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Evaluation of the Etest ESBL and the BD Phoenix, VITEK 1, and VITEK 2 Automated Instruments for Detection of Extended-Spectrum Beta-Lactamases in Multiresistant *Escherichia coli* and *Klebsiella* spp.

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Seventy-four isolates of multiresistant *Escherichia coli* and *Klebsiella* spp. recovered during a 3-year period and 17 control strains with genotypically identified beta-lactamases were tested for the production of extended-spectrum beta-lactamases (ESBLs) by using the Etest and the VITEK 1, VITEK 2, and Phoenix automated instruments. The use of the Etest was evaluated by investigating its accuracy in detecting the ESBLs of the control strains and by comparing interpretation results of laboratory technicians and experts. The accuracy of the Etest was 94%. With the Etest as the reference for the clinical strains and the genotype as the reference for the control strains, the automated instruments detected the ESBLs with accuracies of 78% (VITEK 2), 83% (VITEK 1), and 89% (Phoenix). No significant difference between the systems with regard to the control strains was detected. The VITEK 2 did, however, perform less well than the Phoenix ($P = 0.03$) on the collection of clinical isolates, mainly because of its high percentage of indeterminate test results (11%). No significant difference between the performances of the VITEK 1 and either the VITEK 2 or the Phoenix was found. However, because of its associated BDXpert system the Phoenix showed the best performance. The Etest was found to be an accurate test but was limited by its indeterminate results (4%), its inability to differentiate between K1 hyperproduction and ESBLs, questionable guidelines concerning mutants inside the inhibition zones, and the inability of the technicians to recognize subtle zone deformations.

Members of the *Enterobacteriaceae* commonly express plasmid-encoded β-lactamases (mostly TEM-1, TEM-2, and SHV-1) that confer resistance to penicillins but not to expanded-spectrum cephalosporins. In the mid-1980s, however, bacteria that also express resistance to the expanded-spectrum cephalosporins were detected. Investigation into the mechanism of this resistance revealed that mutations had occurred within the structural genes encoding the older enzymes, which altered the configuration of the enzymes near their active sites. This shape change was found to increase the enzyme's affinity to and hydrolytic ability for oxyimino-cephalosporins (e.g., ceftazidime and cefotaxime) as well as for older penicillins and cephalosporins. These new enzymes were given the name extended-spectrum beta-lactamases (ESBLs) to reflect the fact that they are derivatives of older enzymes but have the capability to hydrolyze a broader spectrum of beta-lactam drugs (24). In recent years, more plasmid-mediated broad-spectrum beta-lactamases, such as the CTX-M-type enzymes (3, 6), have appeared. These enzymes are not evolved from previously known smaller-spectrum beta-lactamases but are thought to be unaltered from their original chromosomally encoded forms. Recent literature generally refers to these acquired enzymes (with exception of the AmpC enzymes and carbapenemases) as ESBLs as well, in contrast to the chromosomally encoded broad-spectrum beta-lactamases (e.g., K1 in *Klebsiella oxytoca* and AmpC in *Enterobacter* spp.) (3, 8, 16, 24). The present study uses this extended definition of ESBL.

Generally, ESBL-producing microorganisms exhibit high-level resistance to benzylpenicillins and narrow-spectrum cephalosporins. The MICs for aztreonam and expanded-spectrum, broad-spectrum, and "fourth-generation" cephalosporins (e.g., cefpirome, cefepime), however, vary greatly, because the various mutations confer different phenotypic expressions. ESBL-mediated resistance, therefore, poses problems for in vitro susceptibility testing, first, because of the limited number of cephalosporins routinely tested in the laboratory, and second, because the MICs may not reach National Committee for Clinical Laboratory Standards (NCCLS) breakpoints for resistance when the standard inoculum of 10^5 CFU/ml is used. Both in vitro and animal studies, however, have shown that the ESBL-producing organisms may become resistant as the inoculum increases (10, 17, 29, 30). Reported treatment failures, suboptimal clinical outcome, and the increased mortality of patients infected with ESBL-producing strains that have MICs in the susceptible range and treated with cephalosporins, suggest that this inoculum effect occurs in clinical infections as well (21, 27, 28). Therefore, it is recommended and generally accepted that ESBL-producing isolates (even when MICs are in the susceptible range) be reported as resistant to all penicillins, cephalosporins, and aztreonam (14, 18, 21). Whether beta-lactam–beta-lactamase inhibitor combinations should be reported as resistant is still unclear (4, 22, 29). An additional reason for detecting the production of ESBLs in the clinical laboratory is the discovery that infections with ESBL-produc-

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ing *Escherichia coli* and *Klebsiella pneumoniae* are associated with significantly longer hospital stays (12, 27) and greater hospital costs (12). The NCCLS recently provided guidelines for performing standard broth microdilution or disk susceptibility testing and confirmatory tests for the detection of ESBL production in *E. coli*, *K. pneumoniae*, and *K. oxytoca* (18). If these guidelines are followed, however, the detection of ESBL production becomes an elaborate and costly procedure in the daily routine of a large microbiology laboratory. Automated susceptibility testing systems with an additional test for the detection of ESBL production, therefore, are a welcome asset in such laboratories. There are three automated systems currently available in Europe that include an ESBL detection test: (i) the VITEK 1 system (bioMérieux S.A., Marcy-l'Etoile, France), (ii) the VITEK 2 system (bioMérieux S.A.), and (iii) the recently developed Phoenix automated microbiology system (Becton Dickinson Biosciences, Sparks, Md.).

