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Detection of Extended-Spectrum Beta-Lactamases among *Enterobacteriaceae* by Use of Semiautomated Microbiology Systems and Manual Detection Procedures[▽]

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Three commercially available microbiology identification and susceptibility testing systems were compared with regard to their ability to detect extended-spectrum β-lactamase (ESBL) production in *Enterobacteriaceae*, i.e., the Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD), the VITEK 2 System (bioMérieux, Marcy l'Etoile, France), and the MicroScan WalkAway-96 System (Dade Behring, Inc., West Sacramento, CA), using routine testing panels. One hundred fifty putative ESBL producers were distributed blindly to three participating laboratories. Conventional phenotypic confirmatory tests such as the disk approximation method, the CLSI double-disk synergy test, and the Etest ESBL were also evaluated. Biochemical and molecular characterization of β-lactamases performed at an independent laboratory was used as the reference method. One hundred forty-seven isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Serratia marcescens*, *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* were investigated. Of these isolates, 85 were identified as ESBL producers by the reference method. The remaining isolates were identified as non-ESBL producers; they were either hyperproducers of their chromosomal AmpC, Koxy, or SHV enzymes or lacked any detectable β-lactamase activity. The system with the highest sensitivity for the detection of ESBLs was the Phoenix (99%), followed by the VITEK 2 (86%) and the MicroScan (84%); however, specificity was more variable, ranging from 52% (Phoenix) to 78% (VITEK 2). The performance of the semiautomated systems differed widely with the species investigated. The sensitivities of the conventional test methods ranged from 93 to 94%. The double-disk synergy test showed the highest specificity and positive predictive value among all test methods, i.e., 97% and 98%, respectively.

In the *Enterobacteriaceae*, resistance to β-lactams is mainly due to β-lactamases that hydrolytically cleave the β-lactam ring, thus rendering the antibiotic inactive. A strategy to prevent hydrolysis caused by wide-spread β-lactamases, like the TEM-1 and SHV-1 enzymes, was the development of intrinsically stable β-lactams, such as the extended-spectrum cephalosporins. However, plasmid-encoded derivatives of these enzymes that show an enhanced spectrum of catalytic activity have been known since the early 1980s (7). Due to alterations at the active site caused by specific point mutations, these extended spectrum-β-lactamases (ESBLs) are also able to hydrolyze oxyimino-β-cephalosporins (e.g., cefotaxime, cefpodoxime, ceftazidime) and aztreonam (6). In addition to the large number of ESBL-TEM and -SHV variants, other plasmid-encoded ESBL such as CTX-M enzymes (http://www.lahey.org/studies/) are now frequently reported (13). The successful spread of ESBLs in a wide range of *Enterobacteriaceae* can be attributed to the fact that the genes coding for ESBLs are often located on self-transmissible or mobilizable broad-host-range plasmids (5). Failures to rapidly and reliably identify ESBL-producing isolates may delay the institution of appropriate infection control measures and further contribute to their uncontrolled distribution. An ESBL is not always detectable in routine susceptibility tests. The enzymes vary in their substrate affinities and in their catalytic efficiencies, and β-lactams differ in their penetration rates into bacterial cells. Some ESBL-producing isolates may appear susceptible or intermediate to one or more β-lactam compounds if tested in vitro. Approximately 40% of ESBL producers are susceptible to at least one oxyimino-cephalosporin using CLSI breakpoints (16). However, even low-level resistance mediated by some ESBLs is converted to full resistance if high inocula of the strain are used for susceptibility testing (27). Furthermore, pharmacokinetic/pharmacodynamic modeling has shown that usual dosing regimens of extended-spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime) may not be effective for many of the ESBL producers classified as susceptible (1). In addition, plasmids with ESBL genes frequently carry a variety of other resistance determinants conferring reduced susceptibilities to other antibiotics, such as aminogly-

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cosides, antifolates, tetracyclines, and fluoroquinolones (18). Recognition of all ESBL producers is of major clinical concern, as inappropriate treatment of invasive infections with cephalosporins can lead to therapeutic failures and adverse clinical outcome (16).

Semiautomated systems are widely used for species identification and susceptibility testing by clinical laboratories to decrease the in-laboratory turnaround time and to improve cost effectiveness. Each system has inherent strengths as well as recognized limitations. Reporting errors by any test system can have serious implications for the clinical outcome of patients. Numerous studies have reported on the accuracies and limitations of various semiautomated systems (10, 20, 23, 26) that have forced manufacturers to periodic updating their product software.

The purpose of this study was to evaluate and compare the routine performance of three commercially available microbiology identification and susceptibility testing systems with regard to detection of ESBL-producing *Enterobacteriaceae*. Conventional phenotypic confirmatory tests were also evaluated. A combination of biochemical methods, including isoelectric focusing, and molecular methods, such as PCR procedures, for the detection β-lactamase genes and DNA sequencing were used as reference methods, as they provide sensitive and specific genotypic and phenotypic analysis of ESBL production.

