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ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF ISOLATED BACTERIA FROM BILE FLUIDS OF PATIENTS WITH GALLSTONE DISEASE IN ISFAHAN CITY (IRAN)

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Abstract: Bacterial infections are one of the important agents in the creation of gallstones in the gallbladder. In recent years the spread of antibiotic-resistant bacteria such as extended-spectrum β-lactamases (ESBL) is increasing and of concern in hospitalized patients worldwide. The purpose of this study was to investigate the antibiotic susceptibility patterns of isolated bacteria from the bile specimens of patients with chronic and acute cholecystitis who had been operated by single-incision laparoscopic cholecystectomy (SILC) in Isfahan (Iran) using an antibiogram susceptibility test and molecular technique. The bile fluids of 91 patients were obtained from the Al-Zahra hospital and were cultured on specific media for the isolation of Gram-negative and positive bacteria and the disk diffusion test was done to determine the antibiotic susceptibility patterns of isolated bacteria. Finally, bacterial DNA was extracted from the bile samples and polymerase chain reaction (PCR) was performed to investigate extended-spectrum β-lactamases genes. The bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus* spp. and *Staphylococcus aureus* were detected in bile specimens cultured with high frequency, and the results showed that biliary infection increased with aging in patients with gallstone disease operated by SILC. The results showed a high frequency of ESBL genes including *TEM*, *SHV*, and *CTX-M* in isolated bacteria (especially *Escherichia coli* and *Klebsiella* spp.). Thus, evaluating the antibiotic susceptibility patterns and screening of ESBLs bacteria in patients with gallstones are essential. Prescribing suitable drugs, designing good strategies, and informing the medical community could decrease bile infection and antibiotic-resistant bacteria in clinical centers and hospitals.

Key words: Gallstones; extended-spectrum β-lactamases (ESBLs); disk diffusion test; molecular technique

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INTRODUCTION

The gallbladder is a sac-like organ located in the upper right of the abdomen, inferior and posterior of the liver. Gallstones develop in the gallbladder or bile duct and can cause sudden pain (biliary colic), infection and inflammation (acute cholecystitis) as well as jaundice and fever (Vitetta et al., 2000; Paterson et al., 2005). Acute cholecystitis is caused by gallstones in the gallbladder and the symptoms are sudden inflammation of the gallbladder and abdominal pain. Cholesterol, calcium bilirubinate, bile pigment and calcium salts such as calcium carbonate and other things found in bile make up gallstones (Pitout et al., 2005; Paterson et al., 2005). In Europe, 30% of females over 60 years of age and in United States 15 million people have gallstones (Abd-Alkareem, 2011). Furthermore, the prevalence of gallstone disease varies depending on the geographic region, and in white adults of developed countries and American Indians it is 10-15%, and 60-70%, respectively (Stinton et al., 2010). The frequency of gallstone disease in black Americans, East Asia and sub-Saharan Africa is reduced (Shaffer, 2006). There are few studies available about the frequency of gallstone disease in Iran (Massarrat, 2001). In Iran (2013), 20-50% of the patients with chronic cholecystitis had a positive bile culture for isolated bacteria (Moazeni Bistgani and Imani, 2013).

Bacteria play an important role in the creation of biofilms, formation of gallstones, and pathogenicity in patients. Bacterial species such as *Pseudomonas*, *enterococci*, *Acinetobacter*, *Staphylococcus aureus*, *Salmonella typhi*, *Proteus*, *Klebsiella*, and *E. coli* are causative pathogens and are related to patients who have gallbladder infection and cholecystitis. The sex and age of patients may correlate with the species of bacteria (Eslami et al., 2007; Hazrah et al., 2004). Antibiotic-resistant genes such as extended-spectrum β-lactamases (ESBLs) are increasingly prevalent worldwide in bacteria isolated from patients with gallstone disease (Naas et al., 2007).

There are many methods including disk diffusion, agar dilution, Etest, broth microdilution, and broth macrodilution used for *in vitro* antimicrobial susceptibility testing. These tests must be performed according to international accepted procedures such as those of the Clinical and Laboratory Standards Institute (CLSI), Deutsches Institut fur Normung e.V. (DIN), British Society for Antimicrobial Chemotherapy (BSAC), European Committee on Antimicrobial Susceptibility Testing (EUCAST), and Comite de l'Antibiogramme de la Scoiete Francaise de Microbiologie (CA-SFM) (Kahlmeter et al., 2006). In this study, evaluation of the antibiotic susceptibility patterns of bacteria isolated from bile samples was done using the disk diffusion test, executed according to the CLSI. The aim of the present study was to determine the antibiotic susceptibility patterns of isolated bacteria from bile samples of patients with gallstone disease (chronic and acute cholecystitis) who had been operated by single-incision laparoscopic cholecystectomy (SILC) in Isfahan city (Iran) using the disk diffusion test and molecular technique.

