Teethaisong, Y, Hobbs, G, Nakouti, I, Evans, K and Eumkeb, G

A nitrocefin disc supplemented with ertapenem for rapid screening of carbapenemase-producing Enterobacteriaceae

http://researchonline.ljmu.ac.uk/7908/

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Teethaisong, Y, Hobbs, G, Nakouti, I, Evans, K and Eumkeb, G (2018) A nitrocefin disc supplemented with ertapenem for rapid screening of carbapenemase-producing Enterobacteriaceae. Diagnostic Microbiology and Infectious Disease. ISSN 0732-8893

LJMU has developed LJMU Research Online for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

http://researchonline.ljmu.ac.uk/
A nitrocefin disc supplemented with ertapenem for rapid screening of carbapenemase-producing Enterobacteriaceae

Yothin Teethaisong a, Glyn Hobbs b, Ismini Nakouti b, Katie Evans b, Griangsak Eumkeb a,*

Running title: Screening of carbapenemases by nitrocefin

a School of Preclinic, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand

b School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, United Kingdom

* Corresponding author.

Associate Professor Dr. Griangsak Eumkeb

Tel.: +66(0)-44224260; Fax: +66(0)-44224650.

E-mail address: griang@sut.ac.th (G. Eumkeb).

Word counts of the abstract: 138 words

Word counts of the body of the text: 2394 words
ABSTRACT

Reliable, simple and rapid methods for laboratory detection of carbapenemases are important for an appropriate antibiotic administration. A nitrocefin disc containing ertapenem for rapid screening of carbapenemase production among Enterobacteriaceae is developed in the present study. A total of 87 molecularly-confirmed Enterobacteriaceae including 31 carbapenemase producers and 56 non-carbapenemase producers, were tested with nitrocefin discs supplemented with and without ertapenem (20 µg/disc). Nitrocefin discs with ertapenem successfully discriminated all 31 carbapenemase and all non-carbapenemase producers within 30 minutes. The sensitivity and specificity of the method were 100%. The minimum inhibitory concentrations (MICs) of ertapenem against all carbapenemase-producing isolates ranged from 1 to ≥ 256 µg/mL. This simple test could help to minimize the treatment failure and control the dissemination of infections caused by carbapenemase-associated resistant bacteria. It is a promising approach that could be performed routinely in any laboratory.

Keywords: Nitrocefin disc, ertapenem, carbapenemase, Enterobacteriaceae
1. Introduction

Since carbapenems have been extensively used for the treatment of infections caused by AmpC and extended-spectrum β-lactamase (ESBL)-producing isolates, an emergence of carbapenem-resistant Enterobacteriaceae (CRE) has been identified (Jacoby, 2009; Perez et al., 2007). Prevalence of CRE has been increasing dramatically, becoming one of the most serious clinical issues with increased morbidity and mortality worldwide (Logan and Weinstein, 2017). The mechanism of resistance to carbapenems, the last line β-lactam antibiotics for the treatment of Gram-negative bacterial infections, is predominantly mediated by enzymes called “carbapenemases” (Bush and Fisher, 2011). Resistance also involves a variety of other mechanisms, including overproduction of AmpC β-lactamase or ESBL combined with decreased porin channel for carbapenem entry, and increased efflux pump activity (Thomson, 2010). Colistin, fosfomycin and tigecycline are among the most frequently used antibiotics for the treatment of carbapenem resistance (Lee and Doi, 2014).

The carbapenemase gene (blaNmcA) was first proven in the chromosome of Enterobacter cloacae in France in 1993 (Naas and Nordmann, 1994). In 1995, a plasmid-encoded blaIMP-1 was described in Japan in Serratia spp. This metallo-β-lactamase (MBL) was able to hydrolyze carbapenem antibiotics (Ito et al., 1995). A few years later, carbapenem-hydrolyzing blaKPC-1 and blaKPC-2 gene were identified in a plasmid of Klebsiella pneumoniae (Smith Moland et al., 2003; Yigit et al., 2001). Furthermore, OXA-48 carbapenemase was also first discovered and identified in an isolate of carbapenem-resistant K. pneumoniae in Istanbul, Turkey (Poirel et al., 2004). Even though numerous clinically significant carbapenemase-encoded genes have been identified recently, they can be molecularly grouped into Ambler class A (KPC and GES), class B (MBL: ...
NDM, VIM, and IMP), and class D with carbapenemase activity (OXA-48-like) (Nordmann, 2014).