MATERIALS AND METHODS

Study design. Three commercially available microbiology identification and susceptibility testing systems were evaluated and compared with regard to their ability to presumptively or definitely detect ESBL production in *Enterobacteriaceae*. The methods tested were the Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD), the VITEK 2 System (bioMérieux, Marcy l'Etoile, France), and the MicroScan WalkAway-96 System (Dade Behring, Inc., West Sacramento, CA). For the purpose of this study, only routine identification and susceptibility testing panels from the three manufactures for gram-negative bacteria were used; specific cards designed as ESBL confirmatory tests were not employed. Unless the system advised a retest, the first test results were used for the evaluation and comparison of the different semiautomated systems. Conventional phenotypic confirmatory tests such as the disk approximation method (DAM), the CLSI double-disk synergy (DDS) test, and the Etest ESBL were also evaluated. Isolates that were identified as ESBL positive by at least one of the six different methods were sent to an independent laboratory for characterization of their β-lactamases by biochemical and molecular methods, which was used as the reference method.

Bacterial strains. A total of 150 isolates of the family *Enterobacteriaceae*, the majority of which were *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Enterobacter cloacae*, were included in the study (see Results for species distribution). Each of the three participating laboratories (Cologne, laboratory A; Hamburg, laboratory B; Heidelberg, laboratory C) contributed 50 nonduplicate isolates collected consecutively over a period of 3 months prior to study entry. Following identification of isolates to species level and as ESBL producers or ESBL nonproducers using the routine methods in use at the participating laboratories, 30 presumptive ESBL-producing *Enterobacteriaceae* isolates and 20 multidrug-resistant isolates that were not ESBL producers were selected at each laboratory. All isolates were sent with their presumptive species identification to the central coordinating laboratory. They were then labeled with consecutive study numbers and redistributed blindly to the participating laboratories. The quality control strains used for this study and included in each day's testing were *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.

MicroScan analysis. The MicroScan WalkAway-96 SI System was used in laboratory B. Identification and antimicrobial susceptibility testing was performed with Neg/BP/Combo 30-B1017-306E combination panels. All procedures were performed according to the manufacturer's directions. The integrated LabPro version 1.12 that includes the Alert expert system uses growth in the presence of cefpodoxime (4 μg/ml) and ceftazidime (1 μg/ml), i.e., at concentrations recommended by the CLSI for ESBL screening (3), as primary indicators for possible ESBL production. MICs obtained for ceftriaxone, cefotaxime, and aztreonam are interpreted according to CLSI breakpoints (3), and results may also trigger rules which alert users to possible ESBL production. These results were considered a positive ESBL screening result for the purposes of our study. Screening with this system is limited to *E. coli*, *K. pneumoniae*, and *K. oxytoca*, i.e., those species that are primarily dealt with in the CLSI guideline. Other *Enterobacteriaceae* isolates which commonly harbor AmpC enzymes but additionally may produce ESBLs, such as *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and members of the *Proteus* group, may also produce a positive screening result. However, the expert system does not support the detection of derepressed AmpC β-lactamases and ESBL production in these organisms and does not alert the user to the possibility of ESBL production.

Phoenix analysis. Identification and antimicrobial susceptibility testing with the Phoenix Automated Microbiology System (version 4.05W) was performed at laboratory C. Phoenix GN Combo Panels 448541 (combined susceptibility and identification card) were inoculated and incubated according to the manufacturer's recommendations. The Phoenix ESBL test incorporated in the panel uses growth in the presence of cefpodoxime, ceftazidime, ceftriaxone, and cefotaxime, with or without clavulanic acid (CA), to detect the production of ESBL. The BDXpert system (version 3.81C) provides a series of rules, which are triggered by various conditions given by the bacterial species identification, result of the ESBL test and MIC data. The BDXpert rules associated with ESBL identification in *E. coli*, *K. pneumoniae*, and *K. oxytoca* include rules no. 1502 and 1505, "isolate is confirmed positive for ESBL," and rule no. 106, "screening tests suggests a possible ESBL producer, confirmatory testing is recommended." Interpretation rules for *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, and *Serratia* spp. include rule no. 1405, "isolate exhibits ESBL resistance," rule no. 1430, "this isolate may exhibit resistance to extended-spectrum beta-lactam antibiotics," and rule no. 1433 (for *Enterobacter* spp. only), "isolate exhibits unusual resistance to third-generation cephalosporins, additional confirmatory testing for possible ESBL or AmpC hyperproduction is recommended." If the ESBL test is negative, then no rule is supplied. A printed report of each test indicates the actual MIC, the breakpoint-based interpretation, the expert system's interpretation, at times therapeutic advice, and the rule applied. Reports were considered a positive ESBL screening result for the purposes of our study if any of the rules listed above were triggered.

VITEK 2 analysis. Identification and antimicrobial susceptibility testing were performed at laboratory A with the VITEK 2 automated system using the ID-GNB and AST-N020 cards (catalogue no. 21312 and 22029, respectively) in accordance with the guidelines of the manufacturer. The antimicrobial susceptibility testing card AST-N020 comprises various β-lactam antibiotics, including cefoxitin, cefotaxime, ceftazidime, and cefepime; it does not include testing of a cephalosporin in the presence of CA. The results were interpreted by using software version 3.02, an advanced expert system (AES) designed to analyze the results generated by the VITEK 2 system. The AES is based on a large number of phenotypes and MIC distributions, which have mainly been derived from the literature. The MIC phenotype found for the test isolate by the VITEK 2 is compared with all the patterns in the database, and the best match is identified. There are six possible phenotypes relevant to β-lactam antibiotics among *Enterobacteriaceae* isolates that are proposed by the AES: wild type, SHV1 hyperproduction, (high-level) penicillinase, (high-level) cephalosporinase, impermeability, ESBL, or a combination of these. Testing was repeated if suggested by the AES. For the purposes of comparative analysis with the other automated systems, all phenotype interpretations that included ESBL as highlighted by the AES were reported as a positive ESBL screening result. Results were reported as EBSL negative, if only phenotypic interpretations other than ESBL were proposed by the expert system.

