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Original Article

A Multipathogen Bile Sample-based PCR Assay Can Guide Empirical Antimicrobial Strategies in Cholestatic Liver Diseases

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Abstract

Background and objectives: Polymerase chain reaction (PCR) techniques provide rapid detection of pathogens. This pilot study evaluated the diagnostic utility and clinical impact of multiplex real-time PCR (mRT-PCR, Septi*Fast*) vs. conventional microbial culture (CMC) in bile samples of patients with chronic cholestatic liver diseases (cCLDs), endoscopic retrograde cholangio-pancreatography (ERCP), and peri-interventional-antimicrobial-prophylaxis (pAP). ***Methods:*** We prospectively collected bile samples from 26 patients for microbiological analysis by CMC and mRT-PCR. Concordance of the results of both methods was determined by Krippendorff's alpha (α) for inter-rater reliability and the Jaccard index of similarity. ***Results:*** mRT-PCR$_{bile}$ and CMC$_{bile}$ results were concordant for only *Candida albicans* (α=0.8406; Jaccard index=0.8181). mRT-PCR$_{bile}$ detected pathogens in 8/8 cases (100%), CMC$_{bile}$ in 7/8 (87.5%), and CMC$_{blood}$ in 5/8 (62.5%) with clinical signs of infection. mRT-PCR$_{bile}$, CMC$_{bile}$, and CMC$_{blood}$ had identical detection results in 3/8 (37.5%) with clinical signs of infection (two *Klebsiella* spp. and one *Enterococcus faecium*). The total pathogen count was significantly higher with mRT-PCR$_{bile}$ than with CMC$_{bile}$ (62 vs. 31; χ^2=30.031, p<0.001). However, pathogens detected by mRT-PCR$_{bile}$ were more often susceptible to pAP according to the patient infection/colonization history (PI/CH) and surveillance data for antibiotic resistance in our clinic (DARC). Pathogens identified by mRT-PCR$_{bile}$ and resistant to pAP by PI/CH and DARC were likely to be clinically relevant. ***Conclusions:*** mRT-PCR in conjunction with CMCs for bile analysis increased diagnostic sensitivity and may benefit infection management in patients with cholestatic diseases. Implementation of mRT-PCR in a bile sample-based diagnostic routine can support more rapid and targeted use of antimicrobial agents in cCLD-patients undergoing ERCP and reduce the rate/length of unnecessary administration of broad-spectrum antibiotics.

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Keywords: ERCP; PCR; Antibiotic prophylaxis; Cholestasis; Cholangitis.

Abbreviations: cCLDs, chronic cholestatic liver diseases; CFU/mL, colony-forming units per mL; CMC, conventional microbial culture; CoNS, coagulase-negative *Staphylococci*; DARC, data for antibiotic resistance in our clinic; ERCP, endoscopic retrograde cholangio-pancreatography; ITBLs, ischemic-type biliary lesions; IQR, interquartile range; LTx, liver transplantation; mRT-PCR, multiplex real-time PCR; pAP, peri-interventional-antimicrobial-prophylaxis; PCR, polymerase chain reaction; PI/CH, patient infection/colonization history; PSC, primary sclerosing cholangitis; RMD, rapid molecular diagnostics; SSC, secondary sclerosing cholangitis.

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Introduction

Endoscopic retrograde cholangio-pancreatography (ERCP) is a mainstay of therapeutic procedures in the vast majority of chronic cholestatic liver diseases (cCLDs), which are associated with either primary or secondary obstructions of the biliary system, leading to reduced or disrupted bile flow. Regardless of the cause, stasis of bile supports exponential growth of micro-organisms, which enter the biliary tree via the portal venous system or by ascent from the intestine and cause increased intrabiliary pressure as well as cholangiovenous reflux along with bacteremia or fungemia.[1]

ERCP-related interventions like balloon dilatation, stenting, lavage or cast and stone extractions help to restore the biliary drainage and are recommended by numerous treatment guidelines for cCLD, e.g., ischemic-type biliary lesions (ITBLs)[2] or primary sclerosing cholangitis (PSC).[3–5] Antibiotic prophylaxis prior to ERCP is not routinely recommended, as it does not significantly reduce the risk of the subsequent emergence of cholangitis in unselected patients.[6,7] However, peri-interventional antimicrobial prophylaxis (pAP) should be administered in patients undergoing ERCP if the likelihood

of achieving the best outcome, i.e. complete biliary drainage, is small. This recommendation refers to complex clinical conditions caused by cCLD, especially in association with hilar tumors, immunosuppression, and pancreatic pseudocysts communicating with the pancreatic duct.[8,9] Currently, local recommendations for pAP still follow relatively heterogeneous guidelines on empirical use of antimicrobial agents. Moreover, reliable results of conventional microbial cultures (CMC) require up to 48 h. Microbiological tests detecting cholangitis-causing pathogens more rapidly would contribute toward more timely and specific administration of antimicrobials. To this end, multiplex real-time PCR (mRT-PCR) assays are promising to complement CMC performance and improve overall diagnostic ability.[10–12]

