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Bile microbiocenosis in cats suffering from acute cholangiohepatitis

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SUMMARY
Commensal microorganisms are responsible for numerous diseases of animals, including diseases of internal organs (gastroenteritis, pneumonia, nephritis, hepatitis, cholecystitis, etc.). Cholangiohepatitis, one of the most common liver diseases in cats, is often fatal. The focus of the study was the bile of cats, suffering from acute cholangiohepatitis. The bile was sampled using non-lethal method guided by USG. The bile amount, taken from cats by percutaneous puncture of the gall bladder, was 2.6 ± 0.85 cm^3. No complications following the cholecystocentesis were observed in the animals. The microbiocenosis of bile from 51 cats was studied. Acute feline neutrophilic cholangiohepatitis is mostly caused by commensal bacteria. The range of bacterial pathogens includes the isolates of *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter cloacae*, *Citrobacter freundii*. The infectious process was caused by two-component associations in 75% of cases, and by three-component associations in 25%. Most common polycomponent bacterial associations included *E. faecalis* + *E. coli* (26.9%), less common – *E. aerogenes* + *E. coli* (15.4%), *P. vulgaris* + *E. coli* (11.5%), *S. aureus* + *E. coli* (11.5%), rarely – *P. aeruginosa* + *E. coli* (7.7%), *S. aureus* + *E. cloacae* (3.9%), *S. aureus* + *E. faecalis* (3.9%), *P. mirabilis* + *E. coli* (3.9%), *S. epidermidis* + *E. coli* (3.9%), *E. coli* + *S. epidermidis* + *E. faecalis* (3.9%), *P. aeruginosa* + *E. coli* + *S. epidermidis* (3.9%), *E. faecalis* + *E. coli* + *C. freundii* (3.9%). The predominant component of the mentioned associations is *E. coli* serovars O101 (28.9%), O41 (2.0%), O141 (15.6%), O26 (13.3%), O138 (13.3%), O15 (6.7%) and O33 (2.2%). It was established that 76.25% of commensal microorganism isolates, recovered from the bile of cats, suffering from feline cholangiohepatitis, were pathogenic for white mice.

Key words: cholangiohepatitis, cats, microbiocenosis, bile, bactobilia.

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Микробиоценоз желчи у кошек при остром холангиогепатите

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РЕЗЮМЕ
Условно-патогенные микроорганизмы являются возбудителями множества заболеваний у животных, в том числе внутренних болезней (гастроэнтерит, пневмония, нефрит, гепатит, холецистит и т. д.). Холангиогепатит – одна из самых распространенных патологий печени у кошек, которая может приводить к летальному исходу. Объектом исследования была желчь кошек, больных острым холангиогепатитом, полученная прижизненно под контролем ультрасонографии. Объем желчи, отобранной у кошек с помощью чрескожной пункции желчного пузыря, составил 2,6 ± 0,85 см^3. Осложнений после

проведения холецистоцентеза у животных не отмечали. Изучен микробиоценоз желчи у 51 кошки. Основной причиной острого нейтрофильного холангиогепатита у кошек являются условно-патогенные бактерии. Спектр бактериальных патогенов представлен изолятами *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter cloacae*, *Citrobacter freundii*. В инфекционном процессе принимали участие двухкомпонентные ассоциации в 75% случаев, а трехкомпонентные – в 25% случаев. Из поликомпонентных бактериальных ассоциаций у больных кошек чаще всего встречались *E. faecalis* + *E. coli* (26,9%), реже – *E. aerogenes* + *E. coli* (15,4%), *P. vulgaris* + *E. coli* (11,5%), *S. aureus* + *E. coli* (11,5%), редко – *P. aeruginosa* + *E. coli* (7,7%), *S. aureus* + *E. cloacae* (3,9%), *S. aureus* + *E. faecalis* (3,9%), *P. mirabilis* + *E. coli* (3,9%), *S. epidermidis* + *E. coli* (3,9%), *E. coli* + *S. epidermidis* + *E. faecalis* (3,9%), *P. aeruginosa* + *E. coli* + *S. epidermidis* (3,9%), *E. faecalis* + *E. coli* + *C. freundii* (3,9%). Доминирующим компонентом указанных ассоциаций является *E. coli* сероваров 0101 (28,9%), 041 (20,0%), 0141 (15,6%), 026 (13,3%), 0138 (13,3%), 015 (6,7%) и 033 (2,2%). Установлено, что 76,25% изолятов условно-патогенных микроорганизмов, изолированных из желчи больных холангиогепатитом кошек, были патогенными для белых мышей.

