CAT
Critically Appraised Topic

Evaluation and Development of a Phenotypic Screening Strategy for Emerging β-lactamases in Gram-negative Bacilli.

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CLINICAL/DIAGNOSTIC SCENARIO

Gram-negative bacteria such as *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter* spp. are able to cause serious infections in hospitalized patients. Treatment of these infections is often complicated due to the increasing bacterial resistance against different classes of antibiotics.
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.
As emerging β-lactamases (ESBLs, AmpC β-lactamases and carbapenemases) are a main cause of multi-drug resistance (MDR) in GNB, early detection and identification of these resistance enzymes will help to optimal antimicrobial therapy and ensure timely introduction of appropriate infection control procedures.
In this work we evaluate phenotypic screening and confirmation methods for emerging β-lactamases in GNB in order to develop an efficient screening strategy. Literature data concerning *direct* screening for MDR-GNB in clinical screening samples by use of selective media supplemented with one or more antibiotimicrobial agent are limited. But, since there is a great interest in clinical laboratories for the use of chromogenic media, this review will focus on the value of these for selective isolation of Gram-negative bacilli with emerging ß-lactamases. Besides, this work discusses different phenotypic screening and confirmation assays to identify these enzymes among *Gram-negative isolates*. Finally, based on the comparison of these methods, recommendations will be made on which phenotypic assays to use for each type of emerging ß-lactamase.

SEARCH TERMS

A literature search was done via Pubmed for citations from January 1980 to March 2011. Only papers published in the English language were considered. MeSH-terms were "ESBL or AmpC or class C beta-lactamase or carbapenemase or KPC or metallo-beta-lactamase or OXA or beta-lactamase" combined with "detection, screening, confirmation, identification, chromogenic agar/medium, Vitek2, Phoenix, Etest, comparison of methods, inducible *Enterobacteriaceae*, *P. aeruginosa*, *Acinetobacter* spp." and "beta-lactamase inhibitors, AmpC inhibitors, carbapenemase inhibitors, metallo-beta-alctamase inhibitos, Modified Hodge".
Performance data within these papers were only considered if PCR was used as the gold standard.
Other literature sources were ECCMIDs abstract publications and recommendations published by the Clinical and Laboratory Standards Institute (CLSI [1]), the European Society of Clinical Microbiology and Infectious Diseases (EUCAST [1]), the British Society for Antimicrobial Chemotherapy (BSAC [3]) and Netherlands Society for Medical Microbiology (NVMM [4]).

APPRAISAL

1. ESBL†

1.1 Screening for ESBL in surveillance samples

Recently, chromogenic media designed specifically for screening and identification of ESBL- producing Enterobacteriaceae, have become commercially available: CHROMagar ESBL (Kanto Chemical, Tokyo, Japan), chromID ESBL (bioMérieux, Marcy l'Etoile, France) and Brilliance ESBL agar (Oxoid, Basingstoke, United Kingdom). Only a few studies evaluated the performance of these media to detect ESBL-producing Enterobacteriaceae in surveillance samples [4][6][7][8][9].

† The term ESBL in this work refers to extended spectrum bèta-lactamases of the molecular class A (Ambler system [10]).

Thereof, the only one evaluating the CHROMagar ESBL [4] found no significant difference in performance between CHROMagar ESBL and chromID ESBL. However, this study has a concern limitation, as it included only a limited number of ESBL producers.

Three studies have been published concerning the chromID ESBL agar [6][7][8][6][9]. The first study [6] was limited by the fact that only ESBL types that are prevalent in Belgium were included. It reported no significant differences in performance compared to a MacConkey agar supplemented with ceftazidime (2mg/L). However, isolates producing a CTX-M type ESBL were better recovered from the chromID ESBL. The authors explained this phenomen by the fact that CTX-M ESBL enzymes are usually susceptible to ceftazidime while chromID ESBL contains cefpodoxime, hydrolysed by CTX-M type ESBLs. The other two studies [7][9], that did include less prevalent ESBL types, compared the performance of chromID ESBL to selective Gram-negative agars supplemented with cefotaxime (CTX) and ceftazidime (CAZ). One [7] found no difference in sensitivity between both agars, whereas the other[9] reported better sensitivity (SN) rates for the chromogenic agar (100% compared to 88%).

