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Diverse efficacy of CarbaNP test among OXA-48 carbapenemase producing *Enterobacterales* in an endemic region

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ABSTRACT

After the first description of OXA-48 type carbapenemase, it has become endemic in Europe, Mediterranean and North African countries in a short time. OXA-48 carbapenemase is the most difficult type to determine and accurate diagnosis is crucial especially in endemic areas.

The CarbaNP test was described as a rapid phenotypic evaluation method of carbapenemases activity. Sensitivity and specifity of this test were high within all carbapenemases genes. In our study, we evaluated the efficacy of CarbaNP test in routine laboratories located in an endemic area of OXA-48 producing *Enterobacterales*.

A total of 53 *Enterobacterales* isolates were included in this study. Antimicrobial susceptibility of the isolates to imipenem, meropenem and ertapenem was determined. Polymerase Chain Reaction (PCR) was carried out for the detection of carbapenemases genes (bla_{KPC}, bla_{NDM}, bla_{BIC}, bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, and bla_{OXA-48}). The Carba NP test was performed as in the protocol described previously.

Altogether 31 isolates (58.4%) were bla_{OXA-48} positive (18 *Klebsiella pneumoniae*, 8 *Escherichia coli*, 2 *Serratia marcescens*, 1 *Enterobacter aerogenes*, 1 *Pantoea agglomerans* and 1 *Morganella morganii*). Among these isolates 3 (5.6%) and 2 (3.7%) isolates were also positive for bla_{VIM} and bla_{SPM}, respectively.

The sensitivity and specifity of CarbaNP test were found 64.5, and 68.2% respectively. It was observed that determination of positive isolates is hard to distinguish and subjective.

The CarbaNP test has suboptimal results and low of sensitivity and specifity for detection of OXA-48 producing *Enterobacterales,* and not suitable for detection of bla_{OXA-48} positive isolates in routine laboratories in endemic areas.

KEYWORDS

carbapenem-resistant *Enterobacterales*, carbapenemase, OXA-48, Carba NP, PCR

INTRODUCTION

Enterobacterales are known as some of the most common pathogens causing both community and hospital-acquired infections, including meningitis, sepsis, pneumonia, peritonitis, and gastrointestinal tracts, urinary, and medical device-associated infections. Carbapenemase-producing *Enterobacterales* (CPE) have already been detected all over the world, with a marked endemicity according to enzyme type. The first CPE (NmcA) was identified in 1993 [1]. Thereafter, a large variety of carbapenemases has been identified in *Enterobacterales* relating to 3 classes of β-lactamases: the Ambler class A, B, and D [2]. Class D enzymes of the OXA-48 type carbapenemase was first described in a *Klebsiella pneumoniae* strain from

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Turkey [3]. After then, OXA-48 producers have been extensively reported from all over the world as a source of nosocomial outbreaks and became the most frequent carbapenemase type reported in countries in Europe, Mediterranean, the Middle East and North Africa [4–8]. So far, these enzymes are the most common circulating ones in this part of the world [9]. In an assesssment on CPE epidemiology in Europe, Turkey has been identified as a country that OXA-48 type carbapenemase is endemic [10].

OXA-48 type carbapenemese is distinctive; because it hydrolyses penicillins but hydrolyses carbapenems and broad-spectrum cephalosporins, such as ceftazidime, and aztreonam weakly. Its activity is not inhibited by EDTA, clavulanic acid, sulbactam, or tazobactam, whereas the activity may be inhibited *in vitro* by sodium chloride [11]. Identification of OXA-48 type carbapenemases are the most difficult among all carbapenemase producers [8]. Although high level resistance to temocillin disk was used to phenotypic identification of OXA-48 CPE, there is still lacking an inhibitor for class D enzymes [12]. Different phenotypic methods such as the Modified Hodge Test (MHT) and disk diffusion tests with different inhibitors lack specifity and sensitivity. Therefore identification of carbapemase producers by using the molecular methods still remains the gold standard method; but this method is inapplicable for many laboratories.

Recently, the CarbaNP test (The Carbapenemase Nordmann-Poirel test) has been devoloped for an early identification of CPE [13]. It is said that the test is rapid (maximum 2 h), cost-effective, easy to apply for the phenotypic detection of variety of CPE including OXA-48 and it seemed as a promising test to fill the identification gap [14]. The goal of this study is to evaluate sensitivity and specificity of the CarbaNP test to detect OXA-48 type CPE especially in endemic areas.

