Eur J Clin Microbiol Infect Dis (2016) 35:1331–1339
DOI 10.1007/s10096-016-2669-x

ORIGINAL ARTICLE

Association of diverse bacterial communities in human bile samples with biliary tract disorders: a survey using culture and polymerase chain reaction-denaturing gradient gel electrophoresis methods

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Received: 2 March 2016 / Accepted: 3 May 2016 / Published online: 18 May 2016

Abstract Bacterial infection is considered a predisposing factor for disorders of the biliary tract. This study aimed to determine the diversity of bacterial communities in bile samples and their involvement in the occurrence of biliary tract diseases. A total of 102 bile samples were collected during endoscopic retrograde cholangiopancreatography (ERCP). Characterization of bacteria was done using culture and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) methods. Antimicrobial susceptibility of the isolates was determined based on the Clinical and Laboratory Standards Institute (CLSI) guidelines and identity of the nucleotide sequences of differentiated bands from the DGGE gels was determined based on GenBank data. In total, 41.2 % (42/102) of the patients showed bacterial infection in their bile samples. This infection was detected in 21 % (4/19), 45.4 % (5/11), 53.5 % (15/28), and 54.5 % (24/44) of patients with common bile duct stone, microlithiasis, malignancy, and gallbladder stone, respectively. *Escherichia coli* showed a significant association with gallstones. Polymicrobial infection was detected in 48 % of the patients. While results of the culture method established coexistence of biofilm-forming bacteria (*Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Enterococcus* spp., and *Acinetobacter* spp.) in different combinations, the presence of *Capnocytophaga* spp., *Lactococcus* spp., *Bacillus* spp., *Staphylococcus haemolyticus*, *Enterobacter* or *Citrobacter* spp., *Morganella* spp., *Salmonella* spp., and *Helicobacter pylori* was also characterized in these samples by the PCR-DGGE method. Multidrug resistance phenotypes (87.5 %) and resistance to third- and fourth-generation cephalosporins and quinolones were common in these strains, which could evolve through their selection by bile components. Ability for biofilm formation seems to be a need for polymicrobial infection in this organ.

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Introduction

Bile is bacteriologically sterile in healthy individuals; however, under some conditions, such as bile duct obstruction, bacteria proliferate within the stagnant bile. Although sources of these infections are not very well known, fecal microbiota seems to be the main responsible agents in these patients [1]. Cholelithiasis, choledocholithiasis, primary sclerosing cholangitis (PSC), and sphincter of Oddi dysfunction (SOD) are the main disorders of the biliary tract, for which their associations with bacterial infections were proposed by several studies [2–4]. The role of bacteria in the formation of biliary tract diseases has been mostly suggested in the case of cholelithiasis as the most common disorder, which accounts for over 90 % of the cases throughout the world [5]. Bacteria play a key role in the pathogenesis of brown pigment stones; however, debate still continues on the involvement of bacteria in

the formation of biliary tract stones. Cholelithiasis occurs due to the presence of bacteria that originate from the gut and ascend to the lower bile duct due to various predisposing conditions [1]. *Escherichia coli* and various other enteric bacteria, such as *Enterococcus*, are the most common bacteria in these infections. These bacteria can use the same surface proteins (named adhesins) to adhere and colonize the biliary tract epithelium [6]. Most studies showed that most cases of bile infection are caused by enteric organisms, such as *E. coli*, *Klebsiella pneumoniae*, and *E. faecalis* [2, 5, 7]. However, since unculturable bacteria comprise nearly 80 % of the human intestinal microbiota, this is particularly important to investigate the involvement of the other bacteria in biliary tract diseases. There are just a few reports about this involvement, which are focused on the microbial flora of pigment gallstones based on the results of sequences of cloned microbial 16S rDNA [8, 9]. Usage of this method or other molecular approaches for the detection and differentiation of bacterial species could increase our understanding of the role of these bacteria and manners of their pathogenesis in human biliary diseases. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is a fast and reliable technique for the genetic fingerprinting of bacterial communities and may be useful for characterizing uncultivated prokaryotes and mixed microbial infections that may be associated with diverse disorders in this organ [10]. The aim of this study was the investigation of diverse bacterial communities in bile samples of patients with distinct disorders in their biliary tract using culture and PCR-DGGE methods. Resistance of these bacteria to common and broad-spectrum antibiotics was also investigated.

