The evaluation of eazyplex® SuperBug CRE test usefulness for the detection of ESBLs and carbapenemases genes directly from urine samples and positive blood cultures

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Research

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Abstract

Background. An increasing antimicrobial resistance of Gram-negative rods is an important diagnostic, clinical and epidemiological problem of a modern medicine. Therefore, it is important to detect multi-drug resistant strains as quickly as possible. The aim of this study was to evaluate eazyplex® SuperBug CRE test for beta-lactamases genes detection among Gram-negative rods, directly from urine samples and positive blood cultures.

Methods. Eazyplex® SuperBug CRE test is based on isothermal amplification of a genetic material and allows for the detection of a selection genes encoding carbapenemases, i.e. KPC, NDM, VIM, OXA-48, OXA-181 and extended-spectrum beta-lactamases from the CTX-M-1 and CTX-M-9 groups. A total of 120 clinical specimens were included in the study.

Results. The test gave valid results for 58 (96.7%) urine samples and 57 (95.0%) positive blood cultures. ESBL and/or carbapenemase enzymes genes were detected in 56 urine and 55 blood samples, respectively.

Conclusions. Eazyplex® SuperBug CRE test can be used for a rapid detection of the most important genes encoded for resistance to beta-lactams in Gram-negative rods also without the necessity of bacterial culture.

Background

Bacteria of *Enterobacterales*, including multi-drug resistant isolates, are one of the most important causes of nosocomial infections. This can be explained in several ways: an easy acquisition of antibiotic resistance genes by Gram-negative rods, a capability of these strains to survive in hospital environment and common application of a wide spectrum antibiotic therapy. The problem of multi-drug resistance of Gram-negative rods is mostly associated with the horizontal gene transfer and possible synthesis of several beta-lactamases from the same or different groups of beta-lactamases (e.g. ESBLs, carbapenemases). It includes mainly enzymes with a wide range of action, cephalosporinases or carbapenemases, which incidence has risen in the last two decades (1–3). These enzymes have evolved significantly since their discovery and several variants of each enzyme can be now identified within one enzyme family.

Numerous phenotypic and genotypic methods used in microbiological laboratory allow for the detection of carbapenemase producing strains. Phenotypic methods always require initial culture of the strain, which extends the time needed to obtain a result. On the other hand these methods are simple to perform and relatively inexpensive, but usually do not allow for the identification of a specific enzyme type or variant (4). An introduction of genetic methods for routine microbiological diagnostics allows for a simultaneous detection of different antimicrobial resistance genes, also within one strain. It often enables the identification of a specific enzyme within bacteria group or family also directly from a clinical sample or a specimen pre-culture.
The evaluated eazyplex® SuperBug CRE test can be used for the detection of ESBL-type enzymes genes from CTX-M-1 and CTX-M-9 groups, as well as VIM-1–37, NDM-1–7, KPC and OXA-48-like (-48, -162, -204, -244) carbapenemases genes.

The study aimed to evaluate the usefulness of eazyplex® SuperBug CRE test for the detection of the most important enzymes genes associated with antibiotic resistance in Gram-negative rods. The results of the evaluated test were also compared with the results of conventional methods applied (phenotypic tests for beta-lactamases detection) and the results of the standard PCR.

**Methods**

The study included 120 clinical specimens: 60 urine samples and 60 positive blood cultures, in which Gram-negative strains producing beta-lactamases with a wide range of action: ESBLs and/or carbapenemases were identified. Each sample included into the study was collected from a specific patient. All the samples included into the study were obtained through routine diagnostic and clinical microbiology laboratory practice. Until the identification and the assessment of drug susceptibility of the strains, urine samples were stored at 4°C (up to 48 h), while positive blood cultures were kept in a culture medium of BACTEC™ FX instrument (Becton-Dickinson, Franklin Lakes, New Jersey, United States of America) at 37°C. The identification of the strains was performed by mass spectrometry using MALDI Biotyper system (Bruker, Karlsruhe, Germany). Antimicrobial susceptibility testing was determined on BD Phoenix™ M50 instrument (Becton-Dickinson, Franklin Lakes, New Jersey, United States of America) using NMIC-402 panels, performed according to the manufacturer’s instructions. The expression of ESBL-type enzymes was assessed simultaneously by the double disc synergy test (5), while carbapenemases activity by the Carba NP test (6). Phenotypic tests with the application of boronic acid and EDTA-supplemented discs, as specific carbapenemases inhibitors, recommended by EUCAST documents, were also applied. For all of the original samples stored, from which Gram-negative beta-lactamase-producing rods were cultured, eazyplex® SuperBug CRE test (Amplex Diagnostics, Gießen, Germany) was performed on Genie II device (OptiGene, Gießen, Germany) according to the manufacturer’s instructions.

