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The Prevalence of Extended Spectrum Beta-Lactamase Genes SHV, TEM and CTX-M in Clinical Isolates of *Proteus mirabilis*

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Abstract

This study investigates the antibiotic resistance patterns in Proteus mirabilis isolates, with a specific focus on the presence of Beta-Lactamase genes, including SHV, TEM, and CTX-M, which are known to contribute to the bacterium's ability to cause various diseases. A total of 100 urine samples were collected from patients confirmed urinary tract infections (UTIs), and out of these, only 20 of those samples were positively identified as *Proteus mirabilis* using cultural characteristics, biochemical examinations, and the Vitek II system. Antibiotic susceptibility was tested for 27 antibiotics. Results showed that isolates exhibited multi-drug resistance ranging from 10 to 19 antibiotics, with 100% resistance to Ampicillin, Erythromycin, Clindamycin, Clarithromycin, Penicillin G, Cephalothin and Cefaclor. Additionally, the isolates revealed high resistance to the third generation Cephalosporines of, including Ceftriaxone (85%), Cefotaxime (75%), Ceftazidime (55%), and Cefoperazone (45%). Using the Double Disk Synergy Test (DDST), eight isolates were confirmed to produce ESβL. Conventional PCR was conducted with specific primers to detect four distinct ESβLs (TEM, SHV, CTX-M-8, and CTX-M-9). Results revealed that CTX-M-8 was found in all eight isolates with phenotypic evidence of ESβL production, indicating that they are the most prevalent type of ESβL. At the same time, those for CTX-M-9, TEM-type and SHV type were 87.5%, 62.5% and 12.5 % respectively. In conclusion, all the examined isolates were found to produce ESβLs, particularly the CTX-M-8 gene.

Keywords: *P. mirabilis*, antibiotics, ESβL, SHV, TEM, CTX-M

انتشار جينات البيتا لاكتاماز ذات الطيف الواسع SHV وTEM وCTX-M في العزلات السريرية لبكتيريا *Proteus mirabilis*

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الخلاصة :

تبحث هذه الدراسة في أنماط مقاومة المضادات الحيوية في عزلات *Proteus mirabilis* ، مع التركيز بشكل خاص على وجود جينات بيتا لاكتاماز، بما في ذلك SHV و TEMوCTX-M، والتي من المعروف أنها تساهم في قدرة البكتيريا على التسبب في أمراض مختلفة. تم جمع ما مجموعه 100 عينة ادرار من مرضى مصابين بعدوى المسالك البولية المؤكدة، ومن بين هذه العينات، تم تشخيص 20 عينة فقط على أنها

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P. mirabilis باستخدام الخصائص الزرعية والفحوصات الكيميائية الحيوية ونظام Vitek II. تم اختبار حساسية العزلات للمضادات الحيوية لـ 27 مضاد حيوي. أظهرت النتائج أن العزلات أبدت مقاومة متعددة للمضادات الحياتية تراوحت بين 10 إلى 19 مضادًا حيويًا ، اذا بلغت مقاومة الأمبيسيلين، الاريثروميسين، الكليندامايسين، الكلاريثروميسين، البنسلين جي، السيفالوثين والسيفاكلور 100%. بالإضافة إلى ذلك، أظهرت العزلات مقاومة عالية للجيل الثالث من السيفالوسبورينات ومنها سيفترياكسون (85%)، سيفوتاكسيم (75%)، سيفتازيديم (55%)، سيفوبيرازون (45%). وباستخدام اختبار تآزر القرص المزدوج (DDST)، تم تأكيد إنتاج ثماني عزلات لـ ESβL. تم إجراء تفاعل البوليميريزالمتسلسل التقليدي باستخدام بادئات محددة للكشف عن اربعة ESβLs مميزة (TEM وSHV وCTX-M-8 وCTX-M-9). أظهرت النتائج وجود CTX-M-8 في جميع العزلات الثمانية مع وجود دليل مظهري على إنتاج ESβLs، مما يشير إلى أنها النوع الأكثر انتشارا من ESβL. وفي الوقت نفسه، كانت تلك الخاصة بـ CTX-M-9 ونوع TEM ونوع SHV 87.5% و62.5% و12.5% على التوالي. وبالنتيجة النهائية وجد ان جميع العزلات التي تم اختبارها تحتوي على جينات الـ ESβLs وخاصة جين CTX-M-8.