Materials and methods

Patients and sample collection

A total of 102 bile samples from patients with common bile duct (CBD) stone (choledocholithiasis), patients with gallbladder stone (cholelithiasis) and patients with other biliary diseases (cholangiocarcinoma, pancreatic carcinoma, PSC, and SOD) were collected during endoscopic retrograde cholangiopancreatography (ERCP) from August 2010 through December 2011. The stones were classified upon the basis of their visual appearance into three categories: cholesterol, black pigmented, or brown pigmented. Two milliliters of the patients' bile aspirates were collected using biliary drainage tubes and needle aspiration in a sterile container. One part of the samples was stored at −20 °C for molecular analysis and the remainder was used for the isolation and characterization of bacteria.

Biochemical characterization of bacteria

The bile samples were immediately cultured aerobically on blood agar and MacConkey agar plates. Colony count and identity of the grown colonies were determined after 24 h of incubation at 37 °C by Gram staining and standard biochemical tests for both Gram-positive and Gram-negative bacteria. Detection of *Helicobacter pylori* infection was performed by culture of bile samples in supplemented *Brucella* agar plates at microaerophilic conditions as described previously [11].

Antimicrobial sensitivity test

Antimicrobial sensitivity of the strains was determined using the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [12].

DNA extraction from bile samples and PCR-DGGE conditions

To detect infection with unculturable or fastidious bacteria, total DNA was extracted from bile samples using the phenol–chloroform method [13]. The extracted DNA was used as a template to amplify universal 16S rRNA gene for Eubacteria using primers 357F-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and 907R-r (5′-CCGTCAATTCMTTTGAGTTT-3′). PCR was performed in applied thermal cyclers (Eppendorf, Hamburg, Germany). The reaction mixture contained 2 μl of the template DNA, 1 μl of each primer (0.5 μM), 1 U Taq DNA polymerase (Gene Fanavaran, Iran), 1 μl dNTPs (200 μM) (Gene Fanavaran), 5 μl of 10× PCR buffer, and 3 μl $MgCl_2$ (2.5 mM) in a total reaction volume of 50 μl. All the PCR amplifications were initiated at 94 °C for 5 min. The reaction was continued by 30 cycles of denaturation at 94 °C for 30 s, followed by annealing at 60 °C for 45 s and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 5 min was included and 5 μl of the PCR products was separated on a 1.2 % (m/v) agarose gel and visualized under UV light after staining with ethidium bromide. The DCode® Universal Mutation Detection System was used according to the manufacturer's instructions (Bio-Rad, USA). The acrylamide concentration in the gel was 10 % and the denaturing gradient was between 40 and 60 %. Fifteen microliters of the PCR products were mixed with equal volumes of loading buffer and loaded into the gel wells. The gels were run in 1× TAE buffer at 60 °C for 16 h at 45 V. After electrophoresis, the gel was stained with ethidium

bromide (10 mg/ml) and visualized under a UV transilluminator [10].

Analysis of PCR-DGGE bands

To determine the presence of unculturable bacteria in the bile samples, the diversity and homology of the separated bands in the DGGE gels were compared. DGGE bands that were unable to be identified by comparison with known bacterial DGGE markers were excised from the gels with a sterile surgical blade and the PCR products were purified using a DNA extraction kit from agarose gel (Thermo Fisher Scientific, USA). The extracts were used as template DNA for reamplification using the same primers. The amplicons were sequenced and the obtained sequences were characterized by comparison to those in the NCBI database using BLAST analysis. Complete coverage of the sequences with a homology equal to and greater than 97 % was used for the detection and identification of bacterial genera in each sample.

Statistical analysis

The data were analyzed using SPSS software version 17; the Chi-square test was applied wherever applicable. Significance was defined as a p-value of <0.05.

