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Science Journal of University of Zakho

Vol. 5, No. 1, pp. 1 – 6, March-2017

p-ISSN: 2410-7549
e-ISSN: 2414-6943

DISTRIBUTION OF EXTENDED SPECTRUM β-LACTAMASE GENES AMONG *PROTEUS MIRABILIS* ISOLATED FROM CLINICAL SPECIMENS IN DUHOK CITY, KURDISTAN REGION, IRAQ

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Received: Oct. 2016 / Accepted: Feb. 2017 / Published: Mar. 2017

ABSTRACT:
Extended Spectrum Beta Lactamase-among *Proteus mirabilis* strains recorded high incidence leaving few therapeutic options of potential infections. The purpose of current study was to assess the prevalence of antibiotic resistance among Extended Spectrum Beta Lactamases (ESBL) producing *P. mirabilis*, in addition to molecular characterization of the ESBL gene-types using PCR. All isolates were fully identified, checked for antibiotic susceptibility and ESBL production using, double disk synergy phenotypic method. Positive ESBL-producing isolates were subjected to PCR assay using specific primers for detection of CTX-M, TEM and SHV genes. The majority of the isolates exhibited absolute susceptibility (100%) to both meropenem and ertapenem and high susceptibility (95%) to imipenem, while co-resistance were expressed toward cefotaxime, ceftazidime, ceftriaxone and other non-lactam antibiotics. Out of 37 isolates, 21(57%) were ESBL-producers and using a double-disc synergy test (DDST). Using molecular-based PCR, CTX-M (81%), TEM (57%) and SHV (24%) were determined among ESBL-positive. CTX-M was predominant and circulating among phenotypic multiple resistant strains. Moreover, coexistence of CTX-M and TEM gene was more frequent combination. The study highlighted the increasing levels of low antibiotic susceptibility among *P. mirabilis* harbored ESBL genes at Duhok city and also confirms that a high level of *bla*CTX-M-positive ESBL isolates is circulating in this area.

KEYWORDS: Proteus mirabilis, ESBL, PCR.

1. INTRODUCTION

1.1 Instructions

In enterobacteriaceae, Extended Spectrum Beta Lactamases (ESBL) are mainly produced by *Escherichia coli*, *Klebsiella pneumoniae*, or *Proteus mirabilis* strains responsible for nosocomial infection. These strains are disseminated worldwide (Mabilat and Courvlin, 1990). *Proteus mirabilis* is unique species among enterobacteriaceae and is the common cause of several types of infections predominantly urinary tract infections (UTI), mainly after prolonged hospitalization. ESBL are enzymes which are formed by gram negative bacilli which facilitate resistance to the penicillins, cephalosporins and the monobactams and are commonly known as Enterobacteriaceae (Kaur and Aggarwal, 2013). *K. pneumoniae* and *E. coli* are well-known pathogens producing ESBL enzymes particularly those isolates from hospital location. Recently, *P. mirabilis* isolates recorded high level of incidence of ESBL-production, namely in hospital environments, leading to uncontrolled and little treatment options of various infections (Tonkic *et al.*, 2010). This study highlighted the diversity of β-lactamases in *P. mirabilis*; firstly TEM defined then AmpC type β-lactamases reported and recently non-TEM and SHV-derived ESBL have been described (Chanal *et al.*, 2000).
Data on ESBL-producing isolates of *P. mirabilis* in Duhok city are limited, so the goals of the current work is to find out the rates of antibiotic-susceptibility among *P. mirabilis* isolates in various clinical specimens among inpatients at Azadi Teaching Hospital, Duhok city and molecular characterization of the ESBL genes of the isolates using PCR assay.

2. MATERIALS AND METHODS

2.1 Bacterial strains

A total of 37 consecutive non-duplicate *P. mirabilis* were isolated from the clinical specimens including urine, wound and the middle ear of patients admitted to Azadi Teaching Hospital, Duhok city from December 2014 to May 2015.

2.2 Bacterial Identification

Bacterial isolates were identified using conventional microscopic and biochemical tests: Gram staining, motility, swarming behavior, indole production, phenylalanine dehydrogenase, ornithine decarboxylase, gas production from glucose, H2S production, urease, tryptophan deaminase, lysine decarboxylase, and citrate and lactose utilization (Koneman et al., 1992).