DAM. A modified method first described by Jarlier et al. was followed in laboratory B (6). After inoculation of the agar plate, cefotaxime (30 μg), ceftazidime (30 μg), cefpodoxime (10 μg), and cefpirome (30 μg) disks (BD Diagnostics, Heidelberg, Germany) were placed 25 to 30 mm (center to center) away from an amoxicillin-CA disk (20 plus 10 mg) placed in the center of the plate. Enhancement of the zone of inhibition in the area between the amoxicillin-CA disk and any one of the four drug disks in comparison with the zone of inhibition on the far side of the drug disk was interpreted as indicative of the presence of an ESBL in the test strain.

DDS test. The DDS test was performed at laboratory A with Mueller-Hinton agar plates (Oxoid GmbH, Wesel, Germany) and disks containing 30 μg of cefotaxime or ceftazidime with and without 10 μg of CA, 10 mg of cefpodoxime with and without 1 μg of CA, and 30 mg of cefpirome with and without 7.5 μg of CA (Oxoid GmbH, Wesel, Germany) were used. The disk tests were performed with confluent growth according to CLSI guidelines for nonfastidious bacteria (3). For the ESBL confirmation test, isolates were considered positive

for ESBL production if zone diameters increased by ≥5 mm for either cefotaxime, ceftazidime, or cefpodoxime and by ≥4 mm for cefpirome when tested in combination with CA versus its zone when tested alone, as indicated by the manufacturer or CLSI (3).

Etest ESBL. The Etest ESBL was performed at laboratory C. Etest strips with gradient concentrations of cefotaxime, ceftazidime, and cefepime at one end and cefotaxime, ceftazidime, or cefepime with CA at the other end were used in accordance with the guidelines of the manufacturer (catalogue no. 16V03228, 16V03258, and 16V03478; AB BIODISK, Solna, Sweden). ESBL production was determined by a ≥3 twofold-concentration decrease in any MIC of cefotaxime, ceftazidime, or cefepime combined with CA versus its MIC when tested alone (3). ESBL production was also identified by the presence of a phantom zone or a deformation of the cefotaxime, ceftazidime, or cefepime inhibition zone independent of the MIC ratios. A result was considered indeterminate when the MICs were outside the range of MICs of the respective Etest ESBL test strip, and a MIC ratio could therefore not be calculated.

Reproducibility testing. Ten isolates from the blinded sample set were tested on three different occasions during the study period by all participating laboratories and with all test systems included. Results were evaluated with regard to identification as EBSL positive or ESBL negative.

Performance analysis. Sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) of each automated system, of each of the conventional phenotypic tests, and for each species/group of species was calculated. The molecular and biochemical characterization of β-lactamases was used as the reference standard.

Molecular and biochemical characterization of β-lactamases. PCRs were conducted for frequently occurring β-lactamases that can confer resistance to extended-spectrum cephalosporins. PCRs were carried out using the *i*Taq polymerase (Bio-Rad Lab., Munich, Germany) under conditions suggested by the enzyme manufacturer and boiled bacteria as template. To detect ESBL-SHV enzymes, the PCR/NheI test was used (15). The bla_{SHV} gene was amplified with primers S1 (5′-ATGCGTTATATTCGCCTGTG-3′) and S2 (5′-GTTAGCGTT GCCAGTGCTCG-3′) using a standard PCR protocol with an annealing temperature of 49°C. The PCR product was subsequently digested with restriction enzyme NheI. Products were separated in 0.7% agarose gels stained with ethidium bromide, visualized under UV light, and digitally recorded. The entire bla_{SHV} gene was sequenced in isolates for which no other ESBL was detected. The detection of $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, and $bla_{CTX\text{-}M\text{-}9}$ group genes was performed with the primers CTX-MA (5′-CGCTTTGCGATGTGCAG-3′) and CTX-MB (5′-ACCGCGATATCGTTGGT-3′) (2) using an annealing temperature of 51°C, amplifying a conserved region of CTX-M type genes. These primers were not used for detection of CTX-M enzymes in *K. oxytoca*, as they would amplify chromosomal bla_{oxy} genes. Therefore, for *K. oxytoca* isolates, a CTX-M multiplex PCR was carried out according to the method of Woodford et al. (29). For detection of bla_{TEM} genes, the primers T1 (5′-ATTCTTGAAGACGAAA GGGCCTC-3′) and T3 (5′-TTGGTCTGACAGTTACCAATGC-3′) were used with an annealing temperature of 55°C. For isolates where no other ESBL was detected, bla_{TEM} PCR products were sequenced. To detect plasmid-encoded AmpC enzymes in species that lack a chromosomally encoded AmpC β-lactamase, such as *Klebsiella* spp. and *P. mirabilis*, degenerated bla_{AmpC}-specific primers were used (21). CMY family-specific primers were used for non-*Citrobacter freundii* strains, and PCR was carried out as described previously (8). For the biochemical characterization of β-lactamases, cells were grown to an optical density at 600 nm of 1.0 and then harvested by centrifugation at 4°C. Cells were resuspended in 0.01 M Tris-HCl buffer, pH 7.0. Sonication on ice yielded crude β-lactamase extracts. These were used for activity tests, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and isoelectric focusing (IEF). β-Lactamase activity was quantified spectrophotometrically according to the method of Peter et al. by measuring the change in absorbance at 485 nm using 50 μM nitrocefin (Oxoid, Basingstoke, United Kingdom) as a substrate and 0.1 M KH_2PO_4/Na_2HPO_4 buffer (pH 7.0) as a test buffer (17). The molecular weight of the β-lactamases was estimated by separation of the proteins by SDS-PAGE in 13% acrylamide gels. IEF was performed in Criterion IEF gels with a pH range of 3.0 to 10.0 using a Criterion Cell (Bio-Rad Lab., Munich, Germany) according to the manufacturer's instructions. β-Lactamase bands were visualized by staining the gels with nitrocefin solution (1 mM). IEF gels were used to evaluate hyperproduction of the AmpC β-lactamase in *E. coli*. In general, isolates were first screened for the possession of ESBL genes by PCR. If negative, protein extracts were loaded onto SDS-PAGE gels to determine whether class A β-lactamases were present, and the crude β-lactamase extracts were also examined by IEF. Sequencing of β-lactamase genes of the SHV and TEM family was performed if no other ESBL could be detected.