Results

Out of the 102 investigated patients, which ranged between 18 and 92 years old (mean 59.5 ± 16.6 years), 52 patients (50.98 %) were female. Among the patients, the clinical problems in descending order were related to biliary stone disease (74/102, 72.6 %), gallbladder stone (15/102, 14.7 %), and malignancy (13/102, 12.7 %). In total, bacterial infection was detected among 41.2 % (42/102) of the patients. Of the culture-positive samples, 59 bacterial isolates were obtained by conventional microbiological methods. Based on the culture results, monomicrobial and polymicrobial infections were detected in 61.9 % (26/42) and 38.1 % (16/42) of the samples, respectively. The bacterial isolates were obtained mainly from patients with gallbladder stones (52.4 %, 22/42). The most isolated bacterium was *E. coli* (35.5 %). The other genera included *Enterococcus* spp. (19.4 %), *K. pneumoniae* (17.74 %), *Pseudomonas aeruginosa* (17.74 %), *Acinetobacter* spp. (6.45 %), and *Staphylococcus epidermidis* (1.6 %). Mean counts of cultivable bacteria per ml of the bile samples were 10^4 colony-forming units (CFU)/ml. The polymicrobial infections were caused by *P. aeruginosa* and *E. coli* in 3/16 (0.19 %), *E. coli* and *K. pneumoniae* in 4/16 (0.25 %), *E. coli*, *K. pneumoniae*, and *Enterococcus* spp. in 1/16 (0.06 %), *P. aeruginosa* and *K. pneumoniae* in 2/16 (0.125 %), *Enterococcus* spp. and *Acinetobacter* spp. in 2/16 (0.125 %), *P. aeruginosa*, *K. pneumoniae*, and *E. coli* in 1/16 (0.06 %), and *E. coli* and *P. aeruginosa* in 3/16 (0.19 %).

Antibiotic susceptibility testing was performed for all cultured bacteria (Table 1). Most of the *E. coli* and *P. aeruginosa* strains were resistant to fourth-generation cephalosporins and quinolones. All of the *Acinetobacter* spp. strains were resistant to the studied third- and fourth-generation cephalosporins. All of the isolates were resistant to co-amoxiclav and 87.5 % of the strains showed multidrug resistance (MDR) phenotypes (*E. coli*, 95.5 %; *Enterococcus* spp., 25 %; *K. pneumoniae*, 63.63 %; *Acinetobacter* spp. and *P. aeruginosa*, 100 %).

Among the characterized stones, the cholesterol stone was more frequent than the other types (63.51 %, 47/74), followed by black pigmented (23/74, 31.08 %) and brown pigmented stones (4/74, 5.41 %). No significant association was found between the presence and types of the stones and the bacterial infections or their genera based on the conventional culture results (p-value > 0.05) (Table 2).

The results of the PCR-DGGE analysis showed the existence of coinfection in 48 % (49/102) of the samples, comprising three to nine different species. Seven culture-negative samples also showed infection by this method. The maximum number of bands was detected in the DGGE patterns related to patients with gallbladder and CBD stones. *Capnocytophaga* spp., *Lactococcus* spp., *Bacillus* spp., *S. haemolyticus*, *Enterobacter* or *Citrobacter* spp., *Morganella* spp., *Salmonella* spp., and *H. pylori* were among the bacteria that were characterized by this method. The frequency of all the bacteria characterized is shown in Table 3. All the obtained sequences were deposited in the GenBank database under accession numbers KC923069 to KC923078 and KJ661224 to KJ661245 (Table 3).

Considering the estimated frequency of biliary diseases, bacterial infection was detected in 21 % (4/19), 45.4 % (5/11), 53.5 % (15/28), and 54.5 % (24/44) of patients suffering from CBD, microlithiasis, malignancy, and gallbladder stone, respectively. Among these bacteria, *E. coli* showed a significant association with the formation of gallbladder stone (p-value = 0.01). No association was determined for other bacterial infections and occurrence of the diseases. Black pigmented stones showed the highest frequency of infection among patients with biliary stones (56.5 %, 13/23) (Table 2).

Discussion

It was hypothesized earlier that different bacterial species may augment the formation of gallstone and biliary diseases through specific enzyme activities or production of biofilm; however, the exact mechanism of this phenomenon is not clear [14, 15]. There are some reports on the association of bacterial infection and gallbladder cancer, PSC, and biliary stone formation [1, 4, 16]. Singh et al. proposed that infection

Table 1 Susceptibility testing to antimicrobial agents of isolates from human bile samples

