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**Direct detection of extended-spectrum- $\beta$ -lactamase-producers in Enterobacterales from blood cultures: a comparative analysis**

**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1826325> since 2023-02-04T12:36:59Z

*Published version:*

DOI:10.1007/s10096-021-04385-1

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# European Journal of Clinical Microbiology & Infectious Diseases

## Direct detection of extended-spectrum- $\beta$ -lactamase-producers in Enterobacterales from blood cultures, a comparative analysis

--Manuscript Draft--

<b>Manuscript Number:</b>	EJCM-D-21-01624R1
<b>Full Title:</b>	Direct detection of extended-spectrum- $\beta$ -lactamase-producers in Enterobacterales from blood cultures, a comparative analysis
<b>Article Type:</b>	Original Article
<b>Keywords:</b>	ESBL; CTX-M; bloodstream infection; sepsis; Gram-negatives; rapid diagnostic test
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<b>Abstract:</b>	<p><b>Introduction:</b> Accurate detection of extended-spectrum-<math>\beta</math>-lactamase (ESBL)-producing Enterobacterales from bloodstream infection (BSI) is of paramount importance for both epidemiological and clinical purposes, especially for optimization of antibiotic stewardship interventions.</p> <p><b>Methods:</b> Three phenotypic methods for detection of ESBL phenotype in <i>Klebsiella pneumoniae</i> and <i>Escherichia coli</i> BSI were compared over a four-month period (May-August 2021) in a main University Hospital from Northern Italy. The methods were the biochemical Rapid ESBL NP®, the immunological NG-Test CTX-M MULTI®, and the E-test technique based on ESBL E-test®.</p> <p><b>Results:</b> One hundred forty-two blood cultures (BCs) positive for <i>K. pneumoniae</i> or <i>E. coli</i> were included. ESBL and carbapenemase phenotype were detected in 26.1% (n=37) and 16.9% (n=24), respectively. Rapid ESBL NP®, NG-Test CTX-M MULTI®, and direct ESBL E-test® positive and negative predictive values with 95% confidence intervals were 1 [0.87-1] and 0.97 [0.92-0.99], 1 [0.87-1] and 0.97 [0.92-0.99], 1 [0.88-1] and 1 [0.96-1], respectively.</p> <p><b>Conclusion:</b> The three phenotypic methods evaluated showed good performance in detection of ESBL phenotype from <i>K. pneumoniae</i> or <i>E. coli</i> positive BCs. Rapid ESBL NP® and NG-test CTX-M® offer the important advantage of a turnaround time of 15 to 45 min and the Rapid ESBL NP test in addition detects any type of ESBL producers.</p>

**Response to Reviewers:**

Turin, 8th November 2021

Editor-in-Chief

European Journal of Clinical Microbiology and Infectious Diseases

We would like to thank the reviewers and the Editorial Team for their helpful suggestions, which in our view have greatly enhanced the quality and strength of our study. We hope that in this revised version the manuscript is now suitable for publication in European Journal of Clinical Microbiology and Infectious Diseases. Please, note that the changes to the original manuscript have been highlighted in the marked version. The response to the Reviewer's comments and ensuing modifications in the manuscript are also clearly indicated in the rebuttal.

Comments from Reviewer and point-by-point answers

Reviewer #1:

1) Author must explain, why this method is advantageous other than TAT, how much sensitive when compared to pure culture based ESBL, MBL Test with Direct detection method?

We thank the referee for this comment. Sensitivity, specificity, positive predictive value, and negative predictive value of the three phenotypic methods for direct detection of ESBL phenotype from positive blood cultures were calculated in comparison to conventional phenotypic testing results. Moreover, direct ESBL detection from positive blood cultures, and rapid phenotypic tests in wider terms, may predict resistance phenotype and should help to shorten time to optimal antibiotic therapy. Obviously, culture-based testing results remain essential and cannot be replaced by rapid phenotypic results. They should continue to be performed to obtain antimicrobial susceptibility and optimize antibiotic management.

2) If more than 2 organisms in the Blood culture, how this could be relevant in interpreting the drug resistance pattern?

We thank the referee for this comment. This study was aimed at evaluating the performance of three methods for direct detection of ESBL producers from *K. pneumoniae* and *E. coli* positive blood cultures. These phenotypic tests were evaluated in case of reliable identification of *K. pneumoniae* or *E. coli* from bacterial pellet of Gram-negative bacilli positive blood cultures (MALDI-TOF score >1.80, please see Boattini et al EJCMI 2021 <https://doi.org/10.1007/s10096-021-04192-8>) and showed to rapidly screen the presence of ESBL phenotype. Obviously, culture-based approach remains essential to confirm rapid phenotypic results, identify polymicrobial blood cultures and should continue to be performed to obtain antimicrobial susceptibility results and optimize antibiotic management.

3) It focus only on ESBL, What about Gram positive drug susceptibility? Explain in better way with multiple reference in the discussion part about this direct method and culture based methods sensitivity and specificity

We thank the referee for this comment. This study was aimed at evaluating the performance of three methods for direct detection of ESBL producers from *K. pneumoniae* and *E. coli* positive blood cultures. Performance evaluation of the three rapid phenotypic tests was calculated in comparison with culture-based testing results. Since we investigated the presence of ESBL phenotype in *K. pneumoniae* and *E. coli* positive blood cultures, evaluation of Gram-positive susceptibility was not the aim of the study.

Reviewer #2:

1) Antimicrobial resistance is a growing problem. Rapid and accurate diagnosis of specific resistance phenotypes such as extended spectrum beta-lactamases (ESBLs) is of interest. The authors compared the performance of three rapid methods to detect ESBLs directly in positive blood cultures. All of them were successful, however, E-test required a longer time to be reported. Except Etest, the performance of the other rapid tests were subject to recent publications. Although the high sensitivity of Rapid ESBL NP were reported in both blood cultures and screening swabs, it was interesting to see a comparison of the other tests in one center.

We thank the referee for these accurate appraisals.

2) The major limitation of the study was similar to other studies about this subject. The clinical impact of detection of ESBLs or a comparison of rapid antibacterial susceptibility testing recommendations of EUCAST were not investigated. We need to see how these tests change the routine antibacterial treatment practice in real life apart from laboratory investigations. As the authors mentioned in the paper there are increasing number of successful rapid antimicrobial susceptibility tools with little

information about impact of daily practise.

We thank the referee for these accurate appraisals. However, we hope the referee agrees about the need to provide to clinicians rapid microbiologic results. Clinical impact of ESBL detection was not the aim of the study but a further study on the topic is ongoing.

3) An other issue is the cost of the tests. Although my personal belief is none of the tests are expensive than unnecessary broad spectrum antibiotics, a brief discussion on the cost (not only the test also for the technician, IT, ..) can be helpful for the laboratories which are intrested in these tests.