2.3 Antimicrobial Susceptibility Testing

The isolated bacteria were identified according to the standard method of biochemical tests. Antibiotic-susceptibility rates against different antibiotics were determined by the method of disc diffusion assay using Mueller–Hinton agar (CLSI, 2006).

2.4 Determination of ESBL Production

Determination of the ability of the production of ESBL was performed through double-disc synergy test (DDST). Disks containing cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg) were placed 25 mm (center to center) from an amoxicillin-clavulanic acid disk (30 and 10 µg, respectively), incubated at 35°C overnight. Probable indication of ESBL production was observed through a synergy between any one of

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the cephalosporins and amoxicillin/clavulanic acid (CLSI, 2006). The ESBL-producing *P. mirabilis* isolates were further analyzed and their ESBLs were molecularly characterized.

2.5 Detection of ESBL Genes by Polymerase Chain Reaction

The genomic DNA was extracted from 21 positive ESBL isolates of *P. mirabilis* using DNA extraction kit (aidgen, Korea). The ESBL genes, TEM, SHV, CTX-M were detected using specific pair of primers for each gene (table 1), (EUROFINS, mwg, operon, Germany). Primer sequences and amplification settings were performed as described by Yun-Tae *et al*. (2006); Adamus-Bialek *et al*. (2009). The presence of the PCR product was confirmed electrophoretically using 1.5% (w/v) of agarose. Molecular marker (100-1500bp) was used to determine the molecular weight of the PCR product.

Table 1. Primers used for the detection of ESBL genes

Primer	Primer sequences	Amplicon size
CTX-M/F CTX-M/R	5′-CGCTTTGCGATGTGCAG-3′ 5′-ACCGCGATATCGTTGGT 3′	551 bp
TEM/F TEM/R	5′-ATAAAATTCTTGAAGAAGACGAAA-3′ 5′-GACAGTTACCAATGCTTAATC -3′	1080 bp
SHV/F SHV/R	5′-TCGTTATGCGTTATATTCGCC-3′ 5′-GGTTAGCGTTGCCAGTGCT -3′	861 bp

3. RESULTS

The majority of *P. mirabilis* isolates 20/37 (54%) were recovered from urine followed by 10/37(27%) isolates from wounds and 7/37(19%) from the middle ear.

3.1 Antibiotic Susceptibility Rates

Table (2) indicates that 21/37 (57%) from the tested isolates were producers of ESBL using a double-disc synergy test, and all of them exhibited absolute susceptibility (100%) to both meropenem and ertapenem and high susceptibility (95%) to imipenem. On the other hand, moderate to low susceptibility rates were expressed against amoxicillin-clavulanic acid, cefepime, ceftazidime, ceftriaxone and cefotaxime which were 61, 38, 19, 19, and 0%, respectively. While low susceptibility rates were observed with other non-Beta-lactams tested antibiotics like ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin and amikacin. Moreover, 66.6% of ESBL isolates expressed Multiple-Drug Resistance (MDR) phenotype. While, non-ESBL-producer isolates recorded high to moderate susceptibility rates toward tested antibiotics used in this study as shown in table (2).

Table 2. Antibiotic susceptibility rates of all 37 *P. mirabilis* isolates included ESBLs-positive and non-ESBL producers

Antibiotics	**Symbol**	**ESBL-positive (n=21 isolates) (%*)**	**Non-ESBL-producer (n=16 isolates) (%*)**
Amoxicillin+clavulanic acid	AMC	61	94
Cefepime	FEP	38	94
Ceftazidime	CAZ	19	94
Ceftriaxone	CRO	19	94
Cefotaxime	CTX	0	81
Imipenem	IMP	95	100
Meropenem	MEM	100	100
Ertapenem	ERT	100	100
Amikacin	AK	57	63
Gentamicin	CN	33	38
Trimethoprim-sulfamethoxazole	SXT	33	25
Ciprofloxacin	CIP	24	25
Amoxicillin	AX	10	31
Nitrofurantoin	F	10	31
Ampicillin	AM	10	19
		66.6%**	40%**

* Susceptibility rate

** Percentage of resistance collectively to all used antibiotics

3.2 Detection of ESBL genes by PCR

The frequency of CTX-M, TEM and SHV singly or multiply among ESBL-positive isolates were, 17/21, 81(%), 12/21, (57%) and 5/21, 24(%), respectively. Co-presence of all of the three gene groups was significantly more encountered among *P. mirabilis* isolates. In 7(33.3%) of the isolates, simultaneous appearance of TEM + CTX-M combination was predominant genes coexistence among isolates comparing with SHV+ CTX-M and SHV+CTX-M+TEM combination that recorded at a rate of 9.5% for each as shown in table (3) figures (1,2, and 3).

