Detection OF β- lactamase genes in clinical *Klebsiella pneumonia* strains isolated from hilla hospitals

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Abstract . This study aimed for is olation and identification of *Klebsiella pneumonia* ,from clinical sample and from different hospitals in Hilla city, during the period from April to October 2017. 122/1056 (12%) isolates were belonged to *Klebsiella pneumonia* were collected. Growth on blood agar, MacConkey agar and Eosin methylene blue agar was identified by cultural, morphological and biochemical tests and confirmed by VITEK 2 system. The results showed that all the tested isolates were resistant to Ampicillin and Amoxicillin 122(100%) ,while 119(98%) for penicillin ,whereas 100(82%) for piperacillin .Resistance to other drug classes varied among the isolates, a higher resistance was also detected with 95(78 %) to ceftazidime 94(77%) to ceftriaxone and 92(75%) to ceftriaxone. The resistance to quinolones, nalidixic acid ,ciprofloxacin and levofloxacin was detected 57(47%), 39(32%), 35(29%), respectively.majority of isolates 42 (34.4%) were obtained from sputum samples this result , 34 (27.8%) from urine , 17 (14 %) from stool , 15 (12.2 %) from burn, 8 ( 6.6 %) from vagina , 4 (3.3%) from wound , 1 (0.8%) from blood ,1 (0.8%) from ear and 0 (0 %) from both eye and throat. High prevalence rate for MDR,XDR and PDR in Merjan Teaching Hospital. The proportion of the patients from Merjan Teaching Hospital 27 (35.5%),16 (44.4%) and 1 (50%) respectively. Detection of β - lactamase genes (blaCTX-M-9, blaCTX-M-101, and blaNDM-1) was performed by the conventional PCR technique. The results revealed that 36 isolates analyzed, blaCTX-M-9 12 (33.3%), blaCTX-M-101 14 (38.9%), and blaNDM-1 (0%).

Keywords: *Klebsiella pneumoniae* , Antimicrobial susceptibility test , β-lactams genes.

Introduction

*Klebsiella pneumoniae* is an opportunistic pathogen associated with both community-acquired and nosocomial infections, including pneumonia, urinary tract infections, septicemia and wound infections, with the increasingly multidrug-resistant (MDR) *K. pneumoniae* being a major public health concern. The prevailing hypothesis is that these bacteria acquire multidrug resistance through horizontal transfer of antimicrobial resistance genes mediated by mobile genetic elements such as integrons (Stalder et al. , 2012).
Klebsiella pneumoniae is one of the most important pathogenic bacteria. It is gram negative, bacilli, non-motile and causative agent of many diseases, such as pneumonia, urinary tract infections, bacteremia, burns and wounds infections and pyogenic liver abscesses (Rahamathulla et al., 2016).

The widespread emergence of multidrug-resistant (MDR) bacterial pathogens is an important public health challenge worldwide (World Health Organization, 2014). Infections with MDR organisms are associated with increased mortality, longer hospital stays and inflated healthcare costs (Lambert et al., 2011; Neidell et al., 2012; Martin-Loeches et al., 2015). Recent data also indicate a trend towards increased antibiotic resistance among cases of community onset infections (Lim et al., 2014; World Health Organization, 2014; Stefaniuk et al., 2016).

Beta-lactams is one of classes of antibiotics contain a 3-carbon and 1-nitrogen ring that is highly reactive. They interfere with proteins essential for synthesis of bacterial cell wall, and in the process either kills or inhibits their growth. More succinctly, certain bacterial enzymes termed penicillin-binding protein (PBP) are responsible for cross linking peptide units during synthesis of peptidoglycan. Members of beta-lactam antibiotics are able to bind themselves to these PBP enzymes, and in the process, they interfere with the synthesis of peptidoglycan resulting to lysis and cell death (Heesemann, 1993). The aim of this study is to isolate and identify clinical K. pneumonia from Hilla hospitals, and detection of β-lactamase genes.

