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Eubacterial Fluorescence In Situ Hybridization and Histologic Features In 25 Dogs with Gallbladder Mucocele

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Gallbladder mucocele (GBM)

Gallbladder (GB)

Cystic mucinous hyperplasia (CMH)

Fluorescence in situ hybridization (FISH)

Cystic mucinous hyperplasia with cholecystitis (CMHC)

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Abstract

Objectives– To detect and localise bacteria in gallbladder mucoceles utilizing fluorescence *in situ* hybridization (FISH). To report clinical signs, clinicopathologic abnormalities, sonographic findings and histopathological findings in FISH+ and FISH- dogs with gallbladder mucoceles.

Materials and Methods – Retrospective review of signalment, clinical signs, clinicopathologic and sonographic findings of 25 cases of histopathologically confirmed gallbladder mucocele. Histopathological sections of GBM were evaluated for cystic mucinous hyperplasia, cystic mucinous hyperplasia with cholecystitis and rupture. The number and spatial distribution of bacteria was determined by eubacterial FISH. Gallbladder contents were cultured in 21 dogs.

Results –Bacteria were detected within or adherent to the gallbladder in eight of 25 (32%) cases Bacterial culture was positive in one dog. Cystic mucinous hyperplasia with concurrent cholecystitis was found in 17/25 (68%) of dogs with gallbladder mucocele.

Clinical significance – FISH was more sensitive for detection of bacteria in gallbladder mucoceles when compared to bacterial culture of bile. Cholecystitis was common in dogs with gallbladder mucocele. Further study is required to elucidate the relationship of cystic mucinous hyperplasia, bacteria and cholecystitis in the aetiopathogenesis and progression of GBM.

Keywords: gallbladder mucocele, bacteria, FISH, cholecystitis

Gallbladder mucocele (GBM) has emerged as a common and clinically important cause of extrahepatic biliary disease in the dog. GBM is most frequently reported in older dogs and

predisposed breeds include the Shetland sheepdog, Pomeranian, miniature schnauzer, cocker spaniel, Chihuahua, and Border terrier (Worley *et al.* 2004, Aguirre *et al.* 2007, Crews *et al.* 2009, Malek *et al.* 2013, Gookin *et al.* 2015, Mitzutani *et al.* 2017, Allerton *et al.* 2018). GBM is characterized by cystic mucinous hyperplasia (CMH) and the accumulation of viscous bile and mucus in the GB (Neer 1992, Besso *et al.* 2000, Pike *et al.* 2004, Worley *et al.* 2004, Aguirre *et al.* 2007, Malek *et al.* 2013). The underlying cause of GBM formation is not well understood (Neer 1992, Besso *et al.* 2000, Pike *et al.* 2004) but may involve the excess secretion of particular gel-forming mucins (Kesimer *et al.* 2015) and dysmotility (Tsukagoshi *et al.* 2012).

Bacterial culture has been reported positive in 3-67% of cases of GBM (Pike *et al.* 2004, Worley *et al.* 2004, Aguirre *et al.* 2007, Mayhew *et al.* 2008, Crews *et al.* 2009, Malek *et al.* 2013, Policelli *et al.* 2017, Mitzutani *et al.* 2017, Policelli Smith *et al.* 2017). This wide variation may reflect the use of perioperative antimicrobial therapy, and differences in sampling and culture based methodologies. The prevalence of bacterial infection in GBM could also be impacted by concurrent comorbidities such as cholecystitis or cholelithiasis, conditions which have reported culture positive rates of 35-50% (Mehler *et al.* 2004, Aguirre *et al.* 2007). Concurrent cholecystitis has been reported in 17-40% of dogs with GBM (Besso *et al.* 2000, Pike *et al.* 2004, Worley *et al.* 2004, Malek *et al.* 2013).

Co-morbidities such as bacterial infection and cholecystitis could impact the progression and outcome of GBM. For example, septic bile peritonitis is significantly associated with mortality in dogs receiving extrahepatic biliary tract surgery for a variety of causes including cholelithiasis, cholecystitis, neoplasia, and trauma (Mehler *et al.* 2004). Mural inflammation, erosion and

ulceration of the GB could compromise the structural integrity of the GB and predispose to rupture. Timely recognition of bacterial infection and cholecystitis could influence the medical management of GBM and reduce perioperative mortality. Further, because cholecystectomy may be postponed in cases of suspected benign GBM, there is a clear need to better understand the relationship of concurrent bacterial infection and cholecystitis to GBM.

Fluorescent in-situ hybridization (FISH) is a culture-independent technique that enables visualization and localisation of intact bacteria in formalin-fixed, paraffin wax-embedded tissues. FISH has been used to document bacteria in a variety of cells and tissues and in some cases has been demonstrated to be more sensitive for detection of bacteria when compared to culture (Simpson *et al.* 2006, Recordati *et al.* 2009, Warren *et al.* 2011, Kornreich *et al.* 2012, Twedt *et al.* 2014). To our knowledge, FISH has not been used previously to detect bacteria in archived GB samples in dogs.