The only study comparing the performance of the Brilliance ESBL agar to chromID ESBL and a MacConkey agar with a ceftazidime (30µg) disk is Huang et al. [8]. Its results are comparable to the study of Paniagua et al. [9], showing that chromogenic agars are more sensitive for ESBL detection in surveillance sample. In this study, both chromogenic media had the same performance data (SN= 95%, specificity (SP)=96%)

1.2 Screening for ESBL among Gram-negative isolates

In 2010, both CLSI [1]and EUCAST [2] published new cephalosporin and aztreonam susceptibility breakpoints for Enterobacteriaceae. When these revised breakpoints are used, routine ESBL testing should be no longer necessary as these breakpoints will detect all clinically important resistance mechanisms (including ESBL, plasmid mediated AmpC). Until implementations of these new guidelines, ESBL testing should still be performed. After implementation, ESBL testing may still be useful for epidemiological and infection control purposes. But at this moment, EUCAST has no recommendations for ESBL-screening and CLSI recommendations for ESBL-screening are limited to *K. oxytoca*, *K. pneumoniae*, *E. coli* and *P. mirabilis*. On the other hand, BSAC [3] and NVMM [4] have recommendations for ESBL-screening among all Enterobacteriaceae, but they lack a method detecting ESBL production in non-Enterobacteriaceae (e.g. *P. aeruginosa*, *Acinetobacter* spp.).
Comparing the strategies proposed by these guidelines for ESBL-screening, no uniform recommendations exist on the choice of indicator antimicrobial agent(s) and cut-off value(s) (Table 1).
The most prevalent ESBL-types in clinical isolates in Europe are TEM, SHV and CTX-M. As some SHV- and TEM-type ESBLs (e.g. TEM-10 and -26) are much more active against ceftazidime (CAZ) than against cefotaxime (CTX) or ceftriaxone (CTR), the use CTX and CTR as the only indicator for ESBL screening can no longer be recommended. Neither can CAZ be recommended as only indicator because many CTX-M ESBL enzymes do not confer resistance to CAZ.
If only one indicator antibiotic would be used for screening, cefpodoxime (CPD) has proven to be the best molecule for screening all types of ESBL producers in clinical specimens [3][4][13][14][15][16]. However, screening with a combination of CAZ with CTX or CTR allows better specificity [4][17].

Guideline	Indicator antimicrobial agent(s)	Cut-off values for DD (mm)	Cut-off values for MIC determination (µg/mL)
CLSI [1]*	Cefotaxime, CTX (30µg)	≤27	≥ 1
(*E. coli, K. pneumoniae*	Ceftriaxone, CTR (30µg)	≤25	≥ 1
K. oxytoca)	Cefpodoxime,CPD (10µg)	≤17	≥ 4
	Ceftazidime,CAZ (30µg)	≤22	≥ 1
	Aztreonam, ATM (30µg)	≤27	≥ 1
(*P. mirabilis*)	Cefotaxime (30µg)	≤27	≥ 1
	Cefpodoxime (10µg)	≤22	≥ 1
	Ceftazidime (30µg)	≤22	≥ 1
BSAC[3]	Cefpodoxime alone (10µg)	≤19	>1
(Enterobacteriaceae)	Cefotaxime/ceftriaxone (30µg)	NA**	NA**
	combined with ceftazidime (30µg)	NA**	NA**
NVMM [4]	at least cefotaxime (30µg)	NA**	>1
(Enterobacteriaceae)	and ceftazidime (30µg)	NA**	>1

Table 1. Recommendations of the Clinical and Laboratory Standards Institute (CLSI [1]), Britisch Society for Antimicrobial Chemotherapy (BSAC [3]) and Netherlands Society for Medical Microbiology (NVMM [4]) on which antimicrobial agent(s) and cut-off value(s) should be used for ESBL-screening. *CLSI recommends the use of more than one antimicrobial agent for screening as it improves the sensitivity of ESBL detection. But CLSI does not specify which antimicrobial agents should be best used. ** Not available.

