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New Delhi Metallo-Beta-Lactamase Ndm-1 Producing *Klebsiella Pneumoniae* in Slovakia

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Abstract

The rapid spread of NDM-1-positive bacteria proved to be a major challenge for the treatment and control of infectious diseases. The aim of our study was to confirm the presence of Carbapenem-hydrolyzing beta-lactamases in the Slovak Republic determinated with molecular-based detection methods. This paper is the first report of MBL NDM-1 in Slovakia. We describe four patients infected by multiresistant strain of *Klebsiella pneumoniae* and hospitalized on I.st Department of Anaesthesiology and Intensive Care Unit of the University Hospital L. Pasteur Kosice. In these cases the New Delhi metallo-beta-lactamase 1-producing *Enterobacteriaceae* was rapidly detected by phenotypic and genotypic methods. The rapid spread of NDM-1-positive bacteria proved to be a major challenge for the treatment and control of infectious diseases.

Keywords: *Klebsiella pneumonia; Enterobacteriaceae;* Penicillinase; Plasmid

Introduction

Carbapenem-hydrolyzing beta-lactamases, i.e., carbapenemases, originated from bacterial clinical isolates are an increasing concern because they often also confer resistance to most other beta-lactam antimicrobial agents. In class of *Enterobacteriaceae*, the carbapenemases are mainly found in the Ambler class A penicillinase or class B metallo-enzyme groups [1]. The major global public health problem that leads to increasing healthcare costs, extra length of hospital stay and treatment failures is the antibiotic resistance. The first information about appearance of the resistant strain to almost all antibiotics in vitro except colistin was associated with Swedish patient, who has been hospitalized in New Delhi in 2009 [2]. This resistance was due to blaNDM-1 – carrying plasmid which was mobile DNA elements with a high capacity for dissemination [3]. Many cases with NDM-1-producing microorganisms have been reported worldwide [4]. A lot of studies point to a connection between NDM-1 carrying microorganisms and Indian subcontinent [1].

The recent European Antimicrobial Resistance Surveillance Network report indicates that the occurrence of antibiotic resistance in gram-positive pathogens appears to be stabilizing or even decreasing in some European countries. Otherwise there are many other reports worldwide signalising increase of antimicrobial resistance in gram-negative pathogens, such as *Escherichia coli* and *Klebsiella pneumoniae*.

We report presence of carbapenemases in patients in Slovak Republic too using molecular-based detection methods.

Material and Methods

Bacterial isolates

K. pneumoniae non-duplicated strains were isolated from the patients of wards with high risk of nosocomial disease transmission in University Hospital L. Pasteur Kosice.

MALDI-TOF MS analysis was performed on a Microflex MALDI Biotyper (Bruker Daltonik) according to a standard sample preparation protocol of Bruker Daltonik [5]. MALDI-TOF mass spectra were subjected to numerical analysis (BioTyper 3.1 software, Bruker Daltonik). The similarity between the MALDI-TOF mass spectra of the isolates and the reference MALDI-TOF mass spectra was indicated by BioTyper scoring, where the score value exceeding 2.000 indicated identification of the genus and probable identification of the species, and score value exceeding 2.500 indicated highly probable identification of the species.

The MALDI-TOF mass spectra-based dendrogram was generated using the correlation distance measure with the average linkage algorithm. (Matrix-Assisted Laser Desorption / Ionization Time-of-Flight mass spectrometry Biotyper, Bruker Daltonics GmbH, Germany). K. pneumoniae strains were confirmed by standard biochemical methods ENTEROtest 16, ENTERO-Rapid 24 (Lachema, Czech Republic).

Susceptibility testing

The antibiotic susceptibility of each isolate and their corresponding *E. coli* transformants was determined using the disc diffusion technique on Mueller-Hinton agar plates with beta-lactam and non-beta-lactam antibiotic-containing discs and interpreted according to the CLSI guidelines. Azide-resistant *E. coli* J53 was used as a host in conjugation experiments [6].

All of clinical specimens were screened for carbapenem non-susceptibility according to European Committee of Antimicrobial Susceptibility Testing [7]. We used both microdilution method and method with the only active agent, respectively. Samples were positively tested for ESBL with the use of the CLSI disk diffusion method [8]. For detection of MBL (metallo-beta lactamase) producing strains it was using two methods: a carbapenem - EDTA combined disc method [9], a Tris-EDTA double-disc synergy method [9]. The modified Hodge test (MHT) was performed according to CLSI guidelines using imipenem, meropenem, and ertapenem disks on Muller–Hinton agar plates. *E. coli* ATCC 25922 was used as a carbapenem susceptible strain and also for MBLs screening.