Materials and Methods

Bacterial isolates

In the present study, a total of 1056 clinical samples were collected during the period of five months from April to October 2017 from patients hospitalized / or attended to different hospitals in Hilla city / Babylon Province, included: Babylon Teaching Hospital for Maternity and Pediatric, AL- Hilla Teaching Hospital, Merjan Teaching Hospital and Chest Diseases Center. All samples were cultured on MacConkey's agar (Himedia) and incubated at 37 C˚ for 24 hrs. Bacterial isolates of K. pneumoniae were identified to the level of species by using the standard biochemical tests according to methods described by Collee et al and MacFaddin (Collee.,1996; MacFaddin, 2000) confirmatory identification was carried out by VITEK 2 system following manufacturer’s instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of resistant K.pneumoniae isolates was performed on Mueller-Hinton agar (Oxoid) plates by using Kirby-Bauer disk diffusion method (Bauer et al,1966). The isolates were tested against the following antibiotics: Ampicillin (10μg), Penicillin (10 μg), Piperacillin (10 μg), Amoxicillin (30 μg), Cefotaxime (30 μg), Ceftazidime (30 μg), Ceftriaxone (30μg), Cefepime (30 μg), Aztreonam (30 μg), Ceftazidime (30μg), Imipenem (10 μg), Meropenem (10 μg), Gentamicin (10 μg), Amikacin (30μg); Kanamycin (30μg) , Nalidixic acid (30 μg) , Ciprofloxacin (5μg) ; Levofoxacin (5μg) ; Trimethoprim- Sulfamethoxazole (25μg) Cefotaxime (30μg), , Rifampin(5 μg) ,Chloramphenicol (30μg), Tetracycline (30μg) and Doxycycline (30μg). The cultures were incubated at 37 Co for 18 hrs under aerobic conditions and bacterial growth inhibition zones diameter were measured and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,2017).
DNA preparation

DNA preparation from bacterial cells was performed by salting out method as described by Pospiech and Neuman with some modification and used as a template for PCR reaction (Pospiech and Neumann, 1995).

Polymerase Chain Reaction Protocols

Genes resistance primers detailed down in table (1) and the volumes and concentration of PCR mixture showed in table (2).

Table (1): Genes Resistance Primers

| Primer name | Oligosequence (5′ 3′) | Product size (bp) | Reference |
|-------------|-----------------------|-------------------|-----------|
| blaCTX-M-9 | GTG ACA AAG AGA GTG | ATG ATT CTC GCC GCT GAA GCC | 857 | Heffernan et al., 2007 |
| blaCTX-M-10 | GCA GCA CCA GTA AAG TGA | GCG ATA TCG TTG GTG GTA CC | 524 | Oliver et al., 2001 |
| blaNDM-1 | TGCCCAATATTATGCACCCG | CGAAACC CGG CATGTCGAG A | 813 | Schmittgen and Livak, 2008 |

Table (2): Volumes and concentration of PCR mixture for each primer used in the current study.

| Primer Name | Final volume in μl for each ingredient | Final concentration of primers in total reaction 25 μl |
|-------------|----------------------------------------|-----------------------------------------------------|
|             | Go Taq® Green Master Mix, 2X | Forward primer | Reverse primer | DNA template | Nuclease-Free-Water |  |
| blaCTX-M-9 | 12.5 μl | 2.5 μl | 2.5 μl | 5 μl | 2.5 μl | 10 pmole/ μl |
| blaCTX-M-10 | 12.5 μl | 2.5 μl | 2.5 μl | 5 μl | 2.5 μl | 10 pmole/ μl |
| blaNDM-1 | 12.5 μl | 2.5 μl | 2.5 μl | 5 μl | 2.5 μl | 10 pmole/ μl |

PCR Cycling Profiles

Polymerase chain reaction assays were carried out in a 25 μl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure as in Table (3).
Table (3): PCR thermocycling conditions for detection of antibiotics resistance genes and virulence factors genes in this study.

