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MOLECULAR CHARACTERIZATION OF CLINICAL DRUG RESISTANT PATTERN IN *KLEBSIELLA PNEUMONIAE* ISOLATES IN HILLA HOSPITALS

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ABSTRACT : This study aimed for isolation and identification of *Klebsiella pneumonia*, from clinical sample and from different hospitals in Hilla city. A total of 1056 samples (423 male and 633 female), collected were included clinical samples (Burns 185(17.5%), wounds 70(6.6%), urine 200(19%), sputum 192(18%), Ear 25(2.4%), blood 46(4.4%), eye 10 (0.9%), stool 194 (18.4%), throat 22(2.1%) and vagina 112(10.6%)). While in Marjan teaching hospital was 390(37%), Al- Hilla teaching hospitals 352(33%), Babylon Teaching hospital for Maternity and Pediatric 212(20%) and Chest Disease Center 102(10%)), during the period from April to October 2017 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.

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-Bauerdisk 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%) to ceftriaxone. The results also revealed that high resistant rates for Azetreonam 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%).

In this study, resistance isolates submitted to molecular detection of some resistance genes (blaCTX-M-1, blaCTX-M-2, dhfr and aacA4) by conventional Polymerase chain reaction (PCR) technique.

Key words : *Klebsiella pneumoniae*, antibiotic resistance genes, Polymerase Chain Reaction.

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).

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; Timothy *et al*, 2017). 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). For many bacterial pathogens, particularly Gram-negative organisms, high rates of antimicrobial resistance present limited therapeutic options for treating serious infections. *Klebsiella pneumoniae* is one of these MDR organisms identifiedas an urgent threat to human health by the World Health Organization, the US Centers for Disease Control and

Prevention and the UK Department of Health. *K. pneumoniae* infections are particularly a problem among neonates, elderly and immunocompromised individuals within the healthcare setting, but this organism is also responsible for a significant number of community-acquired infectionsincluding pneumonia and sepsis (Paczosa and Mecsas, 2016; Quan *et al*, 2016).

Types of resistance

MDR

In literal terms, MDR means 'resistant to more than one antimicrobial agent', but no standardized definitions for MDRhave been agreed upon yet by the medical community. Many definitions are being used in order to characterize patterns of multidrug resistance in Gram-positive and Gram-negative organisms (Falagas *et al*, 2006; Cohen *et al*, 2008; Hidron *et al*, 2008; MacGowan, 2008; Paterson, 2008). One of the methods used by various authors and authorities to characterize organisms as MDR is based on *in vitro* antimicrobial susceptibility test results, when they test 'resistant to multiple antimicrobial agents, classes or subclasses of antimicrobial agent' (Falagas *et al*, 2006; Cohen *et al,* 2008; Hidron *et al*, 2008; Kallen *et al*, 2010).

XDR

Bacteria that are classified as XDR are epidemiologically significant due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being resistant to all, or almost all, approved antimicrobial agents. In the medical literature, XDR has been used as an acronym for several different terms such as 'extreme drug resistance', 'extensive drug resistance', 'extremely drug resistant' and 'extensively drug resistant' (Falagas *et al*, 2008; Park *et al*, 2009; Brink *et al*, 2009; Tseng *et al*, 2007).

PDR

From the Greek prefix 'pan', meaning 'all', pandrug resistant (PDR) means 'resistant to all antimicrobial agents'. Definitions in the literature for PDR vary even though this term is etymologically exact and means that, in order for a particular species and a bacterial isolate of this species to be characterized as PDR, it must be tested and found to be resistant to all approved and useful agents. Examples of current definitions are: 'resistant to almost all commercially available antimicrobials', 'resistant to all antimicrobials routinely tested' and 'resistant to all antibiotic classes available for empirical treatment' (Falagas *et al*, 2006; Kuo *et al*, 2003; Kuo *et al*, 2004). The aim of this study is todetect antibiotic resistant genes ofclinical drug resistant pattern in *Klebsiella pneumoniae* isolates from Hilla Hospitals.