The aim of this study was to evaluate the abilities of the VITEK 1, VITEK 2, and Phoenix systems in detecting ESBL production by a collection of multiresistant isolates of *E. coli* and *Klebsiella* spp. and by a number of strains with genotypically identified beta-lactamases. The results of the automated systems were then compared by using the Etest ESBL (AB Biodisk, Solna, Sweden) as the reference for the clinical strains and the genotype as the reference for the control strains. In addition, the use of the Etest ESBL for the detection of ESBLs in the clinical laboratory was evaluated by investigating its accuracy in detecting the genotypically identified beta-lactamases and by comparing the reading and interpretation results of laboratory technicians and experts.

MATERIALS AND METHODS

Bacterial isolates. The 74 multiresistant *Enterobacteriaceae* isolates (34 *E. coli*, 26 *K. pneumoniae*, and 14 *K. oxytoca* isolates) included in this study were obtained from 49 patients admitted to the University Hospital Utrecht between 1994 and 1996. Ten patients carried more than one species. The main criterion for selection was either a VITEK 1 ESBL-positive result ($n = 42$) or a VITEK 1 ESBL-negative result combined with a phenotype suspect of ESBL production (i.e., resistance to a cephalosporin combined with resistance to at least two other classes of antibiotics) ($n = 25$). Seven multiresistant but cephalosporin-susceptible VITEK 1 ESBL-negative strains from patients from whom another Vitek 1 ESBL-positive isolate had been collected were also included. Isolates of the same species collected from the same patient were included only if the VITEK 1 had yielded opposite outcomes with regard to ESBL production (14 isolates from seven patients).

Controls included 17 isolates with genotypically identified beta-lactamases: eight non-ESBLs (OXA-1, OXA-2, OXA-3, TEM-1 [two strains], TEM-2 [two strains], K1 [KOXY] hyperproduction) and nine different ESBLs (TEM-3, TEM-4, TEM-5, TEM-7, TEM-9, TEM-10, SHV-2a, SHV-4, SHV-5), kindly provided by E. E. Stobberingh (University Hospital Maastricht, Maastricht, The Netherlands). We confirmed the identification of the TEM- and SHV-beta-lactamases by PCR and subsequent sequencing. When these PCRs were performed on the isolates with OXA and KOXY beta-lactamases, no amplicons were obtained.

Detection and identification of bla_{SHV} and bla_{TEM} genes. The presence of bla_{SHV} and bla_{TEM} genes was determined for all clinical isolates for which at least one of the three automated systems or the Etest ESBL yielded discordant results in the ESBL test. In addition, a collection of randomly chosen clinical isolates, for which the outcome of all methods was the same, was analyzed in the same way. The mutants were also analyzed to determine whether single colonies within the inhibition zone of the Etest ESBL carried the same ESBL genotype as the mother strain.

PCR primers for bla_{SHV} and bla_{TEM} gene amplification (GenBank accession no.: for bla_{TEM}, J01749; for bla_{SHV}, AF124984) were bla_{TEM}-F (5′-CGTGTCG CCCTTATTCCC-3′), bla_{TEM}-R (5′-AGGCACCTATCTCAGCGATC-3′), bla_{SHV}-F (5′-ATGCGTTATATTCGCCTGTG-3′), and bla_{SHV}-R (5′-TTAGCG TTGCCAGTGCTC-3′). The PCR conditions for TEM were as follows: 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. Those for SHV were 5 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 s at 57°C, and 2 min at 72°C, followed by an extension of 5 min at 72°C. The amplified DNA was then purified by using MicroSpin G-25 columns (Pharmacia). In addition to the amplification primers, the following primers were used for double-stranded sequencing in an ABI 377 automated sequencer (Applied Biosystems): bla_{TEM}-F (5′-ACAACG ATCGGAGGACCG-3′), bla_{TEM}-R (5′-GCGGTTAGCTCCTTCGGT-5-3′), bla_{SHV}-F (5′-CCGCAGGATTGACTGCC-3′), and bla_{SHV}-R (5′-AGGCGGGT GACGTTGTC-3′). The resulting sequences were then aligned with the gene sequences available from GenBank.

Etest ESBL. The Etest ESBL test was performed in accordance with the guidelines of the manufacturer. The synergic activity of clavulanate with both ceftazidime and cefotaxime was confirmed by means of two different Etest strips containing ceftazidime and cefotaxime with or without clavulanate. Isolates were considered ESBL producers when clavulanate caused a ≥3 twofold-concentration decrease (ratio, ≥8) in the MIC of ceftazidime or cefotaxime, in combination with a ceftazidime MIC ≥1 μg/ml or a cefotaxime MIC ≥0.5 μg/ml, respectively. Additionally, a strain was considered ESBL positive if a phantom zone or a deformation of the ceftazidime and cefotaxime zone could be observed, independent of the ratios or MICs. The outcome of the test was indeterminate when both MICs were outside the test range of the test device. This phenomenon may suggest the presence of an inhibitor-resistant TEM or AmpC enzymes. The outcome was also indeterminate when the result of one strip was ESBL negative and the result of the other strip was indeterminate.

Two technicians from the clinical laboratory and two experts from the manufacturing company interpreted the inhibition zones blinded and independently of each other. The technicians from the clinical laboratory had been asked to read the Etest ESBL package insert thoroughly before conducting the analysis.

VITEK 1 analysis. VITEK cards for identification (GNI) and susceptibility testing (GNS-522) were inoculated and incubated according to the manufacturer's recommendations. The results were interpreted by using software version AMS R09.1. The substrates of the ESBL test were included in the GNS-522 panel, and the interpretation was based on the comparison between the reduction in growth caused by cefotaxime-clavulanate and ceftazidime-clavulanate and that caused by the cephalosporins alone (24). The outcome of the test was either ESBL positive or ESBL negative.

