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Occurrence of TEM and SHV among extended-spectrum-beta lactamase producers of some *Enterobacteriaceae* members

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ABSTRACT

This study aimed for the molecular detection of TEM and SHV genes in Extended-Spectrum beta-lactamases (ESBLs) clinical isolates of *Enterobacteriace (Eshericichia coli, Klebsiella pneumoniae and Proteus spp.).* ESBLs are one of the main cause of resistance of *Enterobacteriace* family to beta-lactam antibiotics. The susceptibility of isolates to different beta-lactam and non-beta lactam antimicrobial agents was performed to identify the proper antimicrobial chemotherapeutics for treatment of infections in case of development of resistance to Extended spectrum beta-lactams. Ninety clinical isolates belong to *Enterobacteriacae* family (54 *E. coli*, 28 *Kl. Pneumonia* and 8 *Proteus spp*) recovered from 220 different clinical specimens (urine, blood, pus and sputum). Thirty (33.3%) out of 90 isolates were ESBLs producers. The ESBLs producers were more frequent among *E. coli* isolates (40.7%), followed by *Proteus* (25%) and *klebsiella pneumoniae* (21.4%). Susceptibility testing to Beta-lactams and non beta-lactams was performed by disk diffusion method to all isolates. The *bla* TEM gene was identified in 14 *of E. coli* isolates at 504 bp. The *bla*SHV-625 gene was identified in two isolates of *Klebsiella pneumoniae* at 625bp. Meanwhile *bla*SHV-1,107 was not detected in any clinical isolates.

INTRODUCTION

Resistance to the extended-spectrum cephalosporins among members of the family *Enterobacteriaceae* has become a growing worldwide problem (Nordman 1998). Extended -spectrum β-lactamase-(ESBL) producing bacteria are emerging pathogens in the community, and that clinical laboratories play a critical role for their detection and control.

Although ESBLs have been described in a range of *Enterobacteriaceae* family from different parts of the world, they are most often identified in *Klebsiella pneumoniae, Escherichia coli and Proteus spp.* (Bush *et al*. 1995). The first plasmid-mediated ß-lactamase was detected in Gram-negative bacteria in Greece in the 1960s and was designated TEM after the name of the patient (Temoneira) who carried the pathogen (Datta and Kontomichalou 1965). TEM-1 is the most commonβ-lactamase in Gram-negative bacteria, it can hydrolyze penicillins (ampicillin).The ß-lactamases are quickly spread to other bacteria, after changes in one or few amino acids (Brunton *et al.* 1986) Compared to the TEMs, the sulphydryl variable (SHV) ß-lactamases are similar in biochemical structure but are more common in *Klebsiella* spp. (Bush *et al*. 1995).

MATERIALS and METHODS:

Bacterial isolates

A total of 220 different clinical specimens (urine, blood, pus and sputum) were obtained from Zgazig-University Hospitals and Al-Ahrar Educational Hospital during the period from October 2010 to April 2012.

These isolates were identified and verified by using standard biochemical reactions according to Collee *et al*. (1996). All isolates were collected under approved ethical procedures. For long term maintenance of isolates, 900 µl of overnight culture in Mueller-Hinton broth of each strain were mixed with 100 µl of glycrol and stored at -80°C.

Susceptibility testing

The antibiotic sensitivity of the isolates to the antimicrobial agents was carried out by the disk diffusion method according to **CLSI (2013)** on Muller-Hinton agar (Oxoid UK). The following disks (Oxoid UK) were used Cefotaxim (CTX, 30µg), Cefoperazone (CEP, 30 µg), Tetracyclin (TE, 30 µg), Ceftriaxone (CRO, 30 µg), Ceftazidime (CAZ, 30 µg), Cefuroxime (CXm, 30 µg), Aztreonam (ATM, 30 µg), Ciprofloxacin (Cip, 30 µg), Nitrofurantoin (F, 300 µg), Sulphamethoxazole / trimethoprim (SXT, 25 µg) and Gentamicin (CN, 10 µg).