Additionally, the chosen strains (*Klebsiella* spp. and *Serratia marcescens*) were cultured on LB Broth (Biocorp, Issoire, France) to confirm the selected genes presence. DNA was extracted from the strains recovered from them (applying Extractme DNA Bacteria Kit, Blirt, Gdańsk, pomeranian, Poland) and $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, $\text{bla}_{\text{SHV}}$, $\text{bla}_{\text{VIM}}$ and $\text{bla}_{\text{NDM-1}}$ genes were detected by PCR, according to the methodology of the previous studies (2, 7, 8).

**Results**

Of 60 urine samples tested: 40 *K. pneumoniae* strains, 13 strains of *Escherichia coli*, three of *K. oxytoca*, three *Enterobacter cloacae* and one *Klebsiella variicola* strain were recovered. All the strains were isolated in monoculture at a titer of ≥ 10⁴CFU/ml. The tests performed for 58 (96.7%) samples were valid. In 51 samples ESBLs genes were detected exclusively, and in additional five ESBLs and carbapenemases
genes were present simultaneously (Table 1). The detection time ranged from 4 min. 30 s to 12 min. 45 s for ESBL enzyme genes, and from 8 min. 15 s to 15 min. 15 s for carbapenemases genes. Thus, the overall sensitivity of the investigated method for urine samples reached 93.3%. Specificity of eazyplex® SuperBug CRE for ESBLs genes detection reached 93.3%, while for NDM and VIM genes – 100%.

Table 1
Beta-lactamases genes detected in eazyplex® SuperBug CRE test performed directly on urine samples (n = 60) and the enzymes activity with the application of phenotypic methods.

| Species recovered from the samples | Beta-lactamases genes detected | No. of isolates | Genes detection time (min:s) | Double disc synergy test result | Carba NP test result |
|-----------------------------------|-------------------------------|----------------|-----------------------------|--------------------------------|---------------------|
| **K. pneumoniae** (n = 40)        | CTX-M-1                       | 32             | 4:30 – 10:15                | (+)                            | (+)                 |
|                                   | CTX-M-1, CTX-M-9              | 2              | 5:30 – 8:30                 | (+)                            |                     |
|                                   | CTX-M-1, NDM                  | 3              | 5:15–15:15                 | (+)                            |                     |
|                                   | Negative result               | 2              | -                           | UI                             |                     |
|                                   | Invalid test                  | 1              | -                           | (+)                            |                     |
| **E. coli** (n = 13)              | CTX-M-1                       | 9              | 6:30 – 11:45                | (+)                            | (+)                 |
|                                   | CTX-M-9                       | 3              | 7:00–8:45                  | (+)                            |                     |
|                                   | CTX-M1, VIM                   | 1              | 5:30, 9:15                 | (+)                            |                     |
| **E. cloacae** (n = 3)            | CTX-M-1                       | 2              | 6:45 – 7:00                | (+)                            | (+)                 |
|                                   | CTX-M-1, VIM                  | 1              | 6, 8:15                    | (+)                            |                     |
| **K. oxytoca** (n = 3)            | CTX-M-1                       | 1              | 9:45                       | (+)                            |                     |
|                                   | CTX-M-9                       | 1              | 7:00                       | (+)                            |                     |
|                                   | Invalid test                  | 1              | -                         | (+)                            |                     |
| **K. variicola** (n = 1)          | CTX-M1                        | 1              | 7:30                       | (+)                            |                     |

UI – uninterpretable due to lack of any growth inhibition zone of the strain

For 58 strains derived from urine samples double disc synergy test results revealed enlargement of the growth inhibition zones between the discs, from the side of the beta-lactamase containing disc, which confirmed presence of ESBLs. For two *K. pneumoniae* strains the results were not interpretable due to lack of any inhibition zone around the discs applied for ESBLs detection. For five strains with positive results of Carba NP test and EDTA-supplemented disc method, positive results were obtained in eazyplex® SuperBug CRE. With the application of PCR, *bla*<sub>CTX-M</sub> were confirmed amongst 39 *K. pneumoniae* strains (including negative and non-validated results of eazyplex® SuperBug CRE), while *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> were detected amongst 34 and 26 isolates, respectively. For *K. variicola* strain the
consent results were obtained with the application of eazyplex® SuperBug CRE and PCR. The results for two *K. oxytoca* strains were concordant for the evaluated test and PCR, while for one strain the non-valid eazyplex® SuperBug CRE result accompanied with the presence of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes in a standard PCR.

