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GALLBLADDER MICROBIOTA IN PATIENTS WITH GALLSTONE DISEASE

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◆ The article presents the results of investigating 20 patients with cholelithiasis. The bile and a piece of the gallbladder were taken to analyze microbiota during a scheduled laparoscopic cholecystectomy. The study of microbiota was carried out with the cultural method and real-time PCR. *Bifidobacterium spp.*, *Bacteroides spp.*, *Lactobacillus spp.*, *E. coli* prevailed in the taxonomic structure of isolated bacteria. Isolated *Enterococcus spp.* had a lot of genes encoding various factors of pathogenicity.

◆ **Keywords:** gallstone disease; bile; microbiota; microflora; dysbacteriosis; *Enterococcus spp.*; *E. faecium*; *E. faecalis.*

МИКРОБИОТА ЖЕЛЧНОГО ПУЗЫРЯ У ПАЦИЕНТОВ С ЖЕЛЧНОКАМЕННОЙ БОЛЕЗНЬЮ

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◆ В статье приведены результаты обследования 20 пациентов с желчнокаменной болезнью, которым во время плановой лапароскопической холецистэктомии проводили забор желчи и участка стенки желчного пузыря для исследования микробиоценоза. Микробиоценоз исследовали культуральным методом и методом полимеразной цепной реакции в реальном времени. В таксономической структуре выявленных из желчного пузыря бактерий преобладали *Bifidobacterium spp.*, *Bacteroides spp.*, *Lactobacillus spp.*, *E. coli.* Большинство выделенных бактерий *Enterococcus spp.* обладали генами, кодирующими различные факторы патогенности.

◆ **Ключевые слова:** желчнокаменная болезнь; желчь; микробиота; микрофлора; дисбактериоз; *Enterococcus spp.*, *E. faecium, E. faecalis.*

Introduction

Gallstone disease (GSD) is one of the most common lifestyle diseases. According to Vasilenko, this is "payment for a long and happy life" [1]. In Europe, cholelithiasis is detected in 9%–21% of the population during ultrasound examinations, and the incidence is 0.63 cases per 100 people annually [2, 3]. Over the past decades, the incidence of GSD, revealed both by ultrasound and autopsy, tends to increase worldwide, which may be due to the change in the stereotype of nutrition and the pandemic of obesity [4, 5].

GSD or cholelithiasis is a multifactorial and multistage disease of the hepatobiliary tract with genetic predisposition, characterized by pathological changes in cholesterol and/or bilirubin metabolism with stone formation in the biliary tract [6].

According to classical concepts, gallstones are formed due to the following three pathological processes:

- changes in the component composition of bile (oversaturation with cholesterol, lack of bile acids, increase in the index of bile saturation with cholesterol);
- nucleation (formation of microscopic crystals of cholesterol monohydrate in bile oversaturated with cholesterol); and
- gallbladder dysfunction (hypokinetic disorders leading to impaired bile discharge) [7].

In the etiopathogenesis of GSD, an important role is played by various risk factors that

Table 1 / Таблица 1

Risk factor of cholesterol and bilirubin stones formation [7]
Факторы риска формирования холестериновых и билирубиновых камней [7]

Risk factor for cholesterol stones	Risk factor for bilirubin stones
Unmodifiable risk factors	
Age	Age
Female sex	
Heredity	
Nationality	
Modifiable risk factors	
High-caloric diet rich in simple carbohydrates	Extensive resection of the stomach (black stones)
Diet low in fiber content	Hepatic cirrhosis
Pregnancy	Crohn disease
Hypodynamia	Chronic hemolysis (black stones)
Obesity, metabolic syndrome	Cystic fibrosis (black stones)
Type 2 diabetes mellitus, insulin resistance	Biliary tract infections (brown stones)
Rapid weight loss, weight fluctuations	
Parenteral nutrition	
Drugs: estrogens, progesterone, octreotide, ceftriaxone, thiazide diuretics	
Bariatric surgeries, gastrectomy	
Chronic hepatitis C	

differentiate depending on the type of gallstones. Table 1 shows the risk factors for cholesterol and bilirubin stone formation.