Phenotypic detection of Extended-spectrum beta-lactamases (ESBLs)

Isolates resistant to ceftazidime antibiotic were tested for ESBLs production by disk diffusion method by using ceftazidim/clavulinic acid (CAZ-CAZ/CAV-30/10µg). The disk of ceftazidime (CAZ, 30 µg) and the disk of (CAZ/CAV, 30/10 µg), were placed on the inoculated Mueller-Hinton agar plates. The plates were incubated at 37°C for 18 h. Plates were examined and diameters of the inhibition zones were measured in mm, and interpreted according to **CLSI (2013).** The increase in diameter of inhibition zone of ceftazidime/clavulanate by 5mm or more over ceftazidime alone indicates ESBLs production.

Extraction of plasmid DNA

Five pure colonies of each organism were inoculated into 5ml of Luria-Bertani broth and inoculated for 20hr at 37 °C. Cells from 1.5 ml of the overnight culture were harvested by centrifugation at 12,000 rpm for 5 minutes. Plasmid DNA was extracted by using plasmid extraction kits obtained from Fermentas, Germany.

Plasmid DNA extracted from ESBLs producers was electrophoresed from agarose gel. The gel was prepared by adding 0.8% agarose to 100 ml TAE buffer and microwaved for few minutes for complete dissolution then 10µg /ml Eithidium-Bromide (Et-Br) were added for DNA staining after slightly cooling of agarose (**Sambrook and Russell 2001).** Extracted plasmid was mixed with 2µl of 10X loading buffer and DNA marker was loaded at first slot. The gel was visualized on UV transilluminator and photographed.

PCR amplification

To amplify TEM and SHV related genes from clinical isolates, the following oligonucleotide primers were used for PCR. *For bla*TEM-504bp gene, the forward primer was (TTGGGTGCACGA GTGGGTTA) and the reverse primer was (TAATTGTTGCCGGGAAGCTA). *For bla*SHV-626 gene, forward primer was (TCG GGCCGCGTAGGCATGAT) and reverse primer was (AGCAGGGCGACAATCCGC G) and for *bla*SHV -1,017bp gene, forward primer was (CGCCGGGTTATTCTTATTTGTCGC) and reverse primer was (TCTTTCCGATGCCGCCGCCAGTCA). A single reaction mixture (50 µl) contained 3µl of both forward and reverse primers, 25µl master mix, 1.5 µl of template and completed to 50 µl by nuclease free water was prepared. In thermal cycler, the following reaction parameters were followed: initial denaturation at 94°C for five minutes, Then denaturation at 95°C for 30 seconds, annealing at 52°C for 30 second for TEM and 68°C for 30 seconds for SHV, Extension at 72°C for one minute for 30 cycles, and final extension at 72°C for 10 minutes. Then the PCR products were separated by electrophoresis in 1.8% agarose gel, stained with eithidium Bromide (10µl for100 ml), and visualized by UV transilluminator and photographed. DNA ladder was used for size determination.

RESULTS:

Identification of isolates

Ninty (90) bacterial isolates were identified as 54 (60%) *E.coli*, 28 (31.1%) *klebsiella pneumoniae* and 8 (8.9%) *Proteus* isolates.

Susceptibility testing

The results of susceptibility are presented in **table (1)** and interpreted according to CLSI (2013)**.** The susceptibility of *E. coli* isolates

towards beta-lactam antibiotics showed high resistance toward Cefuroxime (88.9%) followed by ceftazidime (57.4%) and revealed sensitivity (68.5%) to both ceftriaxone and aztreonam. The susceptibility of *Klebsiella pnumoniae* isolates showed resistance (64.3%) to cefuroxime .while the isolates revealed sensitivity 64.3% and 60.7% respectively to cefotaxime and azteronam. While *Proteus* isolates showed resistance to cefuroxime (62.5%) and revealed sensitivity (62.5%) to ceftazidime.

