Prevalence of bactibilia in apparently healthy dogs

By

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PROJECT TITLE	**Prevalence of bactibilia in apparently healthy dogs**
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APPROVED	Date **27 August 2018**
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Animal Ethics Committee

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KINDLY NOTE:

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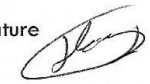
CHAIRMAN: UP Animal Ethics Committee	Signature 

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Nothing in the world can take the place of persistence.
Talent will not; nothing is more common than unsuccessful men with talent.
Genius will not; unrewarded genius is almost a proverb.
Education will not; the world is full of educated derelicts.
Persistence and determination alone are omnipotent.

- *Calvin Coolidge (1872 – 1933)*

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ABBREVIATIONS

AACL	Animal Anti-Cruelty League
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
CI	Confidence interval
EDTA	Ethylenediamine tetraacetic acid
GGT	Gamma-glutamyl transferase
GI	Gastrointestinal
h	hour
H&E	Haematoxylin and eosin
HPF	High-power field
IgA	Immunoglobulin A
IQR	Interquartile range
WSAVA	World Small Animal Veterinary Association

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SUMMARY

Prevalence of bactibilia in apparently healthy dogs

By

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Bacterial cholecystitis in dogs is a histological diagnosis, however in clinical practice bactibilia accompanying bacterial cholecystitis is usually diagnosed based on a combination of bile cytology findings and the isolation of bacteria on bile culture. There is currently a paucity of data available regarding the prevalence of bactibilia in healthy dogs and its subsequent clinical implications. The association between raised liver enzymes and bactibilia, as well as the value of bile culture in determining the likelihood of clinically significant cholecystitis in dogs with suspected biliary disease is unknown.

The aims of this study were to a) determine the prevalence of asymptomatic bactibilia in apparently healthy dogs; b) determine if differences between bactibillic and non-bactibillic healthy dogs occur with regards to serum liver enzymes alkaline phosphatase (ALP), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) activities; and c) determine if differences between bactibillic and non-bactibillic healthy dogs occur with regards to liver and gallbladder histopathology.

A cross-sectional, prospective study was performed at the Animal Anti-Cruelty League Epping. Sixty-five apparently healthy, abandoned dogs euthanased for non-medical reasons were used. Whole blood, aspirated bile, gallbladder wall and liver section samples were collected aseptically from all dogs within 25 minutes of euthanasia. Bile samples of all dogs involved in the study were submitted for cytology as well as aerobic and anaerobic culture. After determining the prevalence of bactibilia, sectioned gallbladder wall and liver samples from all dogs with bactibilia and from 9 dogs without bactibilia (control group) were submitted for histopathological evaluation. Measurement of ALP, ALT and GGT was performed on all blood samples.

Bactibilia was present in 10.77% (7/65) of dogs, with a diagnosis in 9.23% (6/65) of dogs made on cytology and 4.62% (3/65) on bile culture. There was weak agreement between bile cytology and culture (0.408, Cohens kappa; p = 0.001; CI (0.007, 0.824)). No significant differences in median liver enzyme activities and hepatobiliary histopathology were found between the bactibilic and non-bactibilic dogs in this study. However, the possibility of these differences existing in the population could not be excluded based on the results of this study. The reasons for these differences possibly not being identified in this study include the study being underpowered, the cross-sectional nature of the study with median liver enzyme activities and hepatobiliary histopathology only evaluated at a single point in time, and the collection of hepatobiliary histopathology samples after euthanasia which may have led to the histopathological findings not being reflective of liver and gallbladder pathology in the apparently healthy dog population.

This study showed that the prevalence of bactibilia in asymptomatic dogs is 10.77%. Further studies are needed to determine if significant differences in median liver enzyme activities and hepatobiliary histopathology are present between the bactibilic and non-bactibilic dogs.

Key terms: Bactibilia, bile culture, bile cytology, liver enzymes, hepatobiliary histopathology

CHAPTER 1

LITERATURE REVIEW

Bactibilia

In dogs with suspected hepatobiliary disease it is common practice to perform cytological examination and bacterial culture of bile during the diagnostic work-up.[1-5] However, there is a paucity of information regarding the prevalence of bacteria in bile (bactibilia) in healthy canines,[6] making interpretation of the presence of bactibilia difficult. Hence, identifying bactibilia may not indicate hepatobiliary disease if there is a reasonable probability of finding bactibilia in healthy dogs. This can have far reaching effects on the accuracy of the diagnosis, the necessity of treatment and ultimately, the quality of life of the patient.[5]

Abnormal biological states can only be identified once the normal state has been established. It is known that hepatobiliary-enteric circulation of bacteria can occur whereby enteric bacteria gain entry into the portal circulation from the gastrointestinal (GI) tract.[6-9] Bacteria are then delivered via the portal circulation to the liver where they are extracted and inactivated by neutrophils and hepatic Kupffer cells, which line the liver sinusoids (Figure 1).[5,6,10,11] Kupffer cells can phagocytose a single bacterium in less than 0.01 seconds and form such an effective particulate filtration system that almost none of the bacteria from the gastrointestinal tract pass from the portal blood into the general systemic circulation.[12] Any remaining viable organisms that escaped intrinsic killing mechanisms may navigate bile canaliculi and transcend the biliary tract into bile.[5,6,8,10,11] This intrinsic killing mechanism has been shown to be susceptible to being overwhelmed in cats by large portal vein inoculation of bacteria, producing bactibilia in the healthy animal.[9] It was also demonstrated that this situation occurred with lower numbers of bacteria in cats suffering from chronic biliary stasis.[9] A similar finding was demonstrated in a study where intrahepatic enteropathogenic bacteria were detected in Bedlington Terriers fed a raw meat diet.[13] In dogs and cats enteric bacteria are the most commonly detected bacteria involved in hepatobiliary infections,[4,14] with *Escherichia coli (E. coli)*, *Enterococcus* spp., *Bacteroides* spp., and *Clostridium* spp. most commonly isolated.[4,15-17]

There are two main routes whereby bacteria can gain access to the biliary tract, either by ascending infection from the duodenum or as previously mentioned through haematogenous spread via the hepatic portal venous system.[6,11,17] The normal biliary defence mechanisms that protect against bacterial invasion include the mechanical barriers provided by the sphincter of Oddi and tight

junctions between hepatocytes, the continual flushing action of bile, the bacteriostatic effect of bile salts and potent local immunological defence mechanisms involving Kupffer cells and secretory immunoglobulin A (IgA).[5,10,11,18] Mucus, Kupffer cells and IgA defence mechanisms also help to protect against the adhesion and colonisation of bacteria.[10,11] Factors that impair these natural defence mechanisms, such as biliary stasis, impaired immune function and increased biliary pressure, predispose an animal to biliary infection and hepatobiliary disease.[3,5,7,8,11] In humans Kupffer cells, dendritic cells, liver sinusoidal endothelial cells and other liver antigen-presenting cells preferentially mediate tolerizing immunity.[19-21] It has been put forward in the human literature that this tolerizing environment is required in order to maintain a state of immunological homeostasis with regards to intestinal pathogens that gain access to the liver through the enterohepatic circulation.[21] If the tolerizing immunity promotes non-pathological bacterial colonization of in the biliary system is unknown.[21]

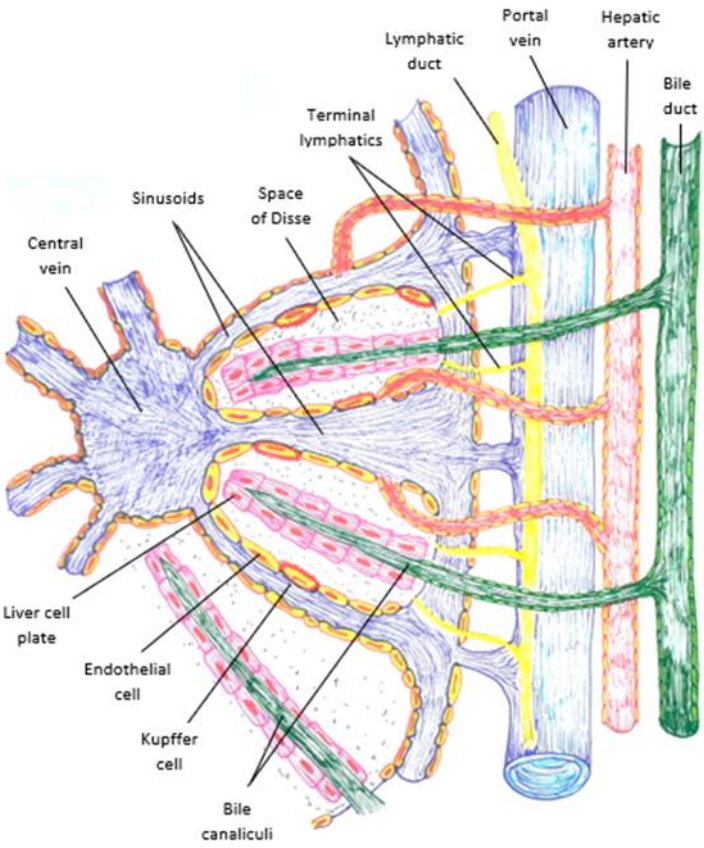

Figure 1. The basic structure of a liver lobule showing the blood vessels, Kupffer cells and bile-collecting system which all play a role in the hepatobiliary-enteric circulation of bacteria (Verwey, E., 2019, veterinarian, personal drawing based on information from Guyton and Hall textbook of medical physiology[12]).

In healthy people it is generally accepted that bile is normally sterile,[6,10,18,22,23] while the situation in dogs and cats is currently far less clear.[6,10] The occasional presence of enteric bacteria in the bile of healthy dogs has been reported and some studies suggest that bacteria could be readily isolated from the normal liver in dogs.[6,7,24,25] It has been put forward that feline and canine bile is sterile in the absence of biliary tree pathology.[9,10,17,26,27] However, two studies report intermittent isolation of bacteria from healthy dogs' gallbladders and the isolation of bacteria from liver biopsies of normal dogs.[6,24] This highlights the need for further studies on the normal canine biliary bacterial flora.[3,6,10]

There is also little data available regarding the clinical implications and prevalence of asymptomatic canine bactibilia.[6,10] The current literature suggests that the presence of bactibilia does not necessarily translate to clinically significant cholecystitis in dogs and humans.[6,22] However the pathogenesis of bacterial cholangitis and cholecystitis is poorly understood in dogs and there is currently no known pathognomonic finding for bactibilia or clinically significant bacterial cholecystitis in dogs.[1,10,17] Studies in humans show that most organisms isolated sterilely from bile through bacterial culture were found not to be true pathogens.[22] A study comparing bactibilia from apparently healthy dogs to dogs with hypercortisolism found, based on conventional bacterial culture techniques, that bile from apparently healthy dogs can harbour bacteria from time to time and that this does not lead to clinically relevant changes.[6] However the sample size of apparently healthy dogs in that study was low (n=6). A retrospective study of bile culture results from dogs and cats with hepatobiliary disease demonstrated that 13 out of 46 bile samples from dogs had positive bacterial culture results.[4] Unfortunately, the clinical significance of positive bile bacterial cultures were not assessed in that study,[4] and the presence of hepatobiliary disease makes it difficult to extrapolate these results to the clinical setting in small animal practice.

