

ORIGINAL PAPER

# RAPID DETECTION OF CARBAPENEMASES AND ESKAPEE<sub>c</sub> BACTERIA

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## ABSTRACT

**Introduction.** Carbapenems have been the ultimate antibiotics for the treatment of infections caused by multidrug-resistant bacteria. However, recently, carbapenems-resistant bacteria have emerged significantly.

**The objective of the study** was the identification of ESKAPEE<sub>c</sub> bacteria and rapid detection of carbapenemase production.

**Materials and methods.** We tried a novel rapid test methodology that detects some carbohydrates metabolism associated with bacterial growth in the presence of imipenem. The formation of acid metabolites is evidenced by a color change of a pH indicator.

**Results.** Carbapenemase production is phenotypically demonstrated in carbapenem-resistant bacterial strains. In the study, carbapenemase production was detected within 3 hours, and identification of ESKAPEE<sub>c</sub> bacteria was completed within 4 hours by carbohydrate metabolism.

**Conclusions.** In conclusion, our cost-effective technique may provide a practical solution for the determination of multi-drug resistance by using the fermentation metabolism in bacteria.

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## RÉSUMÉ

**Détection rapide des carbapénémases et des bactéries ESKAPEE<sub>c</sub>**

**Introduction.** Les carbapénèmes sont les antibiotiques de dernière génération pour le traitement des infections causées par des bactéries multirésistantes. Cependant, récemment, des bactéries résistantes aux carbapénèmes ont émergé de manière significative.

**L'objectif de l'étude.** Nous visons une étude préliminaire pour l'identification des souches pathogènes de la bactérie ESKAPEE<sub>c</sub> et la détection rapide de la production de carbapénémase.

**Matériel et méthodes.** Nous avons essayé une nouvelle méthodologie de test rapide qui détecte la métabolisation de certains glucides associée à la croissance bactérienne en présence d'imipénème. La formation de métabolites acides est mise en évidence par un changement de couleur d'un indicateur de pH.

**Résultats.** La production de carbapénémase est phénotypiquement démontrée dans des souches bactériennes résistantes aux carbapénèmes. Dans l'étude, la production de carbapénémase a été détectée dans 3 heures et

**Keywords:** ESKAPEE<sub>c</sub>, *Enterobacteriaceae*, carbapenemase,  $\beta$ -lactamase, antimicrobial drug

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resistance. L'identification des bactéries ESKAPEEc a été achevée dans 4 heures par le métabolisme des glucides.

**Conclusions.** Cette technique rentable peut fournir une solution pratique pour la détermination de la résistance multi-

## INTRODUCTION

Carbapenems are the antibiotics with the broadest spectrum and rapid bactericidal effect among the beta-lactam antibiotics. Carbapenems bind very strongly to penicillin-binding proteins. Due to their general structure and molecular size, their transport through porine channels and their penetration to the bacterial cells are very good. Thus, carbapenems are widely used as the last resort agents reserved for treatment of infections due to highly multidrug-resistant organisms such as *Enterobacteriaceae*. However, carbapenem-resistant *Enterobacteriaceae* (CRE) infections are seen with increasing frequency and spreading around the world, posing great challenges to patients and clinicians<sup>1-8</sup>. Conventional laboratory diagnosis methods remain time-consuming (24 to 48 h). In recent years, rapid methods were developed for identification of CRE by using carbohydrate metabolism-related to bacterial growth in the presence of a defined concentration of imipenem<sup>2</sup>.

*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli* abbreviated as ESKAPEEc defined by De Angelis as cipher word<sup>3</sup>. This group is responsible for a substantial percentage of severe infections and of multidrug-resistant organisms in hospitals, especially among critically ill and immunocompromised patients. During the last decade, in many hospitals, ESKAPEEc pathogens ranged from 70-80% of total bacterial isolates causing bloodstream infections<sup>4,7</sup>. Even if awareness is increasing in some countries, the lack of basic infection control measures brings about delays in diagnosis. The short-term efficiency of a novel antibiotic discovery prompted pharmaceutical companies to abandon the production process<sup>8,9</sup>.

