Study of the bacterial population of the duodenum and presence of bacteria in the bile of cats with chronic inflammatory bowel disease, cholangitis, pancreatitis, triaditis and small intestinal lymphoma, in comparison to healthy cats

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> Abstract

The etiopathogenetic relationship between the intestinal flora and the presence of bacteria in bile in feline gastrointestinal disorders has not been studied previously. The aim of the study was the bacteriological analysis of duodenal juice and bile in cats with chronic inflammatory bowel disease (IBD), cholangitis, pancreatitis, and their combinations (triaditis), as well as in cats with intestinal lymphoma. In this prospective study 49 sick cats were included, 45 (25 symptomatic, 20 asymptomatic) with histopathological evidence of IBD, and/or cholangitis, and/or pancreatitis and four with intestinal lymphoma, as well as eight healthy cats. Samples of duodenal juice and bile were collected during exploratory laparotomy and cultured under aerobic, anaerobic and microaerobic conditions in order to isolate, enumerate and identify bacteria following standard microbiological guidelines. Comparisons of the bacterial populations of the duodenum among the groups of cats of the study regarding the growth of aerobic (P=0,831), anaerobic (P=0,406) and the total population of bacteria (P=0,752) did not outline any statistically significant differences. A statistically significant difference was noted in cats with triaditis regarding the growth of anaerobic *Clostridium* spp. (P=0,055). The bile samples of the normal and most (48/49, 98%) of the sick cats were bacteriologically negative. However, growth of a strain of *Enterobacter cloacae* was noted in a bile sample of a cat with IBD and pancreatitis. Inflammatory disorders of the small intestine, the liver, and the pancreas are not related to bacterial growth in the bile. In order to confirm the possibility of triaditis being correlated with an overgrowth of anaerobic intestinal, such as *Clostridium* spp., further research using sensitive molecular diagnostics will be necessary.

> Introduction

Canine and feline intestinal flora is composed of several hundreds to thousands of species of aerobic, microaerophilic and obligate anaerobic bacteria, the composition of which is specific and characteristic to each animal with differences even between individuals of the same species. However, its basic composition has not been fully revealed.[1] According to studies based on bacterial culturing,[2,3,4] the main species of bacteria prevalent in the feline small intestine are *Escherichia coli* and strains of the genera *Bacteroides*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Clostridium*, in various percentages depending on the segment of the gastrointestinal tract and its distance from the large intestine. In the stomach bacterial populations ranging from 10^1 to

10^6 cfu/g have been reported, whereas in the duodenum and the jejunum bacterial populations from 10^5 up to 10^9 cfu/ml have been noted in some cats. The number and range of bacterial strains increases in the ileum (10^7 cfu/ml) and even more in the colon (>10^9 cfu/ml).[2,14] Aerobes are detected in higher number in the cranial segments of the intestinal tract, whereas anaerobes predominate in the colon. In cats, however, the number of anaerobic bacteria colonising the small intestine appears to be higher than in dogs.[2,4,5]

In recent years it has been proven that, similar to humans, in dogs and cats, alterations in the composition of intestinal flora are implicated in chronic enteropathies.[6-13] Higher than normal colonisation of enteric bacteria in the proximal segment of the small intestine characterise the bacterial overgrowth syndrome, which is involved in the development of chronic gastrointestinal signs. The standard diagnostic procedure includes culturing intestinal juice collected from the duodenal lumen under aerobic and anaerobic conditions.[14,15] Bacterial overgrowth in the cat is defined as an increase in the bacterial population of the proximal small intestine higher than $1{,}1 \times 10^9$ cfu/ml of intestinal content.[4,14,15] In healthy cats the total bacterial population in the proximal small intestine shows high variation and it may usually surpass the numbers initially set as bacterial overgrowth. Moreover, apart from alterations in the number of bacteria, changes in the bacterial species comprising the intestinal flora are also of great significance. This disorder is described by the term "intestinal dysbiosis".[5,15]

Alterations in the composition of intestinal flora in the proximal small intestine, and the presence of bacteria in bile and their relationship with the etiopathogesis of inflammatory disorders of the feline gastrointestinal tract still remain unclear. The aim of the present study was to reveal the duodenal bacterial composition and the presence of bacteria in the bile of cats with IBD, cholangitis, pancreatitis, or combinations of the aforementioned, including the clinical syndrome of triaditis (IBD, cholangitis and pancreatitis), or with intestinal lymphoma, and to compare all of the above with normal cats. The present study exists in continuation of a previous research project about the clinical, laboratory and histopathologic presentation of the feline triaditis complex,[16] in order to investigate the etiopathogenesis of inflammatory bowel disorders.