The objectives of this study were to 1) detect, count and localise bacteria in GBMs when evaluated by FISH and 2) report clinical signs, clinicopathologic abnormalities, sonographic findings and histopathological findings in FISH+ and FISH- dogs with GBM.

Materials and Methods

Inclusion Criteria and Case Data Review

Electronic medical records (EMR) at Colorado State University were reviewed for cases of histopathologically confirmed canine GBM between December 2010 and January 2015. Dogs were included if their primary diagnosis was gallbladder mucocele with no significant concurrent

96 extra-hepatobiliary disease noted in the EMR, and a complete blood count, biochemical profile
97 and abdominal ultrasound had been performed within 48 hours prior to cholecystectomy.
98 Retrospective case review included signalment, clinical signs, clinicopathological abnormalities,
99 peri-operative outcome, and bacterial culture of bile and antimicrobial use.
100
101 ***FISH***
102 Formalin-fixed paraffin-embedded histological sections (4 μm) were mounted on Probe-On Plus
103 slides (Fisher Scientific) and evaluated by FISH as previously described (Simpson *et al.* 2006).
104 In short, paraffin-embedded biopsy specimens were de-paraffinized by passage through xylene
105 (3 × 10 mins), 100% alcohol (2 × 5 mins), 95% ethanol (5 mins) and, finally, 70% ethanol (5
106 mins). The slides were air-dried. FISH probes 5'-labeled with either Cy3 or 6-FAM (Integrated
107 DNA Technologies) were reconstituted with sterile water and diluted to a working concentration
108 of 5 ng/μl with a hybridization buffer appropriate to the probe. For evaluation EUB338 Cy-3 was
109 combined with the irrelevant probe non-EUB- 338-FAM (ACTCCTACGGGAGGCAGC) to
110 control for non-specific hybridization. Sections were examined on an Olympus BX51
111 epifluorescence microscope and images captured with an Olympus DP-7 camera (Olympus
112 America). The relative number and spatial orientation of bacteria within the section of
113 gallbladder was also recorded.
114
115 ***Ultrasonographic Data***
116 All ultrasound examinations were performed by a board-certified veterinary radiologist or a
117 veterinary radiology resident under the direct supervision of a board-certified veterinary
118 radiologist. Written reports, still images, and video clips of ultrasonographic examinations were

then retrospectively reviewed by a board-certified veterinary radiologist (EKR) blinded to the case data. The appearance of the gallbladder, gallbladder wall, adjacent abdominal structures, and free peritoneal fluid was evaluated. Sonographic features of GBM were defined as stellate or finely striated bile patterns that differed from biliary sludge by the absence of gravity-dependent bile movement (Besso *et al.* 2000). The gall bladder wall was evaluated for echogenicity, presence of oedema, thickening and rupture. A thickened gallbladder wall was defined as more than 2 mm in dogs (Nyland & Hager 1985). GB wall oedema was defined as a thickened GB wall with a hypoechoic layer within the GB wall.

Histopathological Evaluation

Original histopathologic reports (all by board-certified veterinary pathologists) were reviewed to confirm a diagnosis of GBM. Following this, archival formalin-fixed paraffin-embedded tissue blocks were located for 23/25 cases, sectioned at 4 um and stained with hematoxylin and eosin (HE) for blinded review by a board-certified veterinary pathologist (SLP) employing WSAVA criteria for CMH and cholecystitis (Rothuizen 2006). Cholecystitis was defined as the presence of a neutrophilic and/or lymphoplasmacytic infiltrate in the epithelium or wall of the gallbladder +/- fibrosis (Rothuizen 2006) and assigned a grade of mild, moderate or severe. Each case was assigned to one of 4 groups: CMH, CMH with cholecystitis (CMHC), mild, moderate or severe.

Statistical Analysis

Descriptive statistics were calculated for the presence/absence of clinical signs, clinicopathological data, sonographic findings, and histologic findings in FISH+ *versus* FISH- dogs with GBM.

Results

Twenty-six dogs with a histopathological diagnosis of gallbladder mucocele were identified. One dog was excluded due to concurrent hemolytic anemia and so 25 cases were included. Tissue blocks for 23 of 25 cases were available for blinded histopathological review.

Patient Demographics, Clinical and Clinicopathologic Characteristics and Outcome

The median age was 11 (n=25; range, 6-14), with a near even distribution between castrated male 12 (48%) and spayed females 13 (52%). Breeds included mixed (n=10), Shetland sheepdog (n=3), miniature schnauzer (n=2), Pomeranian (n=2), and one each of the following: Australian shepherd, Bernese mountain dog, cocker spaniel, Labrador retriever, Maltese, miniature dachshund, miniature poodle and Yorkshire Terrier. Due to the retrospective nature of this study it was not possible to fully determine the presence or absence of potential medical conditions predisposing to mucocele in every case (Mesich *et al.* 2009, Kutsani *et al.* 2014, Gookin *et al.* 2015). Three mixed breed dogs had a previous diagnosis of hyperadrenocorticism.