Much attention has been recently paid to elucidating the role of microorganisms of various biotopes in maintaining health and developing a wide range of diseases in humans. The gastrointestinal tract (GIT) microbiota was shown to actively participate in the pathogenesis of acute cholecystitis and cholangitis. Furthermore, with regard to the issue related to the presence of microorganisms, the role of gallbladder microbiocenosis under normal and pathological conditions is not completely resolved, which is primarily due to the methodological difficulties of material sampling.

In vitro studies have shown the bactericidal action of bile acids, implemented through damage to bacterial membranes and DNA of microorganisms. This fact was an indirect evidence of a healthy person, such as sterility, and the separation of bacterial flora from the bile of patients with acute surgical diseases of the biliary tract was regarded as pathological [8]. The detection of classical representatives of intestinal microflora in bile, particularly bacteria belonging to the Enterobacteriacea family, under such conditions, was explained by potential duodenobiliary refluxes. In cholelithiasis, Gram-negative intestinal bacteria characteristic of the proximal GIT were also found in gallstones [9].

The use of the latest molecular and genetic methods, such as 16S rRNA sequencing and metagenomic shotgun sequencing, enabled the study of the complex endoecology of the bile ducts (BD). Microbiocenosis has been proven to play an important role in the normal physiology of BD and may also be responsible for the development of various diseases, including GSD [10].

The Institute of Experimental Medicine and I.I. Mechnikov North Western State Medical University conducted a clinical study of microbiocenosis of the intestine and gallbladder by using culture and culture-independent methods to investigate the influence of the GIT microflora on the course of GSD.

Materials and methods

The study included patients with GSD with indications for elective cholecystectomy. During laparoscopic cholecystectomy under sterile conditions, samples from bile and a segment of the gallbladder wall were obtained to investigate the microbiota of these biotopes by using the culture and real-time polymerase chain reaction (PCR) methods.

The gallbladder microbiota was studied in 20 patients (4 [20%] men and 16 [80%] women). The mean age of the patients was 47.4 ± 6.1 years.

The quali- and quantitative compositions of the gallbladder microbiota were studied using real-time PCR using the Colonoflor-16 diagnostic test system. This test system enables the evaluation of the composition of microbiota according to parameters, such as total bacterial mass, content and concentration of *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Bacteroides fragilis* group, *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Clostridium difficile*, *Clostridium perfringens*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Escherichia coli* enteropathogenic, *Enterococcus* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Fusobacterium nucleatum*, *Parvimonas micra*, *Staphylococcus aureus*, *Candida* spp., *Salmonella* spp., and *Shigella spp.*

In addition to PCR analysis, the contents of the gallbladder were inoculated on non-selective (blood agar), differential diagnostic (endo agar, chromogenic UTI agar), and selective (azide agar) media to detect and identify aerobic bacteria.

Research results

The structure of the gallbladder microbiota studied using real-time PCR and the Colonoflor-16 diagnostic test system is presented in Figure 1.

The taxonomic structure of the microorganisms identified was predominated by *Bifidobacterium* spp., *Bacteroides* spp., *E. coli*, and *Lactobacillus* spp., which generally corresponds to the previously obtained data from metagenomic studies on the detection of a variety of gamma proteobacteria and bacteroids in cholesterol stones and bile in patients with cholelithiasis [11, 12].

