



Molecular characterization of extended spectrum β -Lactamases (ESBLs) producing *Escherichia coli* isolated from Cholecystitis

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Abstract

Objectives: Cholecystitis is one of the most widespread bacterial infections with a global extension. These infections are predominantly caused by *Escherichia coli*. The purpose of the study was conducted to isolate and identify bacterial agents for gallbladder patients and identify the frequency of ESBLs genes among *E. coli* isolates obtained from patients with significance bacterial infection.

Method: Eighty isolates were recognized as the significant bacterial infection and diagnosed by growth of at least 10^5 colony forming units (CFU)/ml of pathogens in a culture. The isolates were identified by traditional biochemical tests, and confirmed by the API 20E system. Additionally, identification of *E. coli* was confirmed by PCR technique and sequencing. The susceptibility of bacterial isolates to commonly used antibiotics was performed by disk diffusion. Extend spectrum beta-lactamase was evaluated by double disc synergy test. The Molecular techniques were used to detect the most common four kinds of ESBLs; TEM, SHV, CTX-M, and OXA. Extend spectrum beta-lactamase was evaluated by double disc synergy test.

Results: A total of 80 specimens were collected, of which 69.0% were female and 31.3 % were from males, age ranged 25-65 years. Significant bacterial infection was found in 80 (63.0%) specimens, however the frequency was significantly higher in female than in males (female: 80%; male: 20%) in all age groups. The results of the current study showed that 50 (63%) specimens gave bacterial growth, which are divided to Gram negative bacteria (82%) and Gram positive bacteria (18%). *E. coli* was the most common (36.3%) followed by *Klebsiella pneumoniae* (7.5%), methicillin resistant *Staphylococcus aureus*, MRSA (6.3%), *Enterobacter cloacae* (5.0%), *Enterococci faecalis* (5.0%) and *Salmonella typhi* (2.5%). The isolates appeared high frequency of the resistance to the majority of antibiotics tested (the rates of resistance varied from 9% to 100%), the most effective antibiotic were amikacin and imipenem (86% and 100% susceptible). All 29 *E. coli* isolates were studied, of which 22 (75.9%) exhibited potential ESBL production. All ESBL producing isolates carried at least one *bla* gene; 19 (86.3%) isolates carried *bla*_{CTX-M}, 13 (59.0%) isolates carried *bla*_{SHV}, 8(36.3%) isolates carried *bla*_{OXA} genes and 18 (82.0%) isolates carried *bla*_{TEM} gene.

Conclusions: A higher occurrence of ESBL is producing *E. coli* was observed warranting prompt the need of surveillance for effective management of such MDR strains. Although, MDR rates are high and therapeutic options are limited, some therapeutic options remain for *E. coli* in Al-Najaf such as amikacin and imipenem. DNA Sequencing for these isolates and using phylogenetic tree to known substitution for bacterial, gene bank, and document.

Keywords: ESBL, cholecystitis *E. coli*, PCR technique, Sequencing and Phylogenetic tree analysis

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INTRODUCTION

Cholecystitis can be defined as inflammation of the gallbladder wall. This is primarily caused by the obstruction of the biliary tract due to the presence of gallstones, which is one of the most frequent abdominal emergencies (Endo *et al.* 2017). Most acute calculous

cholecystitis is caused by Gram-negative bacteria, being *E. coli*, *K. pneumoniae*, *Enterobacter spp.* and *S. typhi*

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Table 1. Programs of monoplex PCR thermocycling conditions

Gene name	Temperature (°C)/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
16S rRNA	95/3 min	95/30 sec	60/1 min	72/1 min	72/5 min	30
<i>bla</i> _{TEM}	95/3 min	95/30 sec	50/1 min	72/1 min	72/5 min	35
<i>bla</i> _{SHV}	95/3 min	95/30 sec	56/1 min	72/1 min	72/5 min	30
<i>bla</i> _{CTX-M}	95/3 min	95/30 sec	58/1 min	72/1 min	72/5 min	35
<i>bla</i> _{OXA}	95/3 min	95/30 sec	56/15 sec	72/40 sec	72/5 min	30

