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TITLE: Eubacterial fluorescence in situ hybridisation and histologic features in 25 dogs with gallbladder mucocele

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1 2 2	Eubacterial Fluorescence In Situ Hybridization and Histologic Features In 25 Dogs with Gallbladder Mucocele			
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12				
13	Gallbladder mucocele (GBM)			
14	Gallbladder (GB)			
15	Cystic mucinous hyperplasia (CMH)			
16	Fluorescence in situ hybridization (FISH)			
17	Cystic mucinous hyperplasia with cholecystitis (CMHC)			
18				
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20				
21	Preliminary data from this project was presented at the American College of Veterinary Internal			
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23				
24				
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26 Abstract

28	Objectives- To detect and localise bacteria in gallbladder mucoceles utilizing fluorescence in
29	situ hybridization (FISH). To report clinical signs, clinicopathologic abnormalities, sonographic
30	findings and histopathological findings in FISH+ and FISH- dogs with gallbladder mucoceles.
31	Materials and Methods – Retrospective review of signalment, clinical signs, clinicopathologic
32	and sonographic findings of 25 cases of histopathologically confirmed gallbladder mucocele.
33	Histopathological sections of GBM were evaluated for cystic mucinous hyperplasia, cystic
34	mucinous hyperplasia with cholecystitis and rupture. The number and spatial distribution of
35	bacteria was determined by eubacterial FISH. Gallbladder contents were cultured in 21 dogs.
36	<b>Results</b> –Bacteria were detected within or adherent to the gallbladder in eight of 25 (32%) cases
37	Bacterial culture was positive in one dog. Cystic mucinous hyperplasia with concurrent
38	cholecystitis was found in 17/25 (68%) of dogs with gallbladder mucocele.
39	Clinical significance – FISH was more sensitive for detection of bacteria in gallbladder
40	mucoceles when compared to bacterial culture of bile. Cholecystitis was common in dogs with
41	gallbladder mucocele. Further study is required to elucidate the relationship of cystic mucinous
42	hyperplasia, bacteria and cholecystitis in the aetiopathogenesis and progression of GBM.
43	
44	Keywords: gallbladder mucocele, bacteria, FISH, cholecystitis
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46	
47	Gallbladder mucocele (GBM) has emerged as a common and clinically important cause of
48	extrahepatic biliary disease in the dog. GBM is most frequently reported in older dogs and

49 predisposed breeds include the Shetland sheepdog, Pomeranian, miniature schnauzer, cocker 50 spaniel, Chihuahua, and Border terrier (Worley et al. 2004, Aguirre et al. 2007, Crews et al. 51 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 52 53 mucus in the GB (Neer 1992, Besso et al. 2000, Pike et al. 2004, Worley et al. 2004, Aguirre et 54 al. 2007, Malek et al. 2013). The underlying cause of GBM formation is not well understood 55 (Neer 1992, Besso et al. 2000, Pike et al. 2004) but may involve the excess secretion of 56 particular gel-forming mucins (Kesimer et al. 2015) and dysmotility (Tsukagoshi et al. 2012). 57 58 Bacterial culture has been reported positive in 3-67% of cases of GBM (Pike et al. 2004, Worley 59 et al. 2004, Aguirre et al. 2007, Mayhew et al. 2008, Crews et al. 2009, Malek et al. 2013, 60 Policelli et al. 2017, Mitzutani et al. 2017, Policelli Smith et al. 2017). This wide variation may 61 reflect the use of perioperative antimicrobial therapy, and differences in sampling and culture 62 based methodologies. The prevalence of bacterial infection in GBM could also be impacted by 63 concurrent comorbidities such as cholecystitis or cholelithiasis, conditions which have reported 64 culture positive rates of 35-50% (Mehler et al. 2004, Aguirre et al. 2007). Concurrent 65 cholecystitis has been reported in 17-40% of dogs with GBM (Besso et al. 2000, Pike et al. 66 2004, Worley et al. 2004, Malek et al. 2013).

