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Bacteria and fungi in acute cholecystitis. A prospective study comparing next generation sequencing to culture

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SUMMARY

Objectives: Guidelines for antibiotic treatment of acute cholecystitis are based on studies using culture techniques for microbial identification. Microbial culture has well described limitations and more comprehensive data on the microbial spectrum may support adjustments of these recommendations. We used next generation sequencing to conduct a thorough microbiological characterization of bile-samples from patients with moderate and severe acute cholecystitis.

Methods: We prospectively included patients with moderate and severe acute cholecystitis, undergoing percutaneous or perioperative drainage of the gall bladder. Bile samples were analyzed using both culture and deep sequencing of bacterial 16S rRNA and *rpoB* genes and the fungal ITS2-segment. Clinical details were evaluated by medical record review.

Results: Thirty-six patients with moderate and severe acute cholecystitis were included. Bile from 31 (86%) of these contained bacteria (29) and/or fungi (5) as determined by sequencing. Culture identified only 40 (38%) of the 106 microbes identified by sequencing. In none of the 15 polymicrobial samples did culture detect all present microbes. Frequently identified bacteria often missed by culture included oral streptococci, anaerobic bacteria, enterococci and Enterobacteriaceae other than *Klebsiella* spp. and *Escherichia coli*.

Conclusions: Culture techniques display decreased sensitivity for the microbial diagnostics of acute cholecystitis leaving possible pathogens undetected.

Introduction

Acute cholecystitis is defined as an acute inflammation of the gall bladder. It is one of the most common inpatient diagnoses at surgical departments[1,2] and in more than 90% of patients it arises as complications of cholelithiasis (calculous cholecystitis).[1,2] Bacterial growth in bile is reported in 20% to 70% of patients.[3–8] Bacterial infection is believed to represent a secondary complication and not the initiating event of the disease.[2] Infection is considered an important negative prognostic factor, and antibiotics are included in treatment recommendations for all grades of severity.[4,7,9–11]

Empiric treatment with piperacillin/tazobactam or a cephalosporin +/− metronidazole is recommended for moderate and severe acute cholecystitis irrespective of whether there is growth by culture.[9–11] The microbiological studies constituting the basis for choosing these antibiotic regimens were all performed with conventional culture techniques.[10] For other purulent infections, recent comparisons of microbial detection by culture versus culture-free identification of microbial DNA by next generation sequencing (NGS) have demonstrated that conventional culture detects only a fraction of the bacteria being present.[12,13] The lower sensitivity is particularly pronounced for samples containing anaerobic bacteria and for samples collected after the initiation of antimicrobial therapy.

Incomplete data on the microbial spectrum associated with acute cholecystitis may lead to sub-optimal antibiotic treatment, thus worsening patient outcome. A study from Israel found that

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discordant antibiotic therapy for acute cholecystitis, in most cases because of a non-susceptible *Enterobacter* spp. or *Enterococcus* spp., resulted in a relative risk for in-hospital death of 6.28 compared to patients who received concordant therapy.[7]

The aim of the present investigation was to use NGS to conduct a thorough microbiological characterization of bile-samples from clinically well-characterized patients with acute cholecystitis. We further sought to compare the results from culture-free NGS with results obtained by conventional microbiological culture and discuss discrepancies from a diagnostic and clinical perspective.

Materials and methods

We conducted a prospective, single-center study at Haukeland University Hospital, Bergen, Norway. The study was approved by the regional ethical committee (2015/65). Written informed consent was obtained from all participants.

Patients

From July 2015 to April 2017, we collected bile samples from 36 patients who underwent treatment with percutaneous (34) or perioperative (2) drainage for acute cholecystitis, defined according to the Tokyo Guideline 2013 (TG13) criteria for a definite diagnosis.[14] Clinical details were evaluated by medical record review. Although debated,[15,16] at Haukeland University Hospital acute mild cholecystitis is treated with observation and/or antibiotics, sometimes followed by delayed cholecystectomy 2–4 months later. For moderate and severe disease percutaneous drainage is the treatment of choice. Consequently, only patients with moderate or severe disease were available for inclusion, and percutaneous drainage was the dominating sampling method. As a patient control group, we included bile samples taken at cholecystectomy from 16 patients with cholelithiasis and no signs of ongoing gallbladder inflammation, operated at Voss Hospital, Voss, Norway.