Until 2019, Septi*Fast* (Roche Diagnostics, GmbH, Mannheim, Germany) was a commercially available mRT-PCR assay able to detect 20 different bacterial or fungal microorganisms commonly involved in systemic bloodstream infections (Table 1).[11] The original scope of Septi*Fast* was to rapidly identify bacterial and fungal DNA in the bloodstream of patients suspected of having sepsis. The test was designed to run directly, requiring no prior incubation or preculture preparation of blood samples. It has also been shown to be a reliable method for diagnostic evaluation of bacteremia in association with endocarditis or periprosthetic joint infections, particularly in patients receiving antibiotic prophylaxis.[13] Rapid identification of bacterial and fungal pathogens may be of huge benefit in patients with cCLD who are at risk of, or suspected of having, severe cholangitis and require broad-spectrum antibiotic prophylaxis/treatment and further prompt medical intervention, e.g., ERCP. Our particular interest in investigating the diagnostic utility of Septi*Fast* using bile samples from patients with cCLD was that this step may reduce the rate/length of unnecessary use of broad-spectrum antibiotics. The aim was to contribute toward antimicrobial stewardship and promote targeted treatment earlier in the clinical management pathway. To the best of our knowledge, this is the first study to evaluate the diagnostic utility of an mRT-PCR assay of bile (mRT-PCR_{bile}) as a complementary test to CMC of bile samples (CMC_{bile}) obtained during ERCP and required to provide robust antimicrobial susceptibility information.

Methods

This study was conducted at the University Hospital Essen from May 2016 to August 2018. During that time, bile fluid samples were obtained from within the biliary tract during ERCP in patients with cCLDs. Bile (1.5 mL) was directly collected into a 2.6 mL EDTA monovette (S-Monoette 2.6 mL K3E; Sarstedt AG and Co KG, Nümbrecht, Germany). The residual 2–5 mL bile was used for inoculation of blood culture sets (BD Bactec Lyitc/10 Anaerobe/F and BD Bactec Plus Aerobe/F9240 systems; Becton, Dickinson Co., Sparks, USA). All bile samples were immediately sent to our microbiological laboratory for DNA amplification and bile culture analysis.

Pathogen detection by mRT-PCR of bile samples

We used Septi*Fast* kits and a LightCycler® 2.0 real-time PCR instrument (Roche Diagnostics GmbH, Mannheim, Germany) for DNA assays. The LightCycler Septi*Fast* Test, which was taken off the market a few years ago, is a semi-automated real-time PCR system that was designed for simultaneous detection of DNA of most sepsis-relevant bacterial species (Table 1) in EDTA-preserved blood.[14] Primers, PCR conditions, and formulas are patent protected. The patent information are disclosed in PCT publications WO97/46707, WO97/46712, and WO98/46714) and include descriptions of the methods we used.[15,16] DNA was extracted from the bile samples, processed via mechanical lysis, and purified following the manufacturer's instructions. For real-time PCR amplification in parallel reactions for Gram-positive and Gram-negative bacteria and fungi we used 50 µL volumes of each DNA extract.[17] Detection and identification of the automatically amplified products was performed with specific hybridization probes.[11]

As specified by the manufacturer, the analytical sensitivity of Septi*Fast* is 100 colony-forming units per mL (CFU/mL) for coagulase-negative *Staphylococci* (CoNS), *Streptococcus* spp. and *Candida glabrata*, and 30 CFU/mL for all the other measured micro-organisms.[17,18] Table 1 lists the bacteria and fungi that are detectable by Septi*Fast*. Measures for prevention of contamination were as follows: extraction

Table 1. Pathogens (*n*=20) included in the Septi*Fast* mRT-PCR_{bile} panel

Gram-positive bacteria
1. CoNS
2. Staphylococcus aureus
3. Streptococcus pneumoniae
4. Streptococcus spp.[a]
5. Enterococcus faecium
6. Enterococcus faecalis
Gram-negative bacteria
7. Escherichia coli
8. Klebsiella
pneumoniae
oxytoca
9. Enterobacter
cloacae
aerogenes
10. Pseudomonas aeruginosa
11.Stenotrophomonas maltophilia
12. Serratia marcescens
13. Proteus mirabilis
14. Acinetobacter baumanii
Fungi
15. Candida albicans
16. Candida glabrata
17. Candida tropicalis
18. Candida parapsilosis
19. Candida krusei
20. Aspergillus fumigatus

