Study of Antibiotic Resistance in ESKAPE Bacteria Using β-lactamase and ESBL Genes

Hiba Ahmed Jawade [1], Zahraa Yosif Motaweq [2*], Hawraa Dheyaa Rasool [3], Fatima Hassan Hussain [1]

Abstract

Background: Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species, collectively known as ESKAPE bacteria, pose a significant threat in healthcare settings due to their increasing antibiotic resistance. Understanding the mechanisms underlying their resistance is crucial for developing effective therapeutic strategies. Methods: This study aimed to investigate the phenotypic and genotypic properties of β-lactamase enzymes in ESKAPE bacteria isolated from ulcer infections. Clinical specimens were collected from patients with various ulcer diseases, and bacterial isolates were identified using standard bacteriological methods and the Vitek-2 automated system. DNA extraction, polymerase chain reaction (PCR), and antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method were performed. The presence of specific β-lactamase genes (blaKPC, blaTEM, blaCTX-M, and blaAMPC) was examined through molecular techniques. Results: Among the 104 clinical specimens collected, 88% yielded positive cultures, with Gram-negative bacteria predominating. Antibiotic susceptibility testing revealed high resistance rates, particularly to β-lactam antibiotics, among ESKAPE isolates. Molecular analysis identified the presence of extended-spectrum β-lactamases (ESBLs) in all isolates, with blaKPC and blaTEM being the most prevalent β-lactamase genes. Notably, blaKPC was detected in 72% of E. cloacae, 13.3% of S. aureus, 33.33% of Klebsiella pneumoniae, 50% of Pseudomonas aeruginosa, 0% of E. faecium, and 100% of A. baumannii isolates. Similarly, blaCTX-M and blaAMPC genes showed distinct distribution patterns across the different species. Conclusion: The study highlights the widespread presence of ESBL-producing ESKAPE bacteria in ulcer infections and underscores the importance of molecular techniques for accurate detection of β-lactamase production.

Keywords: ESKAPE bacteria, Antibiotic resistance mechanisms, β-lactamases, ESBL genes (blaKPC, blaTEM, blaCTX-M, blaAMPC), Molecular identification

Significance | This study determined the resistance mechanisms of SKAPE group bacteria. Understanding this bacterial resistance mechanisms, particularly β-lactamase production, is crucial for effective treatment and infection control.

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Introduction

Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species—collectively known as ESKAPE bacteria—are significant contributors to healthcare-acquired infections globally due to their antibiotic resistance (Bhagirath et al., 2019; Hayati et al., 2022). Their ability to evade antimicrobial therapy poses a considerable challenge in treating such infections. These pathogens, characterized by their evasion mechanisms, have led to the term "ESKAPE," highlighting their ability to resist conventional treatment strategies. Additionally, the term has been

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expanded to include "ESCAPE" pathogens, incorporating other clinically relevant bacteria like Clostridium difficile (Peterson, 2009).

Antimicrobial resistance mechanisms in ESKAPE infections can be broadly categorized into four groups: (i) Enzyme-mediated antimicrobial inactivation, where enzymes such as β-lactamases irreversibly destroy antibiotic activity or modify key drug structures to prevent bacterial target site interaction; (ii) Modification of bacterial target sites, hindering antibiotic binding or reducing affinity at the cell level.

Surface alterations such as Van gene cluster-mediated peptidoglycan modification, decreased β-lactam affinity PBP2a expression, and LPS modification, along with internal alterations like methylation of 16S RNA, contribute to antibiotic resistance in ESKAPE bacteria (De Oliveira et al., 2020). Additionally, expression of efflux systems and mutation or loss of outer membrane channels, such as OprD in P. aeruginosa, CarO in A. baumannii, and OmpK36 in K. pneumoniae, can lead to decreased antibiotic accumulation. Moreover, biofilm-embedded cells exhibit better susceptibility to antimicrobial drugs compared to planktonic bacteria, facilitating persistence.

The discovery of penicillin was swiftly followed by the identification of β-lactamase enzymes. To date, over 2,600 different β-lactamases conferring resistance against various β-lactam antibiotics have been described (Naas et al., 2017). In Gram-negative ESKAPE bacteria, β-lactamases remain a primary resistance mechanism, predominantly located in the periplasm where they hydrolyze β-lactam agents before reaching their target, the penicillin-binding protein (PBP), within the cell wall. These enzymes employ different molecular mechanisms, including the hydrolysis of enzymes using Zn2+ atoms (class B) or a serine residue at the active site (class A, C, and D), to bind the antibiotic (Tehrani and Martin, 2018). This study aimed to investigate the phenotypic and genotypic properties of β-lactamase enzymes in ESKAPE Group bacteria isolated from various ulcer diseases, focusing on their resistance to specific antibiotic classes, particularly β-lactams.

