

Cholelithiasis – always infected?

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Tomasz Matyjas^{1ABCDEFG}, Lech Pomorski^{1ADG}, Henryk Witas^{2BD}, Tomasz Płoszaj^{2BDE},
Katarzyna Matyjas^{3CEF}, Krzysztof Kaczka^{1B}

¹Department of General and Oncological Surgery, Medical University of Lodz;
Head of the Department: Prof. L. Pomorski, MD PhD

²Department of Molecular Biology, Medical University of Lodz; Head of the Department: Prof. H. Witas, MD PhD

³Department of Cardiology, Maria Skłodowska-Curie Hospital in Zgierz; Head of the Department: H. Komorowska, MD, PhD

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ABSTRACT

This study aims to present results regarding the presence and identification of bacterial strains found in bile and gallstones located in the gallbladder and bile ducts in patients operated on due to cholelithiasis.

Materials and Methods. Bacterial culture was evaluated in 92 patients. There were 54 women (59%) and 38 men (41%) who underwent surgery on account of cholelithiasis and /or gallstones in bile ducts between 2013 and 2014. Bile and gallstone samples were cultured intraoperatively for bacteria; bacterial strains were identified, and their sensitivity to antibiotics was determined. Molecular methods (NGS and Sanger method) were used to separate bacterial strains in one of the gallbladder stones and the results were compared with bacterial strains grown from the bile.

Results. Bile cultures were positive in 46 patients that is, 50% of the study group. The following bacteria strains were grown: Enterococcus spp. (44%), Escherichia coli (37%) and Klebsiella spp. (35%). Candidiasis accompanied by bacterial infection was detected in 7 patients (15%). Molecular testing of gallstones revealed DNA of Enterococcus spp., Escherichia spp., Streptococcus spp. and Clostridium spp. In the bile culture of the same patient Enterococcus spp. (avium and faecalis) was detected.

CONCLUSION

1. More than one pathogen was grown on samples obtained from 31 patients (70%) with bile infection.
2. The most common pathogens include Enterococcus spp., Escherichia coli and Klebsiella spp.
3. Bacterial infections are often accompanied by a fungal infection (Candida albicans)
4. Bacterial strains grown from a gallstone sample partially corresponded with strains identified in the bile of the same patient.

KEYWORDS:

bile, bile deposits, gallstones, biliary infection, bacteria

INTRODUCTION

Cholelithiasis is a widespread disease. It is estimated that it affects approximately 15 to 18 percent of adults. In Poland cholelithiasis is 2-3 times more common in women. At the age above 60, over 30% of women have deposits in the gallbladder. The etiology of cholelithiasis is complex (2,4,5,6,8). It is suspected that the bile deposits develop in the course of an inflammatory process involving bacteria (1). In a similar manner, gallstones may act as foreign bodies tending to increase bacterial colonization (8). The development of bile deposits may comprise the interaction of both mechanisms: bacterial and nonbacterial. Nevertheless, the role of bacteria in gallbladder deposit formation has not been proven so far (10). At present, it is believed that bile and deposits in the gallbladder may be sterile in the course of asymptomatic cholelithiasis, whereas ductal cholelithiasis is almost always accompanied by an infection (9). This results in specific clinical and therapeutic consequences that find their reflection in treatment, especially regarding the use of antibiotics. So far, despite these assumptions, the clinical significance of the presence of bacteria in the bile deposits remains unknown. Proving the presence of bacterial DNA in bile deposits and determination of the role played by bacteria in their formation may facilitate prognosis of cholelithiasis and decrease the risk of developing this condition. This may enable not only effective prevention but also treatment, and decrease the prevalence of complications resulting from this condition due to the use of antibacterial agents.

This study aims to present results regarding the presence and identification of bacterial strains in bile and gallstones in the gallbladder and bile ducts. The presence of bacterial DNA in bile de-

posits and strain identification will be performed using molecular methods in the following stages of research, which require large logistic and financial outlays.

MATERIAL AND METHODS

The research was conducted on bile samples and gallstones from the gallbladder and bile ducts sampled during surgery from 92 patients with symptomatic cholelithiasis and / or ductal cholelithiasis from different departments of the Voivodeship Hospital in Zgierz between 2013 and 2014. The study group comprised of 54 (59%) women and 38 (41%) men aged 18-88 years, mean 47 +/- 3 years. The patients underwent standard surgical procedures: laparoscopic cholecystectomy - 46 patients (50%) and open cholecystectomy - 16 (17%). Some patients underwent endoscopic retrograde cholangiopancreatography with deposit evacuation (ERCP) - 30 patients (33%). The bile was collected in a sterile manner in two containers containing a culture medium for bacterial growth (BactAlert system): separate for aerobic bacteria and anaerobic bacteria. The obtained cultures were identified and sensitivity to antibiotics was determined (tests were conducted in bacteriological department of laboratory of Regional Specialist Hospital in Zgierz). The bile samples were collected at the same time with gallstones, which were later tested for bacterial DNA with molecular methods. Trial series of molecular tests were performed in the Department of Molecular Biology, Medical University of Lodz. Bacterial DNA was isolated from gallstones cleaned with chemical and physical methods (UV) and pulverized using a cryogenic grinder (SPEX 6770). Isolation of DNA was carried out in a closed system by Roche biorobot (Compact MagnaPure)