> Materials and methods

- Study design

This prospective study involved domestic cats, which were examined in the Department of Internal Medicine of the Companion Animal Clinic, School of Veterinary Medicine, A.U.Th. (February 2008 - February 2011). The study protocol was approved by the Department of the School of Veterinary Medicine (Clinical research ethical approval: Special General Assembly of the Department of Veterinary Medicine no. 430/20-11-2007) and by the appropriate National Department (Approval of clinical research: Veterinary Administration Office of Thessaloniki, protocol no. 13/3657/29.03.2010). No procedure was undertaken in the cats of the study without signed owner consent. For study purposes, two categories of cats were examined: symptomatic cats, presented to the Companion Animal Clinic with chronic clinical signs, which could be attributed to inflammatory bowel disease, including triaditis or intestinal lymphoma (in particular they had persistent or recurrent one or some combination of the following clinical signs: depression, increased or decreased appetite, vomiting, fecal consistency abnormalities, jaundice, weight loss), as well as asymptomatic cats presented for ovariohysterectomy. At least two weeks prior to diagnostics, all of the clinically healthy cats were admitted to a separate area of the hospitalisation ward of the Department of Internal Medicine of the Companion Animal Clinic in order to adapt to their surroundings, be fed exclusively with commercial high quality dry food (base components: 33.8-34.2% protein, 21.9-22.3% fat, 36.9-38.1% carbohydrates, 1.1-1.3%, fibre and 0.80-0.88% calcium in dry matter) (Feline Adult Optimal Care™ Chicken-Dry, Science Plan™, Hill's™) to be monitored and diagnostic tests to be performed.

The selection of cats for the study was made based on the following inclusion criteria: (1) age over one year of both genders and of various breeds, (2) diet with commercial cat food (dry and/or canned) for at least eight weeks prior to the initial physical examination, (3) written owner consent for the exploratory laparotomy, biopsy sampling and collection of biological materials, (4) histopathological evidence of inflammation (enteritis, cholangitis, pancreatitis, or a combination of the above) or intestinal lymphoma with or without compatible clinical signs at the time of physical examination (study group) or normal clinical and histopathological findings (control group).

Exclusion criteria were as follows: (1) presence of clinical or laboratory findings of other pathological conditions, which could affect the liver, the pancreas, or the small intestine, (2) presence of histopathological findings in the liver, the pancreas and the small intestine other than those investigated for the purposes of the study, (3) positive results in fecal parasitological analysis, (4) positive results in serological detection of antibodies against feline immunodeficiency virus (FIV), antigen of feline leukemia virus (FeLV) and antibody against feline coronavirus (FCoV) and feline infectious peritonitis (FIP), (5) abnormal results of total or free thyroxin concentrations in blood serum (T4, Free T4), (6) administration of drugs such as antimicrobials, anti-inflammatories, or immunosuppressants, in the last two weeks prior to admission.

Symptomatic cats: During the study, 302 cats with clinical signs were evaluated, 82 of which fulfilled the inclusion criteria. Based on owner consent for biopsy sampling, 39 cats were fully investigated, 25 of which were included in the study based on the predetermined inclusion criteria.

Asymptomatic cats: During the same time period, 39 cats without clinical signs were fully investigated, following the same diagnostic protocol with symptomatic cats. Fol-

Table 1. Cat study groups

Groups	Number of cats
C	8
CH	6
IBD	13
IBD+CH	15
IBD+CH+P	8
P	1
IBD+P	2
L	4
Total	57

C: controls

CH: cats with histopathological evidence of cholangitis

IBD: cats with histopathological evidence of chronic inflammatory bowel disease

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis

P: cats with histopathological evidence of pancreatitis

IBD+P: cats with histopathological evidence of chronic inflammatory bowel disease and pancreatitis

L: cats with histopathological evidence of small intestinal lymphoma

lowing histopathology results, eleven cats were excluded through the predetermined criteria, eight were found to be normal, whereas in twenty cats histopathological evidence of inflammation was uncovered in the organs under investigation.

Following histopathological results all cats with inflammatory lesions in the intestine, the liver, and the pancreas regardless of the presence of clinical signs at the time of sampling, as well as cats with small intestinal lymphoma, were included in the study group of cats with abnormal findings intended for diagnostic investigation. Asymptomatic cats, with normal histopathological findings in the liver, pancreas, and the intestine constituted the control group.

Thus, in our study, 57 cats were included in total: 49 cats with abnormal findings (45 cats with histopathologicaly evidence of various combinations of IBD, cholangitis, pancreatitis, 4 with intestinal lymphoma) and 8 cats as normal controls.