PCR amplification and sequencing

PCRs were performed with a series of primers designed for the detection of Ambler class A, B and C beta-lactamase genes [8].

The detection of extended spectrum beta-lactamase (ESBL) genes was carried out by PCR with specific primers to detect blaCTX-M, blaSHV, blaTEM genes [10]. *E. coli* strain (CNCTC-7374) and *K. pneumoniae* (CNCTC-1705) strain were used as the control strain in the detection of these genes.

The blaNDM-1 was detected by PCR and verified by gene sequencing and analysis [11]. PCR assays were carried out with a series of primers designed for the detection of several class B beta-lactamase genes, blaIMP, blaVIM, and blaNDM-1 [12]. Strains known to harbor MBL genes were used as positive controls for the PCR analysis.

The nucleotide sequences, deduced amino acid sequences, and phylogenetic relationships were analyzed by using the software package (SeqScape v2.7 and MicroSeq v2.2).

Plasmid analysis

Conjugation assays were performed using each of the four *K. pneumoniae* clinical isolates as donors and an azide-resistant *E. coli* J53 as the recipient strain. Selection of transconjugants was undertaken by plating on Mueller-Hinton agar containing cefoxitin (10 mg/L) and azide (100 mg/L) [12]. Plasmid incompatibility groups were determined by a PCR-based replicon typing method (PBRT) [13].

Results

Isolate P1 originated from 54 years old man who was admitted to ICU because bicycle trauma. The swab from mouth cavity showed presence of Gram negative *Klebsiella* sp. The Hodge test and Double disc synergy test (DDST) were performed for phenotypic evidence of carbapenemase. *K. pneumoniae* MBL was persisting in the sputum. On the fifth and eight day of hospitalisation another examination of swab was performed with confirmation of multi-drug resistant strain of *K. pneumoniae*. The strain was resistant for the most of commonly used antibiotic (i.e.: aminopenicillins, beta-lactam/beta-lactamase inhibitors, aminoglycosides, fluoroquinolones etc.).

Isolate P2 was considered in 29 years old man with basic diagnosis of Hodgkin lymphomas who was admitted to the hospital. He was treated by broad spectrum acting antibiotics against gram-positive and gram-negative bacteria. The patient condition deteriorated on 6th day and it his worsening condition was followed by hepatic and renal failure. On the 7th day the MBL producing *K. pneumoniae* resistant to carbapenem together with *Ps. aeruginosa* were cultivated from nasal mucosa.

Isolate P3 was prepared from 71 years old female admitted on ICU with global respiratory insufficiency and impaired consciousness. There were elevation of inflammatory markers and the performed X-ray showed inflammation of lungs. One week later ESBL producing *K. pneumoniae* species and gram-negative bacteria´s were cultivated from the swab of mouth. Three days later repeated cultivation of mouth swab again confirmed *K. pneumoniae*, which was suspicious for MBL production.

Isolate P4 originated from 70 years old man who was admitted at Department of Hematology and Onco-Hematology with suspicion for acute hemoblastosis. The chemotherapy was initialized after confirmation of diagnosis. At the end of stay at hospital multidrug-resistant strain of *K. pneumoniae* was obtained from nasal mucosa and for this reason the gentamicin was administered. According to phenotype evidence (Hodge test, Synergy disc test) the bacteria was considered as nosocomial MBL producing strain, resistant to all carbapenems.

All 4 isolates (P1,P2,P3,P4) were detected in course of October and November of 2012. The three isolates were positive for blaNDM-1, and sequencing revealed that they were producing NDM-1.

MALDI-TOF mass spectra revealed formation of a cluster harbouring all 4 isolates identified as *Klebsiella pneumoniae* isolates and were identified in agreement with the ribotyping results. There were also assigned the corresponding reference/type strains with a significant BioTyper score. The isolates were evaluated phenotypically for the presence of a MBL, using the metal chelating agent EDTA.

When we measured the zonal inhibition around of the IMP and IMP+EDTA disc, we found that the zones increase of 7 mm. All four P1-P4 grew up to the EDTA disc. The EDTA combined disc test was repeated using CAZ (10 μg) disc and all P1-P4 yielded positive results for all combinations.

The modified Hodge test (MHT) was performed at all four samples using both MEM and ETP. All four samples were MHT positive in both tests and especially the sample P4 showed significant positivity (Figure 1A). Figure 1 B, C illustrates the MBL activity of P2, P3 that was determinated by the carbapenem-EDTA combined disc method.

