DETECTION OF EXTENDED SPECTRUM BETA LACTAMASE (ESBL) IN HEALTH CARE ASSOCIATED INFECTIONS WITH SPECIES EXPRESSING SHV1 AND/OR TEM1 GENE

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Abstract:

Background: Organisms that harbor extended spectrum beta lactamases (ESBLs) is a major challenge for a diagnostic clinical microbiology laboratory.

Objective: To evaluate phenotypic characteristics, initial screening tests and established confirmatory phenotypic methods for detection of ESBLs Klebsiella isolates prevalent in Cairo university hospital.

Patients and Methods: Standard disk diffusion, double disk synergy test (DDST), combined disk method, E test ESBL strip for detection of ESBL expression, as well as PCR analysis for detection of TEM1 and SHV1 genes were done.

Result: Screening of Klebsiella species using oxyimino-cephalosporin disk diffusion method showed 100% resistance (ESBL suspected) to cefpodoxime, 98% to cefotaxime, ceftriaxone and aztreonam, 96% to ceftazidime, and 92% to cefepime. However DDST method detected the presence of ESBL activity in 76% of isolates, and 98% by the combined disk diffusion test. The ESBL activity was detected by E-test method in 46 isolates (92%). TEM1 gene was determined by PCR in (96%), and SHV1 was determined in (90%) of cases.

Conclusion: The combined disk diffusion test is an easy reliable method which showed the highest incidence of ESBL detection followed by E test. However the DDST method showed the lowest ESBL detection rate in comparison to the aforementioned confirmatory tests. The presence of TEM1 and SHV1 genes was confirmed in a high incidence of ESBL isolates.

Key Words: Extended spectrum beta lactamases (ESBLs), E Test, TEM1 and SHV1 genes.
Introduction:

The oxyimino-cephalosporins, became widely used for the treatment of serious infections due to gram-negative bacteria (1). Not surprisingly, resistance to these expanded-spectrum B-lactam antibiotics due to B-lactamases emerged quickly. Because of their increased spectrum of activity, especially against the oxyimino-cephalosporins, these enzymes were called extended-spectrum B-lactamases (ESBLs) (2, 3). Klebsiella pneumoniae has been found to be the most common species to produce extended- spectrum β-lactamases, and in some countries the prevalence of ESBL production approached 50 % (4, 5).

ESBL producers are associated with increased morbidity and mortality, especially amongst patients in intensive care and high-dependency units. These plasmid mediated enzymes mostly evolved via point mutations of the classical TEM-1 and SHV-1 β-lactamases (6, 7). Moreover, another key problem in the detection of ESBL producers is the possibility of low level production of enzyme and effect of the inoculums, resulting in variable diameter zone by disk diffusion testing. Therefore, specific detection methods such as the double disk synergy test (DDST), combination disk method and E-test have been described (8). Accurate laboratory detection is important to avoid clinical failure due to inappropriate antimicrobial therapy (1). In that respect a comparative study was done to detect Klebsiella species

Despite the fact that there are large numbers of gram-negative clinical isolates that harbor extended spectrum beta lactamases (ESBLs), very few clinical laboratories routinely perform ESBL detection, especially in developing countries, due to costs involved and work load (9).

Expressing ESBL activity using 3 different methods; DDST, combined disk diffusion method and E test. PCR analysis using oligonucleotide primers was used to detect TEM1 and SHV1 genes.

Patients and Methods:

The study was conducted in Kasr EL-Aini - Cairo University hospital from December 2006 till May 2007. The study started with 382 gram negative isolates out of 512 examined specimens and 104 Klebsiella species were identified (10). Klebsiella isolates obtained from urine specimens
were cultured on CLED, while pus, blood and sputum specimens were cultured on blood and MacConkey agar and then incubated aerobically at 37°C for 24 hours. During the study period, all strains of Klebsiella isolated were inoculated in broth glycerol and stored at -70°C for subsequent PCR analysis.

Then antimicrobial susceptibilities for the Klebsiella isolates were done by standard disk diffusion (SDD) using commercially available disks (oxoid, Basingstoke, UK) for different antimicrobial agents; cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (ATM), ceftriaxone (CRO), cefepime (CFP), cefoxitin (FOX), amikacin (AK), gentamicin (GM), amoxicillin clavulanate (AMC), piperacillin-tazobactam (TZP), cefoperazone–sulbactam (SCF) and imipenem (IMP) and were determined as per CLSI guidelines 2005 then isolates suspected for being ESBL will be subjected to further confirmatory tests (11).

Screening for ESBL producers was done using CLSI recommendations by using Kirby-Bauer disk diffusion (KB) techniques using CPD, CAZ, ATM, CTX and CRO (oxoid, Basingstoke, UK) to identify potential ESBL harboring isolates (11, 12).

Phenotypic confirmatory test for ESBL production was determined by:

a) Double disk synergy test (DDST) which was performed using disks of 30 µg each of CPD, CAZ, CTX, CRO, ATM (oxoid, Basingstoke, UK),) that were placed at the distance 20-25 mm from AMC (amoxicillin 20 µg and clavulanic acid 10 µg) centre to centre and incubated at 35°C overnight (13). A clearly visible extension of the edge of the inhibition zone of any disk towards the amoxicillin clavulanic disk was interpreted as positive for clavulanic acid synergy (14).

b) Combined disk method was used as recommended by CLSI 2005 (11). A disk of ceftazidime clavulanate (CAZ/CA) (30/10 µg) and cefotaxime clavulanate (CTX/CA) (30/10 µg) were applied to the surface of the inoculated plate. Disks of CAZ and CTX were also applied. A > 5 mm increase in zone diameter for either antimicrobial agents tested in combination with clavulanate versus its zone when tested alone was taken as an indication of ESBL producing isolate (15).

c) E test ESBL strips (AB Biodisk, solna, Sweden) was used. It is a drug impregnated strip,
one end contains a gradient of ceftazidime TZ (MIC test range 0.5 to 32 µg/mL) and the other a gradient of ceftazidime plus a constant concentration of clavulanate TZL (4µg/mL). An ESBL phenotype was defined as: >8 fold reductions in the MIC of ceftazidime in the presence of clavulanate or the presence of phantom or deformity zones (15), or a break point to ceftazidime (MIC of TZ > 1 µg/ml). MIC TZ: MIC TZL ratio >32 : > 4 is considered indeterminate according to the manufacturer recommendations and require further genotypic analysis.

Genotypic test for expression of TEM 1 and SHV1 was carried out as follows:

DNA extraction was done from Klebsiella species (grown on MacConkey agar) using a DNA mini M48 kit (QIAGEN) and 100 µl of extracted DNA was stored at -20°C and ready to use. PCR analysis was done using the following oligonucleotide primers (Sigma-Genosys) that are specific for TEM1 and SHV1 genes. Sequence of primers were

- *bla* SHV1 F 5’ GGCCGCGTAGGCATGATAGA 3’,
- *bla* SHV1 R 5’ CCCGCCGATTTGCTGATTTC 3’,
- *bla* TEM1 F 5’CAGCGGTAGATCCCTTGAGA 3’ and
- *bla* TEM1 R 5’ ACTCCCCGTCTGATAGATAA 3’ (16).

PCR was performed in 50 µl (total volume) of distilled water containing each deoxyribonucleotide at a concentration of 0.25 mM, 1.5 mM MgCl, 0.2 U of Taq DNA polymerase, and 50 pmol of each primer. The temperature profile included an initial template denaturation step consisting of 95°C for 10 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min and a final step consisting of 72°C for 7 min (16, 17). PCR product was analyzed by gel electrophoresis using 2 % agarose, staining gel with Ethidium Bromide. A 50 bp ladder was used, SHV1 gene band was detected at 720 bp, while TEM1 gene band was detected at 650 bp.
Results:

Out of a total of 382 gram negative clinical isolates, 104 Klebsiella species were identified, 79 (76%) were positive for ESBL production by standard disk diffusion screening technique. Only 50 out of the 79 were subjected to full ESBL production detection (the other 29 cases were excluded from our study due to incomplete data). A large number (30%) of our suspected Klebsiella ESBL isolates (by screening detection method) were isolated from patients in 3 ICUs (surgical, neurology, and chest ICU), while 70% were isolated from patients in the 17 different departments.

All the 50 patients with Klebsiella ESBL infections in our study had risk factors such as prolonged hospital stay or repeated hospitalization, and previous treatment with antimicrobial therapy. Forty eight of the patients (96%) were on β-lactam antibiotics and 43(86%) were on third generation cephalosporins only. The 50 isolates of Klebsiella (ESBL) were isolated from 27 pus samples (54%) (24 surgical wounds, and 3 diabetic foot swabs), 9 pulmonary secretions (18%), 8 urinary samples (16%), 5 blood samples (10%), and one Cerebrospinal Fluid (CSF) shunt (2%).

All the 50 Klebsiella (ESBL) species isolates were retested with DDST, combined disk diffusion method, E-test, and PCR (for SHV1 and TEM 1 genes detection). Results of screening of Klebsiella species using oxyimino-cephalosporin disk diffusion method (KB) showed different resistance results to the disks used (Table 1, Fig 1). All isolates were sensitive to imipenem, but showed cross resistance to Ciprofloxacin in 26 isolates (52%) and to amikacin in 14 isolates (28%). However DDST method (between different substrates and inhibition by clavulanic acid) detected the presence of ESBL activity in 76% of the Klebsiella isolates (Fig 1).

Total positive strains for ESBL detected by DDST were 38(76%), while 49 (98%) were revealed by both cefotaxime and ceftazidime combined disks. However 11(22%) isolates were positive by combination disks and negative by DDST.

The ESBL activity was detected by E-test method (Table 1, Fig 2) in 46 isolates (92%). Phantom phenomena was demonstrated in 6(12%) of the isolates. Two isolates were negative for ESBL production by E-test, also showed sensitivity to CAZ and showed no phantom phenomena,
they expressed TEM1 and SHV1 genes. One of these 2 negative isolates was also negative for ESBL production by the combined test, and DDST methods. Two other isolates were indeterminate by E-test. Those indeterminate 2 isolates were negative for ESBL production when tested by DDST but positive by other test methods and expressed TEM1 and SHV1 genes.

Comparing the 48 isolates diagnosed by E test (46 as positive ESBL and 2 as non ESBL); ESBL activity was detected in 36/48(75%) isolates by both DDST (with CAZ) and E-test, 9/48(19%) were not detected by the DDST( positive by E-test) (Table 2). Number of tested isolates positive for ESBL by DDST, Combination methods and E-Test were 37/48(77%).

TEM1 gene was determined by PCR in 48 strains (96%), and SHV1 was determined in 45 isolates (90%)(Table 1 and 2). Forty three isolates (86%) were positive for both SHV1 and TEM1 genes, while five isolates (10%) were positive for TEM1 gene and two isolates (4%) revealed SHV1 gene only. No strain was negative for both TEM1 and SHV1 genes.
(Fig 1) Percent of Klebsiella ESBL by Kirby-Bauer disk diffusion (KB) and Double disk synergy test (DDST). Ceftazidime (CAZ), Cefotaxime (CTX), Ceftriaxone (CRO), Aztreonam (ATM), Cefepime (CFP), Cefpodoxime (CPD)
Table (1): Results of positive ESBL isolated by different test methods:

KB= Kirby-Bauer disk diffusion, Double disk synergy test (DDST), ceftazidime clavulanate (CAZ/CA), cefotaxime clavulanate (CTX/CA).

<table>
<thead>
<tr>
<th>Klebsiella spp.</th>
<th>KB</th>
<th>DDST</th>
<th>Combination disk method</th>
<th>E-Test</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAZ/CA</td>
<td>SHV-1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>CTX/CA</td>
<td>TEM-1</td>
<td></td>
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<tr>
<td>No of positive isolates</td>
<td>50</td>
<td>38</td>
<td>49</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>76</td>
<td>98</td>
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<td>98</td>
<td></td>
<td>96</td>
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</table>
Table (2): Results of phenotypic and molecular gene detection methods in relation to E-test in 48 isolates.

<table>
<thead>
<tr>
<th>E-Test</th>
<th>DDST</th>
<th>Combination</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CAZ/CA</td>
<td>CTX/CA</td>
</tr>
<tr>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Positive n=46</td>
<td>36</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>Negative n=2</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

-ve = negative, +ve = positive., Double disk synergy test (DDST), ceftazidime clavulanate (CAZ/CA), cefotaxime clavulanate (CTX/CA).

*48 isolates only of the 50 examined as two other isolates were determined by E-Test and couldn’t be considered positive or negative as sequencing method wasn’t included in our study.
Fig(2) Muller Hinton agar showing E Test and combined disc of ceftazidime clavulanate (CAZ/CA), cefotaxime clavulanate (CTX/CA), ceftazidime (CAZ) and cefotaxime (CTX).
Discussion:

Extended-spectrum β-lactamases (ESBL) have widely spread throughout the world and are now found in a significant percentage of Escherichia coli and Klebsiella pneumoniae strains in certain countries. However Klebsiella pneumoniae is one of the most common pathogens causing nosocomial infections that affect mainly immunocompromised patients with severe underlying diseases (18).

Specific risk factors include length of hospital stay, severity of illness, time in the ICU, intubation and mechanical ventilation, urinary or arterial catheterization, and previous exposure to antibiotics (1). All patients with Klebsiella ESBL infection in our study had risk factors of prolonged hospital stay and/ or repeated hospitalization, and previous treatment with antimicrobial therapy.

Detection of ESBL producing Klebsiella by disk diffusion method in this study varied with the cephalosporin tested. Cefpodoxime detected ESBL expression in 100 % of tested Klebsiella when used with disk diffusion method and < 10% when used with DDST. DDST method detected the presence of ESBL activity in 70% of our Klebsiella isolates with CAZ, 62% with CTX, and increased to 76% when results obtained using both agents were taken into consideration, such results were in accordance with other studies (19).

Numerous methods have been proposed for the detection of ESBLs in clinical isolates; however none of the methods that rely on phenotypic expression of β-lactamase can detect every ESBL- producing isolate. The failure of either MIC or disk tests alone to accurately detect the presence of an ESBL in all strains of E.coli and K. pneumoniae has been well documented (20, 21). In our study 9(19%) of ESBL producing Klebsiella isolates were positive by E-test and not detected by the DDST, which was in accordance with Dashti et al., who reported that 16% of the ESBL producing isolates were false negative by DDST and positive by E-test (22).

Overall, the DDST method was able to detect ESBL activity in 76% of our Klebsiella ESBL isolates and similarly DDST method detected (75%) of isolates in Kuwait, but higher results were given from United Kingdom (UK) (92.1%) (22). A study done by Bell et al. showed that there was
significant variation between participating countries (nine countries in the Asia-Pacific region) with regard to both prevalence of ESBL-positive strains and the prevalence of non confirmed strains in ESBL confirmatory tests \(^{(23)}\). The combined disk diffusion method in our study detected 98% of ESBL producing Klebsiella isolates which was in accordance with M’Zali et al., who detected 93% of ESBL by the same method \(^{(19)}\).

The commercially available ESBL E test strip is a quantitative technique, and is widely ranged as the (gold standard) for detection in clinical laboratories of ESBL production, it detected 99.2% of the UK test isolates and 98% of the Kuwait isolates when both Cefotaxime and Ceftazidime strips were used in conjunction \(^{(1, 22)}\). In our study 92% of the screened ESBL producing Klebsiella isolated were positive by E test.

TEM 1 gene was detected in 96% of the Klebsiella isolates, while another study showed a lower incidence of 17.3% \(^{(24)}\). However, TEM 1 gene was detected in association with SHV 1 gene in several isolates (68%), thus it was not possible to presume if they were responsible for the ESBL expression, as they could be non-ESBL encoding genes associated with another gene expressing the ESBL phenotype as reported by Livermore 2008 \(^{(25)}\). While also, co-production of several ESBLs in a strain is common \(^{(26)}\).

In the present study 90% of Klebsiella isolates expressed SHV-1 gene, however several groups have reported that the high-level expression of SHV 1 in K. pneumoniae can cause the MIC of ceftazidime to rise to levels at which an ESBL would be suspected, and can cause false-positive in ESBL detection tests \(^{(1)}\). The identification of bla SHV 1 genes in the major part of ESBL –Klebsiella strains cannot affirm those are ESBL-encoding genes and further studies will be necessary to identify them precisely \(^{(24)}\).

It could be concluded that the combined disk diffusion test method revealed the highest percent for ESBL detection and remains a reliable method for detection of ESBLs and only requires two disks to be added to the sensitivity plate, thus enabling all Gram-negative bacteria to be screened in the diagnostic laboratory. The commercially available E test showed high percentage of ESBL detection following the combined disk diffusion test and has been proposed as simple technique for the detection of ESBL production. Although E test requires an extra agar plate
to be inoculated which could be difficult to be incorporated in a routine disk testing system, it is widely ranged as the (gold standard) for detection in clinical laboratories of ESBL production. The standard DDST requires careful spacing of disks for accurate results and showed the least percentage of ESBL detection. Although molecular methods appear sensitive, they are expensive, time consuming, and require specialized equipment and expertise. In this study further sequencing for determination of SHV1 and TEM1 derivatives is recommended.
References:


الكشف الجزيئي للمجال المتمد لبيتا لاكتاماز في العصيات السالبة لجرام الكليبسيلا المعبرة

لجيني الـ TEM و SHV

سهير فتحى هلال - مني عبد العزيز واصف - إيمان كمال بحيري - ندا نبيل نوار - غادة علي زياد

الباثولوجيا الإكلينيكية كلية الطب - جامعة القاهرة

ملخص العربي

إن إنتاج إنزيمات البيتا لاكتاميز يعتبر من أهم أسباب المقاومة البكتيرية للمضادات الحيوية (البيتاالاكتام) حيث أن هذه الإنزيمات تؤدي إلى تحلل حلقة البيتاالاكتام وتعزى المضاد الحيوي غير فعال. وتضم هذه المجموعة أيضا البيتا لاكتاميز الممتد المجال والتي تفرز من بكتريا سالبة الجرام حيث تقوم بتحليل الكفالوسبورن ممتد المجال والآزتيرونام وتتم تثبيط عملها بحمض الكلافيولونك SHV, TEM ومن هذه البكتيريا التي تحمل جينات

تتم المشكلة في علاج العدوى الناتجة عن هذه الأنزيمات أنها تحمل جينات مقاومة مضادات حيوية أخرى غير البيتا لاكتاميز لذلك فإن خيارات العلاج محدودة جدا تقتصر تقريبا على الكاربابينم.

هذا الدراسة تضمنت 104 عينة مختلفة يحتوي كل منها على بكتيريا الكلبسيلا وقد تم تجميعها من عينات مختلفة من معامل البكتيريا بمستشفيات جامعة القاهرة وذلك في الفترة من ديسمبر 2006 حتى مايو 2007 وهذه العينات تم التعرف عليها من خلال الخصائص الشكلية والكيميائية ثم تم فحصها بالكشف المحمي والعينات التي أعطت نتيجة إيجابية لبيتا لاكتاميز الممتد المجال تم تأكيدها بالإختبار المزدوج و القرص المركب و اختبار (ESBL E-test) ثم عمل الكشف الجزئي عن جيني TEM و SHV-1 .

وقد تم الكشف عن البيتا لاكتاميز الممتد المجال في 38 عينة (76%) بواسطة الاختبار المزدوج و وجد أن 49 عينة (98%) تنتج إنزيمات البيتا لاكتاميز الممتد المجال.

و هناك (92%) 46 عينة أعطاها نتيجة إيجابية و تم تأكيدها بإختبار E-test و وذلك أعطى القصر المركب حساسية (100%) للكشف عن البيتا لاكتاميز الممتد المجال بالمقارنة بالإختبار المزدوج (80%) لعدم الكشف عن إنتاج البيتا لاكتاميز الممتد المجال في 11 من العينات.

تم الكشف عن جيني TEM-1 و SHV-1 في (96%) و (90%) من الحالات على التوالي و وجود هذه الجينات مع النمط الظاهري يؤكذ حدوث طفرات نقطة في هذه الجينات التي تؤدي إلى تغيير عميق في النشاط الإنجيمي.