Traditionally, bacterial culture and/or cytology of bile is used to determine the presence of bactibilia.[3-5,14] The identification of bactibilia in dogs and cats suspected to have bacterial cholangitis has the potential to affect clinical decision-making regarding treatment, particularly when bacterial identification is confirmed by isolation on culture.[4,5] Although anaerobic bacteria are cultured less frequently than aerobic bacteria, anaerobic bacteria still represented a significant proportion of bacteria isolated from bile, emphasizing the importance in performing aerobic as well as anaerobic bile culture.[4,10,17] As bacteria can be viable and culturable, viable and non-culturable or non-viable and non-culturable, bacterial culture alone has a poor sensitivity to assess the prevalence of bactibilia.[5] A study performed on dogs and cats with hepatobiliary disease to determine the level of agreement between bile cytology and bile culture showed an 85% agreement.[5] Cytology is advantageous in that

cytological findings for bile samples can be available on the day of sample collection, whereas bacterial culture of bile samples requires 3 to 5 days or longer.[5] Furthermore, previous antimicrobial use, inappropriate storage and transport conditions of specimens for bacterial culture can compromise the results leading to possible false-negative results particularly with regards to anaerobic cultures.[3,5,28] For these reasons, understanding the relationship between detection of bactibilia via cytological examination and detection via bacterial culture would be of clinical benefit.[3,5]

Cytological evaluation of bile is inexpensive and a relatively uncomplicated procedure which yields diagnostically relevant information that usually precedes and complements bile bacterial culture results.[3] However, false negative bile cytology results can occur when low numbers of bacteria is present in bile.[5,14] Therefore, in order to achieve the most accurate results when attempting to detect bactibilia, it is currently recommended to submit bile samples for cytology and bacterial culture.[1,5] Failure to perform both cytological examination and bacterial culture may result in inaccurate bactibilia prevalence results.[3,5,6] This is demonstrated by Kook et al., where two positive bacterial cultures were negative for bactibilia on cytological examination and two bactibilia positive cytological examinations where negative on bacterial culture.[6] Peters et al. had similar findings with three bile samples demonstrating bacteria cytologically, without bacterial growth on bacterial culture.[3] Conversely, two bile bacterial cultures yielded positive results when no bacteria were detected on bile cytological examination.[3] The current bactibilial prevalence study reported here therefore included both cytological evaluation of bile as well as bile culture in order to optimise the accuracy of determining the prevalence of bactibilia.

Bile cytology is also useful in detecting inflammatory cells or organisms other than bacteria in bile.[3] The chemical composition of bile can potentially affect the presence of inflammatory cells and their local function,[3] as bile has been reported to have immunomodulatory functions.[29] These immunomodulatory functions include suppression of leukocyte activation and phagocytosis.[3,29] A case of non-inflammatory bactibilia in a dog with clinically important bacterial cholecystitis has been described. In veterinary literature it is discussed, without listing specific references, that inflammatory cells might also be lacking in bile samples for patients with bacterial cholecystitis in human medicine.[2,5] However, the pathophysiologic nature of cholecystitis in humans is quite different from that in small animals, precluding the ability to make direct comparisons between species.[30] The bactibilial prevalence study reported here also recorded the presence of inflammatory cells in bile on bile cytological examinations in conjunction with bactibilia in a large population of apparently healthy dogs.

Serum liver biochemistry

Biochemical assessment of the liver and gallbladder is vital when diagnosing, monitoring, and evaluating patients with hepatobiliary diseases.[8,31,32] Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) serum enzymes are measurands often evaluated by veterinarians when hepatobiliary disease is expected.[8,32] Serum liver enzyme activities are indicators of two major hepatobiliary processes, namely hepatocellular injury and cholestasis.[31,32]

ALT is a hepatocellular cytoplasmic enzyme in dogs.[31,32] Significant increases in ALT are indicative of hepatocellular injury.[31,32] Mild to moderate increases may be seen with vacuolar hepatopathy, portosystemic vascular anomalies and passive hepatic congestion.[33] Increases in canine serum ALT have the highest sensitivity for hepatic necrosis and hepatic failure,[32] but are less sensitive to cirrhosis, vacuolar hepatopathy, passive hepatic congestion or portosystemic vascular anomalies.[34] ALT is relatively specific for liver disease,[31] with the largest increases of ALT seen in hepatic necroinflammatory diseases like infectious and inflammatory disease,[34,35] which is a potential complication of bactibilia. In cases of extrahepatic biliary tract disease ALT activity is usually mildly to moderately increased.[32]

ALP is a membrane-bound glycoprotein found in canine liver, bone, renal cortex, intestinal and placental cells.[32,33] Clinically important ALP isoforms include the hepatic, bone, intestinal, and corticosteroid-induced forms.[32,36] Increased serum ALP activity is one of the most common biochemical abnormalities reported in dogs.[33,37] ALP activity may increase with hepatic neoplastic, cholestatic or necroinflammatory injury,[33] as is possible with clinically significant bactibilia.[9] However ALP has a high sensitivity but low specificity for hepatobiliary disease in dogs.[33] Yet, if a concurrent serum GGT elevation is identified, specificity for liver disease is significantly improved in dogs.[38]

GGT is found in the cell membranes of various tissues including renal tubular epithelial cells, pancreatic epithelial cells, mammary epithelial cells, biliary epithelial cells and hepatocytes.[31,32] In dogs the largest GGT tissue activity is in the proximal renal tubules and pancreas.[33] However, due to its epithelial location in these tissues GGT loss occurs into urine or the pancreatic lumen rather than the circulation.[32, 34] GGT is classified as a cholestatic liver enzyme, as its activity increases when bile acid solubilization during cholestasis results in the release of this membrane-bound liver enzyme.[32,33] An increase in GGT activity can therefore potentially occur in bactibilic dogs, if bactibilia is accompanied by cholestasis, which is known to occur.[9] GGT is not a sensitive marker of canine liver disease, but it has a higher specificity than ALP.[31,34, 38] Combined, significant increases in ALP and GGT activities has

been described in dogs with conditions that can potentially be accompanied by bactibilia, such as cholestasis, cholangiohepatitis, biliary obstruction and cholecystitis.[39]

Laboratory findings can vary with cholecystitis with elevations of serum liver enzymes being possible.[32,40] In cats with cholecystitis elevations in ALT and ALP correlate well with ultrasonographic changes expected with cholecystitis, and potentially indicate more clinical significance.[41] Lawrence et al. compared clinicopathologic findings between dogs with (n=10) and dogs without (n=30) bacterial cholecystitis or bactibilia, and recorded elevated serum liver enzyme activities without clinical signs of hepatobiliary disease in 50% (2/4) of dogs with bactibilia.[1] However, as no gallbladder histopathology was performed on the bactibilic dogs in the Lawrence et al. study to truly confirm asymptomatic bactibilia and as the sample size of bactibilic dogs was small,[1] one cannot rightfully extrapolate this finding to the general canine population. No significant association between raised ALT and ALP activities and positive bile bacterial cultures were detected in a study investigating the prevalence, identity and antimicrobial susceptibility of hepatobiliary bacteria commonly isolated from dogs and cats with hepatobiliary disease, however there was no predetermined, set time period between blood collection for serum liver enzyme evaluation and collection of culture samples, which was a major limitation in this study.[4] Knowledge of the prevalence of bactibilia and its relation to elevations in ALT, ALP and GGT activities between asymptomatic bactibilic and non-bactibilic dogs would give clinicians the opportunity to more accurately diagnose clinically relevant bactibilia in dogs in conjunction with bacterial culture, cytology and histopathology.

Hepatobiliary histopathology

Histopathological examination of the liver can substantially aid the diagnosis of certain biliary tract diseases like cholestasis,[42] which predisposes animals to clinically significant bactibilial infections,[8,11] and cholangitis.[42] Extrahepatic cholestasis can be defined as either acute, characterized by enlarged oedematous portal tracts and neutrophilic portal infiltrate, or chronic, which in turn is characterized by fibrosis in portal areas and macrophagic, lymphoplasmacytic and neutrophilic inflammatory infiltrates.[42] Neutrophilic cholangitis is the most common type of cholangitis, even though it is rarely reported in dogs, and can arise from ascending infection from the intestine.[42] Neutrophilic cholangitis is histologically characterized by neutrophilic inflammation and oedema in the acute state and a mixed inflammatory cell infiltrate in the chronic state.[42] According to current literature neutrophils should be detected on histopathological examination and bacteria should be detected in bile in order to make a diagnosis of neutrophilic cholangitis.[42] It is recommended that multiple liver lobes are biopsied when

performing hepatic histopathological analyses to increase the probability of obtaining an accurate histopathological diagnosis.[43]

Gallbladder wall histopathological analyses certainly also aid in the diagnosis of clinically significant cholecystitis. Neutrophilic cholecystitis is generally associated with bacterial infection and can sometimes occur in conjunction with neutrophilic cholangitis.[42] Neutrophilic cholecystitis is characterized by neutrophils in the gallbladder epithelium and/or gallbladder wall with ulceration or oedema being possible in the acute stage of this disease.[42] This is in contrast to the chronic stage of neutrophilic cholecystitis where infiltrates consisting of neutrophils, lymphocytes and plasma cells are reported.[42] Lymphoplasmacellular cholecystitis is characterized by lymphocytes and plasma cells in the mucosa of the gallbladder.[42] Dogs with clinically relevant gallbladder disease typically have various degrees and combinations of intramural haemorrhage, inflammation, fibrosis, cystic mucosal hyperplasia, and necrosis.[15,44,45]

There is currently limited information available on the effect of bactibilia on the histopathological findings of the gallbladder and liver in dogs. Proliferative activity has been previously described in bile duct epithelium and gallbladder wall occurring secondary to bacterial infection.[17,46] However bile duct proliferation is not restricted to biliary tract disorders and can be seen with liver parenchymal disorders or primary vascular abnormalities.[42] It has been stated that enzymes released by bacteria may have an inflammatory effect on the gallbladder by deconjugating bile acids, which makes them more noxious to the gallbladder wall epithelium.[6,47]

A retrospective study by Peters et al. investigated the cytological findings of 140 bile samples from dogs and cats and the associated clinical pathological data.[3] Gallbladder wall histopathological analysis, bile cytology as well as bile bacterial culture were performed in 6 clinically ill dogs in the abovementioned study.[3] Of these 6 dogs none demonstrated signs of inflammation on bile cytological inflammation. However, 4 of the 6 dogs had histopathological evidence of inflammation on in gallbladder wall sections, with bacteria detected in 2 of these 4 dogs on both bile cytology and bile bacterial culture. Only one dog had histopathological evidence of bacteria in the gallbladder wall.[3] Unfortunately this study was performed in clinically ill animals making it difficult to extrapolate the results to apparently healthy dogs. Bile cytological examination did not demonstrate inflammatory changes in the majority of bactibilic dogs.[3] According to Peters et al. this suggests that bactibilia may not necessarily indicate true infection, but rather that transient, bactibilial colonization could potentially occur.[3] Other studies have also suggested that transient, self-limiting bactibilia can occur

in clinically healthy dogs.[2,6,11] In clinically sick dogs bactibilia without bile or gallbladder wall inflammatory changes have also been reported.[2,26, 48] This is similar to what has been reported in human literature where the bile of patients suffering from clinically significant cholecystitis may lack inflammatory cells.[2] Although most of the animals in the Peters et al. study demonstrated clinical signs consistent with hepatobiliary disease, these clinical signs could not be clearly attributed to gallbladder disease in those animals where inflammatory changes or bacteria were present on bile cytology, bacterial culture, or both as concurrent, non-hepatobiliary diseases were present in most animals.[3] There was inadequate follow-up information by Peters et al.[3] regarding the resolution of clinical signs or bactibilia after antibiotic therapy, which makes it impossible to determine if the finding of bactibilia was incidental or clinically important in that study.