The antibiotic susceptibility tests performed by disc diffusion method showed that all 4 isolates producing MBL were resistant to cefuroxime, imipenem, meropenem, ertapenem, cefepime, cefotaxime, ceftazidime, tobramycine, amikacin, ciprofloxacin, aztreonam and less resistant to gentamicin and tetracycline. According to the CLSI guidelines, all four isolates were found to be resistant to carbapenems, cephalosporins, and aminoglycosides. On other-hand all four (P1, P2, P3, P4) isolates were susceptible to tigecyclin, and colimicin.

PCR confirmed that isolates contained beta-lactamase genes including the extended-spectrum beta-lactamase genes blaCTX-M, blaSHV, blaTEM and sequencing confirmed the blaNDM-1 gene in three isolates, were carried by plasmids (Figure 2). Sequencing of blaNDM-1 revealed that the coding region was 99, 8% identical to the coding region of the blaNDM-1 gene in other submitted genes from GenBank database.

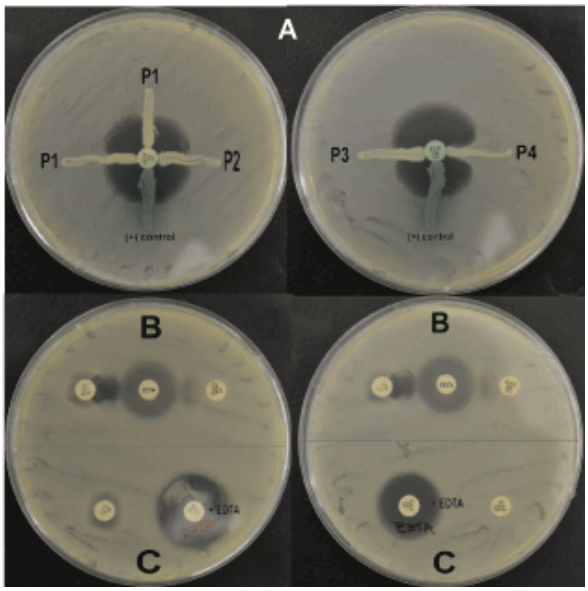

Figure 1: Phenotypic detection of carbapenemases in a *K. pneumoniae* NDM-1 strain isolated from a nasal wash specimen. (A) Modified Hodge test, using IPM, for detection of carbapenemase, performed by the CLSI reference method (P1, P2, P3, P4). (B) EDTA-IMP, CAZ double disc synergy test for MBL. (C) Carbapenem-EDTA combined disc diffusion test for MBL. The *K. pneumoniae* isolate was inoculated to the surface of a MHA plate. Discs containing 10μg/mL IMP and 30μg/mL CAZ plus 10μl of 0.5 M EDTA (pH 7.8) was placed on the agar.

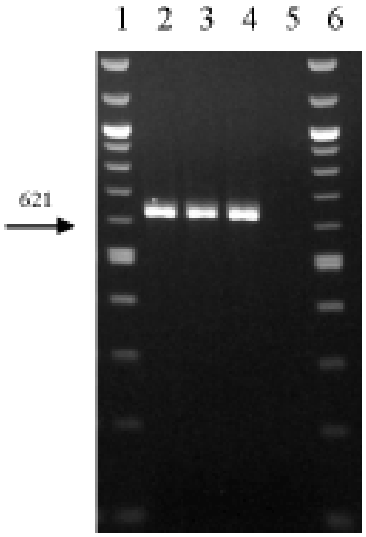

Figure 2: PCR results of detection the *bla*$_{NDM}$*-1* gene from *K. pneumoniae* isolates Lane 1. and 6. –100bp DNA Ladder (BioLabs), lane 2-P1, lane 3-P2, lane 4-P3, lane 5-P4 (negative for gene NDM-1)

Transferability of the blaNDM-1 gene was studied using conjugation experiments with four *K. pneumoniae* isolates signed as the donor, and *E. coli* J53 as the recipient strain. It was obtained transconjugant *E. coli* J53 (pMAR) which was expressing NDM-1. This strain was resistant to all β-lactams, including aztreonam (in this case the synergy was determined with clavulanate), and had decreased susceptibility to carbapenems [MICs of imipenem, meropenem, ertapenem and doripenem being 1, 1, 2 and 0.5 mg/L, respectively (the MICs of these compounds being 0.12 mg/L for the *E. coli* J53 recipient strain)]. The *E. coli* transconjugants were also resistant to tobramycin and chloramphenicol. PCR analysis confirmed that plasmid pMAR co-harboured the blaNDM-1 and blaCTX-M genes. Poirel et al. [14] using this methode of plasmid analysis concluded the velocity of this plasmid as ~250 kb.

