).

Historically, the incidence of diseases restricted to GB is low on veterinary medicine. However, the main conditions in dogs include cholecystitis, mucocele, cholelithiasis and neoplasms (Chebli *et al*., 2000). GB conditions mainly affect elderly animals and endocrinopathies. Gallbladder disorders in elderly animals and patients with endocrinopathies may be related to natural changes of the aging body, endocrine disorders that affect digestive processes, and changes in the composition and circulation of the bile (Chebli *et al*., 2000; Kilpatrick *et al*., 2017). GB neoplasms are uncommon in dogs (Mayhew *et al*., 2002); however, neoplasms, such as carcinoids, adenomas, carcinomas, polyps, and leiomyomas can be seen in GB in dogs and humans (Cullen and Poop, 2002).

The microscopic examination of the GB is considered the gold standard for the final diagnosis, regarding a complete assessment of the tissue architecture. Contrarily, GB biopsy requires an invasive approach, and includes limitations related to the proper sedation/general anesthesia, and trained personnel for advanced techniques (e.g. laparoscopy) (Roquette *et al*., 2008, Oro *et al*., 2010).

On the other hand, abdominal ultrasound examination is the imaging method of choice for the initial diagnostic approach and for the evaluation of the biliary system in general, as it is widely available, safe, minimally invasive, harmless, and low cost, in addition to be performed in real time perspective (Barbosa *et al*., 2011). Among the main indications for ultrasound examination of the bile ducts, the following stand out: suspicion of extrahepatic obstruction, the search for cholelithiasis and mucocele, and the assessment of the thickness of the GB wall (Nyland *et al*., 2015), in addition to assisting in cholecystocentesis, used to decompress the organ or to obtain bile samples for biochemical and microbiological analyzes (Penninck and D'Anjou, 2011).

The association between ultrasound findings and histopathological data is promising for obtaining a more accurate diagnosis and for elucidating factors that predispose to GB conditions (Secchi, 2011). However, studies carried out in veterinary medicine indicate the need for further efforts (Howes, 2011). Therefore, the aim of this study was to identify, quantify, and correlate the different aspects of GB in dogs through ultrasound and histopathological examinations.

MATERIAL E METHODS

In this study, 60 canine cadavers, without any specific bias towards sex, age, or breed, were assessed. These cadavers were part of the routine caseload at the Veterinary Pathology sector. All included dogs were appropriately refrigerated, with a maximum duration of 72 hours in a cold room maintained at temperatures between 0-2°C. Exclusion criteria encompassed cadavers in a significant state of autolysis, which would impede both macroscopic and microscopic evaluations. Additionally, gallbladder samples that, upon histological examination, did not exhibit an intact epithelial layer were also excluded from the study.

Each animal underwent a thorough and meticulous macroscopic examination, which included an assessment of the liver. Subsequently, the liver and bile ducts were carefully extracted to facilitate the ultrasound procedure. A Mindray M5 ultrasound machine, equipped with a linear transducer ranging from 7.5 to 10 MHz, was employed. The selection of frequency was tailored to the specific requirements of each anatomical specimen. To ensure optimal contact between the transducer and the surface being evaluated, a conductive gel was applied. Measurements to ascertain the thickness of both the ventral and dorsal walls were conducted on the body of the GB (Demonaco *et al*., 2016). In the assessment of bile content, echogenicity was categorized based on ultrasound appearance into three groups: anechoic, indicating an ultrasound image without alterations; mobile echogenic, signifying "bile sludge"; and immovable echogenic, indicative of a gallbladder mucocele. Contents designated as hyperechoic were interpreted as mineralized bile components, encompassing microcholeliths and cholеliths. These classifications facilitated the identification and categorization of changes into biliary sludge, cholelithiasis, and gallbladder mucocele.

A GB mucocele was characterized by the detection of echogenic content located at the center of the bladder lumen, followed by the presence of hyperechoic and immobile radiated striations. This could be observed in conjunction with or independently of thickening of the gallbladder (GB) wall, as described by Besso *et al*. (2005). The presence of a double wall was established by the existence of thickening, coupled with different echogenicities between the layers of the GB wall, as outlined by Penninck and D'Anjou (2011). Thickness values exceeding one millimeter were considered indicative of mural thickening of the GB (Hittmair, 2001).

