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Detection of Metallo β-lactamase in *Klebseilla pneumoniae* Isolated From Upper Respiratory Tract of Carrier individuals In Iraq

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ABSTRACT

Klebsiella pneumoniae is an a health care challenge over all the world, due to its resistance to many effective antibacterial, particularly those depend as a standard therapeutic options, Klebsiella carbapenemase producing have been emerged in Indian provinces, this phenomenon then have been disseminated in many countries around the world. The most successful β-lactam hydrolyzing enzymes are belonging to the above antibiotic category is New Delhi metallo β-lactamase, presence of NDM is an indication of multi-drug resistance. K.pneumoniae was isolated from Indian employees in Erbil city and from Iraqi people living in Mosul city, who had previously conducted an interventional surgery at hospitals in India.Of a total of 35 K.pneumoniae isolates, 6 KCP strains were discovered by phenotypic analysis using the modified Hudge test. While molecular screening showed 3 KCP as a true positive result. High molecular sensitivity and specificity, based on Gradiant Multiplex PCR, were found in the comparison with classical diagnosis method.On the other hand, according to the Random Amplification Polymorphic DNA, genetic variation has categorized the six KCP isolates into three clusters due to the existence and absence of random amplicons.

Keywords: Klebsiella pneumonia, NDM, Multiplex-PCR, RAPD-PCR.

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INTRODUCTION

Klebsiella pneumoniae resist large number of antimicrobials and are widely recognized as a significant source of multidrug resistance genes that can disseminate among gram negative genera. Owing to its resistance to "ultimate option" antimicrobials such as last generation quinolones and carbapenems, it has gained notoriety and therapeutic choices are, in turn, limited here. [15,22,25]. Carbapenem antibiotics belong to β-lactam antibiotics category,that are reliant on treating several infections caused by Multidrug resistant (MDR) bacteria. Enzymes are the most prevalent mechanism in the population of pathogenic bacteria involved in an emergence of carbapenem-resistant strains, due to inactivation of β-lactam molecules and occurrence of persistent resistance to β-lactam antibacterial strains.[3,13]. β-lactamases are dividing into two sub groups serine β-lactamase and Metallo β-lactamase(MBL). They were categorized into four groups according to the Ambler classification, three of them (A, C, D)classified as serine β-lactamase as serine β-lactamase and B-class enzymes belonging to the Metallo β-lactamase family, Carbapenemase ezymes belonging to the classes A, B, and D[10]. New Delhi Metallo-β-Lactamase (NDM) is an Ambler class B, that firstly identified in *K. pneumoniae* and *Escherichia coli* during 2008 in Indian sub-continent. Later, NDM producers have been described worldwide. *bla*NDM-1 1 is an NDM-1 encoding gene nomenclature situated on a transmissible plasmid[20,26]. NDM-1 was listed as the first source of new variants such as NDM-2 till to NDM-17, but all of them showed similar enzymatic activities against β-lactam antibacterial[10].Antimicrobial susceptibility test and Huge test consider as perfect tool to observe carbapenem bacterial resistance, while the molecular screening is a confirmatory test to demonstrate responsible genes[1,4,6].

Several studies have been published on isolation and identification of carbapenemase producing Klebsiella (KCP) depending on phenotypic demonstration and molecular detection as a local documented research[18,19,27]. In addition to Hudson, Hu and Arpin as an International studies[2,5,9,6].

The present study focuses on isolation of KCPs from certain individuals in Mosul society, who traveled to India for health care, in addition to Indian workers in Erbil city, as KCP carriers and transporters to our region. As well as to identification of the molecular relationship between isolated strains.

MATERIAL AND METHODS

Samples collection and bacterial identified

A total of 190 upper respiratory swabs (URSs) were obtained from healthy Indian workers(120),and surgical intervention patients(70)who were treated in India, from November 2017 till end of April 2018 Erbil and Mosul cities, respectively. Thirty five isolates of non-duplicated *K.pneumonia* were identified using standard bacteriological technique[7].

Carbapenems Susceptibility Test

The Kirby-Bauer disc diffusion method was performed using Mueller Hinton agar medium supplied by Hi-media, India, to evaluate KCP susceptibilities to certain β-lactam antibiotics (imepenem and meropenem, resistance zone diameter, ≤ 21 mm) As outlined in detail by CLSI[6].