Antibiotics	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*Acinetobacter* spp.	*Enterococcus* spp.
CP	10 (45.45)	11 (100)	9 (90)	3 (75)	2 (16.7)
FEP	19 (86.36)	11 (100)	2 (20)	4 (100)	–
TOB	11 (50)	11 (100)	1 (10)	1 (25)	–
CA	18 (81.81)	2 (18.18)	5 (50)	4 (100)	–
CTX	18 (81.81)	11 (100)	2 (20)	4 (100)	–
LOM	16 (72.72)	3 (27.27)	1 (10)	4 (100)	–
FOX	14 (63.63)	11 (100)	5 (50)	4 (100)	–
MEN	14 (63.63)	6 (54.54)	7 (70)	4 (100)	–
GM	8 (36.36)	0	0	1 (25)	3 (25)
AMC	22 (100)	11 (100)	10 (100)	4 (100)	–
NA	19 (86.36)	9 (81.81)	1 (10)	3 (75)	–
IMP	16 (72.72)	0	0	1 (25)	0
V	–	–	–	–	2 (16.7)
Tei	–	–	–	–	1 (8.3)
C	–	–	–	–	1 (8.3)
Syn	–	–	–	–	0
Lz	–	–	–	–	0
NI	–	–	–	–	2 (16.7)
E	–	–	–	–	3 (25)
AMP	–	–	–	–	3 (25)

CP: ciprofloxacin, FEP: cefepime, TOB: tobramycin, CA: ceftazidime, CTX: cefotaxime, LOM: lomefloxacin, FOX: cefoxitin, MEN: meropenem, GM: gentamicin, AMC: amoxicillin/clavulanic acid, NA: nalidixic acid, IMP: imipenem, V: vancomycin, Tei: teicoplanin, C: chloramphenicol, Syn: quinupristin–dalfopristin (Synercid), Lz: linezolid, NI: nitrofurantoin, E: erythromycin, AMP: ampicillin

with *S. typhi* is associated with gallbladder cancer independent of gallstones formation [17]. In the other study that was performed in Japanese and Thai populations, infection of *H. bilis* showed a significantly higher incidence in bile from the biliary tract and gallbladder cancer patients than patients with gallstones/cholecystitis diseases [18]. In our study, bacterial infection was detected in 41.2 % of the patients with biliary tract diseases, including choledocholithiasis, gallbladder stone, and malignancy. In other studies, the infection rates was found in the range 16–54 % [19, 20]. *Escherichia coli*, *Enterococcus*, *Klebsiella*, and *Pseudomonas* spp. were among the most frequently isolated bacteria, for which their frequency was similar to the results presented by other studies [2, 5, 7, 21, 22]. Among the characterized bacteria, a significant association was observed between the colonization of *E. coli* and biliary tract diseases. Infection of the biliary tract with *C. freundii*, *Salmonella* spp., *Helicobacter* spp., *Enterobacter* spp., *Bacteroides fragilis*, *S. aureus*, *Proteus* spp., and *Acinetobacter* spp. was also reported in different studies; however, their association with biliary tract diseases was not established [23–26].

The role of bacteria in the formation of biliary stones is unclear. In a study by Stewart et al., it was shown that β-glucuronidase-producing bacteria only arise in pigment or mixed stones, while other bacteria are frequently seen in the core of cholesterol stones [27]. Our results confirmed this finding, since infection with *E. coli*, *Enterococcus* spp., *Klebsiella* spp., *Acinetobacter* spp., *Streptococcus* spp., and *Staphylococcus*, which possess β-glucuronidase activity, was more frequent in the pigment stones. The results of our study showed that most of these bacteria originate from the intestinal tract.

Polymicrobial infection was common among our patients (38.1 %), which was higher than that in other reports (4.8–17.2 %) [21, 26, 28]. Polymicrobial infections are generally seen in patients who have biliary sphincterotomy and a stent placed in their bile tract [29]. Biofilm formation seems to be a need for polymicrobial infections, since most of the responsible bacteria, including *Klebsiella* spp., *Pseudomonas* spp., *Enterococcus*, and *E. coli*, showed this characteristic [30]. In the present study, a higher rate of bacterial colonization was observed in patients with black pigment stones (56.5 %) in comparison to the patients who presented cholesterol stones (42.5 %) or brown pigment stones (25 %). These results were in contrast to other reports that proposed the formation of brown pigment stones, but not black pigment or cholesterol stones, secondary to the bacterial infection [9, 15]. This controversy could be explained by the methods that were used for the detection of the bacteria in the bile samples (conventional culture and PCR-DGGE on the DNA extract). The presence of

