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RESEARCH ARTICLE

OCCURRENCE OF ESBL AND MBL GENES IN *Pseudomonas aeruginosa* AND *Acinetobacter baumannii* ISOLATED FROM BAGHDAD, IRAQ

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ABSTRACT

A total of Ninety two clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolated from various infections were tested for extended spectrum β- lactamase (ESBL) and metallo-β-lactamase (MBL) production by double disc synergy and disc potentiating tests. The prevalence of ESBLs was 35.2% and 25% of *A.baumannii* and *P.aeruginosa* respectively, and 41.1% of *A. baumannii* and 20% of *P.aeruginosa* isolates were produced MBLs. The susceptibility to different antibiotics was evaluated by disk diffusion method and MICs of cefixime, imipenem, cefotaxime and ceftazidime were determined. PCR was performed for detection of *bla* $_{TEM}$, *bla* $_{SHV}$, *bla* $_{CTX-M}$, *bla* $_{OXA-1}$, *bla* $_{IMP-1}$ and *bla* $_{VIM-2}$ beta-lactamase genes .The isolates were highly beta- lactam-resistant (MIC ranges of cefixime, imipenem, cefotaxime and ceftazidime were 2 - 512 µg/ml , 4- 512 µg/ml, 32 - >512 µg/ml and 16 - >512 µg/ml respectively) and multidrug-resistant. The results of ESBLs genes detection clarify, that all of ESBLs and MBLs producer isolates of *A.baumannii* and *P.aeruginosa* carried *bla* $_{CTX-M}$ gene, 16.6 % of ESBLs producers and 42.8% of MBLs producers of *A.baumannii* have carried *bla* $_{TEM}$ gene, in contrast, neither ESBLs nor MBLs producers of *P.aruginosa* were carried *bla* $_{TEM}$ gene. *bla* $_{IMP-1}$ type was detected in 42.8 % of *A.baumannii. bla* $_{VIM-2}$ didn't appear in any isolate of ESBLs or MBLs producers.

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INTRODUCTION

Acinetobacter baumannii is a nosocomial pathogen implicated with septicemia, pneumonia, and death (Zurawski *et al*., 2012). *A. baumannii* is truly a global pathogen, as it has been isolated from hospitals throughout the world (Perez *et al*., 2007), as well as in wounded soldiers serving in Iraq and Afghanistan (Schafer and Mangino, 2008; Sahl *et al*., 2013). The worldwide emergence of multi-drug resistant bacterial strains is a growing concern, especially infections caused by Pseudomonas spp. and *P. aeruginosa* in particular. *P. aeruginosa* is an opportunistic pathogen with innate resistance to many antibiotics and disinfectants including anti-pseudomonal Penicillins, Ceftazidime, Carbapenems, Amino glycosides and Ciprofloxacin (Dundar and Otkun, 2010). Infections due to *P. aeruginosa* are seldom encountered in healthy adults; but in the last two decades, the organism has become increasingly recognized as the etiological agent in patients with impaired immune defenses (Begum *et al*., 2013). The extensive use of the third generation cephalosporins like cefotaxime, ceftriaxone and ceftazidime has led to the evolution of newer β-lactamases such as the Extended Spectrum Beta Lactamases (ESBLs). ESBL are Plasmid-mediated enzymes that hydrolyze the oxyimino β-lactams and the monobactams (aztreonam) but have no effect on the cephamycins (cefoxitin, cefotitan) and the carbapenems (Imipenem). Being plasmid mediated, they can be easily transferred from one organism to another (Quale *et al*., 2002). Class B carbapenemases including IMP and VIM termed as metallo beta lactamases (MBLs) have been found so far in *A. baumannii* and these are encoded by different plasmid types (Walsh *et al*., 2002). Genes encoding for MBLs were shown to be carried on large transferable plasmids or were associated with transposons, allowing horizontal transfer of these MBL genes among different bacterial genera and species (Pitout *et al*., 2007). To date, five types of acquired MBL genes (IMP, VIM, SPM, GIM, and SIM) have been identified based on their divergent protein molecular structures (Khosravi *et al* .,2011).While IMP and VIM variants have been reported worldwide, members of SPM, GIM, and SIM are restricted to certain geographical regions (Ellington *et al*., 2007). Determination of ESBL and MBL genes by molecular techniques in ESBL and MBL producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections. The aims of this study were to determine the prevalence of mainly *bla* genes responsible for ESBL & MBL β-lactamases amongst the *P. aeruginosa* and *A. baumannii* from the patients admitted to Some Iraqi medical centers in Baghdad.