Sample material, routine diagnostics and DNA-extraction

Bile fluid was aseptically collected during surgery or percutaneous drainage and injected into a sterile tube. All samples were cultured according to the laboratory's guidelines; 10 µl sample material was spread on plates of blood agar, lactose agar, and fastidious anaerobic agar with and without kanamycin and vancomycin. An aliquot of bile was inoculated into brain heart infusion (BHI) as an enrichment procedure. Blood agars and BHIs were incubated in a CO2-enriched atmosphere for 48 h. Lactose agar was incubated for 24 h. Anaerobe agars were incubated in an anaerobe atmosphere for 48 h. Isolates were identified by MALDI-TOF MS Bruker Microflex (Bruker Biotyper, Bremen, Germany.)

DNA was extracted from each sample using a volume of 400 µl bile as described previously.[17] The eluate was stored at −80 °C for later NGS analysis.

Massive parallel sequencing of 16S rRNA, ITS2 and rpoB *genes*

Sequencing of partial bacterial 16S rRNA and the fungal ITS2-segment were performed from all samples. Sequencing of partial *rpoB*-genes were done whenever 16S rRNA sequencing revealed bacteria from the Enterobacteriaceae family or from the *Enterococcus, Streptococcus* or *Staphylococcus* genera that could be identified at a higher taxonomic level by the selected *rpoB*-gene segments.[13] Amplification and sequencing of 16S rRNA- and *rpoB*-genes was performed as described previously using the Illumina MiSeq system (Illumina, Redwood City, CA).[13] For the fungal ITS2-segment we used the primers recommended by Khot et al.[18] and otherwise followed the protocol as described for 16S rRNA.[13] All primers are listed in Supplementary Table S1.

Negative controls

Each clinical sample was processed together with a parallel negative extraction control consisting of lysis buffer and PCR-grade water. Before sequencing, the negative extraction controls were mixed into three pools. A positive extraction control consisting of *Legionella pneumophila* suspended in PCR-grade water was also included and sequenced in the same run.

Sequence data analysis

After Illumina-sequencing, barcode separated FASTQ-files were processed using the RipSeq NGS software[12] (Pathogenomix, Santa Cruz, CA) where sequences were *de novo* clustered into operational taxonomic units (OTUs) using a similarity threshold of 99%. OTUs containing less than 50 sequences were rejected.[13] Criteria for sequence interpretations are provided in Table 1.

Background DNA

Management of background contaminant bacterial DNA was done as described previously.[13] There was a high consistency across all negative and positive extraction controls for the dominant contaminant bacterial species.

Background contaminant fungal DNA showed a higher variation across negative and positive extraction controls. For management of background fungal DNA, we defined a list of the ten most abundant contaminating fungi based on results from negative and positive extraction controls. Additionally, the laboratory keeps a list of

Table 1
Criteria for sequence interpretations.

Gene	Species	Species-group	Genus
16S[a]	≥99.3% homology with a high-quality reference, and minimum distance >0.7% to the next alternative species.[13]	≥99.3% homology with a high-quality reference, and minimum distance ≤0.7% to the next alternative species.	>97.0% homology with a high-quality reference
rpoB_Ent[b]	≥99.0% homology with a high-quality reference, and minimum distance >1.5% to the next alternative species.[13]	≥99.0% homology with a high-quality reference, and minimum distance ≤1.5% to the next alternative species	Not applicable
rpoB_ESS[c]	≥97.0% homology with a high-quality reference, and minimum distance >2.0% to the next alternative species.[13]	≥97.0% homology with a high-quality reference, and minimum distance ≤2.0% to the next alternative species.	Not applicable
ITS-2	≥99.0% homology with a high-quality reference, and minimum distance >2.0% to the next alternative species	≥99.0% homology with a high-quality reference, and minimum distance ≤2.0% to the next alternative species	Not applicable

[a] V3-V4 region of 16S rRNA-gene.
[b] rpoB-gene sequence targeted at Enterobacteriaceae.
[c] rpoB-gene sequence targeted at Staphylococcus, Enterococcus and Streptococcus species.

common contaminant fungi based on previous sequencing of negative and positive extraction controls. Fungi appearing in higher concentrations than any of these contaminants were accepted as valid identifications.

