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ORIGINAL ARTICLE

Carbapenemase investigation with rapid phenotypic test (RESIST-4 O.K.N.V) and comparison with PCR in carbapenem-resistant *Enterobacterales* strains

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ABSTRACT

Background and Objectives: RESİST-4 O.K.N.V. assay is a lateral immunochromatocraphic test for the identification of oxacillinase (OXA)-48-like, *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), and Verona integron-encoded metallo-β-lactamase (VIM) producing strains. It was aimed to evaluate the performance of the RESIST-4 O.K.N.V. test and to compare it with the reference method polymerase chain reaction (PCR). Also, the objective was to determine the distribution of carbapenemase types of CRE strains isolated in our hospital.

Materials and Methods: Between January 2016-October 2019, 187 strains isolated from clinical samples were included in this study. Bacterial identification was done using MALDI-TOF MS. Antibiotic susceptibility tests were studied with the VITEK-2 automated system. Meropenem minimum inhibitory concentrations (MICs) were determined by the gradient test. All strains were studied with the RESIST-4 O.K.N.V. test and then the strains were selected for the PCR test. bla_{OXA-48}, bla_{NDM}, bla_{KPC}, and bla_{VIM} were investigated with PCR. *K. pneumoniae* NCTC® 13438 (KWIKSTIKTM, Microbiologics®,USA) was used as the positive control, *E. coli* ATTC® 25922 TM (Microbiologics®,USA) and three carbapenem-sensitive clinical isolates were also used as the negative control.

Results: Meropenem MIC_{50} and MIC_{90} values were determined to be >32 mg/L. With PCR bla_{OXA-48}, bla_{NDM}, bla_{KPC}, and bla_{VIM} were detected in 79, 63, 20, and 4 strains, respectively. bla_{OXA-48} and bla_{NDM} were found together in 51 of the isolates. bla_{OXA-48}, bla_{NDM}, bla_{KPC}, and bla_{VIM} were not detected in two strains with carbapenem resistance in susceptibility tests. The sensitivity of the immunochromatographic test was 100% for OXA-48, KPC, and VIM but 84.1% for NDM. Specificity was determined as 100% for OXA-48, NDM, KPC, and VIM.

Conclusion: RESIST-4 O.K.N.V. test showed high sensitivity and specificity in detecting OXA-48, KPC, NDM, and VIM type carbapenemases. However, it should be kept in mind that there may be false-negative results related to NDM.

Keywords: Carbapenemases; Immunochromatographic test; Oxacillinase-48; *Klebsiella pneumoniae*

INTRODUCTION

Infections related to Carbapenem-resistant Enterobacterales (CRE) are among the most important health problems all over the world. The ability for production of ESBL by these microorganisms has led to the use of carbapenems as the last defense line. But the solution created a bigger problem than the

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problem itself, and the emergence of carbapenem resistance caused these pathogens to manifest themselves in MDR bacteria (1, 2). Although this threat appears in almost all members of the family, *Klebsiella pneumoniae* which often causes severe clinical manifestations such as bloodstream infections, pneumonia, complicated urinary tract, and intra-abdominal infections in intensive care unit patients, stands out as the most concerning one, as it leads 40-50% mortality rates (1, 2).

Rapid and accurate identification of carbapenemases could be a critical step that can help to monitor and prevent the spread of CPE (carbapenemase-producing Enterobacterales). Additionally, since the susceptibility of isolates containing different carbapenemases may differ such as OXA like, it may also guide treatment (3). There are phenotypical and genotypical methods such as modified Hodge test, carbapenemase inactivation method (CIM) and modified version of it (mCIM), CarbaNP test, Matrix-Assisted Laser Desorption of Carbapenem Hydrolysis/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS), immunochromatographic lateral flow assay, and polymerase chain reaction (PCR) to detect carbapenemases (4). CIM and mCIM require one night of incubation for the result. Furthermore, while these tests only detect carbapenem hydrolyses, cannot identify the type of enzyme.

A relatively novel test method, RESİST O.K.N.V., is also a method for the identification of carbapenemases (2). The principle of this is to change color in specific areas after contact with the sample of monoclonal antibody absorbed nitrocellulose membranes against OXA-48-like, KPC, NDM, and VIM carbapenemases. Aside from the existence of different methods, the polymerase chain reaction (PCR) is considered the golden standard method for carbapenemase detection, but the need for additional equipment and qualified personnel restricts its routine use in laboratories (3).