We thank the referee for this comment. Accordingly, the following sentence was added to discussion section "All the tests evaluated are low cost and require no specialized equipment or personnel, further studies on cost-benefit analysis are needed however."

Reviewer #3:

1) The authors compared commercial techniques to detect ESBLs production. The results are clear and well described.

We thank the referee for these accurate appraisals.

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Direct detection of extended-spectrum- $\beta$ -lactamase-producers in Enterobacterales from blood cultures, a comparative analysis

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## **Abstract**

**Introduction:** Accurate detection of extended-spectrum- $\beta$ -lactamase (ESBL)-producing Enterobacterales from bloodstream infection (BSI) is of paramount importance for both epidemiological and clinical purposes, especially for optimization of antibiotic stewardship interventions.

**Methods:** Three phenotypic methods for detection of ESBL phenotype in *Klebsiella pneumoniae* and *Escherichia coli* BSI were compared over a four-month period (May-August 2021) in a main University Hospital from Northern Italy. The methods were the biochemical Rapid ESBL NP®, the immunological NG-Test CTX-M MULTI®, and the E-test technique based on ESBL E-test®.

**Results:** One hundred forty-two blood cultures (BCs) positive for *K. pneumoniae* or *E. coli* were included. ESBL and carbapenemase phenotype were detected in 26.1% (n=37) and 16.9% (n=24), respectively. Rapid ESBL NP®, NG-Test CTX-M MULTI®, and direct ESBL E-test® positive and negative predictive values with 95% confidence intervals were 1 [0.87-1] and 0.97 [0.92-0.99], 1 [0.87-1] and 0.97 [0.92-0.99], 1 [0.88-1] and 1 [0.96-1], respectively.

**Conclusion:** The three phenotypic methods evaluated showed good performance in detection of ESBL phenotype from *K. pneumoniae* or *E. coli* positive BCs. Rapid ESBL NP® and NG-test CTX-M® offer the important advantage of a turnaround time of 15 to 45 min and the Rapid ESBL NP test in addition detects any type of ESBL producers.

**Keywords:** ESBL; CTX-M; bloodstream infection; sepsis; Gram-negatives; rapid diagnostic test

## **Introduction**

Extended-spectrum- $\beta$ -lactamase (ESBL)-producing Enterobacterales (EB) bloodstream infections (BSI) represent a worldwide clinical issue, especially given their association with multidrug resistance, severity of illness, poor outcomes, and growing number in the community [1-3]. The spread of plasmids carrying CTX-M-type genes in the community beginning mostly in the 2000s is the main driver of ESBL dissemination in EB and replaced other ESBL enzymes (i.e. mostly TEM, SHV derivatives) that were mostly identified in hospital-acquired EB infections [1,4-5]. However, given the heterogeneity of ESBL family, other-than-CTX-M-type enzymes have been also reported as a source of outbreaks of ESBL-producers [6]. Therefore, accurate detection of ESBLs is of paramount importance for both epidemiological and clinical purposes, given the ability of ESBLs of hydrolyzing penicillins, monobactams and cephalosporins. Several phenotypic tests are available for ESBL detection [7-9]. They are mostly performed on bacterial colonies and associated with non-negligible turnaround time (TAT) contributing to delay obtention of results in critical scenarios as that of management of BSI patients. Various phenotypic tests that can detect ESBLs have been evaluated directly from EB positive blood cultures (BCs) to provide result on the same day of positive BC processing with variable specificity and sensitivity [10-23]. Although the benefit in reducing TAT is recognized [12,23], evidence of detection of ESBL producers directly from EB positive BCs in clinical routine are limited. Therefore, this study was aimed at evaluating the performance of three methods for direct detection of ESBL producers from BSI, none of them being molecular based and all of them being very recently commercialized.

## **Methods**

### *Conventional blood culture routine*

Our laboratory based at the Microbiology and Virology Unit (University Hospital Città della Salute e della Scienza di Torino, Turin, Italy) is open 7 days per week from 8 a.m. to 6 p.m. BACT/ALERT FA and FN Plus BC bottles (bioMérieux, Marcy l'Étoile, France) are incubated in the BACT/ALERT Virtuo (bioMérieux, Marcy l'Étoile, France) at various times each day. Positive BCs are subjected to Gram staining and subculture on appropriate solid culture media. Pathogen identification is performed on overnight subcultures using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker DALTONIK GmbH, Bremen, Germany). Antimicrobial susceptibility testing is performed on overnight subcultures using the MicroscanWalkAway plus system according to the manufacturer's instructions (Beckman Coulter, Brea, CA, USA). Antimicrobial susceptibilities are interpreted according to EUCAST breakpoints as updated in 2021 [24]. A disc-based phenotypic method evaluating inhibitory activity of clavulanate or cloxacillin on broad-spectrum- $\beta$ -lactamases (total ESBL + AmpC Confirm kit, Rosco, Taastrup, Denmark) is used to identify ESBL- and AmpC-producers if cefotaxime and/or ceftazidime minimum inhibitory concentrations (MICs) were  $>1$  mg/L. The Mastdiscs combi Carba plus disc system (Mast Group Ltd, Bootle, UK) is used to assess carbapenemase producers when meropenem MIC value was  $>0.125$  mg/L. Xpert Carba-R assay (Cepheid, Sunnyvale, CA) was also carried out to detect the main carbapenem resistance genes when meropenem MIC value was  $>0.125$  mg/L and/or ceftazidime-avibactam (CZA) MIC value  $\geq 8$  mg/L.

### *Study design*

The bacterial pellets of positive BCs with Gram-negative bacilli obtained using the MBT Sepsityper IVD Kit (Bruker DALTONIK GmbH, Bremen, Germany) from 1 mL of positive BC broth underwent MALDI-TOF MS analysis using the MALDI BioTyper system in accordance with the manufacturer's instructions (Bruker DALTONIK GmbH, Bremen, Germany). In case of identification of *Klebsiella pneumoniae* or *Escherichia coli*, three

phenotypic methods were used in parallel for detection of ESBL phenotype and results were compared with conventional culture-based result over a four-month period (May-August 2021).

The Rapid ESBL NP® (Liofilchem, Roseto degli Abruzzi, Italy) is a colorimetric cefotaxime hydrolysis-based assay able to detect presence/absence of any type of ESBLs or presence of an enzyme or combination of enzymes that can hydrolyze cefotaxime, but which is not inhibited by the addition of tazobactam (i.e. cephalosporinase, ESBL + cephalosporinase, carbapenemase with or without an ESBL) [25-26]. Rapid ESBL NP® test was performed using the bacterial pellet recovered with MBT Sepsityper IVD Kit from 1 mL of positive BC broth, as previously described [11-12,27]. Briefly, after keeping test panel at room temperature for 10 min, 400 µL of lysis buffer were added to the bacterial pellet and vortexed for 5 s. After 15 min, 100 µL of the solution obtained were dispensed into each well (A, B and C) of the test cassette. Then, the panel was covered with the lid provided and incubated at  $36\pm 2^{\circ}\text{C}$  for 20 min in ambient air. Rapid ESBL NP test results were read within 20 min. Positive result for ESBL phenotype was considered if wells A and C remained red and Well B turned orange/yellow (Figure 1, a).

