

Laboratory Detection of Extended-Spectrum-Beta-Lactamase-Producing *Enterobacteriaceae*: Evaluation of Two Screening Agar Plates and Two Confirmation Techniques[∇]

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Received 27 September 2010/Returned for modification 21 October 2010/Accepted 22 November 2010

The worldwide prevalence of extended-spectrum-beta-lactamase-producing ESBL-producing *Enterobacteriaceae* (ESBL-E) is increasing, making the need for optimized detection techniques more urgent. In this study we investigated the performance of two ESBL-E screening and two ESBL-E confirmation techniques. In accordance with the Dutch national guidelines (www.wip.nl), a collection of 642 highly resistant *Enterobacteriaceae* strains, as identified by Vitek2, was used to test the performances of two screening techniques (EbSA ESBL agar plate and ChromID ESBL agar plate) and of two confirmation techniques (MIC-strip ESBL and Vitek2 ESBL test panel). The individual test results were compared by using Etest, followed by a combination disk test if Etest results were inconclusive. Among group 1 isolates (*Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Salmonella* spp., and *Shigella* spp.) 291 (57.6%) were ESBL-E, versus 65 (47.4%) in group 2 (*Enterobacter* spp., *Citrobacter* spp., *Morganella morganii*, *Serratia* spp., and *Providencia* spp.). The sensitivities of all four tests for group 1 were comparable (EbSA, 96.6%; ChromID, 97.3%; MIC-strip, 99.6%; and Vitek2, 95.1%). The specificities of the EbSA and ChromID were the same (93.9%). However, the confirmation techniques produced many inconclusive test results, which reduces the applicability in routine laboratories. Only the two screening agar plates were validated for ESBL testing of group 2 microorganisms. They showed comparable sensitivities; however, the EbSA screening agar plate had a significantly higher specificity (78.6% versus 44.3%). In conclusion the screening agar plates performed better than the two confirmation techniques. The EbSA agar plate had the best overall performance.

The worldwide prevalence of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* (ESBL-E) is increasing rapidly (3, 4). In the annual report of 2008, the European Antimicrobial Resistance Surveillance System (EARSS), available at the RIVM website (http://www.rivm.nl/earss/result/Monitoring_reports/), concluded that resistance of *E. coli* to extended-spectrum cephalosporins has increased significantly since 2001 in nearly all European countries.

Infections caused by ESBL-E are associated with an increase of morbidity, mortality, and health care costs (8, 9). To ensure patient safety, optimal treatment and control of the spread of ESBL-E are essential. Isolation of colonized patients is one of the most important control measures (7) and should be instituted as soon as possible. This requires accurate and rapid laboratory diagnosis.

Different screening and confirmatory techniques have been described in the literature. The objective of this study was to compare two screening agar plates and two confirmatory techniques to detect ESBL-E among a collection of clinically relevant strains. For this we used a well-defined collection of highly resistant microorganisms from a recent study in Dutch hospitals.

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[∇] Published ahead of print on 1 December 2010.

MATERIALS AND METHODS

Strain collection. The strains were part of a collection from a multicenter study containing 892 highly resistant Gram-negative rods. The strains were collected in five university hospitals, eight teaching hospitals, and five general hospitals during a 6-month study period in 2007. The strains were recovered from clinical cultures and screening cultures. The screening samples were predominantly nasal, throat, and rectal swabs (11). In total, 642 highly resistant *Enterobacteriaceae* (HRE) isolates were included in this analysis. If two isolates from one patient were included, the strains were unique regarding the species identification or resistance pattern. The criteria used for definition of HRE are described in the Dutch national guidelines for the control of highly resistant microorganisms (7). Table 1 shows a summary of these criteria.