Statistical analysis

Statistical analyses were performed using SPSS 25 (IBM Corp). Clinical and microbial differences between subgroups were analyzed with Pearson's chi squared test for categorical data. For continuous data the Student́s *t*-test was used for normal distributed variables and Mann-Whitney U test for skewed variables.

Results

Clinical description of patients

Thirty-six patients – 19 (53%) males and 17 (47%) females – were included. The mean age was 70 years (median 72, range 37–94). Clinical and demographic characteristics together with main microbiological findings are presented in Table 2. Patients were categorized as having either moderate (24) or severe (12) acute cholecystitis according to the TG18/TG13 severity assessment criteria[19] (Supplementary Table S2). Compared to the moderate disease group, patients in the severe disease group were older, scored higher on Charlson's comorbidity index[20] and had higher prevalence of *Streptococcus* spp. and Enterobacteriaceae other than *Klebsiella* spp. and *E. coli*. Antibiotic treatment had been initiated for all patients except one prior to sample collection. Piperacillin/Tazobactam was the most frequently administered antibiotic, being part of or the only antimicrobial treatment for 28 patients. Eleven patients were diagnosed with local complications including marked local inflammation and/or perforated cholecystitis. The microbial findings by both NGS and culture from these patients can be found in Supplementary Table S3. Individual clinical characteristics, microbial findings and antibiotic treatment are provided in Supplementary Table S4. One patient died during hospital stay (Patient number 26, Supplementary Table S4). This patient had no detectable microbe in bile, neither by culture nor by sequencing.

Characteristics of the patient-control group are detailed in Table 2. Only three (19%) out of the 16 controls had detectable microbes in bile; *Streptococcus parasanguinis, Bifidobacterium animalis* and *Haemophilus parainfluenzae* were identified in one sample each.

Technical sequencing data

For the 16S rRNA amplicon the mean number of accepted reads per sample was 145,155 (range 28,592–404,981, median 115,519) after removal of short reads (<250 base pairs), small clusters (<50 reads) and chimeras. For the ITS2 amplicon the corresponding number was 23,024 (range 5150–47,568, median 15,514).

Microbial findings

Thirty-one samples (86%) contained bacteria (29) and/or fungi (5) as determined by sequencing (Table 2). Among these, five

Table 2
Characteristic, sequencing and culture results for all patients.

Patient group	
Demographic and clinical characteristics	
Number of patients	36
Male	19 (53%)
Mean age (SD; median; min–max), years	70 (16; 72; 37–94)
Community-acquired	30 (83%)
Mean CCI[a] (SD; median; min–max)	1,7 (1,9; 1,0; 0–8)
Gall bladder stone	30 (83%)
Bile duct stone	10 (28%)
Concomitant acute cholangitis	8 (22%)
Ongoing antibiotic therapy	35 (97%)
Severity grade:	
Moderate	24 (67%)
Severe	12 (33%)
Sequencing and culture results	
Samples with detected microbes by sequencing	31 (86%)
Samples with growth in bile culture	26 (72%)
Samples with detected bacteria by sequencing	29 (81%)
Samples with detected fungi by sequencing	5 (14%)
Polymicrobial samples by sequencing	15 (42%)
Major groups of bacteria detected by sequencing:	
Samples with *Klebsiella* spp.	11
Samples with *E. coli*	10
Samples with Enterobacteriaceae other than *Klebsiella* spp. and *Escherichia coli*	7
Samples with *Enterococcus* spp.	7
Samples with *Streptococcus* spp.	13
Samples with anaerobic bacteria	10
Patient control-group	
Number of patients	16
Male	6 (38%)
Mean age (SD; median; min–max), years	53 (18; 55; 20–79)
Mean CCI[a] (SD; median; min–max)	0,25 (0,5; 0; 0–1)
Samples with detected microbes by sequencing and/or culture	3
Species detected	
Bifidobacterium animalis (detected by)	1 (sequencing and culture)
Streptococcus parasanguinis (detected by)	1 (sequencing and culture)
Haemophilus parainfluenzae (detected by)	1 (sequencing)

[a] CCI = Charlsons comorbidity index.