The NG CTX-M MULTI® assay (NG Biotech, Guipry, France) is a lateral flow immunoassay exploiting monoclonal antibodies specific for the specific detection of CTX-M-type (group 1, 2, 8, 9 and 25) ESBL enzymes only [10-12]. As opposed to the Rapid ESBL NP test, this technique is not aimed to identify all types of ESBL producers. NG CTX-M MULTI® assay was also performed using the bacterial pellet recovered with MBT Sepsityper IVD Kit from 1 mL of positive BC broth. Briefly, five drops of lysis buffer provided with the kit were added to bacterial pellet and vortexed for 5 s. One hundred µL were added to the sample well of the test cassette. Assay results were read within 15 min. Positive result for ESBL phenotype was based on the presence of visible line specific for CTX-M ESBL enzyme (Figure 1, c).

The E-test® (bioMérieux, Marcy l'Étoile, France) is an antimicrobial gradient method that combines the principle of dilution methods with that of diffusion methods to determine the MIC

value. Both ESBL E-test® ceftazidime +/-clavulanic acid and cefotaxime +/- clavulanic acid (CTX/CTXL) are the antimicrobial agents required for ESBL confirmation. To confirm presence of ESBLs in isolates with high-level expression of AmpC  $\beta$ -lactamases it is recommended that an additional ESBL confirmation test is performed with cefepime as the indicator cephalosporin, as cefepime is usually not hydrolyzed by AmpC  $\beta$ -lactamases. Direct ESBL E-test® was performed modifying EUCAST inoculum recommendations for rapid AST from positive BCs [28]. In this modified procedure of E-test®, 200  $\mu$ L of positive BC broth were mixed to 200  $\mu$ L of normal saline and the total suspension was inoculated and spread gently on a cation-adjusted MHA plate. MHA surface was let dry under the fume hood for 2 min. Then, a CTX/CTXL and cefepime+/- clavulanic acid (PM/PML) strips were deposited on the agar surface and the plate was incubated at 37°C in 5% CO<sub>2</sub>. Direct ESBL E-test® results were read after 5 h of incubation only if the growth was confluent and zone edges were clearly visible. Growth of microcolonies inside the entire inhibition zone was ignored. The test was considered positive for ESBL phenotype if  $\geq 8$ -fold reduction was observed in the MIC of CTX and/or PM combined with clavulanic acid compared with the MIC of the CTX and/or PM alone or if a phantom zone or deformed ellipse was present (Figure 1, e).

All identified isolates were further analyzed with a multiplex real-time polymerase chain reaction assay specific for *bla*CTX-M-like genes (ESBL ELITe MGB Kits, ELITechGroup Molecular Diagnostics, Turin, Italy) as previously described [29].

In case of discordant result between the direct phenotypic methods and conventional culture-based diagnostics, phenotypic tests were performed on bacterial colony.

### *Statistical analysis*

Descriptive data are shown as absolute (n) and relative (%) frequencies for categorical data. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV)

of the three phenotypic methods for direct detection of ESBL phenotype from positive BCs with 95% confidence interval (95% CI) were computed using the online calculator at <http://vassarstats.net/clin1.html>.

## Results

Over the study period, 242 positive BCs deemed representative of a single Gram-negative bacilli BSI event were processed. Of these, 58.7% (n=142) were associated with *K. pneumoniae* (n=62) or *E. coli* (n=80) and were included in the study (Table 1). ESBL and carbapenemase phenotype were detected in 26.1% (n=37) and 16.9% (n=24), respectively. All the isolates displaying a carbapenemase phenotype were *K. pneumoniae*, being KPC- (95.8%, n=23) the most prevalent enzyme followed by VIM-type (4.2%, n=1). Among KPC-producers, 21.7% (n=5) were co-producers of CTX-M.

Among the three methods for direct detection of ESBL phenotype, the best performance was obtained by direct ESBL E-test® that showed no false negative/positive results when combining the results of the CTX/CTXL and PM/PML strips (Table 2). Of note, CTX/CTXL strips test failed to detect one isolate with an ESBL phenotype and with low MIC ( $\leq 1$  mg/L) for CTX. Similarly, PM/PML strip test failed to detect one ESBL isolate with low MIC value (2 mg/L) for PM.

The Rapid ESBL NP® and NG CTX-M MULTI® showed similar performance (Table 2). In fact, for both the tests, sensitivity, specificity, positive and negative predictive values with 95% confidence intervals were 0.92 [0.77-0.98], 1 [0.96-1], 1 [0.87-1] and 0.97 [0.92-0.99]. The false negative results obtained with Rapid ESBL NP® were as follows: two were positive for CTX-M according to the PCR results while one presented with both bacterial pellets and colony with hypermuroid phenotype and PM, CTX, and CAZ MICs of 4 mg/L, >32 mg/L, and >32 mg/L, respectively. By Repeating the tests from overnight subcultures, one of the isolates

carrying CTX-M tested positive and showed PM, CTX, and CAZ MICs of 2 mg/L, 32 mg/L, and 2 mg/L, respectively, while the other two isolates tested negative. Moreover, Rapid ESBL NP® failed to provide indications on the presence of other combined mechanisms of cephalosporins resistance in four cases: one KPC-producing *K. pneumoniae* isolate; one CZA resistant KPC-producing *K. pneumoniae* isolate with PM, CTX, and CAZ MICs of 8 mg/L, 4 mg/L, and >32 mg/L, respectively; one VIM-producing *K. pneumoniae*; and one AmpC-producing *E. coli* with PM, CTX, and CAZ MICs of ≤0.5 mg/L, 4 mg/L, and 32 mg/L, respectively. However, by repeating the Rapid ESBL NP® tests from overnight subcultures only the CZA KPC-producing *K. pneumoniae* isolate remained negative.

The three false negative results obtained with the NG CTX-M MULTI® were all negative for CTX-M PCR. Of these, one was the same hypermucooid *K. pneumoniae* strain above-mentioned.

Technical comparison of the three methods used for direct detection of ESBL phenotype from *K. pneumoniae* or *E. coli* positive BCs is summarized in Table 3. ESBL phenotype could be detected within 40 to 45 min (Rapid ESBL NP®), 15 to 20 min (NG CTX-M MULTI®), or 5 h (direct ESBL E-test®).