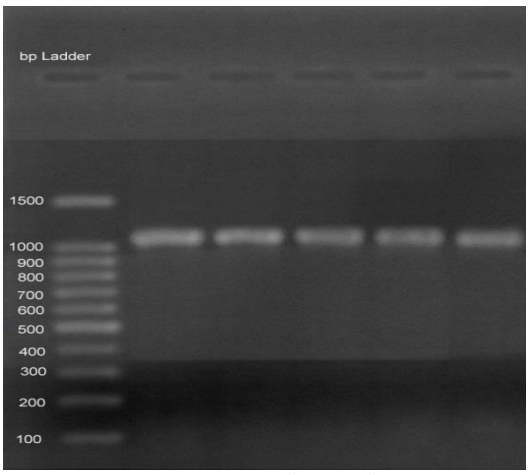

Figure 1. PCR amplification of TEM

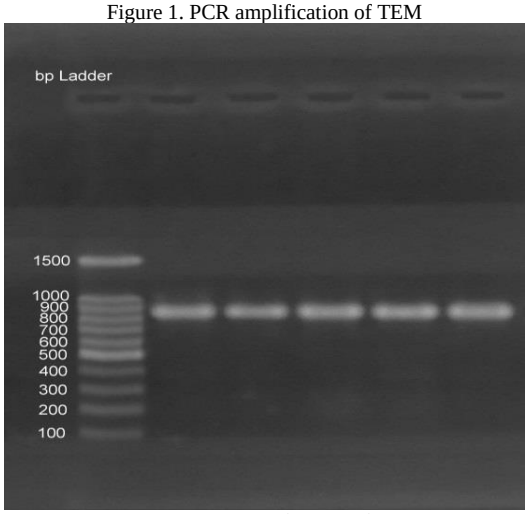

Figure 2. PCR amplification of SHV

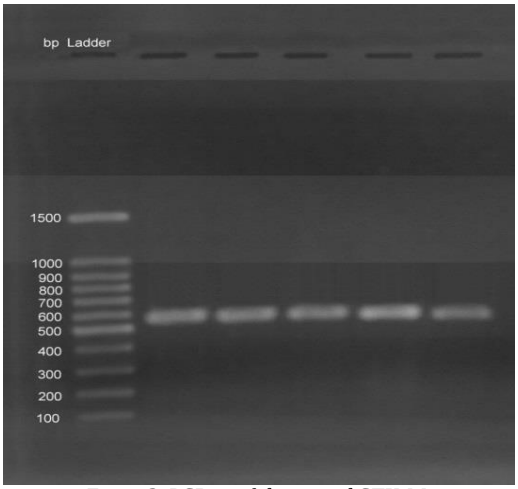

Figure 3. PCR amplification of CTX-M

3.3 Prevalence of ESBL genes among multiple antibiotics-resistant-ESBL-positive P. mirabilis isolates

The gene CTX-M was the most prevalent ESBL gene either singly or in combination with SHV and TEM. CTX-M was the most frequently detected gene within isolates particularly those expressed high resistance toward tested antibiotics in this study. Isolates number 18 and 20 simultaneously recorded 87% and 80% resistance rates and harbored CTX-M as a single gene and TEM+CTX-M+SHV in combination genes, respectively. The least frequently detected genes were SHV, except for isolates number 7 that showed 73% resistance rate and harbored SHV only as shown in table (4).

Table 3. Occurrence of single and multiple ESBL genes among positive-ESBL *P. mirabilis* isolates

ESBL gene types	No. (%) of isolates
TEM only	3 (14.2)
SHV only	1 (5)
CTX-M only	6 (28.5)
TEM+CTX-M	7 (33.3)
SHV+CTX-M	2 (9.5)
TEM+SHV+CTX-M	2 (9.5)
Total	21

Table 4. Prevalence of ESBL genes among 21 multiple-antibiotics resistant-ESBL-positive *P. mirabilis* isolates

