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Comparison of detection methods for extended-spectrum beta-lactamases in *Escherichia coli* strains*

Porównanie metod wykrywania beta-laktamaz typu ESBL u pałeczek *Escherichia coli*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction

Detection of extended-spectrum beta-lactamases (ESBLs) could be a major challenge for microbiologists – the difficulties arise mainly from the phenotypic differences among strains.

Materials and Methods

Evaluation of ESBLs was performed on 42 strains of E. coli by: 1) DDST on MHA, 2) DDST on MHA with cloxacillin, 3) CT on MHA, according to CLSI, 4) CT on MHA with cloxacillin, 5) Etest ESBL (AB Biodisk), 6) CHROMagar™ ESBL (GRASO), 7) ChromID® ESBL (bioMérieux), and 8) automatic system VITEK2 ESBL test (bioMérieux).

Result

Positive results were obtained for 20 strains using method 1, for 18 strains using method 2, 17 by method 3, 14 by method 4, 11 by method 5, 39 by method 6, 40 by method 7, and 15 by method 8. Using Etest ESBL 6.0 non-determinable results were obtained. The most consistent results were obtained when comparing the results of method 3 with results of method 2 (97.6%), and comparing the results obtained using methods 3 and 8 (95.2%).

Conclusions

Based on our study we conclude that the chromogenic media can only be used as a screening method for the detection of ESBLs in *E. coli* rods. Etest is less useful compared to other phenotype methods, due to the impossibility of obtaining results for all the tested strains. Adding cloxacillin to MHA does not increase the frequency of detection of ESBLs in *E. coli* strains. DDST seems to be the most reliable among phenotypic methods for the detection of ESBLs in *E. coli* rods.

Key words: ***Escherichia coli*** • **ESBL** • **beta-lactamases** • **double-disc synergy test**

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Abbreviations used: ChromID® ESBL **–** Agar with chromogenic substrate bioMérieux, **CHROMagar™ ESBL –** Agar with chromogenic substrate GRASO, **CLSI** – Clinical and Laboratory Standards Institute, **CT –** Confirmatory test, **DDST –** Double-disc synergy test, **ESBL –** Extended-spectrum beta-lactamase, **EUCAST –** European Committee on Antimicrobial Susceptibility Testing, **KL –** Cloxacillin, **MCA –** MacConkey agar, **MHA –** Müeller Hinton agar.

Introduction

Escherichia coli is a Gram-negative rod, being the dominant component of the physiological micro-flora of the digestive system of humans and animals. In addition to the commensal *E. coli* strains, within a species are pathogenic strains responsible for diarrhea, wound infections, urinary tract infection, inflammation of meningitis and septicemia.

The most important from the clinical and epidemiological point of view is the resistance of *E. coli* to beta-lactam antibiotics, most often associated with the production of beta-lactamases, including beta-lactamase with an extended spectrum of substrate type ESBL (extended-spectrum beta--lactamases). The term ESBL is defined as mostly plasmid--encoded beta-lactamase, with a mass of about 30 kDa [1], in Class A (group 2be according to the Bush-Jacoby-Medeiros classification) able to hydrolyse penicillins, cephalosporins (except cephamycins) and aztreonam, which are inhibited *in vitro* by the beta-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) [1,2,5,8,10,14,16].

Strains that produce ESBLs are widely distributed, especially in a hospital environment, where they may even cause an endemic. They are responsible for therapeutic failure, especially with the use of beta-lactam antibiotics, and increased morbidity and mortality of patients. The detection of strains producing ESBLs is a challenge for microbiologists, because of the difficulties mainly arising from the phenotypic differences of these strains.

The aim of this study was to compare result of eight phenotypic methods applied for detection of ESBL produced by *E. coli* rods.

Material and Methods

Bacterial strains. The study included 42 strains of *E. coli* isolated from clinical material in the Clinical Microbiology Department of Dr. A. Jurasz University Hospital in Bydgoszcz, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, in the period from January to November 2011.