RESULTS

Bacterial strains. Among the 150 *Enterobacteriaceae* isolates, three isolates for which discrepant species identification results were obtained from the different semiautomated instruments were excluded from further analysis. The remaining 147 isolates (*E. coli*, 62 isolates; *K. pneumoniae*, 29 isolates; *E. cloacae*, 17 isolates; *K. oxytoca*, 16 isolates; *Enterobacter aerogenes*, 6 isolates; *Proteus mirabilis*, 6 isolates; *Morganella morganii*, 5 isolates; *C. freundii*, 4 isolates; *Proteus vulgaris*, 1 isolate; *Serratia marcescens*, 1 isolate) gave concordant species identification results with the three test systems and were used for further study. Twenty isolates (*E. coli*, 12 isolates; *M. morganii*, 5 isolates; *K. pneumoniae*, 2 isolates; *S. marcescens*, 1 isolate) were identified as ESBL negative by all phenotypic test systems. These isolates were considered definitely ESBL negative and not further investigated. The remaining 127 isolates were identified as possible ESBL producers by at least one of the phenotypic test systems and were included in the molecular and biochemical study for detection of β-lactamases (Table 1). Among these 127 isolates, 58 were uniformly reported ESBL positive by all methods, 24 were classified ESBL positive by one or more of the semiautomated instruments but not by the conventional phenotypic tests, and 3 isolates were classified as ESBL producers only by one or two conventional phenotypic tests.

Identification of β-lactamases. Extended-spectrum β-lactamases were confirmed in 85 isolates (67%) among the 127 putative ESBL producers; among these were *E. coli* (43 isolates), *K. oxytoca* (5 isolates), *K. pneumoniae* (23 isolates), *C. freundii* (1 isolate), *Enterobacter* spp. (9 isolates), and *P. mirabilis* (4 isolates). Details are summarized in Table 1. Fifty-nine ESBL-producing isolates (69%) were positive for $bla_{CTX\text{-}M}$ gene-specific PCRs (*E. coli*, $n = 34$ [79%]; *K. oxytoca*, $n = 4$ [80%]; *K. pneumoniae*, $n = 11$ [48%]). The SHV-ESBL-specific amino acid substitution G238S was detected in 23 of the isolates (*E. coli*, $n = 4$; *Klebsiella* spp., $n = 13$; *Enterobacter* spp., $n = 6$). Four bla_{SHV} genes from *K. pneumoniae* lacking the respective mutation were sequenced. Three sequences were identical to $bla_{SHV\text{-}1}$; determination of the β-lactamase activity classified one of the isolates as a SHV-1-hyperproducing strain. The fourth sequence revealed a nucleotide exchange at position 170 (G to A) resulting in the amino acid exchange R61H. This exchange has not been described in SHV-ESBL so far; however, this position is also found to be changed in ESBL SHV-67 (L35Q, R61C) (GenBank accession no. DQ174307). The MIC data available for the isolate from the Etest ESBL points to a weak ESBL phenotype, with a ceftazidime MIC of 2 μg/ml, reduced to 0.25 μg/ml when combined with CA. Therefore, we classified the isolate as a possible ESBL producer. In total, 47 isolates were bla_{TEM} positive. bla_{TEM} genes were sequenced for isolates that had not previously been shown to be ESBL positive, and we detected several TEM enzymes with an ESBL phenotype (TEM-6, TEM-12, TEM-15, TEM-20, TEM-52, TEM-92, and TEM-142 [$n = 1$ for each]) besides the parent enzyme TEM-1 only ($n = 7$).