## **Discussion**

The global CTX-M pandemic underlines the need for rapid ESBL detection to accelerate clinical decision making, infection control measures and finally contribute to improve patient outcomes. Our data make obvious the high prevalence of ESBL producers in *K. pneumoniae* and *E. coli* BSI in our hospital located in the northern part of Italy, confirming the relevant burden of resistance rates to expanded-spectrum cephalosporins in those species in Italy compared to Europe [30]. Therefore, we compared the performance of two rapid tests, the biochemical based Rapid ESBL NP® test very recently commercialized and lateral flow

immunoassay NG CTX-M MULTI® for detection of ESBL producers from BSI and compared their results with the direct ESBL E-test® performed also from BC samples. Our results demonstrated that with the three phenotypic tests evaluated, rapid and reliable detection of ESBL phenotype can be achieved directly from *K. pneumoniae* or *E. coli* positive BCs. We excluded from this comparison the  $\beta$ -Lacta® test (Bio-Rad, Marnes-La-Coquette, France), based on a chromogenic cephalosporin, that is a rapid technique for identification of any type of cephalosporin resistance since it lacks specificity [15]. Similarly, we excluded the direct  $\beta$ -lactam inactivation method despite its claimed sensitivity and specificity, both being 100% since it remains a home-made technique with a long TAT [18-19]. We did not also evaluate MALDI-TOF MS-based approach for detection of ESBL producers (sensitivity 91.1-100% and specificity 91.5-100%) since it required equipment and careful adaptation for its implement in routine microbiology [20-23].

The direct ESBL E-test® allows a reliable detection of ESBL phenotype but with a TAT unsuitable for antibiotic management of BSI patients. The Rapid ESBL NP® and NG CTX-M MULTI® also allowed a reliable phenotypic detection of ESBL producers with a TAT ranging from 15 min to 45 min compatible with antibiotic stewardship. In addition, the Rapid ESBL NP® test offers the advantage to detect any type of ESBL (and not only CTX-M) and to identify combined mechanisms such as carbapenemase production. This is an important point to consider for implementing a carbapenem-containing therapy.

Based on these results, the following strategy for ESBL phenotype detection from *K. pneumoniae* and *E. coli* positive BC is proposed. *Klebsiella* spp or *E. coli* positive BCs should be screened with the NG CTX-M MULTI® assay or the Rapid ESBL NP®. In countries where the prevalence of other-than-CTX-M types ESBL enzymes is important such as those from Asia, South America or Middle East the Rapid ESBL NP® offers the advantage to detect any kind of ESBL producers. In addition, one shall be aware that false detection of ESBL has been

noted by using the NG CTX-M MULTI® assay for several *K. oxytoca* strains that possess specific naturally-occurring ESBLs of OXY type [31]. All the tests evaluated are low cost and require no specialized equipment or personnel, further studies on cost-benefit analysis are needed however.

In conclusion, the three phenotypic methods evaluated showed good performance for detection of ESBL phenotype directly from *K. pneumoniae* and *E. coli* positive BCs. The rapid ESBL tests directly from BC samples might be implemented worldwide in particular for patients hospitalized in acute care facilities for promptly optimizing their antibiotic therapy.

#### **Acknowledgements:**

**Authorship statement:** Boattini M, Bianco G, Cavallo R, Nordmann P, and Costa C designed the study; Boattini M, Bianco G, Comini S, Iannaccone M, and Casale R acquired data; Boattini M and Bianco G analyzed and interpreted data; Boattini M wrote the paper; all authors revised the article critically and approved the final version.

**Funding:** Liofilchem (Italy) kindly provided the Rapid ESBL NP® tests. They were not involved in the study design; the collection, analysis, and interpretation of data; the writing of the paper; and the decision to submit the article for publication.

**Conflicts of interest:** Patrice Nordmann is the main inventor of the Rapid ESBL NP® test. A patent of this test has been taken on behalf of INSERM, University of Paris XI and Assistance Publique Hôpitaux de Paris (France).

**Ethics approval:** This study was conducted in accordance with the Declaration of Helsinki. Formal ethical approval was obtained by our Center's institutional review board (Protocol No. 0029345).

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and material:** The authors confirm that the data supporting the findings of this study are available within the article.

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Direct detection of extended-spectrum- $\beta$ -lactamase-producers in Enterobacterales from blood cultures, a comparative analysis

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## Abstract

**Introduction:** Accurate detection of extended-spectrum- $\beta$ -lactamase (ESBL)-producing Enterobacterales from bloodstream infection (BSI) is of paramount importance for both epidemiological and clinical purposes, especially for optimization of antibiotic stewardship interventions.

**Methods:** Three phenotypic methods for detection of ESBL phenotype in *Klebsiella pneumoniae* and *Escherichia coli* BSI were compared over a four-month period (May-August 2021) in a main University Hospital from Northern Italy. The methods were the biochemical Rapid ESBL NP®, the immunological NG-Test CTX-M MULTI®, and the E-test technique based on ESBL E-test®.

**Results:** One hundred forty-two blood cultures (BCs) positive for *K. pneumoniae* or *E. coli* were included. ESBL and carbapenemase phenotype were detected in 26.1% (n=37) and 16.9% (n=24), respectively. Rapid ESBL NP®, NG-Test CTX-M MULTI®, and direct ESBL E-test® positive and negative predictive values with 95% confidence intervals were 1 [0.87-1] and 0.97 [0.92-0.99], 1 [0.87-1] and 0.97 [0.92-0.99], 1 [0.88-1] and 1 [0.96-1], respectively.

**Conclusion:** The three phenotypic methods evaluated showed good performance in detection of ESBL phenotype from *K. pneumoniae* or *E. coli* positive BCs. Rapid ESBL NP® and NG-test CTX-M® offer the important advantage of a turnaround time of 15 to 45 min and the Rapid ESBL NP test in addition detects any type of ESBL producers.

**Keywords:** ESBL; CTX-M; bloodstream infection; sepsis; Gram-negatives; rapid diagnostic test

## **Introduction**

Extended-spectrum- $\beta$ -lactamase (ESBL)-producing Enterobacterales (EB) bloodstream infections (BSI) represent a worldwide clinical issue, especially given their association with multidrug resistance, severity of illness, poor outcomes, and growing number in the community [1-3]. The spread of plasmids carrying CTX-M-type genes in the community beginning mostly in the 2000s is the main driver of ESBL dissemination in EB and replaced other ESBL enzymes (i.e. mostly TEM, SHV derivatives) that were mostly identified in hospital-acquired EB infections [1,4-5]. However, given the heterogeneity of ESBL family, other-than-CTX-M-type enzymes have been also reported as a source of outbreaks of ESBL-producers [6]. Therefore, accurate detection of ESBLs is of paramount importance for both epidemiological and clinical purposes, given the ability of ESBLs of hydrolyzing penicillins, monobactams and cephalosporins. Several phenotypic tests are available for ESBL detection [7-9]. They are mostly performed on bacterial colonies and associated with non-negligible turnaround time (TAT) contributing to delay obtention of results in critical scenarios as that of management of BSI patients. Various phenotypic tests that can detect ESBLs have been evaluated directly from EB positive blood cultures (BCs) to provide result on the same day of positive BC processing with variable specificity and sensitivity [10-23]. Although the benefit in reducing TAT is recognized [12,23], evidence of detection of ESBL producers directly from EB positive BCs in clinical routine are limited. Therefore, this study was aimed at evaluating the performance of three methods for direct detection of ESBL producers from BSI, none of them being molecular based and all of them being very recently commercialized.