Table 3
Species identified at a higher taxonomic level with use of partial *rpoB*-gene compared to partial 16S rRNA gene sequencing (V3–V4).

	16S rRNA gene sequencing results	*rpoB*-gene sequencing results
1	*Citrobacter werkmanii/Citrobacter freundii/Citrobacter braakii/Citrobacter pasteurii/Kluyvera ascorbata*	*Citrobacter* sp.
2	*Klebsiella michiganensis/Enterobacter ludwigii/Enterobacter asburiae/Enterobcater cloacae/Enterobacter kobei/Citrobacter freundii/Salmonella enterica*	*Enterobacter asburiae/Enterobacter cloacae/Enterobacter kobei*
3	*Enterobacter asburiae/Enterobacter cloacae/Enterobacter hormaechei/Klebsiella michiganensis/Klebsiella oxytoca/Klebsiella pneumoniae/Klebsiella quasipneumoniae*	*Enterobacter cloacae/Enterobacter hormaechei*
4	*Enterococcus gallinarum /Enterococcus casseliflavus*	*Enterococcus casseliflavus*
5	*Enterococcus durans/Enterococcus faecium/Enterococcus hirae*	*Enterococcus faecium*
6	*Escherichia coli/Escherichia albertii/Escherichia fergusonii/Shigella species*	*Escherichia coli/Shigella* sp.
7	*Hafnia alvei/Hafnia paralvei/Ewingella americana*	*Hafnia alvei*
8	*Klebsiella michiganensis/Klebsiella oxytoca/Enterobacter asburiae/Enterobacter cloacae/Enterobacter hormaechei*	*Klebsiella michiganensis*
9	*Klebsiella michiganensis/Klebsiella oxytoca/Enterobacter asburiae/Enterobacter hormaechei/Enterobacter cloacae/Salmonella enterica*	*Klebsiella oxytoca*
10	*Klebsiella aerogenes/Enterobacter asburiae/E. cancerogenes/Enterobacter cloacae/Enterobacter hormaechei/Enterobacter ludwigii/Enterobacter xiangfangensis/Klebsiella pneumoniae/Klebsiella oxytoca/Klebsiella michiganensis/Klebsiella variicola*	*Klebsiella pneumoniae/Klebsiella quasipneumoniae*
11	*Klebsiella pneumoniae/Klebsiella variicola*	*Klebsiella variicola*
12	*Proteus hauseri/Proteus penneri/Proteus vulgaris*	*Proteus vulgaris*
13	*Salmonella enterica/Enterobacter cloacae/Enterobacter kobei/Enterobacter ludwigii/Citrobacter amalonaticus/Klebsiella michiganensis*	*Salmonella enterica*
14	*Streptococcus anginosus/Streptococcus intermedius*	*Streptococcus anginosus*
15	*Streptococcus gordonii/Streptococcus cristatus*	*Streptococcus gordonii*
16	*Streptococcus mitis/oralis group*	*Streptococcus mitis*
17	*Streptococcus mitis/oralis group*	*Streptococcus oralis*
18	*Streptococcus sanguinis group*	*Streptococcus parasanguinis*
19	*Streptococcus salivarius group*	*Streptococcus salivarius*
20	*Streptococcus sanguinis group*	*Streptococcus sanguinis*
21	*Streptococcus salivarius group*	*Streptococcus thermophilus*

samples were culture negative. From the 106 microbial detections made by sequencing (100 bacteria and 6 fungi), only 40 were cultured (38%). The 100 bacteria detected by sequencing represented 53 different species of which 38 were identified to the species level, 14 to a species group level, and 1 to the genus level. The *rpoB* gene improved identification for 21 species (Table 3). Two bacterial identifications were made by culture alone, one *Klebsiella pneumoniae* and one *Staphylococcus epidermidis*. A detailed comparison of identifications made by sequencing versus culture is provided in Table 4. Table 5 provides an overview of the bacterial genera found in each patient and the proportion of samples containing each genus. In patients with polymicrobial infections culture failed to detect one or more microbes in all 15 samples (Supplementary Table S4). For the monomicrobial infections, there was a higher concordance (81%) between culture and sequencing. Only three of the 16 monomicrobial samples were culture negative (Supplementary Table S4).