the most commonly isolated. Other bacteria involved are *P. aeruginosa*, methicillin resistant *Staphylococcus aureus* (MRSA) and *Enterococcus* spp. (Sartelli et al. 2016). The remaining a calculous cholecystitis is generally associated with trauma, parenteral nutrition or sepsis (Walsh et al. 2018). Acute cholecystitis presents in a variety of ways which vary from mild cases to severe cases complicated by peritonitis and sepsis that require emergency correct and effective use of empirical antimicrobial therapy (Sartelli et al. 2016). An ineffective therapy associated with increased mortality in patients and led to three-fold increase in bacterial resistances Adequate empirical antimicrobial therapy is fundamental to reduce bacterial resistance and to improve treatment outcomes (Coccolini et al. 2015). The importance of resistance to antimicrobial drugs continues to evolve periodically, influencing the assessment and treatment of infections in nosocomial and health care–relating condition in the community. The ESBLs are a group of enzymes with the ability to hydrolyze and cause resistance to the oxyimino-cephalosporins (e.g. cefotaxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam), but not the cephamycins or carbapenems and they can be inactivated by clavulanic acid (Bradford 2005, CLSI 2018, EUCAST 2016). Besides, ESBL producing organism's demonstration co-resistance to many other types of antibiotics resulting in harassment of therapeutic options. Resistance genes are disseminated by plasmids or by transposons and can be integrated into DNA elements designated integrons (Cambray et al. 2011). Reports of ESBLs became increasingly frequent, and the most common ESBLs that have emerged recently are CTX-M types (Al-Sehlawi 2012, Bert et al. 2012, Gulamber et al. 2012, Yasmin 2012, Hadi 2015).

MATERIALS AND METHODS

Patients and Bacterial Isolates

A cross-sectional study was directed at the Hospital of Surgery of the digestive system and education in the holy city of Najaf- Iraq, during the study period extending from first October 2017 to December 2018. 80 clinical specimens comprising; fine needle aspiration from tissue, Swab technique for gall bladder wall or bile fluid, and were collected from patient attending to Two hospitals in Al-Najaf; Al-sadder medical city, and Al-Manathirah hospital was included in this study. Standard microbiology techniques were used for isolation and

identification of organisms. The bacterial isolates were confirmed by the API 20E system. Additionally, identification of *E. coli* was confirmed by PCR technique and sequencing.

Antibiotic Susceptibility Pattern

Disk-diffusion tests were carried out with antibiotic containing disks (Bioanalyse) on Mueller-Hinton agar plate (Himedia, India). The results were expressed as susceptible or resistant according to the criteria recommended by (CLSI 2018). The following antimicrobial agents were tested: Ampicillin (10µg), piperacillin (25µg), Amoxicillin-clavulanic acid (30 µg), Cefazidime (30 µg), Cefotaxime (30 µg), Ceftriaxone (30µg), Cefoxitin (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Meropenem (30 µg), Amikacin (30µg), Gentamicin (10µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Tetracycline (30µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Phenotypic Screening for ESBL

Screening of reduced susceptibility to third generation cephalosporins was carried out using Cefotaxime (CTX) and ceftazidime (CAZ) discs and double-disk synergy (DDS) method was used to confirm the presence of ESBLs as recommended by the Clinical and Laboratory Standards Institute (CLSI 2018).

PCR Amplification for Detection of β-Lactamase Genes

Genomic DNA was extracted using a commercial extraction system (Genomic DNA promega Kit). The Genomic DNA extracted from *E.coli* isolates were subjected to 16S rRNA gene and all isolates were screened for the resistance genes CTX-M, TEM, SHV, and OXA by a Monoplex PCR assay using universal primers. Nucleotide sequences of PCR primers used to amplify 16S rRNA and four ESBL genes as previously described by (Dallenne et al. 2010, Hujer et al. 2006, Rasheed et al. 1997, Saladin et al. 2002, Shahi et al. 2013). The protocol used depending on KAPA Biosystem manufacturer's instruction.

RESULTS

In the present study, Out of 50 specimens: 29 isolates of *E.coli* and 31 isolate other bacteria such as (*K. pneumoniae*, *Enterobacter* spp., *S. Typhi*, *MRSA*, and *Enterococci* spp.). 50 positive (62.5%) (50 FNA and 33 gallbladder wall or bile fluid). In Eighty patients, 25 (31%) were male and 55 (69%) were female. A Female was found to be more infected than males. The patient's

