CAT
Critically Appraised Topic

Part I: Evaluation of Three Different Agar Media for Rapid Detection of Extended-Spectrum β-lactamase Producing *Enterobacteriaceae* from Clinical Screening Samples.

Part II: Comparison of Different Phenotypic Assays for Detection of Extended-Spectrum β-lactamase Production by Inducible AmpC-Producing Gram-negative Bacilli.

Author: Elise Willems
Supervisor: Prof J Verhaegen
Search/methodology verified by: Prof J Verhaegen
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CLINICAL BOTTOM LINE

The first part of this study evaluated the performance of BLSE agar (AES Chemunex) and two chromogenic media, chromID ESBL (bioMérieux) and Brilliance ESBL agar (Oxoid) for rapid detection of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* from clinical screening samples. From a total of 139 specimens (69 perineal and 70 nose samples), sixty specimens (43%) yielded no growth on any media and 79 (57%) yielded growth on at least one of the selective media after 18 to 24h of incubation. Overall, 16 ESBL-producing *Enterobacteriaceae* strains (10 *Klebsiella pneumoniae*, 4 *Escherichia coli* and 2 *Citrobacter* species) were isolated from 16 specimens by a combination of all three media after 18 to 24h of incubation. The sensitivities by specimen were 87,5%, 87,5% and 81,3% for BLSE, Brilliance ESBL and chromID medium, respectively. The specificity of Brilliance ESBL and chromID (82,1% and 80,7%, respectively) was significantly higher than the specificity observed on BLSE medium (60,8%). Chromosomal AmpC-producing *Enterobacteriaceae*, mainly *Enterobacter* spp., as well as *P. aeruginosa* isolates accounted for most of the false-positive results. Prolonging the incubation time beyond 24h not significantly increased the sensitivity rate of either of the three evaluated media. It even increased the growth of mixed flora, decreasing specificity. Overall we show that BLSE, Brilliance ESBL as well as chromID are reliable culture media to screen for ESBL-producing *Enterobacteriaceae* directly from clinical samples. Yet, the main advantages of chromogenic media over BLSE reside in their chromogenic character and their significant higher specificity rate, reducing the need for identification work (with ± 70%) and unnecessary ESBL confirmation testing (with ± 40%) in our hospital setting when disregarding all colonies without a correct enterobacterial species chromogenic character. Yielding an excellent NPV of 98% for Brilliance ESBL and 97% for chromID at 24h in patients hospitalized in the intensive care units of the university hospital of Leuven, both media enable rapid exclusion of patients not carrying ESBL-producing *Enterobacteriaceae*.

In the second part of this study we compared the performance of different phenotypic assays to confirm ESBL production by inducible AmpC-producing *Enterobacteriaceae* (*Enterobacter* spp., *Morganella morganii, Providencia* spp., *Citrobacter freundii,* and *Serratia* spp.). Four different phenotypic ESBL confirmation methods (ESBL Etests®, combined double disk synergy tests (CDDST)) on Mueller-Hinton (MH) agar with or without the use of an AmpC inhibitor, cloxacillin were tested against a total of 73 AmpC inducible *Enterobacteriaceae* isolates with presumptive ESBL production and 10 extra well-defined ESBL-producing strains. All strains with presumptive ESBL production were screened for the presence of most common acquired ESBL-encoding genes by a microarray-based diagnostic test. The molecular result was considered the gold standard for evaluation of the other test methods. Among the 83 strains, 25 were ESBL-positive. Our study showed that cefotaxime as only indicator cephalosporin in a CDDST on MH agar is not able to reliably detect ESBL-encoding genes among chromosomal AmpC-producing *Enterobacteriaceae* due to its low sensitivity rate of 52%. Increased sensitivities of the CDDST by the addition of cloxacillin never reached the level of significance. It only significantly increased the specificity rate of the CDDST with CAZ as indicator. Regarding ESBL Etest® strips, the sensitivity rate of the PM/PML Etest® strip (80%) was significantly higher compared to the CT/CTL and TZ/TZL Etest® strips (16% and 32%, respectively). Addition of cloxacillin to the MH agar improved the ESBL detection of each ESBL Etest® strip. The highest sensitivity rate of 96% with a specificity rate of 95% was reached in a combination of PM/PML and TZ/TZL Etest® strips. Combining our results with previous literature, we recommend the CDDST on MH agar supplemented with cloxacillin and cefepime or ceftazidime as indicator cephalosporin as the most cost-efficient strategy to confirm ESBL production in inducible AmpC-producing *Enterobacteriaceae*. Further, we note that microbiologist should bear in mind that the use of Vitek2 AES is not a reliable ESBL-screening method among AmpC-producing *Enterobacteriaceae* .

CLINICAL/DIAGNOSTIC SCENARIO

Infections with multi-drug resistant Gram-negative bacilli (MDR-GNB) have become a great concern as they are associated with higher morbidity, mortality, prolonged hospital stay and rising health care costs[1-2]. As extended-spectrum β-lactamases are a main cause of multi-drug resistance (MDR) in GNB, early detection and identification of these resistance enzymes will help (i) to optimize antimicrobial therapy, (ii) to ensure timely introduction of appropriate infection control procedures to limit the spread of these multidrug-resistant organisms in hospital settings, and (iii) epidemiological surveillance [3-5].

Literature data concerning rapid screening for carriage of ESBL-producing Gram-negative bacilli in high risk populations by use of commercially available, selective (chromogenic) media supplemented with one or more antimicrobial agent(s) are limited [6-10]. To date, only phenotypic ESBL confirmation in *Enterobacteriaceae* with little or no chromosomal β-lactamase activity, such as *Klebsiella* spp., *E. coli* and *Proteus mirabilis*, has been well evaluated and is based on *in vitro* inhibition of ESBL by clavulanic acid (CA). In E*nterobacteriaceae* coproducing a chromosomally encoded inducible AmpC β-lactamase (e.g., *Enterobacter, Serratia*, *Providencia*, *Aeromonas* spp., *M. morganii*, *C. freundii*, *Hafnia alvei*, and *P. aeruginosa*)

clavulanate may act as an inducer of high-level AmpC production. This high-level AmpC production attacks the cephalosporins, thereby masking synergy arising from inhibition of the ESBL, causing false negative results in the inhibitor-based ESBL confirmatory tests. The use of an indicator cephalosporin stable against hydrolysis by most AmpC's (cefepime), as well as addition of an AmpC inhibitor (cloxacillin), have shown to improve ESBL detection in the presence of an AmpC β-lactamase [11-20]. However, studies concerning this topic are limited by the fact that they only include ESBL-producing *Enterobacter* spp., *P. aeruginosa* or less than 2 of other chromosomal AmpC-producing strains with ESBL coproduction.

In the first part of this work we will evaluate three commercially available (chromogenic) agars (BLSE, AES Chemunex; chromID ESBL, bioMérieux; Brilliance ESBL, Oxoid) for rapid detection of ESBL-producing *Enterobacteriaceae* from clinical screening samples. The second part compares the performance of different, commercially available phenotypic assays to confirm extended-spectrum β-lactamase production among inducible AmpC-producing *Enterobacteriaceae* isolates. To our knowledge, this study will be the first study to include a representative set of different, non-duplicated chromosomal AmpC-producing *Enterobacteriaceae* species (*Enterobacter cloacae*, *Enterobacter aerogenes*, *Morganella morganii, Providencia* spp., *Citrobacter freundii,* and *Serratia marcescens*) with different types of ESBL-encoding genes in different *Enterobacteriaceae* species isolates.).