- Groups of cats

Based on histopathological results, the cats of the study were classified into eight groups, which are presented in Table 1. In one cat with cholangitis, one cat with IBD and cholangitis, two cats with simultaneous IBD, cholangitis and pancreatitis, two cats with IBD and pancreatitis, three cats with IBD and a cat with intestinal lymphoma,duodenal juice was not sampled for analysis.

- Histopathological characteristics of study groups

IBD: In total, thirty eight cats had histopathological evidence of the lymphocytic/plasmacytic type of IBD. In all of the latter there was infiltration of lymphocytes, plasmacytes and macrophages in the intestinal mucosa, whereas neutrophils were observed in variable numbers and more rarely, occasional eosinophils. The infiltrations, combined with architectural lesions in the intestinal epithelium, extended in all entire parts (duodenum, jejunum, ileum) with a variable degree of severity.

Cholangitis: Twenty nine cats had histopathological evidence of cholangitis. The of majority lesions was defined by infiltration of portal areas consisting primarily of lymphocytes and to a lesser extent by plasmacells, fibrosis, and hyperplasia of the bile ducts (lymphocytic type of cholangitis). In a small number of cats (5/29), in addition to mononuclear cells neutrophils were present (chronic neutrophilic cholangitis).

Pancreatitis: Eleven cats showed lesions of chronic pancreatitis, characterised by mononuclear cell infiltration and fibrosis. In five of them, there was also a considerable number of neutrophils (three cases were classified as chronic-active pancreatitis and the other two, where necrotic lesions were present, as acute necrotic pancreatitis in combination with chronic pancreatitis).

Lymphoma: Four cats had histopathological findings compatible with intestinal lymphoma. The latter constituted of diffuse aggregations of a uniform population of lymphocytes in the connective tissue and submucosal layer with multifocal infiltrations of the muscularis, whereas in some cases they were also present in the innermost mucosa. Very small numbers of the other types of inflammatory cells were observed, whereas micro-erosions and erosions of the epithelial surface were noted. In one cat an increased number of neutrophils was noted in the connective tissue layer.

- Physical and Diagnostic examinations

In all 57 cats included in the study the history was initially obtained and a general physical examination was performed. Diagnostic procedures (<3 days prior to exploratory laparotomy) for the 57 cats included fecal parasitological examination and the detection of *Giardia* spp. antigen, standard urinalysis, complete blood count, biochemistry in blood serum including: albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA), alkaline phosphatase (ALP), alanine aminotransferase activity (ALT), g-glutamine transferase activity (γGT), aspartate aminotransferase activity (AST), total bilirubin (TBIL), lipase activity, ionised calcium (Ca), phosphorus (P), potassium (K), sodium (Na), blood coagulation profile including prothrombin time (PT) and partial thromboplastin time (PTT) (52/57), serum total thyroxin (T4) and free thyroxin (Free T4), serum feline immunoreactivity of pancreatic lipase (fPLI measured by Spec fPL®)[17] (56/57) trypsin like immunoreactity (fTLI) and testing for viral origin disorders including FIV, FeLV, and coronavirus for FIP. Diagnostic imaging included thoracic and abdominal radiographs, (49/57) and abdominal ultrasonography (56/57). Full thickness biopsy samples were collected for histopathological diagnosis (at least five from each cat: one from the liver, the pancreas, the duodenum, the jejunum, and the ileum) via exploratory laparotomy and were evaluated in a blinded fashion by a specialised veterinary pathologist (P. T.), based on internationally acceptable histopathological criteria.[18-20]

- Intestinal content and bile sampling

Prior to intestinal biopsy, a sample of duodenal juice was obtained from the duodenum. To this purpose, the duodenum was located and by "massaging" its full length any contents present were collected in the middle segment. Duodenal juice was removed by suction through plastic intravenous catheter (Abbocath-T I.V. Catheter 20 G x 1,25", Venisystems™, Abbott, Ireland) attached on a 20 ml syringe.[15] The sample of duodenal juice was immediately placed in a sterile,

glass vacuum blood collection vial without anticoagulant.

During exploratory laparotomy, 1 ml of bile was obtained from the gall bladder, by use of sterile 1 ml syringe and a 25 G needle. In cases when bile could not be aspirated (e.g. increased viscosity), a wider bore needle was used (23-21 G). The samples were immediately transfused to a sterile vacuum glass vial for blood collection, without anticoagulant (Venoject®, Terumo Europe N.V., Leuven, Belgium).