In this study, we aimed to evaluate the performance of the rapid immunochromatographic assay, which is suitable for routine use in the laboratory, and compare the results with the golden standard method, PCR.

MATERIALS AND METHODS

Study design. Between January 2016 and October 2019, 187 bacterial strains isolated from different patients' culture samples that were found to be resistant to ertapenem or meropenem were included in this study. *K. pneumoniae* NCTC® 13438 (KWIKSTIK-TM, Microbiologics®,USA) was used as the positive control, *E. coli* ATTC® 25922 TM (Microbiologics®, USA) and three carbapenem-sensitive clinical isolates were also used as the negative control. The identification and susceptibilitytests of the isolates were performed by using MALDI-TOF MS (Biomerieux, France) and VITEK 2 automated systems, respectively. The MIC of meropenem was also studied by gradient test method. Meropenem MIC_{50} and MIC_{90} values were determined for all isolates. All the susceptibility test results were interpreted according to EUCAST criteria. All isolates were studied with RESIST-4 O.K.N.V. test (Coris Bioconcept, Belgium). After the immunochromatographic assay, a panel of representative isolates was selected for PCR testing.

Immunochromatographic assay. OXA-48, NDM, VIM, and KPC carbapenemase enzymes of all origins were investigated according to the manufacturer's recommendations. For this, a fresh colony was homogenized in tubes with 12 drops of LY-A buffer. Three drops of suspension were then added to the sample well of each of the two cassettes (KPC / OXA-48 and NDM / VIM). The results were evaluated according to the manufacturer's recommendations.

Sample selection for PCR testing. Nine strains not identified as *K. pneumoniae* (*Klebsiella oxytoca*, *Escherischia coli*, *Enterobacter aerogenes*, and *Serratia marcescens*) were included in the PCR study. When immunochromatographic testing was applied to 178 *K. pneumoniae* strains, it was observed that 144 strains were producing OXA-48 and NDM+OXA-48. For this reason, the isolates containing the other types of carbapenemase for diversity were included in the PCR analysis. From a total of 144 strains that produced OXA-48 and NDM+OXA-48 carbapenemase together, systematic sampling was performed. The sample size was determined as 72 when the prevalence was 10% and the margin of error was 5%. Thus, a total of 116 isolates, as well as control strains, were included in the PCR study.

DNA isolation. Before the PCR experiments, DNA isolation of isolates was performed by boiling meth-

od (5). Colonies of bacteria taken from fresh culture were suspended in sterile ultra-pure water, homogenized by vortexing. Tube contents were placed in a heat block for 10 min at 95°C (Dry Bath Incubator, Miulab, China). Then contents were centrifuged for 5 min at 13000 rpm at +4ºC (Mikro200R, Hettich, Germany). Finally, the liquid part was transferred to sterile microtubes and stored at -80°C until it was used.

PCR method. Primers targeting the *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$, and *bla*$_{VIM}$ were used in carbapenem-resistant isolates (6, 7). The final ingredient of the PCR mix was prepared as; X Taq Buffer, 200 mm dNTP, 2.5 mm $MgCl_2$ (Taq polymerase - set dNTP, Applied Biological Materials Inc., Canada), and 50 pmol of primer pairs in a final volume of 25 µL. These mixtures were added to pre-prepared 2.5 µL DNA extracts. The remaining volume is completed with double-distilled H_2O to the thermal cycler device (AriaMx qPCR, Agilent Tech., USA).

Amplification program was applied 35 cycles as; 1 minute at 95°C; 50 seconds at 95°C, the annealing temperature of the primer, 40 sec at 72°C, and the final elongation stage at 72°C for 10 minutes. To examine the resulting PCR products, 1.5% agarose (Agarose, Sigma-Aldrich, Germany) gel was prepared in X Tris-boric acid-EDTA (TBE) buffer with the addition of nucleic acid dye (SafeView™ Classic, Applied Biological Materials Inc, Canada). Then the gel placed in the electrophoresis tank and was kept at 100 volts for 1 hour. Finally, the results were determined in a chemiluminescence imaging device (GEN-BOX imagER CFx, Erbiotek, Turkey) under UV light. Isolates containing relevant genes were used as the positive control (6).

Statistical analysis. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive values (NPV) of the immunochromatographic test were calculated using MEDCALC® by taking the PCR reference method (https://www.medcalc.org/calc/diagnostic_test.php). Also, results were given with 95% confidence intervals (CI).