Ключевые слова: холангиогепатит, кошки, микробоценоз, желчь, бактобилия.

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INTRODUCTION

Opportunistic microorganisms are responsible for numerous animal diseases, including diseases of internal organs (gastroenteritis, pneumonia, nephritis, hepatitis, cholecystitis, etc.) [1]. Cholangiohepatitis, one of the most common liver diseases in cats, is often fatal [2, 3]. This condition is characterized by the development of bacterial or immunity-mediated inflammation process in the hepatic parenchyma and bile ducts, by secondary metabolic changes, intoxication and dehydration [2, 4]. According to the data on hepatobiliary disease prevalence in cats, acute (bacterial, neutrophilic) cholangiohepatitis ranks second after hepatic lipidosis [2, 3, 5].

Acute feline neutrophilic cholangiohepatitis is mostly caused by the following opportunistic bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus* spp., *Bacteroides* spp., *Streptococcus* spp., *Clostridium* spp. [5]. Development and progressing of feline cholangiohepatitis are conditioned by poor bile flow, because under normal physiological conditions the continuous bile flow and concurrent immunological protection, conferred by the epithelial cells of the bile ducts, maintain the hepatobiliary tract sterile [6]. Bile flow disorder and bile stasis create favourable conditions for backward migration of bacterial pathogens from the small intestine [3, 5, 7]. In the view of the above, biliary dyskinesia in the setting of partial obstruction, followed by ascending biliary infection, is the key factor for the development of acute feline bacterial cholangiohepatitis. Acute inflammation process, edema and thickening of bile duct walls, in turn, also aggravate stasis and facilitate pathological influence [8, 9]. A significant growth in bile pressure inside the hepatic ducts leads to weakening of protective immunological mechanisms, which in turn ensures favourable conditions for growth and development of commensal microorganisms. Further on the inflammation process involves bile ducts and hepatic parenchyma; bacterial translocation into systemic circulation occurs leading to bacteremia [10, 11].

Recently in clinical practice non-lethal evaluation of hepatic tissue and bile sterility has been widely used in case of hepatobiliary tract inflammation in cats [12]. Both bile and hepatic parenchyma can be taken for testing; and the procedure itself is performed under ultrasonographic guidance [13]. However bacteriological testing of bile is more informative, than testing of hepatic parenchyma puncture samples [5]. F. Schiborra et al. analyzed the results, obtained in the process of bacterial microflora study of small pet bile and its cytology [12]. Bile bacterial cultures were isolated in 21.3% cases. Most often *Escherichia coli* and *Enterococcus* spp. were isolated from bile, less often – *Clostridium perfringens*, *Bacteroides* spp. and *Actinomyces* spp., *Lactobacillus* spp., *Lactococcus* spp., *Listeria* spp., *Klebsiella* spp., *Salmonella* spp., *Streptococcus bovis* and *Pseudomonas*. Microbial associations in bile from cats and dogs were detected in 43.8% of cases. Cytology revealed bactobilia in 17.3% of cats. Liver samples taken from ill cats using surgery or laparoscopy more likely showed clean cultures, than samples obtained by percutaneous fine needle biopsy [5].

Thus, study of bacterial associations in the bile of cats, suffering from acute cholangiohepatitis is a vital trend in veterinary medicine of small pets.

In the view of the above, the aim of this study was qualitative and quantitative analysis of bile microbiocenosis of cats, suffering from acute cholangiohepatitis.