Clinical signs were present in 18 of 25 (72%) dogs with GBM (Table 1). Change in appetite (*i.e.* hyporexia or anorexia) was most common, 12/25 (48%). Other clinical signs included: vomiting (11/25;44%), lethargy (9/25;36%), diarrhea (6/25;24%), abdominal pain (4/25;16%), jaundice (3/25;12%), polyuria/polydipsia (3/25;12%), fever (2/25;8%), and abdominal distension (1/25;4%).

There were clinicopathological abnormalities in all 25 dogs. Neutrophilia (12/25; 48%) was the most common hematological abnormality. Biochemical abnormalities were present in every dog, with elevated alkaline phosphatase (ALP) activity (22/25; 88%) the most common (Tables 1).

Aerobic and anaerobic culture of bile was performed in 21/25 (84%) cases. Culture was positive in 1/21 dogs, yielding *Escherichia coli* in a dog with "CMHC moderate" and clinical findings of vomiting, neutrophilia with left shift, thrombocytosis, and elevated ALP. Review of medical records revealed that all dogs received perioperative antibiotics: cefazolin (four of 25; 16%), cefoxitin (15 of 25; 60%), and ampicillin-sulbactam (six of 25; 24%). The dog with *E.coli* detected from bile culture was receiving cefoxitin.

Perioperative death occurred in three of 25 (12%) cases. Necropsies were not performed. Clinical signs in these dogs included vomiting alone in one dog, inappetence alone in one dog, and jaundice, vomiting, diarrhoea, lethargy and inappetence in the third dog. One dog had a neutrophilia and another had band neutrophilia with a normal neutrophil count. Two of three dogs were hyperbilirubinemic and hypoalbuminemic. Two of three dogs that died in the peri-operative period had cholecystitis and were FISH+ but the cause of death was not determined. The remaining dog suffered respiratory arrest postoperatively and pulmonary thromboembolism was suspected, but not confirmed.

FISH analysis of GB mucosa

Bacteria that hybridized to the eubacterial FISH probe were detected in eight of 25 (32%) cases. Bacteria were noted adherent to the GB epithelium and/or invasive within the GB mucosa in all

dogs, some dogs also had bacteria visualized within the mucus. Three dogs had less than 10 bacteria visualized; the remainder of the dogs had bacteria visualized as dense clusters or masses (Table 2; Figure 1). FISH analysis of the dog with *E.coli* cultured in the bile revealed masses of bacteria within luminal mucus and adhering to the GB wall (Figure 1, D).

Sonographic Findings

The sonographic appearance of the GB was consistent with mucocele (Besso *et al.* 2000) in 24 of 25 (96%) cases. The dog lacking sonographic features of GBM was presented for vomiting and sonography revealed a thickened GB wall with peritoneal effusion so abdominal exploratory was performed. Seven of 25 (28%) dogs had an abnormal GB wall (hyperechoic [four/25; 16%], thickened [three of 25;12%], edema[four of 25;16%]). Nine of 25 (36%) had peritoneal effusion detected on abdominal ultrasound. One of these dogs had a moderate amount of effusion found diffusely throughout the abdomen; the other eight dogs had trace effusion reported. GB rupture was suspected based on the original ultrasound in two cases. Ultrasound correctly identified GB rupture in one of the two cases. The dog that was incorrectly suspected of rupture presented for lethargy and hyporexia. This dog had hyperbilirubinemia, marked elevations in ALP and ALT, neutrophilia and band neutrophilia, and sonographic evidence of GBM with a thickened, hyperechoic, and oedematous GB wall, and a moderate peritoneal effusion (fluid cytology not performed). Surgical exploration found an intact GB. Histopathological diagnosis was CMHC (moderate), and no bacteria were evident on culture or FISH.

In five of 25 (20%) dogs, cholecystitis was listed as suspected in the ultrasound report based on abdominal ultrasound findings of GB wall abnormality, peritoneal effusion, and/or cystic bile duct. All five of those dogs had histopathological evidence of cholecystitis. .

Histopathological Findings

The original and blinded (23 of 25 cases) histopathological examinations indicated a diagnosis of CMH in all cases. The blinded examination documented CMH alone in eight of 25(32%) cases and CMH with concurrent cholecystitis (CMHC) in 17 of 25(68%). Cholecystitis was classified as mild in eight of 17(47%), moderate in seven of 17(41%), and severe in two of 17(12%) (Figures 2 and 3). In three cases, necrosis of the GB wall was also noted along with cholecystitis (two moderate, one severe). Seven of eight (88%) FISH+ dogs had CMH with concurrent cholecystitis; one dog had CMH alone. Cholecystitis was classified as mild in three FISH+ dogs, as moderate in two FISH+ dogs, and as severe in two FISH+ dogs (Table 1). Rupture of the GB was not apparent histologically in any of the cases. However, rupture of the GB was documented at surgery in two of 25(8%) dogs, both with CMHC.