Materials and method

Study Design

The study was conducted at the Department of Biology, Faculty of Sciences, Bacteriology, and Molecular Laboratories, Kufa University, Iraq. Over a three-month period from October 2023 to December 2023, 104 specimens were collected from patients at Asadder Medical City, Al-Najaf, Iraq, presenting with various types of ulcer infections. Bacteriological methods, including colony morphology assessment, Gram staining, and biochemical assays, were initially employed for bacterial isolation and identification. The automated Vitek-2 compact system, equipped with GP and GN-ID cards containing 45 biochemical assays and one negative control, was utilized to identify all potential bacterial isolates. A total of 164 isolates underwent identification, with some isolates utilizing GP-ID cards for Gram-positive bacteria and GN-ID cards for Gram-negative bacteria, ensuring confirmation by the Vitek-2 system with ID message confidence levels ranging from very good to excellent (probability percentage from 95 to 99).

The research conducted in this study was approved by the Asadder Medical City, Al-Najaf, Iraq ethics committee with approval number. All procedures involving human participants were performed in accordance with the ethical standards of the committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

DNA Purification and Extraction

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

Polymerase Chain Reaction (PCR)

A monoplex PCR approach was employed in Assay A to amplify specific segments of the target genes associated with β-lactamase production. Two β-lactamase genes from each type were selected for amplification individually using this technique (refer to Table 1). In each PCR reaction, 5 µL of master mix was combined with 5 µL of template DNA, along with 2.5 µL of each primer set in a suitable PCR tube. The total volume was adjusted to 25 µL using sterile nuclease-free water, and the mixture was thoroughly vortexed. PCR cycling conditions for amplifying the blaSHV and blaGES genes were as outlined in Table 2. Subsequently, all PCR products underwent analysis by 1% agarose gel electrophoresis supplemented with 3 µL of ethidium bromide dye. The electrophoresis tank was sealed, and an electric current was applied (80 volts for 1.5 hours). Finally, the gel documentation system was utilized to visualize and document the electrophoresis results.

Antibiotic SusceptibilityTest

In this study, a panel of twenty commonly used antibiotics was employed, including Ampicillin (10 µg), Ceftazidime (30 µg), Cefotaxime (10 µg), Azithromycin (15 µg), Ceftriaxone (30 µg), Cefepime (30 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (30 µg), Rifampin (5 µg), Trimethoprim-sulfamethoxazole (25 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Doxycycline (30 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Colistin (10 µg), Vancomycin (10 µg), and Tetracycline (30 µg). The antibiotic sensitivity testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates, following the protocol outlined by Morello et al. (2006).

In brief, the bacterial isolates were allowed to grow overnight at 37°C in Brain Heart Infusion (BHI) broth to achieve a turbidity standard equivalent to 0.5 McFarland. A volume of 0.1 ml of the bacterial suspension was spread uniformly on Mueller-Hinton agar plates to obtain a density of 1.5 × 10^8 CFU/ml before applying the antibiotic discs. After the discs were placed on the agar surface, the plates were allowed to stand for ten to fifteen minutes before being incubated at 37°C for twenty-four hours. The zones of inhibition around each antibiotic disc were measured in millimeters (mm) and interpreted as sensitive (S), intermediate (I), or resistant (R) according to established criteria, as provided by the Clinical and Laboratory Standards Institute (CLSI, 2023).

Results

Microbiological Analysis:

Upon culturing specimens from ulcer infections, it was found that 88% of the samples yielded positive cultures on MacConkey agar and/or blood agar, while 16% showed no growth on either medium. Gram-negative bacteria accounted for 135 out of 152 isolates (88.8%), with 17 of these isolates (11.2%) being Gram-positive bacteria. Additionally, 44 samples exhibited mixed growth. Among the Gram-positive isolates, 15 out of 17 were identified as Staphylococcus aureus. The most prevalent Gram-negative bacteria isolated from ulcer infections were Escherichia coli (34 isolates), followed by Pseudomonas aeruginosa (24 isolates), Klebsiella pneumoniae (11 isolates), and Burkholderia (30 isolates). Other isolated pathogens included Proteus spp. (8 isolates) and Acinetobacter spp. (2 isolates).