MATERIALS AND METHODS

The study was performed from 2015 to 2019 by the Chair for Infectious Diseases, Pathological Anatomy and Judicial Veterinary Medicine under the SEI LPR "Lugansk National Agrarian University" (Lugansk, Lugansk People's Republic). Clinical tests were performed at private veterinary clinics of Donetsk city.

The subject of the testing was the bile of cats, suffering from acute cholangiohepatitis, obtained using non-lethal methods under ultrasonographic guidance. The groups

were formed as the animals were submitted to clinics. The cats were selected for the study based on inclusion and exclusion criteria.

Inclusion criteria: clinical, laboratory and ultrasonographic signs of feline acute cholangiohepatitis.

Exclusion criteria: feline hepatic lipidosis, fatty liver disease, acute hepatitis, aseptic (immunity-mediated) types of cholangitis, oncology in abdominal cavity, positive results for parasites in feces, positive PCR results for infectious peritonitis agents, viral immunodeficiency, viral leukemia and hemotropic mycoplasma.

The acute cholangiohepatitis diagnosis was made in a comprehensive manner, taking into account the history, results of clinical examination, physical examination, morphological and biochemical blood test and ultrasonography [9].

Cholecystocentesis of cats, suffering from acute cholangiohepatitis, was performed under brief multimodal anesthesia. Cats were premedicated using gabapentin orally at the dose of 50 mg/kg; 15 minutes later dexmedetomidine hydrochloride solution was injected intramuscularly (5–10 μg/kg); 20 minutes after that propofol at the dose of 1–2 mg/kg was injected intravenously.

Ultrasound scan of abdominal organs was performed using multi frequency micro-convex probe at the frequency of 6–9 MHz. The optimal site for gall bladder puncture was determined using ultrasonography. The abdominal wall was punctured from the right side. Cholecystocentesis was performed aseptically under ultrasonographic guidance using a syringe (5 cm^3) and a needle 22G (0.7 × 40 mm). Transhepatic access was used. Maximum volume of bile was aspirated into a syringe. The surgical area around the puncture site was treated with 70% ethanol three times. At the end of the procedure the follow-up ultrasonographic scanning of cats' hepatobiliary system was carried out to evaluate the potential damage of the gall bladder.

Bacteriological testing of bile from cholangiohepatitis-affected cats was performed by seeding it on different nutrient media (beef extract broth, beef extract agar, serum broth with glucose and Sabouraud dextrose broth). Following incubation in a thermostat at 38 °C or at room temperature (Sabouraud dextrose broth) for 24–72 hours; colonies of various types were reseeded onto Petri dishes, containing Endo medium, beef extract broth, beef extract agar. Tubes demonstrating no growth of microorganisms were kept in a thermostat at 38 °C for more than 10 days. After studying cultural and morphological properties some typical microorganism colonies were seeded onto beef extract agar, beef extract broth and beef extract semi-liquid agar and incubated at 38 °C for 24 hours. Then tinctorial properties of bacterial cultures using common methods were studied. Microbial mobility was determined by bacterial growth rate in beef extract semi-liquid agar.

All pure bacterial cultures were seeded onto Gissa medium with glucose, maltose, mannose, sucrose, lactose, dulcite and mannitol added. Catalase activity was determined for all bacterial isolates. For this purpose bacterial mass, removed using a loop from the agar surface, was suspended in a slide in one drop of 3% hydrogen peroxide.

Gram-positive cocci were tested for hemolytic and coagulase activity, and growth properties were studied at 45 and 10 °C, pH 9.6, with 40% bovine bile and 6.5% sodium chloride added. To differentiate staphylococci and micrococci oxidation-fermentation test was performed using Hugh and Leifson's medium.

Gram-negative rod-shaped bacteria were additionally tested for fermentation of such hydrocarbons like sorbitol and inositol; lysine decarboxylase, ornithine decarboxylase, β-galactoside and phenylalanine-desaminase activities; ability to synthesize acetylmethylcarbinol, hydrogen sulfide and indole; utilize malonate and sodium citrate.

