

Detection of carbapenemase genes in *Klebsiella pneumoniae* isolates

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SUMMARY

Introduction *Klebsiella pneumoniae* is one of the leading causes of serious hospital-acquired infections worldwide among *Enterobacteriaceae* species. It is the most common producer of carbapenemases in many parts of the world.

Objective The aim of the study was to determine which enzymes were responsible for resistance to carbapenems in *Klebsiella pneumoniae* strains isolated at the Centre of Microbiology of Public Health Institute of Vojvodina.

Methods A total of 29 *Klebsiella pneumoniae* non-duplicated strains resistant to at least one carbapenem isolated from clinical samples of hospitalized patients between November 1st 2013 and April 30th 2014 were studied. The species identification and susceptibility were done using VITEK 2 (bioMérieux, Marcy-l'Étoile, France) system. Phenotypic conformation of carbapenemase production was done by double-disc synergy test. PCR technique was performed for detection of genes encoding production of carbapenemases (*bla*_{KPC}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{OXA-48}).

Results Isolates of *Klebsiella pneumoniae* resistant to at least one carbapenem showed positive on double-disc synergy test between meropenem and dipicolinic acid. All strains positive in phenotypic test contained *bla*_{NDM} gene. In isolates resistant only to ertapenem, neither production of carbapenemases nor presence of genes encoding these enzymes were detected. Among these isolates, nine produced extended-spectrum β -lactamase.

Conclusion The presence of NDM metallo- β -lactamase was determined in all *Klebsiella pneumoniae* isolates resistant to at least one carbapenem.

Keywords: *Klebsiella pneumoniae*; carbapenem-resistance; NDM β -lactamase

INTRODUCTION

Enterobacteriaceae are one of the leading causes of both community- and hospital- acquired serious infections in humans that are associated with increased morbidity and mortality rates and healthcare costs. *Escherichia coli* and *Klebsiella pneumoniae* are responsible for the majority of these infections including urinary tract infections, septicemia, pneumonia, peritonitis, meningitis, and device-associated infections [1]. They are often multidrug resistant (MDR), and infections caused by these strains are a formidable problem to clinicians because therapeutic options are often very limited [2]. Carbapenems had been antibiotics of choice for the treatment of severe infections caused by MDR strains until 1990s, when the first carbapenemase producer in *Enterobacteriaceae* was identified [3]. After that, other types of enzymes that hydrolyze carbapenems were detected and reported worldwide [4]. Emergence and spread of carbapenemase-producing *Enterobacteriaceae* further reduced options for effective treatment of these infections.

Resistance to carbapenems is a result of production of enzymes that belong to the following three classes of β -lactamases: Ambler class

A, B, and D β -lactamases [5]. Among class A β -lactamases, the most clinically significant is *Klebsiella pneumoniae* carbapenemase (KPC) [6]. Class B β -lactamases or metallo- β -lactamases are mostly of the IMP, VIM, and most recently of the New Delhi metallo- β -lactamase (NDM) type [7]. The most important types of class D are OXA-48-like enzymes [8]. Most of these enzymes were identified in MDR *Klebsiella pneumoniae*, but distribution and prevalence vary widely in different regions [9].

OBJECTIVE

The aim of this study was to determine which enzymes were responsible to resistance to carbapenems in clinical isolates of *Klebsiella pneumoniae*.

METHODS

The study was conducted at the Center for Microbiology of Public Health Institute of Vojvodina. A total of 29 non-duplicated strains of *Klebsiella pneumoniae* resistant to at least one carbapenem were isolated from clinical

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samples including blood (nine), urine (nine), wound swab (eight), and tracheal aspirate (three) of patients hospitalized on the various wards of Clinical Centre of Vojvodina and Institute of Child and Youth Health Care of Vojvodina between November 1st 2013 and April 30th 2014. Isolated strains were identified using VITEK 2 Compact system, GN cards (bioMérieux, Marcy-l'Étoile, France). To determine susceptibility to antimicrobial drugs and production of extended-spectrum β -lactamase (ESBL) VITEK 2 Compact system, AST-GN71 and AST-N240 cards were used according to Clinical and Laboratory Standards Institute guideline. Susceptibility to fosfomycin was tested by Etest strip (AB Biodisk, Solna, Sweden) using European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (2013) [10, 11]. All isolates were tested for the presence of the most prevalent enzymes (KPC, VIM, NDM, OXA-48) and genes responsible for the resistance to carbapenems (Table 1). Phenotypic confirmation of carbapenemase production and interpretation of results were done by double-disk synergy test according to manufacturer's instructions and EUCAST guideline [12], using tablets containing meropenem (10 μ g), cloxacillin, dipicolinic acid, boronic acid (Rosco Diagnostica Neo-Sensitabs, Taastrup, Denmark). Enhancement of the zone of inhibition in the area between meropenem disc and the inhibitor-containing disc was considered to be a positive result. Detection of genes encoding production of carbapenemases using PCR technique was performed as follows: extraction of total bacterial DNA was performed in a 2 mL tube containing 1.5 mL of bacteria in a tryptic soy broth culture. It was centrifuged at 13,000 rpm for five minutes, followed by discarding of the supernatant, and the pellets were suspended with 200 μ L DNase/RNase free water (Gibco, Invitrogen, Waltham, MA, USA). The suspension was vortexed briefly, then heated at 100°C for five minutes, and cooled at 4°C for five to 10 minutes. Mixture was then centrifuged for five minutes at 13,000 rpm, and 150 μ L of supernatant was transferred to a new 1.5 mL tube and was ready for use.

