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Estimation of β-Lactamase Phenotypically and Genotypically in ESKAPE Group Isolates from Ulcers Infection in Al-Najaf-Iraq

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Abstract
Jawad HA, Motaweq ZY, Ibrahim SH. 2022. Estimation of β- lactamase phenotypically and genotypically in ESKAPE group isolates from ulcers infections in Al-Najaf-Iraq. Colonizing the ulcers by potentially ESKAPE group bacteria constitutes the threat of their transmission to various human tissues and organs. Thus, there is an increased risk of developing general infections, which would be particularly serious for persons with impaired immunity; such a health risk should be considered. The bacteriological methods, including colonial morphology, Gram stain, and other biochemical tests, first made identification of bacterial isolates. Identification of all suspected bacterial isolates confirmed by the automated vitek-2 compact system using GP and GN-ID cards. The ESKAPE group test's antimicrobial susceptibility results revealed that Enterobacter cloacae, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* have great resistance to the most common antibiotics used in hospitals. All of the isolates, 14 *K. pneumoniae* and 12 *E. cloacae* (100%), were able to grow normally in the presence of ampicillin and amoxicillin and then considered as β-lactam resistant isolates. The result revealed that out of 14 β-lactam resistance *K. pneumonia* isolates only, 13 isolates (92.85%) gave positive results with the direct iodine method. In contrast, β-lactam resistance 5 *E. cloacae*, 1 *Acinetobacter baumannii*, 1 *Enterococcus faecium*, 8 *Staphylococcus aureus* gave 100% positive results. Using Polymerase Chain Reaction (PCR) technique, the genotyping method was used to detect the β-lactamase enzyme (bla_{SHV}, bla_{GES}) using Polymerase Chain Reaction (PCR) technique. Molecular amplification of bla_{SHV} and bla_{GES} genes by using a specific primer for ESKAPE group isolates revealed a positive result for revealed that out of 4 *E. cloacae*, 6 *S. aureus* isolates, 6 *K. pneumoniae*, 6 *P. aeruginosa*, *E. faecium* and 1 *A. baumannii* in this study were given 4(100%), 0 (0%), 3(50%), 5(83.33%), 0(0%), 0(0%) for the bla_{SHV} gene. In contrast, all 100% of ESKAPE group isolates gave negative results for the bla_{GES} gene.

Keywords: β-Lactamase, Bla_{GES}, Bla_{SHV}, ESKAPE Group, Ulcer Infections

Introduction

ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) are globally the leading cause of severe healthcare-acquired infections in hospital settings with limited or no antimicrobial treatment options due to Antimicrobial Resistance (Bhagirath *et al*. 2019; Hayati *et al*. 2022) [4, 20]. This acronym is derived from their ability to "escape" from antimicrobial therapy. Also, this acronym has been further modified as ESCAPE pathogens, with the 'C' referring to *Clostridium difficile* and 'E' for all Enterobacteriaceae, such as *E. coli*, *Proteus* spp., and *Enterobacter* spp. (Peterson 2009) [29].

Virulence factors of nosocomial ESKAPE bacteria represent resistance, pathogenesis, and disease transmission paradigms. There is a range of antimicrobial resistance mechanisms used by the nosocomial ESKAPE pathogens, such as enzymatic inactivation, modification of drug targets, changing cell permeability through porin loss or increase in expression of efflux pumps, and mechanical protection provided by biofilm formation (Santajit and Indrawattana 2016) [32]. Infected and colonized hospital patients can disseminate ESKAPE or other drug-resistant bacteria through their excreta, usually together with active antimicrobial compounds; thus, hospital effluents constitute the ideal pool for exchanging resistance genes between clinical and environmental bacteria (Kamaruzzaman *et al*. 2019) [24].

ESKAPE pathogens have developed resistance mechanisms against oxazolidinones, lipopeptides, macrolides, fluoroquinolones, tetracyclines, β-lactams, β-lactam-β lactamase inhibitor combinations, and antibiotics that are the last line of defence, including carbapenems, glycopeptides, and clinically unfavourable polymyxins. Comparatively, resistance to

lipoglycopeptides is rare and has only recently been documented (Kussmann *et al*. 2018) [25].

Antimicrobial resistance genes may be carried on the bacterial chromosome, plasmid, or transposons (Giedraitienė *et al*. 2011) [18]. Mechanisms of drug resistance fall into several broad categories, including drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability resulting in reduced intracellular drug accumulation, and biofilm formation (Wright 2005) [43].