QUESTION(S)

1) Which commercial available (chromogenic) agar for rapid screening for carriage of ESBL-producing *Enterobacteriaceae* in high risk populations has the best performance; BLSE (AES Chemunex), chromID ESBL (bioMérieux) or Brilliance ESBL (Oxoid)?

2) Which of four different phenotypic assays to confirm extended-spectrum β-lactamase production by inducible AmpC-producing Gram-negative bacilli (*Enterobacter* spp., *Morganella morganii, Providencia* spp., *Citrobacter freundii,* and *Serratia* spp.) has the best performance?

SEARCH TERMS

The literature search for this study was done via the MEDLINE database for citations from January 1980 to April 2012. Only papers published in the English language were considered.

MeSH-terms were "extended-spectrum β-lactamase or ESBL" combined with "cost, impact, outcome, implication, epidemiology, detection, screening, confirmation, identification, chromogenic agar/medium, phenotypic methods, E-test, *Enterobacteriaceae*, AmpC coproducers, *P. aeruginosa*, *Acinetobacter* spp." and "β-lactamase- and AmpC-inhibitor". Analytical performance data within these papers were only considered if molecular methods were used as gold standard. Other literature sources were recommendations published by the Clinical and Laboratory Standards Institute (CLSI [21]) and abstract publications of Interscience Conference on Antimicrobial Agents & Chemotherapy Chicago.

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24. Brilliance ESBL package insert, Oxoid Hampshire, United Kingdom.

MATERIALS & METHODS

Part 1

Hospital Setting and Specimen Collection

The study was performed at the University Hospital of Leuven (UZLeuven), Belgium, from October 2011 to March 2012. A total of 139 perineal and nose samples were processed. 97 specimens were obtained from 48 different patients hospitalized in the chirurgical intensive care units and 42 from 24 different patients hospitalized in the medical intensive care units.

Inoculation and Incubation of the Media

First the culture media were allowed to warm to room temperature. After vortexing (5"), 100µL of homogenized ESwab's (Copan Diagnostics, Murrieta California, USA) Liquid Amies suspension medium was inoculated onto each of the culture media: chromID ESBL (bioMérieux, Marcy l'Etoile, France), Brilliance ESBL (Oxoid, Hampshire, United Kingdom), MacConkey and Drigalski agar of the BLSE bi-plate (AES Chemunex, Bruz, France). All media were incubated at 35°± 2°C in ambient air and examined after 18 to 24 h and 42 to 48h of incubation. T

ChromID ESBL and Brilliance ESBL are media designed for selective isolation and presumptive identification of ESBL-producing Gram-negative bacilli, based on a rich nutrient capacity with a selective mixture of antibiotics, including cefpodoxime. BLSE agar is a commercially available bi-plate made of two selective media, Drigalski and MacConkey, supplemented with cefotaxime (1.5µg/mL^{-1}) and ceftazidime (2µg/mL^{-1}), respectively, enabling the detection of Gram-negative bacteria resistant to these antibiotics.

Identification of ESBL Producers.

For all three agars, the density of growth of each type of colony was scored semi quantitatively (+1, +2, +3, or +4, based on the number of quadrants in which growth was observed). For the chromogenic agars (chromID ESBL and Brilliance ESBL), also the colour of each type of colony was recorded according to the colour chart provided by the manufacturer (chromID ESBL (fig. 1): *Escherichia coli* pink/burgundy; KESC (*Klebsiella*/*Enterobacter*/*Serratia* /*Citrobacter*) group green/brownish-green/blue; PMP (*Proteus*/*Morganella*/*Providencia*) group dark to light brown colonies / Brilliance ESBL (fig. 2): *E. coli* blue/pink; KESC group green; PMP group tan with a brown halo; *Salmonella*, *Acinetobacter* and other resistant organisms colourless). All bacterial strains isolated on either medium were regarded as presumptive ESBL producers and identified by MALDI-TOF. Phenotypic confirmation of presumptive ESBL-producing *Enterobacteriaceae* was performed on a Mueller-Hinton (MH) agar, supplemented with cloxacillin for inducible AmpC-producing *Enterobacteriaceae*, by combined double disks using Neosensitabs tablets (30µg ceftazidime, 30µg cefotaxime, and 30µg cefepime with or without 10µg clavulanic acid) according to CLSI guidelines [21]. Further, non-inducible *Enterobacteriaceae* displaying an ESBL phenotype in the combined double-disk test as well as all inducible AmpC-producing *Enterobacteriaceae* were molecularly characterized by a microarray-based diagnostic test, Check-Point Check-MDR CT 101 (Check-Points Health BV, The Netherlands). This test identifies the presence of

TEM, SHV, CTX-M and AmpC-encoding genes as well as mutations leading to extended-spectrum types of TEM and SHV-enzymes [22].

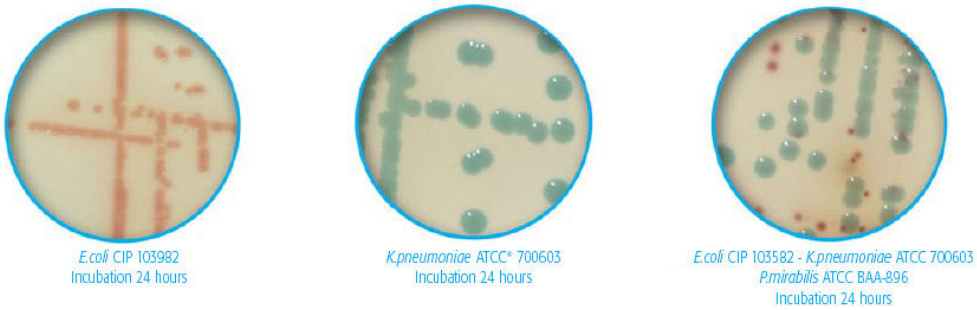

Fig. 1: Interpretation of the colour of each type of colony isolated on a chromID ESBL agar according to bioMérieux: *Escherichia coli* pink/burgundy; KESC (*Klebsiella/ Enterobacter/Serratia /Citrobacter*) group green/brownish-green/blue; PMP (*Proteus/Morganella/Providencia* [23].