MATERIALS AND METHODS

Bacterial isolates

In the present study, a total of 1056 clinical samples were collected during theperiod 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 37^0C 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* (1996) and 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 (30 µg), Cefepime (30 µg), Aztreonam (30 µg), Ceftazidime (30µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), Amikacin (30 µg); Kanamycin (30µg), Nalidixic acid (30 µg), Ciprofloxacin (5µg), Levofloxacin (5 µg), Trimethoprim-Sulfamethoxazole (25µg), Cefotaxime (30µg), Rifampin(5µg), Chloramphenicol (30µg), Tetracycline (30µg) and Doxycycline (30µg). The cultures were incubated at 37^0C 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 DNA extraction kit (Genoiad company) and used as a template for PCR reaction.

Polymerase Chain Reaction Protocols

Polymerase chain reaction was used to amplify the entire sequence of *bla*CTX-1, bla CTX-2, dhfr and aacA4 genes. The primer (Bioneer) (Table 1). PCR was performed in a 50-mL reaction mixture consisting of 5 mL of 106 PCR buffer, 2.5 units of Taq DNA polymerase (Takara), 0.2 mM of dNTPs, 0.4 mM each of the primer, and 1 mL chromosomal DNA. All reaction mixtures were

subjected to 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The resulting PCR product was run in 1.5% agarose gels and electric current was allowed at 70 volts for 2 hr. DNA bands were observed using UV-Transilluminator and photographed with Gel documentation system. 1500 bp DNA Ladder (Bioneer) was used to assess PCR product size.

RESULTS

Bacterial isolates

A total of 1056 samples (423 male and 633 female) collected were included clinical samples (Burns 185(17.5%), wounds 70(6.6%), urine 200(19%), sputum 192(18%) Ear 25(2.4%), blood 46(4.4%), eye 10 (0.9%), stool 194 (18.4%), throat 22(2.1%) and vagina 112 (10.6%) (Table 2), while in Marjan teaching hospital was 390(37%), Al-Hilla teaching hospitals 352(33%), Babylon Teaching hospital for Maternity and pediatric 212(20%) and Chest Disease Center 102(10%)), during the period from April to October 2017 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.

Antibiotic susceptibility test

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-Bauerdisk 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%) to ceftriaxone. The results also revealed that high resistant rates for Azetreonam 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(62.2%), XDR 122/36(29.5%) and PDR 122/2 (1.6%), while sensitive isolates were 122/8(6.5%) (Table 3).

Screening and detection of resistance genes in *K. pneumoniae* isolates

Presence of blaCTX-M-1, blaCTX-M-2, dhfr and aacA4 were determined by PCR technique. Table 3 shows the distribution of resistance genes among the isolates. However, results revealed that of 36 isolates analyzed,

Table 1 : Primers used for PCR and sequencing of drug resistance-associated genes from *K. pneumoniae* isolates.

Target	Primer sequence (5' to 3')		Amplicon size (pb)	Source of reference
	Forward	Reverse		
blaCTX-1	GGT TAA AAA ATC ACT GCG TC	TTA CAA ACC GTC GGT GAC GA	**876**	Jemima and Verghese (2008)
blaCTX-2	ATG ATG ACT CAG AGC ATT CG	TTA TTG CAT CAG AAA CCG TG	**876**	Bauernfeind *et al* (1996)
dhfr	GCC AAT CGG GTT ATT GGC AA	TGG GAA GAA GGC GTC ACC CTC	**357**	Leavitt *et al* (2009)
aacA4	ATG ACT GAG CAT GAC CTT GCG	TTA GGC ATC ACT GCG TGT TCG	**540**	Hujer *et al* (2006)

Table 2 : Number and percentage of *Klebsiella pneumoniae* isolates among different clinical samples.

Clinical sample	No. of samples	No. (%) of *K. pneumoniae* isolates
Burns	185(17.5%)	15 (8.1%)
Wounds	70(6.6%)	6 (8.6%)
Urine	200(19%)	26 (13%)
Sputum	192(18%)	24 (12.5%)
Ear	25(2.4%)	1 (4%)
Blood	46(4.4%)	2 (4.3%)
Eye	10(0.9%)	1 (10%)
Stool	194(18.4%)	39 (20.1%)
Throat	22(2.1%)	No (0%)
Vagina	112(10.6%)	8 (7.1%)
Total	1056 (100%)	122 (100%)

Table 3 : Drug resistance pattern for *K. pneumoniae* isolates.

