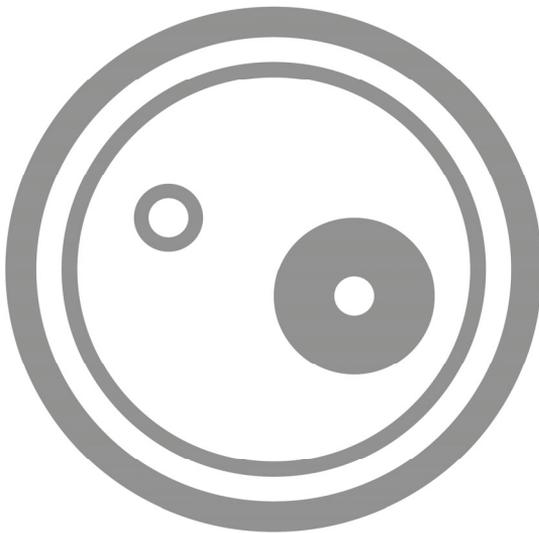# Chapter 2

Detection of ESBL-producing

Enterobacteriaceae

Chapter 2.1

Laboratory detection of extended-spectrum β-lactamase-producing *Enterobacteriaceae*: evaluation of two screening agar plates and two confirmation techniques

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2.1

Abstract

The worldwide prevalence of ESBL-producing *Enterobacteriaceae* is increasing, making the need for optimized detection techniques more urgent. In this study we investigated the performance of two screening- and two confirmation techniques to detect ESBL-production in *Enterobacteriaceae*.

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 with 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-producing *Enterobacteriaceae* 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, was comparable (EbSA, 96.6%; ChromID, 97.3%; MIC-strip, 99.6%; Vitek2, 95.1%). The specificities of the EbSA and ChromID was 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 in group 2 microorganisms. They showed a 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.

Introduction

The worldwide prevalence of extended-spectrum β-lactamase-producing *Entero-bacteriaceae* is increasing rapidly.[1,2] 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 *Escherichia coli* to extended-spectum cephalosporins has increased significantly since 2001 in nearly all European countries.

Infections caused by ESBL-producing *Enterobacteriaceae* are associated with an increase of morbidity, mortality, and healthcare costs.[3,4] To ensure patient safety, optimal treatment and control of the spread of ESBL-producing *Enterobacteriaceae* are essential. Isolation of colonized patients is one of the most important control measures[5] 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-producing *Enterobacteriaceae* 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.

Methods

Strain collection

The strains were part of a collection from a multicentre 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.[6] In total, 642 highly resistant *Enterobacteriaceae* 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 highly resistant *Enterobacteriaceae* are described in the Dutch national guidelines for the control of highly resistant microorganisms.[5] Table 2.1.1 shows a summary of these criteria.

Reference method

The strains were identified as highly resistant *Enterobacteriaceae* 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) using '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.[7] This division is based on the presence of chromosomal AmpC β-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 cefoxitin resistant were also tested for synergy between cefepime and clavulanic acid. This procedure was considered the golden standard.

Table 2.1.1   Definition of highly resistant *Enterobacteriaceae*.

Species	ESBL	CAR	QUI	AMG	COT
Escherichia coli	A	A	B	B	
Klebsiella spp.	A	A	B	B	
Other	A	A	B	B	B

ESBL=extended-spectrum β-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 agent is required to define the microorganism as highly resistant.

EbSA ESBL screening agar plate

The EbSA ESBL screening agar plate consists of a double MacConkey agar plate, containing ceftazidime (1,0 mg/l) on one side and cefotaxime (1,0 mg/l) on the other side. (AlphaOmega, 's-Gravenhage, Netherlands). Both sides contain cloxacillin (400 mg/l) and vancomycin (64 mg/l) for inhibition of AmpC β-lactamase-producing bacteria and Gram-positive bacteria, respectively. Both sides of the screening agar were inoculated with 1 µl (a standard loopful) of a 0.5 McFarland standard suspension of *Enterobacteriaceae*. Subsequently they were incubated aerobically at 35-37°C for 18-24 h in an inverted position. Growth on the agar was considered 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 µl (a standard loopful) of a 0.5 McFarland standard suspension of *Enterobacteriaceae*. Subsequently, the agar was incubated aerobically at 35-37°C for 18-24 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 (BioTrading, 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 one 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 µl was inoculated in each well of the MIC-strip. Subsequently, the MIC-strip was incubated aerobically at 35-37°C for 18-24 h and then a visual check for growth was executed, based on turbidity. The lowest concentration with no detectable growth was considered the minimum inhibitory concentration (MIC). A MIC reduction of 3 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 µg/ml cefepime, 0.5 µg/ml cefotaxime, and 0.5 µg/ml ceftazidime, alone and in combination with clavulanic acid (10 µg/ml, 4 µg/ml, and 4 µg/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 of the two groups were analysed 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*, 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 2.1.2. *E. coli* was the most frequently encountered species in group 1, and *Enterobacter 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.