The macroscopic examination of the gallbladder was conducted after the ultrasound examination, adhering to a systematic protocol. This sequence involved the assessment of intravesical content volume, followed by an evaluation of potential lesions' location and the identification of any lumps and/or cholеliths within the biliary content.

To gather bile contents, the cystic duct was temporarily clamped using a Halstead hemostatic forceps, and carefully detached from the hepatic lobe. Once the GB was removed, the clamping of the cystic duct was released, allowing its contents to be carefully collected into a universal collection bottle. The volume of collected bile was measured using a 10-milliliter (ml) syringe with graduated markings. The clumps or cholеliths were visually examined during the pouring of gallbladder contents into the universal collection bottle. Any small, suspended particles displaying varying shades within the bile content were classified as lumps.

Colleliths were defined by their size, typically larger than two to three millimeters, displaying a slightly rounded shape and a rigid surface (Pelegrini, 2015). Following the thorough emptying of intravesical contents, the GB was carefully sectioned from the cystic duct orifice to the body of the GB. This method enabled the evaluation of macroscopic lesions, including thickening and focal nodulations in its wall. For confirmation of cholеliths, supplementary tests such as physical-chemical analyses of the sample were employed.

For histopathological examination, the sample was immersed in 10% buffered formalin and allowed to fix for a minimum of 48 hours. Following fixation, the gallbladder was sectioned into three segments representing the bottom, body, and neck regions. Subsequently, a specialized veterinary pathologist conducted the evaluation of histopathological slides. The diagnosis of (CMH) was assigned when there was evidence of diffuse hyperplasia of the gallbladder epithelium, accompanied by varying amounts of accumulated mucus, ranging from minimal to moderate.

Cystic dilation of the mucous glands within the gallbladder wall was likewise categorized as cystic mucinous hyperplasia (CMH). The diagnosis of mucocele was assigned when there were random dilations dispersed throughout the gallbladder epithelium, characterized by dilation of the mucinar glands and a higher accumulation of mucus compared to CMH. It's important to note that severity grading for the aforementioned conditions in this study was not undertaken.

The study conducted an analysis to establish the correlation between the data acquired from the histopathological examination and the information gathered during the ultrasound examination. This correlation encompassed factors such as the presence or absence of the "double wall" appearance, characterization of the echogenicity of the biliary content, as well as the presence or absence of CMH and GB mucocele.

To assess the level of agreement between the variables obtained through ultrasound and histopathological examinations, the Kappa agreement test was employed (Silva and Paes, 2012). In addition, we also estimated the degree of agreement between ultrasound and histopathological variables for other analyses. This included assessing correlations between wall thickness and CMH, as well as mucocele, and examining the echogenicity of intravesical content in relation to CMH, mucocele, and biliary sludge. The statistical tests employed for these analyses were the Wilcoxon-Mann-Whitney and Kruskal-Wallis tests, followed by Dunn's multiple comparisons test.

To assess the association between two variables, Spearman's non-parametric correlation coefficient was computed. All statistical analyses were conducted using R software version 3.6.1 (R Core Team, 2019). Isolated alterations observed in only one case were excluded from the analyses. Nonetheless, a descriptive examination of all cases under scrutiny was conducted.

RESULTS E DISCUSSION

A total of 60 canine cadavers, exhibiting no specific bias towards sex, age, or breed, and admitted to the routine Veterinary Pathology sector, were assessed in this study. To derive these findings, a total of 60 dogs were evaluated, comprising 33 males (55%) and 27 females (45%), spanning an age range of 4 months to 10 years. The subsequent results pertain to the Kappa reliability test, which was employed to analyze agreement by juxtaposing the outcomes of both ultrasound and histopathological examinations, both in their collective assessment and on an individual basis.

To assess the overall agreement between test results, Kappa coefficients were initially calculated using the overarching data from the study. For the initial set of data, the presence or absence of abnormalities in both aforementioned examinations was taken into account.