Identification of strains. Identification of *E. coli* strains was based on colony morphology on the MacConkey Agar (MCA, Diag-Med), the ability to ferment lactose, sodium deoxycholate precipitation and the ability to produce tryptophanase. In order to identify lactose-negative strains of *E. coli* cards were used to identify the Gram-negative bacteria (GN), which were read in an automatic system, VITEK 2 Compact (bioMerieux).

Evaluation of ESBL production. As a control in all performed methods, a strain of *Klebsiella pneumoniae*, ATCC 700603, was applied (beta-lactamase-producing) and *E. coli* ATCC 25922 (not producing these enzymes). In order to evaluate the production of ESBLs, we used:

- The double-disc method (DDST) on Müeller Hinton agar (MHA) according to Jarlier et al. [6];
- The double-disc method on cloxacillin MH agar; tests were performed on MHA with cloxacillin (MHA + KL) at a concentration of 200 µg/ml; in the situation of the absence of growth of *E. coli* on MHA with cloxacillin at a concentration of 200 µg/ml, cloxacillin at a concentration of 100 µg/ml was use;
- Confirmatory test (CT) on MHA, according to CLSI;
- Confirmatory test on MHA with cloxacillin at a concentration of 100 µg/ml (CT+KL);
- Etest ESBL (AB Biodisk); in tests, strips impregnated with antibiotics in a concentration gradient were used – cefotaxime (0.25-16 mg/ml) and cefotaxime (0.016-1.0 µg/ml) with clavulanic acid (4 mg/ml; the test was interpreted according to the producer's recommendations,
- Agar with chromogenic substrate (GRASO);
- Agar with chromogenic substrate (bioMérieux);
- Automatic system VITEK2 ESBL test (bioMérieux).

Results and discussion

The results of evaluation of ESBL production in *E. coli* strains, obtained by means of the 8 methods, are summarized in Table 1. The percentages of consistent and inconsistent results obtained for all pairs of methods (each of each) were compared (Table 2).

Using the CHROMagar™ ESBL and ChromID® ESBL for detection of ESBL-positive strains of E. coli the highest number of positive results was obtained, compared to other methods used, respectively 39 and 40 results. The highest percentage of consistent results (respectively 54.8% and 52.5%) was obtained compared with the DDST; for the other methods, this percentage was lower than 50.1%. Comparing the results obtained by these methods gained 88.1% compatibility. These results were different from the results obtained by other methods, which suggests their low reliability. Overdevest et al. [9] evaluated production of ESBL using the chromogenic substrate (bioMérieux) in two groups of *Enterobacteriaceae*. For the first group (n = 291), the authors obtained a sensitivity of 97.3% and specificity of 93.9%, while for the second

Table 1. Number and percentage of results obtained using eight methods for the detection of ESBLs in *E. coli* rods (n = 42)

Method	Number and percentage of positive results *	Number and percentage of negative results **	Number and percentage of non-determinable results ***
CHROMagar ESBL (GRASO)	39 (92.9%)	3 (7.1%)	0 (0.0%)
ChromID® ESBL (bioMérieux)	40 (95.2%)	2 (4.8%)	0 (0.0%)
DDST	20 (47.6%)	22 (52.4%)	0 (0.0%)
CT	17 (40.5%)	25 (59.5%)	0 (0.0%)
Etest ESBL	11 (26.2%)	25 (59.5%)	6 (14.3%)
VITEK 2 ESBL test	15 (35.7%)	27 (64.3%)	0 (0.0%)
DDST + KL	18 (42.9%)	24 (57.1%)	0 (0.0%)
CT + KL	14 (33.3%)	28 (66.7%)	0 (0.0%)

* Positive result – strain producer's ESBLs

** Negative – strain does not produce ESBLs

*** Non-determinable result – according to producer recommendations

group (n = 65) they obtained sensitivity of 98.5% and specificity of 44.3%. For the second group false-positive results for 39 isolates were obtained, whereas for one strain the result was false negative. The results of these studies and the results of our own suggest that chromogenic media for the detection of ESBL-positive *E. coli* strains are characterized by a relatively high sensitivity, but lower specificity, which creates the risk of false-positive results. This is due to the fact that these methods detected not only ESBL enzymes, but also broad-spectrum beta-lactamases such as a SHV-1, for example. Rawat and Nair [12] evaluated ESBL production in Gram-negative bacteria and found that *E. coli* and *K. pneumoniae* rods, in which genes encoding ESBLs were not detected, but SHV-1 production was observed at a high level, gave false-positive results by phenotypic methods.

