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Comparison of Conventional and Molecular techniques with inflammatory markers in differentiation of bacterial and non-bacterial Cholecystitis

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ABSTRACT
Background/aims: The presence of bacteria in bile during surgery or invasive diagnostic procedures predisposes to septic complications.Traditional methods in identifying bacterial agents aretime-consumingand some bacteria are difficult to grow or even non-cultivable. This study aims to compare molecular methods and inflammatory indices with conventional culture method in identification of infection in patients with cholecystitis.
Methods: Across-sectional study was conducted. Twenty-nine bile fluids along with blood samples were taken from patients who underwent laparoscopic cholecystectomy. Bile samples were used for both culture and deep sequencing of bacterial 16S rRNA. blood was used to detect C-reactive protein and Complete blood count.
Results: From Twenty-nine patients with cholecystitis, Culturing of Bile identified only 6 (20.7%) positive for bacterial growth whereas sequencing revealed 27(93.1%). Serum inflammatory markers CRP, WBC, NLR, PLR and SII levels were higher in patients infected with pathogenic bacteria. ROC curve analysis showed that PLRhad the highest discriminatory power (AUC=0.75).
Conclusion: Molecular method was better than conventional culture technique in identifying the presence of bacterial infection. Inflammatory markers had acceptable power in discrimination between infection with pathogenic and non-pathogenic bacteria.

Keywords: cholecystitis; conventional culture technique; 16SrRNA; C-Reactive protein; neutrophil-to-lymphocyte ratio; platelet-to-lymphocyte ratio;Systemic inflammatory index.

INTRODUCTION

The biliary tract disorders fall into two essential illnesses, the first one is cholecystitis (gallbladder inflammation) and the second isthe existence of gallstones in the gallbladder(cholelithiasis). Gallbladder inflammation can beeither acute or chronic, with recurring incidents of acuteinflammation potentially which leads to chronic cholecystitis[1].Different kinds of bacteria can cause Gallbladder infection. Bacteria enter the gallbladder by ascending route from the duodenum by traveling through the hematogenous or enterohepatic circulation, and surviving there in spite of the bile's bactericidal properties[2]. The biliary tract is commonly sterile; but, if cholelithiasis occurs, various microbes might be identified in cultured from the bile or gallbladder wall [3].The correct utilizationof prophylactic antibiotics reduces morbidity and mortality due to infection because the presence of bacteria in bile during surgery or invasive diagnostic procedures predisposes to septic complications like septicemia, hepatic and renal failure, liver abscess, endotoxemia, and disseminated intravascular coagulation [4].Traditional methods are used to identify many bacteria from bile and different sites of the gallbladder in patients with gallstones.The most common isolated species include Citrobacter, E. Coli, Klebsiella, Enterobacter, Acinetobacter, and Pseudomonas aeruginosa[5] . As an alternative to culture, sequencing of the 16S rRNA gene hasemerged as a precise and faster technique widely used forbacterial identification[6]. The 16S rRNA gene, ~1.5 kilobase (kb) in length, has been established to be a useful molecular target since it is present in all bacteria, either as a single copy or in multiple copies, and it is highly conserved over time within a species [7]. On the other hand, variouslaboratory tests for inflammatory markers, such as white blood cell count (WBC) and C-reactive protein (CRP) were used to detect the presence of an inflammatory process [8].White blood cell count (WBC), neutrophil-to-lymphocyte ratio (NLR), Platelet-to-to-lymphocyte (PLR), whichhas the benefits of straightforward retrieval from a standard complete blood count test, easy sampling, with mature detection methods, and rapid resultsare often used as indicators for detection of

bacterial infections clinically [9][10].C-reactive protein is an inflammatory marker forinflammation, and its level increase during bacterial infection [11]. Its homopentameric acute-phase inflammatory protein, an extremely preserved plasmaprotein that exhibits elevated expression during inflammatory conditions such as rheumatoid arthritis, some cardiovascular diseases, and infection.The highest level ofCRP is found in serum, with some bacterial infections increasing levels up to 1,000-fold[12].But after the stimuli end, CRP values diminish dramaticallyover 18–20 h, near the half-life of CRP[13]. So, this study is aimed to compare between conventional culture methods with molecular technique/ inflammatory indicators to diagnose bacterial infection in patients with cholecystitis.