Three isolates, one *E. coli* isolate and two *K. oxytoca* isolates, carried secondary β-lactamases as shown by IEF, but these could not be identified by molecular methods (data not shown). As the ESBL status was therefore not proven, these

TABLE 1. Characterization of presumptive ESBL-producing clinical isolates (n = 124) and results of the MicroScan, Phoenix, VITEK 2, DAM, DDS test, and Etest ESBL[a]

Species	No. of isolates	β-Lactamase	Result by:					
			MicroScan	Phoenix	VITEK 2	DAM	DDS	Etest ESBL
		Non-ESBLs						
E. coli	1	TEM-1	−	−	−	+	−	−
E. coli	1	TEM-1	+	+	−	+	−	+
E. coli	1	TEM-1	+	+	−	+	−	−
E. coli	3	TEM-1, $AmpC_{hyper}$[b]	+	+	−	−	−	−
		ESBLs						
E. coli	25	CTX-M	+	+	+	+	+	+
E. coli	2	CTX-M	+	+	−	+	+	+
E. coli	2	CTX-M	+	+	NA	+	+	+
E. coli	1	CTX-M	+	+	+	+	+	−
E. coli	1	SHV-ESBL, CTX-M	+	+	+	+	+	+
E. coli	3	SHV-ESBL	+	+	+	+	+	+
E. coli	4	TEM-ESBL	+	+	+	+	+	+
E. coli	1	TEM-ESBL	+	+	−	+	+	+
E. coli	1	TEM-ESBL	+	+	−	+	−	+
E. coli	1	CTX-M, $AmpC_{plasmid}$[c]	+	+	+	+	+	+
E. coli	2	CTX-M, $AmpC_{plasmid}$[c]	+	+	−	+	+	+
		Non-ESBLs						
K. oxytoca	1	$Koxy_{wildtype}$	+	+	−	−	−	−
K. oxytoca	1	$Koxy_{hyper}$[b]	+	+	+	+	+	+
K. oxytoca	3	$Koxy_{hyper}$	+	+	−	−	−	+
K. oxytoca	1	$Koxy_{hyper}$	−	+	−	−	−	+
K. oxytoca	2	$Koxy_{hyper}$	+	+	−	+	−	+
K. oxytoca	1	$Koxy_{hyper}$	+	+	−	+	−	−
		ESBLs						
K. oxytoca	1	CTX-M-1-group	+	+	−	−	+	+
K. oxytoca	1	CTX-M-1-group	+	+	−	+	−	+
K. oxytoca	2	CTX-M-1-group	+	+	+	+	+	+
K. oxytoca	1	SHV-ESBL	+	+	+	+	+	+
		Non-ESBLs						
K. pneumoniae	1	$SHV\text{-}1_{hyper}$[b]	+	+	−	+	−	+
K. pneumoniae	1	SHV-1	+	−	−	−	−	−
K. pneumoniae	1	SHV-1	−	−	−	+	−	−
K. pneumoniae	1	$AmpC_{plasmid}$[d]	+	+	+	−	−	−
		ESBLs						
K. pneumoniae	9	CTX-M	+	+	+	+	+	+
K. pneumoniae	1	CTX-M	−	+	+	+	+	+
K. pneumoniae	1	CTX-M	+	+	+	−	+	+
K. pneumoniae	8	SHV-ESBL	+	+	+	+	+	+
K. pneumoniae	1	SHV-ESBL	+	+	+	−	+	+
K. pneumoniae	1	SHV-ESBL	+	+	−	+	−	+
K. pneumoniae	1	SHV-ESBL, $AmpC_{plasmid}$[c]	+	+	+	−	−	+
K. pneumoniae	1	SHV-ESBL, $AmpC_{plasmid}$[c]	+	+	+	+	+	+
		Non-ESBLs						
E. cloacae	1	$AmpC_{wildtype}$	−	−	+	−	−	−
E. cloacae	1	$AmpC_{wildtype}$	−	−	−	−	−	−
E. aerogenes	1	$AmpC_{wildtype}$	−	−	+	−	−	−
E. cloacae	1	$AmpC_{hyper}$	−	−	+	−	−	−
E. cloacae	5	$AmpC_{hyper}$	−	+	−	−	−	−
E. cloacae	2	$AmpC_{hyper}$	−	+	+	−	−	−
E. cloacae	1	$AmpC_{hyper}$	−	+	+	−	−	−
E. aerogenes	2	$AmpC_{hyper}$	−	−	+	−	−	−
C. freundii	3	$AmpC_{hyper}$	−	−	+	−	−	−
		ESBLs						
E. cloacae	1	CTX-M	−	+	+	+	+	−
E. cloacae	1	SHV-ESBL, CTX-M	−	+	+	+	+	−
E. cloacae	1	SHV-ESBL, CTX-M	−	+	+	+	−	−
E. cloacae	1	SHV-ESBL, CTX-M	−	+	+	+	+	+
E. cloacae	2	SHV-ESBL	−	+	+	+	+	+
E. aerogenes	1	SHV-ESBL	−	+	+	−	−	−
E. aerogenes	2	CTX-M	−	+	+	+	+	+
C. freundii	1	CTX-M	−	−	+	+	+	+
		Non-ESBLs						
P. mirabilis	1	Not detectable	−	−	−	−	−	−
P. mirabilis	1	TEM-1	−	−	−	+	−	−
P. vulgaris	1	Chromosomal enzyme	−	−	−	+	+	−
		ESBLs						
P. mirabilis	1	TEM-ESBL	−	+	−	+	+	+
P. mirabilis	2	CTX-M	−	+	+	+	+	+
P. mirabilis	1	CTX-M	−	+	+	+	+	+

[a] +, ESBL positive; −, ESBL negative; NA, no expert analysis available.
[b] Subscript hyper indicates that the respective chromosomal enzyme was hyperproduced.
[c] Isolates were positive for bla_{CMY}-specific PCR.
[d] Isolate was positive for bla_{AmpC} PCR.