Phenotypic Carbapenemase Production estimation

Carbapenemase production was evaluate by Hodge test using meropenem disc 10 µg . MBLs (*blaNDM-1* and *blaNDM-2* genes)production confirmed by synergistic reaction of 10µg imipenem discs add to 290µg EDTA (Sigma Chemicals, USA),which placed onto Mueller-Hinton agar plate after cultivation of bacterial strains by pour plate method. Inhibition zone increased around imipenem EDTA disc(diameter>4mm)in comparison with

imipenem disc alone, which was depended as visible indicator for production of MBLs[16,17].

Molecular detection of β-lactamase genes:

Episomal DNA was extracted from previous positive screening isolates using spin plasmid kit(Bio-basic,Canada)as manufacturer's protocol description, Amplification mixture was subjected to Gradiant thermocycler(Applied biosystem, USA) to demonstrated *blaNDM-1* gene *blaNDM-2* gene via multiplex PCR[8].using the following specific primer sets,NDM-1-F(5′-GTCTGGCAGCA CACTTCCT-3′)and NDM -1 -R(5′-CGTATGAGTGATTGCG GCG-3′) with accession No.(KT956171) and NDM-5-F(ATCACGATCATGCTG GCCTT) and NDM-5-R(TTGCGACTTATGCCAATGCG)with accession No.(MK372393) documented by National Center for Biotechnology information, NCBI[26]. Amplification program included 95 C as DNA unwinding for 180 sec and 35 times of each of denaturation 94 C for 30 sec, hybridization 55 and54 C(respectively)for 30 sec, extension72 C for 30 sec, in addition to final extension72 C for 180sec, for both two primer sets.

Genetic diversity of KCP

Extraction of genomic DNA was conducted as that detailed by promega company, gDNA concentration and purity were carried out by nano-spectrophotometer, Template integrity was estimated according to that documented by[24].KCP variety was identified via Randomly amplification polymorphic DNA(RAPD) for amplification of four random primers (Bioneer, S.Korea), which were included OPAX-01(GTGTG CCGTT),OPAV-08 (TGAGA AGCGG),OPBH-17(CTCTTACGGG) and OPAT-01(CAGTGGTTC C) . Primex was supplied by Bioneer company, amplification mixture was placed in Gradiant thermocycler (Applied biosystem, USA)wells to carry out the following program: Pre-incubation 94 C for 240s,followed with 35 cycles of 93 C for 30s as initial incubation,33,31,27 and 28 C respectively for 30s as primers hybridization temperature and 72 C for 30s as complementary DNA strand extension, then finally 72 C for 180s as a terminal extension.

Outcomes documentation of Genotyping and Genetic diversity analysis

All amplification products were detected and photographed using gel documentary system (ATTA,Japan),all DNA bands were classified to polymorphic and single, they were represented as two-digital data(1,refere to band;0,absence) Then, we calculated a simple matching (Sm) coefficient and a Jaccard algorithm to determine a dendrogram and similarity and dissimilarity. (PASS Software, Oslo,2009). The specificity, sensitivity and diagnostic accuracy of the results were also calculated by applying the following equations: (a / a + b) x 100 = sensitivity, (d / d + c) x 100 = specificity[23].

RESULTS

Bacterial isolation

The present results showed that the percent of *K. pneumoniae* isolation from URSs was 18.4%,35/190(Table1). This finding was nearly to that reported by Bora and Ahmed(2012), who showed that 19/219(8.7%) isolates were revealed positive results for NDM-1 production.

Table (1): Number and percentage of *K. pneumoniae* isolation from URSs.

Isolation sources	No.	Positive results No. (%)	Negative results No. (%)
Clinical samples	190	35(18.4)	155(81.6)
Cal.X^2= 0.82, tab.X^2= 0.604, df =1, significance P<0.01.			

Biochemical reaction revealed that all *K. pneumoniae* isolate showed mucoid lactose fermentation colonies (pink color)on Macconkey agar, with oxidase negative reaction and Gram's negative rods under oil immersion, while IMViC (Indol, Methyl red, vogas proskuaer and Citrate tests) revealed negative result for I and M and positive result for Vi and M[7,21].