MATERIAL AND METHODS

Bacterial strains and susceptibility tests

A total of 53 isolates (34 *K. pneumoniae,* 10 *Escherichia coli,* 2 *Serratia marcescens,* 2 *Citrobacter freundii,* 2 *Enterobacter aerogenes,* 1 *Morganella morganii,* and 1 *Pantoea agglomerans*) isolated from clinical samples of hospitalized patients which were more likely to be positive for OXA-48 were included in this study. All isolates were identified by conventional methods as well as automated BD Phoenix System (Becton Dickinson, USA). Susceptibility of isolates to imipenem, meropenem and ertapenem was determined by microdilution method and interpreted according to the standards of European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimum inhibitory concentration (MIC) breakpoints (version 10.0).

Molecular assays

The primers described by Poirel et al. [15] used for the investigation of carbapenemases gene detection. PCR amplification was carried out for the detection of carbapenemases genes (bla_{KPC}, bla_{NDM}, bla_{BIC}, bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, and bla_{OXA-48}) with an in-house multiplex multiplex PCR method.

The CarbaNP test

The CarbaNP test was performed on strains grown on Mueller-Hinton agar plates as previously described by Nordmann et al. [13]. Two calibrated inoculated loops (10 µL) of the tested strains directly recovered from a Mueller-Hinton agar plates (Becton Dickinson, France) were resuspended in 200 µL Tris HCl lysis buffer (20 mMol/L). Then, the material was mixed by a vortex for 1 min and after incubated at room temperature for 30 min. These bacterial strains were centrifuged at 10.000× at room temperature for 5 min. Thirty µL of the supernatant was mixed in a microwell with 100 µL of a diluted phenol red (Merck, France; 2 mL of 0.5% phenol red solution and 16.6 mL distilled water, pH 7.8), 5% solution containing 0.1 mMol $ZnSo_4$ and 3 mg/mL imipenem monohydrate (Sigma, France). Mixtures in the wells were incubated at 37 °C for a maximum 2 h. The wells changed color to yellow/orange after incubation read as a positive result.

Statistical analysis

SPSS software package (version 17, SPSS Inc., Chicago, IL) was used for the data analysis. Number and percentage of categorical measurement, continuous measurement if they mean and standart deviation were summarized. To compare categorical variables chi-square test were used. Sensitivity, specificity, positive and negative predictive values of the Carba NP test were assessed. The level of statistical significance in all tests were taken as 0.05.

RESULTS

Susceptibility results

According to EUCAST breakpoint tables, 45 isolates (84.9%) showed decreased sensitivity to at least one carbapenem (imipenem, meropenem or ertapenem). 24.5% (13/53) of isolates were resistant to imipenem and 1.8% (1/53) categorized as intermediate. 16.9% (9/53) and 9.4% (5/53) of isolates were resistant and intermediate susceptible to meropenem, respectively. 83% (44/53) of isolates were resistant to ertapenem. While 3 isolates (5.6%) were resistant to both imipenem and ertapenem, 9 isolates (16.9%) were resistant to all carbapenems. All isolates with multiple carbapenem resistance were identified as *K. pneumoniae.*

Molecular assays

31 of total 53 isolates (58.4%) were bla_{OXA-48} positive, 3 (5.6%) and 2 (3.7%) isolates were also positive for bla_{VIM} and bla_{SPM} as well as bla_{OXA-48} respectively. No carbapenemases genes were detected in 22 isolates (41.5%).

The CarbaNP test results

It is found that the sensitivity 64.5%, specificity 68.2%, positive predictive value (PPV) 74% and negative predictive value (NPV) 57.7% of the CarbaNP test to detection of $bla_{\text{OXA-48}}$ CPE.

Bacterial isolates with molecular assay, carbapenem susceptibility and the CarbaNP test results are shown in Table 1.

DISCUSSION

The OXA-48 carbapenemase was first described in *K. pneumoniae* epidemic isolates from Turkey in 2001 and then in several European countries [16]. Recently, OXA-48 carbapenemase has also been identified in *Enterobacterales* isolates recovered from non-European countries, such as India, Senegal, Lebanon and Monocco [5, 7, 17, 18]. Recent epidemiological studies have shown that OXA-48 carbapenemase is now the most prevalent carbapenemase in many European countries. Class D carbapenemase has been found in different *Enterobacterales*, such as *C. freundii*, *Providencia rettgeri*, *Enterobacter cloacae*, and even in *E. coli* [19]. The rate of hospital mortality was 58.3% for blood-stream infections due to OXA-48 producing *Enterobacterales* [20]. The $bla_{\text{OXA-48}}$ gene codes for an oxacillinase that causes resistance to penicillin and reduces susceptibility to carbapenems, but when produced alone, not to expanded-spectrum cephalosporins. The level of resistance is often low and such strains are thus frequently missed in laboratories using automated antimicrobial susceptibility test systems [21].