CoNS, coagulase-negative *Staphylococcus* species including *S. epidermidis, S. haemolyticus, S. hominis, S. pasteuri, S. warneri, S. cohnii, S. lugdunensis, S. capitis, S. caprae, S. saprophyticus,* and *S. xylosus.* [a]*Streptococcus* species including *S. pyogenes, S. agalactiae, S. anginosus, S. bovis, S. constellatus, S. cristatus, S. gordonii, S. intermedius, S. milleri, S. mitis, S. mutans, S. oralis, S. parasanguinis, S. salivarius, S. sanguinis, S. thermophilus, S. vestibularis,* and viridans group streptococci.

of DNA in a safety cabinet, use of MGRADE disposables, and extraction and amplification of DNA in separate rooms. An internal control was added in the extraction step to each sample. In each extraction series, a negative control was included. As positive control, the reagent controls were used. PCR results were available within 4.5 h after the specimens were processed in the laboratory.

Pathogen detection with CMC in bile and/or blood samples

For bile culture analysis, the automated microbial detection platform BacT/Alert 3D system (BioMerieux, Marcy l'Etoile, France) was used, which colorimetrically detects the growth of micro-organisms based on their CO_2 production. Bile Samples were declared as negative if no growth of micro-organisms was detectable after 5 days of incubation. In positive bile cultures, subsamples were taken for Gram staining, plate culturing, and subsequent analysis. Identification and determination of the micro-organisms' antibiotic susceptibilities were tested with the VITEK2 system (BioMerieux). Results of bile cultures were obtained within 72 h. Because of an insufficient amount of bile collected during ERCP, six patients had to be excluded from further consideration, as their samples could be subjected to microbial diagnostics either by CMC_{bile} (n=3) or mRT-PCR_{bile} (n=3) only. Thus, bile samples of 26 of 32 initially enrolled patients were included in the final analysis. In patients developing systemic infections (e.g., cholangitis), conventional microbial culture of blood samples (CMC_{blood}) was also performed. CMC_{blood} samples were processed with the BACTEC 9240 system (BD Bactec Lyitc/10 Anaerobe/F and BD Bactec Plus Aerobe/F 9240 systems; Becton, Dickinson and Co., Sparks, MD, USA). After the inoculation of 5–10 mL of blood, further analysis was similar to the bile culture procedure.

Statistics

Statistical analysis was performed with SPSS (version 21.0; IBM Corp., Armonk, NY, USA). For descriptive statistics, absolute and relative frequencies were calculated for categorical variables. Patients suspected of post-ERCP cholangitis were compared with clinically inapparent patients for systemic infections. Inferential statistics included Fisher's exact tests for categorical variables and Mann-Whitney *U* test for continuous variables. Results were considered significant when $p \leq 0.05$.

To describe the diagnostic concordance between mRT-PCR_{bile} and CMC_{bile} results, we used Krippendorff's alpha test (α) to estimate the interrater reliability,[19] and the Jaccard index to measure similarity.[20,21] Reliability was indicated when α >0.800; non-reliability was indicated when α <0.667.[19] The Jaccard index does not impose any weights. It assigns a value of 1 in case of match and 0 otherwise. *Hafnia* spp. and *Enterococcus casseliflavus* (n=2) were not considered for concordant analysis, as they were not part of the SeptiFast panel. McNemar chi-square tests at a significance level of 5% were performed to compare the overall detection rates of micro-organisms with CMC_{bile} and mRT-PCR_{bile}. The McNemar chi-square test does not test for independence, but for consistency in responses across two variables. It is generally used with paired data, and can directly compare counts.[22]

Ethical approval

This study was performed in accordance following the ethical principles and standards of the 1964 Helsinki declaration and the guidelines of the International Conference for Harmonization for Good Clinical Practice. The study was approved by the local institutional review board (IRB: "Ethik-Kommission am Universitätsklinikum Essen", 18-8482-BO).

Results

Samples from 26 patients with cCLDs like PSC (n=14/26; 54%), biliary disorders after liver transplantation (n=10/26; 38%) and secondary sclerosing cholangitis (SSC; n=2/26; 8%) were included in the final analysis. The median age, body mass index, and Charlson comorbidity index were 56 years, 25 kg/m^2 and 3.5. Most frequent reasons for medication intake (Table 2) were immunosuppressive therapies after LTx (n=10), maintenance therapy for PSC (n=4), hypertension (n=11), and diabetes (n=5). It should be noted that none of the patients was pregnant. Overall, eight patients (n=8/26; 31%) developed clinical signs of post-ERCP cholangitis; of those, seven patients (n=7/8; 88%) were on immunosuppressive therapy after liver transplantation (Table 2).