Reference method. The strains were identified as HRE by the local microbiology laboratories and sent to a central laboratory. In the central laboratory, identification and susceptibility testing of the strains were confirmed using the Vitek2 system (bioMérieux, Marcy l'Etoile, France) with “GN” and “AST-N020” cards. The isolates were divided into group 1 (*Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis*, *Salmonella* spp., and *Shigella* spp.) and group 2 (*Enterobacter* spp., *Serratia* spp., *Providencia* spp., *Citrobacter freundii*, *Morganella morganii*, and *Hafnia alvei*) microorganisms according to the Dutch guidelines for ESBL detection (1). This division is based on the presence of chromosomal AmpC beta-lactamase in the species of group 2 and the resulting decreased sensitivity to ceftazidime or cefotaxime. Confirmation of the presence of ESBL was performed by using Etest (bioMérieux, Marcy l'Etoile, France) on all isolates. If the Etest was inconclusive, a combination disc diffusion test (Rosco, Taastrup, Denmark) was performed to confirm the presence of ESBL. Group 1 microorganisms were tested for synergy between ceftazidime and clavulanic acid and between cefotaxime and clavulanic acid. Group 2 microorganisms were tested for synergy between cefepime and clavulanic acid. Group 1 microorganisms that were ceftazidime resistant were also tested for synergy between cefepime and clavulanic acid. This procedure was considered the gold standard.

EbSA ESBL screening agar plate. The EbSA ESBL screening agar plate consists of a double MacConkey agar plate containing ceftazidime (1.0 mg/liter) on one side and cefotaxime (1.0 mg/liter) on the other side. (AlphaOmega,

TABLE 1. Definition of highly resistant *Enterobacteriaceae*

Species	Definition of resistance ^a				
	ESBL	CAR	QUI	AMG	COT
<i>Escherichia coli</i>	A	A	B	B	
<i>Klebsiella</i> spp.	A	A	B	B	
Other	A	A	B	B	B

^a ESBL, extended-spectrum beta-lactamases; CAR, carbapenems; QUI, fluoroquinolones; AMG, aminoglycosides; COT, co-trimoxazole. A, resistance against an antibacterial agent of the carbapenem group and/or presence of ESBL production is sufficient to define the microorganism as highly resistant; B, resistance against antibacterial agents from at least two of the indicated groups or specified antibacterial agents is required to define the microorganism as highly resistant.

's-Gravenhage, Netherlands). Both sides contain cloxacillin (400 mg/liter) and vancomycin (64 mg/liter) for inhibition of AmpC beta-lactamase-producing bacteria and Gram-positive bacteria, respectively. Both sides of the screening agar were inoculated with 1 μ l (a standard loopful) of a 0.5 McFarland standard suspension of *Enterobacteriaceae*. Subsequently they were incubated aerobically at 35 to 37°C for 18 to 24 h in an inverted position. Growth on either side of the agar plate was interpreted as a positive screening result.

ChromID ESBL screening agar plate. The ChromID ESBL screening agar plate contains a mixture of antibiotics, including cefpodoxim, two chromogenic substrates, and one natural substrate, to enable direct species identification (bioMérieux, Marcy l'Etoile, France). The species identification was not part of our evaluation. The screening agar plate was inoculated with 1 μ l (a standard loopful) of a 0.5 McFarland standard suspension of *Enterobacteriaceae*. Subsequently, the agar was incubated aerobically at 35 to 37°C for 18 to 24 h in an inverted position. Growth on the agar was considered a positive test result.

MIC-strip ESBL. The MIC-strip ESBL is a broth microdilution assay (Bio-Trading, Mijdrecht, Netherlands) which is validated only for group 1 microorganisms. Therefore, group 2 microorganisms were excluded for this analysis. The test consists of 11 wells containing different amounts of antibiotic; 6 wells contained cefpodoxim, 4 wells contained cefpodoxim plus clavulanic acid, and 1 control well contained no antibiotics. Mueller-Hinton broth was inoculated with a 0.5 McFarland standard bacterial suspension, resulting in a 1:200 dilution. Of this dilution, 100 μ l was inoculated in each well of the MIC-strip. Subsequently, the MIC-strip was incubated aerobically at 35 to 37°C for 18 to 24 h, and then a visual check for growth was executed, based on turbidity. The lowest concentration with no detectable growth was considered the MIC. A MIC reduction of three or more dilution steps was considered indicative of ESBL production.