## **Methods**

### *Conventional blood culture routine*

Our laboratory based at the Microbiology and Virology Unit (University Hospital Città della Salute e della Scienza di Torino, Turin, Italy) is open 7 days per week from 8 a.m. to 6 p.m. BACT/ALERT FA and FN Plus BC bottles (bioMérieux, Marcy l'Étoile, France) are incubated in the BACT/ALERT Virtuo (bioMérieux, Marcy l'Étoile, France) at various times each day. Positive BCs are subjected to Gram staining and subculture on appropriate solid culture media. Pathogen identification is performed on overnight subcultures using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker DALTONIK GmbH, Bremen, Germany). Antimicrobial susceptibility testing is performed on overnight subcultures using the MicroscanWalkAway plus system according to the manufacturer's instructions (Beckman Coulter, Brea, CA, USA). Antimicrobial susceptibilities are interpreted according to EUCAST breakpoints as updated in 2021 [24]. A disc-based phenotypic method evaluating inhibitory activity of clavulanate or cloxacillin on broad-spectrum- $\beta$ -lactamases (total ESBL + AmpC Confirm kit, Rosco, Taastrup, Denmark) is used to identify ESBL- and AmpC-producers if cefotaxime and/or ceftazidime minimum inhibitory concentrations (MICs) were  $>1$  mg/L. The Mastdiscs combi Carba plus disc system (Mast Group Ltd, Bootle, UK) is used to assess carbapenemase producers when meropenem MIC value was  $>0.125$  mg/L. Xpert Carba-R assay (Cepheid, Sunnyvale, CA) was also carried out to detect the main carbapenem resistance genes when meropenem MIC value was  $>0.125$  mg/L and/or ceftazidime-avibactam (CZA) MIC value  $\geq 8$  mg/L.

### *Study design*

The bacterial pellets of positive BCs with Gram-negative bacilli obtained using the MBT Sepsityper IVD Kit (Bruker DALTONIK GmbH, Bremen, Germany) from 1 mL of positive BC broth underwent MALDI-TOF MS analysis using the MALDI BioTyper system in accordance with the manufacturer's instructions (Bruker DALTONIK GmbH, Bremen, Germany). In case of identification of *Klebsiella pneumoniae* or *Escherichia coli*, three

phenotypic methods were used in parallel for detection of ESBL phenotype and results were compared with conventional culture-based result over a four-month period (May-August 2021).

The Rapid ESBL NP® (Liofilchem, Roseto degli Abruzzi, Italy) is a colorimetric cefotaxime hydrolysis-based assay able to detect presence/absence of any type of ESBLs or presence of an enzyme or combination of enzymes that can hydrolyze cefotaxime, but which is not inhibited by the addition of tazobactam (i.e. cephalosporinase, ESBL + cephalosporinase, carbapenemase with or without an ESBL) [25-26]. Rapid ESBL NP® test was performed using the bacterial pellet recovered with MBT Sepsityper IVD Kit from 1 mL of positive BC broth, as previously described [11-12,27]. Briefly, after keeping test panel at room temperature for 10 min, 400 µL of lysis buffer were added to the bacterial pellet and vortexed for 5 s. After 15 min, 100 µL of the solution obtained were dispensed into each well (A, B and C) of the test cassette. Then, the panel was covered with the lid provided and incubated at  $36\pm 2^{\circ}\text{C}$  for 20 min in ambient air. Rapid ESBL NP test results were read within 20 min. Positive result for ESBL phenotype was considered if wells A and C remained red and Well B turned orange/yellow (Figure 1, a).

The NG CTX-M MULTI® assay (NG Biotech, Guipry, France) is a lateral flow immunoassay exploiting monoclonal antibodies specific for the specific detection of CTX-M-type (group 1, 2, 8, 9 and 25) ESBL enzymes only [10-12]. As opposed to the Rapid ESBL NP test, this technique is not aimed to identify all types of ESBL producers. NG CTX-M MULTI® assay was also performed using the bacterial pellet recovered with MBT Sepsityper IVD Kit from 1 mL of positive BC broth. Briefly, five drops of lysis buffer provided with the kit were added to bacterial pellet and vortexed for 5 s. One hundred µL were added to the sample well of the test cassette. Assay results were read within 15 min. Positive result for ESBL phenotype was based on the presence of visible line specific for CTX-M ESBL enzyme (Figure 1, c).

The E-test® (bioMérieux, Marcy l'Étoile, France) is an antimicrobial gradient method that combines the principle of dilution methods with that of diffusion methods to determine the MIC

value. Both ESBL E-test® ceftazidime +/-clavulanic acid and cefotaxime +/- clavulanic acid (CTX/CTXL) are the antimicrobial agents required for ESBL confirmation. To confirm presence of ESBLs in isolates with high-level expression of AmpC  $\beta$ -lactamases it is recommended that an additional ESBL confirmation test is performed with cefepime as the indicator cephalosporin, as cefepime is usually not hydrolyzed by AmpC  $\beta$ -lactamases. Direct ESBL E-test® was performed modifying EUCAST inoculum recommendations for rapid AST from positive BCs [28]. In this modified procedure of E-test®, 200  $\mu$ L of positive BC broth were mixed to 200  $\mu$ L of normal saline and the total suspension was inoculated and spread gently on a cation-adjusted MHA plate. MHA surface was let dry under the fume hood for 2 min. Then, a CTX/CTXL and cefepime+/- clavulanic acid (PM/PML) strips were deposited on the agar surface and the plate was incubated at 37°C in 5% CO<sub>2</sub>. Direct ESBL E-test® results were read after 5 h of incubation only if the growth was confluent and zone edges were clearly visible. Growth of microcolonies inside the entire inhibition zone was ignored. The test was considered positive for ESBL phenotype if  $\geq 8$ -fold reduction was observed in the MIC of CTX and/or PM combined with clavulanic acid compared with the MIC of the CTX and/or PM alone or if a phantom zone or deformed ellipse was present (Figure 1, e).

All identified isolates were further analyzed with a multiplex real-time polymerase chain reaction assay specific for *bla*CTX-M-like genes (ESBL ELITe MGB Kits, ELITechGroup Molecular Diagnostics, Turin, Italy) as previously described [29].