Discussion

In this study we evaluated the utility of FISH to demonstrate bacteria in the gallbladder of a group of dogs with GBM. In these dogs, FISH was more sensitive for the detection of bacteria (eight of 25; 32%) than aerobic and anaerobic culture, which was positive in only one of 21 cases.

The importance of the bacteria identified in GBM by FISH is unclear. The accepted standard for diagnosis of bacterial infection in the biliary system is aerobic and anaerobic culture and sensitivity (Neer 1992). Further, this would ideally be correlated with cytologic results in order to attempt to determine whether the bacteria may be transient, iatrogenic contamination, or true biliary infection as healthy dogs have been shown to periodically harbor bacteria in the bile with no obvious clinical relevance (Kook *et al.* 2010). In humans with cholelithiasis and/or chronic cholecystitis there is also a wide variation in the reported rates of bacterial infection (0 to 73%) and controversy over the significance of the results (Lemos *et al.* 2010). FISH alone is unable to definitively prove infection *versus* transient bacteria *versus* iatrogenic contamination. However, in all of our cases bacteria were visualized adjacent to the GB wall or within the GB parenchyma, which would suggest pathogenic behaviour of the bacteria.

In attempting to determine the significance of the bacteria seen by FISH, it is important to consider possible reasons for the discordancy between FISH and bacterial culture results. All dogs in the study were administered perioperative antibiotics, but it is unclear whether antibiotic administration would have influenced culture results. In a report of dogs undergoing cystotomy for urolithiasis the use of perioperative antibiotics did not change culture results when compared to antimicrobial administration following surgery (Buote *et al.* 2012). However, the dogs in our study did not all receive the same antibiotic and there may be a differential effect of antimicrobials on recovery of cultured bacteria. Additionally, it is possible some of the bacteria seen with FISH were not cultivable with routine culture methodologies. Also, the varied method of collection of GB contents/bile may affect the ability to consistently identify bacteria. During the time period of this study, our hospital generally submitted microbiology swabs of GB

contents following cholecystectomy for aerobic and anaerobic bacterial culture. Although there is no concrete evidence to suggest that swabs placed in transport media is inferior to a direct culture of bile or culture of GB tissue, it is possible this could have contributed to the discrepancy between bacterial culture results and the positive identification of bacteria using FISH in this group of dogs.

Generally, identification of concurrent bacterial infection of the bile, GB mucosa or liver in cases of GBM is challenging. Ultrasound-guided percutaneous cholecystocentesis is a common and typically safe procedure for the collection of bile for the purposes of cytologic evaluation and culture (Uno *et al.* 2009, Peters *et al.* 2016, Schiborra *et al.* 2017). However, biliary mucocele is considered by many to be a contraindication to cholecystocentesis as the potential for GB necrosis secondary to GBM makes rupture of the biliary tract possible (Kook *et al.* 2010). A recent publication described 201 dogs that had percutaneous cholecystocentesis performed, six of which had GBM. Two of these dogs had complications from cholecystocentesis, one of which died from bile peritonitis (Schiborra *et al.* 2017). Aspirate of a GBM for collection of bile preoperatively is generally discouraged. Based on the results of this study, FISH could be considered as a complimentary diagnostic tool as it may be more sensitive than bacterial culture of bile in some instances, and has the added benefit of demonstrating the organism within the tissue. If a high suspicion of bacterial infection exists and bacterial culture is negative, FISH could be performed and while awaiting results an appropriate empirical antimicrobial could be administered to the dog. While FISH is unable to give information on antimicrobial susceptibility, the use of specialized probes may enable the identification of the bacterial species to help ensure appropriate choice of antimicrobial in regards to spectrum and penetration of

277 tissue. It remains unclear whether the bacteria seen are pathogenic and potentially contributing to
278 the aetiopathogenesis or progression of GBM, or whether they are transient or of little clinical
279 relevance. However, the number of GBM cases with bacteria visualized by FISH is of interest.
280 Prospective studies utilizing bacterial culture, cytology, and FISH are needed to further evaluate
281 the relationship between GBM and bacteria.
282
283 In our cohort of 25 dogs with GBM, we found that only 32% had histological findings restricted
284 to CMH. Concurrent cholecystitis was a common (17 of 25; 68%) co-morbidity and ranged from
285 mild in seven of 17 (41%) to moderate-severe in nine of 17 (53%) cases. This is higher than
286 previous reports of concurrent cholecystitis in 17 to 40% of dogs with GBM (Besso *et al.* 2000,
287 Pike *et al.* 2004, Worley *et al.* 2004, Malek *et al.* 2013). In theory, cholecystitis in GBM may be
288 a consequence of inadequate GB emptying and subsequent ischemic or pressure necrosis of the
289 GB wall. However, only three of 17 dogs with CMHC in the present study had evidence of GB
290 wall necrosis. This suggests that factors other than wall necrosis, including bile stasis, infarction
291 or bacterial infection (ascending or enterohepatic) may be involved (Aguirre 2010). We found
292 that clinical signs and clinicopathological findings were broadly similar in dogs with CMH and
293 CMHC; however a higher percentage of dogs with CMHC were hyperbilirubinemic versus dogs
294 with CMH alone. Thus the presence of hyperbilirubinemia may alert the clinician to the presence
295 of cholecystitis in an otherwise benign appearing GBM. Furthermore, chronic cholecystitis is a
296 condition that can result in pain, anorexia, vomiting, and weight loss, and the diagnosis may not
297 always be obvious, especially in patients with other concurrent hepatobiliary disease (Aguirre
298 2010). Thus, the high proportion of cholecystitis among the dogs in our study is noteworthy, and