Table 2.1.2   Strain collection.

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

Group 1 microorganisms

The results of the different tests for the group 1 microorganisms are shown in Table 2.1.3A. Of 505 group 1 microorganisms, 294 (58.2%) were suspected ESBL–producers as determined by using the EbSA screening agar. Thirteen isolates were false positives and twelve 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 of the non-interpretable isolates were excluded for sensitivity en specificity calculation. The calculated sensitivity was 99.6%, and the specificity was 91.1%.

Table 2.1.3A Results of different screening tests for group 1 microorganisms.

NVMM guideline	EbSA		ChromID		Phenotypical test MIC strip			Vitek2 test panel		
	ESBL positive	ESBL negative	ESBL positive	ESBL negative	ESBL positive	ESBL negative	Inconclusive	ESBL positive	ESBL negative	Inconclusive
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%*		

* Inconclusive test results were excluded from sensitivity and specificity calculations.

Using the Vitek2 test panel, 291 (57.6%) isolates were suspected ESBL producers. Twenty isolates were false positives and fourteen 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 calculation. 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 performance of both 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 2.1.3B.

Table 2.1.3B   Results of different screening tests for group 2 microorganisms.

NVMM guideline	Phenotypical test			
	EbSA		ChromID	
	ESBL positive	ESBL negative	ESBL positive	ESBL negative
ESBL positive	63	2	64	1
ESBL negative	15	55	39	31
Sensitivity	96.9%		98.5%	
Specificity	78.6%		44.3%	

Of 137 microorganisms, 80 (58.4%) were suspected ESBL producers as determined by 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 both tests was comparable, but he specificity was significantly higher for the EbSA screening agar plate (P=0.001).

Discussion

In this study we evaluated the performance of two ESBL screening agar plates and two ESBL confirmatory tests to detect ESBL-production in *Enterobacteriaceae*. Our evaluation showed that the two screening agar plates had a 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 amount 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 difference 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 test were significantly lower than the specificities of the screening agar plates. This is remarkable, as confirmation techniques should have a higher specificity then screening tests. Therefore, the screenings agar plates are more suited as a confirmatory test 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.[7] 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 positive. They found an overall sensitivity of 100% and a specificity of 84.7%. The sensitivity and specificity that we calculated, was comparable with these results (*P*=0.114 and *P*=0.107, respectively).

Huang et al.[9] 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 harbouring 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 phenotypical reference method. Using a genotypic reference method could affect the results. Cohen Stuart et al.[10] 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

2.1

theoretically affect the growth of bacteria on these media; however, a recent study[9] 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 lower specificity and will therefore require more confirmatory testing, increasing the total laboratory costs and turnaround time for the results.

A recent study from Tumbarella et al.[11] reported that ESBL infections in the bloodstream were associated with longer (7 days) and costlier (mean additional costs, €5026.-) hospital stays and an 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 agar plate in daily practice will help to shorten the period in 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 costs and turnaround time.

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2.1

Chapter 2.2

New diagnostic microarray (Check-KPC ESBL) for detection and identification of extended-spectrum β-lactamases in highly resistant *Enterobacteriaceae*

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Abstract

The performance of a microarray for the detection of extended-spectrum β-lactamases was determined on a collection of 638 highly resistant members of the family *Enterobacteriaceae* collected from patients in 18 hospitals in The Netherlands. The microarray had a significantly higher specificity than the phenotypic assays. It also detects carbapenemases and characterized the resistance genes, providing epidemiological insight.

