Extended-spectrum $\beta$-lactamases of *Escherichia coli* and *Klebsiella pneumoniae* screened by the VITEK 2 system

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The VITEK 2 automated system (bioMérieux) is one of the most widely used instruments in clinical microbiology laboratories for the identification and evaluation of the susceptibility profiles of bacteria including the detection of extended-spectrum $\beta$-lactamases (ESBLs) produced by *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*. Currently, the Clinical and Laboratory Standards Institute recommends the use of ESBL confirmatory tests in addition to standard susceptibility testing. In order to evaluate the accuracy of VITEK 2-positive results regarding clinical isolates of *E. coli* ($n = 110$) and *K. pneumoniae* ($n = 72$), four additional ESBL detection systems were compared: the Phoenix Automated Microbiology System (BD Diagnostic Systems) and the MicroScan WalkAway-96 System (Dade Behring), and two manual systems as confirmatory tests, the Etest (AB Biodisk) and double disc diffusion (DDS) test. Epidemiological data regarding the tested strains were also collected and their susceptibility phenotypes were determined. The four methods resulted in concordant results for 126 of the 182 strains. However, the different tests displayed distinct results: the VITEK 2 system was in disagreement in 23.9% of cases with DDS, in 15.3% with Etest, in 23% with the MicroScan WalkAway-96 System and in 23.6% with the Phoenix Automated Microbiology System. Epidemiological data indicated that the majority of ESBL-positive *E. coli* strains were isolated from patients admitted to internal medicine wards (72.7%), whilst *K. pneumoniae* ESBL-positive isolates were equally distributed between internal medicine wards (45.8%) and intensive care units (45.8%). Most of these strains were isolated from urine. In contrast to ESBL-negative isolates, the ESBL-positive strains displayed multiple drug resistance, namely to quinolones, aminoglycosides and trimethoprim–sulfamethoxazole. No significant resistance to carbapenems was detected. Overall, this study demonstrates the need for a confirmatory test following positive ESBL detection with the VITEK 2 system (panel AST-037), which appears to yield a large number of false-positive results.

INTRODUCTION

Extended-spectrum $\beta$-lactamases (ESBLs) are a large and rapidly evolving group of enzymes able to hydrolyse oxyimino-cephalosporins and monobactams, which can be inhibited by clavulanate, sulbactam or tazobactam (Paterson & Bonomo, 2005). ESBLs are responsible for mediating resistance to $\beta$-lactams and their accurate detection is a major clinical problem, particularly in invasive infections, frequently leading to therapeutic failure and an adverse clinical outcome. These enzymes vary in their substrate affinities and catalytic efficiencies, whilst $\beta$-lactams differ in their penetration rates into bacterial cells (Kaye et al., 2004). Successful spread of ESBL-encoding genes within the microbial genome can be attributed to their common localization on self-transmissible or easily movable broad-range plasmids (Jacoby & Sutton, 1991; Paterson & Bonomo, 2005). Their reliable detection is a prerequisite for the successful management of infection and implementation of valid therapeutic strategies.

Abbreviations: AES, Advanced Expert System; CA, clavulanic acid; CLSI, Clinical and Laboratory Standards Institute; DDS, double disc diffusion; ESBL, extended-spectrum $\beta$-lactamase; ICU, intensive care unit.
However, they are often not detected during routine susceptibility testing, as the expression of phenotypic resistance is multifactorial, depending on the bacterial carrier and test conditions (Spanu et al., 2006). Guidelines from the Clinical and Laboratory Standards Institute (CLSI) recommend the use of a confirmatory test for the detection of ESBLs in routine clinical laboratories in addition to the standard susceptibility testing methods. Each of the distinct semi-automated systems commonly used by the majority of clinical microbiology laboratories presents inherent strengths but variable sensitivity regarding ESBL detection capacity (Stürenburg et al., 2003; Thomson et al., 2007; Wiegand et al., 2007).