MATERIAL AND METHODS

Twenty-nine patients whom they admitted to Al-Safeer and Al-Kafeel hospital in Karbala Province to undergo laparoscopic cholecystectomy between the period (February 2023 to July 2023) were enrolled in this study. Both Bile and blood samples were collected from each patient.

Blood sample wasused to measure CRP and CBC tests and 1ml of bile from the gall bladder were injected aseptically into two sterile sealed containers during surgery. one of them contained shield for 16s RNA Sequencing at a ratio of 100 µl of bile fluid to 300 µl of Shield and preserved in (-29) Cº according to the instructions of the manufacturer of the preservation material. The other container was including brain heart infusion broth (BHI) for bacterial culture which is incubated aerobically for 24 hours at (37)Cº, then re-cultured on MacConkey agar, blood agar and EMB agar. Positive colonies from each plate were bacteriologically identified and confirmed by biochemical tests.

DNA Extraction and PCR

The DNA of bacterial isolates was extracted using the (Genomic DNA purification Kit FAVORGEN)) according to the protocols of the manufacturer.Genomic DNA was subjected to amplification with the primers mentioned in table (1), The primer pair (Macrogen/Korea) to detect the gene of 16sRNA.

Table 1: The primer pairgene of 16sRNA

Primer name	Sequence 5′-3′	Reference
27F	AGAGTTTGATCCTGGCTCA	[14]
1492R	GGTTACCTTGTTACGACTT	

Sequence Editing and Analysis by BioEdit

BioEdit (Sequence Alignment Editor version 4.7.8) a software of the Department of Microbiology, North Carolina State University [15]was used as the general tools for sequence editing and analysis e.g., for locating the putative ORFs in all reading frames, analyzing nucleotide composition, amino acids composition and codon usage ORF for performing multiple sequence alignment.

Similarity Search by BLASTN

DNA sequence was analyzed for sequence similarity to the existing DNA sequences available in the database at National Library of Medicine (NLM), National institute of Health (NIH), and National Center for Biotechnology Information (NCBl). The DNA sequences were used to perform BLASTN search against sequence database [16].

Statistical Analysis:

Data was analyzed using IBM SPSS version 22.0. Discriptive statistics like frequencies, mean and standard error was measured. Normal distribution of numerical data was done. Student T test was used to find the difference between means if the tested variable follows normal distribution and KrusKal-Wallis test was used to analyze difference if the variable does not follow the normal distribution.

Ethical consideration

This study was approved by council of University of Kerbala/college of science/ Department of Biology. Oral acceptance was taken from each patient before sample collection.

RESULTS

Out of the 29 patients involved in the study, 21 were females (72.4%) and 8 were males (27.6%) with a median age of 45years (range 20–74). Six (20.7%) of them had positive cultures, and 23(79.3%)had negative culture (no growth).One (3.4%)patient of 29 had acalculous cholecystitiswhile 28(96.5%) had calculous cholecystitis with single 3(10.3%) stone and multiple stones 25(86.2%)as demonstrated in Table 2.

Table 2: Distribution of patients with Cholecystectomy according to Socio-Demographic Characteristics

Socio-Demographic Characteristics variable		**Male**	**Female**
Sex Distribution			
N=29(%)		8(27.6)	21(72.4)
OR for Female		1.4	
Age groups			
	20-39	3(27.3)	8(72.7)
	40-59	3(33.3%)	6(66.7)
	60-79	2(22.2)	7(77.8)
Mean ± SE		47.25 ± 6.20	47 ± 3.79
Bile Stone			
Calculous - Single stone		0(0.0)	3(100)
Calculous – Multiple stone		7(28.0)	18(72.0)
Acalculous - No stone		1(100)	0(0.0)

Conventional culture technique for bile revealed that 23 (79.3%) of the bile samples were culture negative, whereas only 6 (20.7%) revealed bacterial growth. In a comparison to bile culturing, 16SrRNA sequencing technique reveled the presence of bacteria in 27 (93.1%) of bile samples, while 2(6.9%) samples were free of bacterial DNA as shown in Table (4). PCR technique applied using 16S rRNA universal primers. In all 27-extract sample, fragment 1450 bp was observed emphasizing the presence of 16S rRNA amplification gene as shown in figure (1).