Phenotypic and genotypic screening of the Carbapenemase Production

Of all 35 *K. pneumoniae*, 6(17.1%) isolates showed positive appearance towards Hodge test, but PCR amplification products of *bla*NDM-1 gene and *bla*NDM-2 gene revealed 3(8.6%) isolates with an amplicon size 640 base pair(bp.) and 314bp. respectively associated with Indian workers samples(Figure1,table 2).

Gene	Indian workers	Iraqian Citizen	Tests	Sensitivity(%)	Specificity(%)
*bla*NDM-1,2 genes	3	--------	PCR method	50	50
	4	2	Hodge test	100	100

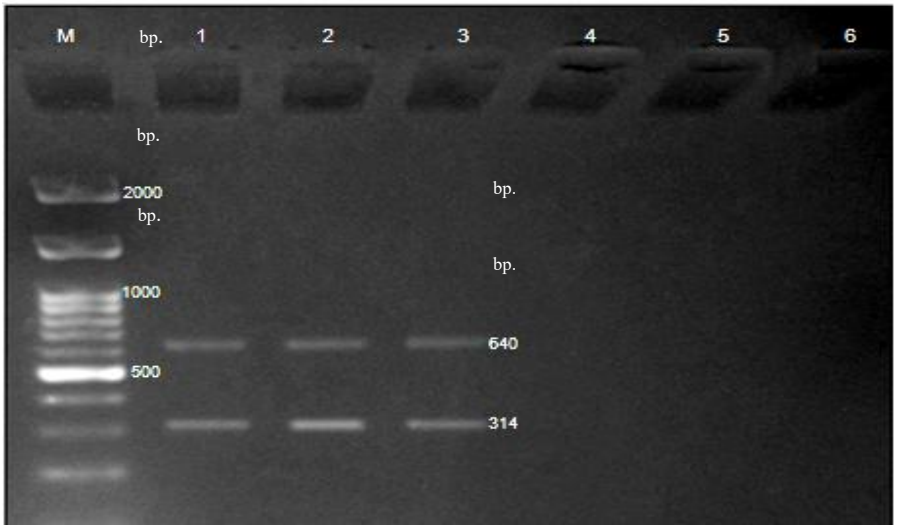


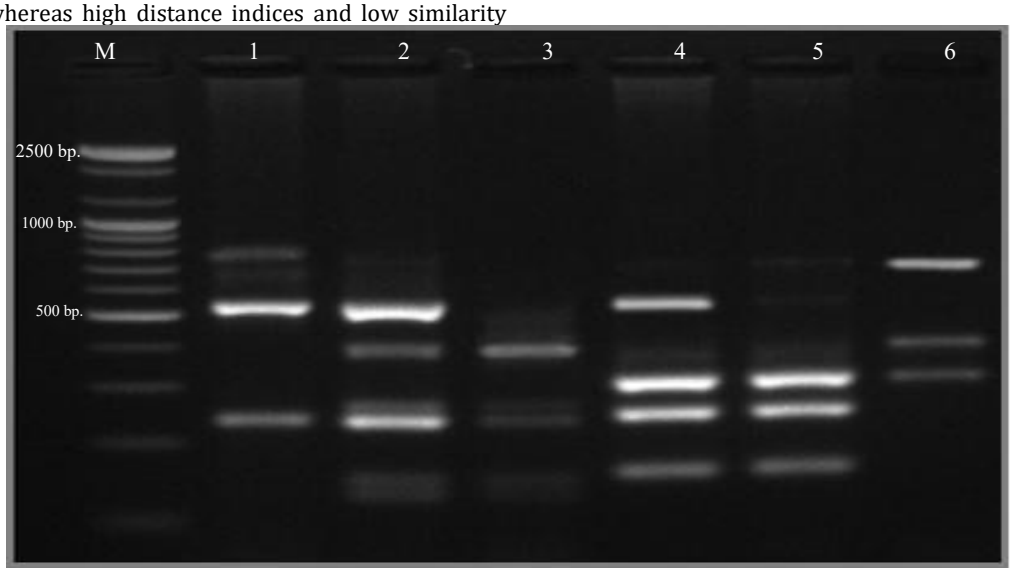
Figure (1): Multiplex amplification products for *bla*NDM-1 and *bla*NDM-2 genes from *K. pneumoniae* isolates. Lanes M, marker 100-bp., lanes (1-3) Amplicon size 640 bp. for *NDM-1* and 314 bp. for *NDM-2*, lanes (4-6) showed negative results.