Table (1): Determination of antimicrobial susceptibility pattern of clinical isolates to beta-lactams and non beta-lactams

Antimicrobial Disk	*E. coli*			*Kl. pneumoniae*			*PROTEUS spp.*		
	R	**I**	**S**	R	I	S	R	I	**S**
	No(%)	**No (%)**	**No (%)**	**No (%)**	**No (%)**	**No (%)**	**No (%)**	**No (%)**	No (%)
CAZ	17 (31.4%)	9 (16.7)%	28 (51.9%)	16 (57.1%)	1 (3.6 %)	11 (39.3%)	4 (50%)	2 (25%)	2 (25%)
CEP	33 (61.1%)	8 (14.8%)	13 (24.1%)	14 (50%)	5 (17.9%)	9 (32.1%)	1 (12.5%)	3 (37.5%)	4 (50%)
CXM	1 (1.8%)	5 (9.3%)	48 (89 %)	6 (21.4%)	4 (14.3%)	18 (64.3%)	1 (12.5%)	2 (25%)	5 (62.5%)
CRO	37 (68.5%)	4 (7.4%)	13 (24 %)	14 (50%)	4 (14.3%)	10 (35.7%)	2 (25%)	3 (37.5%)	3 (37.5%)
CTX	36 (66.7%)	6 (11.1%)	12 (22 %)	18 (64.3%)	2 (7.1%)	8 (28.6%)	3 (37.5%)	2 (25%)	3 (37.5%)
ATM	37 (68.5%)	5 (9.3%)	12 (22 %)	17 (60.7%)	1 (3.6%)	10 (35.7%)	3 (37.5%)	1 (12.5%)	4 (50%)
TE	7 (13%)	10 (18.5%)	37 (68 %)	0 (0%)	4 (14.3%)	24 (85.7%)	3 (37.5%)	3 (37.5%)	2 (25%)
CN	47 (87%)	3 (5.6%)	4 (7.4 %)	18 (64.3%)	1 (3.6%)	9 (32.1%)	3 (37.5%)	2 (25%)	3 (37.5%)
CIP	38 (70.4%)	2 (3.7%)	14 (26 %)	11 (39.3%)	3 (10.7%)	14 (50%)	3 (37.5%)	3 (37.5%)	2 (25%)
SXT	27 (27%)	1 (1.9%)	26 (48 %)	21 (75%)	1 (3.6%)	6 (21.4%)	1 (12.5%)	0 (0%)	7 (87.5%)
F	53 (98%)	0 (0 %)	1 (1.9 %)	10 (35.7%)	7 (25%)	11 (39.3%)	4 (50%)	2 (25%)	2 (25%)

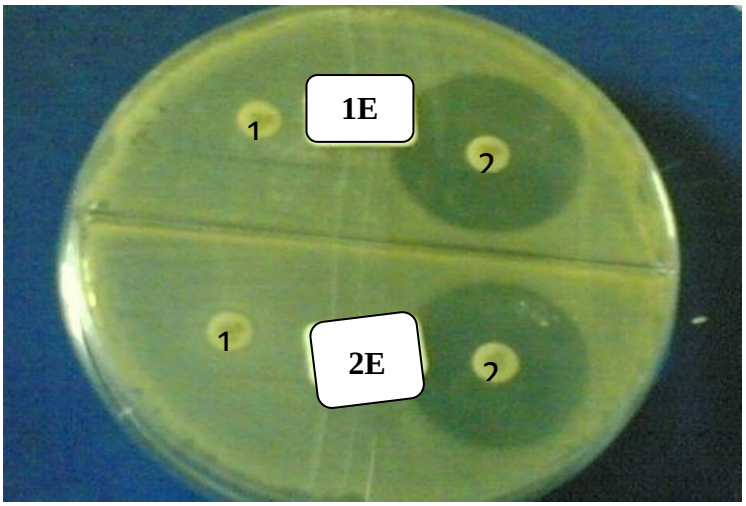
Caz=ceftazidim CEP=cefoperazon CXM= ceforuxim CTX=ccefotaxim CRO=ceftriaxone ATM=aztreonam CIP= ciprofloxacin TE=tetracycline CN=gentimicin F=nitrofurantoin SXT=Sulphamethoxazole-Trimethporime