In case of discordant result between the direct phenotypic methods and conventional culture-based diagnostics, phenotypic tests were performed on bacterial colony.

### *Statistical analysis*

Descriptive data are shown as absolute (n) and relative (%) frequencies for categorical data. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV)

of the three phenotypic methods for direct detection of ESBL phenotype from positive BCs with 95% confidence interval (95% CI) were computed using the online calculator at <http://vassarstats.net/clin1.html>.

## Results

Over the study period, 242 positive BCs deemed representative of a single Gram-negative bacilli BSI event were processed. Of these, 58.7% (n=142) were associated with *K. pneumoniae* (n=62) or *E. coli* (n=80) and were included in the study (Table 1). ESBL and carbapenemase phenotype were detected in 26.1% (n=37) and 16.9% (n=24), respectively. All the isolates displaying a carbapenemase phenotype were *K. pneumoniae*, being KPC- (95.8%, n=23) the most prevalent enzyme followed by VIM-type (4.2%, n=1). Among KPC-producers, 21.7% (n=5) were co-producers of CTX-M.

Among the three methods for direct detection of ESBL phenotype, the best performance was obtained by direct ESBL E-test® that showed no false negative/positive results when combining the results of the CTX/CTXL and PM/PML strips (Table 2). Of note, CTX/CTXL strips test failed to detect one isolate with an ESBL phenotype and with low MIC ( $\leq 1$  mg/L) for CTX. Similarly, PM/PML strip test failed to detect one ESBL isolate with low MIC value (2 mg/L) for PM.

The Rapid ESBL NP® and NG CTX-M MULTI® showed similar performance (Table 2). In fact, for both the tests, sensitivity, specificity, positive and negative predictive values with 95% confidence intervals were 0.92 [0.77-0.98], 1 [0.96-1], 1 [0.87-1] and 0.97 [0.92-0.99]. The false negative results obtained with Rapid ESBL NP® were as follows: two were positive for CTX-M according to the PCR results while one presented with both bacterial pellets and colony with hypermuroid phenotype and PM, CTX, and CAZ MICs of 4 mg/L, >32 mg/L, and >32 mg/L, respectively. By Repeating the tests from overnight subcultures, one of the isolates

carrying CTX-M tested positive and showed PM, CTX, and CAZ MICs of 2 mg/L, 32 mg/L, and 2 mg/L, respectively, while the other two isolates tested negative. Moreover, Rapid ESBL NP® failed to provide indications on the presence of other combined mechanisms of cephalosporins resistance in four cases: one KPC-producing *K. pneumoniae* isolate; one CZA resistant KPC-producing *K. pneumoniae* isolate with PM, CTX, and CAZ MICs of 8 mg/L, 4 mg/L, and >32 mg/L, respectively; one VIM-producing *K. pneumoniae*; and one AmpC-producing *E. coli* with PM, CTX, and CAZ MICs of ≤0.5 mg/L, 4 mg/L, and 32 mg/L, respectively. However, by repeating the Rapid ESBL NP® tests from overnight subcultures only the CZA KPC-producing *K. pneumoniae* isolate remained negative.

The three false negative results obtained with the NG CTX-M MULTI® were all negative for CTX-M PCR. Of these, one was the same hypermucooid *K. pneumoniae* strain above-mentioned.

Technical comparison of the three methods used for direct detection of ESBL phenotype from *K. pneumoniae* or *E. coli* positive BCs is summarized in Table 3. ESBL phenotype could be detected within 40 to 45 min (Rapid ESBL NP®), 15 to 20 min (NG CTX-M MULTI®), or 5 h (direct ESBL E-test®).

## **Discussion**

The global CTX-M pandemic underlines the need for rapid ESBL detection to accelerate clinical decision making, infection control measures and finally contribute to improve patient outcomes. Our data make obvious the high prevalence of ESBL producers in *K. pneumoniae* and *E. coli* BSI in our hospital located in the northern part of Italy, confirming the relevant burden of resistance rates to expanded-spectrum cephalosporins in those species in Italy compared to Europe [30]. Therefore, we compared the performance of two rapid tests, the biochemical based Rapid ESBL NP® test very recently commercialized and lateral flow

immunoassay NG CTX-M MULTI® for detection of ESBL producers from BSI and compared their results with the direct ESBL E-test® performed also from BC samples. Our results demonstrated that with the three phenotypic tests evaluated, rapid and reliable detection of ESBL phenotype can be achieved directly from *K. pneumoniae* or *E. coli* positive BCs. We excluded from this comparison the  $\beta$ -Lacta® test (Bio-Rad, Marnes-La-Coquette, France), based on a chromogenic cephalosporin, that is a rapid technique for identification of any type of cephalosporin resistance since it lacks specificity [15]. Similarly, we excluded the direct  $\beta$ -lactam inactivation method despite its claimed sensitivity and specificity, both being 100% since it remains a home-made technique with a long TAT [18-19]. We did not also evaluate MALDI-TOF MS-based approach for detection of ESBL producers (sensitivity 91.1-100% and specificity 91.5-100%) since it required equipment and careful adaptation for its implement in routine microbiology [20-23].

The direct ESBL E-test® allows a reliable detection of ESBL phenotype but with a TAT unsuitable for antibiotic management of BSI patients. The Rapid ESBL NP® and NG CTX-M MULTI® also allowed a reliable phenotypic detection of ESBL producers with a TAT ranging from 15 min to 45 min compatible with antibiotic stewardship. In addition, the Rapid ESBL NP® test offers the advantage to detect any type of ESBL (and not only CTX-M) and to identify combined mechanisms such as carbapenemase production. This is an important point to consider for implementing a carbapenem-containing therapy.

Based on these results, the following strategy for ESBL phenotype detection from *K. pneumoniae* and *E. coli* positive BC is proposed. *Klebsiella* spp or *E. coli* positive BCs should be screened with the NG CTX-M MULTI® assay or the Rapid ESBL NP®. In countries where the prevalence of other-than-CTX-M types ESBL enzymes is important such as those from Asia, South America or Middle East the Rapid ESBL NP® offers the advantage to detect any kind of ESBL producers. In addition, one shall be aware that false detection of ESBL has been

noted by using the NG CTX-M MULTI® assay for several *K. oxytoca* strains that possess specific naturally-occurring ESBLs of OXY type [31]. All the tests evaluated are low cost and require no specialized equipment or personnel, further studies on cost-benefit analysis are needed however.

In conclusion, the three phenotypic methods evaluated showed good performance for detection of ESBL phenotype directly from *K. pneumoniae* and *E. coli* positive BCs. The rapid ESBL tests directly from BC samples might be implemented worldwide in particular for patients hospitalized in acute care facilities for promptly optimizing their antibiotic therapy.