This prospective study on the prevalence of bactibilia in apparently healthy dogs aimed to investigate the association between bactibilia and histopathological changes in the gallbladder wall and liver of healthy dogs in a large population of dogs, as hepatobiliary histopathological analyses play an important role in diagnosing clinically relevant bactibilia. Yet the diagnosis of biliary tract diseases, in contrast to neoplastic and parenchymal hepatic diseases, depends not solely on histopathological examination, but also on ultrasonography, serum biochemical, bile bacterial culture and cytological analyses.[3,10,15,42] Therefore histopathology of the liver and gallbladder wall, as well as all of the abovementioned tests besides ultrasonography were performed in this study.

Literature review conclusion

As bacterial cholangitis and cholecystitis in dogs may occur more frequently than suggested by current literature these conditions are important differential diagnoses that should not be overlooked in dogs with clinical signs such as icterus, pyrexia and abdominal pain.[8,10,48] Suspicion of these conditions should be confirmed by bile cytology, bile culture, serum biochemistry and histopathology, or ideally by a combination of these tests.[3] Yet, if the prevalence of bactibilia in healthy dogs is unknown the interpretation of the above-mentioned tests becomes difficult. This affects accurate clinical diagnostic and treatment decisions resulting in increased morbidity and mortality of patients suffering from bacterial cholangitis and cholecystitis. This study on the prevalence of bactibilia in apparently healthy dogs and its relation to liver enzymes and histopathology aims to bridge the large knowledge gap on this subject in the literature. It is currently the largest study, to the author's knowledge, investigating the prevalence of bactibilia in healthy dogs and the only study in canines which compares bile cytology, serum liver enzyme activities and hepatobiliary histopathology between healthy bactibilic and healthy non-bactibilic dogs.

CHAPTER 2

OBJECTIVES

1. To determine the prevalence of asymptomatic bactibilia in apparently healthy dogs.

2. To determine if the activities of serum liver enzymes ALP, ALT and GGT differ between bactibillic and non-bactibillic apparently healthy dogs.

3. To determine if liver and gallbladder histopathology differs between bactibillic and non-bactibillic apparently healthy dogs.

CHAPTER 3

RESEARCH HYPOTHESIS

Primary null hypothesis: Asymptomatic bactibilia will not be present in apparently healthy dogs.
Primary alternative hypothesis: Asymptomatic bactibilia will be present in apparently healthy dogs.

Secondary null hypothesis: There will be no significant difference in serum ALP, ALT and GGT activities between bactibilic and non-bactibilic apparently healthy dogs.
Secondary alternative hypothesis: There will be a significant increase in serum ALP, ALT and GGT activities in bactibilic compared to non-bactibilic apparently healthy dogs.

Tertiary null hypothesis: There will be no significant difference in liver and gallbladder histopathology between bactibilic and non-bactibilic apparently healthy dogs.
Tertiary alternative hypothesis: There will be significantly more liver and gallbladder histopathological abnormalities in bactibilic compared to non-bactibilic apparently healthy dogs.

CHAPTER 4

MATERIALS AND METHODS

4.1 Study design

A cross-sectional, prospective, observational study using clustered, random sampling was performed at the Animal Anti-Cruelty League (AACL), Epping, South Africa. Sixty-five apparently healthy, non-client-owned, abandoned dogs euthanased for non-medical reasons, as per standard AACL protocol (Appendix A), were included in this study.

4.2 Study Population

Inclusion Criteria:

Apparently healthy, non-client owned, abandoned dogs presenting at the AACL Epping were considered for inclusion in the study if the following criteria were met:

- Non-client owned, abandoned.
- Older than 6 months (based on mature dentition), of any sex or breed.
- Persons who handed in the dog to the AACL signed a standardised AACL consent form stating that the animal can be rehomed, treated or euthanased within a certain time period determined by the AACL.
- No history of clinical disease in the preceding 10 days.

Exclusion criteria:

- Pregnant animals.[49]
- Aggressive dogs in which restraint for blood collection prior to euthanasia without sedation would be overly stressful and unethical.
- Abnormalities detected on clinical exam immediately prior to euthanasia.
- Gross macroscopic abnormalities on necropsic evaluation of abdominal and thoracic viscera.
- Histopathological evidence of liver or gallbladder wall neoplasia.
- Gross macroscopic hepatovascular abnormalities on necropsic evaluation.
- History of receiving antimicrobial drugs in the preceding 10 days.

The AACL Epping general manager signed a consent form (Appendix B) at the start of the study allowing non-client owned, abandoned dogs at the AACL Epping meeting the inclusion criteria to be included in the study.

4.3 Sample collection and experimental procedures

Dogs were deemed apparently healthy based on history, clinical examination and macroscopic necropsy findings. Histories of the 65 apparently healthy, non-client owned, abandoned dogs involved in the study were collected in the form of copies from all AACL hospital records. These histories included any clinical abnormalities noted by AACL staff and any medication given in the 10 days prior to euthanasia at the AACL. Thorough clinical examinations were also performed by the primary investigator on these dogs immediately prior to euthanasia. The clinical abnormalities evaluated were predetermined and were assessed using a checklist (Appendix C). Cases that met the inclusion and exclusion criteria were enrolled sequentially in the study as they presented for euthanasia. The dogs in this study were euthanased after a set period of time as determined by AACL protocol (Appendix A) after unsuccessful attempts to rehome or rehabilitate the abandoned dogs. The time and the number of animals euthanased were not influenced by the study in any way.

Whole blood was collected aseptically from all dogs in the study via venepuncture of the dog's jugular vein after the clinical examination was performed, immediately prior to (no longer than 10 minutes before) euthanasia. Blood was drawn using a sterile 23 gauge 1.25 inch Terumo® needle[a] and a sterile 5mL Omnifix® syringe[b]. At least 3mL blood was placed into a 6mL BD Vacutainer® serum tube[c]. The serum tube was marked using a permanent marker with a unique patient number and then immediately stored on ice for the following 1 to 4 hours before the sample was centrifuged at the Vetdiagnostix Cape Town laboratory. Serum collected after centrifugation was used to determine the serum ALP, ALT and GGT activities in all dogs forming part of the study.

Euthanasia was carried out using an intravenous injection of Pentobarbital at 100 – 200mg/kg. Bile, liver and gallbladder wall samples were collected aseptically from all 65 dogs within 25 minutes of euthanasia. After euthanasia a ventral midline incision of the linea alba was made with a sterile Swann-Morton® Surgical Scalpel Blade no.15[d] and the gallbladder exposed via sterile blunt dissection by the primary investigator wearing sterile Sempermed® Classic Latex Surgical Gloves[e]. Four mL bile was aspirated through the intact gallbladder wall with a sterile 22 gauge 1.5 inch Terumo® needle[a] attached to a sterile 5mL Omnifix® syringe[b].[8] The needle was withdrawn after releasing the negative pressure on the syringe.

One mL bile was aseptically transferred to an Amies Charcoal swab PS+ Viscose[f] for bacterial culture immediately after sample collection. Culture swabs were marked using a permanent marker with a unique patient number and stored on ice for the following 1 to 4 hours before arriving at the Vetdiagnostix Cape Town branch. Here the samples were stored in a secure fridge at 2 – 8 °C for 1 to 3 hours before they were couriered on ice to the Vetdiagnostix Gauteng laboratory. Samples were stored at the Gauteng branch overnight on ice and placed on bacterial growth mediums there the following morning. Aerobic and anaerobic bacterial culture was therefore performed on bile samples from all dogs within 24 hours of sample collection, minimising the potential of storage errors.[50]

Two direct and two centrifuged bile smears from aspirated bile were made per dog for cytological examination. A direct bile smear was made at the AACL within 20 minutes of sample collection using a drop of bile and sterile Thermo Scientific microscope plain slides[g]. One mL of bile was centrifuged at the AACL and a sediment smear was made within 20 minutes of sample collection using sterile Thermo Scientific microscope plain slides[g]. The remaining 1 mL of the bile sample was placed in a 3mL BD Vacutainer® Ethylenediamine tetraacetic acid (EDTA) tube[c] marked using a permanent marker with a unique patient number and immediately stored on ice for the following 1 to 4 hours until the sample was received at the Vetdiagnostix Cape Town laboratory. At the laboratory another direct and centrifuged bile smear was made using sterile Thermo Scientific microscope plain slides[g].

Liver and gallbladder wall samples were collected, following bile collection, for histopathological examination from all dogs involved in the study. A single deep tissue sample of approximately 2 cm × 2 cm × 1 cm was taken from the centre of three liver lobes (left lateral lobe, right lateral lobe and left medial lobe) and three full thickness gallbladder wall samples (from the neck and body of the gallbladder) were collected by sharp dissection with a sterile Swann-Morton® Surgical Scalpel Blade no.15[d], within 25 minutes of euthanasia. All samples were immediately placed in neutral-buffered 10% formalin. After the prevalence of bactibilia was determined, histopathology was performed on gallbladder wall and liver samples from all dogs with evidence of bactibilia on cytological evaluation and/or bacterial culture as well as on gallbladder wall and liver samples from 9 dogs without bactibilia. The 9 non-bactibilic dogs that formed the control group were selected using a computer based random number selector website (www.random.org).

After samples for serum biochemistry, cytology, bacterial culture and histopathology were collected a necropsy examination was performed by the primary investigator by macroscopically examining all organs in the abdominal and thoracic cavity. The necropsy was performed after sample collection to

reduce the risk of contamination of samples. If morphological abnormalities were detected on necropsy that were not consistent with what can be expected to be a normal variation in a healthy dog, the dog was excluded from the study and the samples previously collected from the dog discarded.

4.4 Assay methodologies

Biochemistry

Whole blood was centrifuged 1 to 4 hours after sample collection. Delaying whole blood centrifugation up to 4 hours would not have affected the stability of ALT, ALP or GGT serum liver enzymes.[51] The samples were centrifuged at 1000 x *g* for 10 minutes using a Heraeus™ Megafuge™ 40 Centrifuge[h], the serum drawn off and placed in 1.5 mL Eppendorf Tubes®[i]. The serum was stored at -20°C, with daily temperature checks performed and recorded. The serum samples were stored for up to 150 days before being allowed to thaw to room temperature immediately prior to ALP, ALT and GGT serum biochemistry being performed using a Thermo Scientific™ Indiko™ Clinical and Specialty Chemistry System[h] and Thermo Scientific™ ALP, ALT and GGT reagents[h]. The analytic method used was subjected to quality control with the performance being adequate at the time of performing the abovementioned tests. Storing serum samples at -20°C for 150 days would not have affected the stability of ALT, ALP and GGT liver enzymes to such a degree that their activities would be clinically different from serum samples on which ALT, ALP and GGT biochemistry was performed on the same day of collection.[51] All serum biochemistry tests were performed by the same senior Vetdiagnostix laboratory technologist to ensure as much consistency as possible. ALT, ALP and GGT values were compared to the internal laboratory reference interval for these measurands. ALT values greater than 60 U/L, ALP values greater than 250 U/L and GGT values greater than 20 U/L were considered elevated.