On the other hand the susceptibility of clinical isolates to non beta-lactams showed that *E. coli* isolates had high sensitivity to nitrofurantoin (98%) followed by gentamycin

(87%) and ciprofloxacin (70.4%). while the same isolates showed resistance (68.5%) to tetracycline. In addition, *Klebsiella pnumoniae* isolates had high sensitivity reached about (75%) to sulphamethoxazole-trimethoprim followed by (64.3%) to gentamycin and showed high resistance toward tetracycline (85.7%) and (57.1%) to ciprofloxacin. *Proteus* isolates revealed sensitivity (62.5%) to nitrofurantoin followed by ciprofloxacin (37.5%) and high resistance (87.5%) to sulphamethoxazole-trimethoprim.

Phenotypic detection of Extended Spectrum beta- lactamases (ESBLs)

Detection of ESBLs was done by Double Disk Diffusion method (**figure 1**) and interpreted according to CLSI (2013). The enhancement in zone of inhibition by 5mm or more of ceftazidim/clavulinic acid over ceftazidime alone is indicative of a positive test. Among the 90 clinical isolates 30 (33%) were ESBLs producers. Whereas (40.7%) *E.coli* isolates were ESBLs producers, (21.4%) of *K. pneumoniae* and 25% of *Proteus* isolates were ESBLs producer



1=ceftazidime 2=ceftazidime/clavulinic E= *E.coli*

Figure 1: presentive of Extended-spectrum B-lactamase screening in *E.coli* isolates 1 and 2.

The susceptibility of ESBLs to non beta-lactams

The ESBLs producers showed high susceptibility to nitrofurantoin (83.3%) and gentamicin (70%) and ciprofloxacin (63.3%). On other hand, isolates revealed high resistant to tetracycline (84.6%) followed by sulphamethoxazole-trimethoprim (56.7%) (**Table 2**)

PCR amplification

The thirty ESBL producers identified as 22 *E. coli*, 6 *K. pneumoniae* and two *Proteus* isolates were subjected to plasmid screening as shown in **figure (2).**

The ESBLs producers were subjected to plasmid extraction and screened by gel electrophoresis. The results revealed (15/30) 50% of ESBLs producers have only one plasmid and (9/30) 30% have two plasmids, (4/30) 13.3 % have 3 plasmids and finally only two isolates (6.7%) have four plasmids

The ESBLs isolates were subjected to amplification of *bla TEM* and *bla SHV* genes. The *bla*TEM -504bp was amplified in 14 out of 22 (63.6%) *E. coli* ESBL producers and in 2 out of 6 (33.3%) *K. pnumoniae* isolates (**Figure 3**). Meanwhile; TEM was not detected in any *Proteus* isolates. The *bla* SHV-626 *bp* was amplified in 2 *K. pneumoniae* isolates as shown in figure (4). While *bla*SHV-1,107 bp was not detected in any isolate in this investigation

Table (2): Susceptibility of ESBLs isolates to non beta-lactams

Non beta-lactams	Susceptibility of ESBLs isolates	
	Resistant %	**Sensitive %**
Ciprofloxacine	36.7	63.3
Tetracycline	84.6	14.9
GentamIcine	30	70
sulphamethoxazoletrimethoprime	56.7	43.3
Nitrofurantoin	13.7	83.3