TABLE 2. Sensitivities, specificities, PPV, and NPV of phenotypic methods for detection of ESBL production with molecular identification as the reference method for all isolates (n = 144)

Detection method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MicroScan	83.5	72.9	81.6	75.4
Phoenix	98.8	52.2	75.0	96.6
VITEK 2	85.9	78.0	84.9	79.3
DAM	94.1	81.4	87.9	90.6
DDS	92.9	96.6	97.5	90.5
Etest ESBL	94.1	84.7	89.9	90.9

TABLE 4. Sensitivities, specificities, PPV, and NPV of phenotypic methods for detection of ESBL production with molecular identification as the reference method for *K. oxytoca* isolates (n = 14)

Detection method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MicroScan	100.0	11.1	38.5	100.0
Phoenix	100.0	0.0	35.7	ND[a]
VITEK 2	60.0	88.9	75.0	80.0
DAM	80.0	55.6	50.5	83.3
DDS	80.0	88.9	80.0	88.9
Etest ESBL	100.0	22.2	41.7	100.0

[a] ND, not determined.

isolates were also excluded, leaving 124 isolates for the comparative performance analysis.

With the exception of one *P. mirabilis* isolate that lacked any detectable β-lactamase activity, non-ESBL β-lactamases were identified in all remaining isolates (n = 38; *E. coli*, 6 isolates; *K. oxytoca*, 9; *K. pneumoniae*, 4; *E. aerogenes*, 3; *E. cloacae*, 11; *C. freundii*, 3; *P. mirabilis*, 2). These were either endogenous enzymes or secondary β-lactamases that were not classified as ESBL (broad-spectrum enzymes such as TEM-1 or SHV-1 and plasmid-encoded AmpCs). For 17 *Enterobacter* spp. and *C. freundii* isolates, SDS-PAGE of the crude β-lactamase extracts showed only one high-molecular-mass band of ~39 kDa, characteristic for the species-specific chromosomal enzymes. Isolates were classified as AmpC hyperproducers if they showed a >10-fold higher specific β-lactamase activity than an AmpC wild-type reference strain, and 14 hyperproducers and 3 wild-type strains were identified. IEF and β-lactamase activities revealed that 8 *K. oxytoca* isolates (57%) were hyperproducing their chromosomal Koxy enzymes (different pI groups).

Performance of semiautomated systems. The sensitivities, specificities, PPV, and NPV of the three semiautomated species identification and susceptibility testing systems for the presumptive and/or definite identification of ESBL-producing *Enterobacteriaceae* are shown in Tables 2 to 7. For the entire collection of isolates (n = 144) (Table 2), the system with the highest sensitivity for the detection of ESBLs was the Phoenix (98.8%), followed by the VITEK 2 (85.9%) and the MicroScan (83.5%). However, the Phoenix system identified ESBL-producing *Enterobacteriaceae* isolates with a specificity of only 52.2% (PPV, 75%), while the specificity was higher with the MicroScan (72.9%; PPV, 81.6%) and the VITEK 2 system (78%; PPV, 84.9%). The performance of the semiautomated systems differed widely with the species investigated. All systems identified ESBL-producing *E. coli* and *Klebsiella* spp., i.e., those species that have been recommended by the CLSI for routine screening, with sensitivities of 100% (Phoenix), 98.6% (MicroScan), and 84.5% (VITEK 2), irrespective of the β-lactamase family involved (Tables 3, 4, 5, and 6). The specificities for the different systems ranged from 51.5% (MicroScan and Phoenix) to 93.6% (VITEK 2). The MicroScan and the Phoenix system were both unable to reliably differentiate between ESBL-producing *K. oxytoca* and Koxy β-lactamase hyperproducers (specificity for ESBL detection, 11.1 and 0%; PPV, 38.5 and 35.7%, respectively). The VITEK 2, in contrast, misidentified only one of eight Koxy β-lactamase hyperproducers as ESBL producers (specificity, 88.90%; PPV, 75%). Also, the Phoenix and the VITEK 2 identified the 10 ESBL producers among *Enterobacter* and *Citrobacter* spp. with sensitivities of 90 and 100%, respectively (Table 7). However, these systems could not reliably identify AmpC producers that were not ESBL producers. The software of the MicroScan expert system, in contrast, does not permit identification of ESBL producers among *Enterobacteriaceae* other than *E. coli* and *Klebsiella* spp.

Performance of conventional tests. The performance characteristics of conventional phenotypic ESBL confirmatory tests are shown in Tables 2 to 7. Their sensitivities ranged from 92.9 to 94.1%. The DDS test showed the highest specificity and PPV among all test methods, 96.6% and 97.5%, respectively. For species other than *K. oxytoca*, the specificity and PPV were both 100%. The performance of the Etest ESBL was comparable to the DAM.