Vitek2 ESBL test panel. The AST-EXN4 card (bioMérieux, Marcy l'Etoile, France) was used in combination with the Vitek2 system. The panel has six wells containing 1.0 μ g/ml cefepime, 0.5 μ g/ml cefotaxime, or 0.5 μ g/ml ceftazidime alone and in combination with clavulanic acid (10 μ g/ml, 4 μ g/ml, or 4 μ g/ml, respectively). Growth in the wells was quantitatively assessed by means of an optical scanner. The proportional reduction in growth in wells containing a cephalosporin with clavulanic acid compared with those containing the cephalosporin without clavulanic acid was considered indicative of ESBL production. This test is validated only for group 1 microorganisms, and therefore group 2 microorganisms were excluded from the analysis.

Statistical analysis. All screening tests were compared to the reference method, the ESBL Etest, combined when needed with the combination disk diffusion as described above. The sensitivities and specificities of the tests were determined; the analysis was done separately for group 1 and group 2 microorganisms. Differences in sensitivity and specificity between the two groups were analyzed with the chi-square test or Fisher's exact test when appropriate. Statistical significance was accepted when the chance for coincidence was less than 5%. All analysis were performed using the Statistical Package for Social Sciences software (SPSS version 17).

RESULTS

There were a total of 642 highly resistant *Enterobacteriaceae* strains, of which 505 (78.7%) were classified as group 1 and 137 (21.3%) as group 2. The distribution of the various species of microorganisms is shown in Table 2. *E. coli* was the most frequently encountered species in group 1, and *Enterobacter*

TABLE 2. Strain collection

Group and species	n (%) of isolates as determined by reference method			
	ESBL positive	ESBL negative	Inconclusive	Total
1				
Total	291 (57.6)	214 (42.4)	0 (0.0)	505 (78.7)
<i>Escherichia coli</i>	190 (56.9)	144 (43.1)	0 (0.0)	334 (66.1)
<i>Klebsiella</i> spp.	98 (79.0)	26 (21.0)	0 (0.0)	124 (24.6)
<i>Proteus</i> spp.	2 (4.8)	40 (95.2)	0 (0.0)	42 (8.3)
<i>Salmonella</i> spp.	1 (33.3)	2 (66.7)	0 (0.0)	3 (0.6)
<i>Shigella</i> spp.	0 (0.0)	2 (100.0)	0 (0.0)	2 (0.4)
2				
Total	65 (47.4)	70 (51.1)	2 (1.5)	137 (21.3)
<i>Enterobacter</i> spp.	58 (64.4)	30 (33.3)	2 (2.2)	90 (65.7)
<i>Citrobacter</i> spp.	7 (23.3)	23 (76.7)	0 (0.0)	30 (21.9)
<i>Morganella morganii</i>	0 (0.0)	9 (100.0)	0 (0.0)	9 (6.6)
<i>Serratia marcescens</i>	0 (0.0)	6 (100.0)	0 (0.0)	6 (4.4)
<i>Providencia</i> spp.	0 (0.0)	2 (100.0)	0 (0.0)	2 (1.5)

spp. were most frequently encountered in group 2. According to the reference method, a total of 356 isolates (55.5%) were confirmed as ESBL producers. Of the ESBL-positive isolates, 291 (81.7%) belonged to group 1 and 65 (18.3%) belonged to group 2. There were two *Enterobacter* spp. for which the ESBL status could not be determined. These two isolates were excluded from the evaluation of the screening agar plates and the confirmatory methods.

Group 1 microorganisms. The results of the different tests for the group 1 microorganisms are shown in Table 3. Of 505 group 1 microorganisms, 294 (58.2%) were suspected ESBL producers as determined using the EbSA screening agar. Thirteen isolates were false positives and 12 false negatives. The calculated sensitivity was 96.6%, and the specificity was 93.9%. Using the ChromID screening agar, 296 (58.6%) isolates were suspected ESBL producers. Thirteen isolates were false positives, and eight were false negatives. The sensitivity and specificity of this test method were 97.3% and 93.9%, respectively.

