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Investigation of the gallbladder for the presence of pathogenic bacteria and their antibiotic susceptibility pattern

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ABSTRACT

Background

The gallbladder is an essential organ in the human body that functions as an antioxidant. Cholangitis and pyogenic cholecystitis are two significant reasons for cholecystectomy. The significance of establishing microbial cultures and antibiotic susceptibility is that suitable antibiotics can be supplied because the infection can lead to severe consequences and death if not treated properly. However, very limited data is available on this matter. Therefore, this study is an attempt to investigate the presence of bacterial pathogens causing gallbladder disease and antibacterial susceptibility patterns to assess the choice of antibiotic. Moreover, both gallbladder's wall and bile samples were investigated for accurate results. None of the past studies have worked on the gallbladder and the bile simultaneously.

Methodology

A prospective analytical study was conducted from July to December 2014. Blood, MacConkey agar, and Robertson cooked medium were used to culture all the clinical samples. After 24 and 48 hours of incubation, culture plates were examined for evident growth. Standard microbiological procedures were used to identify the isolated species. Antibiotic susceptibility tests were performed, and the isolated bacteria were also examined for extended-spectrum beta-lactamase (ESBL) development.

Results

Among 60 patients, 66% were positive for bacteria in the gallbladder wall or bile, or both. Gram-negative bacteria were the most commonly isolated ones. Due to ESBL production, bacteria were mainly resistant to all cephalosporins and Augmentin. However, aminoglycosides, carbapenem, and antibiotics combination were the most effective.

Conclusion

It can be concluded that organisms are more resistant now due to ESBL production, so the old regimen of antibiotics might not be effective in treating them. The considerable culture-positive rate highlights the significance of acquiring bile and gallbladder wall bacterial cultures to administer the proper antibiotics at cholecystectomy. To avoid major complications like gram-negative septicemias, necessary adjustments must be made by the results of antibiotic sensitivity tests.

Keywords: gallbladder, bacterial infection; resistant pathogen; cholecystectomy; antibiotics susceptibility; *Pseudomonas aeruginosa*

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1. INTRODUCTION

The gallbladder is an essential organ in the human body. In addition to bile storage, it also helps in the neutralization of acids produced by the stomach. Bile helps in the emulsification of fats. It also acts as an antioxidant that helps remove toxins from the body [1].

Gallbladder abnormalities are a significant health problem throughout the world. Cholecystitis and cholangitis are common causes of cholecystectomy [2]. Cholecystitis is the inflammation of the gall bladder's wall, while cholangitis is an inflammation and infection of the bile ducts [3]. Both conditions are common causes of emergency admission for acute abdominal pain. Typically, ductal stones produce pyogenic inflammation of the gallbladder, which can result in bacteremia and septicemia [4]. Additionally, it can result in peritonitis, empyema, emphysematous cholecystitis, intraperitoneal abscess, liver abscess, and gallbladder gangrene [4].

Bacterial presence in the gallbladder bile is known as bactibilia. Bacteria can be found in bile, gallbladder walls, and gallstones in patients with infected gallbladders [5]. But in cases of biliary tract obstruction, germs may enter the biliary tract via the papilla of Vater or the portal circulation, which can result in biliary tract infection [6].

Cholecystectomy is a well-established procedure; nevertheless, some complications can occur, such as wound infections, because of endogenous contamination. These infections might result in severe consequences like septicemia and can prolong the hospital stay [7][8]. Post-cholecystectomy wound infections are prevalent, and the prevention of these infections is a concern for most surgeons [9]. It is observed that patients with pathogenic bacteria in the gallbladder are at higher risk of infectious complications and postoperative morbidity than those with no bacterial growth or opportunistic bacteria [10].

Recently, resistant strains with lower antibiotic susceptibility have made antimicrobial therapy difficult and complicated [11]. Multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) organisms have become more common these days. It is also important to note that bacteria, including *Staphylococcus aureus*, *Enterococcus species*, *Enterobacteriaceae*, *Acinetobacter* species, and *Pseudomonas aeruginosa*, frequently cause infections in healthcare settings and are capable of producing multidrug resistance [12]. Selective reporting and suppression of results should be avoided to ensure the correct application of antibiotics. Moreover, bacteria should be tested against all or nearly all antimicrobial agents [13].