1.3 ESBL confirmation

Phenotypic ESBL confirmation tests for routine are based on *in vitro* inhibition of ESBL by clavulanic acid (CA). These tests were tailored to detect ESBLs in *Klebsiella* spp., but are equally applicable to other Enterobacteriaceae with little or no chromosomal β-lactamase activity, such as *E. coli* and *Proteus mirabilis* [17]. However, false negative results can be obtained in (see also Table 2):

(1) strains that coproduce an inducible chromosomal or plasmid-mediated AmpC ß-lactamase. Because AmpC enzymes may be induced by clavulanate (which inhibits them poorly) and may then attack the cephalosporins, masking synergy arising from inhibition of the ESBL [13][19].
(2) *P. aeruginosa*, having not only an inducible AmpC enzyme but also a much higher degree of impermeability than Enterobacteriaceae and efflux-mediated resistance mechanisms.
(3) some ESBLs weakly or not inhibited by clavulanate (e.g. CMT) [20].
(4) carbapenemase co-producing strains due to the fact that these can hydrolyse cephalosporins but are not or poorly inhibited by clavulanate [21][22].

Also false positive results are reported using inhibitor based ESBL detection methods:

(1) mainly in *Klebsiella oxytoca* isolates, hyperproducing the chromosomal ß-lactamase K1 [23].
(2) occasionally in SHV-1 hyperproducers or KPC producers.

Causes of false negative results in ESBL confirmation tests	**Causes of false positive results in ESBL confirmation tests**
Chromosomal AmpC ß-lactamases (*Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp., *Serratia* spp., *P. aeruginosa* and *A. baumanii*) **Plasmid-mediated AmpC ß-lactamases** (described mostly in *K. pneumoniae, K.oxytoca, E. coli,* sometimes in *Salmonella* spp., *Proteus mirabilis*, *Citrobacter freundii and Enterobacter aerogenes*). ***P. aeruginosa*** **ESBLs weakly or not inhibited by CA** (e.g. CMT) **Carbapenemases**	**K1-hyperproducing *K. oxytoca*** **SHV-1 hyperproduction** (*K. pneumoniae* and *E. coli)* **KPC carbapenemase**

Table 2. Causes of false negative and false positive results in ESBL confirmation tests.

Because K1 hyperproducing *K. oxytoca* strains cause false positive results in ESBL confirmation tests based on observed synergy between cefotaxim and clavulanate, these tests are not able to distinguish an ESBL-producing *K. oxytoca* strain from a K1 hyperproducing *K. oxytoca* strain. However, synergy between CAZ and CA in combination with a high MIC for CAZ identifies an ESBL-positive *K. oxytoca* [4]. The typical antibiogram of K1 hyperproducing organisms, characterized by resistance to AZR and cefuroxime (CXM), moderate resistance to CTR, and susceptibility to CAZ, can be useful to differentiate them from an ESBL-producer.

Since coproduction of an AmpC or carbapenemases may cause false negative results in ESBL confirmation assays, they require adaptations when coproduction of these enzymes is suspected.

The next sections describe the most prevelant used confirmation tests in clinical laboratories. For each confirmation test data concerning their ability to detect ESBLs in non-inducible Enterobacteriaceae will be discussed ass well as adaptations that can be made to detect them in AmpC or carbapenemase coproducers. Finally, the recommendations made by the CLSI [1], BSAC [12] and NVMM [4] concerning the use of each of these ESBL confirmation tests are mentioned.

It is important to realise that data concerning the performance of different ESBL confirmation approaches in non-inducible Enterobacteriaceae are based on studies on mainly *E. coli*, *Klebsiella* spp. and some *P. mirabilis* strains. Further evaluation of these tests in other Enterobacteriaceae species, with less prevalent ESBL production (e.g. *Salmonella* spp., *Proteus* spp.) and in strains producing rare ESBL types is necessary.