Ethical approval. This study was approved by the Medical Research Ethics Committee of the Ege University Faculty of Medicine (Approval number: 20-3.1T/57).

RESULTS

K. pneumoniae (n=178) was determined to the majority of the isolates included in the study, while other origins examined included *Klebsiella oxytoca* (n=2), *E. coli* (n=5), *Enterobacter aerogenes* (n=1), and *Serratia marcescens* (n=1).

According to the results of antibiotic susceptibility-tests with Vitek 2 automated system, all the isolates were found to be ertapenem resistant, while meropenem and imipenem resistance were determined as 99%. Meropenem MIC values varied between 1- and >32 mg/L, while meropenem MIC_{50} and MIC_{90} values were determined to be>32 mg/L. Also, imipenem MIC_{50} and MIC_{90} values of 58 isolates were found to be above 32 mg/L. A total of 116 isolates were taken into the PCR study. With PCR, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{KPC}$, and *bla*$_{VIM}$ were detected in 79, 63, 20, and in 4 strains, respectively. *bla*$_{OXA-48}$ and *bla*$_{NDM}$ were found together in 51 of the isolates. In two carbapenem-resistant isolates, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{KPC}$, and *bla*$_{VIM}$ werenot detected. In addition to PCR, carbapenemase types were not detected in these two isolates, which were also investigated by immunochromatographic testing. Immunochromatographic assay and PCR results of all strains are included in Table 1. Also, sensitivity, specificity, accuracy, PPV, and NPV of the immunochromatographic assay for the detection of carbapenemases are summarized in Table 2.

DISCUSSION

Carbapenems are highly effective antimicrobials against Gram-negative bacteria and are preferred as the last line in the treatment of infections caused by these microorganisms (8). As a result of the inappropriate use of antibiotics, carbapenem-resistant strains have emerged. Today, the resistance rates of bacteria are increasing rapidly. Antibiotics used in the treatment of these infections cannot be developed at the same pace. Therefore, control of CRE infections will become even more difficult in the future (8). Rapid detection of carbapenemases contributes to both prevention of spread and lowering mortality rate by ensuring that the patient gets the appropriate antibiotic therapy as soon as possible. Therefore, fast, reliable, and inexpensive methods are needed to detect carbapenemases in laboratories.

In this study; the performance of the RESIST-4

Table 1. Immunochromatographic assay and PCR results of all strains

Carbapenemase Types	Immunochromatographic test results of all strains % (n)	Immunochromatographic test results of PCR studied strains % (n)	PCR results % (n)
OXA-48	80.2 (150)	68.1 (79)	68.1 (79)
NDM	48.1 (90)	45.7 (53)	54.3 (63)
NDM+OXA48	41.7 (78)	35.3 (41)	44 (51)
KPC	10.7 (20)	17.2 (20)	17.2 (20)
VIM	2.1 (4)	3.5 (4)	3.5 (4)
Negative	1.1 (2)	1.7 (2)	1.7 (2)
Total	(187)	(116)	(116)

Table 2. Evaluation of the performance of immunochromatographic assay

	OXA-48 % (95% CI)	NDM % (%95 CI)	KPC % (95% CI)	VIM % (95% CI)	OXA-8+NDM % (95% CI)
Sensitivity	100 (95.44% to 100.00%)	84.1 (72.74% to 92.12%)	100 (83.16% to 100.00%)	100 (39.76% to 100.00%)	80.4 (66.88% to 90.18%)
Specificity	100 (90.51% to 100.00%)	100 (93.28% to 100.00%)	100 (96.23% to 100.00%)	100 (96.76% to 100%)	100 (94.48% to 100.00%)
PPV	100	100	100	100	100
NPV	100	84.1 (75.01% to 90.35%)	100	100	86.7 (78.85% to 91.89%)
Accuracy	100 (96.87% to 100.00%)	91.38 (84.72% to 95.79%)	100 (96.87% to 100.00%)	100 (96.87% to 100%)	91.4 (84.72% to 95.79%)

OXA-48: Oxacillinase-48-like, KPC: *Klebsiella pneumoniae* carbapenemase, NDM: New Delhi metallo-β-lactamase, VIM: Verona integron-encoded metallo-β-lactamase, PPV: Positive predictive value, NPV: Negative predictive value