The importance of bacterial culturing and antibiotic susceptibility lies in the fact that proper antibiotics may be administrated because infection can cause serious consequences such as death, if not appropriately treated [14] [15]. Establishing biliary drainage and antibiotics in infected patients is critical for treating biliary tract infection [16][17]. The efficacy of antibiotics against gallbladder pathogens is limited due to tissue distribution and hepatic excretion, which cause difficulty in treating acute cholecystitis or cholangitis [18]. Because polymicrobial infections predominate in the gallbladder and bile. A minimum inhibitory concentration (CIM90) of 0.5 to 1.0 micro g/mL of gentamicin and ampicillin, respectively, was found to be effective against all Gram-positive and Gram-negative bacteria isolated from the bile. Moreover, it was found that imipenem, fluoroquinolones, and second third-generation cephalosporins were also effective against Gram-negative bacteria. [4]

Therefore, culture reports are valuable for planning antibiotic prophylaxis and treatment. However, more data is needed regarding this topic. Thus, this study attempts to investigate the presence of bacterial pathogens causing gallbladder disease in an antimicrobial susceptibility pattern.

2. METHODOLOGY

2.1 Study design

A prospective analytical study was conducted in the Microbiology Department at the University of Health Sciences, Lahore, from July to December 2014. The study includes both males and females of any age group. Informed consent was obtained from either patients or their guardians.

2.2 Sample collection

A total of 60 gallbladder samples (gallbladder wall + bile) were taken. Gallbladders were dissected from the neck with surgical blades in sterile conditions and ground in a tissue grinder. The bile was aspirated from the lumen of the gallbladder using a syringe under aseptic conditions before dissection.

2.3 Bacterial isolation and identification

All the clinical samples (tissues after grinding in a tissue grinder, and the bile as such) were cultured directly on a MacConkey and blood agar and in a Robertson cooked medium (RCM) broth for the presence of anaerobic bacteria. Culture plates were incubated aerobically and anaerobically at 37 oC. Subcultures on a solid media were also done from RCM broth after 24 hours of incubation for both aerobic and anaerobic cultures. All plates were examined the visible growth after 24 to 48 hours of incubation. Culture plates with anaerobes were incubated for 72 hours. Standard microbiological methods were used to identify the isolated microorganisms. Serological grouping and biochemical testing were conducted to confirm gram staining techniques. For the biochemical testing of Gram-negative bacteria, the analytical profile index API -E, API-NE, (BioMerieux, France) was used. The API 20E system was used to identify Enterobacteriaceae, while the API 20NE system was used to identify non-Enterobacteriaceae. The API 20E strip comprised microtubes filled with dehydrated substrates to demonstrate enzymatic activity and carbohydrate (CHO) fermentation. A bacterial suspension is used to rehydrate the substrates. After incubation, the metabolic end products are detected using indicator systems or reagent addition. The pH indicator changes color when CHO fermentation occurs. Several Analytical Profile Indexes (API) systems have been developed over the past years to achieve high sensitivity, specificity, accuracy, speed, and efficiency in identifying bacteria based on their biochemical reaction abilities. Using a biochemical reaction test kit to diagnose bacteria can provide faster results than a molecular method [19].

2.4 Antimicrobial sensitivity testing

The antimicrobial susceptibility testing (AST) of all clinical isolates was done using Kirby-Bauer disk diffusion assay using commercially available antimicrobial disks (Oxoid, Basingstoke, UK), according to the guidelines provided by the Clinical & Laboratory Standards Institute (CLSI). Then, the antibiotic disks were used for Gram- positive isolates of Penicillin (10µg), Cefoxitin (30µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Erythromycin (15µg), Vancomycin (30µg), Clindamycin (2µg), Gentamicin (10µg), and Teicoplanin (30µg), and for gram negative bacteria Ampicillin (10µg), Cefazolin (30µg), Gentamicin (10µg), Tobramycin (10µg), Amikacin (30µg), Augmentin (10µg), Tazobactam (10µg), Cefuroxime (30µg), Imipenem (10µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), and Tetracycline (30 µg). Sensitivity plates were incubated at 37 oC for 24 hours. The inhibitory zone was interpreted according to CLSI Guidelines [20].