Rapid detection of carbapenemases, especially when due to transmissible carbapenemase-producing strains, is important to properly orient antimicrobial treatment and implement appropriate isolation measures. Some methods for detection have long time-to-results, some are complicated or costly. What's more, some tests lack sensitivity and specificity, making results less reliable<sup>1-6</sup>.

médicamenteuse en utilisant le métabolisme de fermentation chez les bactéries.

**Mots-clés:** ESKAPEEc, *Enterobacteriaceae*, carbapénémase,  $\beta$ -lactamase, résistance aux antimicrobiens

A lot of phenotypic tests are currently used in laboratories such as modified Hodge Test (MHT), modified Carbapenem Inactivation Method (mCIM), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), Carba-NP, lateral flow immunoassays for carbapenemases<sup>7,9</sup>. One of these methods, MALDI-TOF MS, can detect carbapenemase production in a short time by analyzing hydrolytic products using carbapenem in the presence of carbapenemase in bacteria. It has many advantages such as the ability to result in 1-4 hours, high sensitivity and specificity, short test time, the potential to perform resistance analysis in a short time from a blood culture bottle in vital infections such as circulatory system infections.

Clinicians can prescribe antibiotics at doses that minimize the risk of bacteria developing resistance by these methods. Especially, *E. coli* and *K. pneumoniae* strains isolated from blood cultures showed high extended-spectrum beta-lactamases (ESBL) and carbapenem resistance rates which increased significantly over the years. Therefore, each hospital needs to focus on infection control surveillance<sup>10-12</sup>.

**THE OBJECTIVE OF THE STUDY** was the identification of ESKAPEEc bacteria and rapid detection of carbapenemase production.

## MATERIALS AND METHODS

This prospective study was conducted in Ekrem Kadri Unat Research Laboratories of Istanbul University-Cerrahpaşa School of Medicine Department of Medical Microbiology, Turkey. The Ethics committee approval for this study was obtained from the Istanbul University-Cerrahpaşa School of Medicine Ethics Committee of Clinical Research (Decision Number: 211535; Decision Date: June 10, 2016).

### Sample collection

Study strains were the 90 ESKAPEEc strains that were isolated from the blood cultures of hospitalized patients in different departments. Of these, 20 *E. coli*, 20 *K. pneumoniae*, 20 *A. baumannii*,

10 *E. faecium*, 10 *S. aureus*, and 10 *P. aeruginosa*. Reference strains *Klebsiella pneumoniae* (ATCC-BAA-1705) and *Escherichia coli* (ATCC-BAA-2340) were used as control organisms.

The BD Phoenix™ automated system (Becton Dickinson Company, Franklin Lakes, NJ, USA) and MALDI-TOF MS (Bruker; Daltonics, Bremen, Germany) were used to identify organisms and antibiotic susceptibility. The concentration gradient-based E-test (bioMérieux, France) strip method was employed to measure the minimum inhibitory concentration (MIC) values in imipenem in vitro susceptibility tests. The susceptibilities of the strains to carbapenems were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria, as updated in 2019<sup>13</sup>. The breakpoints used were those for imipenem; susceptibility <2 µg/mL, resistance >4 µg/mL.

#### Rapid detection of carbapenemase production in *E. coli*, *K. pneumoniae*, and *A. baumannii*

We used a biochemical method to detect carbapenemase-producing strains in a shorter time and with a cheaper cost. This method based on principle of showing the pH change caused by carbapenem hydrolysis reaction with colour change using a colorimetric indicator such as phenol red.

10 µL of the confirmed strain directly recovered from the antibiogram was resuspended in a Tris-HCl 20 mmol/L lysis buffer (Trizma® hydrochloride, SIGMA; St. Louis, Missouri, US), vortexed for 1 minute and incubated at room temperature for 30 minutes. This bacterial suspension was centrifuged at 10,000 × g at room temperature for 5 minutes. Thirty µL of the supernatant, corresponding to the enzymatic bacterial suspension, was mixed in a 96-well tray with 100 µL of a 1-mL solution made of 3 mg of imipenem monohydrate (SIGMA; St. Louis, Missouri, US), pH 7.8, phenol red solution, and 0.1 mmol/L ZnSO<sub>4</sub> (Zinc sulfate solution, SIGMA; St. Louis, Missouri, US). The phenol red solution was prepared by mixing 2 mL of a phenol red solution 0.5% (wt/vol) with 16.6 mL of distilled water. The pH value was then adjusted to 7.8. A mixture of the phenol red solution and the enzymatic suspension being tested was incubated at 37°C.

