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Research Article

First report of OXA-48-producing Klebsiella pneumoniae strains in Iran

Erstbeschreibung von OXA-48-produzierenden Klebsiella pneumoniae-Stämmen im Iran

Abstract

Carbapenem-resistant *Enterobacteriaceae* are increasingly reported worldwide and cause therapeutic problem in health care facilities. In this study 28 imipenem-resistant *K. pneumoniae* were examined for expression of carbapenemases by phenotypic and genotypic methods. Modified Hodge Test (MHT), CarbaNP test were used for phenotypic detection, and PCR using specific primers for the detection of bla_{OXA-48}-, bla_{KPC}-, bla_{NDM}- and bla_{VIM}-type carbapenemases with specific primers were performed. MHT and CarbaNP tests were positive for all of imipenem-resistant *K. pneumoniae*. The bla_{OXA-48} gene was detected in 27/28 isolates. One isolate was positive for the presence of the bla_{VIM-4} gene. According to our results NP test and MHT have high sensitivity and specificity for detection of those carbapenemases. This study reports the first cases of OXA-48-producing *K. pneumoniae* in Iran.

Keywords: carbapenem, Klebsiella pneumoniae, CarbaNP test, MHT, OXA-48

Zusammenfassung

Carbapenem-resistente *Enterobacteriaceae* sind weltweit ein zunehmendes therapeutisches Problem in Einrichtungen des Gesundheitswesens. In dieser epidemiologischen Untersuchung wurden 28 Imipenem-resistente *K. pneumoniae*-Stämme aus klinischem Material phenotypisch und genotypisch gegenüber ihrer Expression von Carbapenemasen untersucht. Carbapenemase-Bildung wurde phenotypisch mittels eines modifizierten Hodge-Tests (MHT) und eines CarbaNP-Tests und genotypisch mittels PCR unter Verwendung spezifischer Primer zur Detektion von Carbapenemase-Genen der Typen bla_{OXA-48}, bla_{KPC}, bla_{NDM} and bla_{VIM} analysiert. MHT- und CarbaNP-Tests waren bei allen untersuchten Imipenem-resistenten *K. pneumoniae*-Stämmen positiv. Das bla_{OXA-48}-Gen wurde in 27/28 Isolaten nachgewiesen. Bei einem Isolat wurde das bla_{VIM-4}-Gen nachgewiesen. Basierend auf dieser Untersuchung zeigten der NP-Test und der MHT die höchste Sensitivität und Spezifität zum Nachweis des Carbapenemase-Status. Diese Studie ist der erste Bericht über das Vorkommen von OXA-48-produzierenden *K. pneumoniae*-Stämmen im Iran.

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Introduction

Carbapenems are often considered as a last therapeutic choice for the treatment of infections due to multidrug-resistant Gram-negative rods [1], [2], [3], [4], [5]. Emergence of carbapenem-resistant *Enterobacteriaceae* is increasingly reported worldwide and is becoming an important issue in health care systems [4], [6]. Resistance to carbapenems in *K. pneumoniae* is related to two main mechanisms: i) production of extended-spectrum β-lactamase (cephalosporinase or ESBL) associated with porin loss, and ii) production of carbapenem-hydrolyzing β-lactamase such as Ambler's class A carbapenemases (KPC-type), class B metallo-β-lactamase (VIM-, NDM- or IMP-type) or class D carbapenemase OXA-48 [1], [4], [6], [7]. A number of phenotypical methods were used for detection of carbapenemases producer strains such as Modified Hodge Test (MHT) according to CLSI recommendations [8]. One new phenotypic method was described for detection of carbapenemases producer bacteria [1]. This test is currently utilized to identify the carba-

penemase production in Gram-negative bacteria, with a low cost and high sensitivity and specificity, and was performed for rapid detection (≤2 h) of carbapenemases [1], [2]. CarbaNP test is a biochemical test, applicable to isolated bacterial colonies, and is based on in vitro hydrolysis of a carbapenem compound (imipenem). The OXA-48 carbapenemase was first reported from a Turkish isolates in 2004 [7] but recently the OXA-48-producing *Enterobacteriaceae* is repeatedly reported from different parts of the world however mostly from Mediterranean countries [9]. The bla_{OXA-48} has been identified within the Tn1999 composite transposon bracketed by two copies of IS1999 responsible for its mobility and expression [10], [11]. This carbapenemase gene is harbored by an IncL/M-type plasmid in which the Tn1999 was inserted [12]. This highly conjugative plasmid has been found responsible for the widespread of the bla_{OXA-48} genes [12].