Mechanisms facilitating antimicrobial resistance in ESKAPE pathogens can be broadly categorized into four groups: (i) enzyme-mediated antimicrobial inactivation, which either irreversibly destroys the active antibiotic site (e.g., hydrolytic cleavage of the β-lactam ring by β-lactamases) or covalently modifies key structural elements of the drug to hinder the bacterial target site interaction (e.g., aminoglycoside modifying enzymes that catalyse hydroxyl/amino group modifications); (ii) bacterial target site modification, which prevents the binding or which reduces the affinity of the antibiotic molecule at the cell surface (e.g., LPS modification, PBP2a expression with reduced β-lactam affinity, and van gene cluster-mediated peptidoglycan modification) or intracellularly (e.g., 16S RNA methylation); (iii) reduced antibiotic accumulation through the mutation or loss of outer membrane channels (e.g., OprD in *P. aeruginosa*, CarO in *A. baumannii*, and OmpK36 in *K. pneumoniae*) and expression of efflux systems to actively extrude drugs out of the cell (e.g., RND, MFS, MATE, SMR, ABC, and PACE); and (iv) persistence through biofilm-embedded cells which demonstrate a markedly higher tolerance to antimicrobial agents than planktonic bacteria (De Oliveira *et al*. 2020) [12].

β-lactamase enzymes were first identified soon after penicillin's initial discovery and purification. Since then, 2,600 unique β-lactamases enabling resistance to one or more β-lactams (i.e., penicillins, cephalosporins, monobactams, and carbapenems) have been described (Naas *et al*. 2017) [28]. β-lactamases remain the most important resistance mechanism among Gram-negative ESKAPE pathogens, where they are concentrated within the periplasm, thus hydrolyzing the β-lactam agents before reaching the penicillin-binding protein (PBP) target in the cell wall. In addition, β-lactamase hydrolyzes the β-lactam core, which is essential for antibiotic action via two molecular mechanisms: hydrolysis of enzymes that utilize an active site serine residue (class A, C, and D) or Zn2+ atoms (class B) to capture the antibiotic (Tehrani and Martin 2018) [39]. This study was suggested and designed to study the phenotypic and genotypic properties of the β-lactamase enzyme of ESKAPE Group isolated from different ulcer types disease as well as the antibiotic-resistant for certain types of antibiotics, especially to β-lactam.

Material and Methods

The research was carried out at the Bacteriology and Molecular Laboratories, Department of Biology, Faculty of Sciences, Kufa University, Iraq.

Clinical Specimens and Patients

During the study, 104 specimens were taken from patients suffering from different types of ulcer infections at AL-Sadder Medical City/Al-Najaf-Iraq for three months, from September 2021 to November 2021. The bacteriological methods, including colonial morphology, Gram stain, and other biochemical tests, first made identification of bacterial isolates. Identification of all suspected bacterial isolates confirmed by the automated vitek-2 compact system using GP and GN-ID cards which contained 45 biochemical tests and one negative control. Exactly 164 isolates performed identification, and some of these isolates confirmed the identification by the vitek-2 system by using kit GP-ID cards to Gram-positive bacteria and GN-ID cards to Gram-negative bacteria (appendix1), with ID message confidence levels ranging between very good to excellent (Probability percentage from 95 to 99).

Phenotypic Detection of β-Lactamase Production Isolates (Direct Iodine Method)

Detection by Rapid Iodometric Method Several colonies of a young bacterial culture on MacConkey agar was transferred to an Eppendorf tube containing 100 µL of penicillin G solution. The tubes were incubated at 37°C for 30 minutes. Then, 50 µL of the starch solution was added and mixed well with the content of the tube, and 20 µL of iodine solution was added to the tube, which caused the appearance of dark color, and rapid change of this color to white (within few second to 2 minutes) indicated a positive result (Collee *et al*. 1996) [10].

Extended-Spectrum β-Lactamase Production

Initial screening for ESBL production

All bacterial isolates β-lactamase producing were tested for ESBL production by initial screen test. The isolate would be considered a potential ESBL producer if the inhibition zone of ceftazidime disks was 30 mg ≤22 mm (CLSI 2021) [9].