#### **Acknowledgements:**

**Authorship statement:** Boattini M, Bianco G, Cavallo R, Nordmann P, and Costa C designed the study; Boattini M, Bianco G, Comini S, Iannaccone M, and Casale R acquired data; Boattini M and Bianco G analyzed and interpreted data; Boattini M wrote the paper; all authors revised the article critically and approved the final version.

**Funding:** Liofilchem (Italy) kindly provided the Rapid ESBL NP® tests. They were not involved in the study design; the collection, analysis, and interpretation of data; the writing of the paper; and the decision to submit the article for publication.

**Conflicts of interest:** Patrice Nordmann is the main inventor of the Rapid ESBL NP® test. A patent of this test has been taken on behalf of INSERM, University of Paris XI and Assistance Publique Hôpitaux de Paris (France).

**Ethics approval:** This study was conducted in accordance with the Declaration of Helsinki. Formal ethical approval was obtained by our Center's institutional review board (Protocol No. 0029345).

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and material:** The authors confirm that the data supporting the findings of this study are available within the article.

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Turin, 8th November 2021

## **Editor-in-Chief**

### **European Journal of Clinical Microbiology and Infectious Diseases**

We would like to thank the reviewers and the Editorial Team for their helpful suggestions, which in our view have greatly enhanced the quality and strength of our study. We hope that in this revised version the manuscript is now suitable for publication in European Journal of Clinical Microbiology and Infectious Diseases.

Please, note that the changes to the original manuscript have been highlighted in the marked version. The response to the Reviewer's comments and ensuing modifications in the manuscript are also clearly indicated in the rebuttal.

### **Comments from Reviewer and point-by-point answers**

#### **Reviewer #1:**

1) Author must explain, why this method is advantageous other than TAT, how much sensitive when compared to pure culture based ESBL, MBL Test with Direct detection method?

*We thank the referee for this comment. Sensitivity, specificity, positive predictive value, and negative predictive value of the three phenotypic methods for direct detection of ESBL phenotype from positive blood cultures were calculated in comparison to conventional phenotypic testing results. Moreover, direct ESBL detection from positive blood cultures, and rapid phenotypic tests in wider terms, may predict resistance phenotype and should help to shorten time to optimal antibiotic therapy. Obviously, culture-based testing results remain essential and cannot be replaced by rapid phenotypic results. They should continue to be performed to obtain antimicrobial susceptibility and optimize antibiotic management.*

2) If more than 2 organisms in the Blood culture, how this could be relevant in interpreting the drug resistance pattern?

*We thank the referee for this comment. This study was aimed at evaluating the performance of three methods for direct detection of ESBL producers from K. pneumoniae and E. coli positive blood cultures. These phenotypic tests were evaluated in case of reliable identification of K. pneumoniae or E. coli from bacterial pellet of Gram-negative bacilli positive blood cultures (MALDI-TOF score >1.80, please see Boattini et al EJCMI 2021 <https://doi.org/10.1007/s10096-021-04192-8>) and showed to rapidly screen the presence of ESBL phenotype. Obviously, culture-based approach remains essential to confirm rapid phenotypic results, identify polymicrobial blood cultures and should continue to be performed to obtain antimicrobial susceptibility results and optimize antibiotic management.*

3) It focus only on ESBL, What about Gram positive drug susceptibility? Explain in better way with multiple reference in the discussion part about this direct method and culture based methods sensitivity and specificity

*We thank the referee for this comment. This study was aimed at evaluating the performance of three methods for direct detection of ESBL producers from K. pneumoniae and E. coli positive blood cultures. Performance evaluation of the three rapid phenotypic tests was calculated in comparison with culture-based testing results. Since we investigated the presence of ESBL phenotype in K. pneumoniae and E. coli positive blood cultures, evaluation of Gram-positive susceptibility was not the aim of the study.*

**Reviewer #2:**

1) Antimicrobial resistance is a growing problem. Rapid and accurate diagnosis of specific resistance phenotypes such as extended spectrum beta-lactamases (ESBLs) is of interest. The authors compared the performance of three rapid methods to detect ESBLs directly in positive blood cultures. All of them were successful, however, E-test required a longer time to be reported. Except Etest, the performance of the other rapid tests were subject to recent publications. Although the high sensitivity of Rapid ESBL NP were reported in both blood cultures and screening swabs, it was interesting to see a comparison of the other tests in one center.

*We thank the referee for these accurate appraisals.*

2) The major limitation of the study was similar to other studies about this subject. The clinical impact of detection of ESBLs or a comparison of rapid antibacterial susceptibility testing recommendations of EUCAST were not investigated. We need to see how these tests change the routine antibacterial treatment practice in real life apart from laboratory investigations. As the authors mentioned in the paper there are increasing number of successful rapid antimicrobial susceptibility tools with little information about impact of daily practice.

*We thank the referee for these accurate appraisals. However, we hope the referee agrees about the need to provide to clinicians rapid microbiologic results. Clinical impact of ESBL detection was not the aim of the study but a further study on the topic is ongoing.*

3) Another issue is the cost of the tests. Although my personal belief is none of the tests are expensive than unnecessary broad spectrum antibiotics, a brief discussion on the cost (not only the test also for the technician, IT, ...) can be helpful for the laboratories which are interested in these tests.