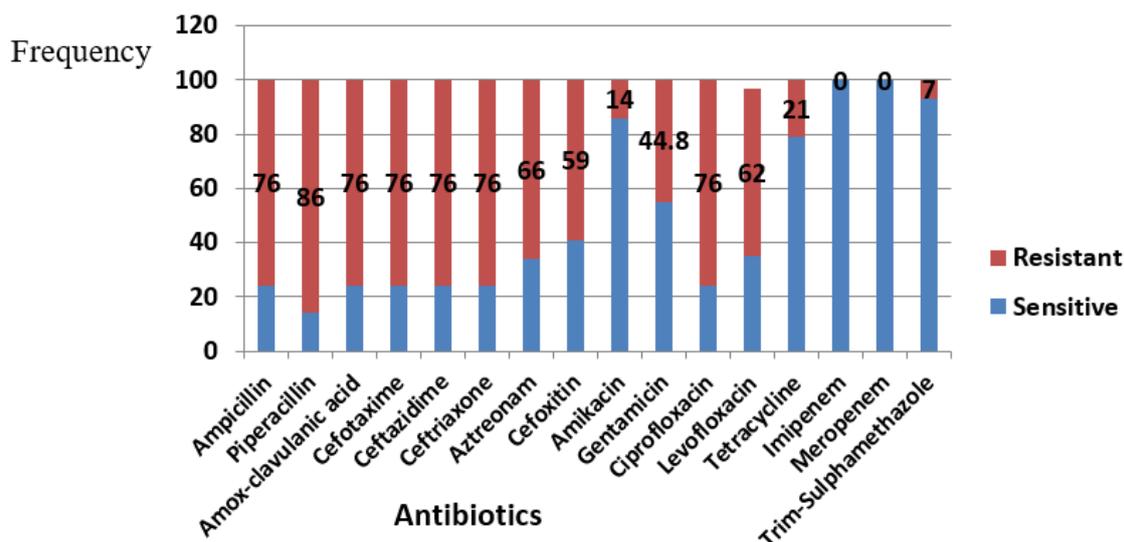


Fig. 1. Sensitivity test for *E. coli*

age ranged from (25-65) years old. The age distribution of the patients. The Patient's age was categorized into four age groups: < 20-35, 36-45, 46-55, and 56-65 years. The Mean \pm SD (42.95 \pm 8.15). The highest incidence was among the (25-35), (36-45) and (46-55) age group (20%), (41.25%) and (27.5%) respectively.

All the 29 *E. coli* isolates obtained from patients with highly significant isolates were tested for their antibiotic susceptibility against the selected 16 antibiotics. Fig. 1 gives the resistance, and susceptibility of the isolated *E. coli* to different antibiotics as represented by the diameter in mm. Aminopenicillin including ampicillin showed a resistance of 90% from all the isolates. The distribution of amoxicillin-clavulanic acid resistance among the isolated *E. coli* showed a high frequency of resistance (76%), and 24% were susceptible. The resistance against third generation cephalosporins was as follows: ceftazidime (76%), cefotaxime (75, 8%), and ceftriaxone (76%), respectively. Beside this diverse resistance to cephalosporins, most of the isolates were resistant to ceftazidime (59%). Of the *E. coli* isolates tested for monobactams (aztreonam), most isolates (66%) were susceptible, while nonsusceptible (34%). The most effective β -lactam antibiotic was imipenem and meropenem; all the isolates were found to be susceptible to this antibiotic. The aminoglycosides resistance rate among the tested *E. coli* isolates ranged from 44.8%-14%, present study showed that amikacin was the most potent aminoglycoside its overall potency over the isolated *E. coli* was 14%, while gentamicin was 44.2%. The isolates exhibited resistances to tetracycline (21%), Trimethoprim- Sulphamethazole (7%) respectively. The quinolones class of antibiotics demonstrated a high frequency of resistance as well, represented by ciprofloxacin (76%) and levofloxacin (62 %).

Table 2. Frequency of TEM, SHV, OXA, and CTX-M Consensus β -lactamases in β -lactamase producing- *E. coli*.

Type of isolate	No. of isolates	Type of β -lactamase enzyme			
		TEM	SHV	CTX-M	OXA
<i>Escherichia coli</i>	22	18	8	19	17
Total	22	82%	36.6%	86.0%	77.2%

Molecular Detection of ESBL Genes

All *E. coli* isolates were further investigated to determine the occurrence and types of extended-spectrum β -lactamases (ESBLs). ESBL production was tested phenotypically by the disk approximation method. However, only the genes in the families of CTX-M, SHV, TEM and OXA were examined in the present study. Detection of these genes (*bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, and *bla*_{OXA}) was performed by PCR technique. The results revealed that out of the 22 *E. coli* isolates, (95.4%) isolates were carrying at least one type of *bla* genes (Table 2). However, the most commonly identified ESBL gene was *bla*_{CTX-M} type in 19 (86.3%) of the tested isolates. PCR amplification using specific primers of *bla*_{TEM} gene showed the gene was predominant in 18 (82%) of analyzing isolates. Occurrence of the *bla*_{SHV} gene was detected in 8 (36.6%) of the tested isolates. Finally, only 17 (77.2%) of the examined isolates harbor a gene for the OXA type enzyme. In addition, the results revealed that one bacterial isolate was carried only one type of β -lactamase genes (*bla*_{SHV}). While, two of the isolates had a combination of two genes, eleven of isolates had a combination of three genes and six of isolates carried a combination of four genes (Table 2).