Isolates	Resistance rates to selected antibiotics %	ESBL harboring genes
1	67	TEM+CTX-M
2	67	TEM+CTX-M
3	53	CTX-M+SHV
4	47	CTX-M
5	60	CTX-M
6	67	TEM+CTX-M+SHV
7	73	SHV
8	60	TEM+CTX-M
9	60	CTX-M
10	40	TEM+CTX-M
11	53	TEM+CTX-M
12	40	CTX-M
13	53	CTX-M
14	67	TEM+CTX-M
15	53	CTX-M
16	47	CTX-M+SHV
17	67	CTX-M
18	87	CTX-M
19	53	CTX-M
20	80	TEM+CTX-M+SHV
21	60	TEM+CTX-M

4. DISCUSSION

Nowadays, there is an increasing use of the broad-spectrum antimicrobial agents, which increased the incidence of ESBL-producing enterobacteriaceae worldwide at a worrying rate. Currently, the major task for infection control teams is the prevention of the appearance and the spread of ESBL-producing enterobacteriaceae (CLSI, 2006). Some isolates of *P. mirabilis* are susceptible to β-lactams, but resistance may be acquired due to the production of β-lactamases (Hassan *et al*., 2013)**.** In the present study, out of 37 isolates of *P. mirabilis*, 21(57%) were ESBL producing using phenotypic double disk method. This result is in accordance with other studies performed in Iraq, in which rates of 34.6, 40, 42 and 55.5% of *P. mirabilis* were ESBL producers, respectively (Jarjees, 2006; Al-Haidari, 2010; Hussein, 2013; Ahmad and Ali, 2014). The result of the current study indicates that our area is among the countries having high level of ESBL production among *P. mirabilis* isolates than other places like Saudi Arabia, Croatia, Poland France and Greece which have 3.1, 12.6, 14.5, 3.3 and 5.9% , respectively (Nijssen *et al.*, 2004; Alghamdi 2006; Empel *et al.*, 2008; Tonkic, *et al.*, 2010; Al-Haidari 2010) .This may be due to the differences in the type and amount of consumption of antibiotics, indicating that patients were infected with ESBL producing *P. mirabilis* which might increase the risk of treatment failure with expanded spectrum β-lactamase antibiotics. These could be also due to the fact that in more developed countries effective policies for the control of antimicrobial agents are present, which successfully prevents the occurrence of ESBL. Additionally, the rates of the drug resistance were higher in the ESBL producers than in the non-ESBL producers in this study. Observations of a high level of antibiotic resistance among strains analyzed in the present study are disturbing, but they are in agreement with previously published results, that showed high susceptibility of ESBL *P.*

mirabilis to meropenem, ertapenem and imipenem (Ojdana *et al*., 2014). The present study also documents that the ESBL producers are also co- resistant to other non- β-lactam antibiotics and their susceptibility was low, to Ciprofloxacin (24%), gentamicin (33%), trimethoprim-sulfa (33%), and are only 10% susceptible to amoxicillin and ampicillin. These results agree with those of Jarjees (2006); Tonkic *et al.* (2010), Hussein (2013); Ahmad and Ali (2014). Moreover, 66.6% of ESBL-positive strains in this study showed simultaneous resistance to both β-lactams and antibiotics of other groups that could defined as multidrug-resistant strains (MDR). This feature is already has been observed by Poirel *et al.* (2000); Luzzaro *et al.* (2001); Ojdana *et al.* (2014), which might be due to an association between ESBL production and resistance to aminoglycosides, because ESBL genes are near or are at close places to multiple resistances gene cassettes that coordinated the occurred expression . In enterobacteriaceae, resistant genes are located close to each other on the plasmids, where genes responsible for resistance to different groups of antibiotics may be located in a close neighborhood thus; they may be transmitted at the same time to other bacteria (Ishii *et al*., 1995; Leverstein-vanHall *et al.,* 2002; Karisiki et *al.,* 2006). So, this is considered as the biggest problem in the treatment of infections caused by gram-negative bacilli.