By studying the production of ESBLs using the DDST, the highest percentage (95.2%) of consistent results was obtained in comparison with the DDST on cloxacillin agar. Valenza et al. [15] and Garrec et al. [4], studying strains of *E. coli* by DDST and using PCR as the reference method, obtained the sensitivity and specificity of the test equal to 100.0%. Results of these studies and our studies indicate that the DDST method is characterized by high sensitivity and specificity, particularly with regard to *E. coli* rods, which confirms the reliability obtained by the means of the results.

Using the confirmatory test according to CLSI for detection of ESBLs, the highest percentage of compatible results (97.6%) was obtained in comparison with the DDST + KL and a slightly lower value (95.2%) compared with the VITEK 2 ESBL test. The lowest percentage of consistent results was obtained in comparison with the results obtained using chromogenic substrates. Garrec et al. [4] and Wiegand et al. [17] evaluated ESBL production by a confirmatory test in a group, respectively, of 61 and 107 strains of *E. coli*, and obtained the sensitivity of greater than 95.0%. They evaluated the usefulness of this test for the diagnosis as satisfactory. Kader et al. [7], studying 776 strains (74.0% *E. coli* and 25.6% *K. pneumoniae*) isolated from urinary tract infections, had the same number of positive results using the DDST and CT. In turn, Rokosz et al. [13] comparing results of CT (cefpodoxime and cefpodoxime with clavulanic acid) with the DDST method obtained 72.5% consistent results. The authors stated that this test is more objective and easier to interpret than DDST, and suggested it can be used as a confirmatory test in difficult diagnostic cases.

In our study, 92.9% consistent results were obtained for these methods. Based on cited results and results described in this work, it appears that CT according to CLSI has similar to DDST diagnostic value for detection of ESBLs in *E. coli* rods. It is worth mentioning that the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends lower antibiotic disc concentration for cefotaxime (5 µg/disc) and ceftazidime (10 µg/disc) in comparison to CLSI [3]. Polsfuss et al. [11] comparing the sensitivity of CT (with above-mentioned antibiotics) according to EUCAST and CLSI in 236 *Enterobacteriaceae* strains revealed that both EUCAST and CLSI had comparable sensitivity. However, the authors showed that CT with cefepime according to EUCAST proved to be the most sensitive for detection and confirmation of ESBL-positive strains regardless of the simultaneous AmpC production. Therefore the authors suggest that changing from CLSI to EUCAST and using cefepime in the CT method could enhance ESBL detection.

Using the Etest ESBL strips (AB Biodisk) resulted in the same percentage of consistent results (78.6%), and also the highest in comparison with the following methods: DDST, DDST + KL, CT + KL and VITEK 2 ESBL tests. The lowest percentage of consistent results, in comparison with a chromogenic substrate, were obtained. This method is rated as easy to perform and interpret, although according to the producer in some cases (as listed in the manual), the result may not be possible to determine.

Table 2. Comparison of consistent and inconsistent results obtained for each pair of methods applied for ESBL detection in *E. coli* strains (n = 42)