PCR reaction was performed with MasterCycler personal (Eppendorf, Hamburg, Germany). The final reaction volume was 25 μ L with 8 μ L DNase/RNase free water (Gibco, Invitrogen), 12.5 μ L PCR Master Mix M752 (Promega, Fitchburg, WI, USA) and 0.5 μ L of each primer. PCR reaction of four genes was performed as two separate multiplex reactions. First reaction included primers for *bla*_{NDM} and *bla*_{KPC}, and second one included primers for *bla*_{OXA-48-like} and *bla*_{VIM}. PCR cycling conditions for *ndm* and *kpc* reaction were one cycle at 95°C for five minutes, 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle at 72°C for three minutes, and holding stage at 4°C. PCR cycling conditions for *oxa48-like* and *vim* reaction were one cycle at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle at 72°C for three minutes, and holding stage at 4°C. The PCR-amplified products were analyzed by 2% agarose (Fisher Scientific International, Inc., Hampton, NH, USA) gel electrophoresis and stained with ethidium

Table 1. Primers for detection of genes encoding carbapenemase production

Primer name	Sequence
<i>bla</i> KPC Fw	ATGTCAGTGTATCGCCGTCT
<i>bla</i> KPC Rv	TTTTCAGAGCCTTACTGCC
<i>bla</i> VIM Fw	GATGGTGTGGTGCATA
<i>bla</i> VIM Rv	CGAATGCGCAGCACCAG
NDM-For	GGCAGTCGCTTCCAACGGT
NDM-Rev	GTAGTGCTCAGTTCGGCAT
OXA48A	TTGGTGGCATCGATTATCGG
OXA48A	GAGCACTCTTTGTGTATGGC

Table 2. Susceptibility of *Klebsiella pneumoniae* to antimicrobial drugs

Antimicrobial drug	<i>Klebsiella pneumoniae</i>	
	No. of isolates tested	No. (%) of resistant isolates
Amoxicillin/clavulanic acid	29	29 (100)
Tazobactam/piperacillin	29	29 (100)
Ceftazidime	29	29 (100)
Ceftriaxone	29	29 (100)
Cefotaxime	29	29 (100)
Cefepime	29	29 (100)
Imipenem	29	15 (51.7)
Meropenem	29	15 (51.7)
Ertapenem	29	29 (100)
Gentamycin	29	22 (75.8)
Amikacin	29	20 (68.9)
Cotrimoxazole	29	29 (100)
Ciprofloxacin	29	28 (96.5)

Table 3. Susceptibility to colistin, fosfomycin, and tigecycline of *Klebsiella pneumoniae* isolates resistant to all carbapenems tested

Antimicrobial drug	<i>Klebsiella pneumoniae</i>
	No. of resistant/tested isolates
Colistin	0/15
Fosfomycin	1/15
Tigecycline	13/15

bromide. Images were documented by a BioDocAnalyze (Biometra, Gottingen, Germany) system.

RESULTS

Table 2 shows results of susceptibility testing of 29 *Klebsiella pneumoniae* isolates resistant to at least one carbapenem.

Among carbapenems, ertapenem showed lower activity than meropenem and imipenem. Percentage of isolates resistant to ertapenem was higher compared to other carbapenems tested. All *Klebsiella pneumoniae* isolates were resistant to eight of 13 antimicrobial agents tested (Table 2).

All isolates of *Klebsiella pneumoniae* resistant to all carbapenems tested were susceptible to colistin, only one isolate was resistant to fosfomycin, while 13 of 15 isolates were resistant to tigecycline (Table 3).

Isolates of *Klebsiella pneumoniae* resistant to all carbapenems tested showed an enhancement of the zone of inhibition in the area between meropenem disc and the disc containing dipicolinic acid that indicated a metallo- β -lactamases production (Figure 1).