The bla_{OXA-48} gene is spreading worldwide but not yet reported from Iran. In this study, we report the very first cases of OXA-48-producing *K. pneumoniae* identified in Iran.

Methods

Bacterial strains and susceptibility testings

In this study, a collection of 28 non-duplicated carbapenem-resistant *K. pneumoniae* isolated from 18 patients has been analyzed. All of these invasive strains were obtained from clinical specimens taken from patients hospitalized in a burn unit from Motahari Hospital, Teheran, Iran between February to August 2011. More than one *K. pneumonia* strain was isolated from 10 patients, but different antibiograms were observed in these isolates. On the other hand different specimens were collected from patients in different days after their hospitalization.

All of these 28 strains were isolated from infected burn wounds. Fifteen patients received meropenem as monotherapy whereas the three remaining patients received cefepime as monotherapy.

These strains were isolated from wounds of burn patients hospitalized in Motahari Hospital (referral center for burn patients in Tehran, Iran) from February to August 2011. These isolates were identified by using the API 20E system (bioMérieux, Marcy l'Etoile, France).

Antibiotic susceptibility testing

The antibiotic susceptibilities of the isolates were determined by the agar disc diffusion method on Mueller-Hinton agar with antibiotic discs MAST Company, UK and interpreted according to Clinical and Laboratory Standards Institute CLSI guidelines [8]. Tested antibiotics included; cefotaxime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), amoxicillin-clavulanic acid (20/10 µg), aztreonam (30 µg), tobramycin (10 µg), gentamicin (10 µg), amikacin (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg) and tetracycline (30 µg). MIC of imipenem, meropenem and cefepime were determined by agar dilution method.

Phenotypic methods for detection of carbapenemase

Modified Hodge Test (MHT): This test was conducted according to the CLSI 2011 guidelines for search of carbapenemase production [8]. *K. pneumoniae* ATCC BAA-1705 were used as positive and *K. pneumoniae* ATCC BAA-1706 as negative controls. Strains with cloverleaf images of inhibition zone were considered as a carbapenemase-producing *K. pneumoniae*.

Use of boronic acid as a KPC inhibitor: Use of boronic acid (BA) as an inhibitor of KPC in combined-disk test with 400 µg/disc BA was carried out.

The stock solution of BA (benzene boronic acid; Sigma-Alderich, Germany) in dimethyl sulfoxcid and distilled water were mixed at a concentration of 20 mg/ml. From this solution 20 µl (containing 400 µg/disk) was added on to commercially meropenem disks.

The test was considered positive when the inhibition zone diameter around the disk containing meropenem and boronic acid was ≥5 mm compared with meropenem alone.

CarbaNP test (Carbapenemase Nordmann-Poirel test): One loop of strains was resuspended to Tris-HCL mmol/L as a lysis buffer from antibiogram plates, vortex for one minute and then incubated at room temperature for 30 minutes. Bacterial suspension was centrifuged at 10,000 xg at room temperature for 5 minutes. 30 µl of supernatant was mixed in 96 tray with 100 µl of imipenem monohydrate solusion (3 mg per ml) pH 7.8, phenolred solution and 0.1 mmol/L $ZnSO_4$.

PCR amplification and sequencing

Rapid DNA extractions were prepared by boiling extraction. PCR experiments were performed using standard conditions and specific primers to search for carbapenemase genes that have been identified previously in *K. pneumoniae* i.e. the bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC} and bla_{OXA-48} (Table 1). Direct sequencing of PCR amplified products was carried out using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea).

Results

Among these 28 isolates, all were resistant to broad-spectrum cephalosporins, carbapenems (imipenem, ertapenem, meropenem), trimethoprim-sulfamethoxazole and quinolones (Table 2). Some of them remained susceptible to amikacin and/or gentamicin, respectively (resistance rates for gentamicin at 90% and resistance rates to chloramphenicol, tetracycline and amikacin at

Table 1: Primers sequencing

Primer	Sequence (5' → 3')	Gene	Product size, bp	References
OXA-48-F	CCA AGCATT TTTACC CGCATC KACC	bla_{OXA-48}	438	[17]
OXA-48-R	GYTTGACCATACGCTGRCTGCG			
KPC-Fm	CTGTCTTGTCTCTCATGGCC	bla_{KPC}	795	[18]
KPC-Rm	CCTCGCTGTGCTTGTCATCC			
NDM-F	GGTTTGGCGATCTGGTTTTC	bla_{NDM}	621	[1]
NDM-R	CGGAATGGCTCATCACGATC			
VIM-F	GATGGTGTTTGGTCGCATA	bla_{VIM}	390	[1]
VIM-R	CGAATGCGCAGCACCAG			
IMP-F	GGAATAGAGTGGCTTAAYTC	bla_{IMP}	232	[1]
IMP-R	TCGGTTTAAYAAAACAACCACC			