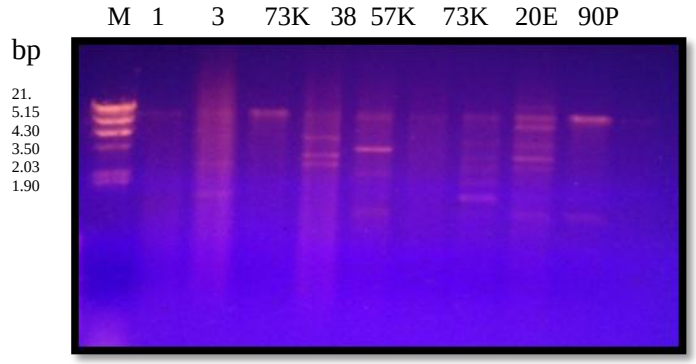


Figure 2: plasmid profiles of representive isolates on agarose gel electrophoresis M=molecular weight marker *E=E.coli K=K. pneumoniae P=Proteus isolates*

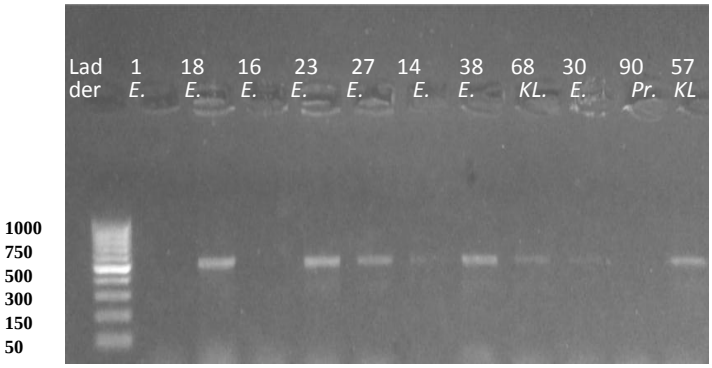


Figure 3: PCR amplification of TEM (504 -bp) genes from of representive clinical isolates on agarose gel electrophoresis. M=molecular weight marker *E=E.coli K=K. pneumoniae P=Proteus*

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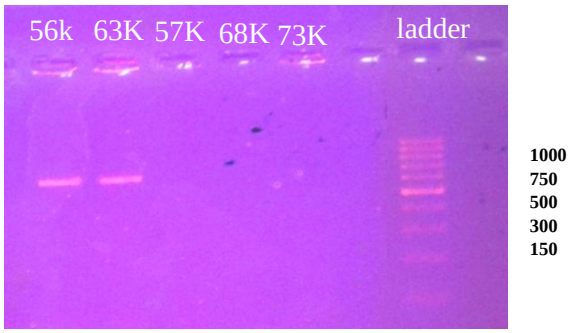

Figure 5: PCR amplification of SHV (626-bp) from plasmid extracts of representive clinical isolates on agarose gel electrophoresis. M=molecular weight marker E=E.*coli K=K. pnumoniae*

DISCUSSION:

Ninety clinical isolates were obtained from different clinical specimens of urine, blood, sputum and pus. The frequency of isolates was as follows: (60% *E.coli, 31.1% Klebsiella pneumoniae and 8.9% Proteus spp.).* These isolation rates were in agreement with that reported by Fam *et al*. (2014) and Alipourfar and Nili. (2010).

The isolates were tested for their antimicrobial susceptibilities by the Disk Diffusion technique and interpreted according to the CLSI (2013). Susceptibility of *E. coli* clinical isolates towards beta-lactam antibiotics (table 1) revealed high sensitivity of isolates to ceftriaxone which was lower than that obtained by Hryniewicza *et al*. (2010). In this study high resistance of *E. coli* isolates to ceforuxime was similar to that reported by Leveristin *et al*. (2003). Moreover, the resistance to ceftazidim was lower than that reported by Hryniewicza *et al*. (2010).

The susceptibility of *K. pneumoniae* isolates toward beta-lactam antibiotics (table1) showed high susceptibility of *K. Pnumoniae* to cefotaxim and azteronam which was lower than that reported by Romanus *et al* (2013). On the other hand, high resistance of *K. pneumoniae* to ceforuximewas in agreement with that reported by Ullah *et al*. (2009) and lower than that reported by Romanus *et al.* (2013). The resistance to ceftazidime in this study was in agreement with that reported by Lee *et al.* (2006).