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MATERIAL AND METHODS

Clinical isolates

Ninety two clinical isolates of *Acinetobacter baumannii* (n=17) and *Pseudomonas aeruginosa* (n=75) recovered from blood, wound, urine, sputum, ear and respiratory tract were obtained from patients admitted to Some Iraqi medical centers in Baghdad between February to September 2012 .The isolates were identified by conventional biochemical methods

Antibiotic susceptibility testing

The isolates were subjected to antimicrobial susceptibility testing using Kirby-Bauer disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines (2009), using commercially available 6mm disks (Bioanalyse/Ankara/Turkey).The susceptibility of the isolates was determined against 11 antibacterial agents by disk diffusion method, They include: cefoxitin(FOX)

(30µg), cefotaxime (CTX)(30µg), ceftriaxone(CRO) (30µg), ceftazidime(CAZ)(30µg), cefixime (CFM)(5ug), cefipime (FEP)(30µg), imipenem (IPM) (10µg),carbenicillin(PY)(25ug), aztreonam(ATM) (30µg), amoxycillin/ clavulanic acid (AMC) (20/10µg),meropenem (MEM)(10µg) ,On Mueller Hinton agar Plate(Lab M Limited Topley House,United Kingdom),using overnight culture at a 0.5 McFarland standard followsed by incubation at 35 ^{o}c for 16 to 18 h.

Minimal Inhibitory Concentrations

The MICs of cefixime, imipenem, cefotaxime and ceftazidime were determined. We used Mueller-Hinton agar with antibiotic concentrations (0.25-512) µg/ml according to the guidelines recommended by the CLSI document.

Detection of ESBL by double disc diffusion synergy method

To detect ESBL production, the double disc diffusion synergy test (DDST) was used as described by Jarlier *et al*. (1988). Mueller Hinton agar was inoculated with standardized inoculum (corresponding to 0.5 McFarland tube) using sterile cotton swab. An Augmentin (20 µg Amoxicillin and 10 µg of Clavulanic acid- AMC) disk was placed in the center of the plate and test disks of 3rd generation Cephalosporins (Ceftazidime- CAZ 30 µg, Ceftriaxone-CRO 30 µg, Cefotaxime-CTX 30 µg) and Aztreonam (ATM 30 µg) disks were placed at 20 mm distance (center to center) from the Amoxicillin-Clavulanic acid disk prior to incubation. The plate was incubated overnight at 35°C. Enhancement of the zone of inhibition of any one of the four drug disks toward Amoxicillin-Clavulanic acid suggested the presence of extended-spectrum beta-lactamases.

Detection of Metallo-β lactamase by disc potentiating test

MBLs production was determined by disc potentiating test as described by Bashir *et al.* (2011), Two imipenem discs were placed on the Mueller Hinton agar; 5 µl of (5M) EDTA solution was added to one of the discs. The inhibition zones of the imipenem and imipenem-EDTA discs were compared after 16-18 hrs of incubation at 35°C. An increase in the zone size of at least 7 mm around the imipenem -EDTA disc was recorded as an MBL-positive isolate.

Plasmid Isolation

Plasmid DNA were isolated using plasmid extraction kit (Promega, USA), and analyzed on 0.8% agarose gel.

Molecular Detection of β-lactamase enzymes from producing isolates using PCR technique

All of ESBLs and MBLs producers isolates were submitted to PCR technique to detection for some genes; *bla* $_{TEM}$, *bla* $_{SHV}$, *bla* $_{CTX-M}$, *bla* $_{OXA-1}$ (encoded for some extended spectrum β- lactamases) ; *bla* $_{IMP-1}$, *bla* $_{VIM-2}$ (encoded for some metallo β -lactamases); DNA amplification was carried with a Gradient PCR System (TechNet-500 /USA). PCR was performed with a final volume of 25 µl. The primers used for PCR amplification are listed in [Table 1]. Each reaction contained 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 mM MgCl2; 1.5 µl each primer; 1.25 U of *Taq* DNA polymerase. Template DNA (2 µl). Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker (Promega/USA) was run with each gel, and the genotype was determined by the size of the amplified product.