2.2

The worldwide prevalence of extended-spectrum β-lactamase-producing members of *Enterobacteriaceae* is increasing rapidly.[1] Controlling ESBL-producing *Entero-bacteriaceae* is difficult, as the resistance genes are located on plasmids and may be transferred between species and even different genera of the *Enterobacteriaceae* family.[2] The rapid laboratory detection of this resistance trait is important to guide antimicrobial therapy and to take appropriate infection control measures.

We evaluated a ligation-mediated amplification in combination with a microarray to detect and characterize ESBL-producing *Enterobacteriaceae* in a contemporary collection of *Enterobacteriaceae* from a representative sample of hospitals in the Netherlands.

Multicenter prospective surveillance was performed in 18 Dutch hospitals during 6 months in 2007. All newly identified patients with highly resistant members of the family *Enterobacteriaceae* were included. The criteria for highly resistant *Enterobacteriaceae* are defined in the Dutch national guideline for the control of highly resistant microorganisms.[3]

Susceptibility tests were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[4] ESBL-production was determined as previously described.[5] All *Escherichia coli*, *Klebsiella spp.*, *Proteus mirabilis*, *Salmonella spp.*, and *Shigella spp.* (group 1) were tested with ceftazidime and cefotaxime with and without clavulanic acid. All *Enterobacter spp.*, *Serratia spp.*, *Providencia spp.*, *Citrobacter freundii*, *Morganella morganii*, and *Hafnia alvei* (group 2) were tested with cefepime with and without clavulanic acid.[6] When Etest results were not conclusive, a disk diffusion test (Rosco diagnostics, Taastrup, Denmark) was performed with the double-disk methodology, using a similar algorithm.

DNA isolation was performed with the Easymaq system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The Check-KPC ESBL array (Check-Points, Wageningen, The Netherlands) was used as previously described.[6] *bla*SHV, *bla*TEM, and *bla*CTX-M genes were amplified by PCR.[7] Sequence analysis and alignments were performed with the Bionumerics 6.01 software program (Applied Maths, Sint-Martens-Latem, Belgium), BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and information from the www.lahey.org/studies website.

All isolates with concordant phenotypic and genotypic ESBL detection results were considered identified correctly. For discordant findings, the results of sequencing were considered the gold standard.

A total of 638 highly resistant members of the family *Enterobacteriaceae* were included (Table 2.2.1). On the basis of the phenotypic test results, 355 isolates (55.6%) were considered ESBL producers. The microarray detected one or more ESBL genes in 345 of the 638 isolates (54.1%).

2.2

Table 2.2.1   Distribution of bacterial species.

Group and species	No. of isolates (%)
Group 1 (n=501)	
Escherichia coli	332 (66.3)
Klebsiella pneumoniae	93 (18.6)
Proteus mirabilis	41 (8.2)
Klebsiella oxytoca	29 (5.8)
Salmonella spp.	3 (0.6)
Shigella sp.	2 (0.4)
Pantoea spp.	1 (0.2)
Group 2 (n=137)	
Enterobacter cloacae	87 (63.5)
Citrobacter freundii	24 (17.5)
Morganella morganii	9 (6.6)
Serratia marcescens	6 (4.4)
Citrobacter spp.	6 (4.4)
Enterobacter aerogenes	3 (2.2)
Providencia spp.	2 (1.5)

The overall phenotypic and genotypic results for 590 out of 638 (92.5%) isolates were concordant. In group 1, the majority (24/30) of discordant results had a positive phenotypic test and a negative microarray result. In group 2, the majority (12/18) had a negative phenotypic test and a positive microarray result.

Table 2.2.2 shows the performance of the phenotypic and microarray tests when the results of sequencing are incorporated into the gold standard. For group 1, the sensitivities were comparable, but the microarray was more specific. For group 2, the microarray was more sensitive, and the specificities were comparable.

Seven of the false-positive phenotypic tests were negative upon retesting, and six of the false-negative test results were positive. Also, both false-positive microarray results were negative upon retesting and five false-negative microarray results were positive on retesting. In Table S2.2.1 in the supplemental material, all discordant findings are shown.