VITEK 2 analysis. VITEK cards for susceptibility testing (GNS AST-N010) were inoculated and incubated by a technician from the manufacturing company according to the manufacturer's recommendations (5). The name of the species of the strain, as determined by VITEK 1, was manually entered into the instrument. The results were interpreted by using software version VTK-R01.02, an advanced expert system (AES) designed to analyze the results generated by the VITEK 2 system (7, 14, 25, 26). The AES is based on over 2,000 phenotypes and 20,000 MIC distributions, which have been derived from the published literature, internal data generated at bioMérieux S.A., and outside experts. Once an organism has been identified and/or tested for antimicrobial susceptibility, the software provided by the AES begins to search the MIC distributions in its knowledge base to ascertain if the results are consistent with any of the phenotypes established for the organism in order to establish a biological validation. Based on this comparison, the AES determines if the identification is consistent with the susceptibility pattern and if the MICs are consistent with a specific phenotype. There are four possible phenotypes relevant to beta-lactam antibiotics: wild type, acquired penicillinase, cephalosporinase, and ESBL. Then, the AES provides one of the following comments: (i) results are fully consistent with identification; (ii) results are not fully consistent with identification; therefore, modify the susceptibility results as suggested by the AES, or retest isolate; (iii) results are not fully consistent; therefore, modify the susceptibility results, or change the identification as suggested by the AES, or retest isolate; and (iv) results are not fully consistent, and the isolate should be retested. In addition to comments i through iv, the AES may also suggest therapeutic corrections in the antibiogram. A printed report of each test indicates the actual MIC, the raw categorizations, the categorizations after interpretation, and the inferred mechanism of beta-lactam resistance. In the present study, the VITEK 2 test was repeated and a confirmatory test for identification was performed in response to comments ii to iv. In the comparative analysis with the other automated systems, all outcomes earmarked by the AES as fully consistent (either in the first or second test) were regarded as definitive outcomes. All others were regarded as indeterminate.

Phoenix analysis. NMIC/ID-5 Phoenix panels (combined susceptibility and identification card) were inoculated and incubated according to the manufacturer's recommendations. The Phoenix ESBL test uses growth response to selected

TABLE 1. Results of the Etest ESBL and the VITEK1, VITEK 2, and Phoenix systems for the control strains[a]

Beta-lactamases	Strain no.	Species	Etest ESBL	VITEK 1	VITEK 2	Phoenix
Non-ESBLs						
TEM-1	1	*E. coli*	−	−	−	−
TEM-1	2	*E. coli*	−	−	Indeterminate[b]	−
TEM-2	3	*E. coli*	−	−	−	−
TEM-2	4	*E. coli*	−	−	−	−
OXA-1	5	*E. coli*	−	−	−[b]	−
OXA-2	6	*E. coli*	−	−	−	Possible[c]
OXA-3	7	*E. coli*	−	−	−	−
K1(KOXY)	8	*K. oxytoca*	+	+	−	−
ESBLs						
TEM-3	9	*E. coli*	+	+	+	+
TEM-4	10	*E. coli*	+	+	+	+
TEM-5	11	*E. coli*	+	+	+	+
TEM-7	12	*E. coli*	+	+	+	−[d]
TEM-9	13	*E. coli*	+	+	+	+
TEM-10	14	*K. pneumoniae*	+	−	+	+
SHV-2a	15	*K. pneumoniae*	+	−	+[b]	+
SHV-4	16	*E. coli*	+	+	+	+
SHV-5	17	*K. pneumoniae*	+	+	+	+

[a] +, positive; −, negative.
[b] Result of repeated test (see also Table 4).
[c] BDXpert supplied rule no. 106: "Screening test suggests a possible ESBL producer; confirmatory testing is recommended."
[d] BDXpert supplied rule no. 1502: suggestion to report the isolate to be resistant to all beta-lactam antibiotics except carbapenems.

expanded-spectrum (cefpodoxime) and broad-spectrum (ceftazidime, ceftriaxone, cefotaxime) cephalosporins, with or without clavulanic acid, to detect the production of ESBL. The result of this test is integrated into the antibiogram through the action of the BDXpert system. This system consists of a series of rules, which are triggered by various conditions, such as the ESBL test or specific bacterial identification and antibiotic susceptibility pattern. Each rule is identified by a numeric code. When a rule is triggered, a cautionary message is appended to the AST report, and where appropriate the interpretations for individual antibiotics are modified from the interpretive result based on the MIC. The BDXpert rule associated with a positive ESBL test for *E. coli*, *K. pneumoniae*, and *K. oxytoca* is rule no. 1505: "Enterobacteriaceae with ESBLs are resistant to all beta-lactam drugs, except carbapenems." If the BDXpert system detects resistance mechanisms related to ESBLs (e.g., AmpC in *E. coli* or K1 in *K. oxytoca*), the supplementary ESBL rule no. 1502 is noted: "Enterobacteriaceae that are susceptible to a carbapenem and resistant to ureidopenicillins and 3rd generation cephems or cefpodoxime or aztreonam are also resistant to all beta-lactams, except carbapenems." Alternatively, rule no. 106 may be provided: "Screening test suggests a possible ESBL producer; confirmatory testing is recommended." If the ESBL test is negative, then no rule is supplied. A printed report of each test indicates the actual MIC, the raw categorizations, the categorizations after interpretation, and the rule applied.

API 20E identification. If the AES of the VITEK 2 detected an inconsistency in the susceptibility results and organism identification, the identification was repeated by using an API 20E test (BioMérieux S.A.).