| Primer Name | Initial denaturation | Temperature (°C) /Time | Cycling condition | Denaturation | Anneling | Extension | Final extension | Cycle Number |
|-------------|----------------------|------------------------|-------------------|-------------|----------|-----------|----------------|--------------|
| blaCTX-M-9 | 94/ 5 min | 94/1 min | 55/1 min | 72/2 min | 72/1 min | 30 |
| blaCTX-M-10 | 94/ 5 min | 94/1 min | 55/1 min | 72/2 min | 72/1 min | 30 |
| blaNDM-1   | 95/ 20 sec | 95/3 sec | 50/2 min | 72/20 sec | 72/1 min | 40 |

Results

Bacterial isolates

A total of 1056 samples were collected during the period from April to October 2017 only 122/1056 (12%) isolates were belonged to Klebsiella pneumonia. Growth on blood agar, MacConkey agar and Eosin methylene blue agar was identified by cultural, morphological and biochemical tests and confirmed by VITEK 2 system.

Antibiotic Susceptibility Test of K. pneumoniae Isolates

Results revealed that only 122/1056 (12%) isolates were belonged to Klebsiella pneumonia. All the 122 isolates of Klebsiella pneumonia were screened for their antibiotic resistance against 24 antibiotics of different classes using Kirby-Bauer disk diffusion method. The results showed that all the tested isolates were resistant to Ampicillin and Amoxicillin 122(100%) ,while 119(98%) for penicillin , whereas 100(82%) for piperacillin .Resistance to other drug classes varied among the isolates, a higher resistance was also detected with 95(78%) to cefotaxime 99(81%) ceftazidime , 94(77%) to ceftriaxone and 92(75%) tocefotaxime. The results also revealed that were high resistant rates for Aztreonam 89(73%), imipenem displayed a lower resistance rate 23(23%), than meropenem 40(33%). Aminoglycosides resistance was variable ,71(50%) to kanamycin ,55(45%) to gentamicin and 37 (30 %) to amikacin. The resistance to quinolones, nalidixic acid, ciprofloxacin and levofloxacin was detected 57(47%), 39(32%), 35(29%), respectively. Percentages of resistance of isolates to the remaining antibiotics were as follows : tetracycline 76(62%), doxycycline 84(69%) and nitrofurantoin 78(64%) each, trimethoprim-sulfamethoxazole 72(59%) and chloramphenicol 51(42.6%).Rifampin resistance 98(80%).

Drug Resistance Pattern for K. pneumoniae Isolates.

Results revealed that MDR isolates 122/76 (16.2%), XDR 122/36(29.5%) and PDR 122/2 (1.6%) ,while sensitive isolates were 122/8(6.5%). (Table 4). Number and percentage of Klebsiella pneumoniae isolates among different clinical samples showed in (Table 5). Distribution of clinical drug resistant pattern in Klebsiella pneumoniae isolates in Hilla Hospitals showed in (Table 6).

Table (4) Drug Resistance Pattern for K. pneumoniae Isolates.

| No. of K. pneumoniae isolates | Type of resistance | Sensitive isolates |
|-------------------------------|--------------------|--------------------|
| MDR                           | XDR                | PDR                |
| 122                           | 16 (16.2%)         | 36 (29.5%)         | 2 (1.6%)    | 8 (6.5%) |
Table (5): Number and percentage of *Klebsiella pneumoniae* isolates among different clinical samples.