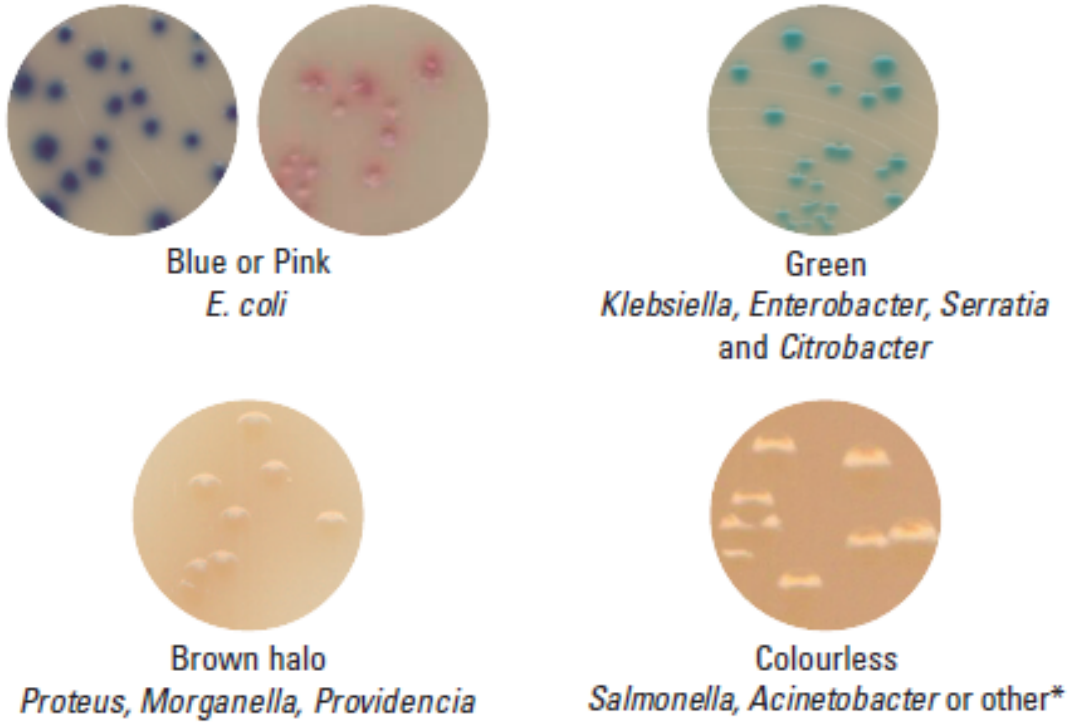

Fig. 2: Interpretation of the colour of each type of colony isolated on a Brilliance agar according to Oxoid [24].

Statistical Analysis

An *Enterobacteriaceae* isolate was categorized as ESBL positive when the ESBL phenotype displayed in the combined double-disk test was confirmed by molecular characterization [21] [22]. Statistical comparisons of the performance data of the different media (sensitivity [SN], specificity [SP], positive predictive value [PPV] and negative predictive value [NPV]) based on the total number of samples were assessed by using nonparametric McNemar test with Yates correction for each performance attribute. A sample was considered ESBL positive when at least one ESBL-positive isolate was recovered from it. A sample was considered ESBL negative when there was no growth on any medium or when the isolate recovered was not an ESBL producer. If a sample yielded a false positive (non-ESBL-producing *Enterobacteriaceae* or non-*Enterobacteriaceae* isolate with the colony color of *Enterobacteriaceae*) as well as a false negative result (no recovery from an ESBL positive sample or growth of an *Enterobacteriaceae*

isolate not producing the expected colony color) on a screening agar, then it was categorized as false positive as well as false negative. If a sample yielded a true positive as well as a false positive result on a screening agar, then it was categorized as true positive as well as false positive.

Part 2

Bacterial Strains

All consecutive nonduplicate strains of *Enterobacter* spp., *Morganella morganii, Providencia* spp., *Citrobacter freundii,* and *Serratia* spp. (AmpC inducible *Enterobacteriaceae)* isolated from clinical samples at the University Hospital (UZLeuven) of Leuven, Belgium, from August 2011 to January 2012 and fulfilling at least one of the following criteria were included in the study: (i) ESBL-screening criteria of the Clinical and Laboratory Standards Institute (CLSI[21], MIC cefotaxime ≥ 1 µg/mL, MIC ceftriaxone ≥ 1 µg/mL or MIC ceftazidime ≥ 1 µg/mL determined by the Vitek2 system), (ii) interpreted by the Vitek2's advanced expert system (AES) as ESBL positive with or without decreased outer membrane permeability (i.e. porin loss). A total of 73 AmpC inducible *Enterobacteriaceae* isolates recovered from clinical samples at UZLeuven were processed, including 31 urine samples, 17 respiratory tract samples(10 sputum, 7 lower respiratory tract aspirates), 11 wound fluids and 14 miscellaneous samples (4 blood cultures, 4 catheters, 2 nose swabs, 2 perineal swabs, 1 ear swab, and 1 peritoneal fluid). The 73 samples were obtained from 73 patients (median age of 65 years, 12 day-92 years) in either an ambulatory- care setting (21%, obtained in the emergency department or polyclinics from patients that were not hospitalized over the past 4 weeks) or a hospital setting (79%), including 19 samples collected from surgical intensive care units, 6 from medical intensive care units, 19 from general medicine units, 11 from general surgery units, 2 from pediatric units, 2 from revalidation, and 1 from a transplantation unit.

In addition, 10 extra molecularly-typed ESBL-producing isolates of AmpC inducible *Enterobacteriaceae* selected from the collection of clinical bacterial isolates of the national surveillance laboratory for multidrug resistant Gram-negative bacilli at the Clinique's UCL de Mont-Godinne were included in the study: *E. cloacae* (N=5), *E. aerogenes* (N=5), *C. freundii* (N=1), *S. marcescens* (N=1), *M. morganii* (N=1) and *P. stuartii* (N=1).

Vitek2 System

Antimicrobial susceptibility testing was performed by Vitek2 system's standard AST-N139 card (bioMérieux, Marcy l'Etoile, France). The AST-N139 card compromises various β-lactam antibiotics, including. cefuroxime, cefoxitin, cefotaxime, ceftazidime, and cefepime. It does not include testing of a cephalosporin in the presence of clavulanic acid (CLA). The results were interpreted by using an advanced expert system (AES, version 5.03) which examines the MIC of each antibiotic to determine potential resistance mechanisms expressed by each patient isolate, for each antibiotic family.

ESBL Etest®

Etest® ESBL detection strips (bioMérieux, Marcy l'Etoile, France) are plastic drug impregnated strips. One side contains a concentration gradient of a cephalosporin (ceftazidime (TZ 0,5-32µg/mL), cefotaxime (CT 0,25-16 µg/mL) or cefepime (PM, 0,25-16µg/mL)), the other contains a concentration

gradient of the same cephalosporin (ceftazidime (0,064-4µg/mL), cefotaxime (0,016-1µg/mL) or cefepime (0,064-4µg/mL)) plus a constant concentration of clavulanate (CLA, 4µg/mL), an ESBL inhibitor. The presence of ESBL is confirmed when we can observe: (i) a ≥ 3 two-fold decrease in the MIC value for any of the three cephalosporins in the presence of clavulanate, (ii) a phantom zone or (iii) a deformation of the CT, TZ or PM inhibition ellipse at the tapering end (bioMérieux Etest ESBL package insert). A result was considered indeterminate when MICs were higher or lower than the predefined range (making it impossible to calculate the MIC ratio) or when one of the tested strips displayed an indeterminate result and the others produced a negative result. All three ESBL Etest strips CT/CTL, TZ/TZL, and PM/PML were tested against each inducible AmpC-producing *Enterobacteriaceae* isolate with presumptive ESBL production on MH agar with or without cloxacillin (250µg/mL), an AmpC β-lactamase inhibitor (AES Chemunex, Bruz, France).

Combined Double Disk Synergy Test

Three different combined double disk synergy tests (CDDST) were tested against each inducible AmpC-producing *Enterobacteriaceae* isolate with presumptive ESBL production. The first was performed on a Mueller-Hinton (MH) agar and depended on comparing zones given by disks containing a 30µg extended-spectrum cephalosporin (cefotaxime (CTX), ceftazidime (CAZ), or cefepime (FEP)) with and without CLA (10µg). Isolates were considered ESBL positive if the inhibition zone around at least one extended-spectrum cephalosporin disk enlarged at least 5 mm in the presence of the ESBL inhibitor (CLA), as recommended by the manufacturer (Rosco Diagnostica, Taastrup, Denmark) and CLSI [21]. The second CDDST only differed from the first by using a MH-agar supplemented with cloxacillin (250µg/mL, AES Chemunex, Bruz, France). The third depended on comparing the inhibition zones around disks containing both cefotaxime and cloxacillin (30µg + 750µg/mL) with and without clavulanate. Isolates were considered ESBL positive when the inhibition zone around the cefotaxime with cloxacillin disk enlarged at least 5 mm in the presence of the ESBL inhibitor (CLA), as recommended by the manufacturer (Rosco Diagnostica, Taastrup, Denmark).