Introduction

The introduction of β-lactam antibiotics into the healthcare system represents one of the most significant advancements in medical science in recent years. These antibiotics are preferred due to their effectiveness, safety, and affordability, as well as the fact that chemical manipulation can increase or decrease their activity [1-3]. In gram negative bacteria, the synthesis of β-lactamase enzymes, which are a diverse group of enzymes capable of inactivating penicillins, cephalosporins, and monobactams, is the primary mechanism by which resistance to β-lactam antibiotics demonstrates. These enzymes irreversibly hydroxylate the β-lactam ring, hydrolyzing it, which renders the antibiotic inactive [4].

There are four primary molecular classes of β-lactamases that are widely recognized: A, B, C, and D. Once established in a region, these enzymes quickly spread over the globe and end up being the main form of resistance [5,6].

P. mirabilis is one of the most common gram-negative bacteria discovered in clinical specimens, capable of causing a range of community- or hospital-acquired diseases, including bloodstream, wound, and urinary tract infections [1, 5]. This bacterium is naturally vulnerable to fluoroquinolones, aminoglycosides, β-lactams, and trimethoprim-sulfamethoxazole due to the production of ESβLs such as TEM, CTX-M, SHV, PER, and CBL type enzymes [7]. However, it is intrinsically resistant to nitrofurantoin and tetracycline. The treatment of *P. mirabilis* infections is becoming increasingly challenging, with 48% of the strains show signs of antibiotic resistance [8]. The number of resistant strains is rapidly increasing, and the available treatments are not keeping up with the demand. Part of a wider family of ESβLs, which provide resistance to a variety of beta-lactam antibiotics, such as penicillins and cephalosporins, is the SHV (sulfhydral variable) beta lactamase gene. It has been shown that *P.mirabilis* possesses SHV-type beta-lactamases, suggesting that these bacteria can acquire resistance through horizontal gene transfer, frequently using plasmids[9]. The second type of ESβLs is TEM which is responsible for over 90% of the ampicillin resistance in Gram-negative bacteria and is usually mediated by plasmid [10]. Meanwhile, CTX-M exhibits greater activity toward cefotaxime and the term “CTX-M” (cefotaximase from Munich) was first introduced in a German report [11].

The occurrence and distribution of ESβLs in *P. mirabilis* strains from long-term care facilities remain unknown. In recent years, ESβLs generating *P. mirabilis* isolates have been recovered worldwide, with a rather high prevalence in specific settings.

This study aimed to investigate the spread and prevalence of ESβLs in *P. mirabilis*, assess the susceptibility of local isolates *of P. mirabilis* from urinary tract of patients to commonly used antibiotics, and use PCR technique to detect the presence of the three common ESβLs genes: TEM, CTX-M, and SHV.

2- Materials and Methods:

2.1 Collection, isolation and identification of clinical specimens

A total of hundred urine samples were collected from patients suffering from UTIs, from Medical City hospitals and Al-Yarmouk Hospital in Baghdad between May 2023 to August 2023 with the approval of the College of Science Research Ethics Committee based on (CSEC/0523/0052). The samples were collected in sterilized containers and transferred into a laboratory for diagnosis. Fifty microliters of each sample were cultured on MacConkey agar (Hi-Media/India) and incubated for 24 hours at 37 ºC. Additionally, gram staining, biochemical tests including (Motility, indole, catalase, oxidase, citrate utilization, urease, gelatinase, methyl red) and the Vitek II system were performed to identify the *P. mirabilis* isolates.

2.2 Antibiotic Susceptibility Test:

The antibiotic sensitivity of the 20 isolates were assessed using 27 different commonly used antibiotic discs. The specific antibiotics tested are presented in Table1.

Table 1: Antibiotics used in this study.