High susceptibility of *Proteus* isolates to both cefotaxim and azteronam but the susceptibility of *Proteus* was lower than that reported by Wang *et al*. (2014) and Adamus *et al.* (2014). On the other hand; resistance of *Proteus* isolates to ceforuxime, ceftazidim and cefoperazone was lower than that obtained by Wang *et al*. (2014).

Bacterial resistance to these beta-lactams has increased dramatically with ESBLs contributing to this increase (Paterson and Bonomo 2005). All isolates were subjected to susceptibility tests to non beta-lactam antimicrobial agents to be used as another choice in treatment in case of resistance or development of resistance of bacteria towards beta-lactams.

In this study susceptibility of *E. coli* isolates to non beta-lactams as nitrofurantoin, Gentamicin and ciprofloxacin (table 1) was higher than the results obtained by Alipourfard and Nilli (2010). *E. coli* isolates showed high resistance to tetracycline and that was in agreement with that reported by sabir *et al*. (2014).

Susceptibility of *K. pneumoniae* isolates to non beta-lactams revealed high susceptibility to sulphamethoxazole-trimethoprime and this sensitivity was higher than that reported by Romanus *et al.* (2013). Susceptibility to Gentamicin was in agreement with that reported by lee *et al.* (2004). *Klebsiella pneumoniae* isolates in this study revealed high resistance to tetracycline which was higher than that reported by lee *et al.* (2004).

Sensitivity of *Proteus* isolates to nitrofurantoin was in agreement with that reported by Romanus *et al*. (2013) while the sensitivity to ciprofloxacin was higher than that reported by Tijjani *et al*. (2012). Resistance of *Proteus* isolates to Gentamicin was in agreement with that reported by Tijjani *et al*. (2012).

In Gram-negative pathogens, beta-lactamases remain the most important contributing factor to beta-lactam resistance, and their increasing prevalence (Medeiros, 1997). In this study, 33.3 % of isolates were ESBLs producers which was lower than that reported by Kaur and Aggarwa (2013) and was higher than that reported by Kumar *et al.* (2014).

The highest percentage of ESBL production was shown among *E. coli* (40.7%) in agreement with that reported by Kumar *et al*. (2014). On the other hand,

25% of *Proteus spp*. and 21.4% of *K. pneumoniae* were ESBL producers. In a study performed by Kumar *et al*. (2014), ESBLs were predominantly present among *Klebsiella spp*. (32.8%), while in *Proteus spp.* Only 1.6% of the isolates were ESBLs producers.

The susceptibility of 30 (33.3%) ESBL isolates showed high resistant to tetracycline and sulphamethoxazole-trimethoprime which is higher than that reported by Morosini *et al.* (2006) and Hosoglu *et al.* (2007).

Susceptibility of ESBLs producers to nitrofurantoin (83.3%) was in agreement with that reported by Hosoglu *et al.* (2007). In addition, the high susceptibility of ESBLs producers to gentamicin and ciprofloxacin were in agreement with that reported by Paterson and Bonomo (2005).

ESBLs are often encoded by genes located on plasmids and on chromosome. These plasmids might carry genes of resistance to other antimicrobial agents. Plasmid DNA extracted from ESBLs producers were screened for the presence of TEM, and SHV genes using the PCR amplification technique. Amplifying fragment of approximate 504-bp for *blaTEM* gene; 626-bp and 1,017-bp for *blaSHV* genes were performed. The *blaTEM* gene, which is responsible for resistance to ESBLs-lactams was present in 14 out of 22 (63.6%) of *E. coli* isolates, 2 out of 6 (33.3%) of *K. pneumoniae* and not detected in any *Proteus* isolates. *The blaSHV* gene (626-bp) gene was detected only in two *k. pneumonia* isolates. The *blaSHV* gene (1,107) was not detected in any clinical isolates. The data presented in this study revealed the predominance of *blaTEM* genes in ESBLs producers in these hospitals.