1.3.1 Double disk synergy test

The double disk synergy test (DDST) is the oldest method for phenotypic confirmation of ESBL-producing organisms, first proposed in the 1980's [24].

In this confirmation test one or more disks containing a third generation cephalosporin or a monobactam are placed at a well-determined center-to-center distance from an amoxicillin/clavulanate disk (20µg/10µg). The test is considered positive when there is an extension of the inhibition zone around a cefalosporin disk towards the amoxicillin/ clavulanate disk. This often results in a characteristic shape-zone referred to as 'champagne cork' or 'keyhole' (Fig. 1).

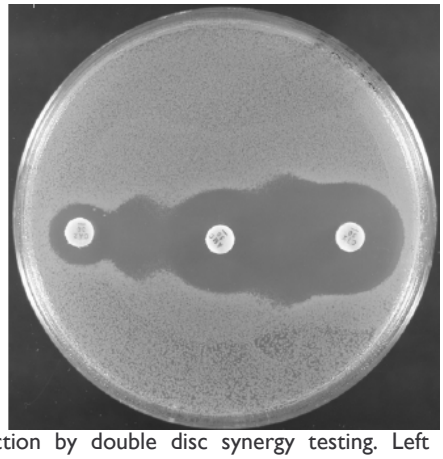

Figure 1. Detection of ESBL production by double disc synergy testing. Left disk: ceftazidime (30 μg); centre disk: amoxycillin/clavulanate (20/10 μg); right disk: cefotaxime (30 μg). The organism is *E. coli* with TEM-5 enzyme.

1.3.1.1 DDST in non-inducible Enterobacteriaceae

In general, using CAZ or CPD alone is not recommended, as false-negative results have been observed with isolates harboring a CTX-M, SHV- or TEM-type ESBL [18][25]. In non-inducible Enterobacteriaceae, the sensitivity of DDST can be improved by using multiple oxymino-cephalosporins or by reducing the distance between the disks of cephalosporins and clavulanate [3][20][22][26][27].

Garrec et al. [27] showed that if a 30mm center-to-center disk spacing is used, the best performance (SN=97%, SP=93%) is reached applying 3 different cephalosporin disks (CTX + FEP + CAZ/CPD/aztreonam (ATM)). By decreasing the center-to-center distance to 20mm, with only a single indicator (CTX or FEP) a 100% sensitivity rate could be achieved. These findings, however, need to be further examined in rare ESBL-types.

DSM-ES is a chromogenic antibacterial susceptibility testing agar changing colour within 4h due to the metabolic activity of growing bacteria. Performing the DDST on this agar can reduce ESBL detection time. However, its performance has only been evaluated in one study. [28].

1.3.1.2 Adapted DDST in AmpC or carbapenemase coproducers

A study of Tzelepi E et al. [29] concerning ESBL detection in clinical isolates of *Enterobacter* spp. characterized by a high prevalence of derepressed AmpC-producers, reported a sensitivity of only 16% if CTX, CTR, CAZ and AZT were applied as indicator antibiotics in the DDST. The use of cefepime, which is stable against hydrolysis by most AmpCs, increased the sensitivity to 61%. By also reducing the center-to-center distance (20mm) between the cephalosporin and clavulanate-containing disks sensitivity increased even more (90%). This is conform to data published by Garrec et al. [27]. Then again, this modified DDST could not detect *A. baumanii* VEB-1 strains (Poirel et al.) that were already reported in Belgium. By performing this modified DDST on a cloxacillin-containing agar plate (200μg/ml), a synergy could be evidenced as a result of cloxacillin inhibiting AmpC beta-lactamases. It has been shown that performing a DDST on a cloxacillin-containing agar plate also improves ESBL detection in *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *S. marcescens* [30][31]. Note however, that larger studies evaluating the performance of DDST on a cloxacillin-containing agar are necessary, as current studies evaluated only a limited number of AmpC coproducing strains.