The sensitivity of the classical cultural method was predictably lower, which may be, *inter alia*, due to the inability to identify anaerobic bacteria. Hence, only representatives of *Enterococcus* spp. were isolated, using the inoculation method, from the gallbladder contents of the patient study group. These bacteria were detected only in

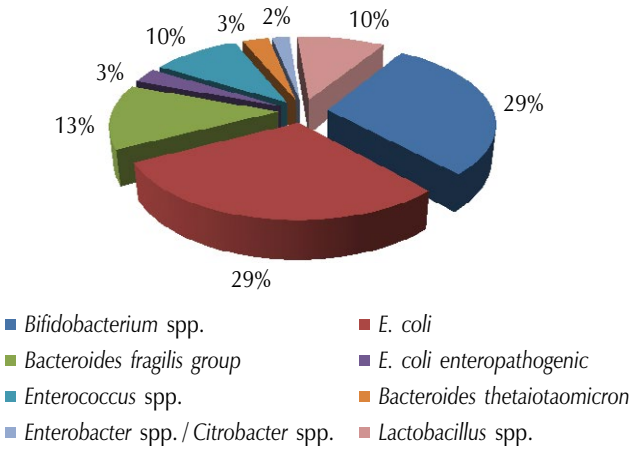

Fig. 1. The structure of bacterial taxa identified by the PCR in the tissues of the removed gallbladder in the patients with gallstone disease

Рис. 1. Структура бактериальных таксонов, идентифицированных методом полимеразной цепной реакции в тканях удаленного желчного пузыря у пациентов с желчнокаменной болезнью

Table 2 / Таблица 2

Genes for pathogenicity factors of *Enterococcus spp.* and their biological significance

Гены факторов патогенности *Enterococcus spp.* и их биологическое значение

Gene for pathogenicity factor	Biological significance
esp	Adhesion and invasion
asa	Adhesion and invasion
efa	Adhesion and invasion
gel	Gelatinase synthesis
spr	Serine proteinase synthesis
fsr	Pheromone synthesis
vanA	Vancomycin resistance

7 of 20 patients (35%). *Enterococcus faecium* was identified in four patients; *Enterococcus faecalis* was identified in two patients; and both types of enterococci were found in one patient.

Enterococci isolated from patients were studied using PCR using previously developed primers to identify the pathogenicity determinants. Table 2 presents the genes for pathogenicity factors of *Enterococcus* spp. and their biological significance, and Table 3 presents the genes for the pathogenicity factors of enterococci isolated from the gallbladder in the study participants.

Enterococci isolated from the gallbladder in patients with GSD were characterized by a wide range of pathogenicity genes. Due to the pathogenicity factors of strains of *Enterococcus* spp., they are known to be capable of causing various diseases. Thus, Duan et al. showed that cytolysin-producing strains of *E. faecalis* have a toxic effect on hepatocytes, increasing the mortality in alcohol-induced liver damage [13].

Table 3 / Таблица 3

Pathogenicity factors of enterococcus strains isolated from the gallbladder in the patients with gallstone disease

Факторы патогенности штаммов энтерококков, выделенных из желчного пузыря у пациентов с желчнокаменной болезнью

Strain number	Species	Pathogenicity factor genes						
		esp	*asa*	*efa*	*gel*	*spr*	*fsr*	*vanA*
1	*E. faecium*	–	–	–	–	–	–	–
2	*E. faecium*	–	+	–	+	+	+	–
3	*E. faecium*	–	+	–	+	+	+	–
4	*E. faecalis*	+	+	+	+	+	+	–
5	*E. faecium*	–	–	–	–	–	–	–
6	*E. faecalis*	+	+	+	+	+	+	–
7	*E. faecium*	–	–	–	–	–	–	+
8	*E. faecalis*	+	+	+	+	+	+	–

Similarly, the role of other pathogenicity factors of enterococci in the development of hepatobiliary system diseases has not been studied, and further study is needed to clarify their role.

Unexpectedly, *E. faecium* strain with vancomycin-resistant gene (*vanA*) was detected in the gallbladder microbiocenosis. In previous studies in the Institute of Experimental Medicine, vancomycin-resistant strains of *E. faecium* have been shown to cause epidemic outbreaks of nosocomial infections in hospitals in the northwest region of Russia [14].