Cytology

Two direct bile smears, one made at the AACL immediately after sample collection and a second made 1 – 4 hours after sample collection at Vetdiagnostix, were made using sterile Thermo Scientific microscope plain slides[g]. A sediment bile smear was made at the AACL within 20 minutes of sample collection by centrifuging bile at 1000 × *g* for 10 minutes using an Ortoalresa Microcen 6500 RPM centrifuge[j]. Another sediment bile smear was made by centrifuging bile at 1000 × *g* for 10 minutes within 1 – 4 hours after sample collection using a Cellspin® I centrifuge[k]. In the process of making the AACL and the Vetdiagnostix bile sediment smears a separate sterile 1 mL Surgiplus syringe[l] was used for each smear respectively to aspirate the bile sediment after the supernatant was decanted off and

sterile Thermo Scientific microscope plain slides[g] were used to make the smear. All the slides were air-dried and stained using Kyro-Quick stain[m] following the standardised Diff-Quik®[52] staining protocol of Vetdiagnostix for bile cytology. Cytology was performed by the primary investigator under the direct supervision and with the direct help of a specialist clinical pathologist using an Olympus CH30RF200 Biological microscope[n]. A total magnification of 1000x was used and the area of a smear visible under this magnification will be referred to as a high-power field (HPF) in this study. The primary investigator received extensive training in examining bile cytology smears by the abovementioned clinical pathologist prior to the commencement of this study. Every cytologically positive finding for bactibilia, inflammatory cells and/or other cells were directly confirmed by the clinical pathologist. The findings of cytological evaluation were recorded using a pre-set work sheet (Appendix E) where desired measurands to be measured were selected as absent or present and quantified to determine the prevalence of bactibilia. The primary investigator and the clinical pathologist were blinded to bile culture and biochemistry results during bile cytological examinations.

Bacterial culture

Bile samples from all dogs were submitted for aerobic and anaerobic culture. All submitted bile samples were plated on MediaMage or SelectaMedia sheep Blood Tryptose Agar[o], MacConkey Agar[o] and Thioglycollate broth[o] growth media within 24 hours of sample collection. The maximum 24-hour time period between sample collection and culture should not have affected the viability of bacteria.[50] The bile was transported in an Amies Charcoal swab PS+ Viscose[f] on ice. Specimens were inoculated on pre-reduced anaerobically sterilized media, processed and incubated in an anaerobic chamber for anaerobic culture. All bile samples were cultured at 35°C for 10 days and examined daily for growth. The presence and type of bacteria was evaluated using semi-quantitative culture method performed by a specialist veterinary bacteriologist. The same bacteriologist evaluated all samples to ensure more consistency in findings when compared to multiple observers. The bacteriologist was blinded to bile cytology and biochemistry results.

Histopathology

Liver and gallbladder wall histopathology was performed at the Vetdiagnostix Gauteng branch. After routine processing at this facility samples were cut in 5-µm sections and stained with Mayer's Haematoxylin and Eosin[p] (H&E) as well as Giemsa[q] stains for histopathological evaluation by a specialist veterinary anatomical pathologist.

Histopathology was performed by a single pathologist to reduce inter-observer variability.[53] Morphologic diagnoses of liver and gallbladder samples were made by the pathologist based on selecting certain standardized microscopic morphological abnormalities based on standardized guidelines from the WSAVA (World Small Animal Veterinary Association) Liver Standardization Group[42] using a pre-set table (Appendix F) which further increased the consistency of diagnoses. The presence of a standardized microscopic morphological diagnosis, the severity thereof (minimal, moderate, or severe) as well as the presence and morphological type of bacteria were indicated on the pre-set table. The liver microscopic morphological diagnoses listed included cholangiohepatitis, acute hepatitis, chronic hepatitis, neoplasia, necrosis, vacuolar degeneration, cholestasis, nodular regeneration, nodular hyperplasia, cirrhosis, hepatocellular atrophy, hepatovascular abnormality and no abnormality. The gall bladder microscopic morphological diagnoses in turn included cholecystitis, oedema, fibrosis and granulation, necrosis and no abnormalities. Inflammatory processes such as cholangiohepatitis, acute hepatitis, chronic hepatitis and cholecystitis were further sub-classified as subacute, acute or chronic with or without neutrophils, lymphocytes, plasma cells, necrosis and/or haemorrhage.

H&E stain as well as Giemsa stain were used on liver and gallbladder wall samples to allow adequate histopathological evaluation and for bacteria within liver and gallbladder wall samples to be identified with greater sensitivity than with H&E staining alone (Last, R., 2018, specialist veterinary anatomical pathologist, personal communication).[54,55]

4.5. Observations

- History.
- Signalment (Appendix C).
- Clinical examination (Appendix C).
- Necropsy findings.
- Cytological examination of bile (presence of bactibilia and presence of inflammatory cells) (Appendix D).
- ALT, ALP and GGT activities.
- Aerobic and anaerobic bacterial culture of bile.
- Histopathology of gallbladder wall and liver samples (Appendix E).

4.6. Data capture and statistical analysis

The data for history, signalment, clinical examinations, necropsy examinations, cytological evaluations and histopathological examinations was initially recorded manually and thereafter captured on Microsoft Excel® spreadsheets. Biochemical data was initially present in an electronic Vetdiagnostix laboratory results report and subsequently captured on a Microsoft Excel® spreadsheet. Statistical analysis was performed using a commercial software package (SPSS Statistics Software, IBM®SPSS® Statistics 245.0^{r}).

The Shapiro-Wilk test was used to assess data for normality. The percentage of dogs with bactibilia on cytological and/or bacterial culture analyses was calculated to determine the prevalence of bactibilia in apparently healthy dogs. The Cohen's kappa coefficient (κ) was used to measure the level of agreement between the qualitative diagnostic methods, namely bile cytology and bile culture. A 95% confidence interval (CI) was calculated. The number of inflammatory cells was compared between bactibilic and non-bactibilic dogs using the Mann-Whitney test. The Mann-Whitney test was used to compare the continuous variables of ALP, ALT, and GGT activities in dogs with bactibilia to dogs without bactibilia. Histopathological changes, if present, secondary to bactibilia, were noted. An ordinal scale was used for histopathological changes and was compared between dogs with bactibilia and the non-bactibilic control group using Mann-Whitney. The hepatic histopathological changes that ordinal scores of no abnormality, mild, moderate and severe were applied to included cholangiohepatitis, acute hepatitis, chronic hepatitis, necrosis, vacuolar degeneration, cholestasis, nodular regeneration, nodular hyperplasia, cirrhosis, hepatocellular atrophy and neoplasia. The same ordinal scores were also applied to gallbladder wall histopathological changes which included cholecystitis, oedema, fibrosis and granulation, necrosis and neoplasia. Descriptive statistics included the median and interquartile range (IQR) for all values unless stated otherwise. In dogs where histopathology was performed the Mann-Whitney test was used to compare age and the Chi-squared test was used to compare sex and breed between the bactibilic dog group and the non-bactibilic dog control group. A P-value of ≤0.05 was considered significant.

CHAPTER 5

RESULTS

Study population

A total of 84 apparently healthy, abandoned dogs euthanased for non-medical reasons were initially included in the study. However, 14 of these dogs were excluded based on abnormalities found on clinical examination, 3 were excluded based on abnormalities on necropsy and 2 were excluded due to their aggressive nature preventing stress-free manual restraint. The remainder of the dogs (n = 65) were found to be apparently healthy based on history, clinical examination and necropsy findings. These 65 dogs consisted of 26 males, of which 2 were neutered and 24 were intact, and 39 females, of which 8 were spayed and 31 were intact. Eight specific breeds and a large number of mixed breed dogs made up the study population (Table 1). The median age of dogs was 1 year (IQR: 1 – 3 years), with 89.23% (58/65) of dogs being younger than 5 years.

None of the 65 dogs in the study population received antibiotics in the 10 days prior to euthanasia. The only medication a small number of dogs received was an acaricide product (Zero® Ticks and Fleas) (Efekto® Garden and Home Pest Control, South Africa) which contains Fipronil, Butylhydroxianisole and Butylhydroxitoluene as active ingredients.

Whole blood samples from all dogs were collected no longer than 10 minutes before euthanasia. All bile, liver and gallbladder wall samples were collected within 25 minutes of euthanasia. The median time from euthanasia to end of bile, liver and gallbladder sample collection was 11 minutes (range 7 – 23 minutes). Four bile cytology smears, two direct and two centrifuged bile smears with one of each made at the AACL and at the Vetdiagnostix laboratory, were made in 63 dogs and three cytology smears were made in 2 non-bactibilic dogs. A centrifuged bile cytology smear for each of the latter 2 dogs could not be made at the AACL due to a 2.5 hour long regional power outage as part of intermittent electrical load-shedding applied in South Africa.

Bactibilia

The prevalence of bactibilia in the study population was 10.77%, with 7 out of the 65 dogs diagnosed with bactibilia on cytological examination and/or bacterial culture. Of the dogs with bactibilia 9.23% (6/65) of dogs were diagnosed on cytology and 4.62% (3/65) on bile culture, with 3.08% (2/65) of dogs testing positive for bactibilia on both bile cytology and bacterial culture. The level of agreement

between bile cytology and culture using Cohen κ analysis demonstrated weak agreement (κ = 0.408, p=0.001, CI (0.007, 0.824)).[56]

The median age of bactibilic dogs, which were positive on bacterial culture and/or bile cytology, was 2Y (IQR: 1 – 2.5). The bactibilic dogs group consisted of 4 intact male dogs and 3 intact female dogs. Mixed breed dogs predominated, with 5 dogs being of mixed breed. One Pit bull and 1 German Shepherd Dog made up the rest of the bactibilic dogs.

Bacteria isolated in the 3 dogs with bactibilia diagnosed on bacterial culture included *E. coli* (2/3) and *Streptococcus minor* (1/3). Bactibilia was also diagnosed on bile cytology in 2 of the abovementioned 3 dogs; specifically, in dogs were *E. coli* (2/3) was isolated. No bacteria were detected on cytology of the 1 dog in which *Streptococcus minor* (1/3) was isolated. Bacteria suspected to be contaminants were isolated from bile samples from 3 different dogs and consisted of *Staphylococcus epidermidis* (2/3) and *Corynebacterium striatum* (1/3). These suspected contaminants were not considered to be indicative of true bactibilia and were not included in the bactibilial prevalence calculations. Bactibilia was diagnosed on cytology in the dog were the suspected contaminant *Corynebacterium striatum* (1/3) was isolated. The bacteria detected on bile cytology in this dog had a different morphology to *Corynebacterium striatum* and was therefore considered not to be *Corynebacterium striatum* bacteria. No bacteria were detected on cytology of the dogs in which *Staphylococcus epidermidis* (2/3) was isolated.

In the 6 dogs diagnosed with bactibilia on cytological examination cocci were present in 2 dogs, while rods were found in the remaining 4 dogs. In 4 of these 6 dogs bactibilia was not present on bile bacterial culture. All bacteria seen on cytology were found to be extracellular. The bacteria were mostly present in relatively low numbers, with ≤2 bacteria per HPF in 5 dogs and 6 – 10 bacteria per HPF in 1 dog.

Inflammatory cells were detected on bile cytology in 10.77% (7/65) of dogs in the study population. Of these 7 dogs, 28.57% (2/7) had bactibilia (diagnosed on cytology and/or culture), while the remaining 71.43% (5/7) were non-bactibilic. In the total number of bactibilic dogs only 28.57% (2/7) showed inflammatory cells on bile cytological examinations, while in non-bactibilic dogs 8.62% (5/58) had inflammatory cells. There was no significant difference in the number of dogs with inflammatory cells between dogs with bactibilia and dogs without bactibilia (p = 0.11). The only type of inflammatory

cell detected was neutrophils. Other cells detected on bile cytology included red blood cells found in 1 dog, in which there was no bactibilia or inflammatory cells on bile cytological examination.