No. of *K. pneumoniae* isolates	Type of resistance			Sensitive isolates
	MDR	XDR	PDR	
122	76 (62.2%)	36 (29.5%)	2 (1.6%)	8 (6.5%)

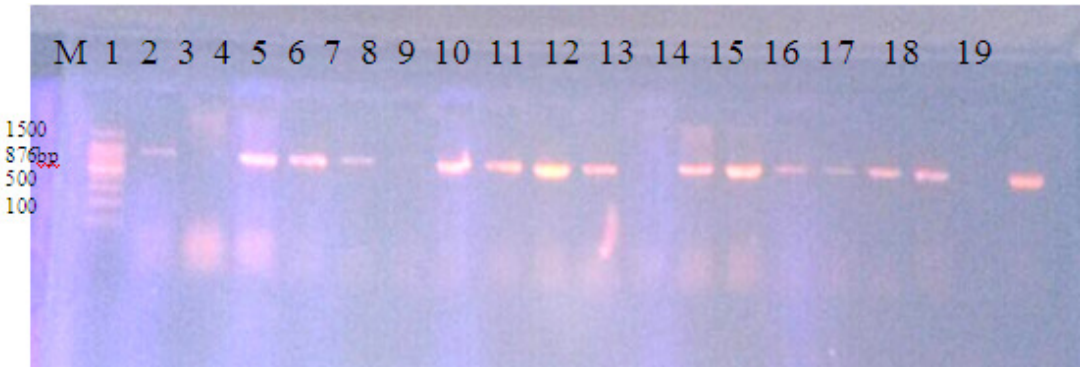

Fig. 1 : Agarose gel electrophoresis of blaCTX-M-1 amplified product.

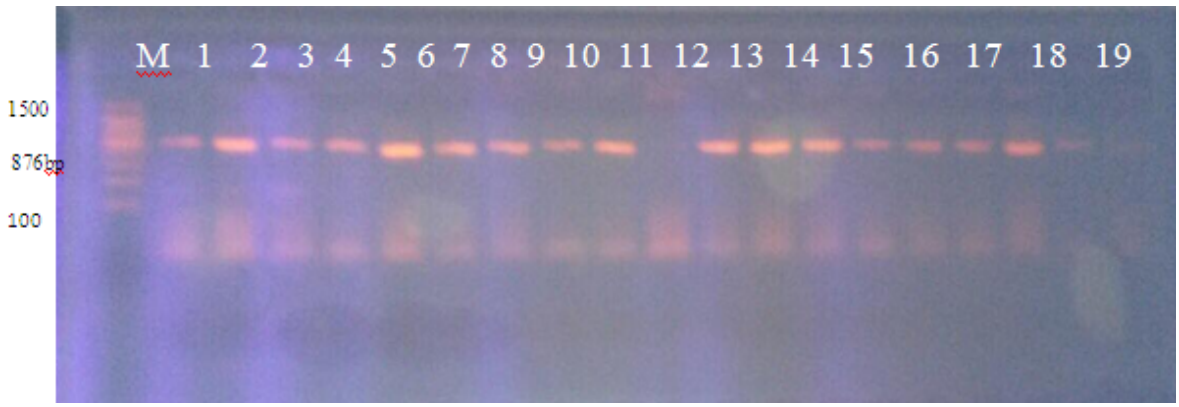

Fig. 2 : Agarose gel electrophoresis of blaCTX-M-2 amplified product.

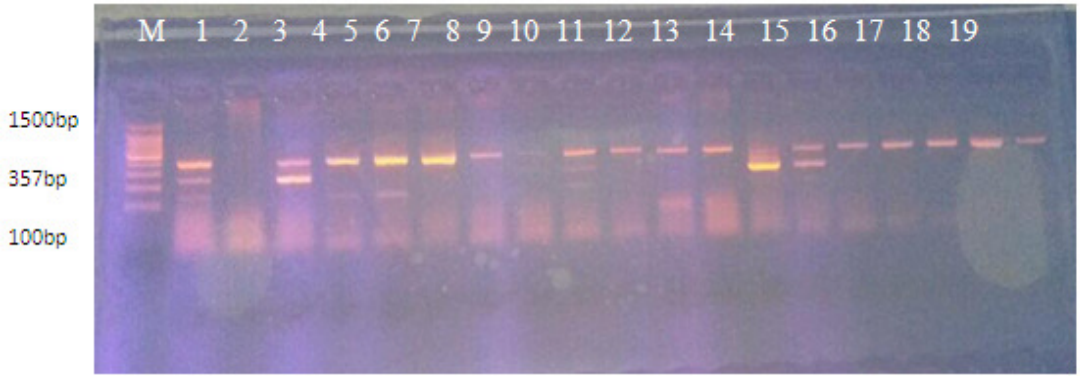

Fig. 3 : Agarose gel electrophoresis of dhfr gene amplified product.