Molecular Characterization of Resistance Mechanisms

All strains were screened for the presence of most common acquired ESBL-encoding genes by a microarray-based diagnostic test, Check-Point Check-MDR CT 101 (Check-Points Health BV, The Netherlands), that identifies the presence of TEM, SHV, CTX-M genes and AmpC genes as well as mutations, leading to extended spectrum types of TEM and SHV-enzymes [22]. The molecular result was considered the gold standard for evaluation of the other test methods.

Statistical Analysis

An isolate was categorized as ESBL positive when the presence of an ESBL-encoding gene was demonstrated by molecular characterization. Indeterminate phenotypic ESBL confirmation results were considered as negative for performance calculations. Statistical comparisons of the performance data (sensitivity and specificity) based on the total number of collected strains were assessed by using nonparametric McNemar test with Yates correction.

RESULTS

Part 1

From a total of 139 perineal and nose samples, 60 (43%) yielded no growth on any media and 79 (57%) yielded growth on at least one of the selective media after 18 to 24h of incubation. Overall, 51 *Enterobacteriaceae* isolates were recovered on 50 specimens (36% of the total sample size) by at least one of the selective media after 18 to 24h of incubation. Forty seven isolates were detected from 46 samples on BLSE versus 36 from 36 samples on chromID and 39 from 39 samples on Brilliance ESBL. *E. coli* as well as *E. cloacae*, *S. marcescens* and *M. morganii* were more frequently isolated on BLSE medium than on chromID or Brilliance ESBL (Table 1). *Enterobacter* spp. (N=20), *K. pneumoniae* (N=10*), M. morganii* (N=5), *Citrobacter* spp. (N=5) and *S. marcescens* (N=5) were the predominant isolates in this study. Nine *Enterobacteriaceae* isolates (4 *C. freundii*, 2 *S. marcescens*, 2 *E. cloacae*, and 1 *P. mirabilis*) recovered on the chromogenic chromID medium and seven *Enterobacteriaceae* isolates (2 *C. freundii*, 2 *M. morganii*, 2 *S. marcescens* and 1 *E. cloacae*) recovered on the chromogenic Brilliance ESBL medium did not produce the expected colony colors. Only two (*C. freundii* species) of all *Enterobacteriaceae* isolates not producing the expected colony color on both chromogenic media, were ESBL positive. As both *C. freundii* stains had the color of an *E.coli* isolate, they caused no false negative screening results on both media.

Table 1: Identification and distribution of bacterial isolates recovered from 139 clinical samples on three different selective media.

Group/species	No. of isolates recovered after 18 to 24h of incubation on:				
	BLSE	chromID	Brilliance ESBL	All three media	One of three media
Escherichia coli	4	2	3	3	4
KESC-group[a]	38	31	33	27	41
Klebsiella pneumoniae	8	9	9	8	10
Klebsiella oxytoca	1	0	1	0	1
Enterobacter aerogenes	5	5	4	4	5
Enterobacter cloacae	12	7	10	6	13
Enterobacter ludwigii	2	2	2	2	2
Serratia marcescens	5	3	2	2	5
Citrobacter freundii	5	5	5	5	5
PMP-group[b]	5	3	3	2	6
Proteus mirabilis	0	1	0	0	1
Morganella morganii	5	2	3	2	5
Total no. of *Enterobacteriaceae*	47	36	39	32	51
Others					
Acinetobacter spp.	2	2	0	0	2
Burkholderia cepacia	0	2	2	0	2
Enterococcus spp.	0	2	8	0	9
Pseudomonas aeroginosa	12	8	10	7	14
Staphylococcus spp.	0	10	1	0	10
Stenotrophomonas maltophilia	6	7	3	3	7
Candida spp.	0	1	12	0	13

[a] KESC, *Klebsiella -Enterobacter -Serratia -Citrobacter* group

[b] PMP, *Proteus -Morganella -Providencia* group

Overall, 16 ESBL-producing *Enterobacteriaceae* strains (10 *K. pneumoniae*, 4 *E. coli* and 2 *C.itrobacter* species) were isolated from 16 specimens by a combination of all three media after 18 to 24h of incubation. The sensitivities by specimen were 87,5%, 87,5% and 81,3% for BLSE, Brilliance ESBL and chromID medium, respectively, with 14 ESBL-positive strains isolated from 14 specimens on BLSE and

Brilliance ESBL versus 13 ESBL-positive organisms from 13 specimens on chromID (Table 2). No significant difference in sensitivity has been found between the three selective media (x^2 for each selective medium compared < critical value of x^2 for a 5% significance level). The NPV of the ESBL-screening media for ESBL detection in patients hospitalized in the intensive care units of the university hospital of Leuven was 97% for BLSE and chromID and 98% for Brilliance ESBL.
Overall, CTX-M-1 enzyme was the most frequently encountered ESBL enzyme (13 isolates) and the sole ESBL enzyme type recovered from all *K. pneumoniae* isolates. SHV-5 ESBLs were found in 2 *C. freundii* isolates and once in an *E. coli* isolate together with a CTX-M-1 enzyme. TEM-derived ESBLs were the least commonly recovered (once in a *E. coli* isolate) (Table 3). There were no marked differences in the recovery rates according to the type of ESBL enzyme between the three tested media. However, the sole TEM-29 *E.coli* isolate was not recovered from chromID after 18 to 24h of incubation. This isolate did grow on chromID after 48h of incubation.

Table 2: Performance data of BLSE (AES Chemunex), chromID ESBL (bioMérieux) and Brilliance ESBL (Oxoid) after 18 to 24h of incubation and prolonged incubation of 48h. SN= sensitivity, SP=specificity.

	$BLSE_{18-24h}$	$BLSE_{48h}$	$ChromID_{18-24h}$	$ChromID_{48h}$	Brilliance $ESBL_{18-24h}$	Brilliance $ESBL_{48h}$
SN (%)	87,50	87,50	81,25	87,50	87,50	87,50
SP (%)	60,80	56,00	80,65	77,42	82,11	77,42
PPV (%)	22,22	20,29	35,14	33,33	38,89	33,33
NPV (%)	97,44	97,22	97,09	97,96	98,06	97,96

A comparison of the density of growth of the 16 ESBL-producing strains recovered on one of three selective media showed identical scores for all ESBL-producing isolates that grew on all selective media (N=12). Of four ESBL-producing isolates that were not recovered on all selective media, three grew only in small numbers (less than 10 colonies) on one selective medium only. One ESBL-producing isolate only recovered on BLSE and Brilliance ESBL, showed the highest density of growth on BLSE (growth till the third quadrant versus only in the first quadrant on Brilliance ESBL).

Table 3: Characterization of ESBLs from 16 *Enterobacteriaceae* isolates growing on any of the three selective media.