| Clinical sample | No. of sample N= 1056 | No. (%) of *K. pneumoniae* isolates N=122 | Patients infected with MDR isolates n= 76 (62.2%) | Patients infected with XDR isolates n=36 (29.5%) | Patients infected with PDR isolates n=2 (1.6%) |
|-----------------|-------------------------|-------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Sputum          | 194                     | 42 (34.4%)                                | 39 (51.3%)                                    | 18 (50%)                                      | 1 (50%)                                       |
| Urine           | 192                     | 34 (27.8%)                                | 16 (21.1%)                                    | 12 (33.3%)                                    | 1 (50%)                                       |
| Stool           | 200                     | 17 (14%)                                  | 11 (14.4%)                                    | 4 (11.1%)                                      |                                               |
| Burn            | 185                     | 15 (12.2%)                                | 7 (9.2%)                                      | 2 (5.6%)                                      |                                               |
| Vagina          | 112                     | 8 (6.6%)                                  | 2 (2.6%)                                      |                                               |                                               |
| Wound           | 70                      | 4 (3.3%)                                  | 1 (1.3%)                                      |                                               |                                               |
| Blood           | 46                      | 1 (0.8%)                                  |                                               |                                               |                                               |
| Ear             | 25                      | 1 (0.8%)                                  |                                               |                                               |                                               |
| Throat          | 22                      |                                            |                                               |                                               |                                               |
| Eye             | 10                      |                                            |                                               |                                               |                                               |
| Total           | 1056                    | 122 (100%)                                | 76 (100%)                                     | 36 (100%)                                     | 2 (100%)                                      |

Table (6): Distribution of clinical drug resistant pattern in *Klebsiella pneumoniae* isolates in Hilla Hospitals.

| Hospital's name                          | No. (%) of *K. pneumoniae* isolates (122) | Patients infected with MDR isolates n= 76 (62.2%) | Patients infected with XDR isolates n=36 (29.5%) | Patients infected with PDR isolates n=2 (1.6%) |
|------------------------------------------|--------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Merjan Teaching Hospital                 | (££ (11.3%))                              | 27 (35.5%)                                    | 16 ( 44.4%)                                   | 1 (50%)                                       |
| Al- Hilla Teaching Hospital              | (£A (10.8%))                              | 23 (30%)                                      | 7 (19.4%)                                     |                                               |
| Babylon Teaching Hospital for Maternity and Pediatric Chest Diseases Center | (\* (14.2%))                              | 18 (23.6%)                                    | 11 (30.5%)                                    | 1 (50%)                                       |
| Total                                    | 122 (11.5%)                               | 76 (62.2%)                                    | 36 (29.5%)                                    | 2 (1.6%)                                      |

*MDR*: multi drug resistant  **XDR**: Extensive drug resistant; ***PDR**: Pandrug resistant

\( \chi^2 = 0.899 \) is no significant in (p value = 0.828 > 0.05)

**Molecular Detection of β - lactamase Genes In Clinical Isolates of *K.pneumoniae*.

Detection of β - lactamase genes (blaCTX-M-9, blaCTX-M-1, and blaNDM-1) was performed by the conventional PCR technique. The results revealed that 36 isolates analyzed, blaCTX-M-9 12 (33.3%) (Figure 1), blaCTX-M-1 14 (38.9%) (Figure 2), and blaNDM-1 0% (Figure 4).
Figure (1): Agarose gel electrophoresis (1.5% agarose, 70 % volt for 2-3 hrs) for \textit{bla}CTX-9 gene product (amplified size 857 bp).

Figure (2): Agarose gel electrophoresis (1.5% agarose, 70 % volt for 2-3 hrs) for \textit{bla}CTX-10 gene product (amplified size 524 bp).

Figure (3): Agarose gel electrophoresis (1.5% agarose, 70 % volt for 2-3 hrs) for \textit{bla} NDM-1 gene product (amplified size 540 bp).
Discussion

Results showed that 114/122 (93.4%) of K. pneumonia isolates were resistant to ampicillin and amoxicillin. This result is in accordance with a previous study in Najaf, Al- Muhannak found that 98.2% of K. pneumonia were resistant to both antibiotics (AL-Muhannak, 2010). This result agreed with study which found that High prevalence of K. pneumonia in sputum samples was demonstrated by other researchers Al- Muhannak (2010), (15.7%), Al- Sehlawi (2012), (16%), (Qi Wang et al., 2013), (75%) and Abd Al-Rhman and Al-Aubydi (2015) in Baghdad, (42.3%).