#### Rapid identification of ESKAPEc bacteria

Ten different saccharides were used in the study, including glucose (D+)Glucose, SIGMA; St. Louis, Missouri, US), lactose (D-Lactose, SIGMA; St. Louis, Missouri, US), sucrose (Sucrose, SIGMA;

St. Louis, Missouri, US), mannitol (D-Mannitol, SIGMA; St. Louis, Missouri, US), mannose (D+) Mannose, ACROS; Geel, Belgium), raffinose (D+) Raffinose, SIGMA; St. Louis, Missouri, US), xylose (L-) Xylose, ACROS; Geel, Belgium), trehalose (D Trehalose, ACROS; Geel, Belgium), cellobiose (D+) Cellobiose; SIGMA; St. Louis, Missouri, US), and adonitol (Adonitol, SIGMA; St. Louis, Missouri, US). The saccharides included in the study were taken into consideration when referring to Bergeyeria's Manual of Systematic Bacteriology, The Proteobacteria, Part B The Gammaproteobacteria<sup>14</sup>.

We prepared a stock bacterial solution for wells (96-well plate) that consisted of a mixture of Luria Bertani (LB Broth, Lennox, SIGMA; St. Louis, Missouri, US), saline (0.09% NaCl in water, w/v), and 0.05% phenol red (w/v) as a colorimetric indicator of the pH value. LB provides essential nutrients that aid cell growth and rapid fermentation of the saccharides in the well. We tested stock solution with LB, added phenol red to a final concentration of 0.05% (w/v), and adjusted to 8.2 pH.

To identify ESKAPEc species, incubate bacteria overnight at 37°C on LB. And then single colonies, and transferred to 100 µL of bacterial stock solution. Mixed by vortexing, then loaded suspension into a well and incubated at room temperature for 15–30 min. Wells were placed within a 37°C incubator and the colorimetric profiles were recorded every 30 mins and 1 hrs.

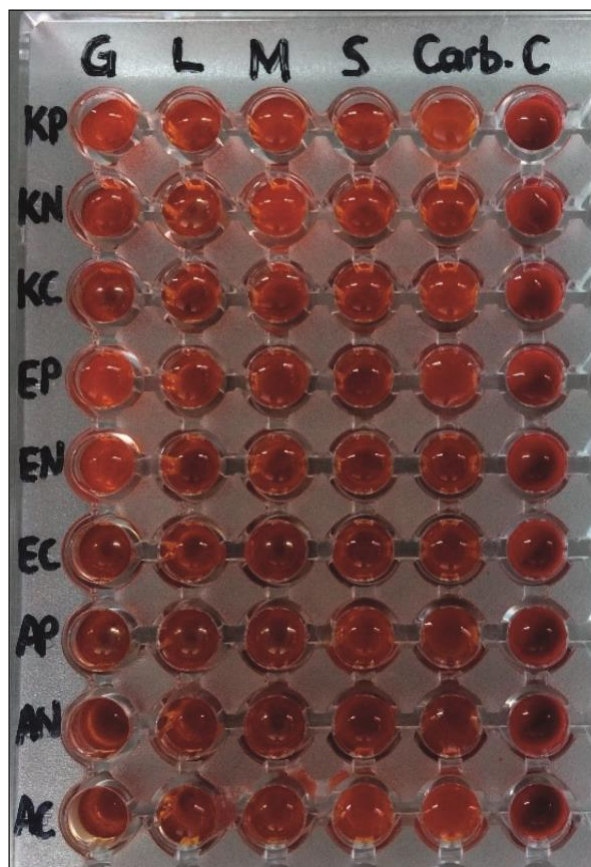
#### Statistical analysis

SPSS Version 23.0 for Windows (IBM Corp., Armonk, NY) was used to perform the statistical analysis. Descriptive statistics were calculated as numbers and percentages for categorical variables. Statistical significance was accepted as p-value<0.05.