Species	Classes of ESBL enzymes (no. of isolates)		
(no. of isolates)	TEM	SHV	CTX-M
E.coli (4)	TEM-29 (1)	SHV-5 (1)[a]	CTX-M-1 (3)[a]
K. pneumoniae (10)			CTX-M-1 (10)
C. freundii (2)		SHV-5 (2)	

[a] Two ESBLs (CTX-M-1 and SHV-5) were present in one *E.coli* isolate.

The specificity was assessed on 123 specimens that were found negative for ESBL-producing *Enterobacteriaceae* by all three media after 18 to 24h of incubation. The specificity of Brilliance ESBL and chromID was 82,1% and 80,7%, respectively, not significantly differing from each other, but both being significantly higher than the specificity observed on BLSE medium (60,8%) (Table 2).
Inducible AmpC-producing *Enterobacteriaceae* isolates, mainly *Enterobacter* spp., accounted for most of the false-positive results on all three media with the highest recovery on BLSE medium. On the other hand, the specificity of all media for rapid screening for carriage of ESBL-producing *E. coli* and *K. pneumoniae* isolates was 100% (Table 4).

Table 4: Mechanism of resistance of *Enterobacteriaceae* isolates recovered on the selective ESBL media with expected colony colors. *Enterobacteriaceae* with molecular confirmed ESBL production, were classified as true positive isolates. Those with no molecular confirmed ESBL production (other), were classified as false positive isolates.

Group/species	No. of isolates recovered after 18 to 24h of incubation on:					
	BLSE		chromID		Brilliance ESBL	
	ESBL	Other	ESBL	Other	ESBL	Other
Escherichia coli	4	0	2	0	3	0
KESC-group[a]						
Klebsiella pneumoniae	8	0	9	0	9	0
Klebsiella oxytoca	0	1	0	0	0	1
Enterobacter aerogenes	0	5	0	5	0	4
Enterobacter cloacae	0	12	0	5	0	9
Enterobacter ludwigii	0	2	0	2	0	2
Enterobacter spp.	0	19	0	12	0	15
Serratia marcescens	0	5	0	1	0	0
Citrobacter freundii	2	3	2	1	2	3
PMP-group[b]						
Proteus mirabilis	0	0	0	1	0	0
Morganella morganii	0	5	0	3	0	1
Total no. of *Enterobacteriaceae*	14	33	13	18	14	20

Non-*Enterobacteriaceae* isolates (N=57) grew also on all three selective media (Table 5) and better on the chromogenic selective media (N=20 on BLSE, N=32 on chromID and N= 36 on Brilliance ESBL). Some of the non-*Enterobacteriaceae* isolates, in particular *P. aeruginosa* isolates recovered on all three media but also *Acinetobacter* spp. on BLSE, *Staphylococcus* spp. on chromID and *S. maltophilia* on chromID and BLSE had a colony color of *Enterobacteriaceae* causing false positive results on these selective media (Table 5). Despite the higher recovery of non-*Enterobacteriaceae* isolates on the chromogenic selective media, less false positive results were observed on the chromogenic media due to non-*Enterobacteriaceae* isolates (N=8 on chromID, N=2 on Brilliance ESBL) compared to BLSE medium (N=19). Organisms other than Gram-negative bacteria were only recovered on the chromogenic media; *Candida* spp. and *Enterococcus* spp. more on Brilliance ESBL and *Staphylococcus* spp. more on chromID.

Table 5: Non-*Enterobacteriaceae* isolates recovered on the three different selective ESBL media with a colony color of *Enterobacteriaceae* (false positive isolates, FP) or without a colony color of *Enterobacteriaceae* (true negative isolates, TN).

Group/species	No. of isolates recovered after 18 to 24h of incubation on:					
	BLSE		chromID		Brilliance ESBL	
	TN	FP	TN	FP	TN	FP
Acinetobacter spp.	0	2	2	0	0	0
Burkholderia cepacia	0	0	2	0	2	0
Candida spp.	0	0	1	0	12	0
Enterococcus spp.	0	0	2	0	8	0
Pseudomonas aeruginosa	1	11	3	5	8	2
Staphylococcus spp.	0	0	8	2	1	0
Stenotrophomonas maltophilia	0	6	6	1	3	0
Total no.	1	19	24	8	34	2

By prolonging the incubation of BLSE, Brilliance ESBL and chromID to 48h only one extra ESBL producing *Enterobacteriaceae* isolate was recovered on chromID, increasing it's sensitivity from 81,3%

to 87,5% (not significant, McNemar test) (Table 2). On the other hand, prolonged incubation resulted in increased growth of mixed flora, decreasing specificity of all three media, being significantly for BLSE and Brilliance ESBL (McNemar test) (Table 2).

Part 2

Among the 73 AmpC-inducible *Enterobacteriaceae* strains, 15 were ESBL producers (20,6%). Overall, *Enterobacter* spp. isolates were the most frequently encountered ESBL producers (N=12), next to *Providencia* species (N=2) and *C. freundii* (N=1). The majority of strains harbored a TEM-26 encoding gene (6 *E. aerogenes* and 2 *Providencia* species isolates). SHV-5 ESBL was found in 2 *E. cloacae* and in one *C. freundii* isolate and one in an *E. cloacae* isolate together with a CTX-M-9 enzyme. TEM-15 (N=1) and CTX-M-1(N=2) were the least commonly recovered ESBL-encoding genes (Table 6). The ESBL-producing strains were in general isolates from patients in hospital setting (93,3%), including general medicine units (N=8), surgical intensive care units (N=2), medical intensive care units (N=1), general surgery units (N=2) and transplantation units (N=1). Only 1 ESBL-producing isolate was recovered from a patient in ambulatory care setting.

Taking into account the 10 extra ESBL-producing AmpC inducible *Enterobacteriaceae* strains of the national surveillance laboratory for multidrug resistant Gram-negative bacilli, *Enterobacter* spp. remained the most frequently encountered ESBL producer and TEM-26 the most prevalent ESBL-encoding gene. TEM-24, SHV-4,-5,-12 and CTX-M-9 encoding genes expressed by these 10 extra strains differed from the ESBL-encoding genes encountered in the AmpC inducible *Enterobacteriaceae* of our hospital. Overall, 25 (30%) of the 83 AmpC inducible *Enterobacteriaceae* strains included in this study were ESBL positive (Table 6).

Table 6: Distribution of the 83 AmpC inducible *Enterobacteriaceae* isolates included in the study and their ESBL-encoding genes.

Enterobacteriaceae isolates	No. of isolates (% of total)	No. of ESBL producers (% of total)	Classes of ESBL enzymes (no. of isolates)		
			TEM	SHV	CTX-M
Enterobacter aerogenes	22 (27)	10 (12)	TEM-15 (1), TEM-24 (1), TEM-26 (6)	SHV-4,-5,-12 (2)[a]	CTX-M 9 (1)[a]
Enterobacter cloacae	25 (30)	8 (10)		SHV-5 (3)[b], SHV-12 (1)[b]	CTX-M-1 (3), CTX-M 9 (3)[b]
Citrobacter freundii	8 (10)	2 (2)	TEM-24 (1)	SHV-5 (1)	
Morganella morganii	17 (20)	1 (1)			CTX-M-1 (1)
Serratia marcescens	4 (5)	1 (1)		SHV-4,-5,-12 (1)	
Providencia spp.	7 (8)	3 (4)	TEM-17 (1), TEM-26 (2)		
Total	83 (100)	25 (30)	43% of ESBL-encoding genes (12)	28,5% of ESBL-encoding genes (8)	28,5% of ESBL-encoding genes (8)

[a] Four ESBL-encoding genes were present is one *E. aerogenes* isolate (CTX-M-9 combined with SHV-4,-5,-12)

[b] Two ESBL-encoding genes were present is two *E.cloacae* isolates (CTX-M-9 ones combined with SHV-5 and ones with SHV-12)

Vitek2 system. The Vitek2 AES system yielded a sensitivity and specificity rate of just 56% and 50% respectively (data not shown). The false negative results involved TEM-like as well as SHV- and CTX-M like genes (Table 8).