Typing by PFGE. To determine whether single colonies within the inhibition zone of the Etest ESBL represented mutants or contamination, the genotypes of these colonies and the mother strain were compared by using pulsed-field gel electrophoresis (PFGE) as previously described (13). Isolates of the same species collected from the same patient, but with VITEK 1 results yielding opposite outcomes with regard to ESBL production, were also typed by PFGE.

Study design. Unless the system advised a retest, the first test results were used for the evaluation and comparison of the different automated systems. The susceptibility patterns of all isolates were compared before the results were included in the analysis. If the susceptibility patterns suggested the loss of plasmid, a new isolate was obtained from the stock and tested. The Etest ESBL results for the clinical isolates, determined by the manufacturers' experts, and the results of control isolate genotyping were used as references in the comparative analyses.

Statistical analysis. The accuracy (i.e., the proportion of the test panel for which the tested method obtained an outcome identical to that of the reference method), sensitivity, and specificity of each automated system were calculated. Differences in proportions were evaluated by using the chi-square test.

Nucleotide sequence accession numbers. The nucleotide sequences of the two new TEM-variants, TEM-HM and TEM-FHM, detected in this study have been deposited in the EMBL data library under accession numbers AY130285 and AY130284, respectively.

RESULTS

Evaluation of tests with regard to control strains. The results of the Etest ESBL and the three automated systems for the 17 control strains are shown in Table 1. Both the Etest ESBL and the VITEK 2 showed 100% sensitivity; sensitivity for the Phoenix was 89%, and sensitivity for the VITEK 1 was

TABLE 2. Agreement of the methods with the reference method for the detection of ESBL production[a]

Method	Control strains (n = 17)				Clinical isolates (n = 70)				Total (n = 87)			
	No. indeterminate (%)	No. accurate (%)	Sens. (%)	Spec. (%)	No. indeterminate (%)	No. accurate (%)	Sens. (%)	Spec. (%)	No. indeterminate (%)	No. accurate (%)	Sens. (%)	Spec. (%)
VITEK 1	0	14 (82)	78	87	0	58 (83)	84	81	0	72 (83)	83	82
VITEK 2	1 (6)	16 (94)	100	87	9 (13)	52 (74)[b]	68	85	10 (11)	68 (78)	74	85
Phoenix	1 (6)	15 (88)	89	87	2 (3)	62 (89)[b]	93	81	3 (3)	77 (89)	92	82
Etest ESBL	0	16 (94)	100	87								

[a] Reference method for control strains, ESBL genotype; reference method for clinical isolates, Etest ESBL. Abbreviations: sens., sensitivity; spec., specificity.
[b] Significant difference (chi-square, P = 0.03).

TABLE 3. Discordant ESBL test results obtained with the Etest ESBL and the VITEK 1, VITEK 2, and Phoenix systems on 29 multiresistant clinical isolates[a]

Strain no.	Species	Etest ESBL	VITEK 1	VITEK 2	Phoenix
18, 19, 20	*K. oxytoca*	+	+	−	+
21	*E. coli*	+	+	−	+
22, 23, 24	*E. coli*	+	+	Indeterminate	+
25, 26	*K. pneumoniae*	+	+	Indeterminate	+
27	*E. coli*	+	+	Insuff. growth	−[c]
28	*E. coli*	+	+	Insuff. growth	+
29[b]	*K. pneumoniae*	+	−	+	−[c]
30[b]	*E. coli*	+	−	+	+
31	*K. pneumoniae*	+	−	Indeterminate	+
32[b], 33[b]	*K. pneumoniae*	+	−	+	+
34	*E. coli*	+	−	−	−
35[b]	*E. coli*	+	−	−	+
36	*E. coli*	−	+	Indeterminate	−
37, 38	*K. oxytoca*	−	+	+	+
39	*K. oxytoca*	−	+	−	−
40	*K. oxytoca*	−	+	−	+
41	*E. coli*	−	−	+	Possible[d]
42[b]	*E. coli*	−	−	−	Possible[d]
43	*E. coli*	Indeterminate	−	+	Possible[d]
44	*E. coli*	Indeterminate	−	+	−[c]
45[b]	*E. coli*	Indeterminate	−	−	−[c]
46	*K. pneumoniae*	Indeterminate	−	−	−

[a] Abbreviations: +, positive; −, negative; insuff., insufficient.
[b] Isolate with identified ESBL genotype TEM-FHM (no. 29, 30, 32, 33, and 35), TEM-HM (no. 42), or SHV-2a (no. 45).
[c] Xpert supplied rule no. 1502: suggestion to report the isolate to be resistant to all beta-lactam antibiotics except carbapenems.
[d] Xpert supplied rule no. 106: "Screening test suggests a possible ESBL producer; confirmatory testing is recommended."

78% (Table 2). The specificity of all four tests was 87%. The specificity of both the Etest ESBL and the VITEK 1 was hampered due to the positive result given to a *K. oxytoca* strain (no. 8) showing hyperproduction of chromosomal K1 (KOXY) beta-lactamase. The VITEK 2 and Phoenix correctly identified this strain as ESBL negative, but each yielded an indeterminate result for another strain. The discordant results of the VITEK 2 and Phoenix are discussed together with the results of the clinical strains in Discussion.

Evaluation of tests with regard to clinical strains. The results of the Etest ESBL and the three automated systems showed agreement for 26 ESBL-positive and 19 ESBL-negative clinical isolates (19 *E. coli*, 19 *K. pneumoniae*, and 7 *K. oxytoca* isolates). At least one method yielded a discordant result for each of the remaining 29 isolates (39%) (Table 3). This will be discussed separately for each method.