Six fungi were identified by sequencing (Table 4) whereof one, a *Candida albicans*, was also cultured. Two samples were monomicrobial containing *C. albicans*; one severe postoperative acalculous cholecystitis after pancreatic cancer surgery who also had *C. albicans* in blood culture, and one community-acquired calculous cholecystitis of moderate severity. The other identified fungi, *Saccharomyces cerevisiae* (2), *C. albicans* (1) and *Candida humilis* (1) were part of poly-microbial infections (Supplementary Table S4). Only the patient with severe postoperative acalculous cholecystitis received antifungal treatment.

Blood culture samples were collected from 24 patients whereof five had a detectable bacteremia (Supplementary Table S4). Antibiograms of all bacteria cultured from bile or in blood culture are provided in Supplementary Table S5.

Discussion

To the best of our knowledge, this is the first study that uses NGS for microbial characterization of bile samples from patients with acute cholecystitis, with the exception of a small study on six patients.[21] This is also the first study to describe the bacteriology of severe acute cholecystitis according to the TG18/TG13 severity grading.[5,8]

Although bactobilia is considered a negative prognostic factor in acute cholecystitis, there is, with the exception of the aforementioned Israeli study,[7] little evidence on the clinical importance of the individual bacterial species. In many of the polymicrobial samples in our study, the relative abundance of the identified bacteria varied widely (Table 5). Some might dismiss the clinical relevance of low abundance species in complex infections, in particular if found be sequencing only. However, several of the bacteria identified were anaerobic, fastidious, slow growing and/or antibiotics-affected, and their failure to survive and grow in the laboratory does not mean that they are eradicated from the infection site nor that they are of lower clinical relevance. We would also like to point out that abundant growth does not necessarily reflect in-vivo dominance but might as well reflect a microbe's ability to thrive and compete during transportation and cultivation. We have frequently observed, also in this study, that bacteria with abundant growth constitute only minor parts of the population as determined by sequencing or that a dominant microbe as determined by sequencing fails to grow. In our opinion, the clinical relevance of individual bacteria in complex infections should not be considered based on relative quantifications or by method of detection. Rather, such inference should be based on in-depth ecological knowledge of each type of infection, including microbial dynamics over time, microbial aggregate formation, metabolic interdependencies and synergisms.[22,23] Complete microbial characterizations as provided in this study represent the first step in obtaining such knowledge but needs to be followed up by both experimental studies and larger clinical studies.

Except for *Klebsiella* spp. and *E. coli*, we found that 50% of species in the Enterobacteriaceae family, including species from the genera *Citrobacter, Enterobacter, Proteus, Hafnia, Salmonella, Serratia, Morganella and Raoultella* remained undiscovered by culture

Table 4
Identified bacteria and fungi from bile samples by sequencing compared to conventional culture.