Pathogen detection mRT-PCR vs. CMC in bile samples

One or more micro-organisms were identified in 20 patients by CMC_{bile} (n=20/26; 77%) and in 25 patients by mRT-PCR_{bile} (n=25/26; 96%). CMC_{bile} and mRT-PCR_{bile} concurrently detected numerous pathogens (n=29) (Table 3, Fig. 1). They were *Candida albicans* 9×, *Enterococcus faecalis* 6×, *Enterococcus faecium* 6×, *Klebsiella pneumoniae/oxytoca* 4×, *Escherichia coli* 1×, *Streptococcus* spp. 1×, *Pseudomonas aeruginosa* 1×, and CoNS 1×.

A total of 33 pathogens were exclusively detected by mRT-PCR_{bile} and not by CMC_{bile} (Table 3, Fig. 1). They were *Streptococcus* spp. 6×, *Enterococcus faecium* 5×, *Enterobacter cloacae/aerogenes* 5×, *Enterococcus faecalis* 4×, *Klebsiella pneumoniae/oxytoca* 4×, *Escherichia coli* 3×, *Candida albicans* 2×, *Pseudomonas aeruginosa* 1×, CoNS 1×, *S. aureus* 1×, and *Stenotrophomonas maltophilia* 1×. Only two pathogens, *Enterococcus faecalis* 1× and *Escherichia coli* 1×, were detected by CMC_{bile} and not by mRT-PCR_{bile}. $CMC_{bile\ and}$ mRT-PCR_{bile} concurrently detected more than one pathogen in approximately one-third of all cases (n=9/26; 35%). Moreover, mRT-PCR_{bile} identified multiple micro-organisms in 12 additional cases.

All patients (n=26) received pAP. The vast majority (n=18/26, 69.2%) received ciprofloxacin and metronidazole (Table 2), and bile culture predominantly identified pathogens that were not covered by those antibiotics (n=21/31, 67.7%). The pathogens were *Candida albicans* 9×, *Enterococcus faecalis* 6×, *Entercoccus faecium* 5×, *Streptococcus anginosus* 1× (Supplementary Table 1). In contrast, mRT-PCR_{bile} frequently detected pathogens that were likely to be susceptible to the administered antibiotics (n=24/62; 38.7%). They were *Klebsiella pneumoniae/oxytoca* 8×, *Enterobacter cloacae/aerogenes* 5×, *Escherichia coli* 4×, *Pseudomonas aeruginosa* 2×, CoNS 2×, *Enterococcus faecium* 1×, *Stenotrophomonas maltophilia* 1×, *and Staphylococcus aureus* 1× (Supplementary Table 1). Here, susceptibility was suggested by surveillance data for antibiotic resistance at our hospital and previous patient history and microbiological records.

Concordance of mRT-PCR and CMC results in bile samples

The total pathogen count detected by mRT-PCR_{bile} (n=62; Table 3) was significantly higher than that detected by CM-

Table 2. Characteristics of patients with and without clinical signs and symptoms of colangitis infection at admission

Characteristic	All patients *n* (%) or median (IQR) [min-max]	No clinical symptoms *n* (%) or median (IQR) [min-max]	Clinical symptoms *n* (%) or median (IQR) [min-max]	*p*-value
Patients	26	18	8	NA
Age, years	56 (46–62) [29–78]	47 (43–61) [29–78]	59 (55–65) [50–72]	0.090*
Body mass index, kg/m^2	25 (23–26) [20–36]	25 (23–27) [20–36]	25 (24–26) [20–27]	0.933*
Sex				
Male	19 (73.1)	14 (77.8)	5 (62.5)	0.635**
Female	7 (26.9)	4 (22.2)	3 (37.5)	0.635**
Chronic cholestatic liver disease				
PSC	14 (53.8)	14 (77.8)	0	<0.000**
Post-LTx	10 (38.5)	3 (16.7)	7 (87.5)	0.001**
Post-LTx/no ITBL	3 (11.5)	1 (5.6)	2 (25.0)	0.215**
Post-LTx/ITBL Type I	3 (11.5)	0	3 (37.5)	0.022**
Post-LTx/ITBL Type II	1 (3.8)	1 (5.6)	0	1.000**
Post-LTx/ITBL Type III	3 (11.5)	1 (5.6)	2 (25.0)	0.215**
SSC	2 (7.7)	1 (5.6)	1 (12.5)	0.529**
Chronic disease				
Charlson comorbidity index	3.5 (2.2–5.7) [0–9.0]	3.0 (2.0–4.7) [0–9.0]	4.0 (3.0–7.2) [2.0–8.0]	0.216*
Immunosuppression	14 (53.8)	7 (38.9)	7 (87.5)	0.028**
Hypertension	11 (42.3)	6 (33.3)	5 (62.5)	0.246**
Diabetes mellitus	5 (19.2)	2 (11.1)	3 (37.5)	0.330**
Pre-interventional antibiotic prophylaxis				
Ciprofloxacin	18 (69.2)	14 (77.8)	4 (50.0)	0.197**
Metronidazole	19 (73.1)	14 (77.8)	5 (62.5)	0.635**
Ceftriaxon	4 (15.4)	3 (16.7)	1 (12.5)	1.000**
Piperacillin/tazobactam	3 (11.5)	0	3 (37.5)	0.022**
Linezolid	2 (7.7)	0	2 (25.0)	0.086**
Cefixim	1 (3.8)	1 (5.6)	0	1.000**
Microbiological findings				
CMC_{bile}	20 (76.9)	13 (72.2)	7 (87.5)	0.628**
Detection of ≥2 pathogens	10 (38.5)	6 (33.3)	4 (50.0)	0.664**
Septi*Fast* mRT-PCR_{bile}	25 (96.2)	17 (94.4)	8 (100.0)	1.000**
Detection of ≥2 pathogens	21 (80.8)	14 (77.8)	7 (87.5)	1.000**