Using the MIC-strip, 285 (56.4%) isolates were suspected ESBL producers. Ten isolates were false positives, and one was a false negative. The results for 117 isolates (23.2%) were not interpretable. The results for the noninterpretable isolates were excluded for sensitivity and specificity calculations. The calculated sensitivity was 99.6%, and the specificity was 91.1%.

Using the Vitek2 test panel, 291 isolates (57.6%) were suspected ESBL producers. Twenty isolates were false positives, and 14 were false negatives. The Vitek2 system did not provide results for 52 isolates (10.3%), mainly due to lack of validation of the bacterial species involved. Some missing results were due to test inconsistencies. These isolates were not included in the sensitivity and specificity calculations. The resulting sensitivity of this test method was 95.1%, and the specificity was 88.1%.

Because of the high proportion of invalid test results with both the MIC-strip and the Vitek2 test panel, we did not perform a statistical comparison of these tests with the two screening agar plates. A comparison of the performances of the two screening agar plates showed that the screening agar plates were comparable in regard to sensitivity ($P = 0.812$) and specificity ($P = 1.000$).

Group 2 microorganisms. The results of the different tests for the group 2 microorganisms are shown in table 4. Of 137

TABLE 3. Results of different screening tests for group 1 microorganisms

NVMM guideline	No. of isolates with indicated ESBL test result									
	EbSA		ChromID		MIC strip			Vitek2 test panel		
	Positive	Negative	Positive	Negative	Positive	Negative	Inconclusive ^a	Positive	Negative	Inconclusive ^a
ESBL positive	281	10	283	8	275	1	15	271	14	6
ESBL negative	13	201	13	201	10	102	102	20	148	46
Sensitivity (%)	96.6		97.3		99.6			95.1		
Specificity (%)	93.9		93.9		91.1			88.1		

^a Inconclusive test results were excluded from sensitivity and specificity calculations.

microorganisms, 80 (58.4%) were suspected ESBL producers as determined using the EbSA screening agar plate. Fifteen isolates were false positives, and two were false negatives. The sensitivity was 96.9%, and the specificity was 78.6%. Using the ChromID screening agar plate, 105 isolates (76.6%) were suspected ESBL producers. Thirty-nine isolates were false positives and one was a false negative, resulting in a sensitivity of 98.5% and a specificity of 44.3%. For group 2 microorganisms, the sensitivities of the two tests were comparable, but the specificity was significantly higher for the EbSA screening agar plate ($P = 0.001$).

DISCUSSION

In this study we evaluated the performances of two ESBL screening agar plates and two ESBL confirmatory tests. Our evaluation showed that the two ESBL-E screening agar plates had good sensitivity for group 1 microorganisms (>96.5%). The two confirmation tests (MIC-strip and Vitek2 ESBL panel) also showed good sensitivity for the group 1 microorganisms; however, both tests had a large number of invalid test results, which limits their applicability in routine use. The specificities of the two screening agar plates were identical and high (93.9%). The two confirmatory tests also showed good and comparable specificities. However, as stated above, the large number of invalid test results reduces the applicability of these two tests. If these invalid test results were included in our calculation and classified as false-positive results, the specificity would be significantly lower than the specificity for the two screening agar plates.

For group 2 microorganisms, the sensitivities of the two screening agar plates were comparable and high (>96.5%). However, the specificity of the EbSA was significantly higher than the specificity of the ChromID ($P = 0.001$). The differ-

ence in specificity for group 2 microorganisms makes the EbSA the most reliable test in this evaluation.

Both confirmation techniques were not validated for group 2 microorganisms. This further reduces the applicability of these tests in daily practice. Moreover, our comparison showed that the specificities of these tests were significantly lower than the specificities of the screening agar plates. This is remarkable, as confirmation techniques should have a higher specificity than screening tests. Therefore, the screenings agar plates are more suited as confirmatory tests than the two “confirmatory tests” that were included in this analysis.

There are few other evaluations of the screening agar plates that we used. Al Naiemi et al. (2) evaluated the EbSA screening agar plate with a collection of 208 *Enterobacteriaceae* strains. The ESBL status of the isolates was determined by genotyping; 70 isolates were found to be positive. They found an overall sensitivity of 100% and a specificity of 84.7%. The sensitivity and specificity that we calculated were comparable with these results ($P = 0.114$ and $P = 0.107$, respectively).