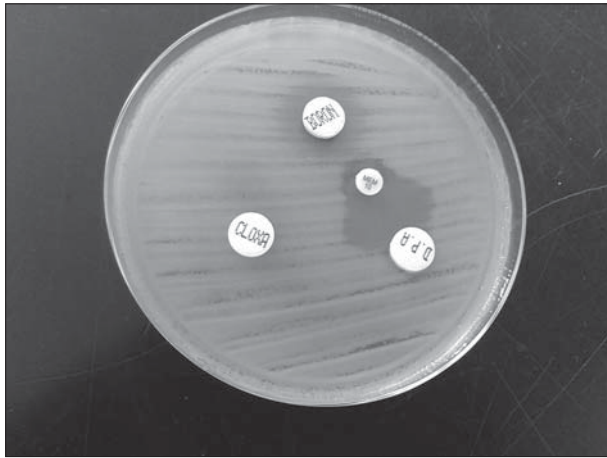


Figure 1. Positive Double Disc Synergy Test. The enhancement of the zone of inhibition in the area between meropenem disc and the disc containing dipicolinic acid.

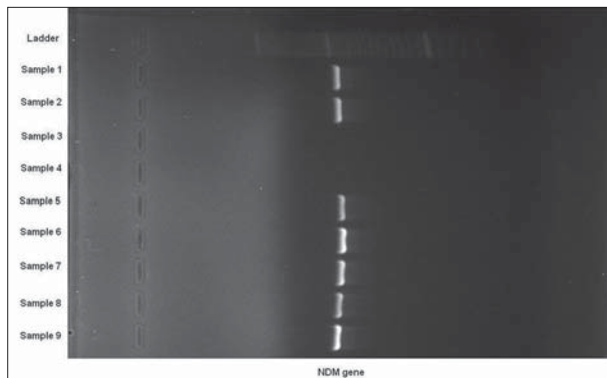


Figure 2. Detection of *bla*_{NDM} gene in *Klebsiella pneumoniae* isolates

All strains positive in phenotypic confirmation test carried *bla*_{NDM} gene encoding NDM β -lactamase detected by PCR technique (Figure 2).

In isolates resistant only to ertapenem, neither production of carbapenemases nor presence of genes encoding these enzymes were detected. Among these isolates, nine out of 14 were ESBL producers.

DISCUSSION

Klebsiella pneumoniae is one of the leading causes of serious hospital-acquired infections worldwide. Emergence of carbapenemase resistant isolates is of particular concern because infections caused by these strains are associated with therapeutic failure and high mortality rates. According to the European Survey on Carbapenemase-Producing *Enterobacteriaceae* working group report, *Klebsiella pneumoniae* has been the most common producer of carbapenemase in majority of European countries. KPC is the most frequently detected enzyme [13]. The first KPC-producing strain was isolated from clinical specimen in North Carolina in 1996 [14]. Subsequently, KPC producers have been reported worldwide causing outbreaks in United States, many European countries, China, and South America [2, 5, 15, 16, 17]. The most important and par-

ticularly problematic carbapenemase in the Ambler class B, with increasing detection in many geographic areas, is the metallo- β -lactamase NDM. It was first isolated in Sweden in 2008, from an Indian patient with urinary tract infection previously hospitalized in New Delhi [7].

Emergence and spread of carbapenemase producers were reported all over the world. Types of enzymes and their frequency of detection differ from country to country. In this study, in all *Klebsiella pneumoniae* strains resistant to at least one carbapenem tested for the presence of the most common carbapenemase, only NDM metallo- β -lactamase was detected. Strains producing this enzyme were isolated in many European countries, but their distribution was not as wide as that reported for KPC- β -lactamase [2]. The largest number of NDM positive isolates was reported in the United Kingdom. Most of these cases had a direct connection to the Indian subcontinent (i.e. India, Pakistan, and Bangladesh). Fewer NDM producers were detected in Denmark, Belgium, France, Austria, Italy, and Germany, which originated most probably from Balkan countries and the Middle East. According to surveillance report of European Center for Disease Prevention and Control, NDM-producing *Enterobacteriaceae* were isolated from persons who had received medical care in Serbia (Kosovo and Metohija), Montenegro, and Bosnia and Herzegovina [18]. Some data indicate that Balkan states and the Middle East are, in addition to the Indian subcontinent, endemic reservoirs for NDM producers [19, 20]. Several isolates were detected in some other countries such as the USA, Canada, China, Japan, and Israel, related to unknown origin [2].