(i) Etest ESBL. Interpretation of the Etest ESBL inhibition zones proved difficult for the multiresistant isolates. MICs should be read at the intersection of the inhibition ellipses with the strips. Due to the presence of mutants along the zone border, however, the intersection was not always obvious. Even the manufacturers' experts had trouble reading the MICs of four isolates. These included four clinical *K. oxytoca* isolates (no. 37 to 40) with one or two mutants in the inhibition zone of the cefotaxime-clavulanic acid strip. Excluding the mutants yielded a ratio for the cefotaxime strip >8, indicating ESBL production. Including the mutants, however, increased the denominator of the ratio in such way that the test results became negative. Three mutants possessed the same PFGE type as the mother strain; the PFGE pattern of the fourth mutant (no. 40) differed from that of the mother strain by two bands. Neither a TEM nor an SHV ESBL genotype was identified in any of these mutants or mother isolates. Repetition of the Etest with the mutants yielded MICs of ceftazidime ranging from 0.75 to 3 mg/liter, MICs of ceftriaxone ranging from 3 to 5 mg/liter, and a negative ESBL test result in both strips. These findings are not compatible with an ESBL or a K1 hyperproducer. Therefore, the mutants most likely lost the plasmid encoding an ESBL, reverted to a non-ESBL genotype, or acquired an additional mutation elsewhere. The experts decided to regard these four isolates as ESBL negative (Table 3).

The results of the Etest were indeterminate for four clinical isolates (4%). Two were *E. coli* isolates (no. 43 and 44) for which the MICs were above the test ranges. The other two were an *E. coli* isolate (no. 45) and a *K. pneumoniae* isolate (no. 46) collected from the same patient. The ratios for cefotaxime/cefotaxime-clavulanic acid were <12, while the ceftazidime strips yielded negative results. A ratio of <12 is indeterminate, because it can imply a ratio of either ≥8 (ESBL positive) or <8 (ESBL negative). None of the automated systems detected ESBL production in isolates 45 and 46.

(ii) VITEK 1 analysis. Susceptibility testing of the 74 clinical isolates gave the following results: 90% of the isolates were resistant or intermediately susceptible to gentamicin, 73% to tobramycin, 3% to amikacin, 84% to cotrimoxazole, 94% to ampicillin, 82% to piperacillin, 83% to amoxicillin-clavulanic acid, 89% to cephalothin, 45% to cefuroxime, 28% to ceftriaxone, 15% to ceftazidime, and 14% to ciprofloxacin. No decreased susceptibility to meropenem was detected.

Inherent to the design of the VITEK 1 system, no indeterminate results were obtained with regard to ESBL production. For seven patients, the VITEK 1 ESBL test yielded opposite outcomes for two consecutive clinical isolates of the same species collected at different times in the hospital. The Etest

ESBL, however, indicated the presence of ESBL production in both members of four pairs of these isolates, indicating a false-negative result of the VITEK 1 (no. 30, 32, 34, and 35). The Etest ESBL confirmed the VITEK 1 results for the other three pairs, indicating the acquisition or loss of the ESBL-encoding gene(s) by the isolates during colonization or infection of the patient.

(iii) VITEK 2 analysis. Of the 91 isolates tested (74 clinical isolates, 17 control strains), the AES found the results for 73 (80%) to be fully consistent with the organism identification, suggesting no biological corrections or necessary repetitions of identification by testing. Two isolates (2%) failed to grow sufficiently in the instrument. The AES reported inconsistencies between the susceptibility results and the organism in 16 isolates (18%) and made the following recommendations: (i) antibiogram correction regarding a single MIC (11 isolates) or repeat of test, (ii) correction of a single MIC or change of identification in the proposed species or repeat of test (1 isolate), and (iii) repeat of the test (4 isolates) (Table 4). The test, therefore, needed to be repeated for 18 (20%) of the 91 isolates tested; this occurred for 17 isolates. Retesting provided full consistency for 7 of the 17 isolates (41%). The outcome for 7 (41%) of the remaining 10 isolates was the same as in the first test, while it changed for the other three strains from "inconsistency" to "repeat the test," from "insufficient growth" to "inconsistency," and from "repeat the test" to "insufficient growth," respectively. Thus, the final result was indeterminate for 10 isolates (11%), including 1 control and 9 clinical isolates (Table 2).

For all 16 isolates for which the AES detected an inconsistency between the susceptibility results and the organism identification, the identification was repeated with the API 20E test. This included two isolates (no. 2 and 5) (Table 4) for which the AES had proposed an alternative species. The API 20E test confirmed the initial identification of all isolates. In conclusion, repetition of the identification test in the case of a detected inconsistency by the AES proved to be of no value and the proposed alternative species were incorrect.

The therapeutic corrections suggested by the AES in this study were limited to two suggestions. The first was to report the ESBL-positive strains as being resistant to all penicillins (including piperacillin) and cephalosporins except cefoxitin. The second was to change the susceptibility report for cefoxitin from S (susceptible) to I (intermediately resistant) (no. 2 and 48) (Table 4).

(iv) Phoenix analysis. Of the 91 isolates tested (74 clinical isolates, 17 control strains), the outcome was indeterminate for 4 (4%), and in those cases rule no. 106 suggested the performance of a confirmatory test. The BDXpert system noted rule no. 1502 for five isolates, i.e., the isolates are resistant to all beta-lactam antibiotics except carbapenems. The outcome of the Etest ESBL was indeterminate for two of these five isolates (no. 44 and 45). The other three (no. 12, 27, and 29) represented three of the four isolates for which the Phoenix failed to detect an ESBL in comparison with the Etest ESBL. This indicates that the BDXpert system was able to compensate partially for the false-negative results.