TABLE 3. Sensitivities, specificities, PPV, and NPV of phenotypic methods for detection of ESBL production with molecular identification as the reference method for *E. coli* isolates (n = 61)

Detection method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MicroScan	100.0	72.2	89.6	100.0
Phoenix	100.0	72.2	89.6	100.0
VITEK 2	81.4	100.0	100.0	69.2
DAM	100.0	83.3	93.5	100.0
DDS	97.7	100.0	100.0	94.7
Etest ESBL	97.7	94.4	94.4	97.7

TABLE 5. Sensitivities, specificities, PPV, and NPV of phenotypic methods for detection of ESBL production with molecular identification as the reference method for *K. pneumoniae* isolates (n = 29)

Detection method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MicroScan	95.7	50.0	88.0	75.0
Phoenix	100.0	66.7	92.0	100.0
VITEK 2	95.7	83.3	95.7	83.3
DAM	87.0	66.7	90.9	57.1
DDS	91.3	100.0	100.0	75.0
Etest ESBL	100.0	83.3	95.8	100.0

TABLE 6. Sensitivities, specificities, PPV, and NPV of phenotypic methods for detection of ESBL production with molecular identification as the reference method for *E. coli*, *K. oxytoca*, and *K. pneumoniae* isolates ($n = 104$)

Detection method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MicroScan	98.6	51.5	81.4	94.4
Phoenix	100.0	51.5	81.6	100.0
VITEK 2	84.5	93.9	96.8	73.8
DAM	94.4	72.7	88.1	85.7
DDS	94.4	97.0	98.5	88.9
Etest ESBL	98.6	72.7	88.6	96.0

TABLE 7. Sensitivities, specificities, PPV, and NPV of phenotypic methods for detection of ESBL production with molecular identification as the reference method for *Enterobacter*, *Citrobacter*, and *Serratia* sp. isolates ($n = 28$)

Detection method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MicroScan	0.0	ND[a]	ND	ND
Phoenix	90.0	33.3	42.9	85.7
VITEK 2	100.0	38.9	47.6	100.0
DAM	90.0	100.0	100.0	94.7
DDS	80.0	100.0	100.0	90.0
Etest ESBL	60.0	100.0	81.8	81.8

[a] ND, not determined.

DISCUSSION

Correct identification of ESBL-positive *Enterobacteriaceae* in due time is mandatory not only for optimal patient management but also for immediate institution of appropriate infection control measures to prevent the spread of these organisms (25). The use of semiautomated systems for identification and antimicrobial susceptibility testing of gram-negative rods is now common practice in many laboratories. The performance of these systems with respect to ESBL identification in comparison to conventional methods such as the DAM, DDS, and Etest ESBL has been studied previously (9, 10, 11, 20, 22, 23, 26). Our investigation differs from those of other researchers in that (i) instead of evaluating a single system, we compared side-by-side the three semiautomated systems that are currently commercially available as well as the three most commonly used manual methods; (ii) we used contemporary isolates that were consecutively collected from routine clinical specimens in a 3-month period prior to this study instead of using a well-characterized strain collection of challenge strains with known β-lactamase types; and (iii) investigators were blinded to whether the isolate was an ESBL producer or not. Our study design is therefore suitable to optimally reflect daily clinical practice.

Of note, we evaluated the semiautomated systems only with regard to both definite and suspected ESBL detection. We did not consider the way different expert systems interpreted or changed MICs, nor did we consider any written alert or clinical advice given by the systems. Separately available confirmation panels were not included in this study. In addition, the DAM, DDS, and Etest ESBL that have been established as confirmatory test methods were not applied in a typical routine two-step procedure but instead were used in parallel to the semiautomated screening methods.

The performance of ESBL detection systems can be significantly influenced by the composition of the strain collection that is used for evaluation (10). As the strains were not collected at one single institution but at three different institutions, a higher diversity of ESBLs could be expected. The variety of different TEM-ESBL enzymes detected in this study supports this assumption. Unlike previous investigators, we also included a considerable number of *K. oxytoca* isolates that turned out to be hyperproducers of Koxy β-lactamase as well as *Enterobacter* and *Citrobacter* isolates that are known for their propensity to overexpress AmpC β-lactamases.

Among the automated systems, the BD Phoenix exhibited the highest sensitivity for the identification of ESBL-producing *Enterobacteriaceae* and detected all but one of the 85 ESBL producers in our collection. But although the test panel that we evaluated included the testing of cephalosporin-clavulanate combinations currently recommended for confirmation of ESBLs, the overall specificity of the method was only 52%. The specificity was particularly low when *K. oxytoca* and *E. cloacae* isolates were evaluated. The MicroScan and the Vitek 2 systems, in contrast, detected only 83.5% and 85.9% of the ESBL-carrying isolates; however, they did so with a higher specificity of 72.9% and 78%, respectively. If only those species were considered that have been recommended by the CLSI for routine screening for ESBLs and for which the semiautomated ESBL test systems have primarily been developed and evaluated, i.e., *E. coli* and *Klebsiella* spp., the performance of the semiautomated systems is not significantly different from the overall performance. In fact, a high percentage of putative ESBL producers were shown to be hyperproducers of their chromosomal enzymes, i.e., 57% of *K. oxytoca*, 52% of *Enterobacter* spp. and *C. freundii*, 6% of *E. coli*, and 4% of *K. pneumoniae* isolates; all were identified as ESBL positive by at least one of the different methods.