2.5 ESBL Testing

2.5.1 Double diffusion synergistic method

A bacterial suspension was prepared with a turbidity of 0.5 McFarland standard and inoculated on Muller Hinton (MH) agar plate. The antibiotic discs were placed on Muller Hinton agar that had been inoculated. Then Amoxicillin/clavulanic acid discs were placed in the center of the plate, with the other antimicrobial discs (third and fourth-generation cephalosporin) positioned 20 mm away from the co-amoxiclav disc. After 24 hours of incubation, plates were tested for an increase in the inhibition zone towards the co-amoxiclav disc side of the third-generation cephalosporin disc zone. It was then compared with the zone found on the side without co-amoxiclav disc, which was declared as an ESBL-producing bacteria **(Figure 1)**.

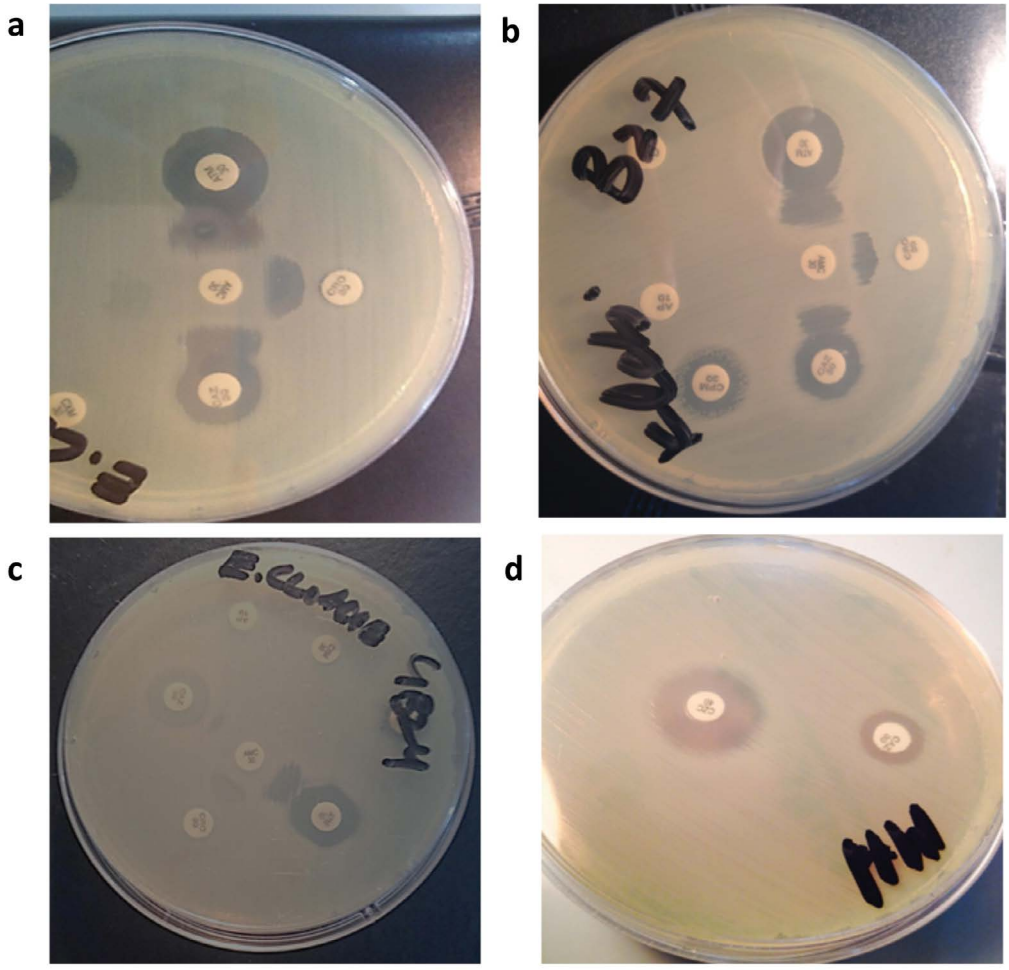

Figure 1: ESBL pattern of bacteria using disk diffusion and phenotypic confirmatory method. a: *E. coli*, b: *K. pneumoniae*, c: *Enterobacter cloacae* d: *P. aeruginosa*.

2.5.2 ESBL detection by a phenotypic confirmatory method

ESBL detection was further confirmed by a phenotypic method for enzyme production using the disk diffusion method according to CLSI guidelines, as shown in **Figure 1**.