Antibiotic Disc	Symbol	Disc Content µg /disc	Antibiotic Disc	Symbol	Disc Content µg /disc
Ampicillin	AMP	10	Tetracycline	TE	30
Carbenicillin	CB	100	Cephalothin	CEP	30
Neomycin	N	30	Azithromycin	AZM	15
Cefoperazone	CPZ	75	Tobramycin	TOB	10
Gentamicin	GEN	10	Ciprofloxacin	CIP	5
Erythromycin	E	15	Aztreonam	AT	30
Imipenem	IPM	10	Penicillin G	P	10 units
Clindamycin	CD	2	Cefaclor	CF	30
Clarithromycin	CLR	15	Ceftazidime	CAZ	30
Norfloxacin	NX	10	Nalidixic Acid	NA	30
Amoxycillin / Clavulanic acid	AMC	30	Ceftriaxone	CTR	30
Chloramphenicol	C	30	Cefotaxime	CTX	30
Cefepime	CPM	30	Fosfomycin	FO	200
Streptomycin	S	10			

Kirby-Bauer disk diffusion method was performed on Muller Hinton Agar [12]. After incubating the plates at 37 °C for 18 hours, the size of the inhibitory zones surrounding the antibiotic disc were measured to determine the sensitivity and resistance, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI,2022) [13].

2.3 Detection of Extended Spectrum β- lactamase (ESβL) production:

The Double disc approximation test (DDST) was used to identify the production of ESBLs. The ability of the most resistant isolates to produce ESβLs was assessed by spreading bacterial suspensions on the surface of Muller Hinton agar plates, as previously reported [14]. An amoxicillin/clavulanic acid (30 µg) disc was placed in the middle of Mueller Hinton agar plate, and then the discs of cefotaxime, ceftazidime, and aztreonam were arranged around the amoxicillin/clavulanic acid (30 µg) disc within 2-3 cm distance. The plates were incubated at 37 °C for 18-24 h. After incubation, synergistic activity between the central disk and any one of the surrounding antimicrobial discs was noted to detect the ESβLs producing isolates.

2.4 DNA Isolation:

Extraction of the genomic DNA from 1 ml of overnight bacterial broth was carried out by using Bioneer DNA extraction kit specific for Gram-negative bacteria.

2.5 Molecular detection of SHV, TEM, CTX-M-8 and CTX-M-9 genes:

2.5.1 Primers and PCR reaction

Four specific sets of primers [15, 16] were used, as detailed in Table 1. Amplifications were performed in a MultigeneTM Gradient Thermal Cycler (Labnet International, Korea). Each PCR reaction was prepared in a final reaction volume 25 µl contains 12.5 µL (1X) GoTaq® Green Master Mix, 10 pmol/µL of each primer (Forward and Reverse), 1µL of 100 ng template DNA and 10.5 µL Nuclease free water. A negative control reaction was included for all amplifications to ensure the reliability of the results.

Table 2: Oligonucleotide primer sequences for antibiotic resistance genes.

Primer	Primer sequence	Product size bp	Reference
SHV-MN1 SHV-MN2	5′-CGCCGGGTTATTCTTATTTGTCGC3′ 5′TCTTTCCGATGCCGCCGCCAGTCA3′	1000	[15], [16]
TEM-1F TEM-1R	5′-AAGCCATACCAAACGACGAG-3′ 5′-ATTGTTGCCGGGAAGCTAGA-3′	100	
CTX-M-8F CTX-M-8R	5′-AGCAAAGTGAAACGCAAAAG-3′ 5′-TCATTCGTCGTACCATAATC-3′	400	
CTX-M-9F CTX-M-9R	5′-CGCTTTATGCGCAGACGA-3′ 5′-GATTCTCGCCGCTGAAGC-3′	500	

For detection the presence of TEM, CTX-M-8 and CTX-M-9 genes among the isolates, PCR reactions were performed with the following parameters: initial denaturation at 94 °C for 10 min., 35 cycles of amplification were applied of 94Cº for 1 min., followed by 1 min. of annealing at 55Cº then extension at 72Cº for 3 min and a final extension at 72Cº for 10 min. The PCR parameters for the detection of SHV gene was: initial denaturation at 94 °C for 5 min. 95Cº for 1 min., annealing at 55Cº for 1 min., followed by extension at 72Cº for 1min. and a final extension of 72 Cº for 10 min repeated for 35 cycles.

To analyze PCR products, electrophoresis was performed 10 µl of PCR mixture was loaded into 2% agarose gels stained with ethidium bromide and separated with a 100 bp molecular size marker (Promega-USA). After electrophoresis, the gels were visualized under ultraviolet light and imaged using the Gel Documentation System (E –Graph - Korea).