Performing the DDST on MH-agar to detect ESBL production in KPC producing strains has an unacceptable low sensitivity as shown by Tsakris et al. (SN=6,8%). A sensitivity of 98.3% can be reached by adding 400μg boronic acid (BA), a class A carbapenemase inhibitor, to the cephalosporin (CTX,CAZ,ATM,FEP) and the amoxicillin/clavulanate disks. However, this BA-based DDST still showed to be less sensitive then the combined DDST. Furthermore, it is also more subjective to interpret [21]. Sometimes the production of an ESBL is masked by the coproduction of a metallo-β-lactamase (MBL). In this case, aztreonam resistance can assist in the phenotypic detection of a coexisting ESBL, as it can not be assigned to a MBL [22]. Unfortunately, to date, only one study [32] describes an adapted DDST to detect ESBL in MBL coproducers. In this study the production of an ESBL masked by a VIM-1 MBL could be detected by means of double inhibition by EDTA and clavulanate. It showed a synergy between the disks containing ceftazidime or cefepime and a disk containing clavulanate and EDTA (4μL of a 0.5 M solution buffered at pH 8).

1.3.1.3 Guideline recommendations

Of the applied guidelines [1][2] [4][12], the use of DDST for ESBL confirmation is only discussed by NVMM [4] and BSAC [12] in non-inducible Enterobacteriaceae. Despite the fact that the DDST is a simple, inexpensive ESBL confirmation method [12][18], it is not recommended by NVMM [4]. This because it can lack sensitivity due to (unpredictable) optimal disk spacing problems [33][34] and the fact that interpretation is quite subjective [18]. The BSAC [12], on the other hand, does recommend this approach but only for *Klebsiella spp*. and *E. coli*.

Up to now, no guideline discusses nor recommends the use of (an adapted) DDST for ESBL confirmation in AmpC or carbapenemase coproducers.

1.3.2 Combined double disk synergy test

The combined double disk synergy test (CDDST) depends on comparing zones given by disks containing an extended-spectrum cephalosporin (30µg) with and without clavulanate (10µg). An ESBL is present when the zone of at least one extended-spectrum cephalosporin disk is sufficiently enlarged in the presence of the inhibitor. Several manufacturers have these combined disks commercially available (e.g. Oxoid, BD, Mast).

1.3.2.1 CDDST in non-inducible Enterobacteriaceae

Concerning the performance of the CDDST as an ESBL confirmation assay in non-inducible Enterobacteriaceae, the literature agrees that using CAZ alone is not recommended as too many false-negative results have been observed (mainly with CTX-M type ESBLs) [16][23][27][33][35][36]. Using CTX together with one or more third or fourth generation cephalosporins in the CDDST has been reported reliable to confirm the most prevalent ESBL-types with sensitivity rates between 94,4 and 100% (Table 3). None of these studies included the cefepime disk nor determined whether the sensitivity of testing CTX alone was significantly improved by concomitant testing of CAZ. Garrec et al. [27] reported only a minor increase in the sensitivity rate of cefotaxime (97%) by concomitant testing of cefepime (100%). As this study did not include rare ESBL types, further confirmation of these results with isolates producing these enzymes may be of interest.

Quicolor agar (Salubris Inc., Massachusetts, USA) is a chromogenic antibacterial susceptibility testing agar changing colour within 4h to 6h due to the metabolic activity of growing bacteria. Performing the CDDST on this agar may reduce ESBL detection time. Currently, it has only been evaluated in one study [37].