Table 2 Frequency of the bacterial infection in bile samples of patients with different biliary diseases

Bacterial species	Type of stone			Biliary disease				
	Cholesterol stone, $n=47$ (%)	Black pigmented stone, $n=23$ (%)	Brown pigmented stone, $n=4$ (%)	Common bile duct stone, $n=19$ (%)	Microlithiasis, $n=11$ (%)	Gallbladder stone, $n=44$ (%)	Malignancy, $n=28$ (%)	*p*-Value	Total (culture/DGGE)
E. coli	9 (19.15)	10 (43.48)	1 (25)	2 (10.52)	1 (9.09)	17 (38.64)	3 (10.71)	0.01	23 (21/23)
Enterococcus spp.	5 (10.64)	3 (13.04)	0	1 (5.26)	1 (9.09)	6 (13.64)	5 (17.56)		13 (12/13)
P. aeruginosa	0	0	0	0	0	0	0		10 (10/0)
K. pneumoniae	8 (17.2)	8 (34.78)	1 (25)	3 (15.79)	0	14 (31.82)	6 (21.43)		23 (11/23)
Acinetobacter spp.	3 (6.38)	2 (8.69)	0	5 (26.32)	5 (45.45)	24 (54.55)	15 (53.57)		49 (4/49)
Streptococcus	1 (2.12)	2 (8.69)	0	0	0	3 (6.82)	0		3 (2/3)
Citrobacter or *Enterobacter* spp.	7 (14.9)	1 (4.35)	0	0	2 (18.18)	6 (13.64)	4 (14.29)		12 (0/12)
H. pylori	1 (2.12)	0	1 (25)	1 (5.26)	0	1 (2.27)	2 (7.14)		4 (0/4)
Salmonella spp.	1 (2.12)	0	0	1 (5.26)	0	0	0		1 (0/1)
Morganella spp.	1 (2.12)	0	0	0	1 (9.09)	0	0		1 (0/1)
Enterobacter spp.	1 (2.12)	0	0	0	0	1 (2.27)	0		1 (0/1)
Staphylococcus spp.	4 (8.51)	3 (13.04)	0	1 (5.26)	1 (9.09)	5 (11.34)	0		7 (0/7)
Bacillus spp.	4 (8.51)	1 (4.34)	0	0	2 (18.18)	3 (6.82)	3 (10.71)		8 (0/8)
Uncultured bacteria	20 (42.55)	13 (56.52)	1 (25)	5 (26.32)	5 (45.45)	24 (54.55)	15 (53.57)		49 (0/49)
Lactococcus spp.	1 (2.12)	2 (8.69)	0	1 (5.26)	0	2 (4.55)	0		3 (0/3)
Capnocytophaga spp.	4 (8.51)	0	0	0	0	4 (9.09)	0		4 (0/4)
Total	20/47 (42.5)	13/23 (56.5)	1/4 (25)	4/19 (21)	5/11 (45.4)	24/44 (54.5)	15/28 (53.5)		

bacteria in the pigment stones, both black and brown, was established by Stewart et al., who showed the colonization of bacteria on the surface and interior of all pigment stones using both culture and scanning electron microscopy methods. However, they did not detect bacteria in the cholesterol stones [27]. To establish the involvement of bacteria in the formation of cholesterol stones, some researchers used a culture-independent molecular genetic approach to detect their DNA in culture-negative cholesterol stones [20, 31]. In a Japanese study, bacterial DNA was detected in 87 % of brown pigment stones, in 57 % of pure cholesterol stones, and in 67 % of mixed cholesterol stones [25]. In agreement with these findings, our results showed a frequency of 42.5 % for bacterial infection in patients with cholesterol stones. Since it was reported that cholesterol stones with a purity of >90 % contain no bacterial DNA, it seems that our patients have had composite cholesterol stones [32]. The association of Gram-positive cocci with the formation of pure cholesterol stones was established by Kawai et al. [25]. However, this association was not confirmed in our study.