This outcome indicates that 83% of the data exhibited concordance, and the Kappa test demonstrated that the level of agreement at 0.63, which is notably high. Conversely, when examining the conditions individually, the Kappa agreement revealed a lower level of correlation.

In cases of GB mucocele, there was an agreement of 65% with a Kappa value of 0.23. In contrast, for cholecystitis and HMC, the test agreement was 56.67% and the Kappa value obtained was 0.061 (Table 1).

Table 1. Contingency table illustrating the Kappa statistical analysis of ultrasound and histopathological examination findings for both general data and individually obtained data. The relative frequency (%) signifies the level of agreement between the data, with the value in parentheses denoting the corresponding Kappa coefficient. Kappa values near zero suggest negligible agreement, while those close to one suggest nearly perfect agreement

	Ultrasound Examination	Histopathological Examination	Kappa Test
Normal	23	20	83% (0,63)
Mucocele	22	21	65% (0,23)
Hyperplasia and Cholecystitis	15	19	56,57% (0,061)

Indeed, there is a notably high level of agreement in the diagnosis of findings observed through both ultrasound and histopathological examinations when focusing solely on the determination of affected or non-affected cases.

Based on these findings, it can be confidently asserted that ultrasound examination serves as a valuable tool for distinguishing between affected and non-affected animals in terms of changes related to the gallbladder. It effectively identifies the presence or absence of such alterations (Oro *et al*., 2010). In this way, the results suggest that

ultrasound examinations can provide valuable guidance for veterinary clinicians in making informed decisions regarding various clinical and surgical treatments. The ability to accurately identify changes related to the gallbladder can significantly influence the course of treatment for affected animals (Besso *et al*., 2005; Woods *et al*., 2012; Ortiz, 2018). It was evident that when examining individual conditions like mucocele, CMH, and cholecystitis, the agreement as measured by the Kappa test was lower. To achieve a more precise correlation, the Wilcoxon-Mann-Whitney test was conducted. This test compared gallbladder mural thicknesses obtained from ultrasound examinations to the histopathological classification of specimens, distinguishing between those with CMH and GB mucocele, and those without any alterations.

In this assessment, classifications indicative of changes yielded higher values compared to classifications considered normal. The graph depicted in Figure 1 illustrates the associations made between altered vesicles and samples testing positive for mucocele and CMH, as well as normal samples associated with specimens without any discernible alterations.

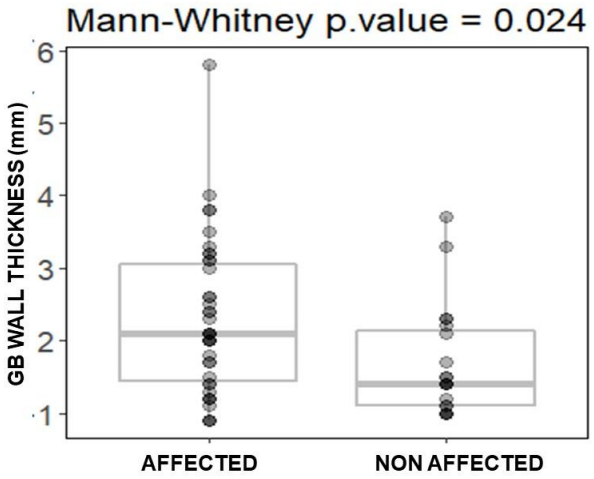

Figure 1. Graph representing the association between GB wall thickness and histopathological and ultrasound classifications of altered specimens (with CMH and GB mucocele) and unaltered specimens. The measured values (points) are presented superimposed on the boxplots for affected and normal vesicles. The value (p) of the tests is found above the graph. A significance level of 0.05 was used to perform the test. The "affected" and "normal" labels represent multiple comparisons (pair-wise test).

While the current study does not establish a definitive correlation between gallbladder wall thickness observed in ultrasound examinations and its histopathological classification, a discernible trend emerged. Animals classified with cystic mucinous hyperplasia (CMH) on histopathological examination tended to exhibit a thicker gallbladder wall compared to those with mucocele. The ultrasound image and histopathological sections pertaining to CMH are presented in Figure 2.