Vials containing bile and intestinal content were immediately placed in a transportation refrigerator (temperature levels of 4-6 °C) and within one hour from sampling they were transported to the laboratory of microbiology, where they were inoculated in the appropriate growth mediums under specific aerobic, microaerophilic and anaerobic conditions, aiming in growth of any bacteria in the samples.

- Culturing of duodenal juice and bile

Isolation and enumeration of bacteria

For the isolation and enumeration, as well as the identification of bacteria standard microbiological guidelines were employed.[21] For the isolation of aerobic and facultative anaerobic bacteria, such as *Enterobacteriaceae* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp. and *Pseudomonas* spp., the mediums Blood Agar, MacConkey Agar, Rogosa Agar and Bile Esculin Agar were used. For the isolation of anaerobic bacteria, such as *Clostridium* spp., *Bacteroides* spp., *Peptostreptococcus* spp. and *Eubacterium* spp., the following mediums were used: Anaerobic Agar acc. to Brewer and TSC-Agar (Tryptose Sulfite Cycloserine Agar, Perfringens Agar). For the isolation of microaerophilic bacterial strains, such as *Campylobacter* spp., the special medium Campylobacter Selective Agar was used.

In order to calculate the bacterial population of samples, the technique of serial dilutions (10^{-1} up to 10^{-6}) and inoculation of every dilution in agar plates with the spread plate method was used. From each dilution usually two agar plates were inoculated for every growth medium. Agar plates were incubated in 37°C in aerobic, anaerobic and microaerophilic conditions, depending on the inoculated substrate. For incubation in anaerobic conditions specialised containers were used (GasPakTM EZ Anaerobe Pouch System, Anaerobe Gas Generating pouch system with indicator, Becton Dickinson, NJ, USA), as well as for incubation of microaerophilic bacteria (GasPakTM Campy Pouch System, BBLTM Microaerophilic Campy pouch system, Becton Dickinson, NJ, USA). The agar plates were daily monitored for the presence of bacterial growth up to 48 hours for aerobic cultures and up to six days for anaerobic and microaerophilic cultures.

In order to calculate the population of bacteria, the colonies that were observed in aerobic, anaerobic and microaerophilic conditions were counted, including the agar plates containing 30-300 colonies. The total of visible colonies from both agar plates that had been inoculated from each dilution and the median of both agar plates were calculated. Finally, the number of bacteria capable of forming colonies was expressed per 1 ml of initial sample (colony forming units, cfu/ml).

Bacterial identification

For the identification of bacterial strains the following were evaluated: (1) growth in special media for isolation and identification of bacteria [(i) Bile Esculin agar (BBLTM Bile Esculin Agar, Becton Dickinson, Maryland, USA): for isolation of *Enterococcus* spp. and differentiation from *Streptococcus* spp. (ii) Campylobacter Selective agar: for isolation of *Campylobacter* spp. (iii) TSC agar: for isolation of *Clostridium* spp. (iv) Rogosa agar: for isolation of *Lactobacilli* (v) Anaerobic agar acc. to Brewer: for isolation of *Clostridium* spp. and other anaerobic or microaerophilic bacteria.] (2) the morphological characteristics of bacteria post staining (Gram stain). (3) the initial biochemical characteristics: catalase testing, oxidase testing, positive or negative growth in MacConkey agar, indole testing, nitric salt induction, production of lecithinase, production of lipase. (4) sensitivity or not to the antimicrobial substance vancomycin, as an additional test beyond Gram staining, for the differentiation between Gram positive and Gram negative bacteria. (5) For specific identification of *Enterobacteriaceae* spp. the API 20 E system was used (API® bioMérieux Inc., Durham NC, USA).

Statistical analysis

The cats of the study were classified into groups using histopathological diagnosis as a criterion. Data processesing and comparisons were made among study groups. Cats with pancreatitis alone, with IBD lesions in combination with pancreatitis and cats with intestinal lymphoma were excluded from statistical analysis, due to small sample size. For the synoptic presentation of statistical results absolute and relative frequencies (percentages %), measures of central tendency (mean, median) and measures of spread-dispersion [(minimum (min)-maximum (max) values and standard deviation] were calculated. For comparisons of means and medians the Kruskal-Wallis and Mann-Whitney tests were employed. For comparisons of proportions (percentages %) z-test was used with Bonferroni correction to the significance level. In all statistical analyses the observed significance level (P-value) was estimated, as appropriate, either with the Exact Method, or a Monte-Carlo simulation based on 10.000 resampling cycles.[22] The level of statistical significance was set at $\alpha=0,05$ ($P\leq0,05$). Statistical analyses were performed by the IBM SPSS v. 20.0 statistical package (USA, Chicago: Illinois) with the Exact Tests subsystem installed. (Statistical pack IBM SPSS v.20.0)