Of 60 positive blood cultures tested: 46 *K. pneumoniae* strains, three strains of each: *K. oxytoca*, *E. cloacae* and *E. coli*, two strains of *S. marcescens* and *Citrobacter freundii*, and one *Proteus mirabilis* strain were recovered. Fifty eight samples (96.7%) gave the strains isolated in monoculture, while from two samples, *K. pneumoniae* and *Enterococcus faecalis* strains were grown simultaneously. For 59 strains derived from positive blood cultures, ESBLs presence was confirmed with the application of a double disc synergy test.

Eazyplex® SuperBug CRE test gave valid results for fifty seven (95.0%) samples of blood pre-cultures; in 50 samples only ESBL enzyme genes were detected, while in four - both ESBLs and carbapenemases, and in one - only carbapenemase genes (Table 2). The detection time for ESBL genes range was from 4 min. 30 s to 10 min. 15 s, while for carbapenemases genes from 4 min. 45 s to 11 min. 45 s. Thus, the overall sensitivity of the investigated method for positive blood samples reached 91.7%. Specificity of eazyplex® SuperBug CRE for ESBLs genes detection reached 93.3%, while for NDM and KPC genes – 100%.
Table 2
Beta-lactamases genes detected with eazyplex® SuperBug CRE while performed on positive blood cultures (n = 60) and the enzymes activity with the application of phenotypic methods.

| Species recovered from the samples | Beta-lactamases genes detected | No. of isolates | Genes detection time (min:s) | Double disc synergy test result | Carba NP test result |
|-----------------------------------|--------------------------------|-----------------|----------------------------|--------------------------------|---------------------|
| K. pneumoniae (n = 46)            | CTX-M-1                        | 39              | 4:30 – 10:15               | (+)                            | (+)                 |
|                                   | CTX-M-1, CTX-M-9, NDM         | 1               | 4:45 – 5:15               | (+)                            | (+)                 |
|                                   | CTX-M-1, NDM                  | 3               | 4:45 – 11:45              | (+)                            | (+)                 |
|                                   | KPC                            | 1               | 9:15                      | UI                             |                     |
|                                   | Negative result                |                 |                           |                                |                     |
|                                   | Invalid test                   |                 |                           |                                |                     |
| E. cloacae (n = 3)                | CTX-M-1                        | 3               | 6:15 – 7:30               | (+)                            |                     |
| E. coli (n = 3)                   | CTX-M-1                        | 1               | 7                         | (+)                            |                     |
|                                   | CTX-M-9                        | 1               | 8:15                      | (+)                            |                     |
|                                   | Invalid test                   | 1               | -                         | (+)                            |                     |
| K. oxytoca (n = 3)                | CTX-M-1                        | 3               | 6 – 7:15                  | (+)                            |                     |
| S. marcescens (n = 2)             | CXT-M-1                        | 1               | 6:30                      | (+)                            |                     |
|                                   | Negative result                | 1               | -                         | (+)                            |                     |
| C. freundii (n = 2)               | CTX-M-1                        | 2               | 6 – 7                     | (+)                            |                     |
| P. mirabilis (n = 1)              | Invalid test                   | 1               | -                         | (+)                            |                     |

UI – uninterpretable due to lack of any growth inhibition zone of the strain

The presence of \( bla_{\text{CTX-M}} \) genes in 44 \( K. pneumoniae \) strains, \( bla_{\text{TEM}} \) in 31 and \( bla_{\text{SHV}} \) in 25 was confirmed by PCR (including negative and non-validated results of eazyplex® SuperBug CRE test). One \( K. pneumoniae \) strain negative in the evaluated test revealed \( bla_{\text{TEM}} \) gene, while for the samples with invalid results in eazyplex® SuperBug CRE test - \( bla_{\text{CTX-M}} \) gene was confirmed with the application of a standard PCR. The results obtained by the evaluated test and PCR methods were consistent for three \( K. oxytoca \) strains. For one \( S. marcescens \) strain the results obtained with eazyplex® SuperBug CRE assay were consistent with the PCR method, while for the second \( S. marcescens \) strain (negative with eazyplex®
SuperBug CRE assay) \( \text{bla}_{\text{CTX-M}} \) and \( \text{bla}_{\text{TEM}} \) genes were detected. For \( E. \text{coli} \) and \( P. \text{mirabilis} \) strains that were both negative in eazyplex® SuperBug CRE test, ESBLs production was confirmed by the double disc synergy test.