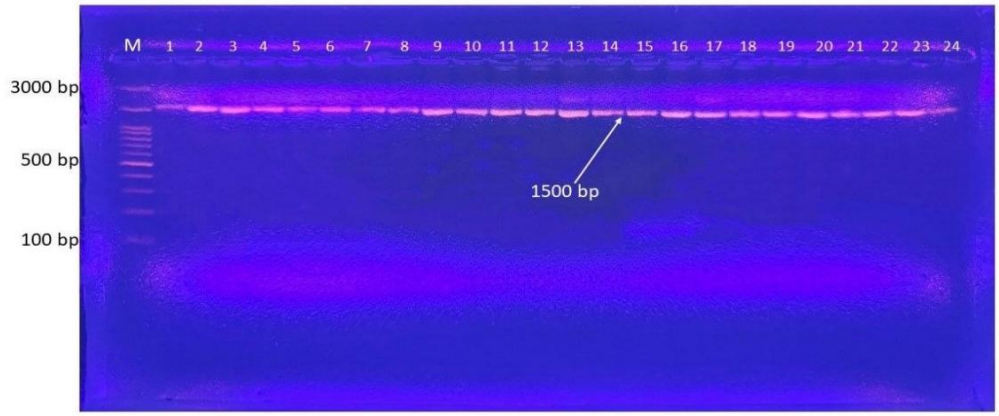

Figure 1: Agarose Gel Electrophoresis of PCR 16S rRNA gene**.**

In the current study, the patients were divided according to their infection into patients with pathogenic bacteria, non-pathogenic bacteria and no bacterial existence as mention in Table (4).

Table 4: Distribution of bacterial species from bile sample by sequencing compared to conventional culture

Bacterial Infection	Identification by sequencing N=29(Percent %)	Growth by culturing N=29(Percent %)
Pathogenic Bacteria	18 (62.1)	
E.coli	7 (24.1)	2(6.9)
Klebsiella pneumoniae	5(17.2)	4(13.8)
Klebsiella variicola	1(3.4)	0
Mycolicibacterium smegmatis	1(3.4)	0
pseudomonas aeruginasa	1(3.4)	0
Pseudomonas anguilliseptica	1(3.4)	0
Staphylococcus aureus	1(3.4)	0
Staphylococcus pasteuri	1(3.4)	0
Non pathogenic Bacteria	9(31.0)	0
Bacillus licheniformis	2(6.9)	0
Bacillus sp.	2(6.9)	0

Bradyrhizobium sp.	3(10.3)	0
Lysinibacillus fusiformis	1(3.4)	0
Exiguobacterium sp.	1(3.4)	0
No bacterial Existence	2(6.9)	23(79.3)
Total	29(100.0)	29(100.0)

Concerning inflammatory markers, non-significant differences between the infectingand non-infected patients was observed. However, Higher level was seen in patients with no growth of bacteria except for Neutrophil count according to conventional technique whereas Higher levels was seen in patients with pathogenic bacteria except for Lymphocyte count according to molecular technique, as shown in Table (5)

Table 5: Comparison of Inflammatory markers mean based on the type of bacterial infection.

Variable		Conventional Culture Technique		Molecular Technique	
		No Growth	Bacterial growth	Pathogenic bacteria	Non-Pathogenic bacteria
CRP	Mean (SE)	15.19 (10.06)	5.48 (3.91)	21.67 (13.45)	1.2950 (37083
	Mean Rank	14.43	17.17	15.22	11.56
Kruskal Wallis Test		0.484		0.258	
WBC	Mean (SE)	8412.38 (692.94)	8040 (674.98)	8985.29(804.99)	6926.25 (460.63)
	Mean Rank	13.33	14.20	14.41	10.00
Kruskal Wallis Test		0.820		0.162	
Lymphocyte	Mean (SE)	32.28 (2.36)	31.88 (7.06)	29.72 (2.59)	37.51 (4.59)
T-test		0.94		0.12	
Neutrophil	Mean (SE)	61.2 (2.51)	62.6 (7.84)	64.42 (2.76)	54.88 (4.84)
T-test		0.82		0.08	