The results showed that higher resistance to Penicillin (Carbenicillin and Ampicillin) 122(100%) and 119(98%) respectively, whereas 100(82%) for piperacillin. This result was higher resistance for amoxi-clav 117(96%). This result was in agreement with a previous study in Hilla by Al- Asady (2009) who found that all 15 (100%) β-lactam resistant Enterobacteriaceae isolates were resistant to ampicillin, piperacillin. Al-Hilli (2010) stated that all K.pneumoniae isolates were resistant (81%) to piperacillin. This result was in agreement with a previous study in Hilla by Abbas, (2013) who found that resistance to penicillins (carbenicillin and ampicillin) of 90(99%) and 86(94.5%), respectively, whereas 75(82.4%) of isolates were resistance to piperacillin, while amoxi-clav (81.3%). Resistance to other drug classes varied among the isolates, a higher resistance was also detected with 99(81%) ceftazidime, 95(78%) to cefotaxime, 92(75%) to ceftriaxone and 89 (73%) to cefepime. Cai et al., (2011) reported that resistance rate of K.pneumoniae isolates to ceftazidime, cefotaxime and cefepime were (70.59%), (88.24%) and (64.71%) respectively. The results also revealed that were high resistant rates for cefoxitin 89(73%), in Najaf, Al-Sehlawi (2012) who found that prevalence rates of cefoxitin resistant was (70.9%) and in Hilla, Abbas, (2013) showed that (78%) rates of cefoxitin resistant.

this study showed that prevalence rates resistant of cefaclor and cefprozil were 95(78%) and 92 (75%) respectively In Hilla, Abbas, (2013).

Azetronam 89(73%), imipenem displayed a lower resistance rate 23(23%), than meropenem 40(33%). In spite of the low level of resistance, this result is higher than that reported by other local studies contacted in Iraq which reported that the susceptibility of K.pneumoniae isolates collected from clinical and environmental samples to imipenem was (100%) (Hadi, 2008; Al- Asady, 2009; Al- Muhannak, 2010 and Al-Hilli, 2010). Aminoglycosides resistance antibiotics in this study agree with Aljanaby and Alhasnawi, (2017) showed that resistant rate for gentamicin and amikacin was (45%) and (25.58%) respectively. The resistance to quinolones, nalidixic acid, ciprofloxacin and levofloxacin agee with study in Indonesia, Radji et al., (2011) showed that resistance results for ciprofloxacin and levofloxacin was (46.9%) and (62.2%) respectively. Rifampin resistance 98(80%). This result agreed with study by Abbas, (2013) in Hilla, found Percentages of resistance for tetracycline, doxycycline, and nitrofurantoin each, trimethoprim-sulfamethozazole and chloramphenicol were (62.6%), (59.3%), (59.3%), (56%) and (39.6%) respectively.
Result from table (3) revealed that MDR isolates 122/76 (26.2%), XDR 122/36 (29.5%) and PDR 122/2 (1.6%) ,while sensitive isolates were 122/8 (6.5%). This result is in accordance with a previous study by Bin found that (61.4)% were MDR isolates,(22%) were XDR isolates and (1.8%) isolates were PDR isolates (Bin et al.,2012) Results revealed that 36 resistant K. pneumonia isolates tested for blaCTX-M-9, blaCTX-M-10, bla-ACT and bla-NDM-1 were determined by PCR technique. Detection of β - lactamase genes (blaCTX-M-9, blaCTX-M-10, bla blaACT, and bla-NDM-1) was performed by the conventional PCR technique. The results revealed that 36 isolates analyzed, blaCTX-M-9 12 (33.3%) this result agree with Pitout and colleagues in 2007 who found 37% CTX-M-9, blaCTX-M-10 14 (38.9%) this result compatible with (Bin et al.,2012) who found that 37.3% resistant isolates of K.pneumoniae carried blaCTX-M-9, and bla-NDM-1 (0%).In Beijing, China, Bin et al.,(2012 )showed that no K.pneumoniae isolates carried bla-NDM-1. The reduction in copy number and loss of the bla-NDM-1 gene in the absence of strong imipenem selection suggests that either the high copy number of this gene or the presence of the gene itself has a negative impact on the fitness of the Klebsiella bacterium that contains this sequence, as discussed by Sole’ et al.,(2012).

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