*Mann-Whitney *U* test, **Fisher's exact test. CMC_{bile}, conventional microbial culture in bile; ERCP, endoscopic retrograde cholangiopancreatography; IQR, interquartile range; ITBL, ischemic-type biliary lesions; LTx, liver transplantation; mRT-PCR_{bile}, multiplex real-time PCR in bile; NA, not applicable; PSC, primary sclerosing cholangitis; SSC, secondary sclerosing cholangitis.

C_{bile} (*n*=31 as shown in Table 3; χ^2 = 30.031, *p*<0.001 as shown in Table 4). CMC_{bile} detected 33.3% (*n*=7) and mRT-PCR_{bile} 95.2% (*n*=20) of all Gram-negative bacteria (*n*=21) found in bile samples. These results reveal a remarkable difference of 61.2% in detection outcome between CMC_{bile} and mRT-PCR_{bile}. Furthermore, CMC_{bile} detected 46.8% (*n*=15) and mRT-PCR_{bile} 96.8% (*n*=31) of all Gram-positive bacteria (*n*=32) found in bile samples. These results illustrate an equally remarkable difference of 50% in detection outcome between CMC_{bile} and mRT-PCR_{bile}. In contrast, the difference in detection outcomes for fungi by CMC_{bile} vs. mRT-PCR_{bile} was small (Table 4, Fig. 1), 81.8% (*n*=9) vs. 100% (*n*=11). The concordance of mRT-PCR_{bile} and CMC_{bile} achieved statistical significance only for detection of *Candida albicans*, with a Krippendorf's alpha inter-rater reliability of 0.8406 and a Jaccard index of similarity of 0.8181. Detection of other pathogens had only modest or low interrater reliability and similarity between mRT-PCR_{bile} and CMC_{bile} (Table 4).

Patients with clinical symptoms of infection (cholangitis)

Among patients with clinical signs of infection (*n*=8/26,

Table 3. Micro-organisms detected in bile samples by mRT-PCR$_{bile}$ and/or CMC$_{bile}$

Pathogen	Bile sample								J-index	IR
	n	Overall positive	mRT-PCR	CMC pos	mRT-PCR | CMC					
					pos|pos	pos|neg	neg|pos	neg|neg		
E. faecalis	26	11	10	7	6	4	1	15	0.5454	0.5714
C. albicans	26	11	11	9	9	2	0	15	0.8181	0.8406
E. faecium	26	11	11	6	6	5	0	15	0.5454	0.5714
K. pneumoniae/oxytoca	26	8	8	4	4	4	0	19	0.5000	0.5750
Streptococcus spp	26	7	7	1	1	6	0	19	0.1428	0.1307
E. coli	26	5	4	2	1	3	1	21	0.2000	0.2609
E. cloacae/aerogenes	26	5	5	0	0	5	0	21	0.0000	−0.0851
P. aeruginosa	26	2	2	1	1	1	0	24	0.5000	0.6531
CoNS	26	2	2	1	1	1	0	24	0.5000	0.6531
S. aureus	26	1	1	0	0	1	0	25	0.0000	0.0000
S. maltophilia	26	1	1	0	0	1	0	25	0.0000	0.0000
Total	286	64	62	31	29	33	2	223	0.4531	0.5514

CMC, conventional microbial culture; CoNS, *coagulase-negative Staphylococcus species; Streptococcus* spp, *Streptococcus species; E. faecalis, Enterococcus faecalis; C. albicans: Candida albicans, E. faecium, Enterococcus faecium; K. pneumoniae/oxytoca, Klebsiella pneumonia/oxytoca; E. coli, Escherichia coli; E. cloacae/aerogenes, Enterobacter cloacae/aerogenes; P. aeruginosa, Pseudomonas aeruginosa; S. aureus, Staphylococcus aureus; S. maltophilia, Stenotrophomonas maltophilia;* J-index, Jaccard index; IR, interrater reliability. Krippendorff's-alpha for interrater reliability and Jaccard index for similarity of the concordance of CMC$_{bile}$ and mRT-PCR$_{bile}$.