Currently, treatment options available for the treatment of infections caused by CRE are limited. Development of diagnostic tools to detect the presence of carbapenemase in Enterobacteriaceae is necessary to guide clinicians for appropriate antimicrobial therapy, as well as to minimize treatment failure (Teethaisong et al., 2016). A number of innovative methods such as genotypic (e.g. multiplex PCR and real-time PCR), phenotypic (e.g. disc-inhibitor synergy test), and biochemical tests (e.g. Carba NP test), have been developed for the detection of carbapenemase-producing isolates (Osei Sekyere et al., 2015; Tamma et al., 2017). However, they are relatively time-consuming, or expensive and difficult to perform.

Nitrocefin, a chromogenic cephalosporin, is a substrate for β-lactamase enzymes. The reaction can visually be observed by a change its original yellow color to red. Nitrocefin assay is one of the most common methods to detect the presence of β-lactamases in both Gram-negative and Gram-positive bacteria ((Kaase et al., 2008)). Carbapenem antibiotics prevent extended spectrum β-lactamase (ESBL) and AmpC β-lactamase-producing bacteria from hydrolyzing nitrocefin (Goessens et. al., 2013 ). The present study, therefore, developed a novel chromogenic method using a nitrocefin disc impregnated with ertapenem for rapid screening of carbapenemase production in Enterobacteriaceae isolates.

2. Materials and methods
2.1 Bacterial isolates
A total of 87 Enterobacteriaceae isolates with confirmed molecular types of β-lactamase enzymes were used in the present study as summarized in Table 1. The bacterial strains used
have been described in our previous study (Teethaisong et al., 2016; Teethaisong et al., 2017), with an addition of KPC-producing K. pneumoniae 4018. Thirty-one carbapenemase-producing isolates (11 MBLs, 9 KPCs and 11 OXA-48), fifty-six non-carbapenemase-producing strains and a β-lactamase-negative E. coli ATCC 25922 were used to investigate nitrocefin discs supplemented with and without ertapenem for the rapid screening of carbapenemase production in Enterobacteriaceae.

2.2 Minimum inhibitory concentration (MIC) determination

The MICs of ertapenem were assessed by a standard broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Clinical Laboratory Standards Institute, 2012). The organisms (18-h culture) were collected by centrifugation and washed twice with sterile saline before adjusting to obtain a 0.5 Mcfarland standard. MIC determination was performed by adding 20 µL of adjusted and diluted organisms (5 x 10⁶ CFU/mL) to the wells containing 20 µL of a doubling dilution series of ertapenem (final concentration ranging from 0.25 - 256 µg/mL) and 160 μL of Mueller-Hinton culture medium. The total volume in each well was 200 µL. The 96-well microtiter plate was then incubated for 20 h at 37˚C. Wells without bacteria and ertapenem were used as controls. The experiment was carried out in triplicate. The lowest concentration showing no visible growth was noted as MIC value. The susceptibility profile of ertapenem was interpreted in accordance with CLSI susceptibility breakpoint: Susceptible ≤ 0.5, intermediate = 1 and resistant > 1 µg/mL (Clinical Laboratory Standards Institute, 2014).

2.3 Disc preparation and experimental procedure

For the preparation of discs, nitrocefin discs (MAST Diagnostic group, UK) were impregnated with and without 10 µL of ertapenem (Sigma-Aldrich) at a concentration of 2 mg/mL to obtain
ertapenem final amount of 20 µg/disc. Following discs had been air-dried in a cabinet, they were stored at -20ºC. The discs were allowed to equiplibrate to room temperature before use. To screen for the presence of carbapenemases in Enterobacteriaceae, two nitrocefin discs (one with and one without ertapenem) were directly placed on 18-h culture colonies grown on LB agar. Following incubation at 37ºC for 30 min, the result was visually observed and interpreted. The development of a red color in the area of both discs with and without ertapenem was denoted as a positive result for carbapenemase, while no color changes in both discs or color change only in nitrocefin without ertapenem disc indicated no carbapenemase production.