Studies in Medicine have traditionally indicated that GB thickening was strongly indicative of cholecystitis (Barbosa *et al*., 2011). However, in order to account for the observed association between CMH and thickening of the GB wall, it's important to recognize that CMH entails diffuse hyperplasia of the GB epithelium, marked by the presence of varying amounts of accumulated mucus. This accumulation contributes to the observed wall thickening.

This data aligns with the observations made by Cullen (2009), who noted that CMH is an infrequent discovery in ultrasound examinations, likely owing to a lack of familiarity with this condition. Given the data obtained in this study through both histopathology and ultrasound, it is recommended that the potential diagnosis of CMH be contemplated in ultrasound reports, especially in cases of gallbladder wall

thickening. This consideration is crucial, as CMH has not been extensively discussed in Veterinary literature up until now.

Mucocele, on the other hand, entails random dilations dispersed throughout the GB epithelium, accompanied by pronounced dilation of the mucin glands and a substantial accumulation of mucus (Hittmair *et al*., 2001; Cullen, 2009; Gaillot *et al*., 2007). The ultrasound representation of this condition was found to be variable, as elucidated by Besso *et al*. (2005) (Figure 2). Mucocele can be categorized into three stages based on the distribution of its echogenic content. Through this classification, it was observed that in the comparison between histopathological examination and the echogenicity of bile content as indicated by ultrasonographic examination, 25% of mucocele cases exhibited anechoic bile content. Indeed, this observation could potentially explain the instances of underestimated mucocele diagnoses. Additionally, it's crucial to acknowledge that cystic mucinous hyperplasia serves as a precursor to the formation of mucoceles. This understanding further underscores the importance of recognizing and addressing cystic mucinous hyperplasia in clinical assessments (Zwiebel, 1998; Choi *et al*., 2014; Parkanzky *et al*., 2019).

Among the histopathological diagnoses identified in this study, cholecystitis was only observed in a single animal, rendering it an isolated case. Given the limited sample size, data pertaining to this condition could not be statistically correlated with the ultrasound examination, as meaningful statistical analysis cannot be conducted with only one sample case.

During the ultrasound examination, the GB wall presented as notably thick, measuring five millimeters. It exhibited a multi-layered and irregular appearance with varying echogenicities. The internal content appeared anechoic according to Choi *et al*. (2014), this presentation would be classified as acalculous cholecystitis.

La Mont *et al*. (1984) noted that hyperechogenicity of the GB wall can be observed in cases of chronic cholecystopathy. Additionally, Barbosa *et al*. (2011) describe that acute inflammatory processes also often lead to mural thickening. However, it is worth mentioning that other authors have identified nonspecific ultrasound findings in similar cases. (Gomez-Pinilla *et al*., 2007; Gaillot *et al*., 2007). The low incidence of cholecystitis observed in this study aligns with existing literature, where this condition is reported to be rare in dogs (Kahn and Line 2013). Moreover, it's worth noting that most animals examined in this study were either young or adults. Given that cholecystitis tends to be more prevalent in elderly patients, the lower incidence observed in this study is further justified by the age distribution of the sample.

The findings in this study align with the observations made by Torres *et al*. (2018) and Barbosa *et al*. (2011), who assert that there is a notable inclination to link an augmented thickness of the GB wall with cholecystitis. With 30% of the cases analyzed here being consistent with CMH, it is reasonable to conclude that this discovery should be considered as a potential differential diagnosis in instances where dogs are suspected of having cholecystitis.

In the context of cholelithiasis, the biochemical analysis unveiled the presence of bilirubin and iron. As elucidated by Prolo *et al*. (2017), a portion of the iron found in the heme group is transported via transferrin, while the remainder undergoes oxidation into biliverdin through the activity of hemoperoxidase. The biliverdin reductase plays a role in converting biliverdin into bilirubin, which is then released into the bloodstream in its unconjugated or indirect form. Here, it binds to albumin and is transported back to the liver.

In the ultrasound examination, it presented as a solid, well-defined structure measuring 6.1mm x 3.0mm. It displayed an exceedingly irregular surface, surrounded by a hyperechogenic halo, and a hypoechogenic center, however, no posterior acoustic shadow artifacts were observed. Nevertheless, in the abdominal ultrasound examination of dogs, the characterization of choleliths typically relies on identifying a hyperechoic structure with a smooth surface, often resulting in pronounced acoustic shadowing, as determined by experienced ultrasound practitioners (Van Geffen *et al*., 2008; Yildiz *et al*., 2019).