Specific tests may help to identify carbapenemase activity phenotypically. The MHT known as cloverleaf method is a time consuming and subjective test, and due to its low specificity (high-level AmpC procuders) and sensitivity (weak detection of bla_{NDM} producers) EUCAST doesn't routinely recommend carbapenemase detection in *Enterobacterales* by MHT [12].

Spread of CPE mostly seen in hospitalized patients infected with *K. pneumoniae*, and community acquisition is increasing especially for OXA-48 producers [19]. Therefore, early and reliable identification of carbapenemase-producing microorganisms is essential to implement contact isolation on time thus, prevent hospital outbreaks.

After the CarbaNP test initially reported as a novel phenotypic method developed for carbapenemase detection, a few years later the Clinical and Laboratory Standards Institute (CLSI) has advocated the use of a modified version of this test for the routine verification of carbapenemases among *Enterobacterales* [22]. The test is based on *in vitro* hydrolysis of imipenem by a bacterial lysate, which is detected by changes in pH values using the indicator phenol red [14]. The CarbaNP test was reported as a rapid, reliable, cost-effective method for detection of carbapenemases, especially of Ambler class A and B, with superior sensitivity [23]. It was reported to be 100% sensitive and specific for the detection of CPE. However, the data were insufficient for OXA-48 type carbapenemase due to studying with small bla_{OXA-48} positive groups [13, 24, 25].

Our experience revealed that the CarbaNP test has 64.5% sensitivity and 68.2% specificity to detect OXA-48 type producer *Enterobacterales* which is lower than other reports [13, 25–27]. This is due to the fact that the isolates we included in the study consisting of OXA-48 type carbapenemases substancially unlike the other studies whose bacteria group contains other type carbapenemases dominantly. Tijet et al. [28] reported that the CarbaNP test had low sensitivity for detection of OXA-48 like enzymes for CPE. It is specified, that the test results were unreliable for accurate identification of OXA-48 type carbapenemases and false-negative results were associated with particulary $bla_{\text{OXA-48}}$ CPE. In the same study, the CarbaNP test also experienced lower sensitivity with mucoid isolates such as *Klebsiella* spp. which is difficult the transfer from the suspension into the wells for analysis. Another study reported that the sensitivity and specificity of the CarbaNP test can be low as 11% for $bla_{\text{OXA-48}}$ positive isolates, whilst it is over 90% for isolates carrying bla_{KPC}, bla_{NDM}, and other β-lactamases [29].

This lack of sensitivity and specifity can be caused by different reasons namely, due to the weak carbapenemase activity and the low level or limited expression of the corresponding genes [14]. Inoculum size of the suspension used for the Carba NP test may also affect the results. Some studies have shown that increasing the bacterial amount to 3 or 4 loopfuls could provide better results which is still an arbitrary measurement [28, 30]. It is also not possible to distinguish true OXA-48-like carbapenemases from OXA-48-like non-carbapenemase variants (e.g. OXA-163) without sequencing. Consequently, false-negative results might be due to the expression of these variants [27, 31]. It is also mentioned that incomplete lysis of the OXA-48 like enzymes during the test procedure can cause false-negative results [25]. To exclude this possibility lysis buffer and imipenem solution prepared freshly and used within a few hours in our study.

There was also no correlation between the carbapenem resistance and the CarbaNP test. This result relates to the importance of membrane permeability changes due to the loss of porin function or expression in the efflux pumps rather than enzyme activity in the emergence of resistant species. It is not unusual to observe highly-resistant species with low enzyme activity. It should be noted that the hydrolysis rate of OXA-48 like enzymes is often lower.