299 may support the recommendation for early cholecystectomy, even in dogs with an otherwise
300 benign-appearing mucocele.
301
302 In our study, three of 25(12%) dogs died in the perioperative period following cholecystectomy.
303 Definitive cause of death was not identified in any case. Two (67%) of these dogs had
304 cholecystitis and were FISH+. The other dog had histopathological evidence of CMH alone and
305 was FISH-. None of these dogs had GB rupture noted surgically or histopathologically. The
306 reason for perioperative death following cholecystectomy for treatment of GBM is not well
307 understood. Although concurrent bacterial infection has not been correlated with perioperative
308 mortality (Besso *et al.* 2000, Pike *et al.* 2004, Worley *et al.* 2004, Aguirre *et al.* 2007, Crews *et*
309 *al.* 2009, Uno *et al.* 2009, Malek *et al.* 2013), the rate of bacterial infection has been variably
310 described (Pike *et al.* 2004, Worley *et al.* 2004, Aguirre *et al.* 2007, Crews *et al.* 2009, Uno *et al.*
311 2009, Malek *et al.* 2013, Mitzutani *et al.* 2017), which may limit the ability to make this
312 correlation. In our two cases of perioperative death with cholecystitis and bacteria detected by
313 FISH, death due to complications of cholecystitis (such as hemodynamic instability) (Amsellem
314 *et al.* 2006, Papazoglou *et al.* 2008) and/or translocation of bacteria from the biliary system and
315 resulting septicemia could be considered as possible causes.
316
317 The limitations of FISH should be considered. A negative FISH result does not exclude the
318 presence of bacteria. Despite enzyme degradation steps, inherent differences in the permeability
319 of different bacteria to FISH probes may lead to a failure to detect some gram positive and acid
320 fast bacteria. The eubacterial FISH probe employed in this study will only detect viable bacteria
321 with intact 16S, it will not recognize dead bacteria. It is important to note the inherent difficulties

of studies that utilize subjective histopathology. The use of standardized scoring schemes are typically employed to reduce subjectivity. However, despite this there is still poor agreement among histopathologists in studies describing hepatic and intestinal lesions in dogs (Jergens *et al.* 2014, Lidbury *et al.* 2017). Finally, the retrospective nature of this study made it difficult to accurately determine the incidence of concurrent diseases previously reported to be associated with GBM (e.g. endocrine disease, hyperlipidemia) (Mesich *et al.* 2009, Kutsani *et al.* 2014, Gookin *et al.* 2015) and postsurgical outcomes.

In conclusion, FISH detected bacteria in eight of 25 (32%) dogs with GBM and was more sensitive for the detection of bacteria than bacterial culture. Additional investigation is needed to further determine the relationship between bacteria and GBM and its relation to aetiopathogenesis or progression of disease, clinicopathologic abnormalities, ultrasound findings, histopathological findings, and outcome. The high proportion of occult cholecystitis may support the recommendation for early elective cholecystectomy in dogs with GBM. Additional investigation is also needed to further elucidate the relationship between GBM and cholecystitis.

No conflicts of interest have been declared.

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Table 1. Selected clinical, clinicopathologic, sonographic and histopathologic abnormalities in dogs with gallbladder mucocele that were FISH- *versus* FISH+