31%), mRT-PCR$_{bile}$ detected pathogens in bile in all cases, CMC$_{bile}$ in seven (87.5%) and CMC$_{blood}$ in five (62.5%). CMC$_{bile}$, mRT-PCR$_{bile}$, and CMC$_{blood}$ delivered identical results in three of these patients (n=3/8, 37.5%). They were *Klebsiella* spp. 2× and *Enterococcus faecium* 1×. In patients with positive CMC$_{blood}$, four had identical hits between CMC$_{blood}$ and mRT-PCR$_{bile}$ (n=4/5, 80%), *Klebsiella* spp. 2×, *Enterococcus faecium* 1×, and *Enterobacter* spp. 1×, whereas three had

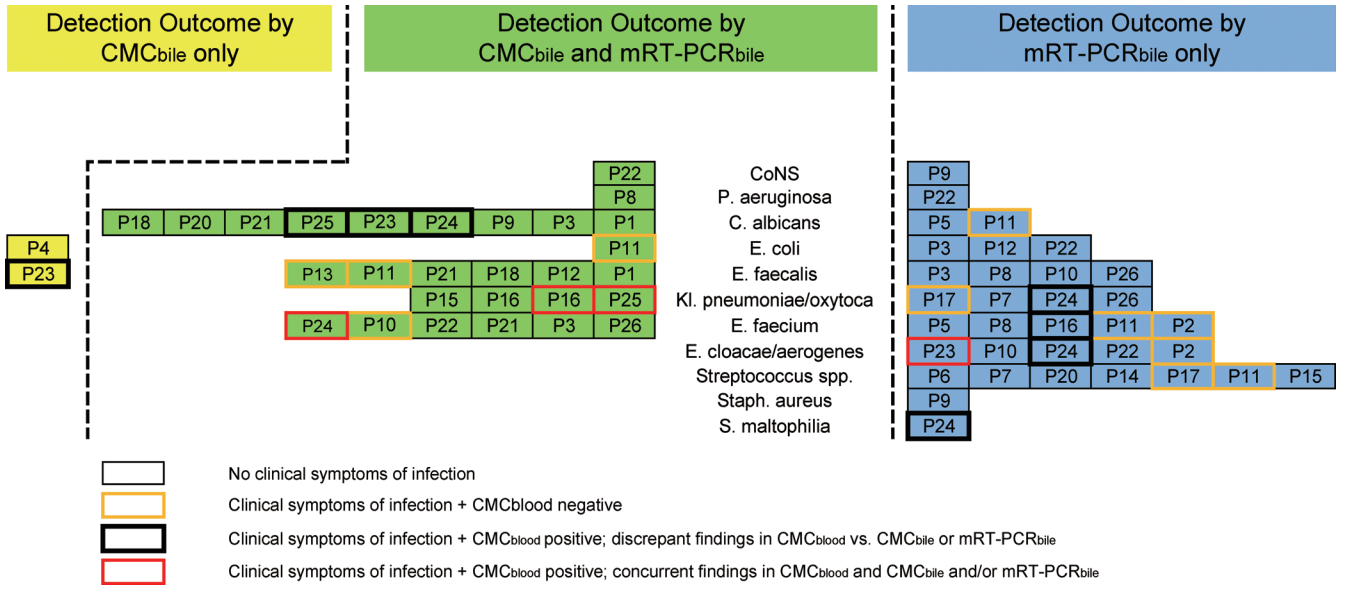

Fig. 1. Pathogen detection in bile samples by mRT-PCR and CMC. mRT-PCR$_{bile}$, multiplex real-time PCR of bile samples; CMC$_{bile}$, conventional microbial culture of bile samples; CMC$_{blood}$, conventional microbial culture of blood samples; CoNS, Coagulase-negative staphylococci; *P. aeruginosa, Pseudomonas aeruginosa; C. albicans, Candida albicans; E. coli, Escherichia coli; E. faecalis, Enterococcus faecalis; Kl. Pneumoniae/oxytoca: Klebsiella pneumoniae, Klebsiella oxytoca; E. faecium: Enterococcus faecium; E. cloacae/aerogenes: Enterobacter cloacae, Enterobacter aerogenes; Streptococcus spp.: Streptococcus species; Staph. aureus: Staphylococcus aureus; S. maltophilia: Stenotrophomonas maltophilia.* P, patient. The figure shows which method successfully identified each pathogen in each patient. While two positive hits were delivered via CMC$_{bile}$ only (yellow heading), 34 positive hits were delivered via mRT-PCR$_{bile}$ only (blue heading). Highlighted frames indicate patients with clinical signs of infection. Yellow frames (P2, P10, P11, P13, and P17) indicate symptomatic patients with negative blood cultures, whereby most of these cases (seven of 11) have only positive hits by mRT-PCR$_{bile}$ and were missed by CMC$_{bile}$. Red frames indicate pathogens that were concurrently detected by CMC$_{blood}$ and CMC$_{bile}$ or mRT-PCR$_{bile}$ (P16, P23, P24, and P25). Black bold frames indicate cases with positive blood cultures and clinical signs of infections, whereby CMC$_{bile}$ or mRT-PCR$_{bile,}$ detected additional pathogens that were not detected by CMC$_{blood}$ (P 16, P 23, P 24, P 25). Especially *C. albicans* was frequently detected in bile samples, but not in blood cultures of patients with signs of clinical infection, e.g., P 23, P24, and P25.