3. Results and Discussion

3.1 *Identification and characterization of P. mirabilis*

After streaking on MacConkey agar, a selective and differentiating medium for isolating and identifying Proteus spp., all isolates exhibited a distinct fish-like odor and formed pale,

round, convex, and smooth colonies. The biochemical tests showed the following results: Motility +ve, indole –ve, catalase +ve, oxidase –ve, citrate utilization –ve, urease +ve, Gelatinase + ve, methyl red +ve, as described by [17]. Additionally, all isolates were identified as *P. mirabilis* using the Vitek 2 identification system's results.

3.2 Antibiotic susceptibility test of P. mirabilis isolates:

The antibiotic susceptibility of *P.mirabilis isolates* was tested against 27 different antibiotics, as detailed in Table 2. The results revealed that all isolates were 100% resistant to Ampicillin, Erythromycin, Clindamycin, Clarithromycin, Penicillin G, Cephalothin and Cefaclor, as well as multi-resistance to antibiotics ranging from 10 to 19 drugs as shown in (figures 1,2).

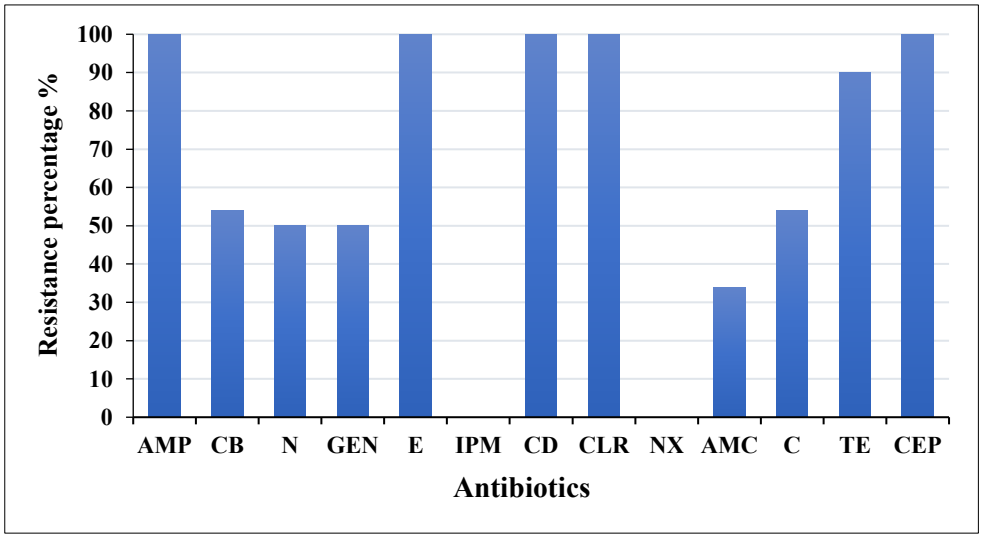

Figure1: Susceptibility of *P. mirabilis* towards different antibiotic discs. Ampicillin(AMP), Carbenicillin(CB),Neomycin(N),Gentamicin(GEN),Erythromycin(E), Imipenem(IPM), Clindamycin(CD), Clarithromycin(CLR), Norfloxacin(NX), Amoxycillin / Clavulanic acid(AMC), Chloramphenicol(C), Tetracycline(TE), Cephalothin(CEP).

The most significant finding was the high resistance of *P. mirabilis* isolates to third generation cephalosporins (3GC), with resistant rate of 85% to Ceftriaxone, 75% to Cefotaxime, 60% to Ceftazidime and 45% to Cefoperazone as shown in Figure (2). Since the first identification of ESBL in *P. mirabilis* in 1987, the clinical incidence of ESβL-positive *P. mirabilis* have been increased [18]. Numerous studies from different countries included United States, United Kingdom, and some European countries reported that susceptibility of *P. mirabilis* towards different antibiotics can vary depending on patient population and the source from which the bacteria were isolated [19].

The therapeutic approaches to control infections caused by *P. mirabilis* isolates must be carefully developed because all of the isolates demonstrated multidrug resistance. On the other hand, 100 % of the isolates were sensitive to Imipenem, and Norfloxacin as shown in Figure 2. The majority of the isolates were susceptible to Cefepime, Tobramycin, and Ciprofloxacin. These findings align with previous studies [20]. Therefore, Imipenem, and Norfloxacin may be considered the preferred medication for treating infections brought on by *Proteus mirabilis*.