The present study was carried out at the University Hospital of São João in Porto (1200 beds), which harbours all medical and surgical specialties, as well as several specialized intensive care units. One hundred and eighty-two Escherichia coli and Klebsiella pneumoniae strains screened as positive for the presence of ESBLs using the VITEK 2 system (bioMérieux) were further evaluated by four other methods: the MicroScan WalkAway-96 System (Dade-Behring) and Phoenix Automated Microbiology System (Becton-Dickinson), both automated, and two manual systems, the Etest (AB Biodisk) and double disc synergy (DDS) test. The aim of this investigation was to compare the ESBL phenotype of bacteria detected as positive by the VITEK 2 system with the results of alternative methodologies, in order to evaluate VITEK 2 reliability. The data obtained were grouped as concordant versus non-concordant results with regard to the VITEK 2 results. In addition, strain epidemiological data and antimicrobial susceptibility profiles were analysed.

**METHODS**

**Strains and study outline.** One hundred and eighty-two clinical isolates of E. coli (n=110) or K. pneumoniae (n=72) characterized as ESBL-positive strains by the VITEK 2 system using its Advanced Expert System (AES) were selected and stored at −70 °C. These isolates were collected from July to December 2009. For the E. coli isolates, 72.7% were recovered from patients admitted to internal medicine wards, 11.8% from intensive care unit (ICU) patients, 9.1% from surgery patients and 6.4% from paediatric patients; 59.1% were isolated from urine, 23.6% from the respiratory tract, 8.2% from exudates, 4.5% from blood and 2.7% from other biological fluids and 1.8% from central venous catheters. For the K. pneumoniae isolates, 45.8% came equally from internal medicine and ICU patients, 7% from paediatric patients and 1.3% from surgical patients; 54.2% were isolated from urine, 19.4% from the respiratory tract, 13.9% from exudates, 6.9% from blood and 5.6% from central venous catheters. Upon thawing and subculturing for 24 h in solid agar medium, four additional tests for the detection of ESBL production were performed: the MicroScan WalkAway-96 System and Phoenix Automated Microbiology System, through their detection panels for ESBLs, as well as two manual tests, the Etest and DDS test using two cephalosporins, cefotaxime and ceftazidime, with and without clavulanic acid (CA; BD Diagnostics). A third cephalosporin (cefeplium) was tested whenever the results by Etest were inconclusive.

**VITEK 2 results analysis.** Strain characterization and antimicrobial susceptibility testing were performed with the VITEK 2 automated system using the ID-GNB and AST-037 cards, in accordance with the manufacturer’s instructions. The antimicrobial susceptibility testing card comprises various antibiotics including cefotaxime, ceftazidime and cefepime, but does not include associations with CA. Final results were analysed using version 3.02 software, an AES specifically designed to evaluate the results generated by the VITEK 2 system. Testing was repeated wherever suggested by the AES. All phenotypic interpretations of ESBLs were reported as a positive ESBL screening result. Strains were reported as ESBL-negative. Phenotypic interpretations other than ESBLs were proposed by the AES.

**MicroScan results analysis.** Strain characterization and antimicrobial susceptibility testing with the MicroScan WalkAway-96 system were performed with the Neg/BP/Combo NM31 panels, according to the manufacturer’s instructions. MICs obtained for cefazidime and ceftazidime with CA were compared with those obtained with the same drugs without CA. Subsequently, strains were considered as ESBL-positive or -negative in accordance with CLSI recommendations (CLSI, 2010).

**Phoenix results analysis.** Strain characterization and antimicrobial susceptibility testing with the Phoenix Automated Microbiology System (version 4.05W) was performed with the GN Combo UNMIC/ID-62 panels, according to the manufacturer’s recommendations. The Phoenix ESBL detection panel incorporates testing for ceftazidime and ceftazidime susceptibility with or without CA. Strains were considered ESBL-positive or -negative in accordance with CLSI recommendations (CLSI, 2010).