Confirmatory Test for ESBL Production

All the β-lactamase producing isolates were also tested for confirmatory ESBL production by Two methods these tests were included:

Disk combination test

Using the disk diffusion approach, the phenotypic validation of probable ESBL-producing isolates was carried out. In addition, we examined the effectiveness of ceftriaxone and ceftazidime both alone and in combination with Tazobactam and clavulanic acid. An ESBL-producing isolate is confirmed by a rise in the inhibition zone for an antibiotic when tested with Tazobactam and clavulanic acid of around 5 mm in diameter (CLSI 2021) [9].

Disk Approximation Test

All β-lactamase producing isolates were tested according to Batchoun *et al*. (2009). Antibiotic disks of cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), and aztreonam (30 µg) were placed 15 mm (edge to edge) around a central disk of amoxiclav (20 µg amoxicillin plus 10 µg clavulanate) on Muller-Hinton agar plates seeded with organism being tested for ESBL production. Plates were incubated aerobically at 37°C for 24 hrs. Any augmentation (increase in diameter of inhibition zone) between the central amoxiclav disk and any of the β-lactam antibiotic disks showing resistance or intermediate susceptibility was recorded, and the organism was thus considered an ESBL producer.

Purification and Extraction of DNA

The boiling procedure was used to extract the DNA of isolates from the ESKAPE group. In a nutshell, the colonies were suspended in 100 µL of sterile distilled water, boiled in a water bath for 15 minutes at 100°C, quickly chilled for an hour at -20°C, centrifuged, and the supernatant was saved for use in the amplification procedures (Shah *et al*. 2017) [33].

Polymerase Chain Reaction (PCR) Assay

A monoplex PCR experiment was used to amplify various segments of the genes under investigation for detecting 2 β-lactamase production genes from each type, and were selected to be amplified separately in the monoplex PCR technique used in this study (Table 1).

Five µL of master mix, 5 µL of template DNA mixed with 2.5 µL each set of primers in a suitable PCR tube, the rest of the total volume was attained to 25 µL by sterile nuclease-free water, the mixture overtaxing well. The PCR for the bla_{SHV} and bla_{GES} genes were planned to include programs in Table 2.

Then, all PCR products were examined using 1% agarose gel electrophoresis and 3 µL of ethidium bromide dye. The cover on the electrophoresis tank was closed, and the electric current was matched (80 volts for 1.5 h). Finally, the gel documentation system was used to identify the electrophoresis data.

Results and Discussion

ESKAPE Group Isolation and Identification in Ulcer Infections

The results showed that 88/104 (84.6%) specimens revealed positive culture on MacConkey agar and/or blood agar, while 16 (15.4%) specimens showed no growth on blood agar and McConkey agar. The culture results showed that 135/15 isolates belonged to Gram-negative bacteria and 17/135 isolates belonged to Gram-positive bacteria (Fig 1). The culture results showed that 44 samples are mixed growth; on blood, agar showed 15/17 isolates belonging to *S. aureus*. Opportunistic and pathogenic Gram-negative bacteria strains isolated from the patients suffering bed ulcer, varicose dermatitis, and diabetic foot ulcers infections are *E. coli* showed that 34, *P. aeruginosa* reached 24, *K. pneumoniae* showed that 24, *A. baumannii* showed that 2, *Burkholderia* showed that 30, *E. faecium* showed that 2, *Providencia regretti* showed that 2, *Proteus* showed that 8, *E. cloacae* showed that 11. Due to biofilm development, which gives the pathogen long-term survival benefits and successfully thwarts elimination by the host immune system or antimicrobial medication therapy, this infection is well adapted to hospital surroundings (Groenewold *et al*. 2018) [19].

The *K. pneumoniae* is an opportunistic pathogen that strikes vulnerable and hospitalized individuals with dangerous infections such as pneumonia, burn infections, soft tissue infections, and urinary tract infections. Numerous virulence factors exist, including a capsule that promotes colonization and confers phagocytosis resistance on the pathogen (Hussein 2009; Riquelme *et al*. 2018) [21, 30]. The pathogen that causes the illness may be anaerobic Gram-negative or/and positive bacteria or other atypical etiological agents, such as viruses, fungi, parasites, etc., that require special media, environmental conditions, and growth techniques, according to the negative results of specimens cultured on MacConkey agar and blood agar features (Jain and Barman 2017) [23].