Variable	Reference Interval	FISH+ n=8 Median (range) Proportion	FISH- n=17 Median (range) Proportion
Clinical signs	–	7/8 (88%)	11/17 (65%)
Neutrophilia	2.6-11 (x10^ 3/ul)	10.8 (2.3-22.6) 3/8 (38%)	12.5 (4.5-20) 9/17 (53%)
Increased band neutrophils	0-0.2 (x10^ 3/ul)	0.3 (0-1.7) 4/8 (50%)	0 (0-0.4) 1/17 (6%)
Thrombocytosis	200-500 (x10^ 3/ul)	405 (167-664) 2/8 (25%)	358 (188-735) 3/17 (18%)
Hypoalbuminemia	3-4.3 (G/dl)	3.3 (1.8-4) 3/8 (38%)	3.5 (2.4-4.1) 1/17 (6%)
Hyperbilirubinemia	0-0.2 (mG/dL)	1.4 (0.1-13.9) 5/8 (63%)	0.2 (0-4.5) 5/17 (29%)
Elevated ALP	15-140 (IU/L)	1554 (62-5579) 7/8 (88%)	786 (69-9718) 15/17 (88%)
Elevated ALT	10-90 (IU/L)	472 (23-2776) 6/8 (75%)	162 (26-1477) 13/17 (76%)
Sonography: Peritoneal effusion	–	2/8 (25%)	7/17 (41%)
Sonography: GB wall abnormality	–	4/8 (50%)	3/17 (18%)
CMH	–	1/8 (13%)	7/17 (41%)
CMHC all	–	7/8 (88%)	10/17 (59%)
CMHC mild	–	3/8 (38%)	5/17 (29%)
CMHC moderate	–	2/8 (25%)	4/17 (29%)
CMHC severe	–	2/8 (25%)	1/17(6%)

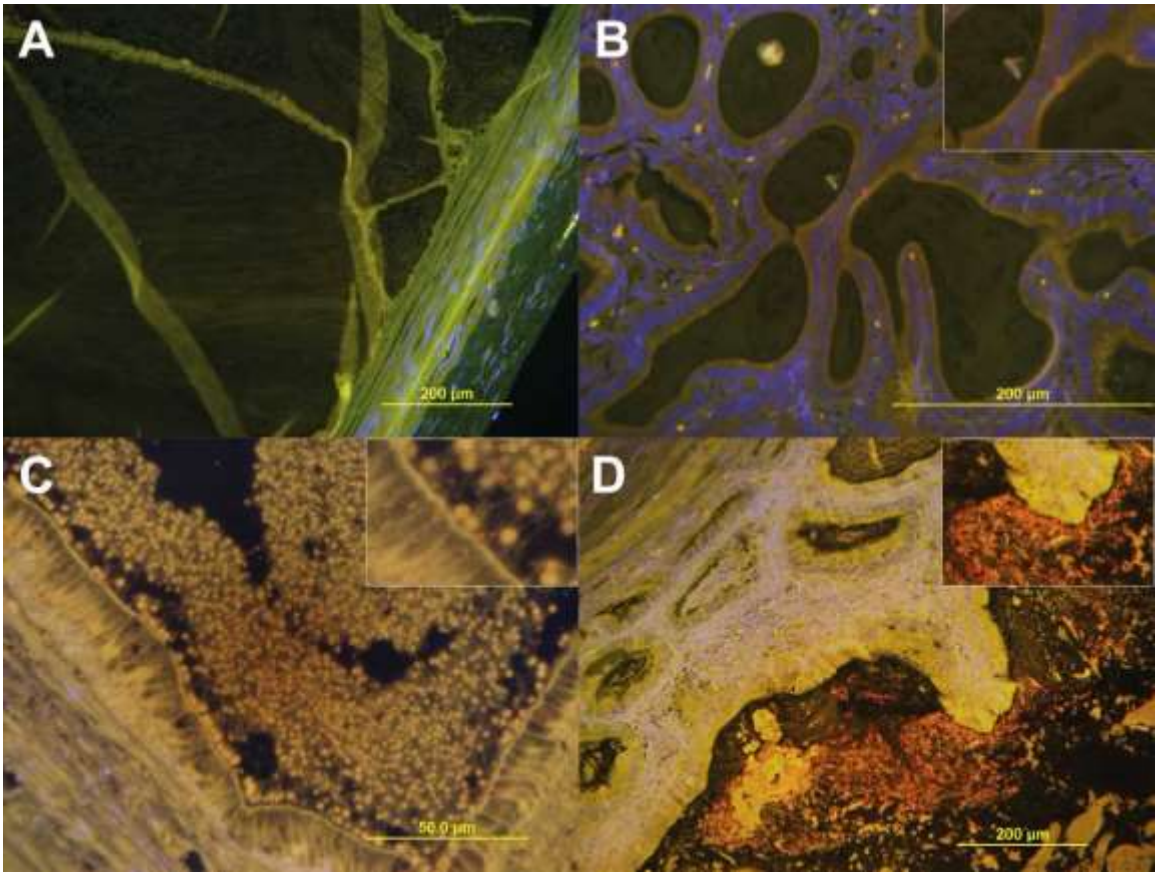
GB wall abnormality includes: hyperechogenicity, increased wall thickness, oedema, or discontinuous wall consistent with rupture. GB=gallbladder; CMH=cystic mucinous hyperplasia alone; CMHC=cystic mucinous hyperplasia + cholecystitis.

Table 2. Number and location of bacteria in FISH+ dogs with GBM.