**Etest results analysis.** Etest strips with gradient concentrations of cefotaxime and ceftazidime alone and in combination with CA were used according to the manufacturer’s guidelines (AB Biodisk). Mueller–Hinton agar plates were used for Etest and incubated at 35 °C for 24 h. ESBL production was implicit by the presence of a phantom zone, by deformation of the cefotaxime and ceftazidime inhibition zones or whenever cefotaxime or ceftazidime MICs decreased by ≥3 twofold dilutions in the presence of CA, according to CLSI guidelines (CLSI, 2010). An indeterminate result was considered whenever MICs fell outside the range of the respective Etest strip, hindering the calculation of MIC ratios. In such cases, cefeplium strips were assayed, both in the presence and absence of CA; ESBL detection followed the criteria described above.

**DSS test results analysis.** Susceptibility testing was performed (McFarland 0.5 standard) on Mueller–Hinton agar (Oxoid) by placing discs on the agar surface containing 30 µg cefotaxime or ceftazidime, with and without 10 µg CA. Plates were incubated at 35 °C for 24 h. According to CLSI guidelines (CLSI, 2010), strains were considered positive for ESBL production whenever zone diameters increased by ≥5 mm for cefotaxime or ceftazidime when tested in combination with CA. This method was considered the gold standard for method comparison (CLSI, 2010).

**Multiplex PCR assay.** In order to characterize the strains that were positive only on the VITEK 2 system, plasmid and genomic DNA extraction was performed according to the methods of Hoffman & Winston (1987) and Sambrook et al. (1989). Considering the most abundant ESBLs, three specific primer sets were designed for the **bla** _TEM_, **bla** _CTX-M_, and **bla** _SHV_ genes that amplified internal fragments of different sizes. The sequences of the primers used were: **bla** _TEM_, 5'- GAGTATTCAACATTTCCGTGTC-3' and 5'-TCATTCAGTTCCGTT-3'; **bla** _CTX-M_, 5'-AGATTTACATTCCGGTGC-3' and 5'-GGCCGAAAATCCTCAAGGATC-3'; and **bla** _SHV_, 5'-GTTTGTGAAATGTTGCAGCGCCG-3' and 5'-GGCCGAAATGTTGCAGCGCCG-3'. Group primer specificity was confirmed using previously sequenced...
Multiplex PCR was performed in a 25 μl reaction mixture containing H2O (Milli-Q grade), 1× PCR buffer (Fermentas) 2.5 mM MgCl2, 0.6 mM each dNTP, variable concentrations of the specific group primers, 50–150 ng plasmid or genomic DNA and 1 U DreamTaq polymerase (Fermentas). Amplification reactions were carried out in a Mastercycler realplex2 (Eppendorf) under the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 30 s, with a final extension step at 72°C for 10 min. PCR products were assessed by electrophoresis (80 V for 1 h) in a 2.5% agarose gel containing ethidium bromide (AppliChem) and exposure to UV light.

**Antimicrobial susceptibility pattern.** The percentage resistance to aminoglycosides (gentamicin), antifolates (trimethoprim–sulfamethoxazole), fluoroquinolones (ciprofloxacin and levofloxacin) and carbapenems (meropenem) was evaluated for ESBL-positive and -negative isolates.

**Epidemiological analysis of ESBL-positive strains.** Considering DDS as the reference CLSI method, an epidemiological study of the bacteria classified as ESBL-positive was undertaken regarding their prevalence and distribution throughout the hospital departments and according to sample provenance. The same approach was undertaken for non-confirmed ESBL strains.

**Quality control.** Quality control of susceptibility testing involved *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 type strains, as recommended by the CLSI.

**Reproducibility.** The strains were tested at least three times on each automated piece of equipment and for each detection test.

**Statistical analysis.** Results obtained from the different tests were compared with results obtained by VITEK 2. In addition, results from the different methods were compared among themselves.

**RESULTS AND DISCUSSION**

Quick and accurate identification of clinical ESBL-positive *Enterobacteriaceae* strains is critical not only for optimal patient management but also for the establishment of appropriate infection control measures, aiming to prevent further spread of resistant micro-organisms (Livermore, 1995; Kaye et al., 2004). ESBLs have been reported in bacteria other than *E. coli*, *K. pneumoniae* and *Klebsiella oxytoca*, but there are currently no CLSI guidelines for the interpretation of such testing. The current available automated methods represent a rapid way of screening for *E. coli*, *K. pneumoniae* and *K. oxytoca* ESBL producers (Wiegand et al., 2007), although with limitations; therefore, a thorough analysis of the antibiogram must be performed for a precise result. Consequently, ESBL detection is highly dependent on the screening and confirmatory methods. Considerable investment has been made recently in advanced automated equipment software, thus improving the informatics tools for ESBL detection.