according to the manufacturer's instructions. The concentration of isolated DNA was assessed by a fluorometer (Qubit 2.0). The presence of bacterial DNA in the isolated material was tested using PCR with primers complementary to the coding region of the 16S rRNA in the bacterial genome. This gene is highly conservative. DNA derived from eukaryotic cells was amplified. PCR was conducted using a GeneAmp PCR system 9700 (Applied Biosystems) and specific products of 800 bp were analyzed by PAGE. In the next step, conventional sequencing was performed (Sanger). Unfortunately, the limitations of this method due to the fact that it analyzes all matrix sequences, unlike the new generation methods, which analyze each matrix separately, prevented more insightful analysis of the obtained sequences. Therefore, the next step was to analyze the sample with a new method of sequencing with device manufactured by Illumina (MiSeq) (testing was conducted by Genomed in Warsaw). Metagenomics was conducted using 16S fragment of rRNA. The obtained results underwent bioinformatic analysis (GreenGenes v13.5) in order to isolate and identify the specific bacterial strains in the sample.

RESULTS

Table 1. presents the number of patients with a confirmed bile infection, diagnosed basing on the bile sampled in the course of individual procedures regarding all patients operated on using the particular method. Bile infection was detected in 46 patients (50%); whereas the patients who underwent ECP with gallstone evacuation constituted 96.5% (29 patients) of all individuals treated with this method due to ductal cholelithiasis.

Table 2. presents data regarding the number of patients in whom the particular bacterial strains were grown from the bile sampled intraoperatively in the context of all patients operated on with the particular method. It indicates that different bacterial strains were grown on samples obtained from one patient.

Table 3. presents the number of patients in whom one or more bacterial species were grown depending on the type of procedure in the context of all patients operated on using the particular method. An infection with more than one bacterial strains was detected in 31 patients out of 46 infected individuals, which constitutes 67.5% of all infected patients.

Table 4. presents bacterial strains identified from the same gallstone by means of analysis of Sanger sequencing method, whereas Figure 1 by means of NGS. Bacterial strains grown at the same time from the bile of the same patient, from whom this gallstone was obtained included *Enterococcus* and *Enterococcus avium faecalis*.

DISCUSSION

Carl Langenbuch performed cholecystectomy for the first time in Berlin in 1882. Although 124 years have passed since this surgery was conducted, treatment of cholelithiasis remains a significant clinical issue due to high prevalence and lack of effective prevention, as well as not entirely satisfactory treatment outcomes and complications such as iatrogenic bile duct damage. A hypothesis was formulated that a primary bile infection may be one of the

Tab. I. The number of positive and negative cultures in patients operated on using various methods.

RODZAJ ZABIEGU	WYNIKI POSIEWÓW ŻÓŁCI		ŁĄCZNIE
	Dodatnie	Ujemne	
Otwarta cholecystektomia	11 (69%)	5 (31%)	16 (100%)
Cholecystektomia laparoskopowa	6 (13%)	40 (87%)	46 (100%)
ECPW z pobraniem złożu	29 (96,5%)	1 (3,5%)	30 (100%)
Razem	46	46	92

Tab. II. Distribution of respective bacterial strains grown on samples obtained from patients operated on with various methods.

WYHODOWANE GATUNKI BAKTERII	LICZBA PACJENTÓW			ŁĄCZNIE
	Otwarta cholecystektomia (11 dodatnich posiewów na 16)	Cholecystektomia laparoskopowa (6 dodatnich posiewów na 46)	ECPW (29 dodatnich posiewów na 30)	
<i>Enterococcus</i> spp.	6 (55%)	2 (33,5%)	12 (41,5%)	20
<i>Escherichia coli</i>	4 (36,5%)	2 (33,5%)	11 (38%)	17
<i>Klebsiella</i> spp.	4 (36,5%)	1 (17%)	11 (38%)	16
<i>Staphylococcus</i> spp.	3 (27,5%)	1 (17%)	6 (21%)	10

Tab. III. The number of pathogen strains grown on the samples obtained from patients with positive cultures operated on with various methods.

LICZBA GATUNKÓW WYHODOWANYCH PATOGENÓW W ŻÓŁCI OD PACJENTA	LICZBA PACJENTÓW			ŁĄCZNIE
	Otwarta cholecystektomia (11 dodatnich posiewów)	Cholecystektomia laparoskopowa (6 dodatnich posiewów)	ECPW (29 dodatnich posiewów)	
1	5 (45,5%)	2 (33,5%)	8 (27,5%)	15
2	2 (18%)	3 (50%)	12 (41,5%)	17
3	3 (27,5%)	1 (16,5%)	6 (20,5%)	10
4	1 (9%)	0 (0%)	3 (10,5%)	4

causes of gallstone formation. So far, however this assumption has not been confirmed (8). Proving the role played by bacteria in cholelithiasis may allow not only for effective prophylaxis, but perhaps also causal treatment with broad-spectrum drug antimicrobial agents (3,4,5) and optionally antifungal drugs. This may result in decrease in the number of complications due to a change in the treatment plan. Detection and identification of bacteria not only in the bile, but also in gallstones justifies further research on a larger group of patients.