Phenotypic Detection of β-Lactamase (Direct Iodine Method)

The direct iodine method detected β-lactamase production in β-lactam resistance 78 ESKAPE isolates. The result in Table 3 revealed that out of 24 β-lactam resistance *K. pneumoniae* isolates only, 21 isolates (92.85%) gave positive results with the direct iodine method, while β-lactam resistance 11 *E. cloacae*, 2 *A. baumannii*, 2 *E. faecium*, 15 *S. aureus* gave (100%) positive results. This result indicated that enzymatic resistance was not prevalent among isolates. However, 2 (7.14%) and 6 (30.76%) were non-β-lactamase producers in this procedure in *K. pneumoniae* and *P. aeruginosa*, respectively.

Iodine reacts with starch to form dark blue complex, which stays without change in the absence of the β-Lactamase enzyme, in the case of β-Lactamase-producing bacteria, the resulting penicilloic or cephalospoic acid will reduce iodine into iodide; consequently, decolonization of starch -iodine complex occurs (changing the color directly to white) if an isolate is a β-Lactamase producer but not if the enzyme is absent (Sykes and Mathew 1979).

The negative results may be because the β-lactamase in isolates required more time to destruct their cell wall and release. Additionally, factors such as temperature and pH may play an important role in enhancing or reducing enzyme activity (Hussein *et al*. 2013) [22]. On the other hand, the negative results may be due to the b-lactamase in ESKAPE isolates requiring more time to destruct their cell wall and release. However, an iodometric method is not recommended for the detection of b-lactamases in Gram-negative bacteria since these enzymes are produced and secreted extracellularly by Gram-positive, whereas Gram-negative accumulate in periplasmic space (MacFaddin 2000) [27].

Table 1: Primers (Macrogen, Europe)

Target gene	Sequence5`-3`	Amplicon size (bp)	Reference
bla_{GES}	F-AGTCGGCTAGACCGGAAAG	307	Dallene *et al*. (2010) [11]
	R-TTTGTCCGTGCTCAGGAT		
bla_{SHV}	F-GGCCGCGTAGGCATGATAGA	714	Ensor *et al*. (2009) [14]
	R-CCCGGCGATTTGCTGATTTC		

Table 2: Amplification conditions of genes were used by PCR reaction

Gene	Temperature (°C)/time					Cycles number
	Initial denaturation	Condition of one cycle			Final extension	
		Denaturation	Annealing	Extension		
bla_{SHV}	95 /3min	94 /30 sec	55/30 sec	72 /90sec	72 /5 min	30
bla_{GES}	94 /2 min	94 /2 min	68 /1 min	72 /1 min	72 /7 min	30

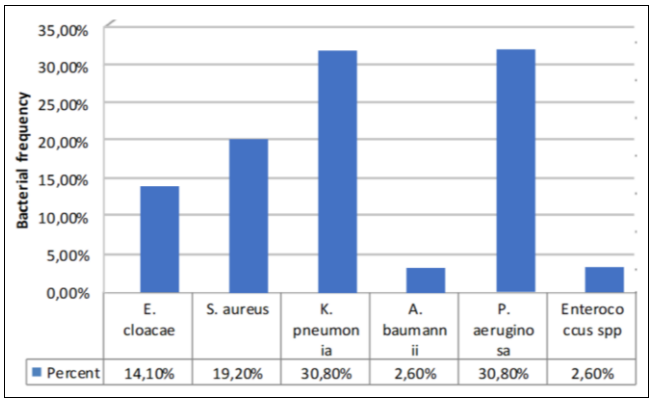

Fig 1: Prevalence of bacterial types in ESKAPE groups

Table 3: Production of β-lactamase by rapid iodometric method

ESKAPE isolate types	No.	No. (%) of positive	No. (%) of negative
K. pneumoniae	24	22 (92.85)	2 (7.14)
P. aeruginosa	24	18 (69.23)	6 (30.76)
E. cloacae	11	11 (100)	0 (0)
S. aureus	15	15 (100)	0 (0)
E. faecium	2	2 (100)	0 (0)
A. baumannii	2	2 (100)	0 (0)

Extended-Spectrum β-Lactamase Production

Initial screening for ESBL production

All 78 bacterial isolates were grown on Muller-Hinton agar supplemented with ampicillin and amoxicillin to test them for β-lactam resistance (each alone). The results indicate that all of the isolates, 24 *K. pneumoniae*, 15 *S. aureus*, 11 *E. cloacae*, and 2 *E. faecium* (100%), were able to grow normally in the presence of these antibiotics and then considered as β-lactam resistant isolates but 20 (85.71%) *P. aeruginosa* growing on Muller-Hinton agar supplemented with ampicillin and amoxicillin) (only 4 (14.28%) did not grow in the presence of amoxicillin). However, all 24 (100%) *P. aeruginosa* growing in the presence of ampicillin, while 2 (100%) *A. baumannii* growing on Muller-Hinton agar supplemented with amoxicillin and 2 (100%) *A. baumannii* not growing on Muller-Hinton agar supplemented with ampicillin as shown in Table 4.