Compared methods	Number and percentage of consistent results	Number and percentage of inconsistent results
CHROMagar ESBL vs DDST	23 (54.8%)	19 (45.2%)
CHROMagar ESBL vs Etest ESBL	14 (33.3%)	28 (66.7%)
CHROMagar ESBL vs CT	20 (47.6%)	22 (52.4%)
CHROMagar ESBL vs VITEK 2 ESBL test	13 (31.0%)	29 (69.0%)
CHROMagar ESBL vs DDST+KL	21 (50.0%)	21 (50.0%)
CHROMagar ESBL vs CT + KL	17 (40.5%)	25 (59.5%)
DDST vs CT	39 (92.9%)	3 (7.1%)
DDST vs Etest ESBL	33 (78.6%)	9 (21.4%)
DDST vs VITEK 2 ESBL test	37 (88.1%)	5 (11.9%)
DDST vs DDST + KL	40 (95.2%)	2 (4.8%)
DDST vs CT+ KL	36 (85.7%)	6 (14.3%)
CT vs Etest ESBL	32 (76.2%)	10 (23.8%)
CT vs VITEK 2 ESBL test	40 (95.2%)	2 (4.8%)
CT vs DDST + KL	41 (97.6%)	1 (2.4%)
CT vs CT + KL	39 (92.9%)	3 (7.1%)
Etest ESBL vs VITEK 2 ESBL test	33 (78.6%)	9 (21.4%)
Etest ESBL vs DDST + KL	33(78.6%)	9 (21.4%)
Etest ESBL vs CT + KL	33 (78.6%)	9 (21.4%)
VITEK 2 ESBL test vs DDST + KL	39 (92.9%)	3 (7.1%)
VITEK 2 ESBL test vs CT + KL	41 (97.6%)	1 (2.4%)
DDST + KL vs CT+ KL	38 (90.5%)	4 (9.5%)
CHROMagar ESBL vs ChromID® ESBL	37 (88.1%)	5 (11.9%)
ChromID® ESBL vs DDST	22 (52.5%)	20 (47.5%)
ChromID® ESBL vs Etest ESBL	19 (45.4%)	23 (54.6%)
ChromID® ESBL vs CT	19 (45.4%)	23 (54.6%)
ChromID® ESBL vs VITEK 2 ESBL test	17 (40.6%)	25 (59.4%)
ChromID® ESBL vs DDST + KL	40 (47.7%)	22 (52.3%)
ChromID® ESBL vs CT + KL	16 (38.2%)	26 (61.8%)

The producer in such cases recommends the use of molecular biology methods for further diagnosis. This fact complicates the course of diagnosis and necessitates the use of other methods for the detection of ESBLs, which delays obtaining a result, implementation of intervention strategies, and treatment of the patient, and increases the cost of diagnosis. Overdevest et al. [9] examined, using Etest ESBL, 291 strains (group 1), where the dominated *E. coli* obtained equal sensitivity of 99.6% and a specificity of 91.1%. In the case of 117 strains non-determinable results were recorded. Garrec et al. [4] examining 107 strains using Etest ESBL obtained a sensitivity of 90.0% and a specificity of 89.0%. The results obtained by other authors, and our own, indicate that the Etest ESBL has a lower sensitivity and specificity, and thus lower reliability of the results obtained, in comparison to results

obtained by DDST and CT. Failure to achieve results for all tested strains further reduces the value of this method as a tool for the detection of ESBLs in *Enterobacteriaceae*.

Using the automated system VITEK 2 Compact for the detection of ESBLs, the highest percentage of consistent results (97.6%) was obtained as compared with CT + KL, and a slightly lower value (95.2%) in combination with CT. The lowest percentage of consistent results was obtained in comparison with chromogenic substrates. Wiegand et al. [17], studying 61 strains of *E. coli* using the system VITEK 2 ESBL test, obtained a sensitivity of 81.4% and a specificity of 100.0%. Garrec et al. [4] obtained values for the sensitivity and specificity of 73.0% and 59.0%, which translates into a high rate of false positive results. The authors evaluated the usefulness of this system for the diagnosis taking into account all species as low. For this reason they recommend using manual methods as a confirmatory test for the strains reported by the system as ESBL-positive. Results obtained by many authors [4, 9, 15, 17] suggest a lower reliability of results obtained by this method, as compared to the DDST or CT, and thus lower usefulness in the diagnosis of rods within *Enterobacteriaceae* rods.