This study presents results of culture growth performed on bile sampled from the gallbladder and bile ducts of patients operated on due to symptomatic cholelithiasis. Bacteria most commonly detected in the bile included *Enterococcus*, *Escherichia*, *Klebsiella*, *Staphylococcus* and *Streptococcus*. *Enterococ-*

cus was the most common (45% of positive cultures), *Escherichia coli* (37,5%) was the second most common, and *Klebsiella* (25%) was the third (Tab.2). Cetta identified the same bacterial strains but with a significantly different prevalence (11). In this trial, the study group was comprised of 960 patients operated on due to cholelithiasis.

As many as 327 of them were men (34%) and 633 (66%) were women. *E. coli* was the most common pathogen (66,8%) whereas, *Klebsiella* and *Enterococcus* were detected in single cases.

Kaufman et al. (1) obtained similar results. In this case, the study group comprised of 75 patients with cholelithiasis (66% women) and ductal cholelithiasis (40% women). In 17 patients (22.5%) bile cultures were positive. Ten of them (16%) suffered from cholelithiasis and 7 (70%) from ductal cholelithiasis. This author also confirmed that the percentage of bile duct infections is significantly higher in ductal cholelithiasis. In our study group, patients with a positive bile culture constituted 27.5 %, whereas bile cultures in the course of ductal cholelithiasis were positive in 96.5%, which proves the previous assumption. In the aforementioned study, *Escherichia*, *Enterococcus*, *Klebsiella* and *Pseudomonas* were most commonly detected. In our work, as well as in the other study in most patients with positive cultures, more than one pathogen was grown (1, 11). When the antibiotic sensitivity was determined, it turned out that targeted therapy requires more than one antibacterial drug or a broad-spectrum drug.

Authors did not observe candidiasis in any of the mentioned articles, while it constituted 15% of positive cultures in our patients, where fungal infection accompanied bacterial infections. Therefore, antifungal drugs may potentially be added to antibacterial drugs before, as well as after surgery.

Initial testing proves the usefulness of molecular methods for analysis of DNA isolated from bile deposits. The research team was able to successfully isolate bacterial DNA, and then multiply with PCR specific for 16S rRNA. In the next step, conventional sequencing was performed (Sanger). Unfortunately, the limitations of this method due to the fact that it analyzes all matrix sequences, unlike the new generation methods, which analyze each matrix separately, prevented a more insightful analysis of the obtained sequences. The image of the obtained sequences proves that there were several genomes of different bacterial strains, which impaired result reading and interpretation. However, in one case we managed to

Tab IV. Bacterial strains identified in a single gallstone with the Sanger sequencing method.

Sekwencjonowanie metodą Sanger	<i>Enterococcus viikkiensis/ Enterococcus sp.</i> <i>Streptococcus parasanguinis/ Streptococcus equinus</i>
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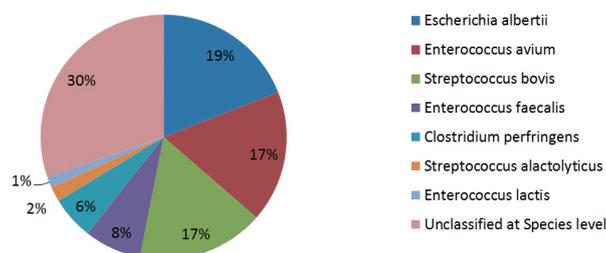


Fig.1. Bacterial strains identified in a gallstone with NGS.

extract and identify DNA of *Enterococcus sp.* *Streptococcus sp.* In order to reach anymore insightful conclusions, it is necessary to use a more effective sequencing method (NGS Next-Generation Sequencing), which would allow to verify the presence of DNA of each bacterial strain. The trial series of NGS performed using the gallstone of the same patient detected *Enterococcus sp.*, *Streptococcus sp.*, *Escherichia sp.*, and *Clostridium perfringens*. Bacterial species extracted from the same gallstone as a result of analysis of Sanger sequencing and NGS such as *Enterococcus sp.* are partially consistent with bacteria grown from the bile of the same patient. This suggests that the gallstone was previously infected in the course of gallstone formation, and not that it was infected as a result of present bile infection. It is also a proof of the body's ability to fight infections, even malicious strains of *Clostridium perfringens*, *Escherichia sp.*, and *Streptococcus sp.*

CONCLUSION

1. More than one pathogen was grown in 67.5% of patients with bile infection.
2. The most commonly grown pathogens are *Enterococcus spp.* (45% of all positive cultures), *Escherichia coli* (37.5%) and *Klebsiella spp.* (25%).
3. Candidiasis accompanied bacterial infection in 15% of cases.
4. Bacterial strains grown on gallstones are partially comparable with bacterial cultures grown on bile in the same patient.

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Corresponding author: Tomasz Matyjas; E-mail tobistm@gmail.com

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