Discussion

Similar to the GIT, the mucous membrane of the biliary tract serves as a chemical, mechanical, and immunological barrier. However, studying the BD microbiota is difficult because it is associated with methodological difficulties of sampling sterile bile. Even in the case of duodenoscopy with cannulation of the common BD, there are risks of contamination of the collected bile by microorganisms that inhabit the upper GIT. In addition, the vast majority of bile microflora cannot be cultured using standard bacteriological techniques.

The taxonomic diversity of gallbladder microbiota has been demonstrated in previous studies. Wu et al. showed that the biliary system microbiota is more diverse than the intestinal microbiota. Similarly, the endoecological system of the gallbladder does not fully correspond to the intestinal microbiota. Thus, it has a lower content of bacteroids [11].

The process of the BD microbiocenosis formation is complex and long. According to one theory of mucosal immunoglobulins, bile acids exert selective pressure during selection of potentially viable intestinal bacterial strains that migrate from the intestinal lumen and form a unique microflora of the biliary tract [15, 16]. In this process, microorganisms that can overcome the natural nonspecific local mechanisms of protection of the macroorganism and those with anti-complementary, anti-lysozyme, anti-immunoglobulin, anti-interferon, proteolytic activity, etc., are selected [17].

The microbiome formed potentially hinders GIT microorganisms, opportunistic and pathogenic bacteria, fungi, and viruses from colonizing the BD [18, 19]. Similar to intestinal bacteria, biliary microbiota is presumed to be capable of deconjugating and hydrolyzing bile acids.

In the presence of structural or functional disorders at the level of the BD, for example, dysfunction of the sphincter of Oddi, microorganisms that inhabit the proximal GIT, including opportunistic and pathogenic microorganisms, can migrate into the biliary tract. Bacterial metabolism products, enzymes, particularly β-glucuronidase, can precipitate bilirubin molecules, leading to biliary sludge and stone formation [20–22].

In our study, a wide range of bacteria localized in the gallbladder, mainly representatives of *Bifidobacterium* spp., *Bacteroides* spp., and *Lactobacillus* spp., was identified in patients with GSD. In addition, a large number of *E. coli*, including enteropathogenic strains, were revealed in the bile of patients. Using the culture method, only Gram-positive aerobic bacteria of the Enterococcus family were identified because of its lower sensitivity in the bile of patients. In most enterococci, genes of pathogenicity factors were identified, including those that determine the reserve antibiotic resistance to vancomycin.

The mechanism of occurrence and role of the microorganisms identified in lithogenesis are discussed and have not been established unequivocally. It should be noted that obesity (body mass index >30 kg/m^2) was noted in 80% of patients enrolled in the study, which is consistent with most epidemiological studies. In this case, several hypotheses that explain the mechanism of communication between dysbiosis and GSD can be proposed. These hypotheses are not mutually exclusive and can complement each other.

Hypothesis 1. The impact of adverse environmental factors, chronic stress, eating disorders, overweight, and obesity lead to a gradual change in the intestinal microbiome and the BD normal microbiota. Activation of bacteria with pathogenicity factors due to the *quorum sensing* mechanism causes the development of subclinical inflammation in the mucous membrane of the GIT, which contributes to a change in the component composition of bile, its kinetics, and stone formation.

Hypothesis 2. Quanti- and qualitative changes in the intestinal microbiome can lead to increased intestinal pressure, intestinal dyskinesia, and duodenostasis, contributing to the occurrence of duodenobiliary refluxes, contamination of bile by