The 345 isolates that produced ESBLs according to the microarray had various types of ESBL genes (Table 2.2.3). More than half of the group 1 isolates from the *Enterobacteriaceae* had *bla*CTX-M-1 family ESBL genes, whereas in group 2, the *bla*CTX-M-1 family of ESBL genes constituted a minority of the genes. The most prevalent ESBL type in group 2 was the *bla*CTX-M-9 family (61.6%). Some isolates contained more than one ESBL gene. *bla*TEM coexisted once with a *bla*CTX-M-1 family ESBL gene and once with a *bla*CTX-M-9 family gene both in group 1 organisms. *bla*SHV was found six times in combination with *bla*CTX-M-1 family in group 1 organisms and nine times in combination with the *bla*CTX-M-9 family in group 1 (n=7) and group 2 (n=2) organisms. One *K. oxytoca* isolate contained three different genes (*bla*TEM, *bla*SHV, and *bla*CTX-M-9 family). In addition to ESBL genes, the microarray detects carbapenemase genes: these genes were detected in two *Klebsiella pneumoniae*

isolates, both from the same patient. This patient, who had been hospitalized in Greece after a traffic accident, was subsequently transferred to a general hospital in The Netherlands and from there to a university hospital. Both hospitals in The Netherlands participated in our survey. Resistance to carbapenems had not been detected in the diagnostic laboratory with the standard laboratory procedures but was confirmed upon retesting. The Etest MIC for ertapenem was 24.0 mg/ml.

Table 2.2.2   Results of the phenotypic tests and microarray testing after resolution of discordant results.[a]

Test, group, and isolates	No. of results		Total no. of results
	ESBL negative	ESBL positive	
Phenotypic tests			
Group I			
ESBL negative	205	5	210
ESBL positive	16	275	291
Total	221	280	501
Group II			
ESBL negative	61	11	72
ESBL positive	5	59	64
Inconclusive	0	1	1
Total	66	71	137
Microarray			
Group I			
ESBL negative	220	8	228
ESBL positive	1	272	273
Total	221	280	501
Group II			
ESBL negative	65	0	65
ESBL positive	1	71	72
Total	66	71	137

[a] The performance of the phenotypic and microarray test when the results of sequencing are incorporated into the gold standard. For phenotypic tests on group 1, the sensitivity was 98.2%, and the specificity was 92.8%. For phenotypic tests on group 2, the sensitivity was 83.1%, and the specificity was 92.4%. For the microarray on group 1, the sensitivity was 97.2%, and the specificity was 99.5%. In group 1, the specificity of the microarray was statistically significantly higher than that of the phenotypic test ($P<0.001$). For the microarray on group 2, the sensitivity was 100%, and the specificity was 99.5%. In group II, the sensitivity of the microarray was statistically significantly higher than that of the phenotypic test ($P<0.001$).

This evaluation showed that the diagnostic microarray was more accurate than the current phenotypic methods to detect ESBL genes in a representative sample of clinical isolates. This microarray has recently been evaluated on three collections of selected isolates containing the majority of known ESBL and KPC genes.[7-9] In the first evaluation,[7] it detected 95% of the isolates that contained an ESBL gene and did not produce false-positive results. The second evaluation[9] included a well-defined collection of ESBL and KPC producers and confirmed the ability of the array to detect most resistance genes. In one case, the array failed to detect a KPC gene, but the authors concluded that plasmid instability was the most likely explanation for this negative result. The third study tested the array on 106 Gram-negative strains.[8] The

following sensitivities and specificities, respectively, were recorded: 98.8% and 100% for *bla*SHV, 100% and 96.4% for *bla*TEM, and 100% and 100% for *bla*CTX-M and blaKPC. These promising results from the analytic evaluations were confirmed in our study. The false-positive phenotypic tests observed in group 1 were often negative upon retesting. This reflects the subjectivity involved with the interpretation of the results. In group 2, chromosomal AmpC production is a known pitfall for the phenotypic methods, resulting in a substantially reduced specificity.[10] As we did not include all known ESBL genes in the sequencing reactions, there is a possibility that some of the false-positive phenotypic test results are in fact true-positive results that were not detected by the molecular tests. However, the ESBL genes that were not included are rare.

Table 2.2.3 Identification of extended-spectrum β-lactamase genes of group 1 and group 2 isolates in *Enterobacteriaceae* by microarray.