Empirical therapy of biliary tract infection covers both Gram-positive and Gram-negative bacteria, which would design against most common causative bacteria according to their resistance rates. Piperacillins, ceftriaxone plus metronidazole, aminoglycosides, co-trimoxazole, and broad-spectrum antibiotics are among the most frequently used antibiotics in this subject [33, 34]. In this study, the antimicrobial susceptibility of *E. coli*, *Acinetobacter* spp., *P. aeruginosa*, and *K. pneumoniae* strains were investigated. The administration of broad-spectrum antibiotics for biliary infections is recommended due to the polymicrobial nature of infections in this organ. However, our results showed high rates of resistance to different antimicrobial agents in this category. Accordingly, treatment regimens based on culture and antimicrobial susceptibility tests are recommended for polymicrobial infections. No susceptible strain to amoxicillin/clavulanate was detected in our isolates, which propose their ineffectiveness for usage, despite the worldwide application of this drug in biliary tract infections. The observed MDR phenotype by most of the isolated bacteria was similarly described in some other studies [32, 35]. This phenotype seems to be bile mediated; however, the resistance of these bacteria to different antimicrobial categories with diverse resistance mechanisms proposes the involvement of resistance genetic markers. Gentamicin was proposed as a more effective drug against the noted infections, since the lowest resistance rate was detected against this antibiotic among the isolates. In agreement with this result, the usage of aminoglycosides for biliary infection in combination with β-lactams was proposed by other researchers [5, 36].

PCR-DGGE is a useful tool to examine the diversity of bacteria in different samples. It allows rapid differentiation of bacteria in a single sample. In the current study, *H. pylori*-

Table 3 Strains and denaturing gradient gel electrophoresis (DGGE) bands identified in this study by means of 16S rDNA sequencing

Number of band	Strains	Accession no.	Fig	Number of band	Strains	Accession no.	Fig
1	*Acinetobacter* spp.	KF208521.1	Fig. 1	18	*H. pylori*	KJ661224.1	Fig. 1
2	*Klebsiella* spp.	KJ661237.1	Fig. 1	19	Uncultured bacteria	KC923074.1	Fig. 1
3	*Salmonella* spp.	KJ661235.1	Fig. 1	20	*Klebsiella* spp.	KJ661240.1	Fig. 1
4	*E. faecalis*	KJ661225.1	Fig. 1	21	Uncultured bacteria	KC923075.1	Fig. 1
5	*E. coli–Shigella*	KJ661233.1	Fig. 1	22	*Citrobacter* spp.	KC923070.1	Fig. 1
6	Uncultured bacteria	KJ661226.1	Fig. 1	23	*Morganella*	KC923072.1	Fig. 1
7	*Citrobacter* or *Enterobacter*	KJ661234.1	Fig. 1	24	Uncultured bacteria	KC923072.1	Fig. 1
8	Uncultured bacteria	KJ661245.1	Fig. 1	25	Uncultured bacteria	KC923076.1	Fig. 1
9	Uncultured bacteria	KJ661236.1	Fig. 1	26	Uncultured bacteria	KJ661244.1	Fig. 1
10	Uncultured bacteria	KJ661230.1	Fig. 1	27	Uncultured bacteria	KC923071.1	Fig. 1
11	*S. haemolyticus*	KJ661231.1	Fig. 1	28	Uncultured bacteria	KJ661241.1	Fig. 1
12	*Streptococcus* spp.	KJ661232.1	Fig. 1	29	*Citrobacter* spp.	KC923069.1	Fig. 1
13	Uncultured bacteria	KJ661238.1	Fig. 1	30	*H. pylori*	KJ661227.1	Fig. 1
14	Uncultured bacteria	KJ661242.1	Fig. 1	31	*H. pylori*	KJ661228.1	Fig. 1
15	*Lactococcus* spp.	KC923078.1	Fig. 1	32	*Bacillus* spp.	KC923077.1	Fig. 1
16	Uncultured bacteria	KJ661239.1	Fig. 1	33	*Staphylococcus*	KJ661229.1	Fig. 1
17	*Enterobacter* spp.	KC923073.1	Fig. 1				