Antimicrobial Susceptibility Testing:

The Kirby-Bauer disk diffusion method was utilized to assess the susceptibility of the isolates to various antibiotics. The results indicated widespread resistance among Enterobacter, Klebsiella, and Pseudomonas isolates to commonly administered hospital antibiotics. Notably, trimethoprim exhibited a resistance rate of 100% in Enterobacter, Klebsiella, and Pseudomonas species. Cefotaxime demonstrated complete resistance in Enterobacter, Klebsiella, and Acinetobacter isolates, with a 92.3% resistance rate in Pseudomonas isolates. High resistance rates were also observed for ampicillin (100%) and ceftriaxone, colistin, and gentamicin (84.61%) in Pseudomonas aeruginosa. However, a notable reduction in resistance rates was observed for tetracycline and imipenem (61.53%).

Molecular Characterization of β-Lactamase Genes:

All isolates from the ESKAPE group were found to harbor extended-spectrum β-lactamases (ESBLs), with various β-lactamase genes identified through molecular analysis. The blaKPC gene was detected in 72% of Escherichia cloacae isolates, 33.33% of Klebsiella pneumoniae isolates, and 50% of Pseudomonas aeruginosa isolates. The blaTEM gene was present in 63.6% of E. cloacae, 33.3% of S. aureus, and 33.3% of K. pneumoniae isolates. Additionally, the blaCTX-M gene was identified in all E. cloacae isolates and 50% of K. pneumoniae isolates. Furthermore, the blaAMPC gene was prevalent across all isolates, with 100% detection in E. cloacae, S. aureus, K. pneumoniae, and A. baumannii.

Distribution of β-Lactamase Genes:

The distribution of β-lactamase genes among the ESKAPE isolates revealed that blaAMPC and blaKPC genes were the most prevalent. These findings underscore the significant presence of ESBL-producing pathogens in ulcer infections, with potential implications for treatment strategies.

Discussion

ESKAPE bacteria are a big problem in hospitals because they resist antibiotics. They include Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species. These bacteria are tricky to treat because they can avoid normal medicines. They cause infections that people get while in the hospital. This study demonstrated the complex mechanism between microbial pathogens and antibiotic resistance.

Isolation and Identification of the ESKAPE Group in Ulcer Infections

The study found that 88% of the specimens showed positive cultures on MacConkey agar or blood agar, while 16% showed no growth on either medium. Among the isolates, 135 out of 152 (88.8%) were Gram-negative bacteria, with 17 of them (30.3%) being Gram-positive (Fig. 1). There were 44 samples with mixed growth, and 15 out of 17 isolates on blood agar were identified as S. aureus. Gram-negative strains isolated from patients with diabetic foot ulcers, varicose dermatitis, and bed ulcers included Enterococcus (2), Proteus (8), E. coli (34), P. aeruginosa (24), K. pneumoniae (11), Acinetobacter (2), Burkholderia (30), and Enterococcus (2).

Klebsiella pneumoniae, a pathogenic opportunistic bacterium, is well-adapted to hospital environments due to its ability to form biofilms, which help it survive and resist elimination by the immune system or antibiotics (Groenewold et al., 2018). It causes infections such as urinary tract infections, burns, pneumonia, and soft tissue infections in hospitalized and weakened patients. The pathogen responsible for the disease could belong to various bacterial families or other unusual agents like viruses, fungi, or parasites, requiring specific growth media, environmental conditions, and testing techniques (Riquelme et al., 2018; Jain and Barman, 2017).

The Kirby-Bauer disk diffusion method was utilized to screen all isolates with commonly used antibacterial drugs. Fourteen antibiotic discs were employed for testing, including macrolides (erythromycin, fumaric acid), carbapenem class (imipenem and meropenem), cephalosporins (ceftazidime), aminoglycoside

Table 1: Primers (Macrogen, Europe)

Target Gene		Sequence5`-3`	Amplicon Size (bp)	Reference
blaCTX	F	GCCATGTGCAGAACCAGTAA	5	Sharma *etal*., (2018)
	R	CCGCAATATGCTTGGTGGTG	5 4	
blaAMPC	F	TGCTCGGCATCTCTTGCTCT	2	Barnaud *etal*., (2001)
	R	CAGCTTGAGCGGCTTAAGGA	0 0	
blaKPC	F	AAA ACG GCA AGA AAA AGC AG	3	Salloum *etal*., (2017)
	R	AAA ACG GCA AGA AAA AGC AG	4 0	
blaTEM	F	ATCAGCAATAAACCAGC	7	Svärd, (2007)
	R	CCCCGAAGAACGTTTTC	6 6	

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

Gene	Temperature (°C)/Time					CyclesNumber
	Initial Denaturation	Condition of one cycle			Final Extension	
		Denaturation	Annealing	Extension		
bla_{KPC}	95 /3min	95 /30 sec	56 /30sec	72 /45sec	72 /5 min	30
bla_{TEM}	94 /5 min	94 /1 min	50 /1 min	72 /1min	72 /7min	30
bla_{CTX}	94 /5 min	94 /1 min	60 /1 min	72 /1min	72 /7min	30
bla_{AMPC}	94 /5min	94 /1 min	56 /1 min	72 /1 min	72 /5 min	30

Table 3. Numbers and percentage of ESKAPE groups isolates in infections Types.