CDDST on Mueller-Hinton agar. For the combined double disk synergy test on MH agar, the sensitivity rate with CTX as only indicator cephalosporin (52%) was significantly lower than those using CAZ or FEP as only indicator cephalosporin (92% and 76%, respectively). The majority (78) of TEM-26 encoding genes, one out of two TEM-24 and SHV-4,-5,-12 encoding genes as well as the TEM-17, the CTX-M-9 combined with SHV-4,-5,-12 encoding genes were not detected by the CDDST with CTX as indicator. A better detection of TEM-26 encoding genes by the CDDST with CAZ as indicator

was responsible for the differences in sensitivity (not significant, McNemar test) between the CDDST with CAZ or FEP as indicator cephalosporin (3/8 isolates with FEP compared to 7/ 8 with CAZ). Adding FEP to CAZ as second indicator cephalosporin did not increase the sensitivity rate of the test. Also, the addition of cloxacillin to the cefotaxime disk (64%) could not significantly increase the sensitivity rate of CTX (52%) (McNemar test). Only two isolates (TEM-26 producing *Providencia stuartii* and CTX-M-1 producing *Morganella morganni*) tested false negative by the CDDST with CAZ as indicator cephalosporin. However, this method has a significantly lower specificity (76%) compared to all other evaluated phenotypic ESBL confirmation assays.

Conclusion: ESBL confirmation in chromosomal AmpC-producing *Enterobacteriaceae* by CDDST on MH agar with CTX as only indicator cephalosporin was not reliable. A CDDST on MH with CAZ as indicator cephalosporin showed a high sensitivity, but a poor specificity rate.

CDDST on Mueller-Hinton agar supplemented with cloxacillin. Performing the CDDST on a MH agar supplemented with cloxacillin yielded 8% indeterminate results due to overlap of large zone diameters. The addition of cloxacillin increased sensitivity rates of the CDDST with CTX (68%) or FEP (92%) as only indicator, but the difference did not reach statistical significance (McNemar test). An interesting finding was that one out of four CTX-M 1-producing *M. morganii* isolates not recovered by any CDDST on MH agar could be detected by addition of cloxacillin (Table 8). Further, it significantly increased the specificity of the CDDST with CAZ as indicator cephalosporin (76% to 93%, Table 7). Either combination of indicator cephalosporins had better performance data than the CDDST with CAZ or FEP as sole indicators.

Conclusion: The CDDST on MH agar supplemented with cloxacillin reached best performance data with CAZ or FEP as sole indicators.

ESBL Etests® on Mueller-Hinton agar. The ESBL Etest method on MH agar yielded 27% to 63% indeterminate results, mainly because MICs were above the level of detection for CT/CTL and TZ/TZL Etests and below the level of detection for PM/PML Etests. Performing ESBL confirmation testing by a CT/CTL, a TZ/TZL strip or a combination of both strips is not appropriate as their sensitivity rates only reached 16%, 32% and 36%, respectively. The highest sensitivity was obtained when a PM/PML was combined with a TZ/TZL strip (SN, 88%, SP, 98%). However, the addition of TZ/TZL did not significantly improve the performance of PM/PML alone (SN, 80%, SP, 100%). In contrast to the other evaluated phenotypic ESBL confirmation methods, none of the ESBL Etests were able to detect the TEM-17like producing *P. stuartii* (Table 8).

Conclusion: ESBL confirmation in chromosomal AmpC-producing *Enterobacteriaceae* by a CT/CTL, a TZ/TZL strip or a combination of both strips on a MH agar is not acceptable due their low sensitivity rates. The PM/PML strip with or without the TZ/TZL strip is the best performing Etests® -based ESBL confirmation in chromosomal AmpC-producing *Enterobacteriaceae* on MH agar.

Table 7: Summary of the performance data of different phenotypic ESBL confirmation assays. SN= sensitivity, SP= specificity, IR= indeterminate results, MH= Mueller-Hinton.

Method	SN (%)	SP (%)	IR (%)
CDDST on MH agar			
CTX	52	91	0
CAZ	92	76	0
FEP	76	93	0
CTX + CAZ	92	76	0
CTX + FEP	76	90	0
CAZ + FEP	92	76	0
CTX + CAZ + FEP	92	76	0
CTX + cloxacillin	64	100	0
CDDST on MH agar supplemented with cloxacillin			
CTX	68	98	8
CAZ	92	93	8
FEP	92	98	8
CTX + CAZ	92	91	8
CTX + FEP	92	97	8
CAZ + FEP	92	93	8
CTX + CAZ + FEP	92	93	8
ESBL Etest on MH agar			
CT/CTL	16	100	61
TZ/TZL	32	98	53
PM/PML	80	100	28
CT/CTL + TZ/TZL	36	98	60
CT/CTL + PM/PML	80	100	60
TZ/TZL + PM/PML	88	98	59
CT/CTL + TZ/TZL + PM/PML	88	98	63
ESBL Etest on MH agar supplemented with cloxacillin			
CT/CTL	68	100	37
TZ/TZL	80	100	34
PM/PML	84	95	52
CT/CTL + TZ/TZL	80	100	39
CT/CTL + PM/PML	88	95	53
TZ/TZL + PM/PML	96	95	52
CT/CTL + TZ/TZL + PM/PML	96	95	52

ESBL Etests® on Mueller-Hinton agar supplemented with cloxacillin. Supplementing cloxacillin to the MH agar decreased the number of indeterminate results for CT/CTL (61% to 37%) and TZ/TZL (53% to 34%) but increased the indeterminate result rate of PM/PML from 28% to 52%. Sensitivity rates were higher than for the respective tests on MH agar. A higher recovery rate of TEM-26, SHV-4,-5,-12 and CTX-M-1 producing *Enterobacteriaceae* by CT/CTL and TZ/TZL after cloxacillin addition was observed (TEM-26; 4-5/8 isolates compared to 1-3/8 isolates. SHV-4,-5,-12: 2/2 isolates compared to 0-1/2 isolates. CTX-M-1: 3-4/4 isolates compared to 0-1/4 isolates). Further, the TEM-15 *E. aerogenes* isolate was discovered by CT/CTL and TZ/TZL after cloxacillin addition. The slight increase in the sensitivity for PM/PML was due to the recovery of the TEM-24 producing *E. aerogenes* (Table 8). Sensitivity was highest when the results of both TZ/TZL and PM/PML strips or of all three strips were combined (96%). The only significant difference in performance was the difference in

sensitivity between the CT/CTL strip and the combinations of at least the TZ/TZL and PM/PML strips (Table 7).

Conclusion: The CT/CTL Etest® strip has a significant lower sensitivity rate than the combination of TZ/TZL with PM/PML, which has the highest sensitivity rate. All other Etest strips or combinations of strips showed good and not significantly differing performance data for ESBL detection in chromosomal AmpC-producing isolates.

Table 8: Summary of sensitivity rates of different phenotypic ESBL confirmation assays for each different type of recovered ESBL-encoding gene. MH= Mueller-Hinton.