67

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

72 ulceration of the GB could compromise the structural integrity of the GB and predispose to 73 rupture. Timely recognition of bacterial infection and cholecystitis could influence the medical 74 management of GBM and reduce perioperative mortality. Further, because cholecystectomy may 75 be postponed in cases of suspected benign GBM, there is a clear need to better understand the 76 relationship of concurrent bacterial infection and cholecystitis to GBM. 77 78 Fluorescent in-situ hybridization (FISH) is a culture-independent technique that enables 79 visualization and localisation of intact bacteria in formalin-fixed, paraffin wax-embedded tissues. 80 FISH has been used to document bacteria in a variety of cells and tissues and in some cases has 81 been demonstrated to be more sensitive for detection of bacteria when compared to culture 82 (Simpson et al. 2006, Recordati et al. 2009, Warren et al. 2011, Kornreich et al. 2012, Twedt et 83 al. 2014). To our knowledge, FISH has not been used previously to detect bacteria in archived 84 GB samples in dogs. 85 The objectives of this study were to 1) detect, count and localise bacteria in GBMs when 86 87 evaluated by FISH and 2) report clinical signs, clinicopathologic abnormalities, sonographic 88 findings and histopathological findings in FISH+ and FISH- dogs with GBM. 89 90 **Materials and Methods** 91 92 Inclusion Criteria and Case Data Review 93 Electronic medical records (EMR) at Colorado State University were reviewed for cases of histopathologically confirmed canine GBM between December 2010 and January 2015. Dogs 94

95 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  $(3 \times 10 \text{ mins})$ , 100% alcohol  $(2 \times 5 \text{ mins})$ , 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/ $\mu$ l 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

All ultrasound examinations were performed by a board-certified veterinary radiologist or a
 veterinary radiology resident under the direct supervision of a board-certified veterinary
 radiologist. Written reports, still images, and video clips of ultrasonographic examinations were

119 then retrospectively reviewed by a board-certified veterinary radiologist (EKR) blinded to the 120 case data. The appearance of the gallbladder, gallbladder wall, adjacent abdominal structures, 121 and free peritoneal fluid was evaluated. Sonographic features of GBM were defined as stellate or 122 finely striated bile patterns that differed from biliary sludge by the absence of gravity-dependent 123 bile movement (Besso et al. 2000). The gall bladder wall was evaluated for echogenicity, 124 presence of oedema, thickening and rupture. A thickened gallbladder wall was defined as more 125 than 2 mm in dogs (Nyland & Hager 1985). GB wall oedema was defined as a thickened GB wall 126 with a hypoechoic layer within the GB wall.

127

# 128 Histopathological Evaluation

129 Original histopathologic reports (all by board-certified veterinary pathologists) were reviewed to 130 confirm a diagnosis of GBM. Following this, archival formalin-fixed paraffin-embedded tissue 131 blocks were located for 23/25 cases, sectioned at 4 um and stained with hematoxylin and eosin 132 (HE) for blinded review by a board-certified veterinary pathologist (SLP) employing WSAVA 133 criteria for CMH and cholecystitis (Rothuizen 2006). Cholecystitis was defined as the presence 134 of a neutrophilic and/or lymphoplasmacytic infiltrate in the epithelium or wall of the gallbladder 135 +/- fibrosis (Rothuizen 2006) and assigned a grade of mild, moderate or severe. Each case was 136 assigned to one of 4 groups: CMH, CMH with cholecystitis (CMHC), mild, moderate or severe. 137

#### 138 Statistical Analysis

139 Descriptive statistics were calculated for the presence/absence of clinical signs,

140 clinicopathological data, sonographic findings, and histologic findings in FISH+ versus FISH-

141 dogs with GBM.

- 142 **Results**
- 143

144 Twenty-six dogs with a histopathological diagnosis of gallbladder mucocele were identified. One

145 dog was excluded due to concurrent hemolytic anemia and so 25 cases were included. Tissue

- 146 blocks for 23 of 25 cases were available for blinded histopathological review.
- 147

## 148 Patient Demographics, Clinical and Clinicopathologic Characteristics and Outcome

149 The median age was 11 (n=25; range, 6-14), with a near even distribution between castrated

150 male 12 (48%) and spayed females 13 (52%). Breeds included mixed (n=10), Shetland sheepdog

151 (n=3), miniature schnauzer (n=2), Pomeranian (n=2), and one each of the following: Australian

152 shepherd, Bernese mountain dog, cocker spaniel, Labrador retriever, Maltese, miniature

dachshund, miniature poodle and Yorkshire Terrier. Due to the retrospective nature of this study

154 it was not possible to fully determine the presence or absence of potential medical conditions

155 predisposing to mucocele in every case (Mesich *et al.* 2009, Kutsani *et al.* 2014, Gookin *et al.* 

156 2015). Three mixed breed dogs had a previous diagnosis of hyperadrenocorticism.