Our results suggest that ertapenem was not appropriate carbapenem for detecting NDM β -lactamase producers because all isolates resistant only to ertapenem were negative in phenotypic confirmation and genotypic tests. Other authors also found low specificity of ertapenem in their studies [6, 21]. Carbapenem resistance is mainly the result of carbapenemase production, but other mechanisms, such as overproduction of ESBL and AmpC β -lactamase associated with porin loss, may cause decreased susceptibility to carbapenems [6, 22, 23]. In this study, among 14 *Klebsiella pneumoniae* isolates resistant only to ertapenem, ESBL production was determined in nine strains.

Carbapenemase resistant *Enterobacteriaceae* are usually resistant to a wide range of antimicrobial drugs. Plasmids carrying genes encoding carbapenemases often carry high number of genes that confer high level of resistance to other antibiotics and can be easily transferred to other bacterial strains and species [9, 20, 24].

The majority of carbapenem resistant *Klebsiella pneumoniae* isolates in this study were MDR, which is in agreement with other reports [9, 19, 20]. All isolates were resistant to β -lactam antibiotics (except meropenem and imipenem) and cotrimoxazole, and only one isolate was susceptible to ciprofloxacin. Lower but still high percentage of resistance to gentamycin and amikacin (75.8% and 68.9%, respectively) was detected. These results are similar to those of other investigations [25, 26]. The most effective antibiotic against all *Klebsiella pneumoniae* resistant to all

carbapenems tested in this study was colistin, followed by fosfomycin. Resistance to tigecycline was detected in almost all isolates tested. Many authors reported very good activity or low percentage of resistance to colistin and fosfomycin. None of the studies reported as high resistance to tigecycline as we have found in our isolates [25, 26].

Treatment of infections caused by MDR *Klebsiella pneumoniae* is a formidable therapeutic problem because there are few potentially effective antibiotics, such as colistin, tigecycline, fosfomycin, and aminoglycosides [27]. Several studies found higher rate of positive outcomes in patients when combination therapy with two or three antibiotics, including colistin, tigecycline, and fosfomycin, was used [28, 29]. Therefore, combination therapy for the

treatment of severe infections caused by MDR isolates is recommended.

CONCLUSION

According to the results obtained, presence of NDM metallo- β -lactamase in all *Klebsiella pneumoniae* isolates resistant to at least one carbapenem was determined. Almost all isolates were multidrug resistant. In order to prevent emergence and spread of multidrug resistant species, it is mandatory to identify carbapenemase-producing isolates and determine mechanisms of resistance to carbapenems in routine laboratory work.

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Доказивање гена резистенције на карбапенеме код изолата *Klebsiella pneumoniae*

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КРАТАК САДРЖАЈ

Увод *Klebsiella pneumoniae* је један од водећих узрока тешких болничких инфекција из фамилије *Enterobacteriaceae* широм света. Она је најчешћа ентеробактерија која продукује карбапенемазе у многим географским подручјима.

Циљ рада Циљ рада био је утврђивање ензима одговорних за резистенцију на карбапенеме *Klebsiella pneumoniae* изолованих у Центру за микробиологију Института за јавно здравље Војводине.

Методе рада Испитивањем је обухваћено 29 примоизолата *Klebsiella pneumoniae* резистентних најмање на један антибиотик из групе карбапенема добијених из клиничких узорака болнички лечених пацијената у периоду 1. новембар 2013 – 30. април 2014. године. Идентификација изолата и испитивање осетљивости на антимикробне лекове вршени су применом Витек 2 система. *Double Disc Synergy Test* коришћен је за фенотипску потврду продукције карбапеназа. *PCR*

методом доказивано је присуство најчешћих гена који кодирају продукцију карбапеназа (*bla_{KPC1}*, *bla_{VIM1}*, *bla_{NDM}*, *bla_{OXA-48}*). **Резултати** Сви изолати *Klebsiella pneumoniae* резистентни најмање на један карбапенем испољили су синергизам између меропенема и дипиколиничне киселине у фенотипском потврдном тесту. Код свих изолата позитивних у овом тесту доказано је присуство *bla_{NDM}* гена, док код изолата резистентних само на ертапенем није потврђена продукција карбапеназа нити присуство гена који кодирају њихову синтезу. Бета-лактамазе проширеног спектра доказане су код 9 од 14 изолата резистентних само на ертапенем.

Закључак Присуство ензима *NDM* метало-β-лактамазе доказано је код свих изолата *Klebsiella pneumoniae* резистентних најмање на један карбапенем. Продукција бета-лактамаза проширеног спектра доказана је код 9 од 14 изолата резистентних само на ертапенем.

Кључне речи: *Klebsiella pneumoniae*; резистенција на карбапенеме; *NDM* β-лактамаза