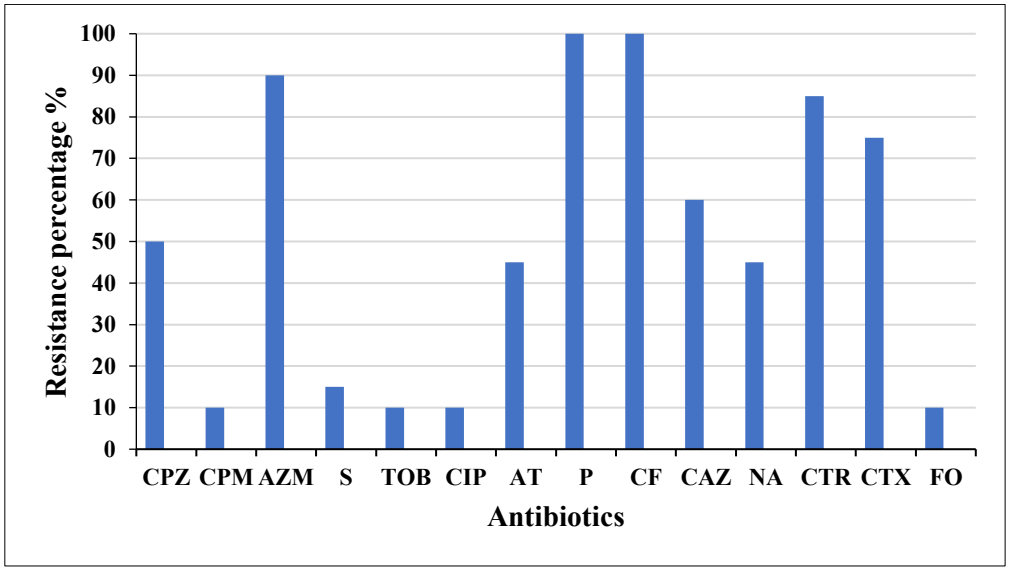

Figure2: Susceptibility of *P. mirabilis* towards different antibiotic groups. Cefoperazone(CPZ), Cefepime(CPM), Azithromycin(AZM), Streptomycin(S), Tobramycin(TOB), Ciprofloxacin(CIP), Aztreonam(AT), Penicillin G(P), Cefaclor(CF), Ceftazidime(CAZ), Nalidixic Acid(NA), Ceftriaxone(CTR), Cefotaxime(CTX), Fosfomycin(FO).

According to the antibiotic susceptibility, *P. mirabilis* isolates exhibited moderate to low resistance to antibiotics that inhibit protein synthesis, including aminoglycosides (streptomycin, tobramycin, and gentamycin), and chloramphenicol, as well as antibiotics that inhibit the synthesis of nucleic acid. These findings were corroborated by the results from previous studies [11, 21].
Several studies have indicated that *P. mirabilis* acquires antimicrobial resistance genes from transferable plasmids, insertion sequences, transposons, and integrons. These mobile genetic elements play a critical role in the horizontal gene transfer of resistance genes into *P. mirabilis* cells [8,22].

Table 3: Antibiotic susceptibility for eight isolates of *P. mirabilis* that produce ESβL

AB NO	Amp	CB	N	CPZ	GEN	E	Ipm	CD	CLR	NX	Amc	C	CPM	TE	CEP	AZM	S	TOB	CIP	AT	P	CF	CAZ	NA	CTR	CTX	FO
1	R	I	I	R	S	R	S	R	R	S	R	R	I	R	R	R	I	S	S	R	R	R	R	I	R	R	S
2	R	S	R	I	S	R	S	R	R	S	S	I	I	R	R	R	I	S	S	I	R	R	R	S	R	R	S
3	R	R	R	R	R	R	S	R	R	S	R	S	I	R	R	R	S	S	S	S	R	R	R	S	R	R	S
4	R	R	S	R	R	R	I	R	R	S	I	S	R	R	R	R	S	S	S	S	R	R	R	S	R	I	S
5	R	R	R	R	R	R	S	R	R	S	R	S	S	R	R	R	S	S	S	S	R	R	R	R	R	R	S
6	R	R	S	R	S	R	S	R	R	S	I	R	R	R	R	R	S	S	S	R	R	R	R	S	R	R	S
7	R	R	R	R	R	R	S	R	R	S	I	I	S	R	R	R	S	R	S	S	R	R	R	S	R	R	S
8	R	I	I	I	S	R	S	R	R	S	S	R	S	R	R	R	I	S	S	R	R	R	R	S	R	R	S