Infection Types	ESKAPE Group					
	E. cloacae	*S. aureus*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*E. cloacae*
Diabetic foot ulcer	8	15	18	2	22	0
Bed ulcer	3	0	2	0	0	0
Varicose dermatitis	0	0	4	0	2	2
Total	11	15	24	2	24	2

Table 4. Antimicrobials Sensitivity Test of *Staphylococcus aureus* and *Enterococcus faecium*.

Antibiotic type	*Staphylococcus aureus*(15)			*Enterococcus faecium*(2)		
	S	I	R	S	I	R
Levofloxacin	1(12.5)	0(0)	14(87.5)	0(0)	0(0)	2(100)
Tetracycline	0(0)	0(0)	15(100)	2(100)	0(0)	0(0)
Vancomycin	0(0)	1(2.5)	14(87.5)	0(0)	0(0)	2(100)
Clindamycin	3(25)	4(25)	8(50)	0(0)	0(0)	2(100)
Chloramphenicol	3(25)	2(2.5)	10(62.5)	0(0)	0(0)	2(100)
Trimethoprim	3(25)	2(2.5)	10(62.5)	0(0)	0(0)	2(100)
Erythromycin	1.(12.5)	0(0)	14(87.5)	1(50)	0(0)	1(50)
Amoxicillin	1(12.5)	0(0)	7(87.5)	0(0)	0(0)	2(100)
Ciprofloxacin	5(37.5)	2(2.5)	8(50)	0(0)	0(0)	2(100)

Table 5. Antimicrobials Sensitivity Test of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Antibiotic type	*Pseudomonas aeruginosa*(24)			*Acinetobacter baumannii*(2)		
	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)
Cefotaxime	0(0)	2(7.69)	22(92.3)	0(0)	0(0)	2(100)
Ciprofloxacin	6(30.7)	0(0)	18(69.2)	2(100)	0(0)	0(0)
Trimethoprim	0(0)	0(0)	24(100)	0(0)	0(0)	2(100)
Gentamicin	3(15.3)	0(0)	21(84.6)	0(0)	0(0)	2(100)
Ampicillin	0(0)	0(0)	24(100)	0(0)	0(0)	2(100)
Meropenem	7(30.7)	0(0)	17(69.2)	2(100)	0(0)	0(0)
Collistin	2(7.7)	1(7.7)	21(84.6)	0(0)	0(0)	2(100)

Table 6. Antimicrobials Sensitivity Test of *Klebsiella pneumoniae* and *Enterobacter clocae*

Antibiotic type	*Klebsiella pneumoniae*(24)			*Enterobacter cloacae*(24)		
	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)
Cefotaxime	0(0)	0(0)	24(100)	0(0)	0(0)	11(100)
Ciprofloxacin	3(21.4)	0(0)	21(78.6)	6(60)	0(0)	5(40)
Trimethoprim	0(0)	0(0)	24(100)	0(0)	0(0)	11(100)
Gentamicin	2(14.3)	0(0)	22(85.7)	0(0)	0(0)	11(100)
Ampicillin	0(0)	0(0)	24(100)	0(0)	0(0)	11(100)
Meropenem	2(14.3)	0(0)	22(85.7)	2(20)	0(0)	9(80)
Collistin	0(0)	0(0)	24(100)	0(0)	0(0)	11(100)
Cefepime	0(0)	2(14.3)	22(85.7)	0(0)	0(0)	11(100)
Ceftriaxone	0(0)	1(7.1)	23(92.9)	0(0)	0(0)	11(100)
Tetracycline	4(28.6)	0(0)	20(71.4)	3(20)	0(0)	9(80)
Levofloxacin	0(0)	2(14.3)	22(85.7)	0(0)	0(0)	11(100)
Impinem	2(14.3)	0(0)	21(85.7)	2(20)	0(0)	9(80)
Doxycycline	3(21.4)	1(7.1)	20(71.4)	4(40)	0(0)	7(60)