Comparative analysis of the automated systems. The results of the comparative analysis between each of the automated systems and the reference method are shown in Table 2. For the comparative analysis of the automated systems, the number of clinical isolates was reduced to 70 after the 4 clinical isolates for which the Etest ESBL found an indeterminate result were removed. No significant difference between the performances of the systems was detected when the control strains were tested. When testing the clinical isolates, however, the accuracy of the ESBL test of the VITEK 2 AES (74%) was significantly lower (chi-square test, $P = 0.03$) than that of the Phoenix (89%). This was mainly due to the high number of indeterminate test results by the VITEK 2, because removal of the isolates from the test panel for which the VITEK 2 provided indeterminate results did increase the accuracy of the VITEK 2 to 85%. No significant difference between the performances of the VITEK 1 and either the VITEK 2 or the Phoenix was found. The specificities of the three tests did not show significant differences. It should be noted, however, that by excluding the mutants in the cefotaxime-clavulanic acid ellipse in the Etest ESBL of the four *K. oxytoca* isolates mentioned above (no. 37 to 40), the specificities of the VITEK 1, the VITEK 2, and the Phoenix rose to 95, 90, and 91%, respectively. This had little influence, however, on the sensitivities of the systems (85, 67, and 92%, respectively).

Identification of the beta-lactamases of the clinical isolates. Fifteen (56%) of the 26 isolates for which all methods yielded a positive ESBL test result were analyzed. An ESBL genotype was identified for 10 (67%) of them: SHV-5 (*K. pneumoniae*), SHV-2a *(K. pneumoniae*, 2 isolates; and *E. coli*), TEM-FHM (*K. pneumoniae*, 2 isolates; and *E. coli*, 2 isolates), TEM-HM (*E. coli*), and the combination of TEM-FHM and SHV-2a (*K. pneumoniae*). TEM-FHM and TEM-HM are new TEMs with amino acid substitutions that have been described before for multiple ESBLs, but not in these particular combinations (http://www.lahey.org/studies/webt.htm). Both mutants possess an Arg164His and Thr265Met mutation, while TEM-FHM contains an additional Lys21Phe mutation. Since the Arg164His mutation is considered to be an important mutation for the production of an ESBL phenotype (3, 11), we considered these TEMs as possible ESBLs. No TEM- or SHV-derived ESBL genotypes could be identified for the remaining five isolates (33%).

Eleven (58%) of the 19 isolates for which all methods yielded a negative or indeterminate ESBL test result were then analyzed. An SHV- or TEM-derived ESBL genotype could not be identified in any of them. Of the 18 isolates with a positive Etest ESBL result and a negative result with at least one of the other tests, 5 (28%) were found to carry an ESBL genotype (TEM-FHM) (Table 3). Of the seven isolates with a negative ESBL-Etest result and a positive result with at least one of the other tests, one was found to carry a TEM-HM genotype (no. 42). Of the four isolates with an indeterminate ESBL-Etest result, one (no. 45) contained an SHV-2a genotype. No ESBL genotype could be identified in the other three isolates.

Evaluation of the Etest ESBL in the routine laboratory. The Etest ESBL was evaluated by comparing the interpretations of the experts with those made by two laboratory technicians. Comparing the MICs found by the experts with those found by the technicians revealed differences for 9% of the strips without clavulanic acid and for 29% of the strips with clavulanic acid; these differences were due to the presence of mutants in the inhibition zone. This resulted in different final test results

TABLE 4. Outcome of VITEK 2 Advanced Expert System for 18 isolates that had had indeterminate outcomes in the first test[a]

Strain no.	Species[b]	Etest ESBL	First VITEK 2 testing				Repeated VITEK 2 testing			
			Outcome	Proposed antibiogram correction[c]	Proposed change in identification	ESBL test result	Outcome	Proposed antibiogram correction	Proposed change in identification	ESBL test result
5	*E. coli*	Neg	Inconsistent	Cefepime (2S to ≤0.5S)	*Citrobacter* spp.[d]	Neg	Full consistency			Neg
47	*E. coli*	Pos	Inconsistent	Amoxicillin-clavulanic acid (4S to ≥16I)	None	Neg	Full consistency			Pos
48	*E. coli*	Pos	Inconsistent	Cefoxitin (≤4S to ≥16I)	None	Neg	Full consistency			Pos
15	*K. pneumoniae*	Pos	Inconsistent	Meropenem (2S to ≤0.5S)	None	Pos	Full consistency			Pos
49	*E. coli*	Neg	Inconsistent	Repeat test			Full consistency			Neg
50	*K. pneumoniae*	Neg	Inconsistent	Repeat test			Full consistency			Neg
51	*K. pneumoniae*	Pos	Inconsistent	Repeat test			Full consistency			Pos
22, 23, 36	*E. coli*	Pos, pos, neg	Inconsistent	Ceftazidime (≤1S to ≥2R)	None	Pos	Inconsistent	Ceftazidime (≤1S to ≥2R)	None	Pos
25, 26, 31	*K. pneumoniae*	Pos, pos, pos	Inconsistent	Ceftazidime (≤1S to ≥2R)	None	Pos	Inconsistent	Ceftazidime (≤1S to ≥2R)	None	Pos
24	*E. coli*	Pos	Inconsistent	Ceftazidime (≤1S to ≥2R)	None	Pos	Repeat test			
52	*K. oxytoca*	Pos	Inconsistent	Cefoxitin (32R to ≤8R)	None	Pos	Not done			
28	*E. coli*	Pos	Repeat test				Insufficient growth			
27	*E. coli*	Pos	Insufficient growth				Insufficient growth			
2	*E. coli*	Neg	Insufficient growth				Inconsistent	Cefoxitin (≤4S to ≥16I)	*Shigella sonnei*	Neg

[a] Abbreviations: pos, positive; neg, negative.
[b] Identification determined by API 20E.
[c] Correction indicated by value of MIC (in micrograms per milliliter); interpretation of the MIC is S, I, or R.
[d] *Citrobacter freundii, Citrobacter braakii,* or *Citrobacter youngae.*

for four isolates. The outcome was false positive for three of the four *K. oxytoca* isolates (no. 37 to 40) mentioned above and false negative for another *K. oxytoca* isolate (no. 52).