Cao *et al.* (2002) reported that *bla*TEM genes were found in (84.3%) of *E. coli* isolates and (38.4%) *of K. pneumonia* isolates and were detected in all *Proteus* isolates.

In one study, the bla SHV-like genes were found in 54% of *K. pneumonia*e isolates and in (44%) *E. coli* isolates and not detected in *Proteus spp*. (Cao *et al.*, 2002).This study indicated that the 20 isolates out of 30 (66.6%) which were ESBLs, 53.3% were carrying *blaTEM* gene, 6.6% were carrying the *blaSHV* gene. Perilli *et al.* (2002) reported that 88% were found to carry either the *blaTEM* or the *blaSHV* gene or both genes .Of those 88% of isolates 32.6% were carrying *blaTEM* genes 35% were carrying the *blaSHV* genes

CONCLUSION

This paper aims to illustrate the spreading of extended-spectrum b-lactamase-(ESBL) producing bacteria among emerging pathogens of *Enterobacteriac*e members in this community. The data revealed the predominance of *blaTEM* genes in ESBLs isolates in these hospitals. According to this investigation, the drugs recommended to be used in treatment of such infection were nitrofurantoin, gentamicin and ciprofloxacine (quinolone, aminoglycides).

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التعرف علي جيني التيم و الشيف بين العزلات الاكلينكية من عائلة الأنتيروبكتريسي والمنتجة لإنزيمات البيتا لاكتاميز

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تهدف هذه الدراسة إلى التوصيف الجزيئي لإنزيمات البيتا لاكتاميز الممتدة المجال والمنتجة بواسطة العزلات الإكلينيكية من عائلة الأنتيروبكتريسي والتي تضم ايشريشيا كولاي والكليبسلا نيومونيا والبروتيس بواسطة الطرق المظهرية مثل طريقة انتشار القرص والطرق الوراثية مثل تفاعل البلمرة المتسلسل وذلك باستخدام البريمرات الخاصه بجينات البيتالاكتاميز.

تسعون من العزلات الإكلينيكية تم الحصول عليها من ٢٢٠ عينة طبية مختلفة (٦٠%) من العزلات تم التعرف عليها كايشريشيا كولاي في حين تم التعرف علي ميكروب الكليبسلانيومونيا (٣١,١%) بالاضافه الي(٨,٩%) ميكروب البروتيس.

خضعت العزلات لاختبارات الحساسيه لكل من المضادات الحيوية البيتالاكتام والمضادات الحيوية منغيرمجموعه البيتالاكتام بواسطة طريقة إنتشار القرص الاجاري. من التسعين عزله إكلينيكية ٣٠ من العزلات بنسبة (٣٣,٣%) منتجة لإنزيمات البيتالاكتاميز الممتدة المجال حيث كانت أعلى نسبة للعزلات المنتجة لإنزيمات البيتالاكتاميز الممتدة المجال بين عزلات الايشريشيا كولاي (٤٠,٧%) تبعها عزلات البروتيس بنسبه (٢٥%) ثم الكلبسيلا نيومونيا (٢١,٤%).

تم عمل مسح للبلازميدات لجميع العزلات المنتجة لإنزيمات البيتالاكتاميز الممتدة المجال والتي تتحكم في مثل تلك الانواع من جينات المقاومه للمضادات الحيوية

و عليها باستخدام جهاز البلمرة SHV و TEMالبلازميدات المستخلصة من تلك العزلات تم فحص وجود جينات الـ المتسلسل.

في ١٤ عينة من ٢٢ عينة من عزلات الإيكولاي بنسبة في عينتين من TEM 504- bpتم التعرف علي جين الـ الكلبسيلا نيومونيا، ولم يتم التعرف عليه في عزلات والبروتيس.

SHV1017- في عينتين من الكلبسيلا نيومونيا،في حين لم يتم التعرف علي وجود SHV626-bpعلاوة على ذلك تم التعرف علي في أي من تلك العزلات الإكلينيكية