Table 7. The percentage of β-lactamase coding genes among ESKAPE Group

ESKAPE Group	*blaCTX-M* No.(%)	bla_{KPC} No.(%)	bla_{TEM} No.(%)	*blaAMPC* No.(%)
***E. cloacae*(11)**	11(100)	8(72.7)	7(63.6)	11(100)
***S. aureus*(15)**	0(0)	2(13.3)	5(33.3)	15(100)
***K. pneumoniae*(24)**	12(50)	8(33.3)	8(33.3)	24(100)
***A. baumannii*(2)**	0(0)	2(100)	2(100)	2(100)
***P. aeruginosa*(24)**	0(0)	12(50)	0(0)	20(83.3)
***E. faecium* (2)**	0(0)	0(0)	0(0)	0(0)

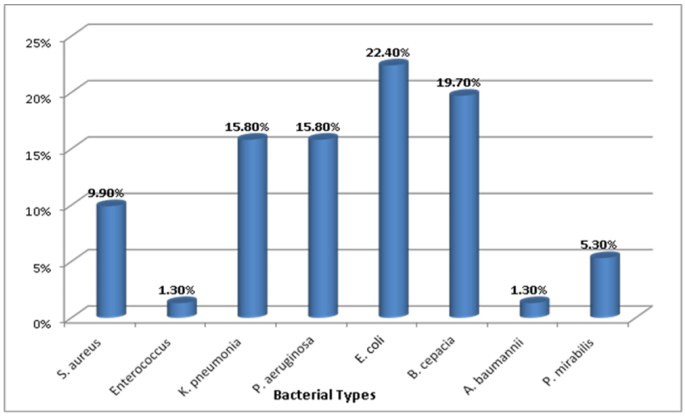

Figure 1. Distribution of bacterial types in ESKAPE groups

Figure 1. Distribution of bacterial types in ESKAPE groups

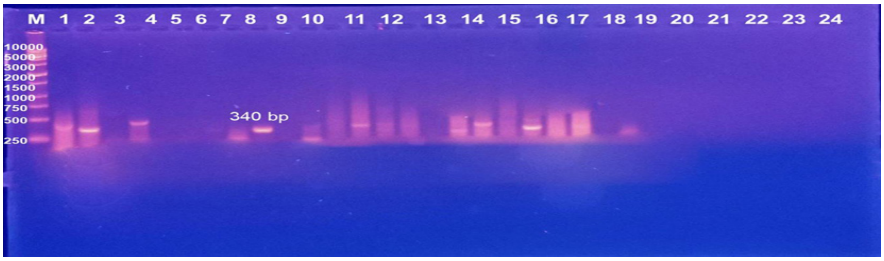

Figure 2. Gel electrophoresis of PCR amplified product of blaKPC gene primers with product 340 bp of ESKAPE group isolates. Lanes (1-25) ESKAPE isolates. (M), DNA molecular size marker (2000-bp ladder).

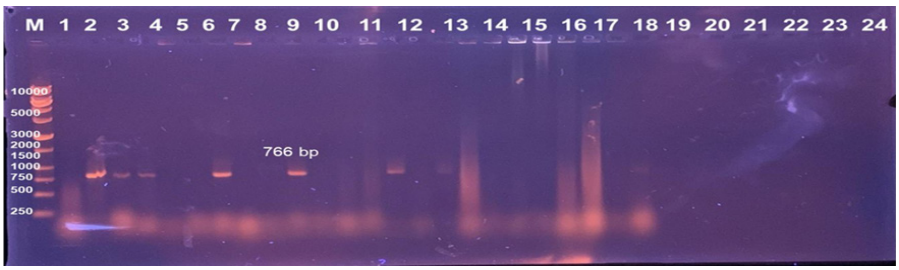

Figure 3. Using blaTEM gene primers and the 766 bp product of the isolates from the ESKAPE group, gel electrophoresis was used to amplify the product.Lanes 1–25 are isolated by ESKAPE. (M), the 2000-bp ladder for DNA molecular size markers.