MATERIALS AND METHODS

Sampling

The bile fluid of 91 patients operated by SILC was obtained from the Al-Zahra hospital (located in Isfahan, Iran) from April 2011 to December 2012. Samples before bacterial culture and DNA extraction were immediately frozen at -70°C.

The protocol and informed consent forms were approved by the Regional Research Ethical Committee of Isfahan University of Medical Sciences. Informed consents were obtained from all patients before their enrolling in the study according to the Helsinki Declaration. Patients were excluded from the study if they had taken antibiotics within two months before sample collection. Details such as sex, age, clinical history, symptoms, and diagnostic tests were extracted from patients' records. The patients were categorized into two groups: 43 individuals (22-76 years old, mean age 42 years, 11 men and 32 women) and 48 individuals (22-79 years old, mean age 49 years, 14 men and 34 women) who underwent laparoscopic cholecystectomy and were diagnosed with acute or chronic cholecystitis, respectively (Table 1). All patients in both groups were submitted to abdominal echography to confirm the presence of cholelithiasis before the cholecystectomy.

Microbiological study

The bile specimens of patients operated by laparoscopic cholecystectomy were obtained and cultured on blood agar (for isolation of Gram-positive bacteria), MacConkey agar and EMB agar for selection of Gram-negative bacteria from Gram-positive. For optimization of *Helicobacter* growth, some of each sample were homogenized in R broth (consisting of *Brucella* broth containing HEPES, $CuSO_4$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, $MgCl_2$, sodium pyruvate, human blood serum, and lysate) and were plated onto fresh R agar medium according to Richards et al. (2011). Plates were incubated at 37°C under microaerophilic conditions for up to 21 days. The rest of the samples were cultured on Selenite F medium by swap for 24 h at 37°C and then cultured again on selective Salmonella-Shigella agar medium.

Antibiotic susceptibility tests

In the present study, disk diffusion tests were done using the Kirby-Bauer method for determination of the antibiotic susceptibility patterns of isolated bacteria from bile specimens of patients using standard antibiotic disks (i2a, Perols Cedex, France) and Mueller-Hinton agar plates (BD, Franklin Lakes, NJ). Isolated bacteria were categorized as susceptible, resistant, or intermediate after determination of inhibitor zone around the disks according to the standards for antimicrobial susceptibility testing published by the Clinical and Laboratory Standards Institute (CLSI) in January 2013. The names of applied antibiotic disks are shown in Table 2.

Table 1. The age groups of patients with gallstone disease (chronic and acute cholecystitis) operated by laparoscopic cholecystectomy

Age group (years)	Chronic cholecystitis Female / Male	Acute cholecystitis Female / Male
21-30	5 / 1	5 / 2
31-40	8 / 2	7 / 3
41-50	10 / 5	9 / 2
51-60	4 / 3	6 / 4
61-70	4 / 2	2 / 0
71-80	3 / 1	3 / 0
Mean age	49	42
Total of patients	34 / 14	32 / 11

Table 2. Antibiotic disks used for determination of antibiotic susceptibility patterns of isolated bacteria from bile specimens by disk diffusion test

Antibiotic	
Amoxicillin	Gentamicin
Ampicillin	Ceftazidime
Piperacillin	Cefotaxime
Imipenem	Cefotetan
Amikacin	Cephalosporins
Ciprofloxacin	Cefazolin
Ofloxacin	Cefepime
Meropenem	Ceftriaxone
Trimethoprim	Cefoperazone
Nitrofurantoin	Gentamicin

DNA isolation

Total DNA was extracted from each bile specimen using a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendation. The quality of extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell (2001).

Gene amplification

PCR using specific primers was performed to determine the ESBLs genes (*TEM*, *SHV*, *CTX-M*) in isolated bacteria. The primer sequences were obtained from AitMhand et al. (2002), Bonnet et al. (2002) and Wiegand et al. (2007) studies and are shown in Table 3.

PCR was performed in a total volume of 25 µL in 0.5 mL tubes containing 1 µg of template DNA, 1 µM of each primer, 200 µM dNTP, 2 mM $Mgcl_2$, 2.5 µL of 10X PCR buffer (20 mM Tris-HCl pH=8.4, 50 mM KCl), and 1 unit of *Taq* DNA polymerase (Takara Bio Inc.). 2 µL of sterile ultrapure deionized water instead of template DNA were used as a negative control. The tubes were placed in a Gradient Palm Cycler (Corbett Research, Australia) for gene amplification. The PCR temperature programs consisted of initial denaturation at 94°C for 5 min; followed by 32 cycles including denaturation at 94°C for 1 min, annealing at 55°C (TEM), 65°C (SHV), and 65°C (CTX-M) for 1 min, elongation at 72°C for 1 min, and a final elongation at 72°C for 5 min was done at the end of the amplification program.