> Results

Duodenal bacterial population

Aerobic and anaerobic bacterial species isolated during culture of duodenal juice per group of cats, are described

Table 2. Growth of aerobic bacterial species (number of cultures in which the particular species was identified) in cultures of duodenal juice per group of cats

Aerobic bacteria	Groups								
	C	CH	IBD	IBD+CH	IBD+CH+P	P	IBD+P	L	Total
Escherichia coli *	3	2	4	3	4	0	1	1	18
Staphylococcus spp.*	3	1	1	7	2	0	0	0	14
Enterobacter spp*	0	0	2	1	1	0	0	1	5
Streptococcus spp.*	1	0	0	2	1	0	0	0	4
Citrobacter spp.*	0	0	2	0	0	0	1	0	3
Enterococcus spp.*	0	0	0	1	0	1	0	0	2
Klebsiella spp*	0	0	0	0	0	0	0	2	2
Bacillus spp.*	0	0	0	1	0	0	0	0	1
Pseudomonas spp.	0	0	0	1	0	0	0	0	1
Proteus spp.*	0	0	0	0	1	0	0	0	1
Serratia spp.*	0	0	1	0	0	0	0	0	1

* facultative anaerobes

C: controls (n=8)

CH: cats with histopathological evidence of cholangitis (n=5)

IBD: cats with histopathological evidence of chronic inflammatory bowel disease (n=10)

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis (n=14)

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis (n=6)

P: cats with histopathological evidence of pancreatitis (n=1)

IBD+P: cats with histopathological evidence of chronic inflammatory bowel disease and pancreatitis (n=1)

L: cats with histopathological evidence of small intestinal lymphoma (n=3)

in Tables 2 and 3. No growth of microaerophilic bacteria of the genus *Campylobacter* spp. was observed in any of the duodenal juice cultures from the cats in this study.

The numerical estimation of the total of aerobes, anaerobes as well as the entire bacterial population from cultures of duodenal juice of cats in this study are reported per group in Table 4.

Comparison of the bacterial population of the duodenum between the feline study groups regarding the growth of aerobics (P=0,831), anaerobics (P=0,406) and the total bacterial population (P=0,752) did not reveal statistically significant differences.

The numerical estimation of the most common aerobic and anaerobic bacterial growth in cultures of duodenal juice of cats in this study is presented per group in Table 5.

Comparisons of the duodenal bacterial population of the cat study groups, regarding the growth of *Escherichia coli*, which was evaluated in aerobic (P=0,317) as well as anaerobic conditions (P=0,313), and *Staphylococcus* spp., in both aerobic (P=0,332) and anaerobic conditions (P=0,279), did not show any statistically significant differences among groups. Statistically significant differences were revealed during comparison of results among groups in regard to growth of anaerobic *Clostridium* spp., as they are presented in Table 6.

Presence of bacteria in bile

Bile cultures of the control group were negative for bacterial growth. From the cat groups with abnormal findings, only one bile culture was positive from the IBD+P group, in which *Enterobacter cloacae* was isolated (Table 7).

Table 3. Growth of anaerobic bacterial species (number of cultures in which the particular species was identified) in cultures of duodenal juice per group of cats

Anaerobic bacteria	Groups								
	C	CH	IBD	IBD+CH	IBD+CH+P	P	IBD+P	L	Total
Clostridium spp.	1	2	2	2	4	0	0	1	12
Bacteroides spp.	0	1	0	3	1	0	0	0	5
Peptostreptococcus spp.	0	0	1	3	0	0	1	1	6
Eubacterium spp.	0	0	0	1	0	0	0	0	1

C: controls (n=8)

CH: cats with histopathological evidence of cholangitis (n=5)

IBD: cats with histopathological evidence of chronic inflammatory bowel disease (n=10)

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis (n=14)

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis (n=6)

P: cats with histopathological evidence of pancreatitis (n=1)

IBD+P: cats with histopathological evidence of chronic inflammatory bowel disease and pancreatitis (n=1)

L: cats with histopathological evidence of small intestinal lymphoma (n=3)

>Discussion

There was wide variability in the duodenal bacterial populations among the cat groups. However, comparisons did not reveal statistically significant differences in the total bacterial populations, as well as in the subgroups of aerobic and anaerobic duodenal bacteria between controls (C group) and cats with abnormal findings of all groups (IBD, Ch, IBD+Ch, IBD+Ch+P, Tables 4, 5 & 6). By reviewing our findings and in comparison to the referred as the feline normal intestinal flora, intestinal bacterial overgrowth was not substantiated in any of the cats in our study. In the control group the small intestinal bacterial population ranged from 0 to $3,7x10^3$ cfu/ml (mean $9x10^2$). Among the groups of sick cats, the most numerous bacterial populations were noted in the triaditis group (IBD+Ch+P, Table 4), ranging from 0 to $7,6x10^5$ cfu/ml (mean $1,2x10^5$).