Many investigators have reported previously on the evaluation of various automated and manual systems (Bradford, 2001; Stürenburg et al., 2003; Spanu et al., 2006; Thomson et al., 2007; Dashri et al., 2009).

In our study, from 182 ESBL-positive clinical isolates, 23 were positive only on the VITEK 2 system, whilst 27 additional isolates were discrepant in at least one other method (Table 1). When comparing the VITEK 2 system results with the four other methods assayed, the percentage discrepancy varied from 15.3 to 27.3%, as depicted in Table 2. Molecular characterization showed that these discrepant isolates were negative for *bla*SHV, *bla*TEM and *bla*CTX-M, suggesting either the occurrence of false-positive results or non-confirmed results, meaning an overdetection of ESBL-positive strains by the VITEK 2 system. Interestingly, Bell et al. (2007) reported a high percentage of non-confirmed positive results for ESBLs and ascribed them to the presence of important β-lactamases such as plasmid-borne AmpC enzyme, which are not inhibited by CA. Therefore, the authors suggested that a positive screening test alone provided insufficient grounds to report resistance.

<table>
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<th>No. of isolates (n=182)</th>
<th>VITEK 2</th>
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The Etest method performed with two cephalosporins with or without CA resulted in 12.6% indeterminate cases, with cephalosporin MICs so high that calculations were impossible. However, when using a third cephalosporin (cefepime), only 2.7% of such cases were considered indeterminate results. The main drawback of the Etest methodology is its inherent high cost, which increases considerably when a third cephalosporin strip is required in the case of a previous indeterminate result. In contrast, the DDS method recommended by the CLSI is easy to implement and interpret, with a much lower cost. Using a DDS test as the reference procedure, we observed an agreement of 76.9% with the VITEK 2 system results (140/182 strains), which improved up to 90% with the WalkAway-96 system (164/182 strains). Interestingly, a higher agreement was observed with the Phoenix system (91.7%; 167/182 strains), although it should be noted that all three automated systems yielded very reproducible and similar results.

Both bacterial species were recovered mainly from the wards of the Department of Internal Medicine, but K. pneumoniae had a higher prevalence in the ICUs. We also observed a slight increase in E. coli prevalence (29%) over K. pneumoniae prevalence (22.2%) in the Ambulatory Department. Concerning the provenance of specimens, both species were most frequently isolated from urine, followed by respiratory secretions, in accordance with previous reports (Cantón et al., 2002; Spanu et al., 2006; Fang et al., 2008; Dashti et al., 2009).

The susceptibility phenotypes of ESBL-positive E. coli and K. pneumoniae compared with ESBL-negative isolates are depicted in Fig. 1. ESBL-positive isolates were more resistant overall to quinolones, trimethoprim–sulfamethoxazole and aminoglycosides, suggesting the coexistence of multiple resistance mechanisms to a much higher extent than has been reported previously (Wiegand et al., 2007). Resistance to carbapenems was

![Fig. 1. Antimicrobial resistance (%) among ESBL-positive (black bars) and -negative (grey bars) clinical isolates of E. coli and K. pneumoniae.](http://jmm.sgmjournals.org)
found in only a few strains, in agreement with recently published epidemiological data regarding antimicrobial resistance (Tamayo et al., 2007).

In conclusion, our study illustrates how distinct methods for ESBL detection can provide discordant results, and highlights the need to evaluate carefully and review individual reports, as well as global data. We demonstrated that the VITEK 2 system overdetected the presence of ESBL-producing bacteria, and therefore there is a need to confirm such results to avoid false-positives. The development of novel, accurate and speedy methods of ESBL detection is a priority in order to face the challenges raised by rapidly evolving ESBL genotypes (Mendonça et al., 2009).

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REFERENCES