specific amplicons were detected in the excised DGGE bands of patients with CBD stones, gallbladder stones, and malignancy. Colonization of *Helicobacter* spp. in the biliary tract was shown as a possible cause of hepatobiliary diseases, including chronic cholecystitis, PSC, gallbladder cancer, and primary hepatic carcinoma [37]. Although the role of *H. pylori* infection in the formation of different types of biliary tract diseases is still unclear, the presence of *Helicobacter* DNA in the bile and biliary tissue of human beings highlights its possible role. Since colonies of *H. pylori* were not detected on the cultures of the positive samples, its real colonization and pathogenesis in this tissue was suspected. In the case of *S. enterica*, while the culture results did not confirm its existence, infection with this pathogen was established in one patient with CBD stones using the PCR-DGGE method. Infection with this pathogen can appear as an active form of inflammatory disease, e.g. cholecystitis, or an asymptomatic form (chronic carrier state). Chronic carriage of different *S. enterica* serovars could occur due to their resistance against the action of bile by regulating the expression of many resistance-related genes and their ability to form biofilms on gallstones [38]. Since there is some evidence that proposes chronic infection and inflammatory response of *Salmonella* spp. (more importantly serovar *S. Typhi*) as a risk factor in the development of premalignant lesions in the gallbladder epithelial cells, more attention should be paid to the detection and management of patients carrying them.

The involvement of other bacterial agents in biliary tract diseases, for which their presence was established by PCR-DGGE, including *Capnocytophaga* spp., *Lactococcus* spp., *Bacillus* spp., *S. haemolyticus*, *Enterobacter* or *Citrobacter* spp., *Morganella* spp., and uncultured bacteria, is not yet known. Most of these bacteria are unculturable in the conventional culture media. A case of ascending cholangitis by *L. lactis* was previously reported in an immunocompetent patient, but the bacterium was isolated from his blood sample. This bacterium is considered as a non-pathogen; however, its involvement in several infections, including liver abscess, endocarditis, deep neck infection, and cerebellar abscess, has been previously reported [39]. Two cases of cholangitis due to septicemia by *Capnocytophaga canimorsus* were also reported in one study. Close contact with dogs was postulated as the source of infection in these patients [40]. In our study, *Morganella* spp. was detected in one patient with microlithiasis; however, its frequency in patients with gallbladder empyema was reported as 7.6 % in a study in Taiwan [41].

The results of this study suggested PCR-DGGE as being a more sensitive method compared with the conventional culture method for the detection of bacterial infection in bile samples of patients. This could be due to limitations of the culture method for the detection of infection of many bacteria which present at an amount $\leq 10^2$ CFU/ml [42]. In our study, the higher frequency of bacterial infection, measured by PCR-DGGE, seems to be due to the capacity of this method for the detection of low counts of common enteric bacteria and fastidious bacteria for which their growth needs specific culture media. However, the detection of even insignificant DNA of non-alive bacteria is considered the main disadvantage of

molecular methods. Inconsistency of results for the culture and PCR-DGGE methods in the cases of *Acinetobacter* and *Enterobacter* or *Citrobacter* spp. was mainly explained by the count of the bacteria in each patient's sample. As shown in Fig. 1, most of the bands associated with *Acinetobacter* and *Enterobacter* or *Citrobacter* spp., which was not detected by the culture method, presented weak sharpness, which proposes the presence of very low counts of the noted bacteria in the bile samples. This is also the case for *Klebsiella* spp. in some of the samples, since while sharp bands were detectable for the culture positive samples, a faint band was detectable for the culture-negative samples. In the case of *Pseudomonas* spp., which showed no DGGE band despite its detection by the culture method in some of the samples, we hypothesized the