The evaluation of the production of ESBLs was also carried out in the context of the presence of AmpC cephalosporinases. For this purpose **DDST + KL** and CT + KL were performed. The highest percentage of consistent results (97.6%) was obtained compared **DDST + KL** with CT. Compared with DDST, 95.2% consistent results were obtained. Two strains recorded as ESBL-positive using the DDST method rated as ESBL-negative by DDST + KL. There were no ESBL-negative strains on agar without KL, and ESBL-positive on agar with KL. Garrec et al. [4], evaluating the DDST method on a medium with KL, at a concentration of 250 mg/ml, achieved a sensitivity equal to 100.0% and a specificity of 98.0%. For analyzed strains, among which the dominant strains were *Acinetobacter baumannii*, defined as cephalosporinase-positive by PCR, the authors obtained an increase in sensitivity of DDST with the addition of a cloxacillin, in comparison to DDST without an antibiotic. This suggests that the use of cloxacillin seems to be reasonable in the diagnosis of ESBL-positive strains among non-fermenting rods, especially *Acinetobacter* sp., as well as rods of the *Enterobacteriaceae* family, especially *K. pneumoniae*, *Enterobacter* sp., *Citrobacter* sp., and *Morganella* sp. In *E. coli*, these enzymes are rarely detected, although according to the latest data, the incidence of the presence of AmpC in these rods is increasing.

Using CT + KL at a concentration of 100 μg/ml 14 (33.3%) positive results and 28 (66.7%) negative results were obtained. The highest percentage of consistent results (97.9%) was compared with the method of VITEK 2 Compact ESBL test. Compared to CT, 92.9% consistent results were observed and in combination with the DDST + KL, 90.5%. For the three strains, positive results were obtained on a medium without KL and negative on a medium with KL. No negative results were obtained in the test without KL, and positive (for the same strain) on a medium with KL, which confirms the results obtained by the DDST + KL and suggests a lack of ability to produce AmpC by tested strains. Garrec et al. [4], evaluating CT + KL at a concentration of 250 mg/ml, obtained a test sensitivity of 100.0% and a specificity of 91.0%.

Our research shows the divergence of results obtained using the 8 methods to detect ESBLs. 100.0% compatibility for any pair of compared methods was not obtained, confirming the existence of difficulties in the diagnosis of strains suspected of ESBL production. Rawat and Nair [12], studying ESBLs produced by Gram-negative bacteria, suggest that the reason for the occurrence of false results obtained by using the most common diagnostic methods for diagnosis of ESBLs are: production of SHV-1 at a high level (false positives), production of cephalosporinases AmpC (false negative), induction of some ESBLs (false negative results), the use of a lower or a higher inoculum of test strain (false negative or positive), variable substrate affinity (false negative or positive) and the presence in one sample of ESBL-positive and ESBL-negative strains (false negative or false positive).

The detection of strains producing ESBLs can be a challenge for microbiologists. Analyzing the results obtained in this study, one should take into account the existence of several factors limiting the ability to draw a firm conclusion. Future research should be conducted on a larger number of strains and the use of molecular methods is needed – it makes it possible to determine the sensitivity and specificity in compared methods, and thus determine credibility of the obtained results and their usefulness in the diagnosis of strains producing ESBLs. It should be taken into account that even if the above-mentioned parameters were determined as the final selection of the best of the used methods, it would still be difficult because, as is apparent from the literature for these same methods, the obtained sensitivity or specificity differs significantly. It may be affected by a variety of steps, starting from the preparation of suspensions, to the interpretation of the obtained results, in particular in relation to manual methods.

Conclusions

Agars with chromogenic substrates could be applicable as screening methods for the detection of ESBLs produced by *E. coli* strains, because these methods also detect beta-lactamases different from ESBLs – broad-spectrum beta-lactamases.

The inability to obtain results for all tested strains by Etest ESBL limits the usefulness of this method as a tool for the detection of ESBLs produced by *E. coli* strains, and the need for a further test for the detection of these enzymes generates additional costs.

Cloxacillin added to the MHA does not increase the frequency of detection of ESBLs in *E. coli* strains.

The DDST method (among evaluable tests) seems to be the most reliable and useful phenotypic method for detection of ESBLs produced by *E. coli* strains.

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