SE=Standard Error;CRP=C-reactive protein; WBC=White blood cell

NLR, PLR, and SII was calculated from dividing the number of neutrophils to lymphocytes,platelets to lymphocyte, and Platelets * Neutrophils / lymphocyte, respectively[17][18]. This study showed significant difference in PLR level and marginal significantdifferences in the level of NLR and SII, according to molecular technique whereas, All the calculated indices were same between infected and non-infected patients according to conventional culture technique,as shown in Table (6).

Table 6: comparison of NLR, PLR, and SII mean values based on the type of bacterial pathogen.

Variable		Conventional Culture Technique		Molecular Technique	
		No Growth	Bacterial growth	Pathogenic bacteria	Non-Pathogenic bacteria
NLR	Mean (SE)	2.41(0.39)	3.02 (1.35)	2.72 (0.44)	2.19 (0.91)
	Mean Rank	13.38	14	14.88	9
Kruskal Wallis Test		0.87		0.062	
PLR	Mean (SE)	10.04(1.11)	10.59 (2.69)	10.67 (1.22)	7.53 (1.00)
	Mean Rank	13.38	14	15	8.75
Kruskal Wallis Test		0.87		0.048	
SII	Mean (SE)	651.59(90.90)	743.22 (276.91)	731.148 (111.90)	443.76(103.19)
	Mean Rank	13.38	14	14.88	9
Kruskal Wallis Test		0.87		0.062	

SE=Standard Error NLR Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII Systemic inflammatory index;

The Receiver operating characteristic (ROC) curve analysis was done to analyze the ability of inflammatory markers to distinguish between infection with pathogenic bacteria from non-pathogenic ones, the results showed that PLA had the highest discriminatory abilityamong the parameters, the AUC, cut-off value, sensitivity, specificity and Significancevalues were shown in Figure (2 B).Based on dividing the patients according to

conventional technique results, AUC for the calculated indices had the same value (poor discrimination) and CRP had the highest power, as shownin figure (2A).

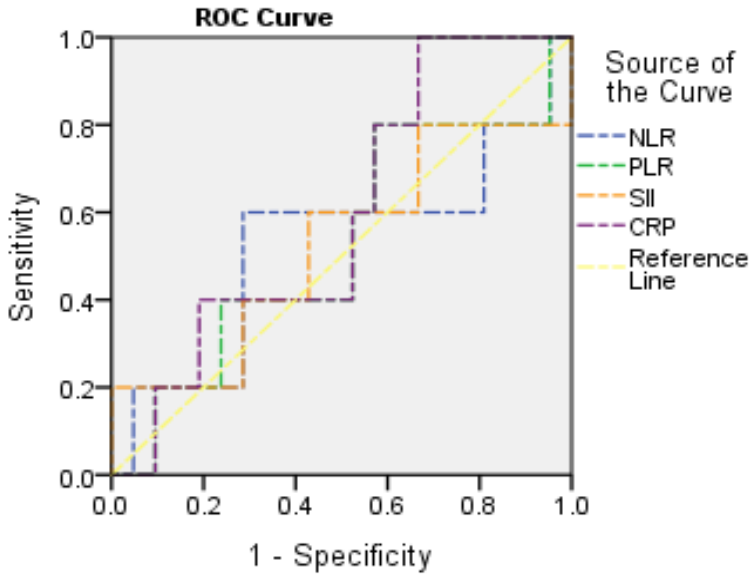

Figure (2A) : Comparative ROC curves for NLR, PLR, SII and CRP according to conventional technique. NLR Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII Systemic inflammatory index;ROC: Receiver operating characteristics.

Table 8: ROC curve for NLR, PLR, and SII.