R: Resistance
S: Sensitive
I: Intermediate

3.3 Molecular detection of TEM, SHV, CTX-M-8 and CTX-M-9:

The high percentage of isolates expressing ESBLs may be attributed to the extensive use of antimicrobial agents, which increases resistance to antibiotic treatment. Consequently, the Double Disk Synergy Test (DDST) was performed, and the results showed that 8 of 20 (40%) *P. mirabilis* isolates were capable of producing ESβL. Their antibiotic susceptibility is shown in Table 3. This result is in accordance with other studies performed in Iraq, which reported ESβL production rates of 40, 42 and 55.5% among *P. mirabilis*, respectively [23, 24].

Molecular characterization of ESβLs genes was carried for the eight isolates by using four sets of primers and the PCR amplification for SHV gene reveled that one isolate out of 8 gave the positive result (12.5%), which appeared in single band with molecular size 1000 bp as compared with DNA ladder 1000 bp (Data not shown). In agreement with our findings, Chanal *et al.,* mentioned that a few numbers of SHV-producing *P. mirabilis* have been reported. Additionally, another study conducted in Iraq, revealed that 5% of the isolates carried the SHV gene [26].

TEM genes are commonly found in gram-negative bacteria. The TEM encoded genes are the cause of about 90% of mpicillin and penicillin resistance in Gram-negative bacteria [27].

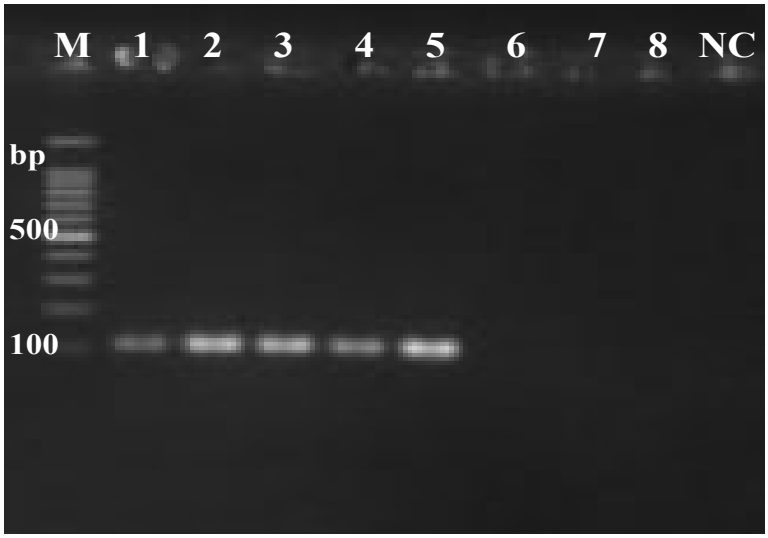

Figure 3: Gel electrophoresis 2 % agarose for amplified TEM gene visualized under U.V. light. Lane: (M:100bp DNA ladder); Lane: 1-8 (*P. mirabilis*) Lane: NC (negative control)

The amplification result for TEM gene showed that the 5 isolates out of 8 gave the positive result (62.5%) and the size of the amplified gene was 100 bp as compared with 100 bp DNA ladder as shown in (Figure 3). The TEM gene has been found to be one of the most prevalent antibiotic resistance genes in multidrug-resistant *Proteus mirabilis* strains by researches conducted in Taiwan and Iraq, with high occurrence rates of 90% and 100% respectively [28,29]. In addition, Al-Kraety et al.(2021) observed an increase in resistance expanded-spectrum cephalosporinsin in *P. mirabilis* clinical isolates, which they linked to the synthesis of ESβLs, including those produced by the TEM gene.

The CTX-M family of ESBLs, poses a growing threat in clinical settings, having been identified in both community and nosocomial infections. CTX-M is a class A enzyme that was originally defined by its preference for breaking down cefotaxime (CTX) over ceftazidime (CAZ). However, in recent years, several derivatives have been found that break down both agents equally [31, 32].

All the isolates gave a result positive and the size of the amplified CTX-M-8 gene was 400 bp Figure (4), while CTX-M-9 that 7 isolates out of 8 gave the positive result (87.5%), the size of the amplified gene was 500bp as compared with 100bp DNA ladder Figure (5).