3. Results

The present study demonstrates the use of a nitrocefin disc with ertapenem for rapid screening of carbapenemase-producing isolates. The performance of the method was determined by a sum of 87 known molecular types of β-lactamase-producing Enterobacteriaceae. The distribution of MICs of ertapenem among 31 carbapenemase producers and 56 non-carbapenemase producers are presented in Fig. 1. In KPC producers, the MICs of ertapenem ranged from 16 to ≥ 256 µg/mL, while in MBL producers, MICs ranged from 8 to ≥ 256 µg/mL. These MIC values indicated that all MBL and KPC producers were resistant to ertapenem. MICs of ertapenem against OXA-48 producers ranged from 1 to 256 µg/mL. These values trended lower than those MICs of KPC and MBL producers. The susceptibility profile of one OXA-48-E. coli was intermediate. Furthermore, the majority of non-carbapenemase producers were susceptible to ertapenem. Five AmpC-producing isolates were found in the intermediate range. The MIC of ertapenem against a reference E. coli ATCC 25922 was ≤ 0.25 µg/mL which was classified as susceptible.
Fig. 1. The distribution of MIC values of ertapenem against distinct types of β-lactamase-producing Enterobacteriaceae. Red dotted line indicates resistant breakpoint according to CLSI guidelines.
The use of nitrocefin discs with and without ertapenem successfully differentiated carbapenemase producers from other types of β-lactamase producers within 30 min as illustrated in Fig. 2. The study demonstrated that the color of both discs with and without ertapenem developed from yellow to red in all 31 carbapenemase producers. In non-carbapenemase producers, the color change from yellow to red was observed only in the area of an ertapenem-free disc. No color change in both discs was seen in a control strain *E. coli* 25922. The sensitivity and specificity of the method were 100%. Interestingly, the diagnostic method developed in this study can detect the presence of OXA-48 carbapenemase enzyme in an ertapenem-intermediate *E. coli*. It can also discriminate the absence of carbapenemase in ertapenem-intermediate isolates harboring hyperexpressed AmpC β-lactamase. Furthermore, some of ESBL and AmpC-producing strains used in the current study were also previously found to be hyperproduction of ESBL and AmpC β-lactamases (Teethaisong et al., 2016 and 2017).

4. Discussion

The first carbapenemase detection assay was based on antibiotic susceptibility profile from disc diffusion method and MIC determination. The interpretation relies on susceptibility breakpoint. Ertapenem is thought to be a good indicator for the detection of carbapenemase-associated resistance in Enterobacteriaceae as it usually shows higher MIC values compared with those of imipenem and meropenem (Nordmann et al., 2012; Vading et al., 2011). Due to reduced sensitivity and specificity, detection of carbapenemase producers based on susceptibility profile is not frequently conducted (Nordmann and Poirel, 2013). Since then, various methods have been developed and described. Carba NP, a biochemical test, is one of the most popular methods that has been extensively used by many laboratories to detect the presence of carbapenemases in Gram-negative bacteria. This test can provide the result in less than 2 h (Dortet et al., 2012).
However, the preparation for manual Carba NP is relatively difficult. In addition to Carba NP, a $\beta$-CARBA$^\text{TM}$ test showed good performance in detection of carbapenemases-producing isolates with sensitivity 100% of KPC, 100% of IMP, 96.4% of VIM, 85.3% of NDM, 80.5% of OXA-48-like carbapenemases. This test exhibited poor specificity for non-KPC Ambler class A and OXA-48-like carbapenemases (Bernabeu et al., 2017). A method developed in the present study is a simple promising method that successfully screen carbapenemase production in Enterobacteriaceae within 30 min. It could be an alternative method for identifying carbapenemase-mediated resistance in Enterobacteriaceae and other Gram-negative bacilli.
Fig. 2. Representative results from nitrocefin disc with and without ertapenem at 30 min of incubation at 37°C. A = nitrocefin disc alone; B = nitrocefin disc supplemented with ertapenem (20 µg/disc). (I), 1) = KPC-2-producing K. pneumoniae 4016; 2) = NDM-1-producing E. coli 4011; 3) = SHV-27 + TEM-53-producing K. pneumoniae 1012; 4) = a susceptible reference strain E. coli ATCC 25922. (II), 5) = IMP-1-producing Klebsiella ozaenae 403; 6) = OXA-48-producing K. pneumoniae 4019; 7) = ACT-32-producing E. cloacae 2009; 8) = TEM-214+SHV-12+ACT-32-E. aerogenes 3009.