2.6 Statistical analysis

All data analyses were done using SPSS software (version 22.0). The detection of organisms and antimicrobial susceptibility pattern was presented in graphs and tables as frequencies and percentages.

3. RESULTS

The total number of samples in this study was 60, including gallbladder's wall and bile. There were 45 female and 15 male patients. **Table 1** shows the number of cases in various age groups. Among 60 patients, 40 (60%) were positive for the presence of bacteria either in the gallbladder's wall or bile or in both. A total of 77 organisms were identified from these forty culture-positive samples, as shown in **Table 2**. From these culture-positive gallbladders, 32 were positive in both the bile and gallbladder's wall, while the remaining eight were only positive for the gallbladder's wall. Nine of the 77 isolates were isolated only from RCM broth, and 68 organisms were isolated from solid media and broth.

Table 1. Cholecystectomy in different group ages with positive cultures.

Age group	Tested cases (N)	Culture-positive cases [N (%)]
Less than 20	3	2 (66)
20-30	12	7 (58)
31-40	21	15 (71)
41-50	13	9 (82)
More then 50	11	7 (64)
Total	60	40

The pathogenic bacteria cultured from the bile and gallbladder's wall are presented in **Table 2**. Nine of those organisms were isolated after subculturing from RCM broth and not by direct culture on solid media.

Table 2. A total number of positive cultures in the gallbladder's wall, bile, or both.

Cultures	N (%)
Culture positives for gallbladder's wall with sterile bile	08 (20)
Culture positives for the bile with sterile gallbladder wall	00 (0)
Culture positives for both gallbladder's wall and bile	32 (80)
Total culture positives	40

The most frequently isolated bacteria were Gram-negative such as *Pseudomonas aeruginosa* 30 (39%), *E. coli* 19 (25%), *K. pneumonia* 10 (13%), *E. cloacae* 09 (12%), *E. faecalis* 03(4%), *Enterobacter sakazakki* 02(3%), *Citrobacter fereuddii* 01(1.3%), *Proteus mirabilis* 01(1.3%) and *Enterobacter aerogenes* 01(1.3%), as shown in **Table 3**.

Table 3. Bacteriology of the gallbladder's wall and the bile.

Organisms	Isolated bacteria from gallbladder's wall (N)	Isolated bacteria from bile (N)	Total isolated bacteria from either gallbladder's wall or bile [N (%)]
Pseudomonas aeruginosa	19	11	29 (39)
E.coli	09	10	19 (25)
Klebsiella pneumonia	06	05	11 (14)
Enterobacter cloacae	05	05	10 (12)
Enterococcus faecalis	03	00	03 (4)
Enterobacter aeruginosa	01	00	01 (1)
Enterobacter sakazakki	01	01	02 (3)
Citrobacter fereuddii	00	01	01 (1)
Proteus mirabilis	00	01	01 (1)
Total	44	33	77

The antibiotics resistant pattern of isolates is given in **Table 4**. It can be shown that *Pseudomonas aeruginosa* were found to be mostly resistant to Tazobactam 34%, Meropenem 24%, less resistant to Amikacin, cefepime, and Aztreonam 7%, Ceftazidime 0%, and Ciprofloxacin 3%. *E. coli* was 100% resistant to Ampicillin, 74% to Augmentin, 42% to Cephalosporin, and 0% to Meropenem, subzone, and Ciprofloxacin **(Figure 1)**.

Table 4. Antibiotics resistance pattern of pathogenic bacteria isolated from the gallbladder and the bile.