Method	Sensitivity rates (%) of different ESBL encoding genes (n=number of isolates encoding the respective gene)										
	TEM-15 (n=1)	TEM-17-like (n=1)	TEM-24 (n=2)	TEM-26 (n=8)	SHV-5 (n=3)	SHV-4,-5,-12 (n=2)	CTX-M-1 (n=4)	CTX-M-9 (n=1)	CTX-M-9 & SHV-5 (n=1)	CTX-M9 & SHV-12 (n=1)	CTX-M9 & SHV-4,-5,-12 (n=1)
Vitek2	0	100	50	37,5	100	50	50	100	100	100	0
CDDST on MH agar											
CTX	100	0	50	12,5	100	50	75	100	100	100	0
CAZ	100	100	100	87,5	100	100	75	100	100	100	100
FEP	100	100	100	37,5	100	100	75	100	100	100	100
CTX + CAZ	100	100	100	87,5	100	100	75	100	100	100	100
CTX + FEP	100	100	100	37,5	100	100	75	100	100	100	100
CAZ + FEP	100	100	100	87,5	100	100	75	100	100	100	100
CTX + CAZ+ FEP	100	100	100	87,5	100	100	75	100	100	100	100
CTX + cloxacillin	100	0	50	25	100	100	100	100	100	100	100
CDDST on MH agar supplemented with cloxacillin											
CTX	100	0	50	25	100	100	100	100	100	100	100
CAZ	100	100	100	75	100	100	100	100	100	100	100
FEP	100	100	100	75	100	100	100	100	100	100	100
CTX + CAZ	100	100	100	75	100	100	100	100	100	100	100
CTX + FEP	100	100	100	75	100	100	100	100	100	100	100
CAZ + FEP	100	100	100	75	100	100	100	100	100	100	100
CTX + CAZ+ FEP	100	100	100	75	100	100	100	100	100	100	100
ESBL Etest on MH agar											
CT / CTL	0	0	50	12,5	67	0	0	0	0	0	0
TZ / TZL	0	0	50	37,5	33	50	25	100	0	0	0
PM / PML	100	0	50	62,5	100	100	50	100	100	100	100
CT / CTL + TZ/TZL	0	0	50	37,5	67	50	25	100	0	0	0
CT / CTL + PM/PML	100	0	50	62,5	100	100	100	100	100	100	100
TZ / TZL + PM/PML	100	0	50	87,5	100	100	100	100	100	100	100
CT / CTL + TZ/TZL + PM/PML	100	0	50	87,5	100	100	100	100	100	100	100
ESBL Etest on MH agar supplemented with cloxacillin											
CT / CTL	100	0	50	50	67	100	75	100	100	100	100
TZ / TZL	100	100	50	62,5	67	100	100	100	100	100	100
PM / PML	100	0	100	62,5	100	100	100	100	100	100	100
CT / CTL + TZ/TZL	100	100	50	62,5	67	100	100	100	100	100	100
CT / CTL + PM/PML	100	0	100	75	100	100	100	100	100	100	100
TZ / TZL + PM/PML	100	100	100	87,5	100	100	100	100	100	100	100
CT / CTL + TZ/TZL + PM/PML	100	100	100	87,5	100	100	100	100	100	100	100

Comparison of the different phenotypic ESBL confirmation assays. Because the main goal of ESBL confirmation is to reach high sensitivity, we performed statistical comparisons among the phenotypic methods with the three highest sensitivity rates for ESBL detection in chromosomal AmpC-producing *Enterobacteriaceae* (Table 9*)*. If the sensitivity of an ESBL confirmation method using one cephalosporin as indicator was not increased by concomitant testing of another cephalosporin, only the confirmation method using one cephalosporin as indicator was considered in the statistical comparison of the methods with the highest sensitivity rates.

The statistical comparison showed that the CDDST on MH agar with ceftazidime as indicator had a significantly lower specificity than all other tests. Other comparisons did not reach the level of statistical significance. Taking into account the cost of all 5 different assays with comparable performance data, the CDDST on MH agar supplemented with cloxacillin and cefepime or ceftazidime as indicator cephalosporin is the most cost-efficient strategy to confirm ESBL production in inducible AmpC-producing *Enterobacteriaceae*.

Table 9: Performance data of the phenotypic methods with the three highest sensitivity rates for ESBL detection in chromosomal AmpC-producing *Enterobacteriaceae*.

Method	SN (%)	SP (%)
CDDST on MH agar		
CAZ	92	**76**
CDDST on MH agar supplemented with cloxacillin		
CAZ	92	93
FEP	92	98
ESBL Etest on MH agar		
TZ/TZL + PM/PML	88	98
ESBL Etest on MH agar supplemented with cloxacillin		
CT/CTL + PM/PML	88	95
TZ/TZL + PM/PML	96	95

Part 1

This evaluation shows that BLSE, Brilliance ESBL and chromID are reliable culture media for the screening of ESBL-producing *Enterobacteriaceae* directly from clinical samples. After 18 to 24h of incubation, the Brilliance and the BLSE agar yielded a higher sensitivity (87,5%) than the chromID (81,3%), but the difference did not reach statistical significance. As previous studies already indicated [6,9], we confirm that prolonging the incubation time beyond 24h not significantly increases the sensitivity rate of either of the three evaluated media for detecting ESBL-producing *Enterobacteriaceae*. It even increases the growth of mixed flora, decreasing specificity.

Concerning the specificity, twice as much false-positive bacteria were detected after 24h of incubation on the BLSE agar than on the chromID or the Brilliance ESBL medium. The difference in specificity between the chromogenic ESBL selective media was not significant. Plasmid- or chromosomally mediated AmpCproducing *Enterobacteriaceae* isolates, primarily accounted for false-positive results on all three media.

On the whole,13.9% (N=10) of the 72 patients were found to be colonized or infected with ESBL-producing *Enterobacteriaceae* isolates. However, this figure does not reflect the actual prevalence of patients carrying ESBL-producing *Enterobacteriaceae* at the intensive care units of our hospital, as only screening samples of patients with reported Gram-negative bacilli in one of their previous clinical specimens were included.

The present study has a concern limitation, in that the number and types of ESBL-producing *Enterobacteriaceae* detected were small. Comparing our findings with larger studies, the sensitivity and specificity of chromID and Brilliance ESBL were lower than reported earlier [6,7,9,10], while the sensitivity of BLSE medium was comparable to that reported by Reglier-Poupet et al. (85%, [9]) (Table 10). Previous studies neither discovered a significant difference in sensitivity between these selective media for detection of ESBL-producing *Enterobacteriaceae* from clinical specimens [7,9,10]. Similarly, studies comparing chromID and Brilliance ESBL [7,10] reported no significant difference in performance between these chromogenic ESBL selective media. Comparable to our findings, Reglier-Poupet et al. [9] discovered that the specificity of chromID was significantly higher than the BLSE agar's. Unlike the study of Glupczynski et al. [6], in our study not all *Enterobacteriaceae* isolates recovered on the chromogenic selective media produced the expected colony colors. However, in contrast to previous studies [7,9], they were not responsible for false negative results, While Huang et al. [7] found that seven out of sixty ESBL-producing *Enterobacteriaceae* that grew on both chromogenic media, had a higher growth density on one of both chromogenic media, our study shows identical density scores for all ESBL-producing isolates that grew on all selective media.