Both the mean and maximum values of bacteria observed in all study groups were found to be within the previously published reference range for the normal feline bacterial flora (10^5-10^8 cfu/ml).[2,14,15] However, in our study a predominance of anaerobic species of the genus *Clostridium* was observed in cats of the triaditis group (IBD+Ch+P, Table 5) compared to the rest of the study groups. *Clostridium* spp. (division *Firmicutes*, family *Clostridiaceae*, including at least 70 different species) constitute most of the cecal flora, however, they can be detected in other intestinal segments performing different functions.[5] They are therefore part of the normal feline small intestinal flora despite their anaerobic nature.[2,15]

The etiopathogenetic connection between abnormal variations in the intestinal flora and induction of inflammation has not yet been clarified.[1] An increase in several bacterial strains of *Proteobacteria*, such as *Escherichia coli*, and a reduction the Firmicutes and especially of the diversity of certain *Clostridium* spp. have both been reported as common disorders.[1,10-13] In dogs with IBD a reduction in the diversity of small intestinal bacterial flora has been observed.[23] Research in cats with IBD has proven the existence of «intestinal dysbiosis» in sick cats, with the *Enterobacteriaceae* spp., *Clostridium* spp., *Bacteroides* spp. and *Streptococcus* spp. corresponding to 91% of bacteria attached to the intestinal mucosa and *Escherichia coli* comprising 30% of the *Enterobacteriaceae* spp..[8] A different study indicated that strains from the genus *Desulfovibrio* predominated in the intestinal flora of cats with IBD, whereas strains of the *Bifidobacterium* and *Bacteroides* genera predominated in the bacterial populations of healthy cats.[7] In our study, at first, it seemed like a paradox that even though cats of the triaditis group (IBD+CH+P, Table 5) had increased populations of *Clostridium* spp. in the duodenum, similar increase was not observed in the rest of the groups. This fact underlines the complicated nature of the etiopathogenetic connection between the three pathological conditions, as well as "chronologically placing" their coexistence from an evolutionary perspective in more advanced stages compared to their combinations in pairs. In the future, sensitive molecular methods could give answers, regarding the increased populations of *Clostridium* spp. evidenced in our study, diversity and their contribution to the pathogenesis of triaditis.

Table 4. Range (min,-max), median (MED), mean (M) and standard deviation (SD) for aerobic, anaerobic and total bacterial species (colony-forming units per millilitre, cfu/ml), grown in cultures of duodenal juice per group of cats

Groups		Duodenal juice bacteria		
		Aerobic[1]	Anaerobic[2]	Total
C	Range	0-3610	0-400	0-3710
	MED	180	0	181.5
	M	841.2	62.9	904.1
	SD	1379.7	140.6	1476.5
CH	Range	0-4700	0-1200	0-5900
	MED	320	10	350
	M	1072	266	1338
	SD	2034.8	524.6	2558
IBD	Range	0-120000	0-8500	0-128500
	MED	130	0	160
	M	12873	868	13741
	SD	37683.6	2682.1	40362
IBD+CH	Range	0-26000	0-1600	0-27600
	MED	835	14.5	880
	M	5526.4	173.5	5699.9
	SD	9907.8	428.1	10230.6
IBD +CH+P	Range	0-430000	0-330000	0-760000
	MED	935	270	1855
	M	72470	55390	127860
	SD	175157	134532.6	309686.7
P	Range	1400	8	1408
	MED	1400	8	1408
	M	1400	8	1408
	SD	-	-	-
IBD+P	Range	3800	600	4400
	MED	3800	600	4400
	M	3800	600	4400
	SD	-	-	-
L	Range	520-180000	0-48	520-180000
	MED	2740	0	2788
	M	61086.7	16	61102.7
	SD	102987.9	27.7	102974.4

[1]aerobic and facultative anaerobic bacteria

[2]strictly anaerobic bacteria

C: controls (n=8)

CH: cats with histopathological evidence of cholangitis (n=5)

IBD: cats with histopathological evidence of chronic inflammatory bowel disease (n=10)

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis (n=14)

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis (n=6)

P: cats with histopathological evidence of pancreatitis (n=1)

IBD+P: cats with histopathological evidence of chronic inflammatory bowel disease and pancreatitis (n=1)