Serum liver enzymes

All serum biochemistry was performed in four batches on two separate days. Due to the sheer number of samples all the serum biochemistry could unfortunately not be performed in a single batch on the same day. Samples were thawed from frozen in batches and ALT, ALP and GGT biochemistry were performed immediately after the samples were adequately thawed. No sample was thawed and refrozen.

The data did not follow a normal distribution. No significant difference in the serum enzyme activity was seen between all bactibilic dogs and non-bactibilic dogs for ALT (40.0 U/L (IQR: 19.5 – 44.5 U/L); 28.5 U/L (IQR: 21.0 – 43.0 U/L), p = 0.88), ALP (50.0 U/L (IQR: 26.5 – 54.0 U/L); 54.0 U/L (IQR: 35.0 – 85.0 U/L), p = 0.26) and GGT (3.0 U/L (IQR: 0 – 5.0 U/L); 0 (IQR: 0 – 3.0 U/L), p = 0.22) respectively (Figure 2 – 4).

No significant difference in the serum enzyme activity was seen between dogs with bactibilia on cytological examination and dogs without bactibilia on cytological examination for ALT (32.0 U/L (IQR: 14.5 – 54.8 U/L); 29.0 U/L (IQR: 21.0 – 44.0 U/L), p = 0.77), ALP (50.5 U/L (IQR: 19.3 – 72.0 U/L); 53.0 U/L (IQR: 35.0 – 85.0 U/L), p = 0.50) and GGT (3.5 U/L (IQR: 0 – 7.3 U/L); 0 U/L (IQR: 0 – 3.0 U/L), p = 0.13).

No significant difference in the serum enzyme activity was found between dogs with and dogs without bactibilia on bacterial culture for ALT (40.0 U/L (IQR: 26.5 – 44.5 U/L); 28.5 U/L (IQR: 21.0 – 43.0 U/L), p = 0.94), ALP (31.0 U/L (IQR: 21.0 – 40.5 U/L); 54.0 U/L (35.0 – 85.0 U/L), p = 0.08) and GGT (0 U/L (IQR: 0 – 2.0 U/L); 0 (0 – 3.0 U/L), p = 0.83).

In bactibilic dogs, serum ALT and GGT activities were increased above the reference interval in 14.29% (1/7) and 14.29% (1/7) of dogs respectively, with ALT measuring 99 U/L (reference interval 10 – 60 U/L) and the GGT at 11 U/L (reference interval 1.2 – 6.4 U/L). In non-bactibilic dogs 8.62% (5/58) of dogs had elevated ALT activities and 3.45% (2/58) had elevated GGT activities, with ALT measuring 61 U/L, 66 U/L, 68 U/L, 83 U/L and 154 U/L and the GGT measuring 7 U/L and 7 U/L respectively. ALP was not elevated above the reference interval in any of the bactibilic or non-bactibilic dogs.

Hepatobiliary histopathology

Histopathology was performed on liver and gallbladder wall section samples from all bactibilic dogs (7/65) and from 9 non-bactibilic dogs (9/65) forming the control group. The non-bactibilic control group had a median age of 1 year (IQR: 0.75 – 2.00 years). There was no significant difference in age ($p = 0.536$) and breed ($p = 0.880$) between these two groups. There was a significant difference in sex ($p = 0.047$) between the bactibilic group and non-bactibilic control group (Table 2).

There were no significant differences in histopathological findings for each category evaluated between the dogs with bactibilia and those without bactibilia (Table 3).

The liver and gallbladder microscopic morphological abnormalities found, based on WSAVA Liver Standardization Group guidelines,[42] included cholangiohepatitis, vacuolar degeneration, cholestasis and nodular hyperplasia; and cholecystitis and oedema respectively. However, in each case of cholangiohepatitis and cholecystitis these morphological abnormalities were classified as subacute, without the presence of necrosis or haemorrhage, and with predominately lymphocytes and plasma cells present. There was no evidence of acute hepatitis, chronic hepatitis, liver necrosis, liver nodular regeneration, liver cirrhosis, hepatocellular atrophy, liver neoplasia, gallbladder fibrosis and granulation, gallbladder necrosis or gallbladder neoplasia in any of the bactibilic or non-bactibilic dogs.

In bactibilic dogs 42.86% (3/7) had mild cholangiohepatitis, 28.57% (2/7) had moderate cholangiohepatitis, 42.86% (3/7) had mild vacuolar degeneration, 42.86% (3/7) had moderate vacuolar degeneration, 14.29% (1/7) had mild liver cholestasis, 14.29% (1/7) had moderate liver cholestasis, 28.57% (2/7) had mild nodular hyperplasia, 14.29% (1/7) had moderate nodular hyperplasia, 14.29% (1/7) had mild cholecystitis, 85.71% (6/7) had moderate cholecystitis, 14.29% (1/7) had mild gallbladder wall oedema and 85.71% (6/7) had moderate gallbladder wall oedema.

In the non-bactibilic control group 55.55% (5/9) had mild cholangiohepatitis, 22.22% (2/9) had moderate cholangiohepatitis, 55.55% (5/9) had mild vacuolar degeneration, 33.33% (3/9) had moderate vacuolar degeneration, 22.22% (2/9) had mild liver cholestasis, 22.22% (2/9) had moderate liver cholestasis, 44.44% (4/9) had mild nodular hyperplasia, 11.11% (1/9) had moderate nodular hyperplasia, 44.44% (4/9) had mild cholecystitis, 55.55% (5/9) had moderate cholecystitis, 44.44% (4/9) had mild gallbladder wall oedema and 55.55% (5/9) had moderate gallbladder oedema.

H&E and Giemsa stains of liver section samples did not reveal any bacteria in the bactibilic group or the non-bactibilic control group. However, bacteria were detected on both H&E and Giemsa stains in the gallbladder walls of 28.57% (2/7) of bactibilic dogs and in 11.11% (1/9) of dogs in the control group. In all 3 of the above-mentioned dogs, cocci were detected on the stained gallbladder wall sections. However, in both bactibilic dogs only rods were detected either on both bile cytology and bacterial culture (1/2) or on bile cytology alone (1/2). Inflammatory cells were present on bile cytology smears of only 1 of the 2 (1/2) bactibilic dogs with gallbladder wall bacteria on histopathology. The non-bactibilic dog with bacteria in the gallbladder wall was 1 of 2 dogs in the control group in which inflammatory cells were detected on bile cytological examination. One (1/7) other bactibilic dog, which did not have bacteria on stained gallbladder sections, had inflammatory cells on bile cytology. Table 3 provides a summary of the bile culture, bile cytology and serum biochemistry results associated with the histopathological changes of dogs in the bactibilic and control groups.

Table 1. Signalment of the study population

Age (years)	Median (IQR)		1 (1 - 3)
Sex	Male	Intact	23
		Neutered	2
		Total	26
	Female	Intact	31
		Spayed	8
		Total	39
Breed	Mixed breed		40
	Pit bull Terrier		11
	German Shepherd Dog		5
	Jack Russell Terrier		4
	Boerboel		1
	Rhodesian Ridgeback		1
	Rottweiler		1
	Border Collie		1
	Boxer		1

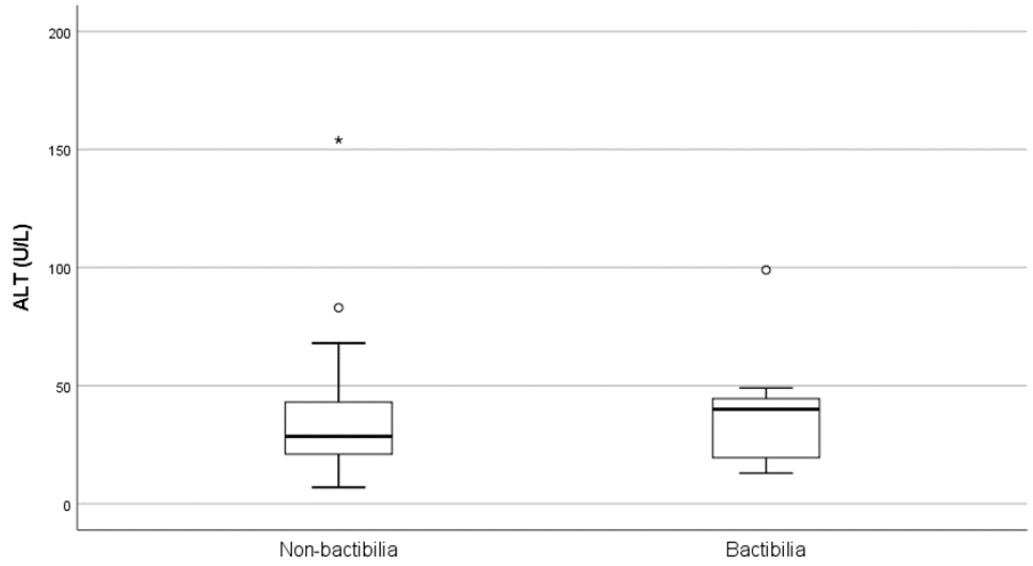

Figure 2. Box plot graph of ALT activities in bactibilic dogs and non-bactibilic dogs as diagnosed on bile culture and/or bile cytology. There was no significant difference between bactibilic and non-bactibilic groups ($p>0.05$). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending 1.5 times the IQR from the upper and lower quartiles. Mild outlier values (more than 1.5 x IQR) are denoted by an open circle (o) or star (*).

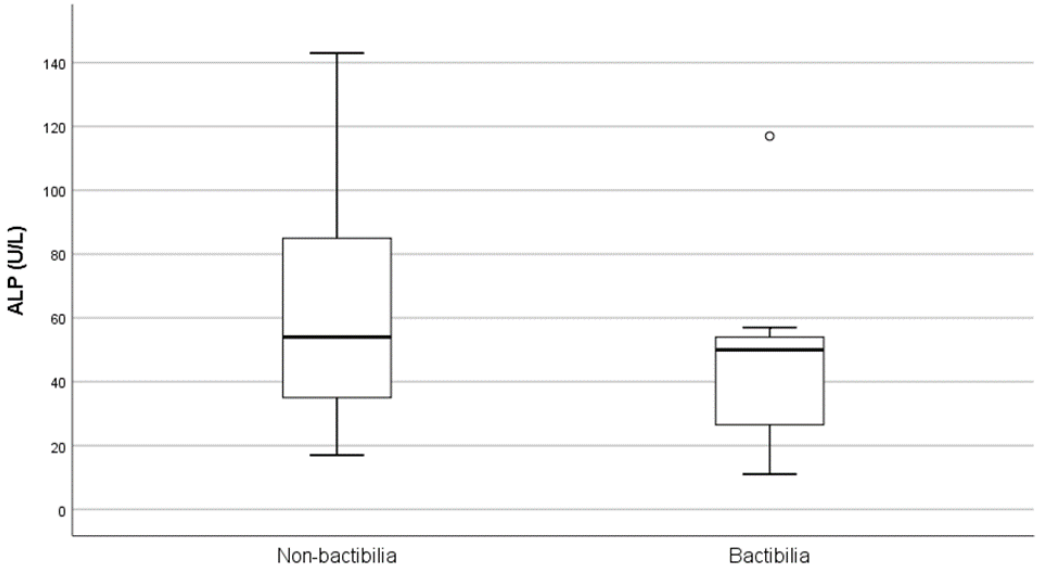

Figure 3. Box plot graph of ALP activities in bactibilic dogs and non-bactibilic dogs as diagnosed on bile culture and/or bile cytology. There was no significant difference between bactibilic and non-bactibilic groups ($p>0.05$). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending 1.5 times the IQR from the upper and lower quartiles. Mild outlier values (more than 1.5 x IQR) are denoted by an open circle (o).