To suppress *Proteus* culture mobility before testing 96% ethanol was added to bacterial flasks, containing peptone-beef extract agar for 3–5 minutes and then removed. To identify and differentiate Pseudomonas the tested cultures were reseeded onto King's medium in flasks, containing peptone-beef extract agar and kept in a thermostat at 42 °C for 24–48 hours.

The further identification and differentiation of isolated microorganism cultures were performed using conventional methods according to Bergey's Manual of Systematic Bacteriology[1]. *Escherichia* serogroups were determined using "O" *Escherichia coli* Agglutinating Sera.

Bacterial pathogenic properties were studied by biological assay in white mice. Each isolate at the dose of 0.5 cm^3 was injected intraperitoneally to three 14–16 g white mice. The cultures were deemed pathogenic if one or more mice died within 5 days after infection.

All animal experiments were performed in strict compliance with interstate standard on laboratory animal keeping and handling GOST 33216-2014, adopted by Interstate Council on Standardization, Metrology and Certification and pursuant to the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

The final stage of the bacteriological test was focused on species composition and ratios of different pathogenic bacteria species, causing feline acute cholangiohepatitis.

The obtained digital data were processed using personal computer and licensed software MS Excel and Statistica 7.0[2].

RESULTS AND DISCUSSION

The volume of bile, collected from cats by percutaneous puncture of the gall bladder was 2.6 ± 0.85 ml. No complications following the cholecystocentesis were observed in the animals. To study etiological role of bacterial microbiocenosis in the development of acute cholangiohepatitis bacteriological tests were performed. 80 cultures of opportunistic bacteria were isolated from the bile of 51 diseased cats (Table 1).

Out of all isolated microorganisms 59 were Gram-negative and 21 Gram-positive. The most frequent isolates were *E. coli* (45); *E. faecalis* (13) were recovered less often, *S. aureus* (5), *E. aerogenes* (4), *P. aeruginosa* (4), *P. vulgaris* (3), *S. epidermidis* (3), *E. cloacae* (1), *P. mirabilis* (1) and *C. freundii* (1) were isolated seldom. The data obtained are compliant with other studies, published in the past [5, 12].

The results of isolation frequency of opportunistic microorganism monocultures and associations from the bile of acute cholangiohepatitis-affected cats were especially important for practical veterinary medicine.

In the course of the study it was established that 5 species of opportunistic microorganisms were isolated as associations and 25 as monocultures (Table 2).

[1] Bergey's Manual of Systematic Bacteriology. Vol. 1–5. ed. G. M. Garrity. 2nd ed. NY: Springer-Verlag; 2001–2012.

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Table 1
Species composition of bacteria, responsible for feline acute bacterial cholangiohepatitis

Таблица 1
Видовой состав бактерий, вызывающих развитие острого бактериального холангиогепатита у кошек

Microorganism species	Isolates	
	Absolute number	%
Gram-negative		
Citrobacter freundii	1	1.2
Enterobacter aerogenes	4	5.0
Enterobacter cloacae	1	1.2
Escherichia coli	45	56.3
Proteus mirabilis	1	1.2
Proteus vulgaris	3	3.8
Pseudomonas aeruginosa	4	5.0
Gram-positive		
Staphylococcus aureus	5	6.2
Staphylococcus epidermidis	3	3.8
Enterococcus faecalis	13	16.3
Total	80	100.0

As part of associations the most frequent isolates were *E. coli* (43.6%), less often – *E. faecalis* (18.2%), seldom – *S. aureus* (9.1%), *E. aerogenes* (7.2%), *P. vulgaris* (5.5%), *P. aeruginosa* (5.5%), *S. epidermidis* (5.5%), *C. freundii* (1.8%), *E. cloacae* (1.8%) and *P. mirabilis* (1.8%). As monocultures *E. coli* (84.0%) were often recovered, and *E. faecalis* (12.0%) and *P. aeruginosa* (4.0%) were isolated rarely. It should be added that *C. freundii*, *E. aerogenes*, *E. cloacae*, *P. mirabilis*, *P. vulgaris*, *S. aureus* and *S. epidermidis* were not isolated as monocultures from the bile of acute cholangiohepatitis-affected cats.