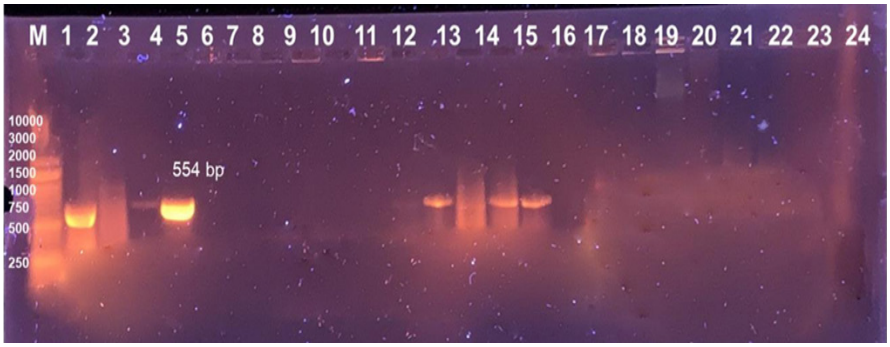

Figure 4. Gel electrophoresis of blaCTX-M gene primer amplified product using 554 bp of isolates from the ESKAPE group. Lanes 1–25 are isolated by ESKAPE. (M), the 2000-bp ladder for DNA molecular size markers.

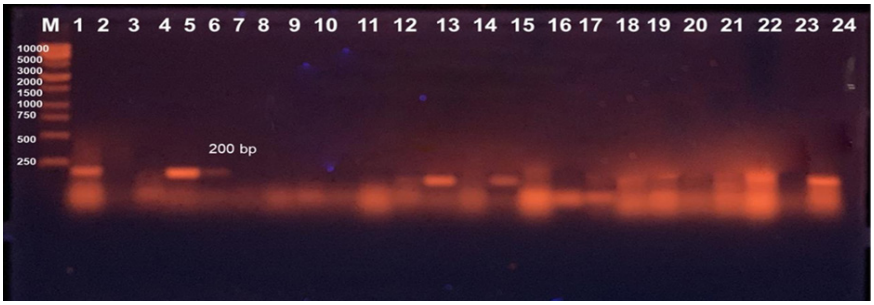

Figure 5. PCR amplified product of blaAMPC gene primers with product 200 bp of isolates from the ESKAPE group electrophoresed on gel. Lanes 1–25 are isolated by ESKAPE. (M), the 2000-bp ladder for DNA molecular size markers.

(gentamycin), tetracyclins (doxycycline, tetracycline), ansamycin (rifampin), phenicols (chloramphenicol, piperacillin), macrolides (fusidic acid, erythromycin), etc.

The phenotypic susceptibility of 24 K. pneumoniae isolates, 24 Pseudomonas isolates, 11 Enterobacter isolates, 15 Staphylococcus isolates, 2 Enterobacter isolates, and 2 Acinetobacter isolates was determined using the Kirby-Bauer disk diffusion method. Results were compared with reference inhibition zones established by the Clinical and Laboratory Standards Institute (CLSI) in 2023 and analyzed based on the diameter of the inhibition zones.

According to the findings, most hospital-administered antibiotics showed inefficacy against Enterobacter, Klebsiella, or Pseudomonas aeruginosa. Trimethoprim exhibited a consistently high resistance rate of 100% in Enterobacter, Klebsiella, and P. aeruginosa. Cefotaxime resistance was observed in 100% of Enterobacter, Klebsiella, and Acinetobacter isolates, and in 92.3% of Pseudomonas isolates. P. aeruginosa displayed complete resistance to ampicillin, and 84.61% resistance to ceftriaxone, colistin, and gentamicin. Resistance percentages for doxycycline, levofloxacin, and cefepime were 76.32%. This study showed a 61.53% reduction in the resistance rates of tetracycline and imipenem.

The overuse and misuse of prescribed medicines are frequently linked to the development of antibiotic resistance. The resistance of K. pneumoniae and Enterobacter cloacae continues to be a major clinical therapeutic concern, as evidenced by the increasing resistance of these bacteria to drugs (Balaban et al., 2004).

The emergence of higher-level resistance may be indicated by bacterial subpopulations or minimum inhibitory concentrations (MIC) (Balaban et al., 2004). Consequently, addressing all factors contributing to the rise in antibiotic resistance is crucial.

The findings revealed that all isolates of Klebsiella pneumoniae (K. pneumoniae) and Enterobacter cloacae (E. cloacae) exhibited higher resistance to penicillins, including ampicillin, penicillin G, amoxicillin, and piperacillin. This resistance may stem from antibiotics failing to reach the target penicillin-binding proteins (PBPs) or from isolates producing β-lactamases (Harwood et al., 2000). Additionally, Round et al. (2011) discovered that heat shock pretreatment could partially prevent penicillin-triggered lysis. In vivo stresses such as inflammation, phagocyte respiratory bursts, and temperature changes can elevate penicillin resistance levels. Moreover, the exchange of plasmids encoding antibiotic resistance genes between microbial cells in contact may further increase bacterial resistance to various antibiotics (Juhas et al., 2015).