The insufficient discrimination of Koxy hyperproducers from ESBL producers by the Phoenix has been described previously (20) and was attributed to an incorrect placement of ceftazidime in the ESBL test algorithm (26). However, relying on ceftazidime susceptibility alone for the discrimination between Koxy hyperproducers and ESBL producers can be misleading. CTX-M enzymes, more closely related to Koxy enzymes than to other plasmid-encoded ESBLs (12), are spreading rapidly in many parts of the world (13). Many of these enzymes are characterized by ceftazidime susceptibility. Therefore, some CTX-M enzymes show a hydrolytic profile different from other plasmid-encoded ESBLs, e.g., many TEM enzymes (13). The VITEK 2 system reliably differentiated between Koxy hyperproducers and ESBL-producing *K. oxytoca.* The VITEK 2 misidentified only one of the Koxy hyperproducers included in our strain collection as ESBL positive but misclassified two ceftazidime-sensitive *K. oxytoca* isolates carrying CTX-M enzymes as presumptive Koxy hyperproducers.

The MicroScan's expert system discriminates between ESBL producers and Koxy hyperproducers on the basis of ceftazidime susceptibility of the latter. The MicroScan panel used in this study correctly identified all ESBL-producing *K. oxytoca* isolates but misclassified 7 of 8 Koxy hyperproducers as ESBL positive and did not point to the possibility of Koxy production.

Currently, the CLSI does not recommend screening *Enterobacteriaceae* isolates other than *E. coli* and *Klebsiella* spp. for ESBL production. Screening of *P. mirabilis* is only recommended for isolates recovered from the bloodstream (3). However, as shown here, ESBL production in *Enterobacter*, *Citrobacter*, and *Proteus* spp. is no longer an exception, and methods to detect these organisms are needed. Automated detection of ESBLs in these species which produce inducible, overexpressed, or plasmid-encoded AmpC β-lactamases can be challenging, as high levels of AmpC can mask the inhibition of ESBL by CA. Our study showed that the BD Phoenix and the VITEK 2 system detected ESBLs among *Enterobacter* spp. and *C. freundii* isolates with sensitivities of 90% and 100%, respectively. However, both systems showed a rather low specificity with numerous false ESBL-positive results reported. The MicroScan's software, in contrast, did not allow detection of ESBLs in *Enterobacter* spp. and *C. freundii*.

Unlike previous investigators who have evaluated ESBL screening systems primarily with regard to their sensitivity for ESBL detection, we also analyzed specificity and predictive values. Considering the necessary isolation procedures, once an organism has been identified as ESBL positive, we think that both specificity and PPV, although dependent on the background ESBL prevalence, are also valuable performance indicators for semiautomated ESBL test systems.

The performance characteristics of conventional methods for the detection of ESBLs that are based on agar diffusion (DAM, DDS test, and Etest ESBL) were better than those of the automated systems. Overall, the conventional test methods showed comparable sensitivities, ranging from 92.9% to 94.1%. Single-indicator cephalosporin usage is known to result in low sensitivity (4, 14, 28), but even with three (Etest) or four (DAM, DDS) different indicator cephalosporins, we did not achieve 100% sensitivity with any of these tests. However, only the DDS test was also highly specific (96.6%), while the other methods were less specific (81.4% and 84.7%, respectively), leading to a high number of false-positive isolates among the presumptive ESBL producers in our collection. Misidentification of extended-spectrum β-lactamases in *K. oxytoca* by several test systems is a well-known phenomenon (19). In our survey, the Etest in particular was not able to reliably differentiate between ESBL-positive and Koxy-hyperproducing *K. oxytoca* isolates, i.e., 87.5% (7/8) of the hyperproducers in our survey were classified as ESBL positive, supporting previous results that both cefotaxime and cefepime ESBL Etests frequently misidentify Koxy-hyperproducing isolates as ESBL producers (19, 24). Although the ESBL prediction of the DDS is also based on the increase in susceptibility resulting from the inhibition of the enzyme by CA, this test performed better, with a specificity of 88.9%.

The three conventional test methods used in this study, all relying on clavulanate synergy, included one extended-spectrum cephalosporin each (cefepime or cefpirome) that is resistant to AmpC hydrolysis, thus allowing for the detection of ESBLs in *Enterobacter* and *Citrobacter* spp. All three test methods showed similar sensitivities (60 to 90%) for detection of ESBLs in *Enterobacter* spp. and *C. freundii*. However, in the present study, the cefepime ESBL Etest was less accurate than reported previously (24).

In summary, the performance of the three semiautomated systems for the detection of ESBL-positive enterobacteria was variable, particularly with organisms such as Koxy-hyperproducing *K. oxytoca* and AmpC-producing *Enterobacter* and *Citrobacter* spp. Considering the rather low specificity observed in the current study, we recommend the use of a manual test for confirmation once an organism is reported positive for ESBL production by any of the semiautomated systems. Alternatively, one can use one of the test panels developed by the manufacturers of the semiautomated systems specifically for confirmation of ESBL production. Integration of an ESBL confirmation test into the routine test panels of the semiautomated systems would considerably reduce the time to accurate ESBL detection in the laboratory and might contribute to earlier institution of optimal antibiotic therapy and adequate infection control procedures.

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