RESULTS AND DISCUSSION

Acinetobacter baumannii and *Pseudomonas aeruginosa* are noted for their intrinsic resistance to antibiotics and for their ability to acquire genes encoding resistance determinants. Foremost among the mechanisms of resistance in both of these pathogens is the production of Beta-lactamases and aminoglycoside-modifying enzymes. In the present study, Ninety two clinical isolates of *Acinetobacter baumannii* (n=17) and *Pseudomonas aeruginosa* (n=75) were isolated from various infections from patients admitted to Some Iraqi medical centers in Baghdad. The resistance patterns for the *Acinetobacter baumannii* isolates are shown in [Table 2]. High level of resistance of *A. baumannii* clinical isolates to most of the β-lactam antibiotic classes, with a resistance rate has reached to 100% for Cefoxitin, Ceftriaxone, Amoxicillin/clavulanic acid, Cefepime and Azteronam. Carbapenem class represented by Imipenem and Meropenem, also has

Table 1. Primers used for detecting ESBLs and MBLs genes among *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates

Gene	Sequence of forward Primer (5´- 3´)	Sequence of reverse primer (5´- 3´)	Product bp	Reference
bla$_{TEM}$	ATGAGTATTCAACAT TTCCG	TTAATCAGTGAGGC ACCTAT	861	Grimm *et al.* (2004)
bla$_{SHV}$	ATTTGTCGCTTCTT TACTCGC	TTTATGGCGTTACCTT TGACC	1018	Jemima and Verghese (2008)
bla$_{CTX}$	CGCTTTGCGATGTGCAG	ACCGCGATATCGTTGGT	550	Shacheraghi *et al.* (2010)
bla$_{IMP}$	CATGGTTTGGTGGTT CTTGT	ATAATTTGGCGGACT TTGGC	488	Sung *et al*. (2008)
bla $_{VIM}$	ATTGGTCTATTTGACC GCGTC	TGCTACTCAACGACTG AGCG	780	Sung *et al*. (2008)
bla$_{OXA}$	ACACAATACATATCAA CTTCGC	AGTGTGTTTAGAATG GTGATC	885	Yao *et al.* (2007)

Table 2. Resistance percentages of *A.baumannii* and *P. aeruginosa* isolates against Beta-lactam Antibiotics

Bacteria / Antibiotics		*A.baumannii(n= 17)*			*P.aeruginosa (n =75)*		
		Resistant	Intermedite	Susceptible	Resistant	Intermediate	Susceptible
IMP	no. (%)	9 (52.9)	0 (0)	8 (47.1)	6 (8.0)	4 (5.4)	65 (86.6)
MEM	no. (%)	15 (88.2)	2 (11.8)	0 (0)	15 (20.0)	13 (17.3)	47(62.7)
CAZ	no. (%)	16 (94.1)	1 (5.9)	0 (0)	22 (29.3)	7 (9.3)	46(61.4)
FOX	no. (%)	17(100)	0(0)	0 (0)	61 (81.3)	9 (12.0)	5 (6.7)
CRO	no. (%)	17(100)	0(0)	0 (0)	63 (84.0)	1 (1.3)	11 (14.7)
AMC	no. (%)	17(100)	0(0)	0 (0)	63 (84.0)	0 (0)	12 (16.0)
FEP	no. (%)	17(100)	0(0)	0 (0)	62 (82.7)	1 (1.3)	12 (16.0)
ATM	no. (%)	17(100)	0(0)	0 (0)	50 (66.6)	7 (9.3)	18 (24.1)
CTX	no. (%)	16(94.1)	1(5.9)	0 (0)	50 (66.6)	12 (16.0)	13 (17.4)
CFM	no. (%)	16(94.1)	1(5.9)	0 (0)	60 (80.0)	2 (2.6)	13 (17.4)
PY	no. (%)	15(88.2)	1(5.9)	1(5.9)	75 (100)	0 (0)	0 (0)

appeared a high level of resistance with percentages reached to 88.2 % for Meropenem and 52.9 % for Imipenem. It is worthy to notice that from 17 isolate of *A.baumannii*, 9 isolates (52.9 %) were resistant to both Imipenem and Meropenem. On other hand, belong to *Psedomonas aeruginosa*, it had showed a varied levels of resistance to β-lactam antibiotic classes when a resistance rate reached to 100% for Carbencillin; 80 % for Cefixime, 84% for Amoxicillin/clavulanic acid and Ceftazidime (29.3 %) (Table 2). Locally study done by AL-Saleem (2013) found that *A.baumanni* clinical isolates developed 97.3% of resistance to Aztreonam and Ceftriaxone, 89.5% to Ceftazidime, 58.2% to Imipenem and Meropenem. Also, study by Al-Mashadani (2010) to same bacterium revealed the resistance percentage to Cefotaxime, Ceftazidime and Ceftriaxone were 100% (for each). In contrast, to Imepenem, Al-Khafaji (2006) mentioned that all clinically identified *A. baumanni* isolates were completely susceptible to it. Bashir *et al*. (2011) reported that percentage of resistance for Imipenem was 13.4 % by *P.aeruginosa* isolates in tertiary care hospital in Kashmir. The *A. baumannii* isolates had MIC >512 µg/ml for cefotaxime, While *P. aeruginosa* isolates had MICs between 128 to 256 µg/ml to this antibiotic. Determination of MIC to Cefixime reveals that all isolates of *A. baumannii* showed high level of resistance(100%) with MIC (>512)µg/ml. MIC for *P. aeruginosa* has revealed varied values, 11 isolates(14.6%) were resisted with high level of MIC (>512) µg/ml, 48 isolates (64.0%) with MIC ranged from (8 – 512)µg/ml, for the other remaining 16 isolates (21.3%), they were susceptible with MIC values less than 4 µg/ml. 13 isolates (76.5%) of *A. baumanni* with MIC ranged from 32 -512 µg/ml to imipenem, 4 isolates(23.5%) were equal to breakpoint (16 µg/ml). *P.aeruginosa* had resisted imipenem antibiotic, 26 isolates (34.6%) ranged with MIC values from 32 -512µg/ml, 8 isolates (10.6%) were equal to breakpoint (16 µg/ml). All isolates were tested for phenotypic producing of extended spectrum β- lactamase (ESBLs), 6 (35.2%) and 5 (25%) of *A.baumannii* and *P.aeruginosa* respectively had ability to produce ESBLs. ESBLs are primarily producing by the Enterobacteriaceae family of Gram-negative organisms, in particular *Klebsiella pneumonia* and *Escherichia coli*. They are also producing by non fermentative Gram-negative organisms, such as *A. baumannii* and *P. aeruginosa*. These enzymes can be carried on bacterial chromosomes, that is, inherent to the organism, or may be plasmid-mediated with the potential to move between bacterial populations. This has clear implications regarding spread of infection and infection control (Dhillon and Clark, 2012).

Bali *et al.* (2010), mentioned that ESBLs isolates have able to hydrolyze 3rd and 4th generation cephalosporins and monobactam and the percentage of ESBLs-producing isolates of *A.baumannii* were 5.2 %. Study done by Sniha *et al.* (2007) in India for ESBLs production, it was appeared in 28 % of the isolates . Chaudhary and Payasi (2012) reported that 83.6% of *A.baumannii* isolates were positive for ESBLs – producing test. In the study of Aggarwal *et al*. (2008), they reported that the ESBLs production in *P. aeruginosa* isolates were 20.27%. The same isolates submitted to ESBLs enzymes detection, were used to detect for metallo β-lactamase enzymes by CDT method, the results revealed that 7 (41.1%) of *A. baumannii* isolates and 4 (20%) of *P.aeruginosa* isolates showed positive results. The MBLs efficiently hydrolyze all β-lactams, except for aztreonam. Therefore, detection of MBL-producing gram-negative bacilli is crucial for the optimal treatment of patients and to control the spread of resistance (Lee *et al*., 2003). Al-Grawi (2011) reported that percentage of metallo-β lactamase producers of *P. aeruginosa* were 68%. Bashir *et al*. (2011) mentioned that 11.6% of *P.aeruginosa* isolates were produced of MBLs enzymes by using combination disc method (CDT). All of ESBLs and MBLs producing isolates have tested in order to detect the plasmid pattern of each isolate, the results have clarified that 12 of 13 *A.baumannii* isolates (92.3%) and 8 of 9 *P.aeruginosa* isolates (88.9%) had plasmid bands. The results of ESBLs genes detection clarify, that all of ESBLs and MBLs producer isolates of *A.baumannii and P. aeruginosa* carried *bla* $_{CTX-M}$ gene (Fig 1), One isolate (16.6%) of ESBLs producers and 3 isolates (42.8%) of MBLs producers of *A.baumannii* have carried *bla* $_{TEM}$ gene (Fig 2), in contrast, neither ESBLs nor MBLs producers of *P.aruginosa* were carried *bla* $_{TEM}$ gene .While *bla* $_{OXA-1}$ gene appeared in 14.8% of *A.baumannii* isolates and 100% of *P.aeruginosa* isolates (Fig 3) which were producing MBLs, and didn't found in any isolates of the both which were ESBLs producing. The *bla* $_{SHV}$ gene didn't appeared in any isolate of ESBLs and MBLs producers. Al-Grawi (2011) found that among isolates of *P.aeruginosa* which were Cefotaxime resistant, 72 % were CTX-M gene harboring. Study of Al- Kaabi (2011) in Baghdad mentioned that percentage of *bla* $_{CTX-M}$ gene was 83.3 %, while *bla* $_{TEM}$ gene appeared in all isolates (100%) in *P.aeruginosa* isolates. Liao *et al.* (2010) reported that all the *P.aeruginosa* isolates had *bla* $_{TEM}$, and these results don't concur with our results.

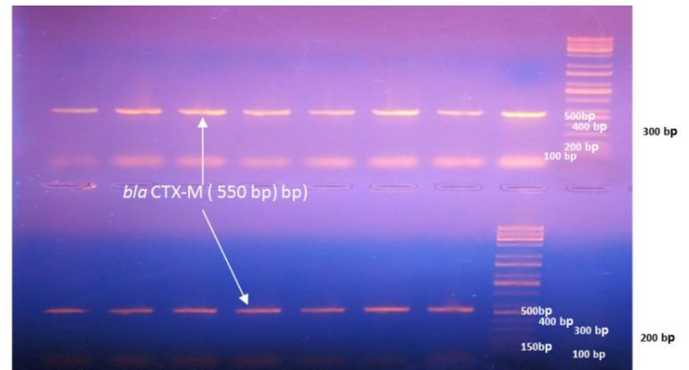

Fig. 1. Gel electrophoresis (1% agarose, 7v/cm) of *bla* $_{CTX-M}$ (550bp) using chromosomal DNA. Line M: 100 bp DNA ladder

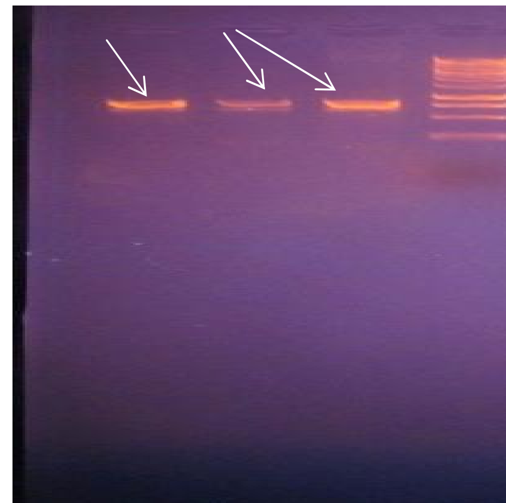

Fig. 2. Gel electrophoresis (1% agarose, 7 v/cm). of *bla* $_{TEM}$ (861bp) using chromosomal DNA. Line M: 1kbp DNA ladder

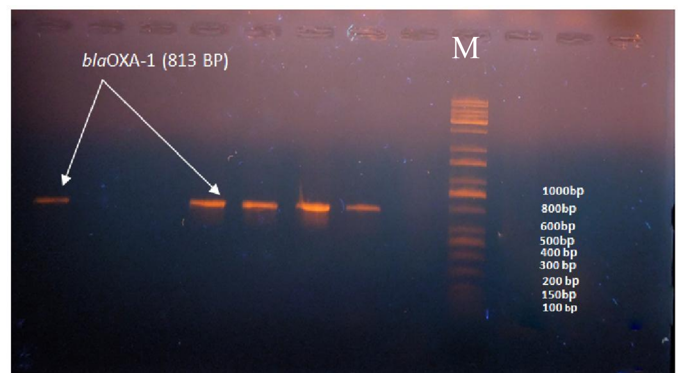

Fig. 3. Gel electrophoresis (1% agarose, 7 v/cm) of *bla* $_{OXA-1}$ (813 bp), using plasmid DNA. Line M: 100bp DNA ladder

Recent studies indicate that the CTX-M enzymes predominate among the ESBLs of community strains, CTX-M ESBLs probably originated from *Kluyvera* species and these enzymes are mostly found in members of the Enterobacteriaceae. Recently, the emergence of these enzymes has been reported in *Acinetobacter baumannii* (Nagano *et al*., 2004). Hujer *et al*. (2006) motioned that Military medical facilities treating patients injured in Iraq and Afghanistan have identified a large number of multidrug-resistant (MDR) *Acinetobacter baumannii* isolates. From among genes which responsible for β-lactamases encoding, they have been found that Percentage of genes were detected were 40% of TEM gene and 1% of SHV gene, whereas all isolates hadn't CTX-M gene. The result of our study had demonstrated that *bla* $_{IMP-1}$ type was detected in 3 isolate (42.8%) of *A.baumannii* (Fig. 4), while it didn't found in any isolate of *P. aruginosa* isolates . *bla* $_{VIM-2}$ didn't appear in any isolate of ESBLs or MBLs producers. Acquired metallo-_-lactamases (MBLs) are emerging worldwide as powerful resistance determinants in Gram negative bacteria. So far, six MBL enzyme types have been described in clinical isolates of *Pseudomonas aeruginosa* -VIM type, IMP type, SPM type, GIM type, AIM type, and, most recently, the NDM type found among clinical isolates from Serbia, a Balkan country (Sardelic *et al*., 2012).

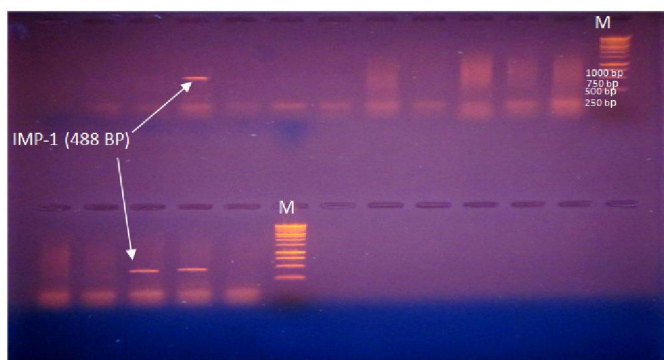

Fig. 4. Gel electrophoresis (1% agarose, 7 v/cm) of *bla* $_{IMP-1}$ (488bp), using chromosomal DNA. Line M: 1kbp DNA ladder

In the study of Franco *et al*. (2010) they recorded that PCR detection of MBL was positive in 21 strains (30.4%) of *P.aeruginosa,* 17 (81%) of which were positive for *bla* $_{SPM-1}$ and 4 (19%) of which were positive for *bla* $_{VIM-2}$.The other genes (*bla* $_{IMP-1}$, *bla* $_{IMP-2}$, *bla* $_{VIM-1}$) were not detected.

Conclusion

In conclusion, we report here the characterization of highly beta-lactam-resistance *in P. aeruginosa* and *A. baumannii* isolates. Thus, *bla* gene may have been spreading in Gram negative rod in Baghdad-Iraq. This emphasizes the necessity of early recognition of MBL and ESBL producing isolates, rigorous infection control, and restricted clinical use of broad-spectrum β-lactams.

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