	AUC	**Std. Error**	**MIN**	**MAX**	**Cut-Off**	**Sensitivity**	**Specificity**	Sig**nificance**
CRP	.590	.128	.339	.842	2.205771	60	72.4	.537
NLR	.524	.166	.199	.848	8.398979	60	47.6	.871
PLR	.524	.150	.230	.817	553.424325	60	57.1	.871
SII	.524	.163	.204	.843	1.205000	60	47.6	.871

AUC=Area under curve**;** SE=Standard Error; MIN=Minimum;MAX=Maximum**;** NLR Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII Systemic inflammatory index

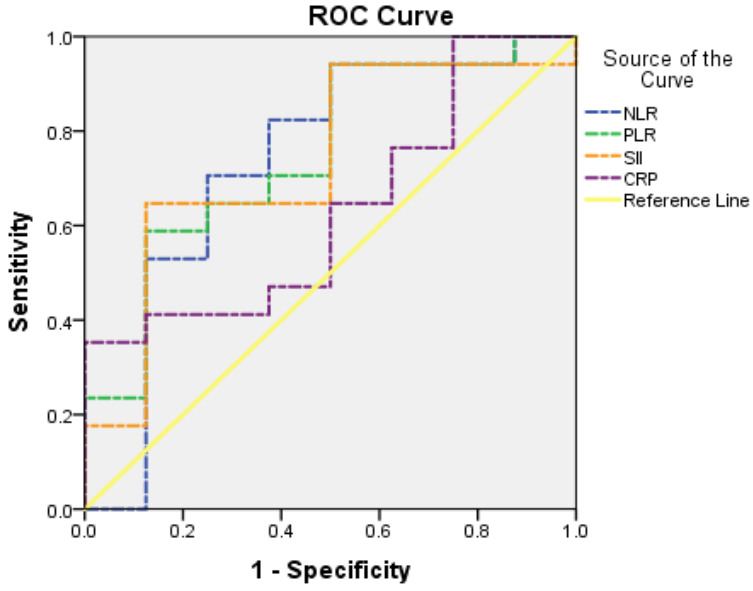

Figure 2B : Comparative ROC curves for NLR, PLR, SII and CRPaccording to molecular technique.

NLR Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII Systemic inflammatory index;ROC: Receiver operating characteristics**.**

Table 7: ROC curve for inflammatory markers according to molecular technique.

	AUC	**SE**	**MIN**	**MAX**	**Cut-Off**	**Sensitivity**	**Specificity**	Sig**nificance**
CRP	.631	.105	.425	.838	0.8200	66.7	45.5	.243
NLR	.654	.113	.433	.875	7800.0000	52.9	66.7	.205
PLR	.307	117	.077	.537	34.2000	47.1	33.3	.112
SII	.706	.114	.483	.929	59.1500	76.5	55.6	090

AUC=Area under curve**;** SE=Standard Error; MIN=Minimum;MAX=Maximum**;** NLR Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII Systemic inflammatory index

DISCUSSION

Cholecystitis may reflect a serious life-threatening situation. It's could be led to sepsis and death as well as there is a greater rate of postoperative morbidity and infectiouscomplications in patients with pathogenicbacteria in gallbladder bile than in patients with no bacterial growth or opportunistic bacteria. Therefor early identification and intervention of bacterial infection are of enormous significance.

In this study higher incidence of calculus cholecystitis was observed among female patients compared to male (21\8) patients with Odds ratio 1.4. Similar finding has been observed in prior study[19].Estrogen hormone, a sex hormone, is believed to stimulate thehydroxymethylglutaryl-coenzyme (HMG-CoA) reductaseleading to increased synthesis of cholesterol, andprogesterone is believed to reduce the gallbladder motilitypromoting bile stasis and concretion. Post-menopausalwomen who are prescribed hormone replacement therapy have also reported higher incidence of gallbladder calculi[1][20]

Concerning the age of the patients, higher frequency of patients with cholecystitis was observed in the age category 20-39 years (11/29, 37.93%). Comparable observations were documented in several previous studies in whichmaximum cholecystitis patients were between 30 to 39 years with a mean of41.70 +/- 14.64 years in one study [21] and 35 to 45 years inanother study[22] . The mean age of patients was 45.90 years [23]and 43.56years [24] in other studies.

