



## NOTE

Bacteriology

# Screening of bacterial DNA in bile sampled from healthy dogs and dogs suffering from liver- or gallbladder-associated disease

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**ABSTRACT.** Although the biliary system is generally aseptic, gallbladder microbiota has been reported in humans and some animals apart from dogs. We screened and analyzed the bacterial deoxyribonucleic acid in canine gallbladders using bile sampled from 7 healthy dogs and 52 dogs with liver- or gallbladder-associated disease. PCR screening detected bacteria in 17.3% of diseased dogs (9/52) and none in healthy dogs. Microbiota analysis of PCR-positive samples showed that the microbial diversity differed between liver- and gallbladder-associated disease groups. Thus, a specific bacterial community appears to occur at a certain frequency in the bile of diseased dogs.

**KEYWORDS:** bile, bile microbiota, gallbladder, hospitalized dog, laboratory dog

The biliary system is typically maintained in a sterile condition by the continuous flushing action of bile, the antimicrobial effects of bile salts, and by the sphincter of Oddi, an anatomical barrier to bacterial invasion [4]. Although the presence of gallbladder microbiota has been reported in healthy humans and animals [3, 5, 6, 14], the hostile environment of the biliary system to bacteria and the limited number of studies because of difficulties in accessing biological samples mean that the presence of bacteria in the gallbladder remains controversial.

Bile samples from dogs are more accessible than those from humans and other animals because the samples can be obtained from dogs in animal hospitals and from laboratory dogs. Bile can be the optimal sample to study gallbladder microbiota. However, there have been few studies on gall bladder microbiota in dogs [5]. In the present study, we screened the presence of bacterial DNA in bile by PCR and analyzed the bile microbiota in positive samples, using healthy laboratory dogs and hospitalized dogs.

Seven healthy laboratory beagles and 52 hospitalized dogs were used in this study (Supplementary Table 1). The hospitalized dogs were being treated for liver-associated disease (16 dogs with hepatic microvascular dysplasia, 20 dogs with port systemic shunt, and 1 dog with hepatic fibrosis) and for gallbladder-associated disease (2 dogs with cholecystitis, 1 dog with cholecystitis/biliary sludge, 5 dogs with biliary sludge, and 7 dogs with gallbladder mucocele).

Bile was collected from the gallbladder of the anesthetized dog by sterile technique with ultrasound-guided cholecystocentesis, or directly from a gallbladder by cholecystocentesis at surgery, with the owner's consent. The care and handling of the animals were in accordance with the Azabu University Animal Experiment Guidelines. All experiments were reviewed and approved by the Ethics Committee of Azabu University (approval number: 180220-2).

DNA was extracted directly from 0.4 ml of bile into 12 µl of TE buffer (10 mM Tris-HCl, 0.2 mM ethylenediaminetetraacetic acid, pH 7.5) using an ISOSPIN Fecal DNA kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Various amounts of DNA, ranging from ca. 50 ng to 1 µg, were extracted and were measured using a spectrophotometer (NanoDrop ND 1000, Thermo Scientific, Wilmington, DE, USA) (Supplementary Table 1).

The 16S ribosomal RNA (rRNA) gene was amplified by PCR using KAPA2G™ Fast HotStart ReadyMix with dye (Nippon Genetics, Tokyo, Japan) and the following primer set: (Pro341F, 5'-CCTACGGGNBGCASCAG-3'; Pro805R, 5'-GACTACNVGGGTATCTAATCC-3') [9, 11]. We optimized the PCR conditions, which showed that no false-positive PCR