Indicator antibiotics	**Sensitivity**	**Evaluated ESBL-types**	**References**
Cefotaxime Ceftazidime Cefpodoxime Cefpirome	94,4%	TEM-6 SHV-types not further specified TEM-12 CTX-M types not further specified TEM-15 TEM-20 TEM-52 TEM-92 TEM-142	Wiegand I. et al., J Clin Microbiol. 2007 [23].
Cefotaxime Ceftazidime	100%	TEM-3 TEM-19 SHV-2 TEM-4 TEM-20 SHV-7 TEM-5 TEM-21 SHV-12 TEM-7 TEM-24 SHV-18 TEM-9 TEM-25 CTX-M-5 TEM-10 TEM-26 CTX-M-9 TEM-11 TEM-52 CTX-M-14 TEM-12 TEM-61 CTX-M-15 TEM-15 TEM-71 TEM-16 TEM-88	Jacoby G. A. et al., J Clin Microbiol. 2006 [16].
Cefotaxime Ceftazidime	98,5%	CTX-M-1 SHV2/2a CTX-M-2 SHV-5 CTX-M-3 SHV-12 CTX-M-9 SHV28 CTX-M-15 SHV-52 CTX-M-28 SHV-128	Tofteland S. et al., J Clin Microbiol. 2007 [35].
Cefotaxime Cefepime	100%	CTX-M, TEM and SHV-types not further specified	Garrec H. et al., J Clin Microbiol. 2011 [27].

Table 3. Overview of performance data of the CDDST using different indicator antibiotics to detect most prevalent ESBLs in non-inducible Enterobacteriaceae.

1.3.2.2 Adapted CDDST in AmpC or carbapenemase coproducers

Comparable to DDST, ESBL confirmation by CDDST in chromosomal or plasmid-mediated AmpC coproducers improves by using cefepime and even more by also performing this on a cloxacillin-containing agar [23][27][31]. In a study of Garrec et al. [27] all ESBL–producing *Enterobacter* spp. were identified by a CDDST performed on a cloxacillin-containing agar with cefepime as only indicator. Jiang et al. [31] state that the sensitivity of the CDDST with CAZ and CTX as indicators in *P. aeruginosa* increases from 58.8% to 97% by performing the test on a cloxacillin-containing agar instead of a MH-agar. As these studies are limited by the fact that they only *Enterobacter* spp. and *P. aeruginosa* , further evaluation of combined double disk synergy testing on cloxacillin-containing agars in inducible non-*Enterobacter* spp. and strains producing a plasmid-mediated AmpC is still necessary.

Addition of 3-aminophenyl boronic acid (BA, 400µg) on antibiotic-containing disks (cefotaxime/ceftazidime with or without clavulanate) has been reported to potentiate the sensitivity and specificity of the combination disk method when this method is applied to organisms with chromosomal or plasmid-mediated AmpC production [20][38][39][40]. CDDST with BA-addition is considered positive for ESBL production if the difference in zone diameter for CTX+BA+CA (or CAZ+BA+CA) and CTX+BA (or CAZ+BA) is at least 5mm (3 mm for *E.coli*).

Tsakaris et al. [21] published a sensitivity rate of only 66,9% for ESBL detection in KPC producing strains using the CDDST as described by the CLSI. Therefore, this strategy is not recommended for ESBL detection in KPC

producing strains. Addition of BA (400µg) to the CAZ, CTX, CAZ+CA, CTX+CA disks enhanced the sensitivity rates significantly (100%).

Included bacterial strains	Indicator antimicrobial agents	Sensitivity	Specificity	Apllied cut-off value	References
E. cloacae *E. freundii* *S. marcescens* *C. freundii*	CTX CAZ	98.4%	100%	5 mm	Jeong SH., Int J Antimicrob Agents 2008.
Klebsiella spp. *E. coli*	CTX CAZ	100%	95%	3 mm	Song W., J Clin Microbiol 2007.
Klebsiella spp. *Salmonella* spp. *P. mirabilis*	CTX CAZ	100%	NA*	5 mm	Song W., Diagn Microbiol Infect Dis 2007.

Table 4. Overview of the performance data of the CDDST with addition of BA (400µg) to all cephalosporin disks with and without CA to detect ESBLs in AmpC coproducing strains.* Not available

1.3.2.3 Guideline recommendations

CLSI [1], NVMM [4] and BSAC [12] recommend the use of the CDDST for ESBL confirmation. It must be notified that CLSI guidelines are restricted to *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis* strains, despite ESBLs have already been reported in many other bacteria (e.g. inducible Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp.). NVMM and BSAC recommend to use cefepime and cefepime/clavulanate for ESBL detection in inducible Enterobacteriaceae. However, Garrec et al. [27] already showed that the sensitivity of this approach is only 85% in Enterobacter spp..