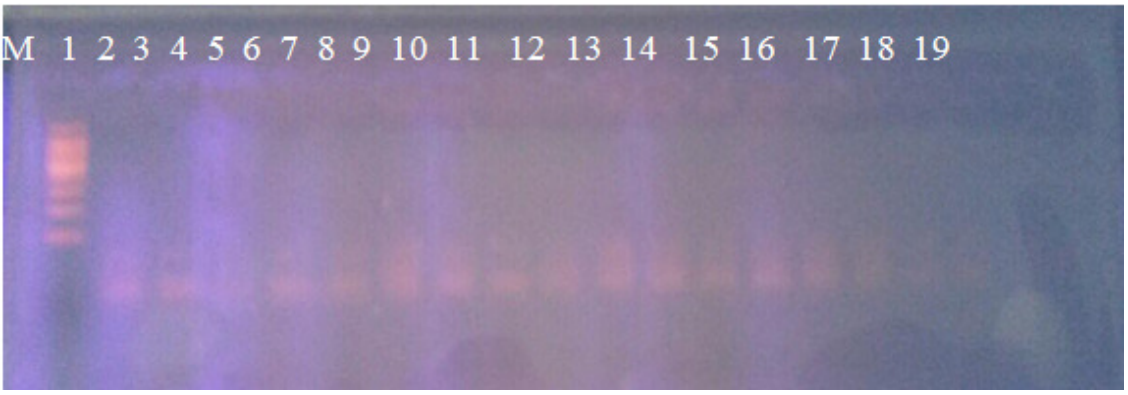

Fig. 4 : Agarose gel electrophoresis of aca4 gene amplified product (negative results).

18 (50 %) isolates yielded amplification products with blaCTX-M-1, blaCTX-M-2 31(86%), dhfr gene was observed in 28(77.8%) isolates and aacA4 gene no isolate have this resistance gene (Figs. 1, 2, 3 and 4).

DISCUSSION

Results showed that 122 (12%) isolates were identified as *K. pneumoniae*. This result is in agreement with a previous local study in Hilla by Al-Saedi (2000), who found that *K. pneumonia* isolates comprised (15.3%) from 725 clinical samples (Al-Saedi, 2000). In another study, Al-Sehlawi reported that the detection rate of *K. pneumoniae* was (14%) among all pathogens isolated from clinical samples in Najaf hospitals (Al-Sehlawi, 2012). However, the majority of *K. pneumoniae* isolates 39/194 (32%) were obtained from stool samples (Table 1). *K. pneumoniae* are Gram- negative bacteria, which are part of the normal human intestinal flora and are frequently spread via fecal-oral contamination. High prevalence of *K. pneumoniae* in stool samples was demonstrated by other researchers, Al- Saedi (2000) in Hilla (14%), Ali *et al* (2010) in Jordon, (20%) and Sarojamma and Ramakrishna (2011) in India (50%). In sputum, *K. pneumoniae* was detected in 24/192 (12.5%) of samples. Increasing prevalence of *K. pneumoniae* in sputum was observed by other researchers, Al-Muhannak (2010) (15.7%) and Al-Sehlawi (2012) (16%).

Result revealed that 114/122 (93.4%) of *K. pneumoniae* 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. pneumoniae* were resistant to both antibiotics (AL-Muhannak, 2010).

Result from Table 2 revealed that MDR isolates 122 /76 (62.2%), 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 (2012) found that (61.4%) were MDR isolates (22%) were XDR isolates and (1.8%) isolates were PDR isolates.

Results revealed that 36 resistant *K. pneumonia* isolates tested for blaCTX-M-1, blaCTX-M-2, dhfr and aacA4 were determined by PCR technique. 19(53%) of blaCTX-M-1, 28(78%) of blaCTX-M-2, 15(42%) of dfrh and non isolates carry for aacA4 gene (0%). Result is in accordance with a previous study by Bin (2012) found that 44.1% was dfrh gene.

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