Overall we show that BLSE, Brilliance ESBL as well as chromID are reliable culture media to screen for ESBL-producing *Enterobacteriaceae* directly from clinical samples. Yet, the main advantages of chromogenic media over BLSE reside in their significant higher specificity and thus a lower number of false-positive results after 24h. This is due to a lower recovery of non-ESBL producing *Enterobacteriaceae* on the chromogenic media compared to the BLSE medium and their chromogenic

character. The first reduces the need for unnecessary ESBL confirmation testing (with 45% for chromID and 39% for Brilliance ESBL compared to BLSE) and the chromogenic character enables presumptive identification of *Enterobacteriaceae* within 24h and reduces unnecessary identification work (with 58% for chromID and 89% for Brilliance ESBL compared to BLSE) when disregarding all colonies without a correct enterobacterial species chromogenic character. Yielding an excellent NPV of 98% for Brilliance ESBL and 97% for chromID at 24h in patients hospitalized in the intensive care units of the university hospital of Leuven, both media enable rapid exclusion of patients not carrying ESBL-producing *Enterobacteriaceae*.

Table 10: Performance data of selective screening media for detection of ESBL-producing *Enterobacteriaceae* in clinical samples as reported by previous studies.

References	BLSE		chromID		Brilliance ESBL	
	SN (%)	SP (%)	SN (%)	SP (%)	SN (%)	SP (%)
Glupczynski et al. [6]			97,7	89,0		
Huang et al. [7]			86,4	95,5	94,9	95,7
Reglier-Poupet et al. [9]	85,0	82,0	88,0	94,4		
Saito et al. [10]			88,2	92,9	100,0	93,3

Part II

Correct identification of ESBL-positive *Enterobacteriaceae* in due time is not only required for optimal patient management but also to ensure timely introduction of appropriate infection control procedures to limit the spread of these multidrug-resistant organisms in hospital settings, and for epidemiological purposes [3-5].

In this study four different phenotypic ESBL confirmation assays were evaluated regarding their sensitivity and specificity to detect ESBL production among chromosomal AmpC-producing *Enterobacteriaceae* isolates. Previous studies have already tested the ability of phenotypic methods to detect ESBL production in chromosomal AmpC-producing *Enterobacteriaceae* [11, 12, 15, 17, 18, 20]. Our study is original as it is the first including a representative set of different, nonduplicated chromosomal AmpC-producing *Enterobacteriaceae* species (*E. cloacae*, *E. aerogenes*, *M. morganii, Providencia* spp., *C. freundii,* and *S. marcescens*) with different types of ESBL-encoding genes in different *Enterobacteriaceae* species isolates.

Conform with previous studies [11,17], the evaluation of the performance of different indicator cephalosporins in the CDDST on MH Agar employing the criterium of at least 5mm increase in zone diameter in the presence of CA to confirm ESBL production in chromosomal AmpC-producing *Enterobacteriaceae*, showed that CTX as only indicator cephalosporin is not able to reliably detect ESBL-encoding genes in these species due to its low sensitivity rate of 52%. Even though FEP is more stable than CAZ against hydrolysis by most AmpC's and in contrast to the previous studies [11,17] , our study shows the highest sensitivity rate with CAZ and not CEP as indicator cephalosporin. However, the difference in sensitivity was not significant (McNemar). A better detection of TEM-26 producing *E. aerogenes* and *Providencia* spp. isolates by the CDDST with CAZ as indicator was responsible for its higher sensitivity. In previous studies [11,17], the higher sensitivity rate of FEP was

due to better recovery of SHV-type encoding *Enterobacter* spp. as they were the only or the majority (10/11) of ESBL-producing isolates in these studies. However, in our study all SHV-producing isolates (Table 6) were equally encountered by CAZ and FEP. This may be explained by a difference in test performance according to the type of SHV-encoding gene. As the study of Garrec et al. [11] does not determine the type of encountered SHV-encoding genes and the study of Towne et al. only included SHV-12 producing *Enterobacter* spp., which were not solely encountered in our study (Table 6), further studies including more species with different types of SHV-encoding genes are necessary to explore this. Adding FEP to CAZ as second indicator cephalosporin did not increase the sensitivity rate of the test. Anyhow, a CDDST with CAZ as only indicator has a significantly lower specificity (76%) compared to all other evaluated phenotypic ESBL confirmation assays.

ESBL detection using cloxacillin as AmpC inhibitor showed higher sensitivity for the CDDST with CTX as indicator cephalosporin when the inhibitor was added to MH agar than when it was added to the CTX disk. However, the difference in sensitivity did not reach the level of statistical significance. Unlike Garrec et al. [11], increased sensitivities of the CDDST by the addition of cloxacillin never reached the level of significance. It only significantly increased the specificity rate of the CDDST with CAZ as indicator in our evaluation.

Regarding ESBL Etest® strips, the manufacturer recommends to test cefotaxime and ceftazidime ESBL Etest® strips on MH agar in first-line, followed by the cefepime ESBL Etest® strip in case of an inconclusive result from the first two strips. Our results agree with previous studies [11,18] that this strategy is not appropriate to confirm ESBL production in chromosomal AmpC-producing *Enterobacteriaceae* as the sensitivity rate of the proposed first-line method only reaches a sensitivity rate of 31-36%. Furthermore, we confirmed that the sensitivity rate of the PM/PML Etest® strip (80%) was significantly higher compared to the CT/CTL and TZ/TZL Etest® strips (16% and 32%, respectively). Previous studies reported sensitivity rates for the PM/PML Etest® strip ranging from 60% to 100% [11, 12,18, 20]. This wide range can perhaps be explained by differences in type of ESBL enzymes included in each study. While Garrec et al. reported that additional testing of other ESBL Etest® strips did not increase the performance of the PM/PML Etest® strip, we observed an 8% increase (80 to 88%) in sensitivity when the PM/PML Etest® strip was combined with the TZ/TZL strip. However, this increase was not statistically significant.

Our evaluation demonstrates that the addition of cloxacillin to the MH agar improves the ESBL detection of each ESBL Etest® strip. The highest sensitivity rate of 96% with a specificity rate of 95% was reached in a combination of PM/PML and TZ/TZL Etest® strips. All other Etest® strips or combinations of strips showed good and not significantly differing performance data for ESBL detection in chromosomal AmpC-producing *Enterobacteriaceae,* except for the CT/CTL Etest® strip which has a significant lower sensitivity rate than the combination of TZ/TZL with PM/PML.

This study also determined the performance of Vitek2's advanced expert system (AES, version 5.03) as ESBL-screening system. Like Garrec et al. [11], we note that microbiologist should bear in mind that this is not a reliable ESBL-screening method among AmpC-producing *Enterobacteriaceae* (sensitivity =

56% and specificity = 50%). In contrast, some authors have reported a high sensitivity for Vitek2 AES [12, 20]. This could be explained by a different Vitek2 AES software version.

Combining our results with previous literature, we recommend the CDDST on MH agar supplemented with cloxacillin and cefepime or ceftazidime as indicator cephalosporin as the most cost-efficient strategy to confirm ESBL production in inducible AmpC-producing *Enterobacteriaceae*.

We observed differences among the phenotypic tests for ESBL confirmation according to the type of β-lactamase produced. However, to more reliable confirm these observations and to evaluate if there also exist a difference among the tests according to the type of species, more studies including equal number of different strains containing each type of ESBL are necessary. In addition, some rare types of ESBL enzymes have not been tested, and further confirmation of our results with isolates producing these enzymes may be of interest.