One patient (3.4%) of 29 had acalculous cholecystitis while 28(96.5%) had calculous cholecystitis. However, most of the patients had multiple stones (25/29, 86.2%).This resultwas in agreement with other studies, which showed thathighernumber of cases were calculus[1][25].

The infection of cholecystitis could be caused by any type of bacteria either Gram positive or gram-negative bacteria. In thecurrent study, noticeable enrichment of Enterobacteriaceae was discovered by employing multiple technologies.Several genera of Enterobacteriaceae,including E. coli, Klebsiella pneumonia, Salmonella, were found to be the main pathogenic bacteria in bile fluid [26].The high rates of Gram-negative bacteria might possibly because of the intestinal origin of the bacteria as itemigrates from the intestine to the bile ducts, especiallywhen the gallbladder contains a stone. Furthermore, the useof multiple drugs and antibiotics may have an effect on theresistance of some bacterial species and their colonization[1]. In this study, the most common bacteria were E. coli 7(24.1%), followed by Klebsiella6 (20.6%). Parekh et al., has been documented that the most commonorganism isolated was E. coli(15.8%) followed by Pseudomonas(3.85%) and Klebsiella(2.56%)[26]. Similarly,Shrivastavaet al., has documented that the most bacterial isolates were E. coli(59%), followed byKlebsiella (25.6%)[27]. However,the cause behind the low rate of bacterial growth is that several of the identified bacteria were required special ingredients and their failure to survive and grow in the laboratory does not mean that they are eradicated from the infection site nor that they are of lower clinical relevance[11].

This study revealedthat culturing of bile specimen with conventional technique shows low rate of bacterial growth in comparisonto molecular technique. Similar study shows thatthe frequency of bacteria detected with PCRis much higher and differs strongly from theconventional culture[28] .This might possibly because of the presence of bacterialspp. that require substances which favoured the growth of them[29] .

Inflammation is the body's natural immune response to harmful stimuli like pathogens, injuries, cancers, and metabolic stress. The primary purpose of this inflammatory response is to quickly address and manage any threats to survival and also working to restore a balanced state of health[17]. Leukocytes and CRP are increasingly used to measure the severity of acute cholecystitis. This study revealed elevation of CRP in pathogenic group rather than non-pathogenic group. Similar finding was documented by Escadafalet al(2020)[30].The performance of CRP for distinguishing bacterial from non- bacterial infections varied considerably across studies with areas under the receiver operating curve (AUC) ranging from 0.62 to 0.91. this study showed acceptable ability of CRP in distinguishing infection with pathogenic from non-pathogenic

bacteria according to molecular technique whereas lower ability was found according to conventional culture technique. The high variability in performance across studies may be due to a number of factors, including differences in the clinical presentation of the population studied, the degree of patient severity, the definition used for bacterial infections (the gold standard used), geographical location of the study, the age of the patients, the specific bacterial pathogens causing infection, concomitant infections and presence of other conditions causing elevated CRP[30].The NLRis assumed to play a major role in inflammation, NLR could be used as indicator of systemic inflammation[4].Turhan et al.(2022),found a significant differencein NLR and PLR levels in association with elevated complication risk in cholecystitis. These new markers are cost-effective and efficient at detecting inflammation; however, they cannot assess the clinical severity of the disease[17]. Also, a recent published study indicated that the levels of NLR and PLR are linked to the diagnosis of acute cholecystitis[31]. Our study's finding is that higher NLR,PLR and SII levels were associated with patients infected with pathogenic bacteria and that the PLR had the highest discriminatory ability. This analysis showed that these rates provide valuable insights that can be used to predict gallbladder infection in patients who have been definitively diagnosed with acute or chronic cholecystitis. This, in turn, aids in preventing morbidity and mortality through timely intervention with appropriate antibiotic.

CONCLUSION

Culture methods may have lower sensitivity than Molecular technique for diagnosing microbiological causes of cholecystitis, which could lead to missed pathogens. CRP and WBC values may be useful in assessing the infection, Inflammatory markers could be used to predict gallbladder infection in patients with cholecystitis which may leads to prevent morbidity and mortality.

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