Our results were similar to that finding by[20].who found that 15/275(5.5%)isolates of *K. pneumoniae* revealed positive outcome for NDM-1 gene Table(2). Other study carried out by Hasan[18], showed that 14(10.9%) isolates of *K. pneumonia* were carbapenemase producers. On the other hand, phenotypic investigation and molecular screening showed high specificity and sensitivity of PCR in comparison with modified Hodge test results. Conventional PCR is a sensitive and accurate technique, which need significant requirements to be optimized[10].

Table 2: comparison between modified Hodge test and PCR results

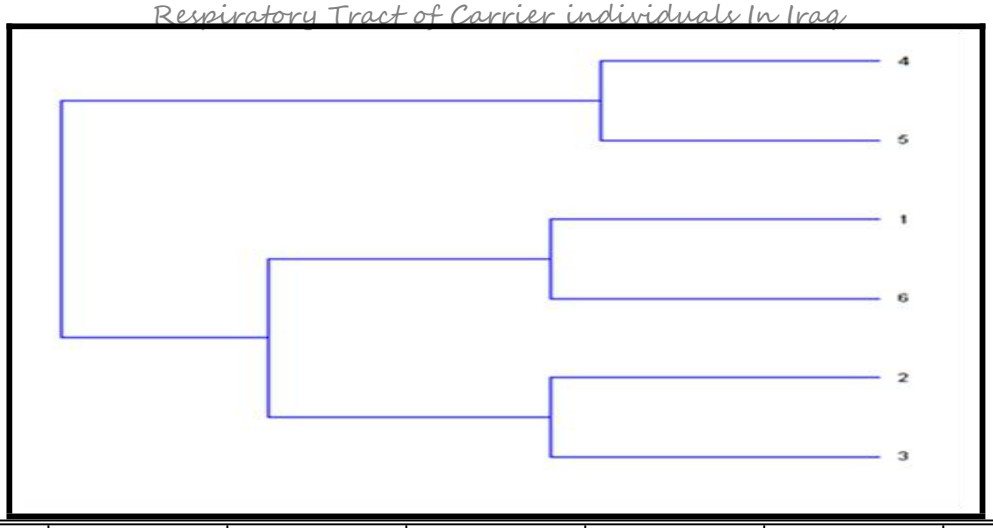
Genetic diversity of KCP

Figure two represented gDNA fragments for six KCP isolated strains, genetic diversity revealed high similarity with low distance indices between the strains 4 and 5, whereas high distance indices and low similarity were noticed between 2 and 4 strains(Table 2).Random amplification polymorphic DNA is an essential tool to determine the genetic relationship among microorganisms[14].



Figure(2):Random PCR amplicons for six KCP isolates. Line M is a DNA marker (100bp.), Lines 1-6 are a random gDNA fragments for KCPs

Table (3) : KCP similarity and dissimilarity



	1	2	3	4	5	6
1	0	0.47619	0.42857	0.42857	0.47619	0.33333
2	0.47619	0	0.33333	0.52381	0.47619	0.33333
3	0.42857	0.33333	0	0.47619	0.42857	0.38095
4	0.42857	0.52381	0.47619	0	0.2381	0.38095
5	0.47619	0.47619	0.42857	0.2381	0	0.42857
6	0.33333	0.33333	0.38095	0.38095	0.42857	0

The dendrogram among the phenotypically diagnosed KCPs showed genetic relationship with two mainly clusters, the first is single have two isolated strains(4 and 5),the second have two sub divisions each of them represented with two isolated strains 1,6 and 2,3 respectively(Figure 3).Random amplification of gDNA is an essential tool to detect the genetic diversity among microorganisms[12].

Figure (3): Phylogenetic tree of six KCPs

RECOMMENDATION

Depending on our result we recommended that

1. Iraqi government must depend clinical testing program for checking all incomes passengers to the country at the borders, in order to control the administration and prevention of non endemic diseases.
2. Recommended for all hospital especially intensive care units to check out medical staff for detect any variant strains they which carrier them, in order to maintain hospital care units clear from any health care problem .

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