#### RESULTS

The colour of the plate-wells turned from red to yellow that were producing carbapenemase, whereas isolates that did not produce carbapenemase remained red. The colour changed approximately 1 h after incubation for KPC producers began. Incubation for 2 h was sufficient for obtaining colour change to carbapenemase producers. (Figures 1, 2). All tests were performed in triplicate, giving identical and reproducible results.





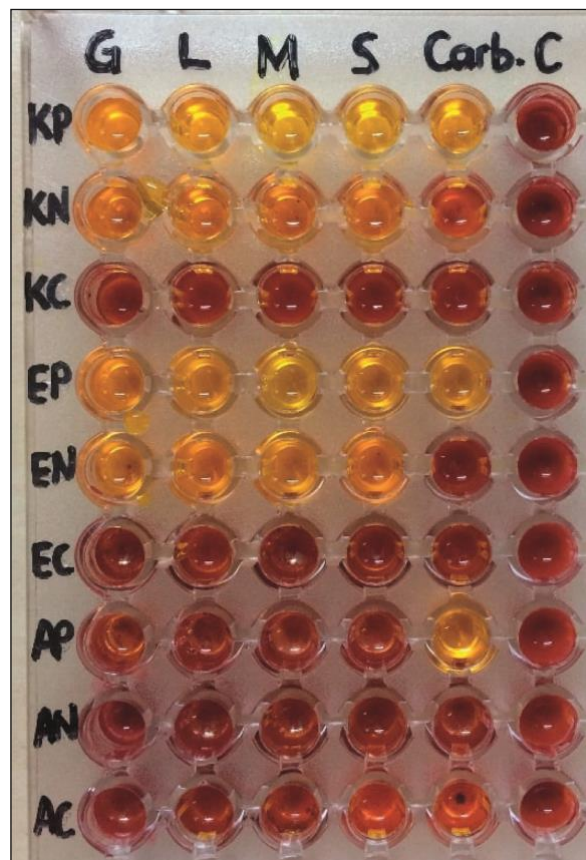
Out of the 60 isolates (20 *E. coli*, 20 *K. pneumoniae*, 20 *A. baumannii*), the test for carbapenemase production was positive in 30 isolates and 30 isolates were negative. Out of 30 imipenem resistant isolates tested for carbapenemase production using our test method, 30 isolates were positive for carbapenemase production ( $p < 0.05$ ).

Glucose, lactose, mannitol, and sucrose, as well as mannose, raffinose, xylose, trehalose, cellobiose,

**Figure 1.** All plate-wells red before incubation of the strains.

**Legend:**

*Vertical abbreviation:* KP, Carbapenemase positive *Klebsiella pneumoniae*; KN, Carbapenemase negative *Klebsiella pneumoniae*; KC, Control strains of *Klebsiella pneumoniae*;



EP, Carbapenemase positive *Escherichia coli*; EN, Carbapenemase negative *Escherichia coli*; EC, Control strains of *Escherichia coli*; AC, Carbapenemase positive *Acinetobacter baumannii*; AN, Carbapenemase negative *Acinetobacter baumannii*; AC, Control strains of *Acinetobacter baumannii*.

*Horizontal abbreviation:* G, glucose; L, lactose; M, mannitol; S, sucrose; Carb, Carbapenemase; C, control well.

**Figure 2.** Colour change in the wells after 2 h of incubation.

**Legend:**

*Vertical abbreviation:* KP, Carbapenemase positive *Klebsiella pneumoniae*; KN, Carbapenemase negative *Klebsiella pneumoniae*; KC, Control strains of *Klebsiella pneumoniae*; EP, Carbapenemase positive *Escherichia coli*; EN, Carbapenemase negative *Escherichia coli*; EC, Control strains of *Escherichia coli*; AC, Carbapenemase positive *Acinetobacter baumannii*; AN, Carbapenemase negative *Acinetobacter baumannii*; AC, Control strains of *Acinetobacter baumannii*.

*Horizontal abbreviation:* G, glucose; L, lactose; M, mannitol; S, sucrose; Carb, Carbapenemase; C, control well.

**Table 1.** Results after incubation of 2 hours.

	Glucose	Lactose	Maltose	Sucrose	Mannitol	Rafinose	Xylose	Trehalose	Cellobiose	Adonitol	Control
<i>Acinetobacter baumannii</i>	□	□	□	□	□	□	□	□	□	□	□
<i>Escherichia coli</i>	□	□	□	□	□	□	□	□	□	□	□
<i>Klebsiella pneumoniae</i>	□	□	□	□	□	□	□	□	□	□	□
<i>Staphylococcus aureus</i>	□	□	□	□	□	□	□	□	□	□	□

<i>Enterococcus faecium</i>	□	□	□	□	□	□	□	□	□	□	□
<i>Pseudomonas aeruginosa</i>	□	□	□	□	□	□	□	□	□	□	□

Legend: +: positive; -: negative; ±: half-positive (variable).

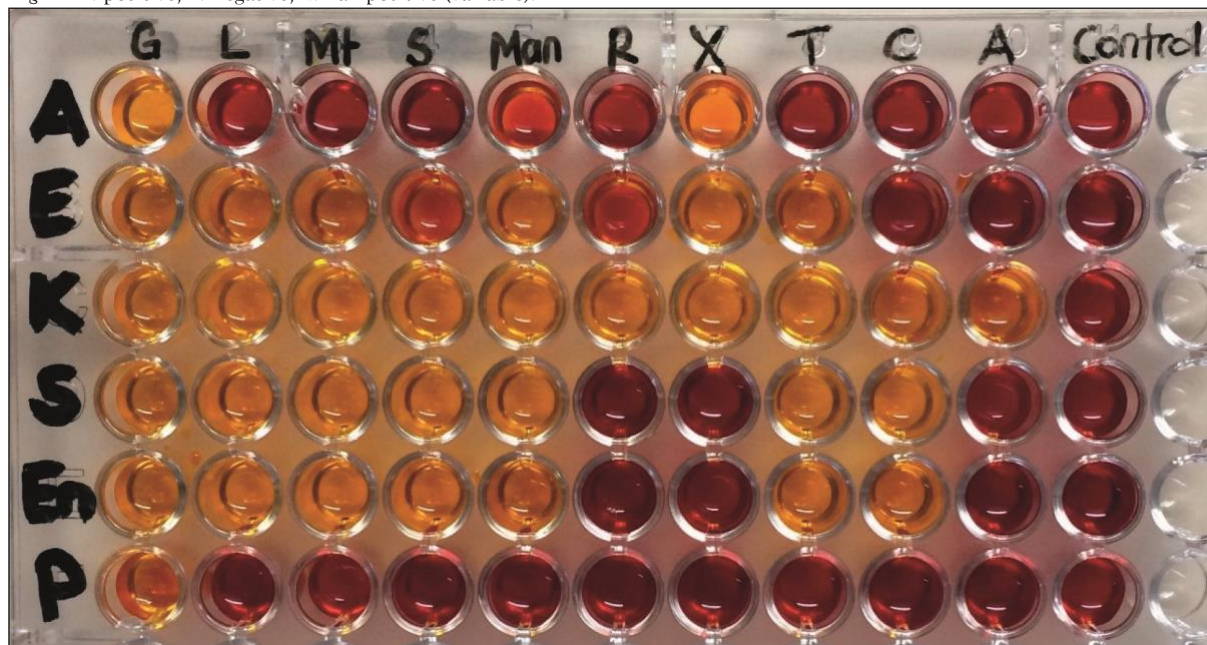


Figure 3. Colour change in the wells after 4 h of incubation.

Legend: Vertical abbreviation: A, *Acinetobacter baumannii*; E, *Escherichia coli*; K, *Klebsiella pneumoniae*; S, *Staphylococcus aureus*; En, *Enterococcus faecium*; P, *Pseudomonas aeruginosa*. Horizontal abbreviation: G, glucose; L, lactose; Mt, mannitol; S, sucrose; Man, mannitol; R, raffinose; X, xylose; T, trehalose; C, cellobiose; A, adonitol.

and adonitol, were used for the rapid identification of bacteria according to their fermentation properties. Thus, it is aimed to distinguish the identification of bacteria by using different saccharides. In the last row, the control group is located (Figure 3). After incubation of 1 h, the colour change was started in isolates except *A. baumannii* and *P. aeruginosa*, but no yellow colour was observed in any well. After 2 h of incubation, the yellow colour was observed in the wells of some saccharides. The results are shown in Table 1.

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to the first well to differentiate *S. aureus* and *E. faecium*, which were determined to have the same effect on the saccharides. The test is easy to perform; bacteria are simply mixed with H<sub>2</sub>O<sub>2</sub>. If bubbles appear, due to the production of oxygen gas, the bacteria are catalase-positive (*Staphylococcus aureus*). If no bubbles appear, the bacteria are catalase-negative (*Enterococcus faecium*). *A. baumannii* is separated from *P. aeruginosa* by its effect against xylose (Figure 3).

All identified strains by MALDI-TOF-MS were correctly identified by our test method.

## DISCUSSION

The increase in multidrug-resistance of ESKAPEEc bacteria has made it a global problem that complicates the treatment in hospital infections and increases the cost of mortality and treatment. Therefore, rapid detection of carbapenemase-producing bacteria has clinical importance<sup>1,15</sup>. The study describes a colorimetric assay for detection of carbapenem hydrolysis and rapid identity of ESKAPEEc. Nordmann et al.<sup>2</sup> showed a rapid detection of carbapenemase by a method based on beta-lactam ring hydrolysis. In the study, we followed a similar methodology. And then we focused on methods that can show antimicrobial resistance as well as identification of ESKAPEEc bacteria.

The gold standard for demonstration of carbapenemases is molecular techniques. However, these techniques are expensive. Detection of carbapenemase is the first step in preventing the spread of carbapenemase<sup>16</sup>.

Nonetheless, the exact determination of the type of carbapenemase is not necessary for the treatment of the patient or in the prevention of



outbreaks. Therefore, these types of molecular techniques can be more useful in reference laboratories<sup>17</sup>. Phenotypic detection methods for the mechanism of carbapenem resistance are tests that can be easily applied and are useful tests for obtaining infection control measures. Some studies showed that the presence of carbapenemase in septicemia patients caused *E. coli* and *K. pneumoniae* in increasing morbidity and mortality patients. Also, the other factor of increase in mortality is the coexistence of Extended-Spectrum Beta-Lactamase (ESBL) and carbapenemases in some isolates<sup>12,18-20</sup>.

In the study, we showed carbapenem-hydrolyzing  $\beta$ -lactamases. The H<sup>+</sup> ions of the beta-lactam ring were revealed and the pH was lowered. The increase in acidity caused a colour change of the phenol red solution. Thus, the presence of carbapenemase was interpreted by observing the colour change. The study showed that the rapid detection of antibiotic-resistant *E. coli* and *K. pneumoniae* bacteria and the presence of carbapenemase can be performed more easily and cheaply in hospitals. Bayraktar et al.<sup>12</sup> reported that resistance rates of 439 *K. pneumoniae* isolates to imipenem were significantly increased ( $p < 0.001$ ).

Marturano and Lowery<sup>21</sup> found that ESKAPEE<sub>c</sub> constitutes nearly half the bacteria in bloodstream infections and is strongly associated with higher lengths of stay in hospital, cost of care, and mortality with non-ESKAPEE<sub>c</sub> pathogens. We observed a stable condition for wells colour in approximately 4 hours in the tests we showed the ESKAPEE<sub>c</sub> bacteria identification. Reducing the time from onset to detection is important for diagnostic testing. In particular, rapid detection of a pathogen is an important step in improving patient outcomes and in reducing costs associated with treating infections<sup>21-24</sup>.

Many studies aim to find simple methods to quickly detect antimicrobial resistance and evaluate the cumulative antimicrobial resistance index. De Socio et al.<sup>23</sup> they were found that antimicrobial-resistance patterns in clinical samples especially blood culture isolates progressively increased from 2014-18 in their training hospital. If no interventional measure is taken, a peak to resistance rates of the ESKAPEE<sub>c</sub> group could be expected in the next 8-15 years.

Mulani et al.<sup>25</sup> focused on the importance of a combinatorial approach for ESKAPEE<sub>c</sub> infections. According to the study, it is crucial that two or more therapies be used together to overcome their individual limitations before being converted into clinical practice.

The increasing frequency of carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections also has been reported from various countries worldwide. CRAB rates for Turkey ranged from 50-80%. European countries such as Greece, Italy, Spain, Germany, and Sweden reported that the CRAB rates 85%, 60%, 45%, 8%, 4%, respectively. These results showed that it is especially important to investigate the spreading of CRAB isolates in hospitals in our country<sup>26-30</sup>.

Other studies have been done for the detection of carbapenemases (typically imipenem) directly from clinical samples and positive blood cultures (e.g. MALDI-TOF-MS, CARBA-NP,  $\square$ -CARBA). Each technique has certain strengths and weaknesses regarding performance, associated costs, and turnaround time. Additional equipment and software are required for both MALDI-TOF hydrolysis testing and molecular analysis. Detection of carbapenemases from blood cultures using an immunochromatographic assay has been recently reported<sup>30-33</sup>.

Many clinical laboratories use methods based on PCR to detect carbapenemase genes to eliminate the problems related to phenotypic identification methods and at the same time to shorten the reporting time. However, multiple and simultaneous PCR methods have also been reported, which allow the detection of the carbapenemase gene type and reduce the time required for detection<sup>34,35</sup>. PCR and hybridization-based kits have been developed commercially for typing the parent carbapenemase genes. These methods can be used directly in clinical samples, but their diagnostic benefits should be evaluated both in different places and systematically<sup>36</sup>.

Microarray technology has also taken its place in the list of fast and reliable molecular techniques for the diagnosis of factors causing multi-drug resistance. Recently, it has been reported that bla genes including almost all carbapenemase genes have been successfully detected in a single tube by Check-MDR CT102 (Check-Points Health BV, Wageningen, Netherlands) microarray<sup>36,37</sup>. The problem we encounter in all molecular methods because the resistance genes to be determined have been determined beforehand, it should be taken into account that molecular methods may missed new gene types.

Continuity of studies for rapid detection is a requirement. This test can be performed in any laboratory in the world by the methods described by our study.

In the present study, carbapenemase was determined within 3 hrs, and the ESKAPEEC identification was completed within 4 hrs. This is consistent with other studies<sup>2,6</sup>. To evaluate the positive and negative aspects of the test, studies are needed in clinical samples from various selective cultures.

## CONCLUSIONS

Reliable and simple tests are needed for routine laboratories to detect carbapenemases. In addition, rapid tests required for the correct implementation of infection control policies in the hospital environment. The study's output can play an important role in antimicrobial stewardship strategies because it is known that the rate of antimicrobial resistance increasing steadily, especially due to overconsumption.

## Author Contributions:

Conceptualization, O.A. and F.K.Ç.; methodology, O.A. and F.K.Ç.; software, validation, O.A. and F.K.Ç.; formal analysis, O.A. and F.K.Ç.; investigation, O.A. and F.K.Ç.; resources, –; data curation, O.A. and F.K.Ç.; writing–original draft preparation, O.A. and F.K.Ç.; writing– review and editing, O.A. and F.K.Ç.; visualization, O.A. and F.K.Ç.; supervision, O.A. and F.K.Ç.; project administration, O.A. and F.K.Ç.; All the authors have read and agreed with the final version of the article.

## Compliance with Ethics Requirements:

“The authors declare no conflict of interest regarding this article”

“The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study”

“All institutional and national guidelines for the care and use of laboratory animals were followed”

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**Ethics Committee Approval:** Ethics committee approval for this study was obtained from the Istanbul University-Cerrahpaşa School of Medicine Ethics Committee of Clinical Research (Decision Date: June 10, 2016; Decision Number: 211535).

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