In our opinion the CarbaNP test is difficult to apply and interpret for routine laboratories particularly in the areas where OXA-48 type carbapenemases are endemic. Because determination of the color changes in wells was completely based on visual which makes the test results subjective. Adjusting the pH value for 7.8 of phenol red was crucial because of the color changes were affected by the pH value. And different shades of orange for positive $bla_{\text{OXA-48}}$ isolates has been difficult to interpret and to distinguish from other negative wells. Chan et al. [32] mentioned that the CarbaNP test is tecnically challenging and the detection of $bla_{\text{OXA-48}}$ isolates remains a problem due to the dark orange color

Table 1. A total of 53 isolates were given with molecular assay, carbapenem susceptibility and the CarbaNP test results

Strain	β-lactamases	Carbapenem susceptibility			The Carba NP test result/color
		IMP	MP	ETP	
K. pneumoniae	OXA-48	S	S	R	+/orange
K. pneumoniae	OXA-48	S	S	R	+/orange
K. pneumoniae	OXA-48	S	S	R	−/red
K. pneumoniae	OXA-48	S	S	R	+/orange
K. pneumoniae	OXA-48	R	I	R	+/orange
K. pneumoniae	OXA-48	R	R	R	+/orange
K. pneumoniae	OXA-48, SPM	S	S	R	−/red
K. pneumoniae	OXA-48	R	R	R	−/red
K. pneumoniae	OXA-48	R	R	R	+/orange
K. pneumoniae	OXA-48, VIM	S	S	R	+/orange
K. pneumoniae	OXA-48	S	S	S	−/red
K. pneumoniae	OXA-48	R	R	R	−/red
K. pneumoniae	OXA-48	S	S	S	+/orange
K. pneumoniae	OXA-48	S	S	S	−/red
K. pneumoniae	OXA-48	R	R	R	+/orange
K. pneumoniae	OXA-48	S	S	R	+/orange
K. pneumoniae	OXA-48	R	R	R	−/red
K. pneumoniae	OXA-48	S	S	R	+/orange
E. coli	OXA-48	S	S	R	+/orange
E. coli	OXA-48	S	S	R	−/red
E. coli	OXA-48	S	S	R	+/orange
E. coli	OXA-48	S	S	R	+/orange
E. coli	OXA-48, VIM	S	S	R	+/orange
E. coli	OXA-48	S	S	R	+/orange
E. coli	OXA-48	S	S	R	+/orange
E. coli	OXA-48	S	S	R	−/red
S. marcescens	OXA-48, VIM	S	S	R	−/red
S. marcescens	OXA-48	S	S	R	−/red
E. aerogenes	OXA-48, SPM	R	R	R	+/yellow
P. agglomerans	OXA-48	I	S	R	+/orange
M. morganii	OXA-48	R	S	S	+/orange
K. pneumoniae	–	S	S	S	+/orange
K. pneumoniae	–	R	R	R	+/orange
K. pneumoniae	–	S	I	R	+/orange
K. pneumoniae	–	R	I	R	−/red
K. pneumoniae	–	S	S	S	−/red
K. pneumoniae	–	S	S	R	−/red
K. pneumoniae	–	S	S	R	−/red
K. pneumoniae	–	S	S	R	+/orange
K. pneumoniae	–	S	S	R	−/red
K. pneumoniae	–	R	R	R	−/red
K. pneumoniae	–	S	S	S	−/red
K. pneumoniae	–	S	S	S	−/red
K. pneumoniae	–	S	S	R	−/red
K. pneumoniae	–	S	S	R	−/red
K. pneumoniae	–	S	S	R	−/red
K. pneumoniae	–	S	S	R	+/orange
E. coli	–	S	S	S	−/red
E. coli	–	S	I	R	+/orange
E. aerogenes	–	R	I	R	+/orange
E. aerogenes	–	S	S	R	−/red
C. freundii	–	S	S	R	−/red
C. freundii	–	S	S	R	−/red

* S: susceptible, I: intermediate, R: resistant.

which is often observed in the carbapenemase negative wells. In our study the color change was yellow only in one isolate (*E. aerogenes*) which is positive for both bla_{OXA-48} and bla_{SPM}. Dortet et al. [33] modified the CarbaNP test by replacing the lysis buffer with a hyperosmotic NaCl solution for *Acinetobacter* species and the results have been clearer for OXA-48 type carbapenemase.

As a result, the CarbaNP test has suboptimal results to detect OXA-48 producing *Enterobacterales* compared to those originally described with other carbapenemase producers, and has low sensitivity and specifity. Therefore, the test is not reliable to detect OXA-48 producing *Enterobacterales* especially in endemic areas.

Conflict of interest: The authors declare no conflict of interest.

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