Huang et al. (6) evaluated the ChromID with a collection of 156 *Enterobacteriaceae* strains. Eight fully susceptible isolates were inhibited, and all 98 ESBL producers were detected. Fifty isolates harboring other resistance mechanisms were also recovered. These results are comparable to our data, considering the fact that they did not divide the *Enterobacteriaceae* into two groups.

To our best knowledge, an evaluation of the MIC-strip and Vitek2 ESBL test panel (AST-EXN4) confirmation techniques has not been described before.

Our study has a few limitations. First, we used a phenotypic reference method. Using a genotypic reference method could affect the results. Cohen Stuart et al. (5) recently evaluated an ESBL microarray and compared it with the ESBL Etest method that we used. They found limited numbers of false-positive (2/106) and false-negative (2/212) ESBL Etest results compared with sequencing. Consequently, if we had used a genotypic method, the sensitivities and specificities obtained could have been slightly different. Second, in daily practice the screening agar plates will be inoculated directly with patient materials, because they are produced to use as screening techniques. This could theoretically affect the growth of bacteria on these media; however, a recent study (6) showed a sensitivity and specificity of 94.9% in a collection of 528 clinical samples with the ChromID screening agar plate.

Our evaluation shows that both media can detect ESBL-producing *Enterobacteriaceae* reliably. The ChromID has a

TABLE 4. Results of different screening tests with group 2 microorganisms

NVMM guideline	No. of isolates with indicated ESBL test result			
	EbSA		ChromID	
	Positive	Negative	Positive	Negative
ESBL positive	63	2	64	1
ESBL negative	15	55	39	31
Sensitivity (%)	96.9		98.5	
Specificity (%)	78.6		44.3	

lower specificity and will therefore require more confirmatory testing, increasing the total laboratory costs and turnaround times for the results.

A recent study by Tumbarello et al. (10) reported that ESBL infections in the bloodstream were associated with longer (7 days) and costlier (mean additional costs, €5026.00) hospital stays and increased 21-day mortality (29.7% versus 6.1%). These findings indicate the need to use reliable and fast detection tests for ESBL. Use of the EbSA in daily practice will help to shorten the period of inadequate therapy, which is related to a higher mortality.

In conclusion, in our study the EbSA screening agar plate was the best screening test to use. The ChromID also performed adequately, but it had a lower specificity, which increases the laboratory cost and turnaround time.

ACKNOWLEDGMENTS

We are indebted to all members of the TRIANGLE study group: E. Lommerse and L. Spanjaard, Academic Medical Centre, Amsterdam, Netherlands; B. Vlamincx, Antonius Hospital, Nieuwegein, Netherlands; A. Vos, Canisius Wilhelmina Hospital, Nijmegen, Netherlands; M. Wulf, Catharina Hospital, Eindhoven, Netherlands; M. Vos, Erasmus University Medical Center, Rotterdam, Netherlands; R. Wintermans, Fransiscus Hospital, Roosendaal, Netherlands; G. Andriess, Lievensberg Hospital, Bergen op Zoom, Netherlands; J. van Zeijl, Medical Center Leeuwarden, Leeuwarden, Netherlands; E. van der Vorm, Reinier de Graaf Groep, Delft, Netherlands; A. Buiting, Sint Elisabeth Hospital, Tilburg, Netherlands; P. Sturm, University Medical Center Nijmegen, Nijmegen, Netherlands; H. Blok and A. Troelstra, Department of Medical Microbiology and Infectious Diseases, University Medical Center, Amsterdam, Netherlands; and A. Kaiser and C. Vandenbroucke-Grauls, VUmc University Medical Center, Amsterdam, Netherlands.

bioMérieux, Marcy l'Etoile, France, supported the study by providing the ChromID agar plates and the Vitek2 AST-EXN4 cards. BioTrading, Mijdrecht, Netherlands, supported the study by providing the

MIC strips. AlphaOmega, 's-Gravenhage, Netherlands, supported the study by providing the EbSA ESBL agar plates.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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