Interestingly, the identified plasmid carried both blaNDM-1and blaCTX-M-15 genes, in contrast to what was previously observed among a collection of NDM-1- and CTX-M-15-producing isolates of worldwide origin (Australia, France, Germany, India, Iraq, Kenya, Oman and Switzerland), in which these two genes were located on two different plasmids [15]

Four detected samples had risk factors which were typically associated with the acquisition of KPC and MBL producing bacteria. These included prolonged hospitalization and ICU stay, hospitalisation at high risk wards of the hospital, using invasive devices, immunosuppression, and therapy with multiple antibiotic agents before initial culture. What is regarding to NDM-1, the three patients never visited Indian subcontinent or other countries where these microorganisms previously were described. Only the first detected – patient (P2) with positive KPC and MBL producing bacteria visited India several times. The resistant strain was transmitted to ICU where all of patients were hospitalised and the infection developed when the patients were exposed to carbapenemas.

Discussion

Resistance to carbapenems is of great concern because the carbapenems are considered to be antibiotics of last resort to combat infections by multidrug resistant bacteria, especially in ICUs and high risk wards.

NDM-1 is a novel MBL encoded by the blaNDM-1 gene, which confers resistance to all beta-lactams, with the exception of aztreonam [2]. Many strains that harbor blaNDM-1 are also aztreonam resistant, presumably by a different resistance mechanism [3].

This NDM beta-lactamase is one type of metallo-beta-lactamase which is found only in members of *Enterobacteriaceae* and differs from other beta lactamases as Extended-Spectrum-Beta-Lactamases (ESBLs) and AmpC by being resistant to the carbapenems. KPC is also a beta lactamase that is a serine beta-lactamase which is resistant to carbapenems [16].

New Delhi metallo-beta-lactamase (NDM-1)-positive *K. pneumoniae* was imported from India and spread to the United Kingdom in 2010 [17]. Similarly 50 % prevalence of metallo-beta-lactamases were reported in *K. pneumoniae* blood isolates from Greece [EARS report, 2011].

More recently, NDM-1 cases have been identified across the globe, including in Canada, Europe, Japan, Africa, and Australia [18], and all of these cases were associated with travel to India or Pakistan, or with medical care in these countries.

The genes conferring such resistance usually reside on large plasmids, which frequently carry additional resistance determinants that confer cross-resistance to the fluoroquinolones and aminoglycosides [19]. As a consequence, prior use of any of these antibiotic classes may predispose for carbapenemase production. It also appears that the prior exposure is not the alone risk factor but the risk also increases with increasing time of prior treatment.

Most plasmids detected in NDM-1-positive bacteria can be easily transferred to many different gram-negative bacteria, posing a serious threat for the uncontrollable spread of multidrug-resistant bacteria. *K. pneumoniae* is the most important cause of nosocomial infections, and it can spread rapidly in the hospital environment [20].

The genes that encode these enzymes are a source of concern, as they usually are carried by mobile genetic elements with a high capacity for horizontal dissemination [21].

Molecular testing is recommended to characterize the MBL genes of *Enterobacteriaceae* [1], but PCR is not available universally. We used phenotypic methods that could efficiently detect the mechanism of resistance. Yan et al., [9] evaluated three methods, DDST, combined-disc test and E-test for this procedure. They concluded that the E-test is not applicable to carbapenem-susceptible MBL-carrying organism (MIC ≤ 4μg/ml).

In March of 2011 Shahcheraghi et al. [22], detected a multiple drug-resistant *K. pneumoniae* isolate that was resistant to all tested antibiotics except colistin. Using PCR method it was considered that this isolate was containing blaNDM-1, blaCTX-M, blaSHV and blaTEM. This was the first report of the detection of MBL NDM-1 in Iran which was corresponding to our first capture of NDM-1 gene by *K. pneumoniae* in hospitalised patients from Slovakia. In other studies, the plasmid carrying blaNDM-1 also harbored other beta-lactamase genes. These evidences would suggested that the plasmid carrying blaNDM-1 carries several antibiotic resistance-conferring genes. The rapid transfer of this plasmid to other pathogen bacteria is probably able to severely disseminate the antibiotic resistance among clinical bacteria [22].

In conclusion, infections due to carbapenem-resistant bacteria are difficult for treatment and rapid identification of MBL-producing Gram-negative species is crucial both for adjustment of appropriate medication and for timely implementation of infection control measurements. Phenotypic methods can be useful for routine detection of carbapenemase production, particularly when PCR is not available immediately.

Our study is the first report discussing about the occurrence of NDM-1-producing *K. pneumoniae* strains in Slovakia, with a link to previous visit of one patient in India. This constitutes a warning for authorities to reinforce strict control measures for preventing the spread of such multidrug-resistant strains.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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