ESBL gene type	No. of genes (%)		Total no. of genes (%)
	Group 1	Group 2	
TEM	27 (9.5)	3 (3.8)	30 (8.2)
SHV	47 (16.5)	20 (25.3)	67 (18.4)
CTX-M-1	153 (53.7)[a]	6 (7.6)[a]	159 (43.7)
CTX-M-2	0 (0)	4 (5.1)	4 (1.1)
CTX-M-9	58 (20.4)[a]	45 (57.0)[a]	103 (28.3)
CTX-M-8/M25	0 (0)	1 (1.3)	1 (0.3)
Total	285 (100)	79 (100)	364 (100)

[a] The differences between the values for group 1 and group 2 are statistically significant for CTX-M-1 and CTX-M-9.

In conclusion, there were only a few failures of the array to detect ESBL genes, and the specificity of the microarray was superior to the phenotypic tests, which makes this commercially available microarray a highly reliable tool to detect and identify ESBL genes in the clinical setting. In addition, it also detects the presence of carbapenemase genes, which are nowadays considered to be the most important threats of antimicrobial resistance.[11] Finally, the array identifies the type of ESBL that is present, as shown in Table 2.2.3. The epidemiology of ESBL-producing *Enterobacteriaceae* is rapidly changing, and it is poorly understood how the resistance genes are spreading and which reservoirs are involved.[12] This new tool will likely improve insight into the epidemiology of resistance genes, which may be an aid in the further control of resistance.

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Table S2.2.1 Resolution of discordant results by sequencing.

Species	Phenotypical test: initial results			Phenotypical test repeated	Microarray ESBL		Microarray: Wildtype TEM/SHV	Sequencing	Interpretation
	Combined disk	E-test	total		Initial	Repeated			
E. coli	-	-	-	-	TEM	-	-	-	FP microarray
E. coli	+	-	+	+	-	-	TEM	bla_{TEM-1} and $bla_{CTX-M-39}$	FN microarray
E. coli	+	+	+	+	-	$bla_{CTX-M-1}$	-	$bla_{CTX-M-15/28}$	FN microarray
E. coli	+	+	+	+	-	$bla_{CTX-M-1}$	-	$bla_{CTX-M-15/28}$	FN microarray
E. coli	-	+	+	+	-	-	-	Negative	FP phenotypical tests
E. coli	-	+	+	+	-	-	-	Negative	FP phenotypical tests
E. coli	+	+	+	+	-	-	-	Negative	FP phenotypical tests
E. coli	+	+	+	-	-	-	-	Negative	FP phenotypical tests
E. coli	+	+	+	+	-	-	TEM	bla_{TEM-1}	FP phenotypical tests
E. coli	+	+	+	+	$bla_{CTX-M-1}$	$bla_{CTX-M-1}$	TEM	bla_{TEM-1}	FP phenotypical tests
E. coli	+	-	-	+	$bla_{CTX-M-1}$	$bla_{CTX-M-1}$	-	$bla_{CTX-M-15/28}$	FN phenotypical tests
K. pneumoniae	-	+	+	-	-	-	-	bla_{SHV-33}	FN microarray
K. pneumoniae	+	+	+	-	-	-	-	bla_{SHV-28}	FN microarray
K. pneumoniae	+	+	+	+	-	$bla_{CTX-M-9}$	TEM/SHV	bla_{TEM-1}, bla_{SHV-1}, and $bla_{CTX-M-9}$	FN microarray
K. pneumoniae	+	+	+	+	-	$bla_{CTX-M-9}$	SHV	bla_{SHV-1} and $bla_{CTX-M-9}$	FN microarray
K. pneumoniae	+	+	+	+	-	$bla_{CTX-M-1}$	-	$bla_{CTX-M-1}$	FN microarray
K. pneumoniae	+	+	+	+	-	-	SHV	bla_{SHV-1}	FP phenotypical tests
K. pneumoniae	-	+	+	+	-	-	SHV	bla_{SHV-1}	FP phenotypical tests
K. pneumoniae	+	+	+	-	-	-	-	Negative	FP phenotypical tests
K. pneumoniae	-	+	+	+	-	-	-	Negative	FP phenotypical tests
K. pneumoniae	-	+	+	-	-	-	SHV	bla_{SHV-1}	FP phenotypical tests
K. pneumoniae	-	+/-	-	-	bla_{SHV} & KPC*	bla_{SHV} & KPC*	TEM	bla_{TEM-1} and bla_{SHV-12}	FN phenotypical tests
K. pneumoniae	+	-	-	-	$bla_{CTX-M-9}$	$bla_{CTX-M-9}$	SHV	bla_{SHV-1} and $bla_{CTX-M-9}$	FN phenotypical tests
K. pneumoniae	-	-	-	-	$bla_{CTX-M-1}$	-	SHV	bla_{TEM-1} and $bla_{SHVM-11}$	FN phenotypical tests
K. oxytoca	-	+	+	+	-	-	-	K1 hyperproducer	FP phenotypical tests
K. oxytoca	-	+	+	+	-	-	-	K1 hyperproducer	FP phenotypical tests
K. oxytoca	-	+	+	+	-	-	-	K1 hyperproducer	FP phenotypical tests
K. oxytoca	+	-	-	+	bla_{TEM}	bla_{TEM}	-	bla_{TEM} & K1 hyperproducer	FN phenotypical tests