Antibiotics	Pseudomonas aeruginosa N=29	*E. coli* N=19	*Klebsiella pneumonia* N=11	*Enterobacter cloaceae* N=10	Enterococcus faecalis N=03	*Enterobacter aerogenes* N=01	*Citrobacter feraudii* N=01	*Proteus Mirabulis* N=01	*Enterobacter sakazakki*
Ampicillin		19/19	11/11	10/10	2/3	1/1	1/1	1/1	2/2
Amikacin	1/29	0/19	0/11	0/10		0/1	0/1	1/1	0/2
Gentamycin					3/3				
Augmentin		14/19	07/11	10/10		1/1	1/1	1/1	2/2
Cefuroxime		8/19	07/11	10/10		1/1	1/1	1/1	2/2
Cefepime	2/29	8/19	04/11	10/10		1/1	1/1	1/1	2/2
Ceftriaxone		8/19	04/11	10/10		1/1	1/1	1/1	2/2
Ceftazidime	0/29	8/19	04/11	10/10		1/1	1/1	1/1	2/2
Aztreonam	2/29	8/19	04/11	10/10		1/1	1/1	1/1	2/2
Meropenem	7/29	0/19	0/11	0/10	2/3	0/1	0/1	0/1	0/2
Ciprofloxacin	1/29	0/19	02/11	06/10	2/3	0/1	0/1	1/1	2/2
Tazobactam	10/29	4/19	02/11	1/10	1/3	0/1	1/1	0/1	2/2
Subzone		0/19	0/11	0/10		0/1	0/1	1/1	0/2
Septran		4/19	06/11	04/10		1/1	0/1	1/1	0/2
Vancomycin					0/3				
Linezolid					0/3				
Tetracycline		2/19	04/11	04/10	3/3	1/1	0/1	1/1	0/2

Table 5 shows that out of 19 *E. coli* isolates, four were found to be ESBL-producing phenotypically. *Klebsiella pneumonia* was 100% resistant to Ampicillin and more sensitive to Meropenem and subzone. Out of the 11 *Klebsiella Pneumonia*, four were found to be ESBL-producing. *Enterobacter cloacae* most resistant *Enterobacteriaceae* and 100% resistant to Ampicillin, Cephalosporin and Augmentin but 100% sensitive to Meropenem and subzone. Out of the 13 *Enterobacter spp.*, 100% were found to be ESBL-producing. For the Gram-positive organisms, *Enterococcus faecalis* was 100% resistant to Gentamycin and 0% to Vancomycin and Linezolid.

Table 5. ESBL-producing bacterial isolates.

Organisms	Total No. of isolates	ESBL producing isolates
E. coli	19	04
Klebsiella pneumonia	11	04
Enterobacter cloacae	10	10
Enterobacter aeruginosa	01	01
Enterobacter sakazakki	02	02
Total		17

4. DISCUSSIONS

The literature has inconsistent reports on bacterial infection in cholecystitis and cholangitis patients [21]. It has been proposed that the inconsistent reports were caused by variations in patient age grouping, cultural techniques, and sampling methods [21]. Although the gallbladder is typically sterile, it is easily contaminated when stasis due to obstruction occurs. In this study, 60 samples of gallbladders, including their bile as content, were collected, with a gender ratio of 1:4 males to females. It was shown that the positive culture rate was higher in the middle-aged female class. In general, 70% of patients were the most symptoms and were around 40-60 years old. This agrees with results reported earlier in previous studies [15][22].

The results also showed a 66% culture-positive rate in either the gallbladder's wall or the bile, which is more prevalent than in previous studies in other parts of the world [15][9]. Pakistan is a developing country with high antibiotic resistance, posing a significant global and regional threat. Recently, both multi-drug-resistant (MDR) and extensively drug-resistant (XDR) bacteria have been identified in Pakistan. Antibiotic resistance results from increased prescribing and easy availability in Pakistan [23]. The resistance of bacteria due to increased antibiotic usage might be a factor in the high burden of bacterial prevalence. Moreover, anaerobic culturing was also performed in this study, an additional parameter that has been investigated. However, our anaerobic culturing rate was 0%, in agreement with what has been reported earlier by Csendes et al., [21]. On the other hand, Al Harbi et al., reported that anaerobic culturing yielded a 10% rate [9].

It is worth noting that collecting organisms from specimens is a challenging task because, in Pakistani surgical practice, cholecystectomy is done after conservative antibiotic treatment in cases of acute cholecystitis. Hence, this method might have caused a decrease in the positive rate in our results [24].

The results also show that the most prevalent Gram-negative bacteria were *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumonia*, and *Enterobacter cloacae*, which made up 95% of the isolates, which is in agreement with the previous studies, which reported 70% to 90% Gram-negative rate [24][15].

An important finding in this study is the identification of *Enterobacter cloacae* in 5 samples. This isolate shows antibiotics resistant to almost all cephalosporin and other antibiotics due to the ESBL phenomenon we have also seen in all gram's negative isolates. Some other studies have also studied this resistant pattern of Enterobacter Cloacae [9]. The third generation of cephalosporin is mostly resistant to these organisms. As compared to other *Enterobacteriaceae*, *Enterobacter cloacae* has a high mortality rate. Hence, to treat these organisms, genome sequencing would be beneficial*(Fraser, 2010 #64)* [25]. It is worth noting that the gallbladder's wall-positive culture rate was more than the sterile bile.