The high percentage may be due to patients' frequent use of β-lactam antibiotics. However, this result is higher than that of Al-Sehlawi (2012) [3], who found that 88% of clinical *K. pneumoniae* isolates were resistant to ampicillin and amoxicillin.

Gram-negative bacteria are intrinsically resistant to penicillin-G by virtue of their double membrane structure, which prevents the antibiotic from accessing the cell wall target. Moreover, the acquired resistance to β-lactams operates through different mechanisms; production of β-lactamases, changes in the outer membrane permeability, or alterations in the PBPs (Wilke *et al*. 2005) [41]. Reduced permeability through porin loss may reduce the steady state of periplasmic drug concentrations and thereby reduces PBP inactivation. Therefore, decreased permeability may act synergistically with the expression of β-lactamases or active efflux to confer higher levels of β-lactam resistance (Livermore and Woodford 2006) [26].

The development of antibiotic resistance is often related to the overuse and misuse of the antibiotic prescribed. One of the developing nations, Iraq, allows the sale of all antibiotics over the counter, which promotes self-medication. Ampicillin, amoxicillin, and other penicillin derivatives frequently prescribed for pediatric patients usually predispose them to antibiotics (Stock and Wiedemann 2001) [36]. However, β-lactam resistance, mostly associated with transmissible plasmids, can be transferred between different bacterial species among hospital isolates (Carattoli 2008) [8]. Current results agree with previous reports by Gamboa and Leong (2013) [16]. Also, Aljanaby and Alhasani (2016) [2] pointed out that *K. pneumoniae* isolates gave high resistance to ampicillin and amoxicillin.

Table 4: Detection of β-lactamase in the ESKAPE group using Amoxicillin and Ampicillin concentrations

ESKAPE group types	No. of isolates	Amoxicillin		Ampicillin	
		No. (%) of positive	No. (%) of negative	No. (%) of positive	No. (%) of negative
K. pneumoniae	24	24 (100)	0 (0)	24 (100)	0 (0)
P. aeruginosa	24	20 (84.61)	4 (15.38)	24 (100)	0 (0)
E. cloacae	11	11 (100)	0 (0)	11 (100)	0 (0)
S. aureus	15	15 (100)	0 (0)	15 (100)	0 (0)
A. baumannii	2	1 (50)	1 (50)	2 (100)	0 (0)
E. faecium	2	2 (100)	0 (0)	2 (100)	0 (0)

Confirmatory Detection of Extended-Spectrum β-Lactamases (ESBLs)

ESKAPE group isolates resistant to a β-lactam antibiotic are suspected to be high producers of ESBLs; therefore, all were subjected to an ESBLs production test. The performance of the test isolates in the ESBL initial screen disk test was assessed using ceftazidime disks. According to the CLSI (2021) [9], the isolate is considered to be a potential ESBL producer if the inhibition zone of ceftazidime disks (30 µg) was ≤ 22 mm. The study found that 13/24 (54.2%) isolates of *K. pneumoniae* were ESBL positive during the initial screening using a ceftazidime disk, and 12/24 (50%) isolates of *P. aeruginosa* and 5/11 (45.5%) *E. cloacae* and 2/2 (100%) *E. faecium* were ESBL positive during the initial screening using ceftazidime disk, which considered as suspected of ESBL producing studied isolates. and 0/8

(0%) *S. aureus* and 0/2 (0%) *A. baumannii* gave negative result for ESBL (Table 5).

The disk combination method performed the detection of ESBL production in studied isolates. This method compared ceftazidime and ceftriaxone disks to cefotaxime and aztreonam disks. The isolate was considered an ESBL producer when the inhibition zone of combined disks was more than or equal to 5 mm more than the disk's inhibition zone alone.