157

158 Clinical signs were present in 18 of 25 (72%) dogs with GBM (Table 1). Change in appetite (*i.e.* 

159 hyporexia or anorexia) was most common, 12/25 (48%). Other clinical signs included: vomiting

160 (11/25;44%), lethargy (9/25;36%), diarrhea (6/25;24%), abdominal pain (4/25;16%), jaundice

161 (3/25;12%), polyuria/polydipsia (3/25;12%), fever (2/25;8%), and abdominal distension

162 (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* 

173 detected from bile culture was receiving cefoxitin.

174

175 Perioperative death occurred in three of 25 (12%) cases. Necropsies were not performed. Clinical 176 signs in these dogs included vomiting alone in one dog, inappetence alone in one dog, and 177 jaundice, vomiting, diarrhoea, lethargy and inappetence in the third dog. One dog had a 178 neutrophilia and another had band neutrophilia with a normal neutrophil count. Two of three 179 dogs were hyperbilirubinemic and hypoalbuminemic. Two of three dogs that died in the peri-180 operative period had cholecystitis and were FISH+ but the cause of death was not determined. 181 The remaining dog suffered respiratory arrest postoperatively and pulmonary thromboembolism 182 was suspected, but not confirmed.

183

#### 184 FISH analysis of GB mucosa

Bacteria that hybridized to the eubacterial FISH probe were detected in eight of 25 (32%) cases.

186 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

188 bacteria visualized; the remainder of the dogs had bacteria visualized as dense clusters or masses

189 (Table 2; Figure 1). FISH analysis of the dog with *E.coli* cultured in the bile revealed masses of

190 bacteria within luminal mucus and adhering to the GB wall (Figure 1, D).

191

# 192 Sonographic Findings

193 The sonographic appearance of the GB was consistent with mucocele (Besso *et al.* 2000) in 24 of

194 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

196 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

198 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

200 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

202 lethargy and hyporexia. This dog had hyperbilirubinemia, marked elevations in ALP and ALT,

203 neutrophilia and band neutrophilia, and sonographic evidence of GBM with a thickened,

204 hyperechoic, and oedematous GB wall, and a moderate peritoneal effusion (fluid cytology not

205 performed). Surgical exploration found an intact GB. Histopathological diagnosis was CMHC

206 (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.

211

210

# 212 Histopathological Findings

213 The original and blinded (23 of 25 cases) histopathological examinations indicated a diagnosis of

214 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%)

217 (Figures 2 and 3). In three cases, necrosis of the GB wall was also noted along with cholecystitis

218 (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

221 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.

223

225

## 224 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.

231 The importance of the bacteria identified in GBM by FISH is unclear. The accepted standard for 232 diagnosis of bacterial infection in the biliary system is aerobic and anaerobic culture and 233 sensitivity (Neer 1992). Further, this would ideally be correlated with cytologic results in order 234 to attempt to determine whether the bacteria may be transient, iatrogenic contamination, or true 235 biliary infection as healthy dogs have been shown to periodically harbor bacteria in the bile with 236 no obvious clinical relevance (Kook et al. 2010). In humans with cholelithiasis and/or chronic 237 cholecystitis there is also a wide variation in the reported rates of bacterial infection (0 to 73%) 238 and controversy over the significance of the results (Lemos et al. 2010). FISH alone is unable to 239 definitively prove infection versus transient bacteria versus iatrogenic contamination. However, 240 in all of our cases bacteria were visualized adjacent to the GB wall or within the GB 241 parenchyma, which would suggest pathogenic behaviour of the bacteria. 242 243 In attempting to determine the significance of the bacteria seen by FISH, it is important to 244 consider possible reasons for the discordancy between FISH and bacterial culture results. All 245 dogs in the study were administered perioperative antibiotics, but it is unclear whether antibiotic 246 administration would have influenced culture results. In a report of dogs undergoing cystotomy 247 for urolithiasis the use of perioperative antibiotics did not change culture results when compared 248 to antimicrobial administration following surgery (Buote et al. 2012). However, the dogs in our 249 study did not all receive the same antibiotic and there may be a differential effect of 250 antimicrobials on recovery of cultured bacteria. Additionally, it is possible some of the bacteria 251 seen with FISH were not cultivable with routine culture methodologies. Also, the varied method 252 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.