The technicians also failed to recognize the phantom zone or ellipse deformation in about 30% of the strips. The experts detected this phenomenon in 48 strips (19 ceftazidime and 29 cefotaxime) used on 37 *E. coli* and *K. pneumoniae* isolates, while the technicians recognized it in 33 strips used on 30 isolates. This did not result in any false-negative results of the tests, because either the technicians detected the phenomenon on the other strip or the calculated ratios already indicated the presence of an ESBL.

In conclusion, there was full agreement on the Etest ESBL results for all *E. coli* and *K. pneumoniae* isolates interpreted by the laboratory technicians and experts. However, the agreement was 80%, the sensitivity was 91%, and the specificity was 50% for the 15 *K. oxytoca* isolates.

DISCUSSION

The three automated systems detected ESBL production with an accuracy ranging from 78% (VITEK 2) to 83% (VITEK 1) to 89% (Phoenix). The comparative analysis showed no significant difference between the outcomes of the systems when the control strains were tested. With regard to multiresistant isolates of *E. coli* and *Klebsiella* spp., however, the VITEK 2 performed worse than the Phoenix ($P = 0.03$), while no significant difference was found between the performance of either of these systems and that of the VITEK 1. The significant difference between the VITEK 2 and the Phoenix can be explained by the high percentage of indeterminate test results reached by the VITEK 2. The AES detected inconsistencies in 20% of the first tests. When this happened, it was impossible to make a well-founded choice between the options provided by the AES, i.e., make either a correction in the identification or a biological correction in the MIC. This problem was also noted by Sanders et al. (26). As a result, repeat testing in the VITEK 2 and a confirmatory test on the identification were indicated in all cases, leading to an important delay of results and an increase in costs. Full consistency was obtained on repeat testing for only 41% of the isolates, leaving 11% of the initial 91 tested isolates with an indeterminate result. Furthermore, in retrospect, none of the confirmatory identification tests were indicated and both identifications suggested by the AES were incorrect. In line with these findings, the most recent update of the VITEK 2 software (R02.01) does not any longer suggest a change in identification in the case of inconsistencies.

Unexpectedly, the performances of the newly developed VITEK 2 and the older VITEK 1 were comparable. The substantial extra effort required from the staff and the increased material costs needed to attain these same results, however, make the VITEK 2 a less attractive alternative.

There was no significant difference in the accuracy of the ESBL tests of the VITEK 1 and the Phoenix. The Phoenix did, however, tend to have a higher sensitivity (92 versus 83%). Moreover, for three of the four ESBL-positive isolates that were missed by the Phoenix, the associated BDXpert system suggested therapeutic corrections in the antibiogram comparable with those applied to an ESBL-positive isolate. The BDXpert system also recommended that a confirmatory test be performed on half of the false-positive results; the VITEK 1 (inherent to the design of the system) did not do so. In conclusion, the Phoenix performed better than either of the VITEK systems on ESBL detection.

The main limitation of the present study was the use of the Etest ESBL as the reference method for testing the clinical isolates. Although it fulfils the criteria for a confirmatory test for ESBL detection as recommended by the NCCLS (18), the Etest ESBL is not a microdilution or disk susceptibility test. It could not differentiate between a chromosomal K1 (KOXY) beta-lactamase or an ESBL in *K. oxytoca* strains. However, only 3 of the 29 isolates with discordant results were *K. oxytoca* isolates with Etest ESBL-positive results, for which only the VITEK 2 gave ESBL-negative results. This shortcoming, therefore, may have posed just a small relative disadvantage to the specificity of the VITEK 2 but generally had limited influence on the outcome of the comparative analysis. Other shortcomings of the Etest ESBL were the indeterminate results for four isolates (5%) and the questionable outcomes for another four. The latter were clinical *K. oxytoca* isolates with sporadic mutants in the inhibition zone of the cefotaxime-clavulanic acid strip. When these mutants were excluded from the analyses, reading of the MICs yielded a positive test result, indicating the production of ESBLs. Including these mutants in conformity with the package insert and the advice of the experts, however, resulted in a negative outcome. The experts' decision to include the mutants decreased the specificities of all of the automated systems to some degree but had little influence on their sensitivities.

In strong support of the use of the Etest ESBL as the reference method, however, was the 100% sensitivity obtained with the control strains, including six different TEM-type and three different SHV-type ESBLs. In addition, the Etest ESBL showed 100% sensitivity for TEM-FHM and the SHV-type ESBLs detected in the clinical isolates. Only once was the Etest ESBL possibly false negative, due to the negative result for one of the two isolates with a TEM-HM. Taken together, these results justify in our opinion the use of the Etest ESBL as a reference method for the comparative analysis with clinical isolates. Previous studies of eight different TEM-like and four different SHV-like ESBLs showed that the Etest ESBL had a sensitivity of 81% (32), while testing only SHV-like ESBLs yielded a sensitivity of 52% (19). Those studies, however, used only a ceftazidime strip. The manufacturers of the Etest ESBL have since added a cefotaxime strip to the test, and the criteria for interpreting the test results have been extended. Besides a changed ratio, minimal MICs for ceftazidime and cefotaxime have also been introduced and the deformation of the inhibition ellipse has been added as a single criterion for the presence of an ESBL.