The results were as follows out of the 78 ESKAPE isolates β-lactamase producers, 19/24 (79.2%) *K. pneumoniae*, 20/24 (83.3%) *P. aeruginosa*, 3/15 (20%) *S. aureus*, 6/11 (54.5%) *E. cloacae*, 2/2 (100%) *E. faecium*, 0/2 (0%) *A. baumannii* exhibited zones enhancement with clavulanic acid, confirming their ESBL production.

Also, all isolates were further confirmed by the disk approximation method. In this method, augmentation of the inhibition zone between a 30 µg antibiotic disks (ceftazidime, ceftriaxone, cefotaxime, and aztreonam) toward amoxicillin-clavulanate disk (20/10 µg) was interpreted as synergy, indicating the presence of an ESBL, (Table 5).

The results of the screening test revealed that 9 (37.5%) *K. pneumoniae* isolates gave positive ESBLs production test, versus 3 (27.3%) *E. cloacae*, 9 (37.5%) *P. aeruginosa*, 1 (6.7%) *S. aureus*, 1 (50%) *A. baumannii* gave positive results since the inhibition zone of synergism has been recognized clearly.

Despite the discovery of ESBLs at least three decades ago, there remains a low level of awareness regarding their laboratory detection and clinical significance. Moreover, the failure to detect these enzymes has contributed to their uncontrolled spread and sometimes therapeutic failures (Yang and Zhang 2008) [44].

ESBL detection is not carried out in many microbiology units in developing countries, including Iraq, and this could be attributed to the lack of awareness and the lack of resources and facilities to conduct ESBL identification.

However, the standard Kirby-Bauer disk diffusion method did an initial screening for reduced susceptibility to third-generation cephalosporins and aztreonam. The isolate was considered positive for screening tests when the zone diameter of any of the indicators met the CLSI criteria (CLSI 2021) [9], and additional phenotypic tests are mandatory to ascertain the production of ESBL.

Over the past two decades, the dramatic escalation of antimicrobial resistance among Enterobacteriaceae worldwide reflects the emergence and widespread dissemination of novel extended-spectrum β-lactamases (ESBLs) (Salabi *et al*. 2013) [31]. Among Gram-negative bacteria, hydrolysis of the β-lactam ring by β-lactamase(s) is the most common mechanism of resistance to β-lactam antibiotics (Bush and Jacoby 2010) [5], but additional mechanisms (e.g., alterations in porin channels, efflux pumps) may contribute to or amplify resistance (Bush 2011; Cantón *et al*. 2012) [6, 7].

The results in partial agreement with the previous study of Shakib *et al*. (2018) [34], showed that 88.6% *of K. pneumoniae* isolates produced ESBL. Moreover, Ghasemi *et al*. (2013) [17] found that 60% of *K. pneumoniae* isolates ESBL producers in Shiraz, Iran. Also, agreement with Aljanaby and Alhasani (2016) [2] pointed out that the presumptive test for ESBL production was positive for 65.5%.

The identification of ESBL producers is a major challenge for the clinical microbiology laboratory, due to the affinity of ESBL-producing isolates to the different substrates is variable and makes their detection difficult. Additionally, some ESBL isolates may appear susceptible to third-generation cephalosporins *in vitro* (Aggarwal and Chaudhary 2004) [1].

However, not all of the screening-positive isolates in the present investigation were ESBL producers. As a result, aztreonam and third-generation cephalosporins may resist alternative mechanisms. In addition, a false negative result in the ESBL detection test could occur from clavulanate's ability to generate hyperproduction of the AmpC β-lactamase in organisms that also produce ESBL and AmpC. This hyperproduction would cause the third-generation cephalosporin to be hydrolyzed (Thomson 2010) [40].

There are a number of instances whereby the screening tests are positive, but the confirmatory tests are negative or indeterminate (Steward *et al*. 2001) [35]. However, the coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic challenges. The ability to detect and distinguish AmpC and ESBL-producing organisms has epidemiological significance and may also have therapeutic importance (Al-Sehlawi 2012) [3].

The CSLI (2021) recommends that for all confirmed ESBL-producing strains, the test results should be reported as resistant for all penicillins, cephalosporins, and aztreonam, regardless of the routine susceptibility test results. In contrast, β-lactam/β-lactamase inhibitor combinations (for example: piperacillin/tazobactam and amoxicillin/clavulanate) are reported as susceptible.