In qualitative terms 12 variants of bacterial associations were isolated (Table 3). When determining quantitative and qualitative composition of microbiocenoses of cats, suffering from acute cholangiohepatitis, 26 opportunistic microorganism associations were detected (2–3 associates).

The recovery ratio of two-component associations from the bile of acute cholangiohepatitis-affected cats was 75.0% and three-component associations – 25.0%. The most frequent associates of polycomponent opportunistic bacteria associations were *E. coli* + *E. faecalis* (26.9%), less often – *E. coli* + *E. aerogenes* (15.4%), *E. coli* + *P. vulgaris* (11.5%), *E. coli* + *S. aureus* (11.5%), seldom – *E. coli* + *P. aeruginosa* (7.7%), *E. cloacae* + *S. aureus* (3.9%), *E. faecalis* + *S. aureus* (3.9%), *E. coli* + *P. mirabilis* (3.9%), *E. coli* + *S. epidermidis* (3.9%), *E. coli* + *E. faecalis* + *S. epidermidis* (3.9%), *E. coli* + *P. aeruginosa* + *S. epidermidis* (3.9%), *E. coli* + *E. faecalis* + *C. freundii* (3.9%). It should be noted that these were the first obtained data on microbiocenosis quantitative and qualitative composition of the bile from acute cholangiohepatitis-affected cats.

It must be stressed that the most essential component of such associations was *Escherichia*.

The most frequent *Escherichia* serovars isolated from the bile of diseased cats were O101 (28.9%), less often – O41 (20.0%) and O141 (15.6%), seldom – O138 (13.3%), O26 (13.3%), O15 (6.7%) and O33 (2.2%) (Table 4).

Table 2
Isolation frequency of opportunistic microflora monocultures and associations from the bile of cats, suffering from acute cholangiohepatitis

Таблица 2
Частота изоляции монокультур и ассоциаций условно-патогенных микроорганизмов из желчи кошек, больных острым холангиогепатитом

Microorganism species	Isolates recovered			
	As associations		As pure culture	
	number	%	number	%
Citrobacter freundii	1	1.8	0	0
Enterobacter aerogenes	4	7.2	0	0
Enterobacter cloacae	1	1.8	0	0
Escherichia coli	24	43.6	21	84.0
Proteus mirabilis	1	1.8	0	0
Proteus vulgaris	3	5.5	0	0
Pseudomonas aeruginosa	3	5.5	1	4.0
Staphylococcus aureus	5	9.1	0	0
Staphylococcus epidermidis	3	5.5	0	0
Enterococcus faecalis	10	18.2	3	12.0
Total	55	100.0	25	100.0

Pathogenic properties of 80 opportunistic bacteria cultures, isolated from the bile of acute cholangiohepatitis-affected cats, are shown in Table 5.

It was established that out of 80 isolates of opportunistic bacteria, recovered from the bile of diseased cats, 61 isolates were pathogenic for white mice and 19 cultures were non-pathogenic (76.25 and 23.75% correspondingly). It should be noted that all *P. aeruginosa* and *C. freundii* isolates were pathogenic for white mice.

Moreover, 39 out of 45 *E. coli* isolates (86.7%) were pathogenic for white mice, and *S. epidermidis, E. cloacae* and *P. mirabilis* cultures were non-pathogenic. The data obtained indirectly support the hypothesis that non-pathogenic strains can transform to pathogenic ones under certain conditions. We deem that the promising areas of further studies are biological properties of opportunistic bacteria in cats, suffering from acute bacterial cholangiohepatitis, and development of highly effective methods to treat this pathology.