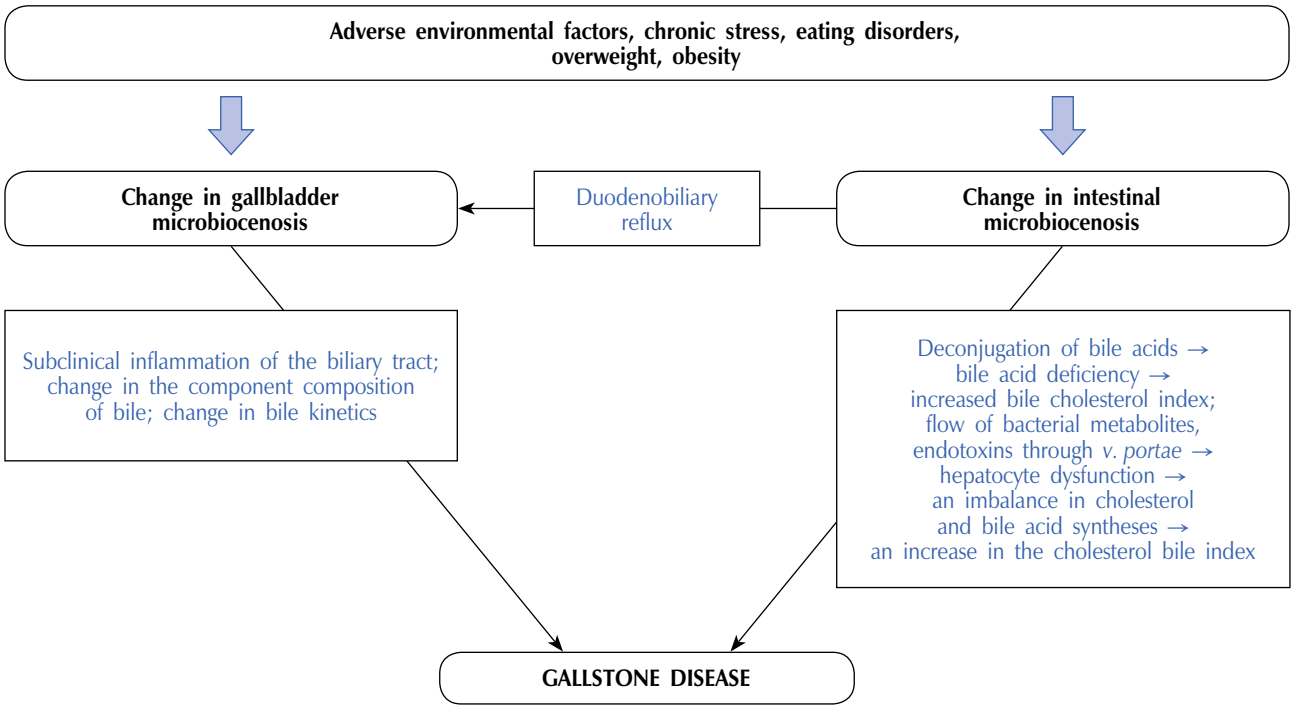

Fig. 2. Possible mechanisms of the influence of dysbiotic disorders on the development of gallstone disease

Рис. 2. Возможные механизмы влияния дисбиотических расстройств на развитие желчнокаменной болезни

opportunistic intestinal bacteria, inflammation, changes in the composition and kinetics of bile, and stone formation.

Hypothesis 3. Intestinal dysbiosis causes excessive deconjugation of bile acids and disrupts their enterohepatic circulation, leading to deficiency of bile acids and an increase in the cholesterol index.

Hypothesis 4. A change in the intestinal microbiome results in a change in the flow of biologically active substances, bacterial metabolites, and endotoxins through the portal system, resulting in a change in the metabolic activity of hepatocytes and an imbalance in cholesterol and bile acid syntheses.

Figure 2 shows the relationship of microbiocenosis and GSD.

Conclusion

Studying the BD microbiota under normal and pathological conditions is difficult, which may be due to the methodological difficulties of collecting material in compliance with sterility.

According to the results of preclinical and clinical studies, the BD microbiocenosis is significant in the development of various pathological conditions. Given the prevalence of diseases of this localization, particularly cholelithiasis, the study of the mechanism of the biliary tract microbiocenosis formation and change seems to be an important task for disease prevention.

To test the existing hypotheses, as well as our hypotheses, large-scale prospective cohort studies are required, which would enable more detailed evaluation of the norm and determine cause–effect relationships between changes in the composition of the digestive tract microbiota and development of cholelithiasis.

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