Regarding third-generation cephalosporins, including cefotaxime, ceftriaxone, and ceftazidime, it was observed that the majority of K. pneumoniae and E. cloacae isolates exhibited strong resistance to these drugs, indicating the presence of extended-spectrum β-lactamases (ESBLs). The synthesis of ESBLs may also contribute to aztreonam resistance in K. pneumoniae and E. cloacae isolates (CLSI, 2023).

Enterobacteriaceae have demonstrated resistance to several antimicrobial drugs in recent years, attributed mainly to the widespread use of antibiotics and the ease with which these microbes can develop resistance (Thomson, 2010). Enhanced efflux of antimicrobial agents from cells could result in less of the agent being present inside cells, potentially leading to increased bacterial survival (Piddock, 2006).

Isolates from the ESKAPE Group: Molecular Identification of Certain *β*-Lactamase Genes

Extended-spectrum β-lactamases (ESBLs) were detected in all ESKAPE group isolates, with their types and prevalence determined through further investigation. ESBL production was examined phenotypically using the disk approximation method. However, this study focused solely on two genes from the β-lactamase families (KPC, TEM, CTX-M, and AMPC).

Detection of blaKPC Gene:

The results revealed that among Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Klebsiella pneumoniae (K. pneumoniae), Pseudomonas aeruginosa (P. aeruginosa), Enterococcus faecium (E. faecium), and Acinetobacter baumannii (A. baumannii) isolates in this study, the blaKPC gene was detected in 72%, 13.3%, 33.33%, 50%, 0%, and 100% of cases, respectively.

Hospital infections associated with K. pneumoniae have been reported in several countries (Nordmann et al., 2011), highlighting the importance of early identification of carbapenemases in carriers and/or infected individuals to prevent outbreaks. House flies have been implicated as potential carriers of K. pneumoniae, an antibiotic-resistant bacterium (Ranjbar et al., 2016). Carbapenemases (KPCs) in K. pneumoniae are the most prevalent transmissible class, primarily found in clonally expanding strains worldwide and circulating in Enterobacteriaceae (Walsh, 2010).

In contrast to KPC, New Delhi metallo-ß-lactamase (NDM)-producing strains spread rapidly and extensively. The promiscuous plasmid that mediates Enterobacteriaceae is not related to the dominant clonal strains (Logan and Weinstein, 2017).

Detection of *bla*$_{TEM}$ gene

The most prevalent β-lactamase in Gram-negative bacteria, encoded on plasmids, is identified through molecular detection of the blaTEM gene using specific PCR primers. The blaTEM gene was found in 63.6% of Escherichia coli (E. coli), 33.3% of Staphylococcus aureus (S. aureus), 33.3% of Klebsiella pneumoniae (K. pneumoniae), 0% of Pseudomonas aeruginosa (P. aeruginosa), 0% of Enterococcus faecium (E. faecium), and 100% of Acinetobacter baumannii (A. baumannii). The experiment's results demonstrated positive amplification.

According to Schmid et al. (2013), TEM-1 is the most common bla-encoded enzyme found in human clinical isolates globally, but it is not considered an ESBL. However, ESBL characteristics are

conferred by numerous TEM-1 derivatives (Paterson and Bonomo, 2005). According to Machado et al. (2005), the blaTEM gene was present in only 31% of isolates that produced ESBL in Spain.

Detection of *bla*$_{CTX-M}$ gene

The amplification of the blaCTX-M gene by PCR revealed its encoding of class A extended-spectrum β-lactamases (ESBLs), which can pose therapeutic challenges. The findings indicated that the blaCTX-M gene was present in 100%, 0%, 50%, 0%, 0%, and 0% of Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Klebsiella pneumoniae (K. pneumoniae), Pseudomonas aeruginosa (P. aeruginosa), Enterococcus faecium (E. faecium), and Acinetobacter baumannii (A. baumannii), respectively.

ESBLs are significant contributors to the resistance of Gram-negative bacteria to ß-lactam antibiotics. These enzymes, encoded on plasmids, confer resistance to penicillins and first, second, and third generation cephalosporins, including cefotaxime, ceftriaxone, and ceftazidime. TEM, SHV, and CTX-M are the three primary genetic groups of ESBLs found in clinically significant Gram-negative bacteria (Khan et al., 2020). The widespread use of third-generation cephalosporins, particularly ceftriaxone and cefotaxime, may contribute to the predominance of CTX-M, or it may be linked to a high level of gene mobilization encoding CTX-M. Barlow et al., (2008) reported that compared to other Class A β-lactamases, the blaCTX-M genes have been mobilized to plasmids nearly ten times more frequently. The prevalence of the CTX-M type of ESBLs gene may indicate its widespread occurrence in India's northeast. Muzaheed et al., (2009) further revealed a high frequency of CTX-M genes in K. pneumoniae and E. coli from Southern India.