The evaluation of the Etest ESBL for the detection of ESBL production in *E. coli* and *Klebsiella* spp. in the clinical laboratory revealed that the reading of the inhibition zones was frequently complicated by the presence of mutants along the zone border, causing discrepancies in the MICs obtained by experts and technicians. Neither these differences in MIC reading nor the failure of the laboratory technicians to recognize the ellipse deformation or phantom zone in 30% of the cases influenced the outcome of the test results. The lack of

clear guidelines regarding sporadic mutants inside the inhibition zone of the cefotaxime-clavulanic acid strip, however, did lead to discordant test results between technicians and experts. In conclusion, the Etest ESBL may be an accurate test if the following conditions are fulfilled. First, guidelines regarding sporadic mutants inside the inhibition zone of the cefotaxime-clavulanic acid or ceftazidime-clavulanic acid strip should be defined. In the present study, the experts decided to include the mutants in the reading of the MICs. In view of the therapeutic consequences, however, it is questionable whether the test result of a single mutant should be allowed to prevail over the other 10^8 CFU/ml tested. Second, besides thoroughly reading the package insert, laboratory technicians should be trained in the recognition of subtle ellipse deformations and phantom zones before conducting the analyses. Third, it should be decided whether additional tests need to be done in order to differentiate between *K. oxytoca* strains with hyperproduction of a K1 beta-lactamase or an ESBL (e.g., additional susceptibility tests for aztreonam) (16). This differentiation will probably not have therapeutic implications, but it will reveal whether the enzyme is plasmid encoded or chromosomal. If it is plasmid encoded, especially on R-plasmids, horizontal transfer to other bacteria can be anticipated and more-stringent hospital control measures may be taken as a result. Many hospitals have already experienced outbreaks of ESBL-producing *Enterobacteriaceae*, and subsequent molecular analysis of those isolates has often indicated the occurrence of horizontal transfer (1, 13, 33).

The results of the present study show that the detection of ESBLs in this collection of multiresistant isolates posed problems for both the automated systems and the Etest. All of the automated methods performed less well in this study than in the few previously published studies (7, 15, 24–26; D. Turner, M. Gosnell, J. Sinha, V. Kenney, T. Wiles, and J. Reuben, Abstr. 101th Gen. Meet Amer. Soc. Microbiol., abstr. C-2261, 2001). First, this may be a result of the character of the ESBLs in the present collection. The isolate panel tested in this study included multiresistant isolates collected within one university hospital over a period of 3 years. As a consequence, the diversity of the identified ESBL genotypes was limited. Genotyping revealed four different ESBLs (two that were TEM-like and two that were SHV-like) among the 55 clinical isolates analyzed, three of which were represented among the isolates with discordant ESBL test results. No TEM or SHV ESBL was detected in the majority of the isolates with discordant results or in one-third of the isolates for which all methods yielded a positive test result. It is plausible, therefore, that other ESBLs were circulating in the hospital at the time of this study. Because of the diverse combinations of MICs towards the different cephalosporins that were expressed by these strains (data not shown), however, it is unlikely that this was a single enzyme. If these ESBLs had not been previously identified, they were probably absent from the databases used for the development of the expert systems. This would explain, at least in part, the high number of discordant results. Second, the suboptimal result may be a consequence of the multiresistant character of the isolates. Multiresistance in *Enterobacteriaceae* is often associated with the presence of plasmids, which may encode different (extended-spectrum) beta-lactamases (1, 2, 6, 9, 13, 28, 35, 36). Simultaneous expression of the different beta-lactamases, possibly in combination with outer membrane porin changes (2, 23, 34), may result in an uncommon phenotype not recognized or recognizable by the automated systems. For example, the presence of an ESBL may be masked by the concurrent expression of an AmpC-type enzyme in the same strain, because no inhibitory effect of clavulanic acid can be detected due to the clavulanate-insensitive AmpC-type enzyme (2, 31). It is doubtful, therefore, whether any method based on the phenotype of the beta-lactamase(s) produced will be 100% sensitive or specific for the accurate detection of all ESBLs produced by multiresistant *Enterobacteriaceae*. Although nucleotide sequencing is considered the standard for the determination of the specific beta-lactamase gene present in a strain (3), this method also has its limitations for the detection of ESBLs: (i) the results of the sequencing may vary depending on the methods used (4); (ii) the results are dependent on the number of ESBL families (e.g., TEM, SHV, OXA, or CTX-M) and other beta-lactamases (e.g., AmpC or metallo-beta-lactamases) that are sought and on the available knowledge concerning the biochemical properties of the encoded beta-lactamase, especially if the sequence results reveal new (combinations of) amino acid alterations; (iii) sole detection of the gene does not predict the level of expression, since this is also dependent on the strength of the promoters, the gene copy number, and other genes (8, 20); and (iv) the phenotypic profile may be influenced by outer membrane porin changes, the inocula tested, and the growth conditions used.

We conclude that the ESBL test of the Phoenix system performed better than that of the other automated systems tested in detecting the ESBLs produced by multiresistant *E. coli* and *Klebsiella* spp. in our hospital. The Etest ESBL was found to be an accurate test that can be reliably interpreted by technicians in a clinical laboratory, with the prerequisites that they are well trained and some additional guidelines are introduced for reading and interpreting the test. To date, there are more than 100 ESBLs known worldwide (http://www.lahey.org/studies/webt.htm). Since they are subject to rapid evolution and are involved in a dynamic epidemiology (8), it should be noted that the results of this study or any other study addressing the same question might not be applicable to every laboratory worldwide at any moment in time.

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