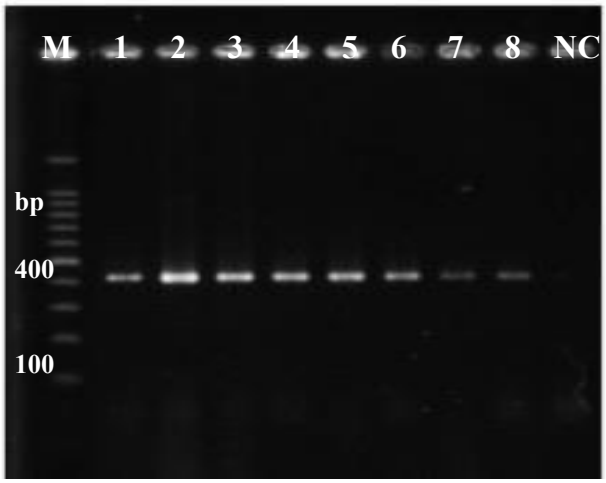

Figure 4: 2 % Agarose gel electrophoresis for amplified CTX-M-8 gene, bands were visualized under U.V. light. Lane:(M:100bp DNA ladder); Lane: 1-8 (*P. mirabilis*) Lane: NC (negative control)

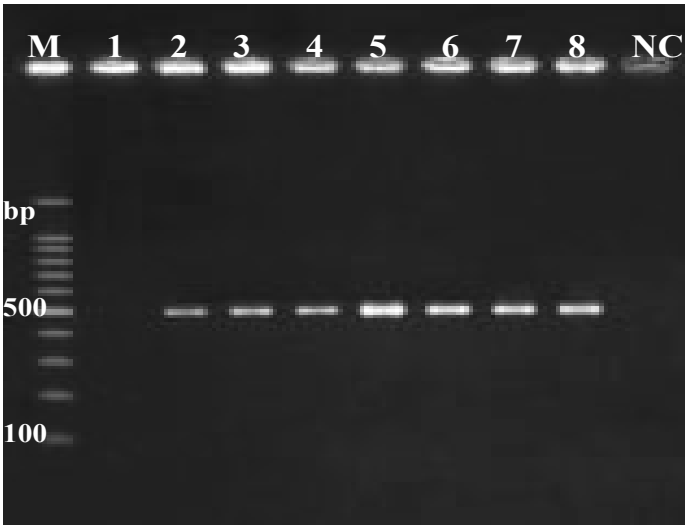

Figure 5: 2 % Agarose gel electrophoresis for amplified CTX-M-9 gene, bands were visualized under U.V. light Lane: (M:100bp DNA ladder); Lane: 1-8 (*P. mirabilis*) Lane: NC (negative control)

It has been reported that CTX-M variants are now the most common β-lactamases produced by gram-negative pathogens in most of Europe, Latin America, and East Asia [31,33,34].Also, the study of [35] agreed with our results and they reported that CTX-M type are currently the most prevalent form of ESβL.

The frequency of CTX-M-8 and CTX-M-9 genes in uropathogenic *P. mirabilis* isolates has been reported in recent investigations, highlighting the growing worry regarding antibiotic resistance in the bacteria.

The extended spectrum beta lactamase CTX-M family holds special significance since it contributes to the development of resistance against third-generation cephalosporins, which are frequently employed in the treatment of urinary tract infections [36, 37]. It is important to note that this investigation focused solely on uropathogenic *P. mirabilis*. To validate and extend the findings, additional research involving more *P. mirabilis* isolates from different sources is necessary.

Conclusion

Treating bacterial infections globally has become a significant challenge due to inappropriate antibiotic use, which has led to the emergence of bacterial resistance and an increase in cases where antibiotic treatments fail. This study found that 12.5 % of our ESBL-positive isolates, had the SHV gene, 62.5% had the TEM gene, 87.5% had the CTX-M-9 gene and 100% had the CTX-M-8 gene. This could support the theory that CTX-M is gradually taking the place of earlier beta lactamases. To stop the emergence of antibiotic resistance, strict policies regarding antibiotic use and stewardship initiatives are essential.

5- Ethical Clearance

This study received approval from the ethical Committee of the College of Science/University of Baghdad, according to the reference number (CSEC/0523/0052).

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