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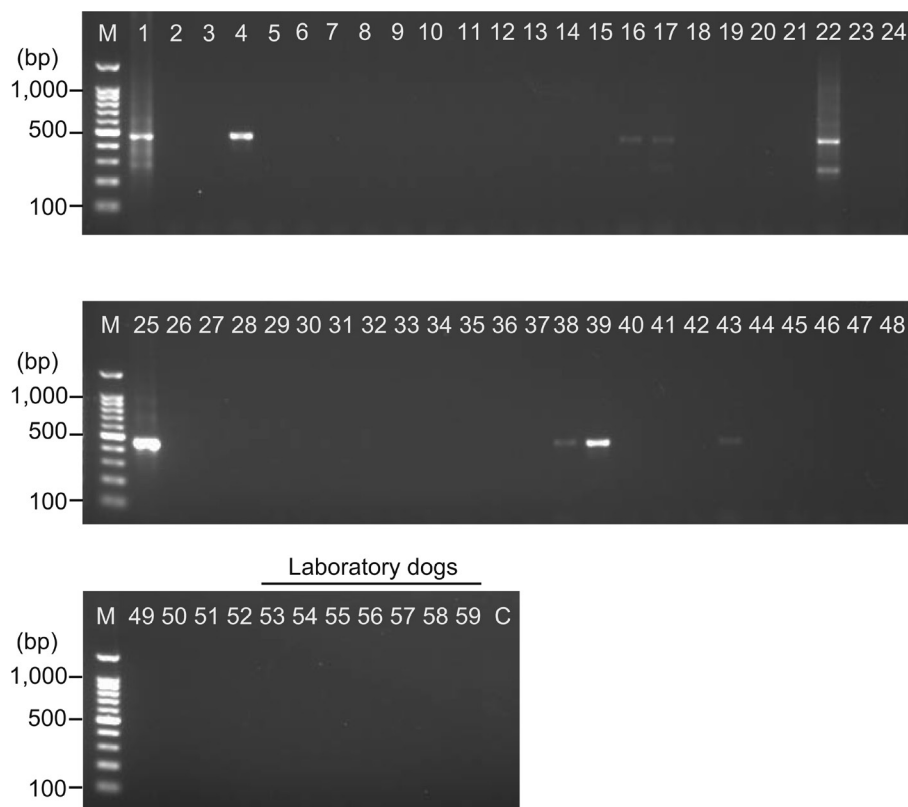
#These authors contributed equally to this work.

(Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

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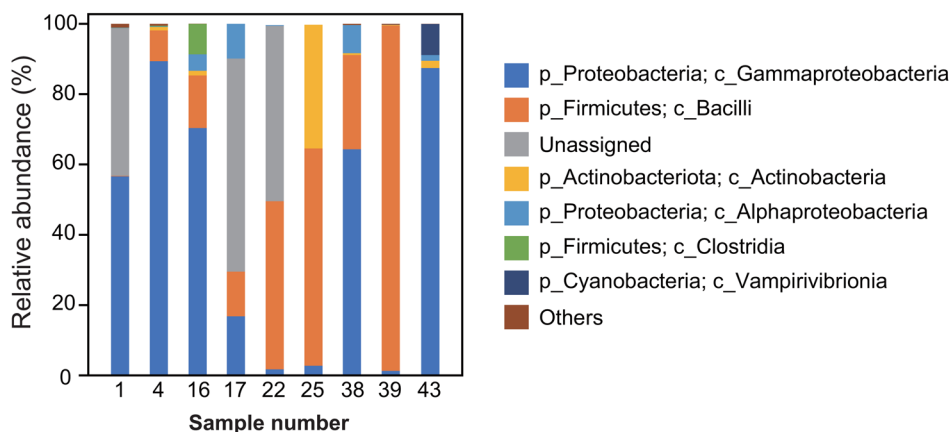
**Fig. 1.** PCR screening to detect bacterial DNA in bile samples. After amplification of the 16S rRNA gene, the PCR products (5  $\mu$ l) together with a molecular standard were electrophoresed in 2% agarose gels. The expected amplified product of 16S rRNA gene is ca. 430 bp. The molecular standard is shown in the leftmost lane of each agarose gel, indicated as “M” at the top of the lane. The sample number described in [Supplementary Table 1](#) is shown at the top of each lane. As a negative control, the product of PCR amplification from TE buffer was also electrophoresed, designated as “C” at the top of the lane. The PCR amplification was observed in sample Nos. 1, 4, 16, 17, 22, 25, 38, 39, and 43.

amplification was produced in the negative control ([Supplementary Fig. 1](#)). One microliter of DNA solution or TE buffer as a negative control was used for each PCR reaction mixture (25  $\mu$ l). The thermocycler heating conditions were 95°C for 1 min, followed by 25 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 5 sec, and finally 72°C for 1 min. The experiment was performed in triplicate. No PCR amplification was observed in the laboratory dogs. PCR amplification was observed from 9 dogs in 52 hospitalized dogs ([Fig. 1](#)), which indicated that bacteria appeared to be present in the bile of 17.3% of diseased dogs.