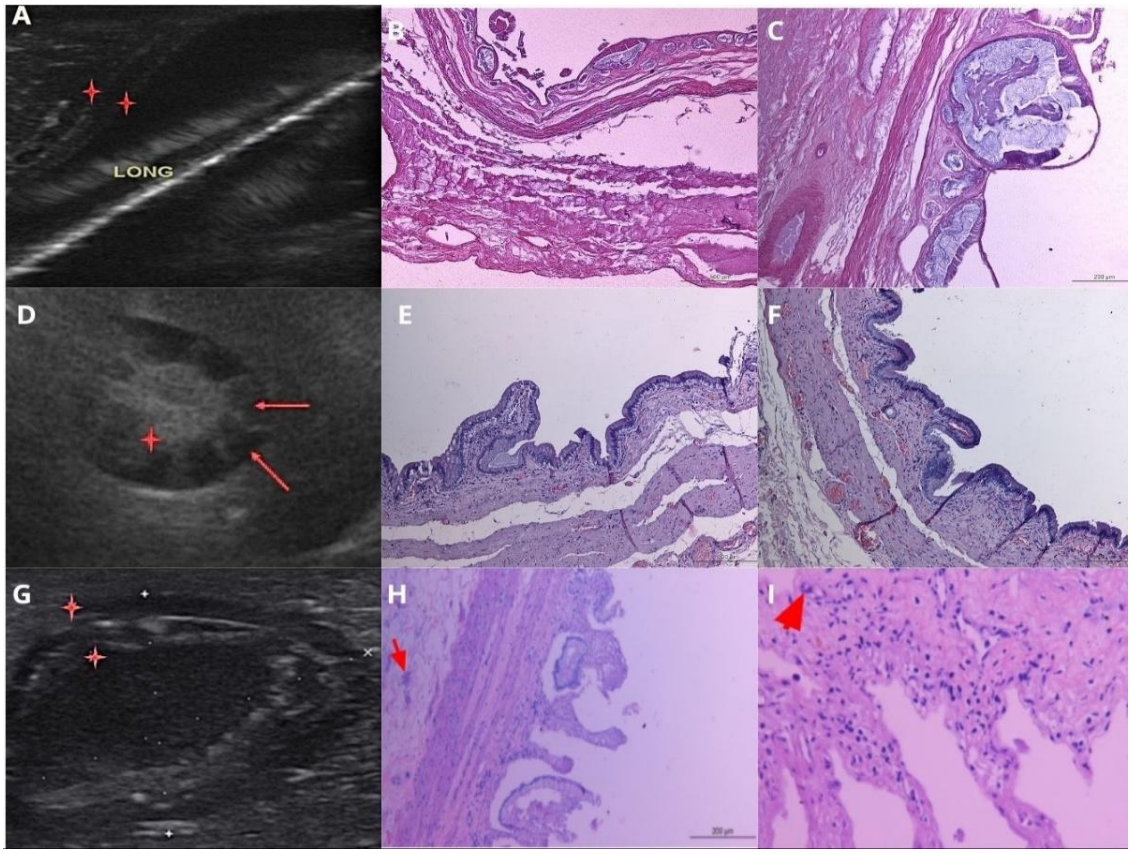

Figure 2. Ultrasound image in a longitudinal plane of anatomical specimen of GB from a dog. (A) Note the GB showing anechoic content, a thickened wall measuring 3.3 millimeters, and a double-wall appearance consistent with edema (red asterisks). (B) Histological section of the GB showing hyperplasia of the mucinar cells in the mucous layer, as well as the presence of multifocal ectasia in the mucinar glands, which are filled with a moderate amount of mucinar content (red arrow). (HE stain, 500µm). (C) It refers to a specific area of the image "b" and and explains that it demonstrates a mucinar gland with a significant amount of intraluminal mucous content (red arrow) (HE staining, 200µm). (D) Transverse plane ultrasound image of a GB anatomicasl specimen from a dog with mucocele. It notes a distended gallbladder, echogenic biliary sludge in the central portion of the lumen (asterisk), presence of a continuous and irregular border along the inner periphery of the GB (red arrows). (E and F) Histological section featuring epithelial projections and accumulation of mucus in close proximity to the GB epithelium (HE staining, 200µm). (G). Transverse plane ultrasound image of a GB anatomical specimen from a dog with cholecystitis. The GB wall is thick, measuring 5mm and exhibits multiple irregular layers and different echogenicities (red asterisks). These features are compatible with severe inflammation and mural edema. (H) A gallbladder specimen showing that the epithelial surface exhibits heightened cellularity, attributed to an inflammatory infiltrate (red arrow) (HE staining, 200µm). (I) A higher magnification view of a previous histological section, emphasizing the presence of an inflammatory infiltrate in the lamina propria (arrowhead) (HE staining, 50µm).

This observation can be explained by the diversity in the presentation of choleliths, which may exhibit varying degrees of echogenicity and posterior acoustic shadowing, as noted by Lee *et al*. (2017). This variability can be attributed to the composition of bile components involved in the formation of choleliths (Yildiz *et al*., 2019). Indeed, from the literature reviewed, there is no explicit indication of a direct association between bilirubin and iron-containing choleliths and the presence or absence of posterior acoustic shadowing. It is important to highlight that gallbladder choleliths may not always generate posterior acoustic shadowing. This variation underscores the complexity and diversity in the characteristics of choleliths, which can be influenced by their composition and individual properties (Yildiz *et al*., 2019). Studies have indicated that as the size and calcium content of stones increase, the likelihood of subsequent acoustic shadowing also rises (Gaillott *et al*., 2007). Additionally, as emphasized by Swartz-