Table 4. Detection of Gram-positive bacteria, Gram-negative bacteria, and fungi in bile samples by mRT-PCR and CMC

Test system	Isolates, *n* (%)			
	Gram-positive bacteria	Gram-negative bacteria	Fungi	All
mRT-PCR	31/32 (96.8)	20/21 (95.2)	11/11 (100)	62/64 (96.8)
CMC	15/32 (46.8)	7/21 (33.3)	9/11 (81.8)	31/64 (48.4)
				χ^2=30.031, p<0.001

CMC, conventional microbial culture; mRT-PCR, multiplex real-time PCR. [2]Chi-square and McNemar tests.

identical hits between CMC_{blood} and CMC_{bile} (n=3/5, 60%), *Klebsiella pneumoniae* 2× and *Enterococcus faecium* 1× (Fig. 1).

Discussion

Apart from comparable performance in identification of *Candida albicans*, we found major discrepancies between both methods, i.e., mRT-PCR_{bile} and CMC_{bile}, in their ability to detect micro-organisms in bile samples. The distribution of organisms identified by CMC_{bile}, mRT-PCR_{bile}, or both methods is shown in Figure 1. Statistical comparisons between the CMC_{bile} and mRT-PCR_{bile} outcomes stratified by pathogen were precluded by the small number of results and patients. However, there are certain noteworthy findings. The majority of the *Candida albicans* isolates (n=9/11) were detected by both CMC_{bile} and mRT-PCR_{bile}, with only two isolates added by mRT-PCR_{bile} only. In contrast, all the *Streptococcus* spp. isolates (n=7/7) were detected by mRT-PCR_{bile}, with only one isolate identified by both CMC_{bile} and mRT-PCR_{bile}. CMC_{bile} identified *Enterococcus* in one additional case and *Escherichia coli* in another, whereas mRT-PCR_{bile} alone failed to detect those pathogens. Overall, regardless Gram stain classification, detection rates for both unique microbes and microbial clusters were significantly higher with mRT-PCR_{bile}.

The findings are in line with previous studies comparing these methods using various specimens. In blood samples, higher detection rates for unique bacteria or fungi as well as combined microbial clusters, were reported for mRT-PCR_{bile} compared with CMC_{blood}.[23,24] Of note, Sancho-Tello *et al*.[13] described higher detection rates for micro-organisms in different purulent fluids, including bile samples, for mRT-PCR compared with CMC. A modest concordance between the two detection techniques was reported. Tajeddin *et al*.[25] used a PCR denaturing gradient gel electrophoresis to assay bile samples from patients with biliary tract disorders. Compared with CMC, that PCR method also delivered positive single and multiple microbial hits at higher frequencies in bile fluids. However, in urine samples of patients with suspected urinary tract infection, Lehmann *et al*.[12] reported equally high pathogen detection rates with mRT-PCR and with CMC.

To the best of our knowledge, this is the first study to use mRT-PCR to test bile samples from patients with chronic cholestatic diseases. Only 1.5 mL of bile fluid was needed for analysis of micro-organisms with mRT-PCR compared with the 2–5 mL that is required for CMC_{bile}. Furthermore, the Septi*Fast* mRT-PCR assay results were available within 4–5 h compared with the 24–72 h required to generate CMC_{bile} reports along with antibiograms after specimen sampling. The Septi*Fast* mRT-PCR assay detects DNA of micro-organisms present at low counts. Thus, it is not surprising that mRT-PCR assays have had a high specificity/accuracy performance in various clinical contexts in previous studies.[13,14,18] Therefore, mRT-PCR assays seem to be valuable to rule out fungobilia and bactobilia in bile samples. At the same time, the non-quantitative detection of DNA fragments, e.g., from degraded pathogens, colonizing micro-organisms, nonpathogens, or facultative pathogens, render the interpretation of positive mRT-PCR results challenging, and led to a sensitivity in previous studies that lagged far behind its specificity.[13,14,18] In our cohort, the small number of patients developing clinical signs of cholangitis (n=8/26, 30.7%) limited the interpretation of the true sensitivity of both methods.