The nitrocefin disc supplemented with ertapenem perfectly detected carbapenemase producers with low ertapenem MIC values, particularly OXA-48-like-producing isolates. However, this method needs to be further validated with carbapenem-susceptible isolates as well as with other carbapenemase variants. It also requires larger sample sizes of carbapenemase-producing isolates to confirm the robustness of this assay. The performance of the assay should be investigated with other carbapenemase-producing Gram-negative bacteria such as Pseudomonas spp. and Acinetobacter spp. Subjectivity in interpretation of the results is not only the common issue of this method, but it is also a problem of all other chromogenic methods (Tijet et al., 2013).

In conclusion, the nitrocefin with ertapenem disc is very simple to perform and provides rapid results to identify carbapenemase producers, with high sensitivity and specificity. This assay can be carried out routinely in any microbiological laboratory. It could facilitate successful treatment of carbapenemase-mediated resistance and indicate the isolates to be further evaluated by PCR or sequencing for molecular epidemiological surveillance. This method is also potentially important for follow-up investigations.
Acknowledgements

This work was supported by Suranaree University of Technology (SUT) and by Office of the Higher Education Commission under NRU Project of Thailand (FtR.07/2560).

References


Clinical Laboratory Standards Institute (2014) Performance standards for antimicrobial susceptibility testing; Twenty-fourth informational supplement update : CLSI document M100-S24. CLSI, Wayne, PA


detection methods for carbapenemase-producing Gram-negative bacteria. J Appl
Microbiol, 119: 1219-1233. 10.1111/jam.12918

Curr Opin Pharmacol, 7: 459-469. 10.1016/j.coph.2007.08.003

resistance to imipenem in Klebsiella pneumoniae. Antimicrob Agents Chemother, 48: 15-

Smith Moland, E, Hanson, ND, Herrera, VL, Black, JA, Lockhart, TJ, Hessain, A, Johnson, JA,
Goering, RV, & Thomson, KS (2003) Plasmid-mediated, carbapenem-hydrolysing beta-
lactamase, KPC-2, in Klebsiella pneumoniae isolates. J Antimicrob Chemother, 51: 711-

Tamma, PD, Opene, BN, Gluck, A, Chambers, KK, Carroll, KC, & Simner, PJ (2017)
Comparison of 11 phenotypic assays for accurate detection of carbapenemase-producing

with resazurin agar plate assay for early phenotypic screening of KPC, MBL and OXA-

Teethaisong, Y, Evans, K, Nakouti, I, Tiamyom, K, Ketudat-Cairns, JR, Hobbs, G, & Eumkeb,
G (2017) The performance of a resazurin chromogenic agar plate with a combined disc
method for rapid screening of extended-spectrum-beta-lactamases, AmpC beta-


Fig. 1. The distribution of MIC values of ertapenem against distinct types of β-lactamase-producing Enterobacteriaceae. Red dotted line indicates resistant breakpoint according to CLSI guidelines.
**Fig. 2.** Representative results from nitrocefin disc with and without ertapenem at 30 min of incubation at 37°C. A = nitrocefin disc alone; B = nitrocefin disc supplemented with ertapenem (20 µg/disc). (I), 1) = KPC-2-producing *K. pneumoniae* 4016; 2) = NDM-1-producing *E. coli* 4011; 3) = SHV-27 + TEM-53-producing *K. pneumoniae* 1012; 4) = a susceptible reference strain *E. coli* ATCC 25922. (II), 5) = IMP-1-producing *Klebsiella ozaenae* 403; 6) = OXA-48-producing *K. pneumoniae* 4019; 7) = ACT-32-producing *E. cloacae* 2009; 8) = TEM-214+SHV-12+ACT-32-*E. aerogenes* 3009.
### Table 1
Summary of carbapenemase-producing and non-carbapenemase-producing Enterobacteriaceae used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Carbapenemase producers (n = 31)</th>
<th>Non-carbapenemase producers (n = 56)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambler class A KPC</td>
<td>Ambler class B NDM</td>
</tr>
<tr>
<td>E. coli</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>K pneumoniae</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. cloaca</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>M. morganii</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C freundii</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K oxytoca</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>K ozaenae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9</strong></td>
<td><strong>11</strong></td>
</tr>
</tbody>
</table>

* One VIM-1 + SHV-12 and one VIM-1 + SHV-102 co-expressions