Analysis of PCR products

The PCR products were analyzed in 2% agarose gel electrophoresis. The electrode buffer was TBE (10.8 g of Tris-base 89 mM, 5.5 g of Boric acid 2 mM, EDTA (pH=8.0) 4 ml of 0.5 M EDTA (pH=8.0). A 1 kb DNA ladder (Fermentas, Germany) was used to determine the length of the amplified fragments. PCR products (10 µL) were applied to the gel. Finally, gels were stained with ethidium bromide. A constant voltage of 80 V for 30 min was used for product separation. After electrophoresis, images were obtained using UVIdoc gel documentation systems (Uvitec, UK).

RESULTS

The cultures of bile specimens of 91 patients operated by SILC on specific culture media showed a high-frequency of Gram-negative bacteria including *Escherichia coli* (*E. coli*) in 78 cases (85.7%), *Klebsiella pneumoniae* in 38 cases (41.7%), *Proteus* spp. in 30 cases (32.96%),

Table 3. Primer used for amplification of ESBL genes in resistant bacteria

Genes	Primer name	Sequence	Product length (bp)	GenBank Accession number
TEM	T1-F T3-R	5′-ATTCTTGAAGACGAAAGGGCCTC-3′ 5′-TTGGTCTGACAGTTACCAATGC-3′	1082	KF826293.1
SHV	NI1-F NI2-R	5′-GCCCGGGTTATTCTTATTTGTCGC-3′ 5′-TCTTTCCGATGCCGCCGCCAGTCA-3′	1016	CP006662.1
CTX-M	CTX-MA-F CTX-MB-R	5′-CGCTTTGCGATGTGCAG-3′ 5′-ACCGCGATATCGTTGGT-3′	551	KF876133.1

Bacteroides spp. in 30 cases (32.96%), and *Pseudomonas aeruginosa* in 23 cases (25.27%). Of Gram-positive bacteria, a high rate of *Staphylococcus aureus* was found in 27 cases (29.67%). Furthermore, low frequencies of *Enterococcus* spp., *Salmonella enterica* (*S. Typhi*), *Citrobacter providencia*, *Acinetobacter* spp., *Clostridia* spp., *Streptococcus faecalis*, and *Aerobacter* were detected (Table 4). The mean age of patients with positive and negative bile cultures was 54.2±14.3 and 55.6±14.5 years, respectively, and significant differences based on the Student's t-test ($P<0.05$) were observed.

The bile specimen cultures using the disk diffusion test showed that 41 (52.56%), and 22 (57.89%) of isolated *E. coli* and *K. pneumonia* were ESBLs, respectively. The amplified ESBL genes using PCR technique revealed fragments with a length size of 1082, 1016, and 551bp for *TEM*, *SHV*, and *CTX-M* genes, respectively (Fig. 1). Analysis of the PCR products showed that *TEM*, *SHV*, and *CTX-M* genes were presented in 29 (70%), 38 (92%), and 33 (80%) of 41 isolated *E. coli* samples, respectively, while these genes were identified in 31 (75%), 39 (95%), and 34 (83%) of 22 *K. pneumonia* isolates from bile fluid samples of patients with gallstone disease, respectively.

DISCUSSION

Bacterial infections are important agents in the creation of gallstones in the gallbladder when bile flow is slowed or blocked (Petakovic et al., 2002). In recent years, antibiotic-resistant bacteria including ESBLs are of concern in hospitalized patients worldwide. In this study, we cultured the bile specimens of patients operated by SILC on specific culture media and the antibiotic susceptibility patterns of the isolated bacteria were evaluated by disk diffusion test and molecular technique. After the culturing of 91 bile samples on specific media, Gram-negative bacteria including *E. coli*, *K. pneumoniae*, *Proteus* spp., *Bacteroides* spp., and *Pseudomonas aeruginosa* in 85.7%, 41.7%, 32.96%, 32.96%, and 25.27% of cases were

Table 4. Frequency of isolated bacteria from bile samples of patients with gallstone disease operated by SILC

No. of Patients (%) Total: 91 cases	Species of bacteria
78 (85.7)	*Escherichia coli*
38 (41.7)	*Klebsiella pneumoniae*
3 (3.29)	*Aerobacter*
11 (12.08)	*Salmonella enterica (S. Typhi)*
30 (32.96)	*Proteus* spp.
12 (13.18)	*Citrobacter providencia*
9 (9.89)	*Acinetobacter* spp.
23 (25.27)	*Pseudomonas aeruginosa*
30 (32.96)	*Bacteroides* spp.
8 (8.79)	*Clostridia* spp.
17 (18.68)	*Enterococcus* spp.
27 (29.67)	*Staphylococcus aureus*
4 (4.39)	*Streptococcus faecalis*