The bile DNA of 9 PCR-positive samples was analyzed by 16S rRNA amplicon sequencing. The V3–V4 region of the 16S rRNA gene amplified by PCR was analyzed using Illumina MiSeq with a MiSeq Reagent Kit v3 (600 cycles; Illumina, San Diego, CA, USA), as described elsewhere [12]. A total of 418,124 reads was obtained. The sequencing data have been deposited in bioproject PRJDB12698. The accession numbers of the samples range from DRR331811 to DRR331819. The sequence data were processed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) v2021.8.0 [1]. Microbial taxonomy was assigned using a Naïve Bayes classifier trained on the SILVA 138 99% database [7]. Observation of the relative abundance of bacterial community at the class level ([Fig. 2](#)) showed that the majority of bacteria were generally from the classes *Gammaproteobacteria* and *Bacilli*, and an unassigned class.

Although the PCR-positive samples were limited in number, the microbial diversities were compared between liver-associated and gallbladder-associated groups ([Supplementary Fig. 2](#)). The PCR-positive samples were comprised of 5 cases of liver-associated disease (2 hepatic microvascular dysplasia, 2 port systemic shunt, and 1 hepatic fibrosis) and 4 cases with gallbladder-associated diseases (1 cholecystitis, 1 gallbladder mucocele, 1 biliary sludge and 1 cholecystitis/biliary sludge). Statistical analysis was performed using R software package version 3.6.1 [8]. On examining the alpha diversity using Shannon’s index, no significant difference was observed (Mann–Whitney *U* test,  $P > 0.05$ ). However, on examining the beta diversity using the weighted and unweighted UniFrac distance metrics, significant differences were observed (permutational multivariate analysis of variance,  $P < 0.05$ ). These suggested that a bacterial community occurred in canine gallbladders that is specific to the overall type of disease affecting the animal.

Because dysfunction of the biliary system and/or liver can cause a stagnant bile environment, bacteria is likely to flow back via the duodenum–bile-duct route and to remain and replicate in the bile. Several recent studies in humans have reported that



**Fig. 2.** Bile microbiota of PCR-positive samples. Relative abundances of the bacterial community are shown in bar plots, and taxonomies are shown in different colors as described on the right. Taxa with relative abundances <1.0% are assigned as “others”. The sample number is shown under each bar plot. Sample Nos. 4, 16, 38, 39, and 43 were classified as the liver-associated disease group, in which the dogs suffered from a port systemic shunt, hepatic microvascular dysplasia, port systemic shunt, hepatic fibrosis, and hepatic microvascular dysplasia, respectively. Sample Nos. 1, 17, 22, and 25 were classified as the gallbladder-associated disease group, in which the dogs suffered from cholecystitis, gallbladder mucocele, biliary sludge, and cholecystitis/biliary sludge, respectively.

the presence of a biliary microbiota is associated with biliary diseases such as cholecystitis, cholangiocarcinoma, and cholesterol gallstones [2, 6, 10, 13]. In human major biliary bacterial phyla were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* in distal cholangiocarcinoma and the onset of common bile duct stones [2, 6, 10, 13]. Although our study included diverse liver disease in addition to biliary tract disease, canine biliary bacterial phyla were mainly composed of *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, which were partly similar to those found in human bile.

In this study, we detected bacterial DNA in the bile from some diseased dogs but not in that from healthy dogs and found that the bile microbiota can differ depending on the affected organs in the diseased dogs, although the sample number was limited. Therefore, a bacterial community specific to the overall type of disease appears to occur at a certain frequency, rather than an autochthonous microbiota being present in the bile of dogs.

**CONFLICTS OF INTEREST.** The authors declare no conflicts of interest.

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