Table 2: Antibiotic susceptibility patterns

Antimicrobial resistant pattern	Number of strains	Percent
All tested antibiotics	16	57.2
All tested antibiotics except T	3	10.5
All tested antibiotics except GM	1	3.6
All tested antibiotics except C	1	3.6
All tested antibiotics except AK	1	3.6
All tested antibiotics except GM and AK	2	7.4
All tested antibiotics except T and C	1	3.6
All tested antibiotics except AK and C	3	10.5

T: Tetracycline, GM: Gentamicin, C: Chloramphenicol, AK: Amikacin

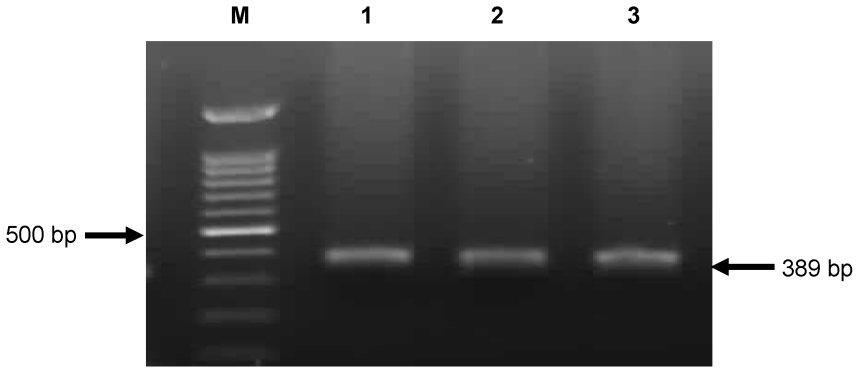

Figure 1: PCR amplification fragments for detection of OXA-48 gene among *Klebsiella pneumoniae* isolates
M: 1kb DNA size marker; lane 1: Positive control; lane 2 and 3: Positive strains.

82%, 86% and 79%, respectively). Antimicrobial susceptibility testing results showed that all of the strains were resistant to imipenem, meropenem, and cefepime. Nine and eleven out of 28 isolates had MIC more than 64 µg/ml against imipenem and meropenem, respectively. Nineteen of 28 strains had MIC more than 128 µg/ml against cefepime.

Eight different antimicrobial resistant patterns were observed among isolates likely indicating several clone are disseminating within the burn unit (Table 2). The results show that MHT and CarbaNP tests were positive for all carbapenem-resistant *K. pneumoniae* but none of them showed at least 5 mm increase in diameter of inhibition zone around meropenem plus BA comparison with meropenem alone.

PCR analysis following by sequencing showed the presence of the bla_{OXA-48} gene in 27 isolates (Figure 1). Whereas a single isolate was positive for bla_{VIM-4} gene. No bla_{KPC}, bla_{NDM} or bla_{IMP} genes were detected in this collection.

Discussion

Different *K. pneumionieae* with different antibiograms were isolated from one patient. Despite the *K. pneumonia* were isolated with same antibiogram among different patients. Isolates with same antibiotic-resistant pattern may derive from identical clone.

The OXA-48-type carbapenemases have been reported from France [4], Spain [6], Netherlands [13], Lebanon [4], Morocco [14] and Oman [15] and is becoming one of the main resistance mechanisms in *K. pneumoniae* [10]. The bla_{OXA-48} gene has been identified in 90.5% of *K. pneumoniae* isolates in six different Spanish hospitals [6]. To best of our knowledge, this study constitutes the very first report of OXA-48-producing *K. pneumoniae* from Iran. Detection of OXA-48-producing *Enterobacteriaceae* can be important, because such strains may often remain susceptible to third and fourth generation cephalosporins and monobactams and also this characteristic can make difficulty for detection of them [10]. After the recent identification of the bla_{KPC} and the bla_{NDM-1} genes in a burn unit in Teheran, the identification of the bla_{OXA-48} carbapenemase gene is worrisome [5], [16].

Overall, this study identifies for the first time OXA-48-producing *K. pneumoniae* strains from Iran. Therefore, the spread of OXA-48 producers may be more widespread than expected and can be expected in any countries of the Middle East.

Notes

Competing interests

The authors declare that they have no competing interests.

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