CONCLUSION

The microbiocenosis of bile, collected from acute cholangiohepatitis-affected cats, is represented by the following isolates: *E. coli*, *E. faecalis*, *S. aureus*, *E. aerogenes*, *P. aeruginosa*, *P. vulgaris*, *S. epidermidis*, *E. cloacae*, *P. mirabilis* and *C. freundii*. The prevalence of two-component associations was 75.0%, and of three-component associations – 25.0%. The most frequent biliary bacterial associations were *E. coli* + *E. faecalis* (26,9%), *E. coli* + *E. aerogenes* (15.4%), *E. coli* + *P. vulgaris* (11.5%), *E. coli* + *S. aureus* (11.5%) were detected less often, *E. coli* + *P. aeruginosa* (7.7%), *E. cloacae* + *S. aureus* (3.9%), *E. faecalis* + *S. aureus* (3.9%), *E. coli* + *P. mirabilis* (3.9%), *E. coli* + *S. epidermidis* (3.9%), *E. coli* + *E. faecalis* + *S. epidermidis* (3.9%), *E. coli* + *P. aeruginosa* + S. *epidermidis* (3.9%), *E. coli* + *E. faecalis* + C. *freundii* (3.9%) were detected on rare occasions. In most cases the essential component of these associations was *E. coli* serovars O101 (28.9%), O41 (20.0%), O141 (15.6%), O138 (13.3%), O26 (13.3%), O15 (6.7%) and O33 (2.2%). It was established that 80.0% of opportunistic bacteria isolates, recovered from the bile of cats, suffering from acute cholangiohepatitis, were pathogenic for white mice. Herewith all *P. aeruginosa* and *C. freundii* isolates, and most *E. coli* isolates (86.7%) were pathogenic.

Table 3
Structure of bacterial associations, responsible for feline acute cholangiohepatitis

Таблица 3
Структура ассоциаций бактерий, вызывающих острый холангиогепатит у кошек

Number of microbe associates	Number of associations	Species composition
2	7	*E. coli* + *E. faecalis*
	4	*E. coli* + E. *aerogenes*
	3	*E. coli* + P. *vulgaris*
	3	*E. coli* + S. *aureus*
	1	*E. cloacae* + S. *aureus*
	1	*E. faecalis* + S. *aureus*
	1	*E. coli* + P. *mirabilis*
	1	*E. coli* + S. *epidermidis*
	2	*E. coli* + P. *aeruginosa*
3	1	*E. coli* + *E. faecalis* + S. *epidermidis*
	1	*E. coli* + *P. aeruginosa* + S. *epidermidis*
	1	*E. coli* + *E. faecalis* + C. *freundii*

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Table 4
Serological typification of *E. coli* isolates, recovered from the bile of cats, suffering from acute cholangiohepatitis

Таблица 4
Серологическая типизация изолятов *E. coli*, изолированных из желчи кошек, больных острым бактериальным холангиогепатитом

Serogroups	Number of isolated cultures	
	Absolute number	%
015	3	6.7
026	6	13.3
033	1	2.2
041	9	20.0
0101	13	28.9
0138	6	13.3
0141	7	15.6
Total	45	100.0

Table 5
Pathogenicity of bacterial isolates, recovered from the bile of acute cholangiohepatitis-affected cats, for white mice

Таблица 5
Патогенность для белых мышей изолятов бактерий, выделенных от кошек при остром бактериальном холангиогепатите

Microorganism	Cultures studied	Pathogenic		Non-pathogenic	
		Number of isolates	%	Number of isolates	%
Citrobacter freundii	1	1	1.6	0	0
Enterobacter aerogenes	4	3	4.9	1	5.3
Enterobacter cloacae	1	0	0	1	5.3
Escherichia coli	45	39	63.9	6	31.4
Proteus mirabilis	1	0	0	1	5.3
Proteus vulgaris	3	2	3.3	1	5.3
Pseudomonas aeruginosa	4	4	6.6	0	0
Staphylococcus aureus	5	2	3.3	3	15.8
Staphylococcus epidermidis	3	0	0	3	15.8
Enterococcus faecalis	13	10	16.4	3	15.8
Total	80	61	100.0	19	100.0

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