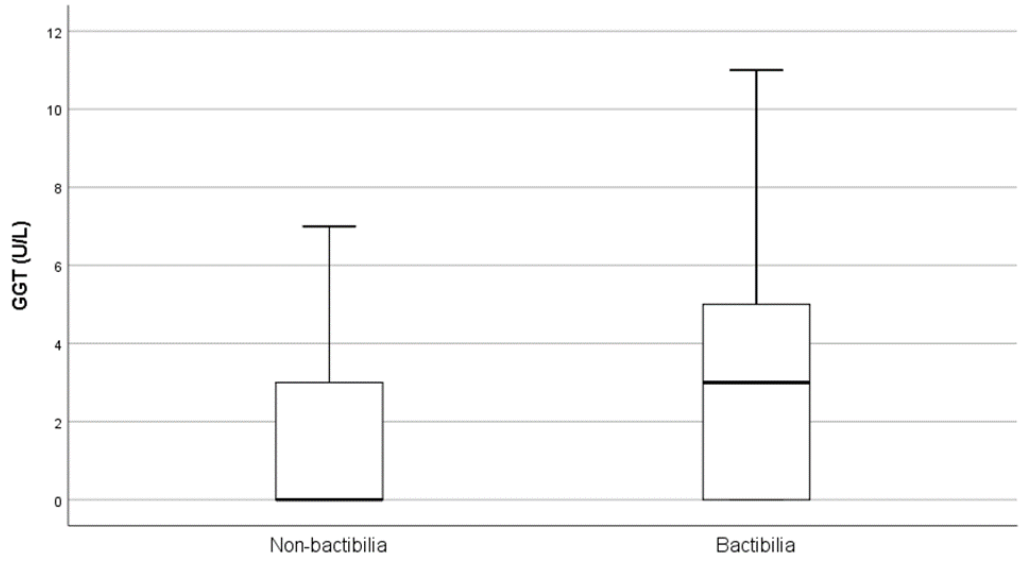

Figure 4. Box plot graph of GGT activities in bactibilic dogs and non-bactibilic dogs as diagnosed on bile culture and/or bile cytology. There was no significant difference between bactibilic and non-bactibilic groups ($p>0.05$). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending 1.5 times the IQR from the upper quartiles. Mild outlier values (more than 1.5 x IQR) are denoted by an open circle (o).

Table 2. Signalment of bactibilic dogs compared to the signalment of the non-bactibilic dogs control group

			Bactibilic dogs	Non-bactibilic dogs control group
Age (years)	Median (IQR)		2 (1 - 2.5)	1 (0.75 - 2)
Sex	Male	Intact	4	3
		Neutered	0	0
		Total	4	3
	Female	Intact	3	6
		Spayed	0	0
		Total	3	6
Breed	Mixed breed		5	6
	Pit bull Terrier		1	1
	German Shepherd Dog		1	2

Table 3. Histopathological abnormalities and associated bacterial culture, cytology and biochemistry results

	Bactibilia present		Inflammatory cells on cytology	Raised serum liver enzymes	Hepatic abnormalities				Gallbladder wall abnormalities		Bacteria present in gallbladder wall	
	Cytology	Culture			Cholangio-hepatitis *	Vacuolar degeneration*	Cholestasis*	Nodular hyperplasia*	Cholecystitis*	Oedema*	H&E stain	Giemsa stain
Bactibilic dogs												
1	cocci	x	✓	x		Moderate			Mild	Mild	x	x
2	cocci	x	x	x	Mild				Moderate	Moderate	x	x
3	rods	x	x	ALT & GGT		Moderate			Moderate	Moderate	x	x
4	rods	rods	x	x	Mild	Mild	Mild	Mild	Moderate	Moderate	x	x
5	rods	rods	x	x	Moderate	Mild	Moderate	Moderate	Moderate	Moderate	cocci	cocci
6	x	cocci	x	x	Mild	Moderate		Mild	Moderate	Moderate	x	x
7	rods	x	✓	x	Moderate	Mild			Moderate	Moderate	cocci	cocci
Non- bactibilic dogs (control group)												
1	x	x	✓	x		Moderate		Moderate	Moderate	Moderate	x	x
2	x	x	x	x	Mild	Mild			Moderate	Moderate	x	x
3	x	x	✓	x		Moderate		Mild	Mild	Mild	cocci	cocci
4	x	x	x	x	Moderate	Mild	Mild		Moderate	Moderate	x	x
5	x	x	x	x	Moderate	Mild	Mild	Mild	Mild	Mild	x	x
6	x	x	x	x	Mild		Moderate		Mild	Mild	x	x
7	x	x	x	x	Mild	Mild			Moderate	Moderate	x	x
8	x	x	x	x	Mild	Mild	Moderate	Mild	Moderate	Moderate	x	x
9	x	x	x	x	Mild	Moderate		Mild	Mild	Mild	x	x

* p-values for Mann-Whitney test comparing severity of histopathological changes in bactibilic dogs compared to non-bactibilic dogs: Cholangiohepatitis p = 1.00, Vacuolar degeneration p = 0.84, Cholestasis p = 0.61, Nodular hyperplasia p = 0.76, Cholecystitis p = 0.35, Oedema p = 0.35.

CHAPTER 6

DISCUSSION

This study strived to determine the prevalence of bactibilia in healthy canines and the data suggests that asymptomatic bactibilia may occur in apparently healthy dogs. Although study results did not identify significant differences in liver enzyme activities and hepatobiliary histopathological abnormalities between bactibilic and non-bactibilic healthy dogs in this study, the possibility of these differences existing in the population could not be excluded based on these results and further studies are needed to determine if these differences are present between the bactibilic and non-bactibilic dogs.

The prevalence of bactibilia in apparently healthy dogs was 10.77% in this study, which is higher than the bactibilial prevalence determined in healthy dogs in the Kook et al. study, where 2 positive bacterial cultures were identified from 7 sampling times in 6 dogs and cytological evidence of bactibilia once.[6] The current prevalence of bactibilia study is the largest study, to our knowledge, investigating the prevalence of bactibilia in healthy dogs.

The prevalence of bactibilia in healthy dogs identified in this study supports the hypothesis that asymptomatic bactibilia may be a normal phenomenon in healthy dogs and the presence of bacteria in bile without concurrent inflammation or biochemical changes may not affect their health.[6] These results are similar to those of the Niza et al. study which demonstrated that the liver of healthy dogs may harbour a variety of different bacterial organisms, without causing clinically detectable manifestations of disease.[24] Yet, the bactibilial prevalence study reported here was a cross-sectional study with bile samples only collected once from all dogs involved in the study at a single point in time. Although bactibilic dogs in this study did not have concurrent biliary inflammation or biochemical changes at the time of sample collection this may or may not be the case long term. Further studies where sequential bile, blood and hepatobiliary section sampling in apparently healthy dogs is performed are needed to adequately determine if bactibilia is transient and if bactibilic dogs remain asymptomatic or progress to develop hepatobiliary disease over time.

Experimental and clinical work suggest that continuous hepatobiliary exposure to enteric bacteria occurs during hepatobiliary-enteric circulation of bacteria.[6-9,24] All the bacteria isolated in this study, except for *Staphylococcus epidermidis* and *Corynebacterium striatum* which are believed to be

contaminants, are enteric in origin, further supporting this pathogenesis and the pathogenesis that ascending bacterial infection from the duodenum can occur. A few bacterial organisms may escape intrinsic killing mechanisms or may be present in bile prior to being excreted with bile into the gastrointestinal tract.[6] These bacteria can be detected on bile culture or cytological examinations, as demonstrated in this study. Clinically significant bacterial cholangitis and cholecystitis are unlikely to occur in dogs where biliary tract obstruction is absent and the bacteriostatic properties of bile acids are maintained.[6,11] If cholestasis or further disturbance of the biliary defence mechanisms occur, dogs may become susceptible to cholangitis,[3,5,10] as is seen in experimentally manipulated cats.[9] Biliary obstruction and the presence of bactibilia are the two predisposing factors in the development of biliary tract infections.[2,57] The pathogenic potential of the bacteria in bile may play a role in the potential risk of a patient developing clinical disease. In humans certain strains of bacteria, like *E. coli* and *Salmonellae*, are considered to be very bile resistant.[58] The ability of pathogenic and commensal bacteria to tolerate bile is likely to be important for their survival and subsequent colonization of the gallbladder.[58] Yet, it has been shown bactibilia alone does not cause clinical cholangitis,[2] which resonates with the results of this study which suggests that bactibilia can indeed be asymptomatic. This finding can be extrapolated to suggest that antimicrobial treatment might be unnecessary in asymptomatic dogs with bactibilia.

No specific breed predilection for bactibilia was found in this study. However, most dogs were mixed breed dogs and the signalment of dogs involved in this study were skewed as dogs with a certain signalment are more likely to become abandoned and present for euthanasia at welfare organizations.[59] Dachshunds, Shetland Sheepdogs, Border Terriers and American Cocker Spaniels have been identified in previous studies as predisposed to biliary disease.[1,15,44,48,60] None of these breeds were present in this study. The majority of dogs (89.23%) involved in the study were younger than 5 years of age, making it difficult to speculate if a certain age group of dogs are more prone to bactibilia as all age groups were not equally represented in the sample population. A significant difference in sex between the bactibilic dog group and the non-bactibilic dog control group was found in this study, with significantly more female dogs found in the control group (6/9) compared to the bactibilic group (3/7). However, due to the small sample size of the bactibilic group (n=7) and the non-bactibilic control group (n=9) this finding cannot be extrapolated to the population. Further studies are needed to determine if certain breeds, age groups and dogs of a certain sex are predisposed to asymptomatic bactibilia.

Bacteria isolated in the 3 dogs with bactibilia diagnosed on bacterial culture included *E. coli* (2/3) and *Streptococcus minor* (1/3), both of which are enteric bacteria. This is similar to bacterial isolates in other studies, where *Escherichia coli*, *Enterococcus* spp., *Bacteroides* spp., *Klebsiella* spp., *Streptococcus* spp., and *Clostridium* spp. were the most common true-positive bacterial isolates.[4,14,15,17] Knowledge of gastrointestinal flora that may ascend into the biliary tree is important in the context of interpreting culture results, given that this is the most commonly recognized route of entry in dogs.[5,14] In 3 dogs in this study bacteria that are not enteric in origin,[61,62] *Staphylococcus epidermidis* (2/3) and *Corynebacterium striatum* (1/3), were also isolated. These bacteria are most likely contaminants. Even though sample contamination is theoretically unlikely to occur in a study such as this study where a standard sterile sample collection technique was used, the possibility of sample contamination cannot be definitively excluded. Bacteria identified as contaminants comprised 9% of all biliary isolates in the Wagner et al. study and were primarily those associated with the skin, most likely the result of collection or specimen-handling contamination.[4] Isolates are generally considered contaminants based on bacterial identification, amount of growth, and length of time for growth detection.[4] *Staphylococcus epidermidis* is known to colonize the skin and mucous membranes of mammals and has not been identified as a true-positive bile bacterial isolate in previous studies.[4,61,63,64] Very few colonies of *Staphylococcus epidermidis* were detected only after 8 days of culture in two bile samples in this prevalence study reported here, suggesting that these bacteria most likely are contaminants. *Corynebacterium striatum* is known as an opportunist of man and animals.[62] In the dog in which *Corynebacterium striatum* was isolated only a single colony was isolated from 1 of the 4 growth media that this sample was inoculated on, which suggests that these bacteria most likely are contaminants.