DNA Sequencing Results

DNA sequencing analysis results analysis for 16SrRNA gene *E. coli* isolates was showing a genetic relationship to NCBI BLAST *Escherichia coli* isolate (MH127501.1). The local *Escherichia coli* gall bladder isolates were shown closed related to NCBI-Blast

Statistical Analysis

One way ANOVA test was applied to determine the statistical significance of the data. *P* value of < 0.05 was considered significant.

DISCUSSION

The biliary tract is normally sterile, but bile-tolerant bacteria are frequently isolated from patients with cholecystitis. The present study has shown *E. coli* isolates were most common pathogens isolated with patients suffering from Cholecystitis in the study. This finding similar to with that reported by who showed that *E. coli* were the most common organism isolated from patients with Acute Cholecystitis (18.6%). Of the total positive (50 cultures), 100% reported a single microbiological agent, and protected Specimen from contamination gallbladder wall (mix microorganism, and surgical surface contamination). Second, the FNA assures that bacteria are from the tissue, not a bile source, while 32.75% showed polymicrobial activity. Also, due to bile salts are antibacterial compounds that disrupt bacterial membranes, denature proteins, chelate iron and calcium, cause oxidative damage to DNA, and control the expression of eukaryotic genes involved in host defense and immunity. Bacterial species adapted to the mammalian gut are able to endure the antibacterial activities of bile salts by multiple physiological adjustments that include remodeling of the cell envelope and activation of efflux systems and stress responses. Resistance to bile salts permits that certain bile-resistant pathogens can colonize the hepatobiliary tract, and an outstanding example is the chronic infection of the gall bladder by *Salmonella enterica* (Cueto-Ramos *et al.* 2017, Urdaneta and Casadesús 2017).

In recent years, antibiotic-resistant bacteria, including ESBLs gene are of concern in hospitalized patients worldwide. In Bacteria isolates, β -lactamases remain the most important Contributing factor to β -lactam resistance and their increasing prevalence and evolution represent a serious challenge for clinical microbiology laboratories. In addition to increasing resistance to β -lactam antibiotics, resistance to other commonly used antibiotics is increasing. The present study showed decreasing in resistance to amikacin, tetracycline, and imipenem, while, show rising at the rate of resistance to a third generation cephalosporin class represented by ceftriaxone, ceftazidime, cefotaxime in addition to monobactams class represented by aztreonam compared with these previously recorded by Al-Hilali (2015) in Najaf. The significant increasing among our community isolates mostly because, they are inexpensive and can be acquired easily without a

medical prescription, resistance is probably due to haphazard antibiotics usage, which could result in plasmid-mediated antibiotic resistance found to be common in *E. coli* (Taneja *et al.*, 2008). The present result is congruent with the results reported in Nigeria Mobaleghi *et al.*, 2012), who found more than 90% resistance of their *E. coli* isolates to ampicillin. On the other hand, most countries reported resistance to aminopenicillins in 50% to 66.5% of *E. coli* with Austria and Estonia decreasing from already low trends (Gonsalves, 2011). The reason of ampicillin resistance to *E. coli* isolates is may be due to the production of TEM and / or SHV β -lactamase, be genetically localized on the chromosome or on a plasmid. This result is similar to TEM, SHV, OXA and CTX-M enzymes have been reported as the most frequent β -lactamase found in ampicillin-resistant *E. coli* (Al-Hilali 2015, Fayroz-Ali 2012, Hadi 2015).

Resistance antibiotic may evolve through mutations in chromosomal DNA or rapid acquisition of mobile genetic elements carrying resistance determinants (Mokracka *et al.* 2012). In Enterobacteriaceae such as *E. coli*, resistance is mostly achieved by horizontal transfer of antibiotic resistance genes are often located on mobile genetic elements such as transposons, plasmids, and integrons (Carattoli *et al.* 2009). The presence of ESBL positive *E. coli* was of great concern as ESBLs mediated the hydrolysis and breakdown of a diversity of β -lactams and oxyimino-cephalosporins (King-sun *et al.* 2007). Therefore, in this part of the study attempting to find out the presence of genes encodes enzymes responsible for resistance to many β -lactam antibiotics.

DNA sequencing was performed for the study of confirmative identification of bacterial isolates based on multiple sequence alignment analysis and phylogenetic tree relationship analysis study of local bacterial isolates with NCBI-Gen bank Global isolates and determination of genetic changes based on single nucleotide substitution analysis, as well as submission the local isolates in NCBI-GenBank database.

CONCLUSION

The most common bacteria of symptomatic cholelithiasis isolated were *E. coli* followed by *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Salmonella typhi* and MRSA and *Enterococci faecalis*. Isolates are multi-drug resistant and pose a challenge to antibiotic therapy. Amikacin, ceftazidime, imipenem, and meropenem may be the effective choice of drugs in case of resistance to β -lactams and fluoroquinolones class of antibiotics.

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