L: cats with histopathological evidence of small intestinal lymphoma (n=3)

Table 5. Range (min,-max), median (MED), mean (M) and standard deviation (SD) for the most common bacterial species (colonies per milliliter, cfu/ml), grown in cultures of duodenal juice under aerobic and anaerobic conditions per group of cats

Groups		Duodenal juice bacteria				
		Aerobic conditions		Anaerobic conditions		
		E. coli	*Staphylococcus*	*E. coli*	*Staphylococcus*	*Clostridium*
C	Range	0-3500	0-360	0-1100	0-130	0-400
	MED	0	0	0	0	0
	M	756.3	82.5	175	31.3	50
	SD	1386.5	132.7	380.5	55.1	141.4
IBD	Range	0-45000	0-25000	0-7200	0-600	0-8500
	MED	0	0	0	0	0
	M	5041	2500	758.2	60	867
	SD	14083.3	7905.7	2264.5	189.7	2682.5
CH	Range	0-4700	0-320	0-2500	0	0-1200
	MED	0	0	0	0	0
	M	1008	64	534	0	264
	SD	2069.1	143.1	1101.5	0	525.8
IBD+CH	Range	0-18000	0-18000	0-1500	0-16000	0-40
	MED	0	75	0	0	0
	M	1302.1	2601.4	120	1284.3	4
	SD	4806.4	5756.0	400.1	4246.4	11.2
IBD+CH+ P	Range	0-32000	0-500	0-400000	0-50	0-310000
	MED	685	0	87	0	270
	M	5795	85.5	66929	16	52056.7
	SD	12852.5	203.1	163171.7	24.8	126367.8
P	Range	0	0	0	0	0
	MED	0	0	0	0	0
	M	0	0	0	0	0
	SD	-	-	-	-	-
IBD+P	Range	2200	0	700	0	0
	MED	2200	0	700	0	0
	M	2200	0	700	0	0
	SD	-	-	-	-	-
L	Range	0-2600	0	0-52	0	0-220
	MED	0	0	0	0	0
	M	866.7	0	17.3	0	73.3
	SD	1501.1	-	30	-	127

C: controls (n=8)

CH: cats with histopathological evidence of cholangitis (n=5)

IBD: cats with histopathological evidence of chronic inflammatory bowel disease (n=10)

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis (n=14)

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis (n=6)

P: cats with histopathological evidence of pancreatitis (n=1)

IBD+P: cats with histopathological evidence of chronic inflammatory bowel disease and pancreatitis (n=1)

L: cats with histopathological evidence of small intestinal lymphoma (n=3)

The microbiological analysis of bile, concerning the diagnosis of cholangitis, usually includes culture in aerobic and anaerobic conditions as well as antibiotic sensitivity testing in order to indicate the proper therapeutic regimen.[24-27] Culturing bile is preferred to culturing liver biopsy samples or gall bladder wall samples, because of improved rates of microorganism detection.[28] Despite the ruling hypothesis that bile in healthy cats is microbiologically sterile,[25,29] some researchers claim that bacterial translocation from duodenum to bile can occur in healthy as well.[28] In our study, however, no bacterial growth was noted in bile cultures from healthy controls.

Regarding the types of feline cholangitis, in the acute neutrophilic cholangitis, isolation of mostly *Enterobacteriaceae* spp. originating from the duodenal flora in the bile is a common occurrence and confirms the diagnosis.[24,25,28,30-35] Bacterial translocation from the intestinal tract to the gall bladder can occur either through reflux of bile from the duodenum, or through the hematogenous or lymphic routes.[25] It is maintained that inflammatory bowel disease and pancreatitis can predispose to cholestasis, resulting in reflux of pancreatic secretions and/or bacteria towards the liver.[32,36] In chronic neutrophilic cholangitis, bile culture is negative in most cases. It is theorised that this occurs

Table 6. Results of comparisons of medians (MED) of bacterial species (colonies per milliliter, cfu/ml), grown in cultures of duodenal juice per group of cats

Groups	Anaerobic *Clostridium* spp (cfu/ml)
C	0[a]
CH	0[a]
IBD	0[a]
IBD+CH	0[a]
IBD+CH+P	270[b]
MSSDO 0,05	270
Kruskal-Wallis P	0.05

MSSDO: Minimum Statistically Significant Difference Observed at a significance level of P=0,05

a, b: In the same column of the table medians followed by common letter (superscript) do not differ significantly according to the results of a series of Mann-Whitney tests. A statistically significant difference exists between medians with different superscript letter.