	Total number of identifications by sequencing (% of all microbial detections)	Growth by culture
Total identifications	106	40
Gram negative[a]	41 (39%)	23
Klebsiella	11 (10%)	9
pneumoniae/quasipneumoniae	3	3
Michiganensis[c]	3	2
Oxytoca[c]	3	2
Variicola[c]	2	2
Escherichia coli[b,c]	10 (9%)	9
Campylobacter	4 (4%)	0
Concisus	1	0
Concisus/mucosalis	1	0
Curvus	1	0
Rectus/showae	1	0
Citrobacter	3 (3%)	1
Species[c]	2	1
Amalonaticus/farmeri	1	0
Haemophilus parainfluenzae	3 (3%)	0
Enterobacter	2 (2%)	1
Asburiae/cloacae/kobei[c]	1	1
Cloacae/hormaechei[c]	1	0
Morganella morganii	2 (2%)	1
Hafnia alvei[c]	1 (1%)	0
Proteus vulgaris	1 (1%)	1
Pseudomonas aeruginosa/otidis	1 (1%)	1
Raoultella ornithinolytica/planticola	1 (1%)	0
Salmonella enterica[c]	1 (1%)	0
Serratia marcescens	1 (1%)	0
Gram positive[a]	35 (33%)	14
Streptococcus	15 (14%)	6
Anginosus[c]	3	1
Salivarius[c]	3	2
Sanguinis[c]	2	1
Gordonii	1	1
Massiliensis	1	1
Mitis[c]	1	0
Mutans	1	0
Oralis[c]	1	0
Parasanguinis[c]	1	0
Termophilus[c]	1	0
Enterococcus	11 (10%)	5
Faecalis	4	2
Faecium[c]	4	2
Avium/raffinosus[c]	2	1
Casseliflavus[c]	1	0
Lactobacillus casei/paracasei/rhamnosus	4 (4%)	3
Actinomyces	5 (5%)	0
Gerencseriae	1	0
Naeslundii/oris	1	0
Naeslundii/oris/johnsonii	1	0
sp. (oral taxon 848)	1	0
Turicensis	1	0
Anaerobic	24 (23%)	2
Clostridium perfringens	5 (5%)	2
Fusobacterium nucleatum	5 (5%)	0
Bifdobacterium	4 (4%)	0
Animalis	2	0
Dentium	1	0
Longum	1	0
Veillonella	3 (3%)	0
Dispar/parvula	2	0
Parvula/tobetsuensis/dentocariosa	1	0
Intestinibacter bartletti	3 (3%)	0
Slackia exigua	1 (1%)	0
Dialister invisius	1 (1%)	0
Bilophila wadsworthia	1 (1%)	0
Propionibacterium acidifaciens	1 (1%)	0
Fungus	6 (6%)	1
Candida	4 (4%)	1
Albicans	3	1
Humilis	1	0
Saccharomyces cerevisiae	2 (2%)	0

[a] One *K. pneumoniae* and one *S. epidermidis* detected exclusively by culture is not included in table.
[b] Not distinguishable from *Shigella* spp.
[c] *rpoB* sequencing provided identification at a higher taxonomic level than 16S rRNA gene sequencing.

Table 5
Heatmap of all bacterial genus identified in each patient. Only samples containing bacteria are included in the table.

Bacterial ID	Number of samples containing the bacterium (%)[a]	1	2	4	7	8	9	10	12	15	16	17	18	19	20	21	22	23	24	25	27	28	29	30	31	32	33	34	35	36
Streptococcus spp.	13 (45%)	5,6	100		0,6			100	21,5	0,2	100						59,6	0,1						88,5	100				84,2	0,5
Klebsiella spp.	11 (38%)	2,3			46,4	100	42,0		0,2	91,0		100		11,6				6,2		32,2										75,4
Escherichia coli	10 (34%)	90,3		100	53,0				20,8				100								100		100			5,6	51,3	100		
Enterococcus spp.	7 (24%)	1,0					37,7		0,1					88,3				11,6		41,0							46,3			
Clostridium perfringens	5 (17%)														100			0,8								68,5	2,3			12,9
Fusobacterium nucleatum	5 (17%)	0,1							42,8								7,0	47,4												11,2
Campylobacter spp.	4 (14%)									0,1							0,5	0,4		10,0										
Lactobacillus spp.	4 (14%)									0,3							7,4		100							23,8				
Actinomyces spp.	3 (10%)	0,3								2,2							5,1													
Bifidobacterium spp.	3 (10%)									0,5							0,6	0,2												
Citrobacter spp.	3 (10%)	0,1					1,8											0,3												
Haemophilus parainfluenzae	3 (10%)																0,3					100						15,8	
Intestinibacter bartlettii	3 (10%)													0,1				0,1								2,1				
Veillonella spp.	3 (10%)	0,3															2,8	7,1												
Enterobacter spp.	2 (7%)									3,3						100														
Morganella morganii	2 (7%)																	0,6		9,6										
Bilophila wadsworthia	1 (3%)								14,6																					
Dialister invisus	1 (3%)																	0,1												
Hafnia alvei	1 (3%)						0,1																							
Propionibacterium acidifaciens	1 (3%)																4,0													
Proteus vulgaris	1 (3%)																	25,1												
Pseudomonas spp.	1 (3%)						18,4																							
Raoultella spp.	1 (3%)									2,3																				
Salmonella enterica	1 (3%)																							11,5						
Serratia marcescens	1 (3%)																			7,3										
Slackia exigua	1 (3%)																12,8													

[a] Percentage in parenthesis represents the proportion of samples, out of all bacterial sequencing-positive samples, containing the bacterium.