The gene CTX-M variants have displaced TEM and SHV enzymes as the predominant β-lactamases producers that reflects remarkable increase in this genotype in this area of Iraq. This is consistent with many published reports worldwide, for example, a study in Japan reported the prevalence of CTX-M genotypes among ESBL-producing *P. mirabilis* isolates (Leverstein-vanHall *et al*., 2002; Nakamura *et al*., 2012. A similar finding reported in Poland by Ojdana *et al.* (2014), Polish assistants ensured also that their isolates of *P. mirabilis* were generally producing CTX-M (Empel *et al*., 2008). Similar data was reported by Moghaddam *et al*. (2014). This study is inconsistent to a study in Baghdad, Iraq in which high prevalence of TEM was reported (Al-Jubori *et al.,* 2012). Moreover, studies from France, India, Croatia, Italia and China illustrated the predominance of TEM genotype (Chanal *et al.,* 2000; Tonkic *et al.,* 2010, Kaur and Aggarwal, 2013, and Huang *et al.,* 2014). Furthermore, some other studies concluded the absolute circulation of the TEM genotype (Endimiani *et al.,* 2005; Perilli *et al.,* 2002). While TEM ESBL was not detected in a study conducted in Saudi Arabia (Hassan and Abdalhamid, 2014). SHV genotype was less encountered in the present study; similarly other studies did not succeeded in detecting this gene (Al-Jubori *et al*., 2012; Hassan *et al.,* 2013; Ojdana *et al.,* 2014). The presence of more than one ESBL in a single isolate was frequently detected in addition to the coproduction of all the three genes (Coexistence of CTX-M and TEM genes) in 7/21 (33%). This scenario was also clarified by Hassan and Abdalhamid (2014). Also, TEM genotype was predominant genotype among ESBL *P. mirabilis* producers in other studies performed in France, India, Croatia and Italy (Chanal *et al*., 2000; Tonkic *et al*., 2010; Nakamura *et al.,* 2012; Kaur and Aggarwal, 2013). The coexistence of CTX-M and TEM highlights the growing of the complexity of antibacterial resistance problems, and the reasons for this situation require further investigation.

The analysis of the resistant *bla* genes revealed that *P. mirabilis* isolates carrying the *bla*CTX-M gene, was the most prevalent phenotype of resistance towards selected antibiotics. It is noteworthy that isolates number 18 and 20 simultaneously recorded 87% and 80% resistance rates and harbored CTX-M as a single gene and TEM+CTX-M+SHV in combination, respectively. In addition, the dominance of CTX-M gene-type among high resistance isolates and multiple-resistant drugs (MRD) was revealed. Same data were reported by Nijssen *et al.* (2004); Ojdana *et al.* (2014); Huang *et al.* (2014), who attributed it to the likelihood of further distribution of *bla* CTX-M gene among multiple-resistant *P. mirabilis* strains. In this study, SHV gene-type was the least frequently detected gene; only detected in isolate number 7 which showed 75% resistance to tested antibiotics. The variability of dissemination of ESBL in *P. mirabilis* retells us it's significant to detect the genotype and the antimicrobial susceptibility pattern which are critical in the treatment of infections caused by ESBL-positive bacteria.

5. CONCLUSION

The increasing levels of resistance to antibiotics by ESBL-producing *P. mirabilis* strains in Duhok city are alarming and denote severe helpful questions. This study demonstrates the presence of a high level of *bla*CTX-M-positive ESBL isolates which are circulating in this area. The tendency of multidrug-resistant profiles associated with the recovery of the *bla*CTX-M gene is worrying. An indiscriminate use of the higher antibiotics should be restricted as far as possible and further monitoring and screening studied for ESBL production must be included in all clinical laboratories in this area.

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کورتیا لێکولینێ:

بەلاڤ بوونا جینێن بێتا لاکتمەیز یا بەرفرە ل ناڤ بەکتریایێن Proteus mirabilis ل نمونەیێن جهی ل باژێرێ دهوکێ / هەرێما کوردستانا عیراقێ بوونا ئەنزیمێ بێتا لاکتیمەیزێ جورێ کەڤانێ بەرفرە (ESBL) ل ناڤ میکروبێ Proteus mirabilis تومارەکا مەزن ئەنجام دایە کو بویە ئەگەرێ نزمکرناکارێن ئەنتی بایوتیکا ب تایبەتی ل تووشبونێن ب هێز،ئارمانجا ڤێ ڤەکولینێ هەلسانگاندنا خوراگریا ئەنتی بایوتیکانە ل سەر میکروبێ Proteus mirabilis خودان ئەنزیمێ بێتا لاکتیمەیزێ جورێ کەڤانێ بەرفرە) ESBL)، هەروەسا نیاسینا سالوخەیێن جینێن نە دیار ل ڤان ئەنزیمان دا،ب رێکا PCR. هەمی بەکتریایێن جوداکری بو خوێندنێ هاتنە نیاسین و دەستنیشانکرن و هەروەسا تاقیکرنێن خوراگرتنا بەکتریا بو ئەنتی بایوتیکان و بەرهەمئینانا ئەنزیمێ بیتا لاکتیمەیەز ب رێکا double disk synergy phenotypic هاتنە ئەنجام دان، هەمی بەکتریایێن جوداکەر پێکئینەرێن ئەنزیمێ ب رێکا PCR جینێن نەدیار وەك CTX-M,TEM, SHV هاتنە دەستنیشانکرن ، ب رێژا ١٠٠% ئەڤ میکروبێن جوداکەر هەستیار بوون بو ئەنتی بایوتیکێن meropenem و ertapenem و ب رێژا ٩٥% هەستیار بوون بو imipenem ، هەروەسا هەستیارەکا هاڤسەنگ دگەل جورێن cefotaxime, Ceftazidime, ceftriaxone ، ژ ٣٧ جوداکەرا ب تنێ ٢١ (٥٧%) ژ جورێن) ESBL) بوون بریکا double-disc synergy، بەلێ رێژێن دی(٨١% ژ CTX- M،57% ژ TEM، و ٢٤% ژ ESBL (SHV هاتنە نیاسین . هەروەسا ئاشکرا بوو کو جینێ CTX- M ژ هەمیان بەلاڤتر بوو و هەردوو جینێن CTX- MوSHV پێکڤە یێ بەربەلاڤ بوو، ڤێ خوێندنێ ئاستێ بلندێ تووشبوونێ ب میکروبێ Proteus mirabilis ژ جورێن(ESBL) ل باژێرێ دهوکێ ئاشکرا کر،هەروەسا دیار کر کو رێژا بلندا وان ژ هەلگرێن جورێ جینێ CTX- M بوون.

خلاصة البحث:

الهدف من الدراسه الحاليه هو معرفة مدى انتشار نزيم البيتا لاكتميز واسع الطيف في جرثومة *Proteus mirabilis* والذي ادى الى ترك مجال ضيق للمضادات الحياتيةالمستخدمه لعلاج الاصابات القوية,اضافة الى الوصف الجزيئي لانواع الجينات المشفرة لهذا الانزيم باستخدام طريقة PCR . تم تشخيص جميع العزلات بشكل كامل وبما فيها فحص حسياسيتها تجاه المضادات الحياتية وانتاج انزيم البيتا لاكتميز واسع الطيف باستخدام طريقة القرص الثنائي المتساند.كذلك تم اخضاع العزلات المنتجة لهذا الانزيم لتقنية PCR باستخدام باديئات متخصصة لتحديد الجينات المشفرة لهذا الانزيم مثل جين CTX-M,TEM, SHV.لقداظهرت هذه الدراسة بان الغالبية المطلقة لهذه العزلات كانت حساسة (١٠٠%) تجاه المضادين meropenem و ertapenem وذات حساسية عالية (٩٥%) تجاه imipenem بينما كانت ذات مقاومة معتدلة نحو مضادات حياتيةاخرى مثل الcefotaxime, Ceftazidime وceftriaxone ومضادات اخرى من نوع اللا بيتالاكتم. من بين ٣٧ عزلة كانت ٢١(٥٧%) موجبة لانتاج هذا الانزيم باستخدام طريقة القرص الثنائي المتساند.استخدام الطرق الجزيئية المبنيه على تقنية PCR بينت نسب الجينات الثلاث كالاتي (81%CTM)-M(وTEM)57%و (SHV)24% ضمن الحالات المنتجه لهذا الانزيم. كان جين CTX-M الاكثر سيادة وانتشارا بين العزلات المقاومة للمضادات الحياتية . اضافة الى ذلك كثرتواجدالجينين CTX- MوSHV معا ضمن العزلات المقاومة.كذلك سلطت الدراسة الضوء على المستويات العالية لجرثومة *Proteus mirabilis* المقاومة للمضادات والحاويه للجينات المنتجة لانزيم بيتا لاكتميز واسع الطيف في مدينة دهوك وكذلك اكدت الدراسة بان نسبة عالية من العزلات المنتجة لهذا الانزيم والحاوية على جينCTX- M كانت الاكثر تواجدا في هذه المنطقة.