Basile *et al*. (2007), it is crucial to consider the nucleation time as a significant factor in the formation of gallbladder stones.

Another notable observation pertained to the histopathologically confirmed normalcy of the GB wall. This suggests that the particular cholelith in question had not resulted in any discernible mural lesions. This observation can be rationalized by the fact that choleliths in the process of formation within the GB typically do not inflict damage upon the vesicle wall. However, with the introduction of precipitated components in the bile that contribute to cholelith formation, coupled with the stasis of GB, calculous cholecystitis can subsequently manifest (Harvey *et al*., 2007; Kahn and Line, 2013). Consequently, this sequence of events may lead to obstructive posthepatic jaundice, a condition that was not observed in the animal under study.

The occurrence of lithiasis in dogs is generally regarded as infrequent, largely attributed to the lower concentrations of cholesterol and free calcium in their bile contents. In contrast to humans, who have a higher susceptibility to stone formation due to specific dietary components like simple sugars and saturated fats, dogs are less predisposed to this condition (Center, 2009).

It's crucial to emphasize that the elevated incidence of GB disorders identified in this study may not necessarily mirror the broader epidemiological landscape of dogs treated at the Veterinary Hospital. Hepatobiliary disorders, in general, have a low prevalence among dogs (Chebli *et al*., 2000). One plausible explanation for the diagnosis of hyperplasia, mucocele, and cholecystitis being confirmed in 66.6% of the evaluated patients is that the histopathological examination was regarded as the gold standard for diagnosis (Veronese *et al*., 2007). Indeed, this examination facilitates the clarification of changes in the GB that are often underdiagnosed (Center, 2009).

CONCLUSION

Ultrasound examinations can be an excellent tool for identifying or excluding gallbladder abnormalities. However, it's important to note that similarities in imaging findings between conditions like CMH, mucocele, and cholecystitis have revealed that ultrasound may not be the most ideal method for making diagnostic differentiations in these cases.

It is important to consider cystic mucinous hyperplasia as a potential differential diagnosis when observing gallbladder wall thickening on ultrasound. Additionally, one should also consider the possibility of diagnosing cholelithiasis in the presence of echogenic structures, even in the absence of posterior acoustic shadowing artifacts.

More studies must be carried out to deepen the understanding of the correlation between imaging and pathological findings to lead diagnostic conclusion.

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