In line with previous reports, the most frequently detected micro-organisms by either mRT-PCR or CMC_{bile} were *Candida albicans*, *Enterococcus faecium* and *Enterococcus faecalis*.[26–28] In our cohort, pAP did not cover those pathogens (0/26 of *Candida albicans* cases and 2/26 of *Enterococcus* spp. Cases) (Table 3, Supplementary Table 1). Despite that in only 1/8 patients suspected of chalngitis, *Enterococcus faecium* was isolated in CMC_{blood} specimen, and *Candida albicans* and *Enterococcus faecalis* were not detected in any CMC_{blood} specimens from patients with clinical signs of cholangitis. Therefore, we assume that the detection of *Enterococcus* spp. and *Candida albicans* in bile samples are of low clinical significance, and rather represent colonization of the bile tract that requires no specific antibiotic treatment in the absence of clinical signs of acute cholangitis. However, that assumption cannot be extrapolated to other microbial strains that are considered susceptible to pAP. mRT-PCR_{bile} detected such pathogens at higher rates compared with CMC_{bile} in our cohort (Table 3, Supplementary Table 1). That is in line with previous studies reporting higher microbial detection rates for DNA detection kits vs. conventional culture methods in patients receiving antibiotics in different clinical settings.[13,24,29] The pAP in our cohort must be considered effective against bacteria like *Escherichia coli, Pseudomonas aeruginosa* or *Klebsiella pneumoniae* in most cases (Supplementary Table 1). Those microbial strains are not only common causative pathogens for acute cholangitis,[30] but also seem to promote polymicrobial bile infections because of their biofilm forming abilities.[25] In our patients with clinical signs of cholangitis, mRT-PCR_{bile} and CMC_{bile} concurrently identified *Klebsiella* spp., but CMC_{bile} failed to detect *Enterobacter cloacae*, which was identified by mRT-PCR_{bile} and CMC_{blood} (Fig. 1).

With regard to the high specificity previously reported for DNA detection kits, the lack of detection of pathogens in bile samples by mRT-PCR_{bile} represents, in light of our data, a true negative result, and is unlikely an effect of brief prophylactic administration of broad-spectrum antibiotics. Although this observation/postulation already guides our empiric practice in post-interventional antibiotic management of patients with cCLDs, it lacks broad evidence, and therefore should be further addressed in large studies in the future. To date, only a handful of studies reported clear benefits for rapid molecular diagnostics (RMD) in antibiotic treatment management.[31] Our single-center experience suggests that it is safe to discontinue standard antibiotic prophylaxis with ciprofloxacin or ceftriaxone if mRT-PCR_{bile} detects no pathogens other than *Enterococcus* spp. or *Can-*

dida albicans in the patient´s bile fluid. According to previous research, significant shortcomings, e.g., low sensitivity and limited antibiotic resistance detection in whole blood of patients with suspected blood stream infection and/or sepsis, represent reasons for discontinuation of RMD production by companies.[31] However, conjunctional use of mRT-PCR assays and CMC in bile samples seems more performant if longitudinally applied and if antimicrobial prophylaxis/therapy for patients with cCLD takes into account the patient infection/colonization history (PI/CH) and the surveillance data for antibiotic resistance-in our clinic (DARC). In this study, longitudinal bile sample-based CMC results, which existed only for a small minority of patients, were excluded from analysis because of a lack of parallel longitudinal mRT-PCR_{bile} tests. Finally, but important, mRT-PCR_{bile} cannot detect anaerobic micro-organisms; thus, it is not suitable to guide decisions regarding the use of metronidazole as part of post-interventional antibiotic treatment strategies. Including anaerobic bacterial species in the mRT-PCR_{bile} panel would presumably be of major interest in the evaluation of bile samples in similar clinical settings and would render this spectrum of detectable pathogens more complete for covering the clinical picture. Intervention studies of the potential added value of mRT-PCR assays in bile diagnostics of comparable populations, such as patients with cCLD undergoing ERCP, and focusing on clinically relevant outcomes such as reducing the risk for cholangiosepsis and/or the selective pressure for antibiotic resistance are needed. To this end, researchers should be aware of other commercially available assays such as VYOO (SIRS-Lab, Jena, Germany) and the SepsiTest (Molzym, Bremen, Germany), which have already been successfully tested as molecular diagnostics of sepsis in critically ill patients and therefore seem promising for application as RMD alternatives to Septi*Fast* in bile.[32]

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization (AK), methodology (AK, AC, MJ), software (MKÖ), validation (SD, PMR, AC), formal analysis (AK, DH, MJ), investigation (AK, SD, AD, MJ), resources (AK, AD, PMR), data curation (HR, MKÖ, DH, MJ), writing the original draft (MJ, AK), review and editing (MJ, SD, HR, AK), visualization (AK), supervision (HR, AC), and project administration (AK).

Data sharing statement

The data used to support the findings of this study are included within the supplementary information file, and are also available from the corresponding author upon request.

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