None of the consulted guidelines discusses the use of (an adapted) CDDST for ESBL confirmation in carbapenemase coproducers.

1.3.3 Etest® ESBL detection strips

Etest® ESBL detection strips 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), cefotaxime (0,016-1µg/ml) or cefepime (0,064-4µg/ml)) plus a constant concentration of clavulanate (4µg/ml). The presence of ESBL is confirmed when we can observe (1) a ≥ 3 two-fold decrease in the MIC value of the tested drug in the presence of clavulanate or (2) a phantom zone or (3) deformation of the CT, TZ or PM inhibition ellipse at the tapering end (Figure 2-5) (bioMérieux Etest ESBL package insert).

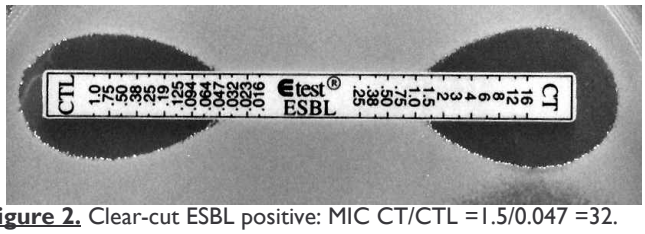

Figure 2. Clear-cut ESBL positive: MIC CT/CTL =1.5/0.047 =32.

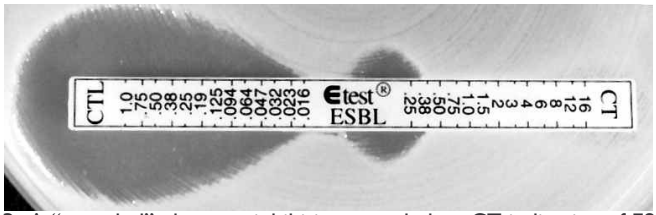

Figure 3. A "rounded" phantom inhibition zone below CT indicative of ESBL.

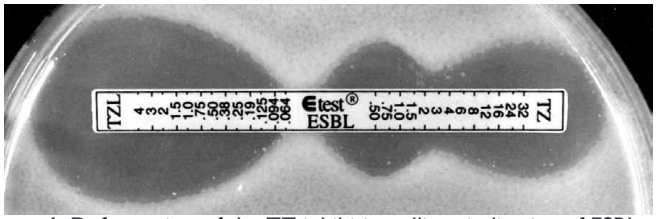

Figure 4. Deformation of the TZ inhibition ellipse indicative of ESBL.

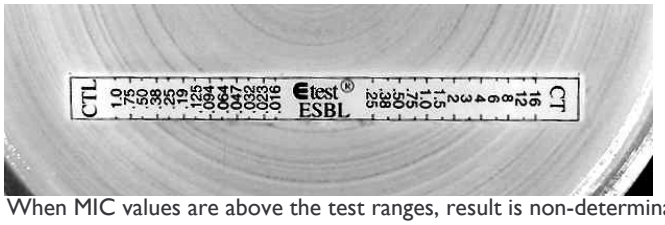

Figure 5. When MIC values are above the test ranges, result is non-determinable.