Dog	<10 bacteria	≥10 bacteria	Adherent	Invasive	Within Mucus
1	X			X	
2		X	X	X	
3		X	X		X
4	X		X		
5		X		X	X
6		X	X		X
7		X	X		X
8	X			X	X

X symbols denote the dogs listed (dogs 1-8; y-axis) had the characteristics in the x-axis (<10 bacteria, *etc*) in their individual tissue sample

Figure 1. FISH analysis of gall bladder mucocele.
(**A**) FISH of GMB and CMH with Cy3-EUB-338 (red) 6-FAM-Non-EUB-338 (green) reveals a smooth villus lining with no bacteria visualized (**B)** FISH of GBM CMHC (moderate) with Cy3-EUB-338 (red) and 6-FAM-Non-EUB-338 (green) reveals the presence of four bacteria (red) within the gallbladder epithelium (insert) (**C**) GBM CMHC (severe) with clusters of bacteria (red) within intraluminal debris and the gallbladder epithelium (insert) (**D**) GBM CMHC (moderate) with masses of bacteria (red) within the gallbladder mucus and adjacent to the gallbladder wall (insert). DAPI (4′,6′-diamidino-2-phenylindole) stained nuclei are blue



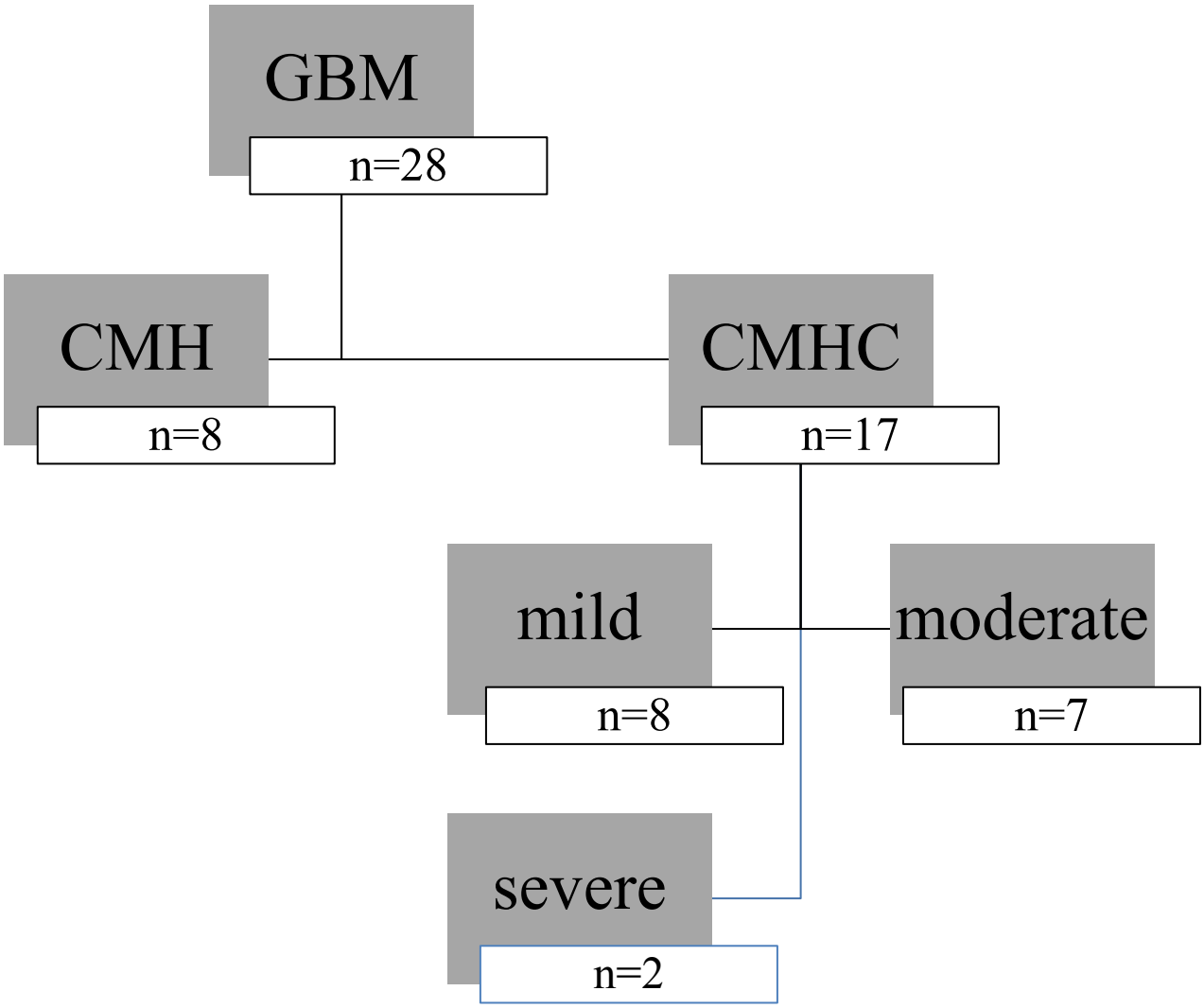


Figure 2. Histopathological characteristics in 25 dogs with GBM. GBM gallbladder mucocele, CMH cystic mucinous hyperplasia alone, CMHC cystic mucinous hyperplasia with cholecystitis