Molecular Detection of Some β-Lactamase Genes in the ESKAPE Group Isolates

All ESKAPE group 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 two genes in the families β-lactamase ($_{SHV}$ and $_{GES}$) were examined in the present study. Detection of these genes (bla_{SHV} and bla_{GES}) was performed by PCR technique.

The results revealed that out of 4 *E. cloacae*, 6 *S. aureus* isolates, 6 *K. pneumoniae*, 6 *P. aeruginosa*, 1 *E. faecium*, and 1 *A. baumannii* in this study were given 4 (100%), 0 (0%), 3(50%), 5(83.33%), 0(0%), 0(0%) for the *blaSHV* gene, respectively (Fig 2).

While all 100% of ESKAPE group isolates were gave negative results for bla_{GES} gene.

ESBLs among the isolates of *S. marcescens*, *Enterobacter* spp., and *C. freundii* have been described from several countries worldwide and have become more prevalent (Ferreira *et al*. 2011) [15]. Initially, the ESBLs among these species were typical TEM or SHV enzymes (Dhillon and Clark 2012) [13]. However, some risk factors for acquiring the ESBLs gene may be pressure from the surroundings by antimicrobial agents. Furthermore, selective pressure is created by using antibiotics as feed additives in animal farming and agriculture (Woodford *et al*. 2013) [42].

The results revealed that out of 12 *E. cloacae* isolates, 33.3% harbor the bla_{SHV} gene, similar results conducted by Szabó *et al*. (2005) [38] reported that 33.3% and 30.9% of *E. cloacae* carried bla_{SHV}, respectively. In Iraq, Aljanaby and

Alhasani (2016) [2] showed that *bla*$_{SHV}$ was detected in 87.5% of *K. pneumoniae* isolates.

Table 5: ESBLs producing in ESKAPE group isolates by confirmation methods

Species	No. of isolates	No. (%) of positive	No. (%) of negative
K. pneumoniae	24	16 (64.28)	8 (35.71)
P. aeruginosa	24	16 (69.23)	8 (30.76)
S. aureus	15	3 (12.5)	12 (87.5)
E. cloacae	11	7 (60)	5 (40)
A. baumannii	2	0 (0)	2 (100)
E. faecium	2	2 (100)	0 (0)

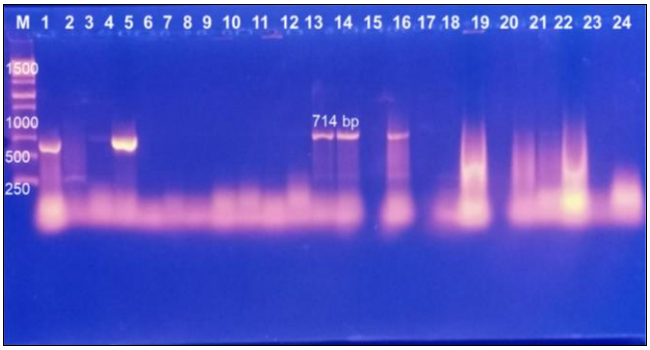

Fig 2: Gel electrophoresis of PCR amplified product of *bla*$_{SHV}$ gene primers with product 714 bp. Lanes 1-4 *E. cloacae*, 5-10 *S. aureus*, 11-16 *K. pneumoniae*, 17-22 *P. aeruginosa*, 23 *E. faecium* and 24 *A. baumannii* isolates. (M), DNA molecular size marker (2000-bp ladder)

The present study revealed that no *bla*$_{GES}$ gene was identified in all tested isolates, either due to the absence of the *bla*$_{GES}$ gene or the presence of another subtype of a gene that could not be targeted by the primer used in this study.

The main reason for the prevalence of β-Lactamases in Iraq may be due to the wide usage of certain third-generation cephalosporin antibiotics. A high prevalence of Enterobacteriaceae that generate ESBL is observed in Asia. The high rate of ESBL production in the developing world is certainly concerning; the lack of resources for efficient infection control and the restricted availability of efficient antibiotics certainly impact reducing the morbidity and mortality brought on by these infections.

In conclusion, regarding the important role of the ESKAPE group as a causative agent in nosocomial infections and the widespread presence of ESBL. *Bla*$_{SHV}$ group I was highly prevalent among ESKAPE group isolates compared with other *bla*$_{GES}$ groups. The study revealed that the PCR technique was more accurate than phenotypic methods for detecting β-lactamase production in ESKAPE isolates.

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