C: controls (n=8)

CH: cats with histopathological evidence of cholangitis (n=5)

IBD: cats with histopathological evidence of chronic inflammatory bowel disease (n=10)

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis (n=14)

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis (n=6)

Table 7. Distribution (n, %) of the positive and negative results of the bile cultures per group of cats

Groups		Bile culture	
		Negative	Positive
C	number	8	0
	%	100%	0%
CH	number	6	0
	%	100%	0%
IBD	number	13	0
	%	100%	0%
IBD+CH	number	15	0
	%	100%	0%
IBD+CH+P	number	8	0
	%	100%	0%
P	number	1	0
	%	100%	0%
IBD+P	number	1	1
	%	50%	50%
L	number	4	0
	%	100%	0%
Total	number	56	1
	%	98,2%	1,8%

C: controls (n=8)

CH: cats with histopathological evidence of cholangitis (n=6)

IBD: cats with histopathological evidence of chronic inflammatory bowel disease (n=13)

IBD+CH: cats with histopathological evidence of chronic inflammatory bowel disease and cholangitis (n=15)

IBD+CH+P: cats with histopathological evidence of chronic inflammatory bowel disease, cholangitis and pancreatitis (n=8)

P: cats with histopathological evidence of pancreatitis (n=1)

IBD+P: cats with histopathological evidence of chronic inflammatory bowel disease and pancreatitis (n=2)

L: cats with histopathological evidence of small intestinal lymphoma (n=4)

due to i) either the bacteriostatic properties of bile, ii) or because the initial bacterial invasion was restricted by the immune system, iii) or due to previous use of antimicrobials, and iv) in cases when bacteria are not the immediate cause of the inflammatory disorder.[24,37-39] However, even in chronic cholangitis of non-bacterial origin, chronic infiltration of bile ducts by inflammatory cells results in a risk for secondary hepatic infection by *Enterobacteriaceae* spp., such as *Escherichia coli*.[26]

The lymphocytic type of cholangitis appears to originate from an immune-mediated aetiopathogenetic mechanism.[40-42] However, there is also a theory that this particular type of cholangitis represents the chronic stage of acute neutrophilic cholangitis or an ascending (originating from the duodenum) bacterial infection.[31,37,38] There is only a small amount of data on which the hypothesis of a primary bacterial infection can be based.[42] Two studies have been published concerning a small group of cats with cholangitis/cholangiohepatitis in which bacterial DNA of the *Helicobacter* genus has been detected, although the pathophysiological significance of this finding has yet to be clarified.[43,44] It is worthy of note that until the present day, there is no evidence to support the involvement of *Helicobacter* spp. to IBD and pancreatitis in cats.[8,45] Furthermore, in an experimental study, moderate inflammation in zone 1 of the feline liver was caused after infection with *Bartonella* spp.[46] Even though there is considerable evidence of an immune-mediated mechanism causing cholangitis, the actual etiopathogenesis of the disorder remains a mystery.[42]

The results of our study do not support the hypothesis of a primary microbial infection, considering that all the bile samples from cats with cholangitis were found to be bacteriologically sterile. From cats lacking histopathological evidence of cholangitis, only a single bile sample was found positive with growth of the bacterium *Enterobacter cloacae*. This was a cat with pancreatitis and IBD (Table 7, IBD+P group) together with cholestasis, without histopathological evidence of cholangitis or feline hepatic lipidosis. As previously mentioned, in this particular case cholestasis, as a result of obstruction in bile flow due to pancreatitis, became a risk factor for the translocation of *Enterobacter cloacae*, which is part of the normal small intestinal flora, toward the gall bladder. Unfortunately, culture of duodenal content was not performed on this cat; therefore its intestinal flora is unknown. Such a hypothesis, however, cannot explain the inflammation in the bile duct system observed in 29 cats with histopathological evidence of cholangitis in our study, in which bile cultures were negative. The possibility of bacterial translocation toward the liver and consequently the pancreas via the common bile and pancreatic duct, does not exclude the theory of an immune response to such bacterial invasion.

To summarise, the present study indicated that inflammatory disorders of the gastrointestinal tract relating to triaditis as well as intestinal lymphoma do not seem to be pathogenetically related to the bacterial flora of the duodenum or any presence of bacteria in bile. The intestine and the liver play a particularly significant role in immunity. This complicated system of the intestinal flora may affect several functions as well as the global health and every disruption in its interactions with the local intestinal immune mechanisms could lead to gastrointestinal disease.[1,6-10] In conclusion, an immune-mediated mechanism could be involved in the development of triaditis in cats.[42,47] Modern molecular methods of analysis are expected to give more answers in the investigation of any correlations between intestinal flora and its variability with histopathological lesions of inflammation in the intestine, liver and pancreas.

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