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563 **Figure 3.** Photomicrograph of gallbladder biopsy sections of dogs with gallbladder mucocele.
564 Hematoxylin-eosin stained section showing (**A**) no significant cellular infiltrates within the
565 gallbladder epithelium with thin villus projections (arrow) into luminal mucus (CMH) (**B**) mild
566 cellular infiltrates within the gallbladder epithelium with thickened, more cellular villus
567 projections (arrow) into luminal mucus (CMHC-mild) (**C**) moderate cellular infiltrates within the
568 gallbladder epithelium with thicker and loculated villus projections (arrow) into luminal mucus
569 (CMHC-moderate) (**D**) marked cellular infiltrate with areas of necrosis within the gallbladder
570 epithelium with blunting, thickening, and cellular infiltration of the villus projections (arrow)
571 into the luminal mucus (CMHC-severe)
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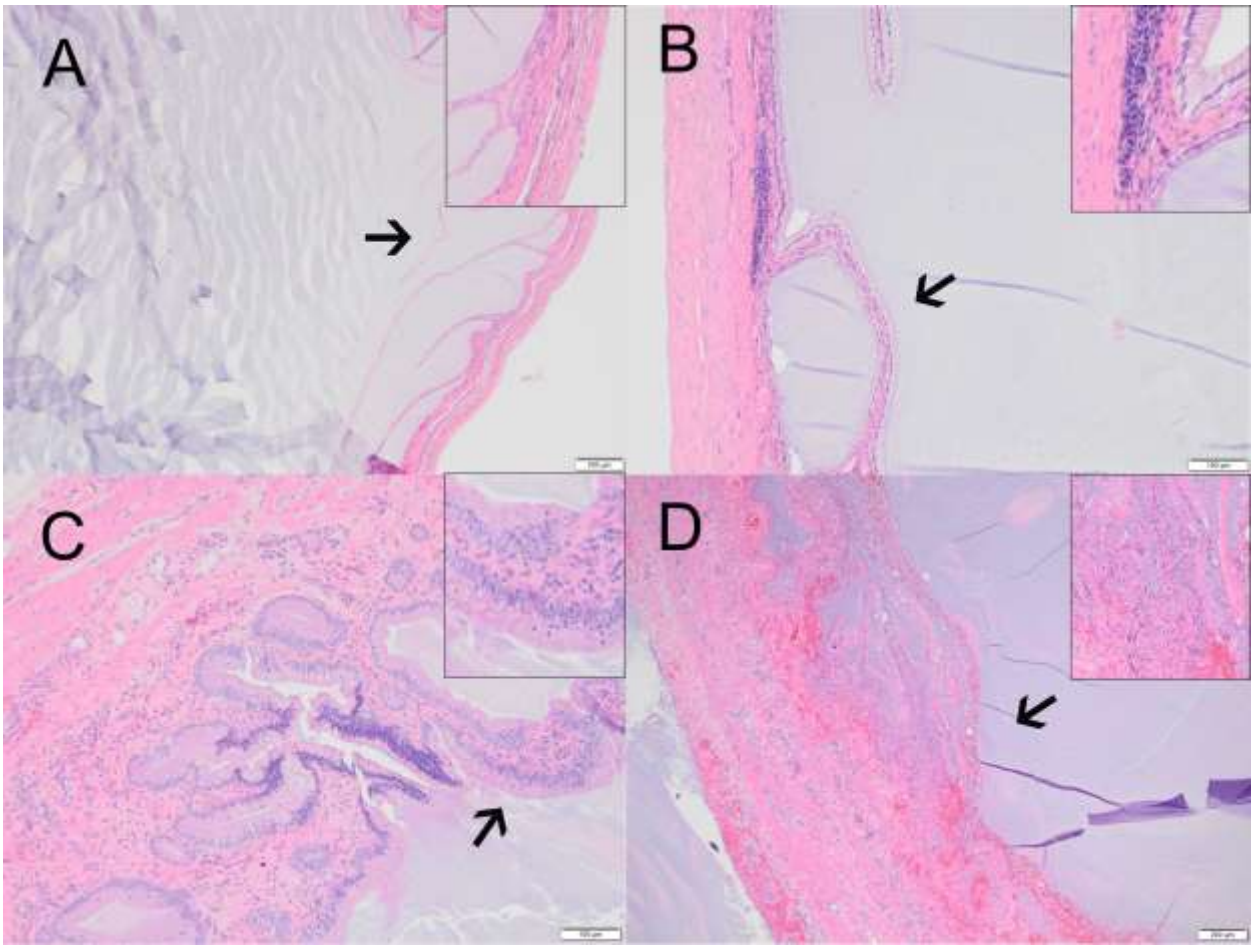

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