259

260 Generally, identification of concurrent bacterial infection of the bile, GB mucosa or liver in cases 261 of GBM is challenging. Ultrasound-guided percutaneous cholecystocentesis is a common and 262 typically safe procedure for the collection of bile for the purposes of cytologic evaluation and 263 culture (Uno et al. 2009, Peters et al. 2016, Schiborra et al. 2017). However, biliary mucocele is 264 considered by many to be a contraindication to cholecystocentesis as the potential for GB 265 necrosis secondary to GBM makes rupture of the biliary tract possible (Kook et al. 2010). A 266 recent publication described 201 dogs that had percutaneous cholecystocentesis performed, six of 267 which had GBM. Two of these dogs had complications from cholecystocentesis, one of which 268 died from bile peritonitis (Schiborra *et al.* 2017). Aspirate of a GBM for collection of bile 269 preoperatively is generally discouraged. Based on the results of this study, FISH could be 270 considered as a complimentary diagnostic tool as it may be more sensitive than bacterial culture 271 of bile in some instances, and has the added benefit of demonstrating the organism within the 272 tissue. If a high suspicion of bacterial infection exists and bacterial culture is negative, FISH 273 could be performed and while awaiting results an appropriate empirical antimicrobial could be 274 administered to the dog. While FISH is unable to give information on antimicrobial 275 susceptibility, the use of specialized probes may enable the identification of the bacterial species 276 to help ensure appropriate choice of antimicrobial in regards to spectrum and penetration of

tissue. It remains unclear whether the bacteria seen are pathogenic and potentially contributing to
the aetiopathogenesis or progression of GBM, or whether they are transient or of little clinical
relevance. However, the number of GBM cases with bacteria visualized by FISH is of interest.
Prospective studies utilizing bacterial culture, cytology, and FISH are needed to further evaluate
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

may support the recommendation for early cholecystectomy, even in dogs with an otherwisebenign-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

The limitations of FISH should be considered. A negative FISH result does not exclude the presence of bacteria. Despite enzyme degradation steps, inherent differences in the permeability of different bacteria to FISH probes may lead to a failure to detect some gram positive and acid fast bacteria. The eubacterial FISH probe employed in this study will only detect viable bacteria with intact 16S, it will not recognize dead bacteria. It is important to note the inherent difficulties

322	of studies that utilize subjective histopathology. The use of standardized scoring schemes are
323	typically employed to reduce subjectivity. However, despite this there is still poor agreement
324	among histopathologists in studies describing hepatic and intestinal lesions in dogs (Jergens et al.
325	2014, Lidbury et al. 2017). Finally, the retrospective nature of this study made it difficult to
326	accurately determine the incidence of concurrent diseases previously reported to be associated
327	with GBM (e.g. endocrine disease, hyperlipidemia) (Mesich et al. 2009, Kutsani et al. 2014,
328	Gookin et al. 2015) and postsurgical outcomes.
329	
330	In conclusion, FISH detected bacteria in eight of 25 (32%) dogs with GBM and was more
331	sensitive for the detection of bacteria than bacterial culture. Additional investigation is needed to
332	further determine the relationship between bacteria and GBM and its relation to
333	aetiopathogenesis or progression of disease, clinicopathologic abnormalities, ultrasound findings,
334	histopathological findings, and outcome. The high proportion of occult cholecystitis may support
335	the recommendation for early elective cholecystectomy in dogs with GBM. Additional
336	investigation is also needed to further elucidate the relationship between GBM and cholecystitis.
337	
338	No conflicts of interest have been declared.
339	
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487 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 <sup>^</sup> 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 <sup>^</sup> 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%)
СМН	_	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%)

489

490 GB wall abnormality includes: hyperechogenicity, increased wall thickness, oedema, or

491 discontinuous wall consistent with rupture. GB=gallbladder; CMH=cystic mucinous hyperplasia

492 alone; CMHC=cystic mucinous hyperplasia + cholecystitis.

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

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

# 520 Figure 1. FISH analysis of gall bladder mucocele.

521 (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

- 525 (red) within intraluminal debris and the gallbladder epithelium (insert) (**D**) GBM CMHC
- 526 (noderate) with masses of bacteria (red) within the gallbladder mucus and adjacent to the
- 527 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,

546 CMH cystic mucinous hyperplasia alone, CMHC cystic mucinous hyperplasia with cholecystitis 

- 562
- **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
- gallbladder epithelium with thicker and loculated villus projections (arrow) into luminal mucus
   (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)
- 570 epithenum with blunting, thickening, and certaial initiation of the vind 571 into the luminal mucus (CMHC-severe)
- 572