Previous studies also revealed that immediate gram stain aids in selecting antibiotic cover during surgery. Therefore, it is an easy and practical way of reducing post-operative sepsis while avoiding unnecessary antibiotics administration. This has also been confirmed in this work. Moreover, Al Hrabi et al., found that rapid Gram stains offer a method of choosing individuals who need antibiotic treatment during surgery. Thus, this provides a valuable technique to reduce post-operative sepsis while preventing unnecessary antibiotic administration [9].

The spectrum of an antibiotic's activity against common biliary infections and its pharmacokinetic features in terms of tissue distribution and hepatic/biliary excretion are factors that determine the effectiveness of antibiotic therapy in acute cholangitis or cholecystitis [18]. In this study, *E. coli*, *Klebsiella pneumonia*, and *Enterobacter cloacae* were ESBL producers, showing resistance to Ampicillin and Cephalosporin. The transformation of the gallbladder epithelium has been attributed to the persistence of infection that causes chronic inflammation, the production of certain toxins by specific bacteria, and metabolites that can potentially cause cancer [26]. Most bacterial antibiotic resistance mechanisms can be divided into three categories: altering the antibiotic target site, altering or destroying the antibiotic molecule, and inhibiting antibiotic binding to the target site using an elimination method. Additionally, bacteria typically pick up resistance genes through transformation (absorption of resistant genes from the environment), transduction (transfer of resistant genes from bacteriophages), and bacterial conjugation (absorption of resistant genes between resistant bacterial strains) [27].

According to some researchers, active biliary excretion of beta-lactam, aztreonam, fluoroquinolones, and aminoglycosides returns gradually once biliary blockage is relieved [4]. According to this study, Aminoglycosides, Carbapenem, Quinolones, and a combination of drugs like Subzone and Tazobactam were found to be more sensitive. However, unlike other studies, Ampicillin, Augmentin, and Cephalosporin were less sensitive due to ESBL production. While Glycopeptides and medication combinations like Subzone and Tazobactam were effective against Gram-negative bacteria, they were not in this study. Traditional antibiotic therapy frequently uses two or more agents due to the prevalence of polymicrobial infections in bile. This includes combining an aminoglycoside, beta-lactam, and an agent like clindamycin or metronidazole [4].

According to studies by expert committees, antibiotic prophylaxis was required for all patients needing surgery for acute and chronic cholecystitis due to the common occurrence of bacterial growth in the bile and the gallbladder's wall [28]. The aminoglycosides or cephalosporin are the best options

for the initial antibiotic, which should be effective against common pathogenic bacteria and aerobic Gram-negative coliforms. Penicillin should be given to the regimen if Gram-positive bacilli are detected on the stain. After two or three days following the cholecystectomy, antibiotics can be stopped without problems. The treatment should last 5 to 7 days in patients with gangrene, perforation, or substantial local infection. Prophylactic antibiotics should only be administered as an urgent preoperative dose in cholecystectomy cases due to chronic cholelithiasis, avoiding the protracted administration of prophylactic antibiotics in any of these patients [29, 30]. Although cephalosporin is frequently used in medicine, it is unclear when is the best time to provide the drug and how long it should last. Previous research found inconsistent and expensive antibiotic use in people with acute cholecystitis [31][5].

The current study has a few limitations, including a low sample size. The minimum inhibitory concentration of antibiotics was not determined in this study. Further studies are needed on the antibiotic sensitivity pattern of bacteria isolated from the gallbladder and to find an effective antibacterial agent against these bacteria.

5. CONCLUSION

We have concluded that organisms are more resistant now due to ESBL production, so the old regimen of antibiotics would not be effective in treating them. The many culture-positive rate highlights the significance of acquiring bile and gallbladder's wall bacterial cultures at the time of cholecystectomy to administer the proper antibiotics. To avoid major complications like Gram-negative septicemias, necessary adjustments must be made by the results of antibiotic sensitivity tests.

Conflict of interests: All the authors have no known conflicts of interest.

Ethical Approval: The study was approved by the Ethical review committee of the University of Health Sciences Lahore Pakistan.

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