(Table 4). These bacteria are generally considered clinically relevant and there is evidence to support their role in the pathogenesis of acute cholecystitis.[24] For *Enterobacter* spp. there is also a possible association with a poorer patient outcome.[7] The capability of acquiring or inducing antibiotic resistance, and a high frequency of multi-resistant clones among some Enterobacteriaceae, increases the likely clinical benefit of identifying these bacteria.[25]

Only five out of eleven enterococci were found by conventional culture (Table 4 and Supplementary Table S4). The clinical significance of enterococci in acute cholecystitis and in intra-abdominal infections in general remains uncertain. Most empiric guidelines for antibiotic treatment of acute cholecystitis do not include specific enterococcal coverage,[9,11] except for the Tokyo Guidelines' recommendation of adding vancomycin for severe cholecystitis.[10] However, in complicated acute cholecystitis and/or severely ill patients it is recommended to use microbiology culture results to guide antimicrobial treatment.[10,11,26] This implies that if the enterococci found only by sequencing in our cohort had also been found by culture, it might have led to an adjustment of antibiotic treatment. As mentioned, failure to culture microbes does not mean that they have been eradicated from the infection site. Future studies addressing the relevance of enterococci should therefore not rely on culture-based diagnostics alone but also include molecular approaches like sequencing or PCR.

Anaerobic bacteria may be sub-optimally covered by monotherapy with a third-generation cephalosporin whereas Piperacillin/tazobactam provides good coverage of anaerobic bacteria. In this study, NGS detected 24 anaerobic bacteria from 10 samples whereof only two (8%) were also detected by culture (Table 4). The two most common anaerobe species were *Clostridium perfringens* and *Fusobacterium nucleatum*. *Clostridium perfringens* is known for its pathogenicity and its ability to cause emphysematous cholecystitis. *Fusobacterium nucleatum* has to the best of our knowledge not previously been reported in acute cholecystitis but is considered an important anaerobe pathogen in both odontogenic infections, pleural empyemas and brain abscesses.[12,13]

In healthy individuals, the bile is considered to be sterile,[27–29] but gallstone disease might lead to bacterial colonization. Culture-based studies report bacteria in between 9% and 54% of patients with gallstone disease without infection.[6,28,29] Two NGS-based studies addressing this issue report conflicting results. One study found a very high rate of colonization (100%) and suggest the existence of a bile core microbiome comprising 208 Operational Taxonomic Units (OTUs)/species.[30] Another study found the rate of colonization to be 13% with a mean bacterial diversity of 5 OTUs per sample.[31] Both studies fail to explain how they addressed the problem of contaminant background DNA, chimera formation and sequencing noise. These are fundamental challenges in microbiome studies and will significantly inflate microbial diversity if not considered properly.[32,33] In our patient control group only three (19%) bile samples were colonized, each with a single bacterial species (Table 2), providing little support for the existence of a bile microbiome.

Some limitations to this study should be noted. It is a single center investigation with a relatively low number of patients, and the general validity of our results therefore needs confirmation by other studies. The patients in our cohort were also of higher mean age than in historic studies on moderate and severe cholecystitis which may in part explain the higher rate of bactobilia observed.[4,5,29] Due to the severity of the disease, antibiotic treatment had been initiated for most patients prior to sample collection. Although bacterial DNA is very stable in undrained purulent infections this might still have impacted the observed relative abundancies of species in the polymicrobial infections.

We have shown that culture-based methods alone are insufficient in the microbiological diagnostics of moderate and severe acute cholecystitis, leaving more than 60% of the microbes undetected. The clinical consequences of not detecting or treating all these bacteria should be further addressed in future studies as should eventual consequences for empiric treatment recommendations. Yet, clinicians should be aware of the risk of leaving clinical important bacteria untreated if antimicrobial treatment is customized based on culture results only. For anaerobic bacteria, the low recovery rate may imply that anaerobic coverage should be considered regardless of a negative anaerobic culture. This and other studies emphasize the need for rapid and reliable culture-independent microbial detection and susceptibility testing in diagnostic microbiology.

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Declaration of Competing Interest

O.K. contributed to the development of the RipSeq software and is a minor shareholder of Pathogenomix Inc.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.09.015.

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