There were conflicting bile culture and cytological results in this study, with the level of agreement between bile cytology and culture using Cohen κ analysis demonstrating a κ value of 0.408 (p=0.001, CI (0.007, 0.824)), which translates to a weak level of agreement between cytology and culture in asymptomatic bactibilic dogs.[56] This is similar to what was recorded in the Kook et al. and Peters et al. studies.[3,6] Bacteria was more commonly detected via bile cytology than via bacterial culture in the bactibilial prevalence study reported here, similar to what has previously been reported in other studies.[3,5] It is not clear whether the discrepancy between bile culture negative and cytology positive results points to suboptimal culture conditions. Yet, bile was cultured aerobically and anaerobically, within 24 h of collection, for 10 days duration, which should be sufficient for bacterial growth.[50] It is possible that low numbers of culturable bacteria in these healthy dogs may have resulted in negative culture results.[5] None of the dogs involved in this prevalence study received any antibiotics in the 10

days prior to sample collection, which is a longer period than what has been documented in dogs involved in other important bactibilia studies.[3,5] Another possible explanation would be suppression of bactibilia by bile growth-inhibiting factors, such as lysozyme and lactoferrin, which act as local defence mechanisms against bacterial infection in humans.[6,65] Bile has a bacteriostatic effect and some bacteria such as *Helicobacter spp.* are difficult to culture.[3] Microscopic detection of bacteria provides no information regarding bacterial viability and it is known that dead and certain live bacteria are not culturable by routine culture methods.[5,66,67] Therefore, some of the bacteria detected on bile cytology in this study may not have been viable and/or culturable. 16S ribosomal RNA gene sequencing techniques can be used to detect some unculturable bacterial groups and has been used successfully in humans to detect specific bacterial *spp.* in bacteraemic cholecystitis which have not been identified before with conventional bile culture techniques.[68,69] As 16S ribosomal RNA gene PCR is not yet readily available in veterinary private practice and due to the significant costs involved in performing these tests, 16S ribosomal RNA gene sequencing was unfortunately not performed in this study. Further studies employing bile cytology, bile bacterial culture and 16S ribosomal RNA gene PCR to detect bactibilia are needed to further investigate the prevalence of bactibilia in apparently healthy dogs.

In the suspect true-bactibilia positive dog where *Streptococcus minor* bacteria were isolated on bacterial culture, but no bacteria detected on bile cytological examination, sample contamination could potentially have occurred. A low bile bacterial load in this dogs was however considered to be a more likely explanation as it is an enteric bacterium in dogs and as *Streptococcus* spp. are common true-bactibilial isolates in other studies investigating bactibilia.[4,70]

Inflammatory cells were detected on bile cytology in 10.77% (7/65) of dogs in the study population, of which 2 dogs had bactibilia. Therefore, the percentage of bactibilic dogs with inflammatory cells was 28.57% (2/7), compared to non-bactibilic dogs were 8.62% (5/58) of dogs had inflammatory cells. No significant difference in the number of dogs with inflammatory cells between dogs with bactibilia and dogs without bactibilia was found, further suggesting that bactibilia can be asymptomatic in apparently healthy dogs. Neutrophils were the only inflammatory cells seen in this study. This is similar to a previous larger-scale study by Peters et al. where poorly preserved or degenerate neutrophils predominated in samples (n=3) and bactibilia was seen in only one of the 3 cases with inflammatory cells.[3] In samples over 12 h old a deterioration of cellularity and cell preservation was observed in smears made in their laboratory when compared to fresh bile smears.[3] In the Pashmakova et al. study 12 bile samples in which bacteria were identified microscopically had no microscopic evidence of inflammation; however, all 5 samples in which inflammatory cells were identified also contained

extracellular bacteria. Inflammatory cells were lacking in most samples in which bacteria were identified microscopically.[5] The presence of inflammatory cells in bile can be negatively affected by the chemical properties of bile. Bile can suppress leucocyte activation and cause phagocytosis of leucocytes.[5] Although this possibility has not been extensively described in the veterinary literature, a case report does exists of non-inflammatory bactibilia in a dog with clinically relevant bacterial cholecystitis.[2,5] Fresh bile smears together with bile fluid should be submitted to laboratories for analysis in order to prevent potentially inaccurate bile cytology results, as the cell count and bacterial overgrowth can decrease in bile fluid over time.[3]

The lack of significant differences in median liver enzyme activities between bactibilic and non-bactibilic healthy dogs found in this study supports the suspicion of the asymptomatic nature of bactibilia in a small proportion of healthy dogs. Lawrence et al. compared clinicopathologic findings between dogs with (n=10) and dogs without (n=30) bacterial cholecystitis or bactibilia, and recorded elevated serum liver enzyme activities without clinical signs of hepatobiliary disease in 50% (2/4) of dogs with bactibilia.[1] However, the sample size of bactibilic dogs was small making extrapolation to the general canine population difficult. This prevalence study of bactibilia in healthy dogs recorded here had similar results to a study characterizing the prevalence, identity, and antimicrobial susceptibility of common hepatobiliary isolates from dogs and cats with hepatobiliary disease,[4] where no significant correlation between raised ALT and ALP activities and positive bile bacterial cultures were detected. The lack of a significant difference in the proportion of dogs with elevated serum liver enzymes between bactibilic and non-bactibilic dogs in the bactibilial prevalence study reported here also supports the suspicion that asymptomatic bactibilia may be present in apparently healthy dogs. However, this study only evaluated serum liver enzyme activities at a single point in time due to the cross-sectional nature of the study, and does not provide any information on the trend of serum liver enzyme activities over time in apparently healthy bactibilic dogs compared to non-bactibilic dogs. Even though no significant differences in serum liver enzymes ALT, ALP and GGT activities were detected between bactibilic and non-bactibilic healthy dogs in this study at the time of sample collection, the possibility of these differences developing over time cannot be ruled out. Therefore, the possibility of no significant differences in serum liver enzyme activities between bactibilic and non-bactibilic dogs existing in the population could not be excluded based on the results of this study alone. Further studies are needed to definitively determine if significant differences in serum liver enzymes ALT, ALP and GGT activities occur between bactibilic and non-bactibilic healthy dogs.

The results of this study support the suspicion that there are no significant hepatobiliary histopathological differences between asymptomatic bactibilic dogs and non-bactibilic dogs. None of the bactibilic and non-bactibilic dogs' hepatobiliary histopathological analyses revealed neutrophilic inflammation, haemorrhage or necrosis. Cholangitis has been previously defined as the demonstration of neutrophilic infiltration of portal areas, with or without extension into the hepatic parenchyma.[10] Cholecystitis in turn has been defined as infiltration of neutrophils into the gallbladder wall.[10] In a retrospective study investigating gallbladder histopathology in dogs with gallbladder disease or rupture, 11 bactibilic dogs had predominant histologic changes of necrosis (10/11), thrombosis (7/11), haemorrhage (7/11), and suppurative inflammation (7/10).[48] The findings of 1 or more combinations of necrosis, thrombosis, haemorrhage, and mucosal hyperplasia of the gallbladder is believed to reflect the primary pathologic features of canine gallbladder disease, and a significant relationship has been found previously between the histologic identification of gallbladder necrosis and gallbladder rupture.[48] Therefore, based on previous studies, the lack of neutrophils,[17] haemorrhage, thrombosis and necrosis in liver and gallbladder wall sections in this prevalence study suggests that the histological changes detected were most likely not related to the presence of bactibilia.

Although no significant hepatobiliary histopathological differences were found between the bactibilic and non-bactibilic dogs in this study, the possibility of these differences existing in the population could not be excluded based on the results of this study alone. Liver and gallbladder wall samples were collected after euthanasia in this study, which might have had an effect on the histopathological findings detected in these samples. Samples collected after Pentobarbital euthanasia in mice have been reported to have subtle euthanasia and post-mortem related histopathological changes when compared to samples collected ante-mortally or under anaesthesia.[71,72] Thus, the histopathological changes reported here might also not be truly equivalent to the histopathological changes present in the healthy dog study population. Similarly, it is not known what effect post-mortem collection of bile will have on the inflammatory cell and bacterial composition of bile in dogs. However, all samples were collected within 25 minutes of euthanasia making the time for changes to occur relatively short, decreasing the likelihood of bacterial contamination and inflammatory bile changes. Thus, although all dogs, bactibilic and non-bactibilic, were submitted to the same euthanasia and sample collection protocol and might demonstrate similar euthanasia and post-mortem related changes, further studies are needed to definitively determine if significant differences in hepatobiliary histopathology occur between bactibilic and non-bactibilic healthy dogs.

H&E and Giemsa stains of the gallbladder wall demonstrated bacteria in 2 bactibilic dogs and 1 non-bactibilic dog in the non-bactibilic control group. These results show a discrepancy between the rod-shaped bacteria detected in bile (seen on cytology in 2 dogs and on bacterial culture in 1 dog) and the cocci-shaped bacteria detected in stained gallbladder wall sections in the 2 bactibilic dogs that had bacteria in their gallbladder walls. This is in contrast to previous studies where the same bacterial isolates were detected in the bile and gallbladder wall cultures of clinically ill dogs (n=5).[10] However, dogs with hepatobiliary disease are equally likely to have single or multiple bacterial species isolated on hepatobiliary cultures,[4] which could explain why different bacterial morphologies were detected in these two bactibilic dogs in this study. Bile or gallbladder wall cultures in Tamborini et al. yielded a far higher proportion of positive bacterial culture results than liver cultures,[10] in agreement with Wagner et al.[4] Similar findings regarding a lower prevalence of bacteria in the liver sections compared to a higher prevalence of bacteria in gallbladder wall sections on histopathological analyses was found in bactibilic dogs and in the non-bactibilic control group dogs in this study.

Several limitations inherent to the clinical nature of this study were present. The greatest limitation to declaring a true bactibilial prevalence in this study was the inadequate sample numbers to show a true prevalence of 10%, which was based on only 65 dogs. Sampling to estimate a 10% population prevalence of bactibilia, using a 95% confidence interval and desired absolute precision-allowable error of 5%, a calculated sample size of 138 dogs would have been necessary to render this study statistically significant to test the previously mentioned hypotheses. However, due to cost and time constraints only 65 healthy dogs could be enrolled in this study. Thus, this study is underpowered with regards to the prevalence of bactibilia, but still shows bactibilia is prevalent in apparently healthy dogs. The inadequate sample size is also a limitation with regards to the secondary and tertiary aims of this study, as failure to demonstrate differences in serum liver enzyme activities and hepatobiliary histopathology between bactibilic and non-bactibilic dogs could have been influenced by the underpowered nature of this study and thus might not be a truly accurate reflection. Another limitation of this study is that bile, liver enzyme activities and hepatobiliary histopathology were only evaluated at a single point in time. No information is therefore available on the possible trends in bactibilia, serum liver enzyme activities and hepatobiliary histopathology over time and what effect bactibilia has on the latter two measurands over time. No conclusions can therefore be drawn regarding whether or not bactibilia remains asymptomatic over time. Further studies where sequential bile, blood and hepatobiliary section sampling in apparently healthy dogs is performed are needed to adequately determine if bactibilia is transient and if bactibilic dogs remain asymptomatic or progress to develop hepatobiliary disease over time. The collection of bile, gallbladder wall and liver

samples after euthanasia is also a limitation of this study. This might have had an effect on the histopathological findings detected in these samples and these findings might not be truly reflective of the hepatobiliary histopathological findings in the apparently healthy dog study population. As a result of all of the abovementioned limitations, which may have led to differences in serum liver enzyme activities and hepatobiliary histopathology between bactibilic and non-bactibilic dogs possibly not being identified in this study, the secondary and tertiary null hypotheses of this study could not be rejected. Thus, even though no significant differences in median liver enzyme activities and hepatobiliary histopathology were found between the bactibilic and non-bactibilic dogs in this study, the possibility of these differences existing in the population could not be definitively excluded. Further studies are needed to determine if significant differences in median liver enzyme activities and hepatobiliary histopathological abnormalities are present between the bactibilic and non-bactibilic dogs.