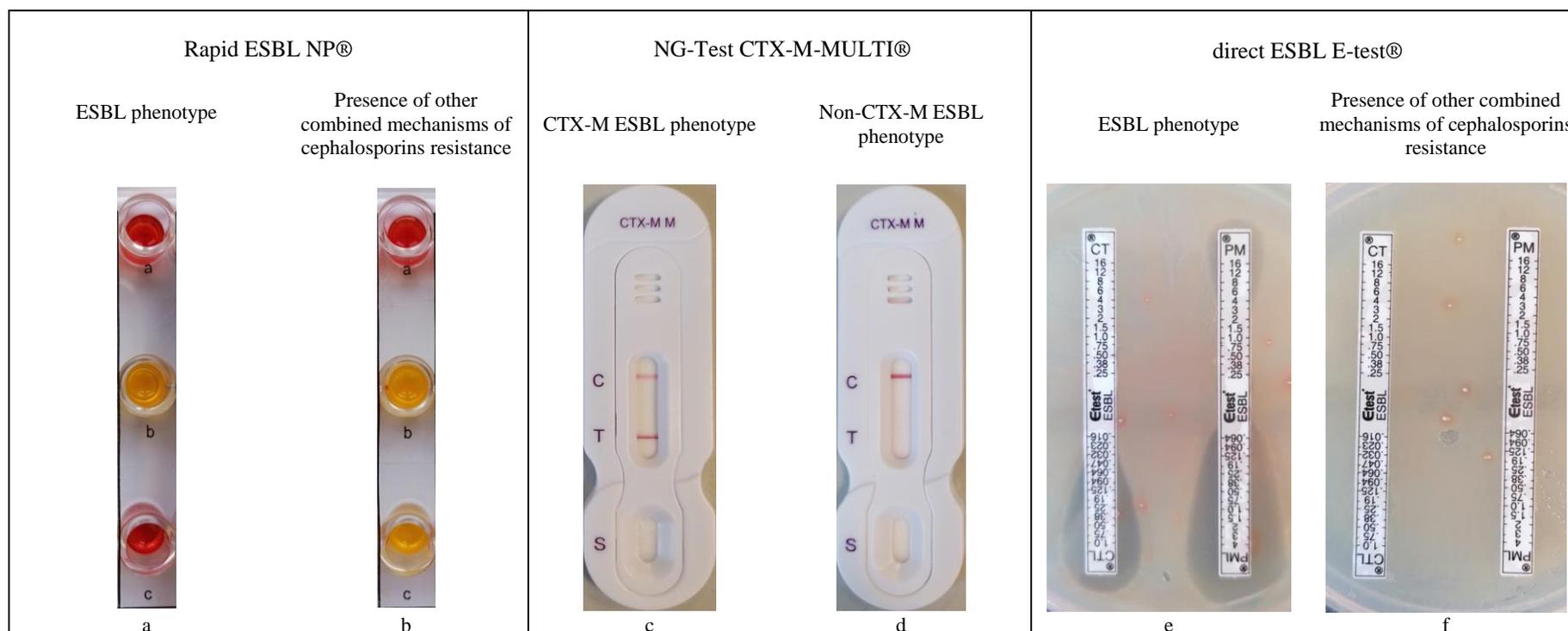
*We thank the referee for this comment. Accordingly, the following sentence was added to discussion section “All the tests evaluated are low cost and require no specialized equipment or personnel, further studies on cost-benefit analysis are needed however.”.*

**Reviewer #3:**

1) The authors compared commercial techniques to detect ESBLs production. The results are clear and well described.

*We thank the referee for these accurate appraisals.*

Figure 1. Several phenotypic test results obtained directly from *K. pneumoniae* or *E. coli* positive blood cultures.



Rapid ESBL NP®: (a) ESBL phenotype, (b) presence of other combined mechanisms of cephalosporins resistance; NG-Test CTX-M-MULTI®: (c) CTX-M ESBL phenotype, (d) Non-CTX-M ESBL phenotype; direct ESBL E-test®: (e) ESBL phenotype, (f) presence of other combined mechanisms of cephalosporins resistance.

Table 1. Characterization of *Klebsiella pneumoniae* and *Escherichia coli* isolates recovered in this study.

	ESBL phenotype	Carbapenemase phenotype	Non-ESBL-and/or-carbapenemase phenotype	Total
	26.1 (37)	16.9 (24)	57 (81)	100 (142)
<i>Klebsiella pneumoniae</i>	25.8 (16)	38.7 (24)	35.5 (22)	43.7 (62)
<i>Escherichia coli</i>	26.3 (21)	0	73.7 (59)	56.3 (80)

Table 2. Performance of rapid methods for direct detection of ESBL phenotype in *Klebsiella pneumoniae* and *Escherichia coli* bloodstream infections.

Direct detection of ESBL phenotype		Conventional phenotypic results n = 142		Sensitivity	Specificity	PPV	NPV
		Positive	Negative				
Rapid ESBL-NP®	Positive	34	0	0.92 [0.77-0.98]	1 [0.96-1]	1 [0.87-1]	0.97 [0.92-0.99]
	Negative	3	105				
NG CTX- M MULTI®	Positive	34	0	0.92 [0.77-0.98]	1 [0.96-1]	1 [0.87-1]	0.97 [0.92-0.99]
	Negative	3	105				
Direct ESBL E-test®	Positive	37	0	1 [0.88-1]	1 [0.96-1]	1 [0.88-1]	1 [0.96-1]
	Negative	0	105				

Abbreviations: PPV positive predictive value, NPV negative predictive value. 95% confidence intervals are shown in parentheses.

Table 3. Technical comparison of rapid methods used for direct detection of ESBL phenotype in *Klebsiella pneumoniae* and *Escherichia coli* bloodstream infections.

	Rapid ESBL-NP®	NG CTX-M MULTI®	Direct ESBL E-test®
Pre-procedural steps	Recovery of bacterial pellet using MBT Sepsityper IVD Kit® from 1 mL of positive BC broth	Recovery of bacterial pellet using MBT Sepsityper IVD Kit® from 1 mL of positive BC broth	None
Procedural steps	Leave a panel at room temperature (10 min), add 400 µL of lysis buffer to one of the empty vials together with the bacterial pellet. After 15 min, dispense 100 µL of the solution obtained into each well (A, B and C) of the test cassette, cover the panel with the lid provided and incubate at 36±2°C for 20 min in ambient air	Add five drops of lysis buffer provided with the kit to bacterial pellet, vortex for 5 s, add 100 µL to the sample well of the test cassette	Add 200 µL of positive BC broth to 200 µL of normal saline and inoculate on a cation-adjusted MHA plate. Spread it gently over the agar surface. Let dry under the fume hood for 2 min. Put CTX/CTXL and PM/PML strips on the agar surface, incubate at 37°C for 300 min in 5% CO <sub>2</sub>
Ease of use	Easy to perform, no special reagents or media necessary beside the test kit	Easy to perform, no special reagents or media necessary beside the test kit	Easy to perform, no special reagents or media necessary
Test reagents and materials	Test kit	Test kit	CTX/CTXL and PM/PML E-test® strips, normal saline (~3.0 mL aliquot), cation-adjusted MHA plate
Interpretation of positive result for ESBL phenotype	If wells A and C remained red and Well B turned orange/yellow	Presence of visible line specific for CTX-M ESBL enzymes	≥8-fold reduction in the MIC of the cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone OR presence of a phantom zone OR presence of deformed ellipse
Time to perform test (min)	25-30	5	5
Time to results (min)	40	~15	~300
Estimated cost per test (€)	~3	~7	~10
Strong point	Short TAT; detection of all types of ESBL producers; able to indicate the	Short TAT	High sensitivity and specificity; able to indicate the presence of other

	presence of other combined mechanisms of cephalosporins resistance (cephalosporinases, carbapenemases)		combined mechanisms of cephalosporins resistance
Main limitation	Low inoculum size may reduce test performance	Unable to detect other-than-CTX-M types ESBL enzymes; unable to detect other combined mechanisms of cephalosporins resistance (cephalosporinases, carbapenemases)	Long TAT unsuitable for antibiotic stewardship

Abbreviations: BC: blood culture; CTX/CTXL: cefotaxime and cefotaxime plus clavulanic acid; PM/PML: cefepime and cefepime plus clavulanic acid; MHA: Mueller-Hinton agar; TAT: turnaround time.