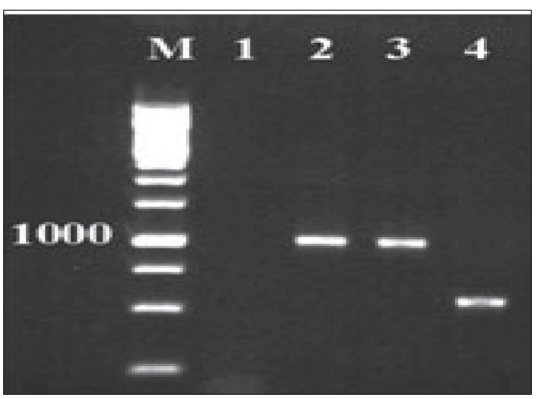

Fig. 1. Two percent agarose gel electrophoresis of PCR products using oligonucleotide primers for detection of *TEM*, *SHV*, and *CTX-M* genes, respectively. Lane M – 1 kb DNA ladder (Fermentas, Germany); lane 1 – blank (negative control); lanes 2, 3 and 4 – *TEM* (1082bp), *SHV* (1016bp), and *CTX-M* (551bp) genes, respectively

observed, respectively. Gram-positive bacteria such as *S. aureus* were detected with high frequency (29.67%), while other infectious bacteria including *Enterococcus* spp., *Salmonella enterica* (*S. Typhi*), *Citrobacter providencia*, *Acinetobacter* spp., *Clostridia* spp., *Streptococcus faecalis*, and *Aerobacter* were observed in low frequency. High rates of ESBL bacteria including *E. coli* and *Klebsiella* spp. using the disk diffusion test were observed in the Al-Zahra hospital (Isfahan, Iran). Furthermore, the amplification of ESBL genes (*TEM*, *SHV*, and *CTX-M*) in isolated ESBL-positive bacteria including *E. coli* and *Klebsiella* spp. showed a high frequency of these genes in patients with gallstone disease operated by SILC. ESBL bacteria have been reported from different regions of the world including Australia, China, Saudi Arabia, Israel, and a variety of North African countries (Cheng and Chen, 1994; AitMhand et al., 2002; Borer et al., 2002; Paterson et al., 2005). ESBLs have also been documented in 30-60% of *Klebsiella* from intensive care units in Brazil, Colombia, and Venezuela (Mendes et al., 2000; Otman et al., 2002; Paterson et al., 2005). The study of Valceano et al. (2005) on the incidence of biliary tract infections in benign gallbladder disease showed Gram-negative bacteria such as *E. coli*, *Proteus* spp., and *Klebsiella* spp. to be the most frequent, similar to the results of our study. But their research showed that the Gram-positive bacrteria such as *Streptococcus viridans* and *Staphylococcus* spp., were not so frequent, while we observed a high frequency of *S. aureus* in the bile samples. Yagi et al. (2000) isolated ESBLs in clinical isolates of *K. pneumoniae* and *E. coli* using minimal inhibitory concentrations (MICs) of oxyimino-cephalosporins and confirmation by double-disk test in Japan and showed that these bacteria produced ESBLs genes. Naas et al. (2007) identified a high presence of CTX-M-type extended-spectrum-β-Lactamase genes in France using real-time PCR and pyrosequencing, pointing to high frequencies of ESBL genes. The study of Ballal et al. (2001) showed that *E. coli*, *Klebsiella*, and *Bacillus fragilis* are predominant, while in the study of Al Harbi et al.(2001), *E. coli* (26.1%), *Enterococcus facials* (15.6%), and *P. aeruginosa* are the most common isolated organisms. These findings indicated that *E. coli* and *Klebsiella* are the common organisms detected in biliary infection. The study of Moazeni Bistgani and Imani (2013) in Iran on the antibacterial susceptibility patterns of isolated bacteria from patients with cholelithiasis showed that 37.87% of cases had positive bile cultures and *E. coli* and *Enterobacter* were the most common isolates (26%), while in our study, *E. coli* and *K. pneumoniae* are more frequent.

CONCLUSIONS

In the current study, antibiotic susceptibility tests and PCR indicated a high frequency of Gram-negative and positive bacteria such as *E. coli*, *K. pneumoniae*, and *S. aureus* in the bile specimens of patients operated by SILC. Furthermore, biliary infection increased with aging in gallstone disease patients operated by SILC and indicated that the evaluation of antibiotic susceptibility patterns in isolated bacteria and screening of ESBL bacteria in bile samples are essential in hospitals.

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