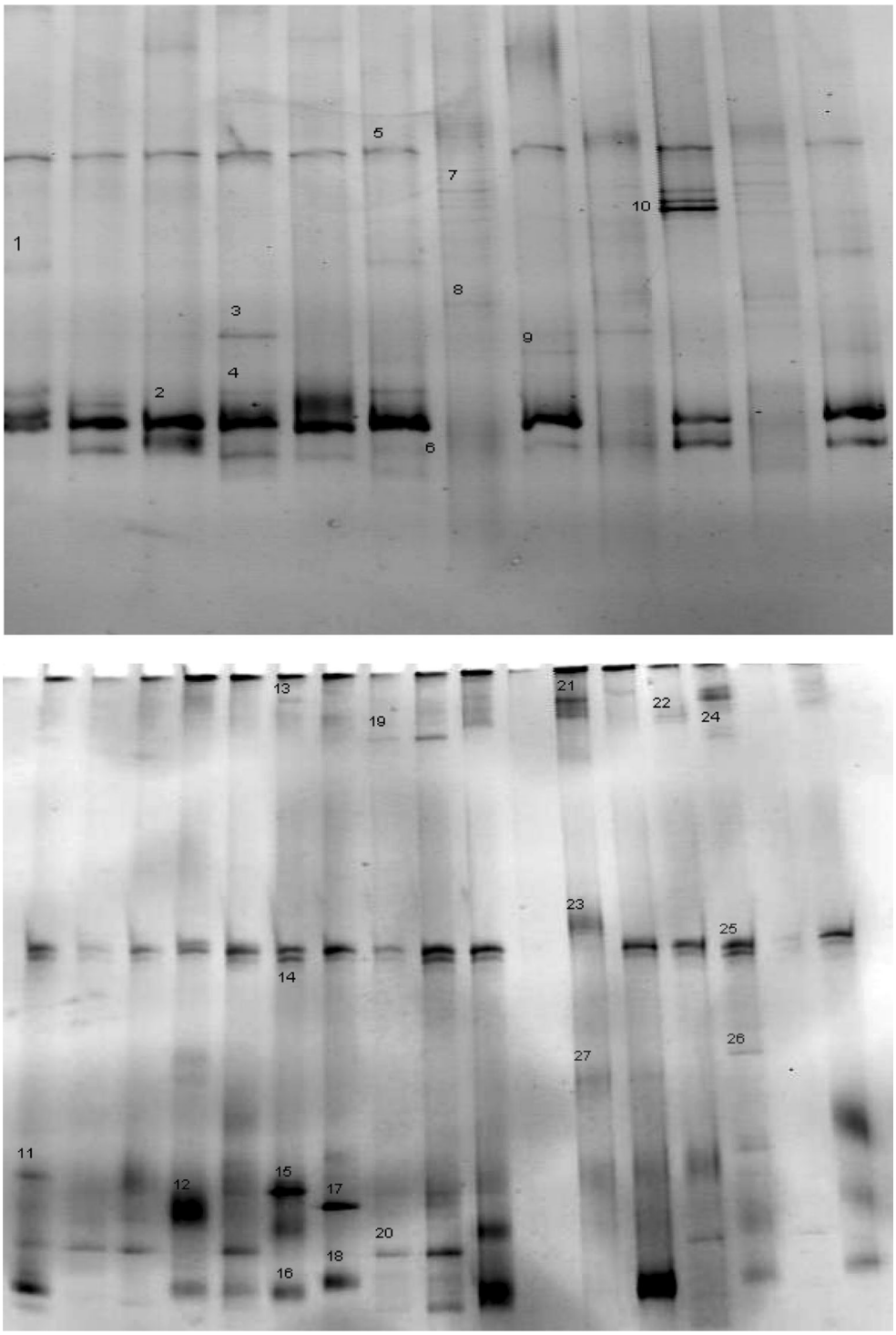

Fig. 1 Denaturing gradient gel electrophoresis (DGGE) analysis of the bacterial diversity in the human bile samples

inability of PCR-DGGE for the differentiation of specific bands of this bacterium from other bacterial products as main reason.

Conclusion

The results of this study showed high rates of bacterial infections in the studied patients. Most of the characterized bacteria belonged to members of the human fecal microbiota. Biofilm formation by these bacteria seems to be a need for polymicrobial infections that was established in most of the patients. While more than 16 different bacterial genera were characterized, infection with *Escherichia coli* was proposed as the main risk factor for progression of the biliary diseases. A direct association between resistance to bile components and broad-spectrum antimicrobials was postulated, since multidrug resistance (MDR) phenotypes were observed in 87.5 % of the isolates. The in vitro sensitivity of these bacteria to gentamicin proposed aminoglycosides as the most effective therapeutic agent against infections of the biliary tissue. Further studies using molecular techniques will provide new insights on the pathophysiology of hepatobiliary disorders and the involvement of microbes or their metabolites in this subject.

Acknowledgments The study was supported by a research grant (number RIGLD 564) from the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The authors appreciate all their colleagues in the Foodborne and Waterborne Diseases Research Center and the ERCP unit at Ayatollah Taleghani Hospital.

Compliance with ethical standards

Funding This study was supported by the Shahid Beheshti University of Medical Sciences, Tehran, Iran, with grant number RIGLD 564.

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Informed consent Informed consent was obtained from all individual participants for the information included in this article.

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