Detection of *bla*$_{AMPC}$ gene

The findings revealed that the blaAMPC gene was present in 100%, 100%, 100%, 83.3%, 0%, and 100% of Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Klebsiella pneumoniae (K. pneumoniae), Pseudomonas aeruginosa (P. aeruginosa), Enterococcus faecium (E. faecium), and Acinetobacter baumannii (A. baumannii), respectively.

The primary mechanism of resistance to carbapenems is the development of carbapenemase enzymes. Additional resistance mechanisms include porin modification, drug efflux pumps, and changes in penicillin-binding proteins, as well as the production of other enzymes with poor carbapenemase activity, including extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases (Logan and Weinstein, 2017; Aslam et al., 2020). Carbapenemases are classified into classes A, B, and D of β-lactamases based on their molecular structures using the Ambler classification method (Meletis, 2016). The presence of AmpC enzymes or a decrease in the permeability of the outer membrane could contribute to cefoxitin resistance (Yang et al., 2012).

Manchanda and Singh (2003) reported that in an earlier study conducted in India, 39% of AmpC producers were vulnerable to cefoxitin antibiotic disks, while 61% of them were resistant to it. AmpC α-lactamases are among the most significant α-lactamases in Gram-negative bacteria, but current understanding of AmpC β-lactamases is still somewhat limited. Reliable identification of AmpC is essential for providing epidemiological data and enhancing clinical infection management in all hospitals. Enterobacteriaceae's decreased susceptibility to cefoxitin may indicate AmpC activity, but further testing is necessary for confirmation. Rudresh and Nagarathnamma (2011) conducted a study in India and found that out of 160 clinical isolates of Enterobacteriaceae, 80 produced AmpC, but only 56/80 (or 70%) of these producers were recognized using the modified three-dimensional test.

Many Enterobacteriaceae species have chromosomally encoded class C cephalosporinases (AmpCs), which can be overproduced (Jacoby, 2009). Additionally, AmpCs can be self-transferable and plasmid-mediated (pAmpCs). The six groups of AmpCs include the CMY-2 group, the MIR-1 and ACT-1 group, the DHA group, the ACC-1 group, the CMY-1 group (also known as MOX-1), and the FOX-1 group. Overexpression of AmpCs in combination with porin deficiency or deletion may lead to carbapenem resistance (Bush, 2010).

Distribution of bla genes among ESKAPE Group

Table 7 showed the distribution of genes among ESKAPE isolates, along with the percentage of these genes. The results indicate that blaAMPC and blaKPC genes were the most prevalent β-lactamase genes in the ESKAPE group.

The synthesis of enzymes known as cefotaximases (CTX-M), which exhibit significantly higher activity against cefotaxime than against ceftazidime, may contribute to the high resistance to ceftazidime and cefotaxime (Walther-Rasmussen and Hoiby, 2004). Furthermore, the presence of multiple α-lactamases in a single isolate could lead to complex phenotypic expression, such as the synthesis of AmpC enzymes resistant to aztreonam and third and fourth-generation cephalosporins (Hammond, 2004).

Cefepime is commonly used as a fourth-generation antibiotic in hospital regimens. Compared to other local studies reporting 93.2% resistance to cefepime, this study's relatively lower resistance rate (68%) was noted (Al-Muhannak, 2010).

However, it is evident that ESBL organisms are widely distributed among the Enterobacteriaceae family globally. While TEM, OXA, and CTX-M are the most common ESBL enzyme types, other ESBL enzymes, such as VEB and PER, exist with varying frequencies among bacteria (David and Robert, 2005). The presence of other ESBL genes within the bacterial genome not considered here may contribute to discrepancies between the findings of this study and

others. Earlier investigations identified blaCTX-M-3 as the two most prevalent ESBL genes in Serratia marcescens (Elvira et al., 2011).

Conclusion

The study determined the widespread presence of ESBL-producing ESKAPE bacteria in ulcer infections and showed the importance of molecular techniques for accurate detection of β-lactamase production. The prevalence of β-lactamase genes underscores the urgent need for comprehensive antibiotic stewardship programs and the development of novel therapeutic approaches to combat multidrug-resistant infections caused by ESKAPE pathogens.

Author contribution

H.A.J., H.D.R., F.H.H. collected samples, performed tests, and wrote the article, Z.Y.M. conducted the study design, edited the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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