O.K.N.V assay in detecting OXA-48, NDM, VIM, and KPC carbapenemases were evaluated. The sensitivity and specificity of the test in detecting OXA-48, VIM, and KPC were 100% similar to other studies (9-12). Resist-4 O.K.N.V. test was also used in the study of Kolenda et al. and its sensitivity for NDM was 95.3%. Researchers reported false-negative results in two strains that produce NDM. The researchers concluded that due to low ertapenem and meropenem MIC values in these two isolates, NDM enzyme production may also below (13). Similarly, in our study, false-negative results were observed in the detection of NDM. However, differently, the meropenem MIC values of the strains used were quite high and were found above 32 mg/L. In 10 strains that were produce OXA-48 and NDM together, with the RESIST-4 O.K.N.V. test, NDM could not be detected in these strains. Therefore, the sensitivity of the test was 84.1% for NDM. Similarly, in the study of Baeza et al. NDM false negativity was detected in the strains producing OXA and NDM together, as in our study (3). Saleh and his colleagues reported false-negative results related to NDM in their study. The researchers concluded that several factors were effective on the sensitivity of the test in NDM detection. Among these factors, the removal of the bacterial colony studied near the carbapenem disk, the density of the inoculum used, and the amount of zinc in the MHA content was counted (14). In contrast, in our study, 5% sheep blood agar was used instead of MHA. In our study, VIM and OXA-48 were detected together in a single strain with the immunochromatographic test and confirmed by PCR. None of the OXA-48, NDM, KPC, VIM carbapenemases were detected with both PCR and immunochromatographic test in two strains that were found to be ertapenem and meropenem resistant by antibiotic susceptibility test.

According to RESIST-4 O.K.N.V test results, the OXA-48 enzyme was the most common carbapene-

mase type with a rate of 80.2%. The rate of detected NDM, KPC, and VIM carbapenemases were 48.1%, 10.7%, and 2.1%, respectively. OXA-48 and NDM were detected together in 41.7% of the isolates by immunochromatographic test. However, considering that the sensitivity of the immunochromatographic test in detecting NDM was lower (80.4%) compared to PCR, it was thought that the number of strains carrying the combined gene could be higher than that detected. Our country is an endemic region for OXA-48 carbapenemase. Accordingly, in our study, the most detected carbapenemase was identified as OXA-48. In a study conducted in our region, the most common carbapenemase in *K. pneumoniae* strains isolated from blood cultures was reported as OXA-48 with a rate of 87.7% and NDM with a rate of 23.1%. In the same study, KPC was identified in four strains (15). In another study conducted in our region in 2017, no KPC-producing strain was found (6). In this study, 20 strains were found to be producing KPC. These results point to a remarkable increase in KPC secreting strains in our region. All the strains producing KPC carbapenemase were identified as *K. pneumoniae* of which six isolated in 2018 and 14 in 2019. Sağıroğlu et al. reported 52.9% NDM and 39.2% OXA-48 in their study. They detected OXA-48 and NDM together in 5.9% of the strains. They also identified KPC in two strains and did not report the VIM gene in any strains (16). In the study of Demir et al. *Enterobacteriaceae* spp. analysis of isolates with multiplex PCR was carried out and $bla_{\text{OXA-48}}$ was reported in 86% of isolates, $bla_{\text{NDM-1}}$ in 10.5%, and bla_{VIM} in two isolates. bla_{IMP} and bla_{KPC} have not been detected in any origin (17). In the study of Çizmeci et al. the VIM-5 gene was detected in two of the strains, while OXA-48 was detected most frequently, and NDM-1 was detected second frequently (18).

CONCLUSION

With this research, it was seen that there were changes in carbapenemase types and ratios seen in our hospital compared to previous years. The strains producing VIM were identified for the first time in this study, and it was observed that there was an increase in the number of strains producing KPC in 2019. There was also an increase in strains carrying more than one carbapenemase gene together.

Fast and accurate detection of carbapenemases in laboratories is important for infection control and prevention. Resist-4 O.K.N.V test was determined to be more than 90% accurate in OXA-48, KPC, VIM, and NDM detection. The practical and easy prep phase of the test, its ability to yield results within 15 minutes, highlights the use of this test in carbapenemase detection. In the detection of NDM producing strains alone, it was observed that the sensitivity of the immunochromatographic test was better compared to the strains that produced NDM and OXA-48 together. However, it should be kept in mind that false-negative results may be obtained in NDM while studying the strains in which NDM accompanied other resistance genes with this test.

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