Five strains, isolated from positive blood cultures, suspected of producing carbapenemases in the Carba NP test, were also positive with the application of eazyplex® SuperBug CRE test. In 4 strains of \( K. \text{pneumoniae} \), production of metallo-beta-lactamases was confirmed by means of EDTA-supplemented discs, and the production of NDM-1 carbapenemase by PCR. For one \( K. \text{pneumoniae} \) strain, the synthesis of KPC was confirmed by the test with boronic acid. In one \( K. \text{pneumoniae} \) strain, producing KPC-type carbapenemases, no growth inhibition zones were observed around the discs when the phenotypic methods for ESBLs detection were applied. Neither the presence of the genes encoding ESBL from the CTX-M1 and CTX-M9 group was confirmed in eazyplex® SuperBug CRE test, nor \( \text{bla}_{\text{CTX-M}} \), \( \text{bla}_{\text{TEM}} \) or \( \text{bla}_{\text{SHV}} \) genes were detected by PCR.

**Discussion**

In the last several years, the frequency of multi-drug-resistant bacterial strains isolation has increased. This is mainly due to irrational antibiotic therapy. Antimicrobial pressure on bacterial strains causes the emergence of new mechanisms of antibiotics resistance. Therefore, it is crucial to obtain a reliable result of antimicrobial resistance presence in the shortest possible time.

It is commonly known that the application of a test which simultaneously detects ESBL and carbapenemase enzymes, or their genes, significantly shortens the time of a standard microbiological diagnostic. In addition, results of phenotypic test performed for carbapenemase producing strains are often ambiguous or difficult for interpretation. For example, in the results of a double disc synergy test the enlargement of the growth inhibition zones, characteristic for ESBL-positive strains, sometimes does not have a typical shape or does not exist at all. Thus, it requires the application of other methods which sometimes significantly extends the time to give a final result.

Eazyplex® SuperBug CRE test is based on isothermal amplification of a genetic material and detects the genes for the following enzymes: KPC, NDM, VIM, OXA-48, CTX-M-1, CTX-M-9 and OXA-181. In the available literature, the first studies on eazyplex® SuperBug CRE test application appeared in 2015 (1, 9). The mentioned studies were performed on 94 and 450 carbapenemase positive Gram-negative rods strains, respectively. The available research results indicated a high sensitivity and specificity of eazyplex® SuperBug CRE tests in the detection of resistance mechanisms genes in Gram-negative rods (1, 2, 9). The study published by Hinić et al. (10), also in 2015, described the use of eazyplex test for the detection of genes of ESBL-positive Gram-negative rods directly in urine samples. The mentioned study evaluated 50 urine samples, in 30 of which the presence of ESBL was confirmed. Thus, the overall sensitivity of the method reached 100% and the specificity − 97.9%.
In the present study ESBL and/or carbapenemase enzymes were detected in more than 93% of urine samples. The mean detection time of ESBL enzyme genes from urine samples was 7 min. 45 s, while for carbapenemases genes – 9 min. 45 s. For two urine samples with negative results in eazyplex® SuperBug CRE test, the $bla_{TEM}$ gene presence was confirmed by PCR. Noteworthy, this gene is not detected by the evaluated test. In the second strain, three different genes encoding ESBL enzymes were detected by the applied confirmatory PCR. False-negative result of eazyplex® SuperBug CRE test for the second strain might have resulted from the low number of gene copies, below the test detection limit. However, the manufacturer assures that the evaluated test efficiently detects also a small number of gene copies.

Recently, Fiori et al. (11) evaluated the usefulness of eazyplex® SuperBug CRE test for ESBL enzymes and/or carbapenemases genes detection directly in positive blood cultures. The mentioned authors detected the presence of CTX-M and/or KPC and/or VIM-like enzymes genes in 151 of the pre-cultured blood samples among 321 episodes of bloodstream infections. The results obtained by this method allowed for the reduction of time to effective antibiotic therapy administration in patients with *E. coli* or *K. pneumoniae* bacteraemia. The cited authors also highlight the proposed algorithm for combination of a mass spectrometry identification, directly from a blood sample, and the detection of a resistance mechanism with eazyplex® SuperBug CRE test which significantly shortens diagnostics procedure and time to get the final results.