2.2

Table S2.2.1   (continued)

Species	Phenotypical test: initial results			Phenotypical test repeated	Microarray ESBL		Microarray: Wildtype TEM/SHV	Sequencing	Interpretation
	Combined disk	E-test	total		Initial	Repeated			
Klebsiella spp.	-	+	+	-	-	-	SHV	$bla_{SHV\text{-}1}$	FP phenotypical tests
P. mirabilis	+	+	+	+	-	-	TEM	$bla_{TEM\text{-}1}$	FP phenotypical tests
Citrobacter spp.	+	+	+	+	-	-	-	Negative	FP phenotypical tests
Citrobacter spp.	-	+	+	+	-	-	-	Negative	FP phenotypical tests
E. cloacae	+/-	+	+	+	-	-	-	Negative	FP phenotypical tests
E. cloacae	+	+	+	+	-	-	-	Negative	FP phenotypical tests
E. cloacae	-	+	+	-	-	-	-	Negative	FP phenotypical tests
E. cloacae	-	-	-	+	$bla_{CTX\text{-}M\text{-}9}$	$bla_{CTX\text{-}M\text{-}9}$	-	$bla_{CTX\text{-}M\text{-}9}$	FN phenotypical tests
E. cloacae	+	-	-	+	bla_{SHV}	bla_{SHV}	TEM	$bla_{SHV\text{-}12}$	FN phenotypical tests
E. cloacae	-	-	-	+	$bla_{CTX\text{-}M\text{-}9}$	$bla_{CTX\text{-}M\text{-}9}$	-	$bla_{CTX\text{-}M\text{-}9}$	FN phenotypical tests
E. cloacae	-	-	-	+	bla_{TEM}	bla_{TEM}	-	$bla_{TEM\text{-}12}$	FN phenotypical tests
E. cloacae	+/-	+/-	+/-	+/-	$bla_{CTX\text{-}M\text{-}9}$	$bla_{CTX\text{-}M\text{-}9}$	TEM	$bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$	FN phenotypical tests
E. cloacae	-	+/-	-	-	$bla_{CTX\text{-}M\text{-}9}$	$bla_{CTX\text{-}M\text{-}9}$	-	$bla_{CTX\text{-}M\text{-}9}$	FN phenotypical tests
E. cloacae	+	-	-	-	$bla_{CTX\text{-}M\text{-}9}$	$bla_{CTX\text{-}M\text{-}9}$	-	$bla_{CTX\text{-}M\text{-}9}$	FN phenotypical tests
E. cloacae	-	-	-	-	$bla_{CTX\text{-}M\text{-}9}$	$bla_{CTX\text{-}M\text{-}9}$	-	$bla_{CTX\text{-}M\text{-}9}$	FN phenotypical tests
E. cloacae	-	-	-	-	bla_{SHV}	bla_{SHV}	TEM	$bla_{SHV\text{-}12}$	FN phenotypical tests
E. cloacae	+	-	-	-	bla_{SHV}	bla_{SHV}	TEM	$bla_{SHV\text{-}12}$	FN phenotypical tests
E. cloacae	-	-	-	-	bla_{SHV}	bla_{SHV}	-	$bla_{SHV\text{-}12}$	FN phenotypical tests
E. cloacae	-	-	-	-	bla_{SHV}	bla_{SHV}	-	$bla_{SHV\text{-}2a}$	FN phenotypical tests
Morganella spp.	-	-	-	-	bla_{SHV}	-	-	-	FP microarray