The limited history of dogs involved in this study available to the primary investigator is a limitation in this study. In most dogs, history was only available for the 10 days prior to euthanasia, with some dogs having longer histories available. This is a limitation as it is not known if certain dogs involved in the study have had hepatobiliary disease in the time period prior to 10 days before euthanasia. Another limitation of this study is that hepatobiliary histopathology was performed on only 9 non-bactibilic dogs and not on all non-bactibilic dogs in this study, due to cost constraints. This is a limitation as abnormalities or bacteria might have been present in the liver and gallbladder walls of the 47 non-bactibilic dogs that did not undergo histopathological analyses which could have affected the study results.

This study confirms that bactibilia does occur in a small number of apparently healthy dogs, even though bile is generally considered sterile. In clinically ill animals, bactibilia may signify the presence of bacterial cholangitis or cholecystitis and warrant therapeutic intervention.[5] Bacterial cholecystitis is a serious hepatobiliary disease in dogs, which if left untreated, can lead to potentially life-threatening consequences such as gallbladder rupture, cholangiohepatitis, and sepsis.[2,48,73] Both diagnostic and clinical data should be carefully interpreted to guide clinical decision making regarding treatment of individual dogs with bactibilia.[5] This study suggests bactibilia may be an incidental finding in dogs not symptomatic and without elevations in liver enzymes or hepatobiliary histopathological changes, as has been previously speculated in veterinary literature.[5] Whether these dogs progress to have clinical biliary disease is unknown.[1,6] Further studies are needed to determine if asymptomatic bactibilia predispose dogs to developing clinically detectable cholecystitis. Although no significant

differences in median liver enzyme activities and hepatobiliary histopathology were found between the bactibilic and non-bactibilic dogs in this study, the possibility of these differences existing in the population could not be excluded based on the results of this study alone. Further studies are needed to determine if significant differences in median liver enzyme activities and hepatobiliary histopathology are present between the bactibilic and non-bactibilic dogs.

CHAPTER 7

CONCLUSIONS

The prevalence of bactibilia in apparently healthy dogs is 10.77%.

Further studies employing bile cytology, bile bacterial culture and 16S ribosomal RNA gene PCR to detect bactibilia are needed to investigate the prevalence of bactibilia in apparently healthy dogs.

Further studies are needed to determine if significant differences in serum liver enzymes ALT, ALP and GGT activities occur between bactibilic and non-bactibilic healthy dogs.

Further studies are needed to determine if significant differences in hepatobiliary histopathological findings occur between bactibilic and non-bactibilic dogs.

Further studies are needed to determine if certain breeds are predisposed to asymptomatic bactibilia.

Further studies are needed to determine if ultrasonographic abnormalities are present in dogs with asymptomatic bactibilia.

Further studies are needed to determine if bactibilia predispose dogs to developing clinically detectable cholecystitis.

FOOTNOTES

[a] Terumo Medical Corporation, USA

[b] B. Braun Medical (Pty) Limited, South Africa

[c] Becton Dickinson, UK

[d] Swann-Morton®, England

[e] Semperit®, Austria

[f] Deltalab, Spain

[g] Thermo Fisher Scientific, ZA

[h] Thermo Fisher Scientific, USA

[i] Eppendorf, Germany

[j] Ortoalresa, Spain

[k] Tharmac®, Germany

[l] Surgiplus, China

[m] Kyron Laboratories, South Africa

[n] Olympus Optical Co. Ltd., Japan

[o] MediaMage, South Africa

[p] Climaks Scientific, South Africa

[q] Merck Group, USA

[r] SPSS Inc.

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APPENDIX A:

The AACL Epping protocol for surrendered dogs, stray dogs and euthanasia – a brief description:

Surrendering dogs to AACL:

People who surrender their own dogs to the AACL Epping sign a standardized consent form on admission of the dog to the AACL stating that:

1. The dog can immediately be rehomed or be transferred to a rehoming facility.
2. The dog can be euthanased immediately as per AACL decision based on the state of the dog on admission.
3. The dog can be kept in hospital until it is rehomed or until space at the rehoming facility becomes available

Reuniting stray dogs with their rightful owners:

Dogs that are brought to the hospital as strays without any owners are:

1. Scanned for a microchip.
2. Posted on social media networks and other platforms in an attempt to find the dog's owner.
3. Kept for 10 days pending possible reunion with owner. If the owner does not come forward within 10 days the previously mentioned protocol for surrendered dogs will then apply to the stray.

Euthanasia of dogs at AACL:

Dogs are euthanased one by one in a private room specifically dedicated to this purpose. No dog is allowed to witness another dog being euthanased. In the case of a litter of puppies being euthanased they may be kept in a crate in the same room as where the euthanasia of a litter mate is taking place, however they are still not allowed to witness the euthanasia process.

Dogs are restrained by an experienced kennel hand during euthanasia to ensure as little stress and discomfort to the dog occurs. Dogs that are extremely stressed may receive sedatives prior to euthanasia*.

Dogs are humanely euthanased by an AACL veterinarian by intravenous injection of Pentobarbital.

The bodies of euthanased dogs are disposed of by an outside pet cremation and burial service company called R.I.Pets.

**These dogs were not used in this study as sedation might affect the test results and the animals might stress unnecessarily during blood collection prior to euthanasia.*

APPENDIX B:

Consent form for the prevalence of bactibilia in apparently healthy dogs study:

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

I agree that surrendered dogs or stray dogs that have not been claimed or successfully rehomed after a standardized, predetermined time period by the Animal Anti-Cruelty League (AACL) and are euthanased by AACL staff may be selected to serve as healthy dogs for a study to aid in evaluating the prevalence of bactibilia (bacteria in bile) in apparently healthy dogs.

I understand that sample collection may only take place if a dog is euthanased after a set period of time as normally determined by the AACL. The decision to euthanase an animal may solely be made by the AACL staff after unsuccessful attempts to rehome or rehabilitate the abandoned dog, and is in no way influenced by this study. The dog will in no way be harmed nor will the AACL be charged for any study related procedures.

I understand that the primary investigator, Dr Elize Verwey, can be contacted at any time with any query relating to the study with the contact details listed below:
Dr E. Verwey
Tygerberg Animal Hospital, Bellville, South Africa, 7550
Tel: 021 919 1191

I, Venessa Strauss (name)
General Manager. (position at AACL), hereby give permission that surrendered dogs; or stray dogs that have not been claimed by their owners after a set time period may participate in this clinical study in association with the University of Pretoria should they be euthanased as per AACL staff decision after a predetermined period of time and unsuccessful rehabilitation or rehoming. The trial has been explained to me and I understand that this study will in no way harm the dogs involved in the study. Furthermore, I understand that no additional costs will be incurred by the AACL in respect of this trial for the collection of samples. I give permission to the primary investigator of this study, Dr Elize Verwey (8706200046088), to collect blood samples immediately prior to euthanasia, to perform a post-mortem examination and to collect bile, gallbladder wall and liver samples after euthanasia.

Signed at AACL Epping on the 26th day of Sept 20 18

Signature Strauss

Work Tel: 0215346426

Cell No: 0823254638

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APPENDIX C:

PATIENT LABEL

(Includes unique number)

Clinical examination form

Clinical signs*	Present	
	Yes	No
Inappetance		
Vomiting		
Diarrhoea		
Coughing		
Pyrexia		
Icterus		
Dehydration		
Emaciation		
Infected wounds		
Abscess		
Seizures		
Neurological abnormalities		
Generalised lymphadenopathy		
Gingivitis / oral disease		
Generalised skin lesions		

**Present on clinical examination immediately prior to euthanasia or present within 5 days of sample collection.*

Age:

Sex:

Breed:

Intact or neutered/spayed or pregnant:

Antibiotic exposure in the previous consecutive 10 days:

Gross macroscopic abnormalities detected on necropsic examination of abdominal and thoracic viscera:

APPENDIX D:

PATIENT LABEL (Includes unique number)

Cytological evaluation form

Cytological finding	Present	Absent
Bacteria		
White blood cells		
Red Blood cells		
Other cells		
Other organisms		
Debris		
Crystals		

If other cells are present please list the cells:

If bacteria are present please complete the following:

Bacterial morphology		
Rods	Cocci	Other

If bacteria of a different morphology than rods or cocci are seen please describe:

Bacterial findings		
Intracellular	Extracellular	Both

Quantification of bacteria*				
1	2	3	4	5
< 2 per HPF	3 – 5 per HPF	6 -10 per HPF	11 – 20 per HPF	>20 per HPF

**An estimation of the number of bacteria seen in the form of number per high-power field (HPF).*

Quantification of white blood cells[×]				
1	2	3	4	5
< 2 per HPF	3 – 5 per HPF	6 -10 per HPF	11 – 20 per HPF	>20 per HPF

[×] *An estimation of the number of white blood cells seen in the form of number per high-power field (HPF).*

	White blood cells present				
	Neutrophils	Macrophages	Lymphocytes	Eosinophils	Other[ɬ]
< 2 per HPF					
3 – 5 per HPF					
6 -10 per HPF					
11– 20 per HPF					
>20 per HPF					

[ɬ] *If other white blood cells are present please list:*

APPENDIX E:

PATIENT LABEL

(Includes unique number)

Hepatic histopathological abnormalities:

Morphological abnormalities

Microscopic morphological diagnosis*	Severity score			
	No abnormality	Mild	Moderate	Severe
Cholangiohepatitis				
Acute hepatitis				
Chronic hepatitis				
Necrosis				
Vacuolar degeneration				
Cholestasis				
Nodular regeneration				
Nodular hyperplasia				
Cirrhosis				
Hepatocellular atrophy				
Neoplasia				
No abnormality				

** Based on the World Small Animal Veterinary Association (WSAVA) Liver Standardization Group guidelines.*

Inflammatory hepatic abnormalities

Abnormality	Subacute	Acute	Chronic	Neutrophils	Lymphocytes	Plasma cells	Necrosis or haemorrhage
Cholangio-hepatitis							
Acute hepatits							
Chronic hepatitis							

Bacterial prevalence

Bacteria present	Yes		No	
	H&E stain	Giemsa stain	H&E stain	Giemsa stain
Cocci				
Rods				
Other				

If bacteria of a different morphology than rods or cocci are seen please describe:

Gall bladder wall histopathological abnormalities:

Morphological abnormalities

Microscopic morphological diagnosis*	Severity score			
	No abnormality	Mild	Moderate	Severe
Cholecystitis				
Oedema				
Fibrosis and granulation				
Necrosis				
Neoplasia				
No abnormality				

Inflammatory gallbladder wall abnormalities

Abnormality	Subacute	Acute	Chronic	Neutrophils	Lymphocytes	Plasma cells	Necrosis or haemorrhage
Cholecystitis							

Bacterial prevalence

Bacteria present	Yes		No	
	H&E stain	Giemsa stain	H&E stain	Giemsa stain
Cocci				
Rods				
Other				

If bacteria of a different morphology than rods or cocci are seen please describe: