Comparison of Three Phenotypic Methods for Detection of Extended-Spectrum β-Lactamase (ESBL) Producing Klebsiella Pneumoniae

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Abstract

Through a period extended from October to December, 2016. Fifty six of Klebsiella pneumonia isolates were collected from some hospitals in Baghdad. The isolates of K. pneumonia were obtained from various clinical specimens including: 39(69.65%) isolates from blood, 6(10.71%) isolates from urine,6(10.71%) isolate from burns, 3(5.36%) isolate from sputum and 2(3.57%) isolates from ear swabs. Our result shows a high level resistance of K. pneumonia clinical isolates to wards most of the antibiotics under test, all K. pneumonia isolates had the highest resistance rate 100% to Ampicillin followed by (87.6%) to Cefazolin, while the lowest level resistance of K. pneumonia isolates was to Imipenem which had 17.86% resistance rate. Three methods were used for phenotypic detection of ESβL producing Klebsiella pneumoniae, first one was done by Vitek-2 system and the results showed that out of 56 isolates, 45 (80.35%) isolates were gave positive results for ESβL producing while 11(19. 65%) isolates of K. pneumonie were gave negative results. While in second method (disc combination method) the results showed that out of the 56 K. pneumonia isolates, 48(80.35%) isolates were positive ESBL producing, while 8(19. 65%) isolates of K. pneumonie were negative by two sets of cartridges (D52C) discs. In the third method the results of CHRO Magar technique, 51(91.07%) of the 56 isolates gave positive result which suspected ESBL producers while only 5(8.93%) isolates gave negative result. Eventually, CHRO Magar technique was recorded a high percent of ESBL detection while method by using Vitek-2 system was recorded a low percent of ESBL detection. From study we noticed that a high percent of ESBL producing K. pneumoniae isolates compared with non ESBL producing K. pneumoniae isolates under test by all previous confirmatory methods used in this study.

Keywords: Klebsiella pneumoniae, Extended-Spectrum β-Lactamases (ESBL), phenotypic detection

Introduction

Klebsiellapneumoniae (K.pneumoniae), a member of the family Enterobacteriaceae, is a rod-shaped, Gram-negative, lactose-fermenting bacillus with a prominent capsule [1]. It is an opportunist microorganism which causes serious diseases such as septicemia, pneumonia, bacteremia [2 and 3], meningitis, wound infections, and purulent abscesses, at various sites [4]. K.pneumoniaeis mainly responsible for hospital-acquired urinary tract infections and intra-abdominal infections [5].

Antimicrobials have been widely used to treat K. pneumoniae infections in humans. However, increasing antimicrobial resistance, especially that mediated by extended-spectrum β-lactamases (ESBL) has been reported in recent years and has become a serious problem [6]. Most Gram-negative bacteria produce Beta-lactamase (β-lactamase) enzymes which are their major defense mechanism against β-lactam antibiotics [7]. Infections due to these ESBL-positive Klebsiella isolates because increased morbidity and mortality [8]. The detection of ESBL-mediated resistance in Gram-negative bacilli is of paramount importance because of its clinical implications and the limited therapeutic options [9].
The aim of this study was to compare three phenotypic methods that can be routinely applied in most microbiological laboratories for their ability to detect ESBL production by Klebsiella pneumoniae.

Materials and Methods

Collection of Klebsiella pneumoniae Isolates

During a period of three months extended from October, 2016 to December, 2016. Fifty six Klebsiella pneumoniae isolates were collected from several hospitals in Baghdad including: Fatima Al-Zahraa hospital, Ibn Al-Balady hospital, Baghdad Teaching Hospital/Medical City, The Burn Specialist Hospital/Medical City and Teaching Laboratories/Medical City. Klebsiella pneumoniae isolates were isolated from different specimens including: blood, urine, sputum, burns and ear swabs.

Identification of Klebsiella pneumoniae Isolates

Morphological Examination

Initial identification of Klebsiella pneumoniae isolates based on morphological characteristic of the colonies including colony shape, colony texture, color, edges and hemolysis were studied depending on bacterial growth on MacConkey agar and Blood agar [10].

Also identification of Klebsiella pneumoniae isolates based on morphological characteristic of the colonies color was studied depending on bacterial growth on CHRO Magar orientation medium (which prepared according to the manufacturer's instructions fixed on the container).

Identification of Klebsiella pneumoniae with VITEK® 2 Compact System

Klebsiella pneumonia isolates were identified at species level by using VITEK® 2 systems with the using Identification- Gram Negative Bacteria (ID-GNB) cards according to the manufacturer's instructions.

Antibiotic Susceptibility Test

Antibiotic susceptibility test towards different antibiotics was done by Vitek-2 system with the using Antibiotic Sensitivity Test Number 69(AST 69) cards according to the manufacturer's instructions.

Phenotypic Detection of ESβL Production

Vitek-2 System

Phenotypic detection of ESβL producing Klebsiella pneumoniae was done by Vitek-2 system with the using Antibiotic sensitivity test Number 69(AST 69) cards according to the manufacturer's instructions.

Disc Combination Method

All of the fifty six Klebsiella pneumonia isolates were screened for ESβL production by CLSI phenotypic confirmatory test of disc combination method. In this method we used D52C discs, for the detection Extended Spectrum Beta–Lactamases (Mast/UK).

Two paired sets of cartridges (D52C) were used in this study, set one contain ceftazidime (30µg) alone and ceftazidime + clavulanic acid (30 µg /10 µg) while set two contain cefotaxime (30µg) alone and cefotaxime + clavulanic acid (30 µg /10 µg). The procedure was done according to the manufacturer’s instructions and it summarized as follows:

- A suspension equivalent in density to a McFarland 0.5 opacity standard has been prepared.
- Spread the suspension uniformly across the surface of susceptibility test agar plate by using a sterile swab.
- MAST ID ESβL Detection Discs were placed onto the inoculated medium by using a sterile needle or forceps, and then incubated at 35-37ºC for 18 to 24 hours.
- The diameter of inhibition zone around the discs were measured and recorded.
- The reference strain of Escherichia coli ATCC 25922 was used as negative control in this test. According to the manufacturer's instructions, the zone of inhibition for the cefotaxime discs were compared to that of cefotaxime Plus clavulanic acid combination discs. An increase in zone diameter of ≥ 5mm in the presence of clavulanic acid from the set of MAST ID ESβL Detection Discs indicates the presence of ESβL in the test organism.

Detection of ESβL by CHRO Magar Technique

CHRO Magar ESBL Supplement added to CHRO Magar Orientation media (prepared according to the manufacturer's instructions fixed on the container).
To make a supplement solution, 570 mg of the supplement powder has been added to 10 ml of purified sterile water, then vortex this supplement to homogenize and then add this supplement solution to melted CHRO Magar Orientation cooled at 45°C. Stir it to make CHRO Magar ESBL, then poured into sterile petri dishes. CHRO Magar ESBL Supplement allows the detection of ESBL-producing bacteria while inhibiting the growth of other bacteria. ESBL CHRO Magar plates were streaked in the same day of preparation by overnight growth of K. pneumoniae, and incubated at 37 °C for 24 hr, according to manufacturer procedure. Growth of blue colonies indicated to suspected ESBL producer. The reference strain of E. coli ATCC 25922 was used as negative control in this test.

**Results**

**Collection and Identification of Klebsiella Pneumonia Isolates**

Fifty six of *Klebsiella pneumonia* isolates were collected from some hospitals in Baghdad city including: Fatima Al-Zahraa hospital, Ibn Al-Balady hospital, Baghdad Teaching Hospital/ Medical City, The Burn Specialist Hospital/ Medical City and Teaching Laboratories/ Medical City, through a period extended from October to December, 2016. The isolates of *K. pneumonia* were obtained from various clinical specimens including: 39(69.65%) isolates from blood, 6(10.71%) isolates from urine, 6(10.71%) isolate from burns, 3(5.36%) isolate from sputum and 2(3.57%) isolates from ear as shown in Figure -1.

![Figure 1: Percentage of Klebsiella pneumonia collected isolates according to type of specimens](image)

Initial identification of *Klebsiella pneumoniae* isolates based on morphological characteristic of the colonies on MacConkey agar, blood agar and CHRO Magar orientation *K. pneumoniae* isolates appear large, mucoid and pink on MacConkey agar, while on blood agar they appear large, mucoid without hemolysis colonies (10), and the colonies appeared as metallic blue on CHROMagar (Figure-2). After that all isolates were identified at species level with VITEK® 2 system by using Identification- Gram Negative Bacteria (ID-GNB) cards and the results showed all isolates under study belong to *Klebsiella pneumoniae* subsp. *pneumoniae*.

![Figure 2: Klebsiella pneumonia colonies on MacConkey agar (A), Blood agar (B) and CHRO Magar orientation (C), after 24 hours of incubation at 37°C](image)
Antimicrobials Susceptibility

All K. pneumonia isolates were evaluated for antimicrobial susceptibility with Antibiotic sensitivity test Number 69 (AST 69) cards which contain 15 antibiotics including; Ampicillin, Amoxicillin/ clavulanic acid, Ampicillin/sulbactam, Pepracillin/Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, imipenem, Gentamycin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantion and Trimethoprim/ sulfamethoxazole. Data presented in figure -3, shows a high level resistance of K. pneumonia clinical isolates towards most of the antibiotics under test. The present study revealed that all K. pneumonia isolates had the highest resistance rate 100% to Ampicillin followed by (87.6%) to Cefazolin, on the other hand the lowest level resistance of K. pneumonia isolates was to Imipenem which had 17.86% resistance rate.

![Antibiotic resistance of 56 Klebsiella pneumonia isolates](chart1.png)

R= Resistance I= Intermediate S= Sensitive

Figure 3: Antibiotic resistance of 56 Klebsiella pneumonia isolates

Phenotypic Detection of ESβL Production

Three methods were applied to detect phenotypic production of ESβL in all fifty six Klebsiella pneumonia isolates under study.

Detection of ESβL by Vitek-2 System

Phenotypic detection of ESBL producing Klebsiellapneumoniae was done by Vitek-2 system with the using Antibiotic sensitivity test Number 69 (AST 69) cards. Result showed that out of 56 isolates, 45 (80.35%) isolates of K. pneumonia were gave positive results for ESBL producing while 11(19.65%) isolates of K. pneumonia were gave negative results for ESBL producing as listed in table -1. Out of 45 positive ESBL producing K. pneumonia isolates, 31(68.89%) isolates from blood, 6(12.33%) from urine, 3(6.67%) from Sputum, 2(4.44%) from ear and 3(6.67%) from burns as shown in Figure 4.

![Percentage of positive ESβL producing K. Pneumonia isolates according to specimen type](chart2.png)

Figure 4: Percentage of positive ESβL producing K. Pneumonia isolates according to specimen type
On another hand out of 39 K. pneumonia isolates collected from blood specimens during this study, 31 (79.49%) isolates were gave positive results for ESβL producing and 8 (20.51%) isolates of K. pneumonia were gave negative results for ESβL producing by Vitek-2 system, while all six isolates from urine specimens gave positive results for ESβL producing (100%). Also the three isolates from sputum specimens and the two isolates from ear specimens were 100% ESβL producing, but out of 6 isolates from burns specimens only 3 isolates gave positive results for ESβL producing (50%) by Vitek-2 system method (Table -2).

Detection of ESβL by Disc Combination Method

In the disc combination method two paired sets of cartridges (D52C) discs were used, set one contain ceftazidime (30µg) alone and ceftazidime + clavulanic acid (30 µg /10 µg) while set two contain cefotaxime (30µg) alone and cefotaxime + clavulanic acid (30 µg /10 µg). Cefotaxime disc was combined with clavulanic acid and compared with cefotaxime disc alone, also ceftazidime disc was combined with clavulanic acid and compared with ceftazidime disc alone. The isolate was considered ESβL producer, when the inhibition zone of combined discs were ≥ 5mm increased than inhibition zone of disc alone.

Results in this regard showed that out of the 56 K. pneumonia isolates, 48 (80.35%) isolates of K. pneumonia were positive ESβL producing, while 8 (19. 65%) isolates of K. pneumonia were negative ESβL producing by two sets of cartridges (D52C) discs (ceftazidime /ceftazidime + clavulanic acid and cefotaxime / cefotaxime + clavulanic acid) as shown in Figure -5 and table -1. Out of 48 positive ESβL producing K. pneumonia isolates, 33 (68.75%) isolates from blood, 5(10.42%) from urine, 3 (6.25%) from Sputum, 2(4.16%) from ear and 5 (10.42%) from burns (Table 2).

Detection of ESβL by CHRO Magar Technique

From CHRO Magar confirmatory test (CHRO Magar Technique) of ESβL-producing isolates, 51(91.07%) of the 56 isolates gave positive result which suspected ESβL producers. All these isolates showed over night growth with blue colonies on the ESβL supplemented CHRO Magar orientation medium (Figure 6 and Table 1). On other hand, 5(8.93%) isolates gave negative result (Table -1). Out of 51 positive ESβL producing K. pneumonia isolates, 34 (66.67%) isolates from blood, 6(11.76%) from urine, 3(5.89%) from Sputum, 2(3.92%) from ear and 6(11.76%) from burns (Table 2).
Results revealed that, ESBL-production was verified with the three different methods (Table -1). Eventually, CHRO Magar technique was recorded a high percent of ESBL detection while method by using Vitek-2 system was recorded a low percent of ESBL detection as shown in Figure 7. This study noticed that a high percent of ESBL producing *K. pneumoniae* isolates compared with non ESBL producing *K. pneumoniae* isolates under test by all previous confirmatory methods used in this study.

![Figure 7: Percentage of ESBL producer and non ESBL producer K. pneumonia isolates](image)

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<th>Phenotypic detection method</th>
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<th>Negative ESβL (%)</th>
<th>Total N (%)</th>
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<td>Disc combination method</td>
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<td>CHROMagar Technique</td>
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Table 2: Phenotypic detection of ESβL producing *K. pneumonia* isolates

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Discussion

Isolates of *Klebsiella pneumonia* are ubiquitously present and reported worldwide. In recent years, *K. pneumoniae* have become important pathogens in nosocomial infections [11]. Although it is a member of the intestinal flora is isolated as the causative agent in severe infections such as bacteremia [3], it is also an important nosocomial pathogen involved in urinary tract infections, hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP) [12], surgical-wound infection, and septicemia [13].

The results of anti bio gram have been shown that the local *K. pneumonia* isolates were possess highly resistance towards most antimicrobial under study, in other sensing were multi-drug resistant pathogens, especially towards Cephalosporins.

A study carried out in India by Shilpa et al., showed that 30 *K. pneumoniae* isolates were highly resistant to Ticarcillin/clavulanic acid (81.48%), Tobramycin (58.06%) and Trimethoprim/Sulfamethoxazole (50.0%), while the isolates were sensitive to Ciprofloxacin (68.90%), Gentamicin (62.50%) and Imipenem (56.66%).

In a local study done by Aziz and Al-Jubori (2016), reported that the highest resistance percentage was towards Ampicillin (95.83%) followed by Ceftazidime (87.50%), however, 29.17% of *K. pneumoniae* isolates were resistant to imipenem. Other local study noticed that the highest resistance rate was found to ampicillin, amoxicillin, ceftazidime, ceftriaxone and cefepime with 100%, tetracycline and levofloxacin 66.6%, tobramycin and ciprofloxacin with 50%. On the other hand 100% of isolates were susceptible to gentamicin, which was the most effective drug [14].

A study carried out in Iran showed that that 79 isolates (54.8%) were resistant to at least one of the third generation cephalosporines, in which 43.7%, 45.8% and 50% were resistant to ceftazidime, cefpodoxime and ceftaxime, respectively. The rates of resistance to other antibiotics were as follows: 144 (100%) of the *K. pneumoniae* isolates were resistant to amoxicillin, 63 (43.7%) to cotrimoxazole,72 (50%) to ceftaxime, 63 (43.7%) to ceftazidime, 66 (45.8%) to cefpodoxime, 61 (42.3%) to cefteriaxon, 58 (40.2%) to ceftizoxime, 54 (37.5%) to aztreonam, 52 (36.1%) to cefepime, 31 (21.5%) to levofloxacin, 25 (19.4%) to gentamicin, 23 (15.9%) to both amikacin and imipenem, and 17 (11.8%) to meropenem [15].

Our results revealed that, ESBL-production was verified with the three different methods, in which CHROMagar technique was recorded a high percent of ESBL detection while method by using Vitek-2 system was recorded a low percent of ESBL detection. Also this study noticed that a high percent of ESBL producing *K. pneumoniae* isolates compared with non ESBL producing *K. pneumoniae* isolates under test by all previous confirmatory methods used in this study.

In a local study done by Al-Charrakhet et al. Reported that three methods were used for detection of ESBLs production. In which,
out of the 38 β-lactamase producing isolates, only 4 (10.5%) ESBL-producers were detected by disk approximation method, 6 (15.7%) isolates were detected by screening tests of NCCLS, and 8 (21%) ESBL-producers were detected by determination of MIC of cefotaxime with and without clavulanate. Among the three ESBL detection methods mentioned previously, determination of MIC with and without clavulanate, was the most accurate method in detection of ESBL-producing isolates.

**Klebsiella pneumoniae** is the most common extended spectrum β-lactamases (ESBLs) producing organism (15). ESBLs have evolved remarkably during the last 20 years and the organisms producing these genes are responsible for increasing in nosocomial infections, morbidity and mortality, especially amongst patients in intensive care and high dependency units (16). These phenotypes usually exhibit multi-drug resistance that is not always detected in routine susceptibility tests. The inability to detect such complex resistance phenotypes is a serious challenge facing clinical laboratories and may have been a major factor in the uncontrolled spread of ESBL-producing organisms and related treatment failures (17). The rate of ESBL production in bacteria differs greatly all over the world, and it has been changing rapidly. The correct detection of ESBL producing microorganisms is a challenge for the laboratories, requiring not only phenotypic tests, but also genotypic tests for all genes associated with β-lactamase production (18; 19).

The results of our study showed that there is a high percentage of *K. pneumoniae* isolates under study were producing ESBLs enzymes. A study done by Mansur et al. (2016) have been reported that 61.3% of *K. pneumoniae* isolates were positive for ESBLs by E-Test ESBL method, but by using combined discs, cefotaxime/ clavulanic acid 89.4% of *K. pneumoniae* isolates were identified as β-lactamase producing isolates.

In a local study done by Hussein et al. have been reported that the most common ESBL producing gram negative isolates were *K. pneumonia* (82.05%) isolated from blood specimens of patients, also Andrew et al., have been reported the most common species presenting ESBL producing activity gram negative bacteria were *K. pneumonia* isolates (92%). Other local study showed phenotypic confirmatory test for ESBL production were positive for 62.5% (20/32) of the *K. pneumonia* isolates resistant to third-generation cephalosporins [20]. A local study done in Al-Qadisiya city revealed that the increase of ESBL producing *Klebsiella pneumoniae* (36.7%) isolated from urinary tract infection in Al-Diwaniya Teaching Hospital [14].

A study in Iran showed that *K. pneumonia* was a potent ESBL producer (71.4%) in comparison to *E. coli* (57.7%) under study [21]. In Asian countries, the ESBL frequency varied from 66.7% in India [22], 54.7%-61% in Turkey, 41% in United Arab Emirates [23], 31.7% in Kuwait [24], and 72.1% in Iran [25], but was lower in America and Europe [26].

Detection of ESBL is not routinely carried out in many microbiology units of service laboratories. This could be attributed to lack of awareness or lack of resources and facilities to conduct ESBL identification. [27]. Presence of isolates that harbor these enzymes in clinical infections making the selection of an effective antibiotic difficult and can result in treatment failure if one of the β-lactam drugs, including extending Spectrum β-lactamase, is used [28].

**References**