In our study, the mean detection time of ESBL enzyme genes from the positive blood cultures was 6 min. and carbapenemases genes – 7 min. 30 s, when compared to an overnight incubation of the phenotypic test for particular antimicrobial resistance mechanism detection. Also, Rödel et al. (12) research indicates a possible reduction of time and rationalization of antibiotic therapy in case of patients with sepsis using eazyplex test. However, the mentioned authors used eazyplex® MRSA test to detect *mecA* and *mecC* genes among *Staphylococcus aureus* and *Staphylococcus epidermidis* strains.

Slightly over 4% of the tests performed in the present work were not valid. This can be explained by several reasons: sample overload, specimen composition, consistency, the presence of some inhibitory substances in urine or blood samples (patients from whom the samples were collected from often take a number of medicines), the presence of more than one strain in a single sample, the presence of several genes encoding for several resistance mechanisms simultaneously or particular genes mutations. It may lead to the necessity of repeating the whole procedure which additionally increases the cost and time of the investigation or to the underestimation and oversight of resistance mechanism presence, if not consequently repeated.

Noteworthy, currently the number of recognized beta-lactamases is estimated at around 200 or more. In the double disc synergy tests, it is not possible to identify a specific ESBL family, or whether the strain produces one or more ESBL-like enzymes. Nor is it possible to determine with this method whether the enzymes belong to the same or different ESBL families. In standard PCR method, it is possible to detect many different ESBL enzymes genes, but this requires more complex approach, usually longer time and
often requires few PCR reactions (or multiplex version) to detect specific genes, which also affects the costs. Moreover, whether a strain produces more than one type of ESBLs is of epidemiological significance only and does not clinically affect antibiotic therapy. Hence, the available commercial tests (including eazyplex® SuperBug CRE) usually detect the most common antibiotic resistance mechanisms among isolated strains - the selected genes encoding for ESBL and/or carbapenemases.

In the present study a great advantage of eazyplex® SuperBug CRE test ability to obtain a result directly from a clinical sample was confirmed. A short duration of the test and a small sample volume needed to perform are also important. However, the test has some limitations. It detects only ESBL enzymes from the CTX-M-1 and M-9 groups. On the other hand, these are the most common ESBL enzymes in *K. pneumoniae*, and the strains with this resistance mechanism are isolated with the highest frequency not only in Poland, but also worldwide (13, 14). Moreover, the evaluated test does not detect all beta-lactamases genes, like ampC-like enzymes but the dissemination of ampC-positive *Klebsiella* spp. isolates, at least in our department, does not exceed 1% (data not shown) and thus it was not the issue of the present work.

Noteworthy, the test detects only some chosen enzymes genes also among carbapenemases. Interestingly, the strains of Gram-negative rods, derived from the patients in our hospital, that express beta-lactams resistance mainly synthesize class B carbapenemases, mostly NDM- or VIM-type. Detection of the genes for both mentioned carbapenemases is available in the test evaluated. Of note, not all types of carbapenemases have been detected among the strains of Gram-negative rods so far in our hospital. Since the first detection of carbapenemase producing strain in our unit, only one KPC-positive and one OXA-48 positive strain have been identified. It confirms that the strains expressing this particular resistance mechanism are very rare in our department.

Eazyplex® SuperBug CRE test is a relatively expensive tool for its use in a routine diagnostic directly for clinical specimens. This approach would significantly increase the cost of a standard microbiological investigation. However, its application might be reasonably taken into account in the diagnostic of some particular cases of infections, like: earlier colonization of the patients with ESBL- or carbapenemase-producing strains, confirmed contact with the infected person or presence of the epidemic outbreak locally. It seems that in such situation the benefits of using an expensive test are favourable for obtaining the result in a short time. Moreover, the possibility of introducing a pre-emptive treatment, also taking into account the phenotype of the strain, seems to be a very reasonable approach.

**Conclusions**

Eazyplex® SuperBug CRE test can be a useful tool for a rapid and reliable identification of resistance mechanisms genes in Gram-negative rods directly from urine and pre-cultured blood samples.

**Declarations**
Acknowledgement
Not applicable.

Ethical statement
The samples were obtained through standard clinical and diagnostic practice. This study received ethical approval from the Bioethical Commission of Ludwik Rydygier Collegium Medicum in Bydgoszcz Nicolaus Copernicus in Torun, agreement no. 367/2019.

Consent for publication
Not applicable.

Availability of data and materials
Supplementary data is available at https://repod.icm.edu.pl/dataverse/umk-medical-sciences as https://repod.icm.edu.pl/dataset.xhtml?persistentId=doi:10.18150/W6IXRP

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
Alicja Sękowski: conceptualization, methodology, data analysis, formal analysis, resources, writing and editing of the original draft.

Tomasz Bogiel: writing and editing of the original draft

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