1.3.3.1 Etests® in non-inducible Enterobacteriaceae

Different studies evaluated the reliability of Etest® ESBL detection strips to detect the most prevalent ESBL-types [23][33][35][36]. Using only a TZ/TZL Etest® a sensitivity of 95,2% was reported to detect mainly SHV-type ESBLs [36]. Probably the sensitivity rate would have been lower if more CTX-M types (CAZ=S) were included. Two other studies [23][35] did include strains containing CTX-M type ESBL's. Wiegeland et al. [23] reported a sensitivity of 98,6% using the CT/CTL combined with the TZ/TZL and PM/PML Etests®. Tofteland et al. [35] found 100% sensitivity using CT/CTL combined with TZ/TZL Etests® (see Table 4 for more details). These studies, however, did not determine whether the sensitivity of CT/CTL Etest® was significantly improved by concomitant testing of the TZ/TZL Etests® as recommended by the manufacturer. A recent article [27] did look into this and reported no significant improved sensitivity rates. Moreover, the highest sensitivity, specificity and lowest rate of indeterminate results (due to MIC values higher than those measurable on the strips) were obtained by testing the cefepime Etest® alone (97%, 79% and 2% respectively).
Performing Etests® for ESBL detection on a Quicolor agar, as described above, can reduce the ESBL detection time. However, currently its performance is only evaluated in one study [37].

Etest® strips	Sensitivity	Evaluated ESBL-types	References
CT/CTL TZ/TZL PM/PML	98,6%	TEM-6 SHV-types not further specified TEM-12 CTX-M types not further specified TEM-15 TEM-20 TEM-52 TEM-92 TEM-142	Wiegand I. et al., J Clin Microbiol. 2007.
TZ/TZL	95,2%	TEM-3 SHV5 TEM-7 SHV1 TEM-12 SHV-7 TEM-26 SHV-12	M'Zali, F.H., J Antimicrob Chemotherapy 2000.
CT/CTL TZ/TZL	100%	CTX-M-1 SHV-2/2a CTX-M-2 SHV-5 CTX-M-3 SHV-12 CTX-M-9 SHV28 CTX-M-15 SHV-52 CTX-M-28 SHV-128	Tofteland S. et al., J Clin Microbiol. 2007
PM/PML	97%	CTX-M, TEM and SHV-types not further specified	Garrec H. et al., J Clin Microbiol. 2011

Table 4. Overview of performance data of Etest® ESBL detection strips using different indicator antibiotics to detect most prevalent ESBLs in non-inducible Enterobacteriaceae.

1.3.3.2 Etests® in AmpC or carbapenemase coproducers

Different studies evaluated the performance of the Etest® ESBL detection strips in strains coproducing AmpC ß-lactamases [20][27][42][43][44]. In these studies only the most frequently reported AmpC hyperproducers (*Enterobacter* species) or plasmid mediated AmpC producers (*K. pneumoniae* and *E. coli*) were tested. The CT/CTL and TZ/TZL Etests® yielded high numbers of non-determinable or negative results and thus showed an inability to detect ESBLs in this group of isolates. The PM/PML Etest®, on the other hand, was found to be particularly useful for detecting ESBLs in AmpC producing bacteria. However, when this PM/PML Etest® was applied on a MH-agar for ESBL detection in Enterobacter spp. only 69% of ESBL producers could be identified. On the other hand, on a cloxacillin-containing agar, the PM/PML Etest® provided a 100% sensitivity rate [27]. Improvement of ESBL detection in plasmid mediated AmpC producers or in inducible non-Enterobacter spp. by performing a PM/PML Etest® on a cloxacillin-containing agar has not yet been evaluated.
Studies evaluating the performance of Etest® ESBL detection strips in carbapenemase coproducers are lacking.

1.3.3.3 Recommendations of guidelines

For the recommendations concerning the use of Etests® for ESBL confirmation in the consulted guidelines [1] [4] [12][22] we refer to section 1.3.2.3, as the recommendations are comparable to those made concerning the use of CDDST for ESBL confirmation.

1.3.4 Semi-automated systems

In Belgium, the most prevalent used automated susceptibility testing systems in clinical laboratories are Vitek (bioMérieux) and Phoenix (BD).
Nowadays, in Vitek2 systems the ESBL confirmation test is incorporated in some of their susceptibility testing cards. The Vitek2 uses growth in the presence of CTX, CAZ and FEP with and without CA to detect the production of ESBL.
In the Phoenix system the ESBL confirmation test is also incorporated in some susceptibiltiy testing panels, using the same principle as in Vitek2 but with CTX, CTR, CAZ and CPD as indicator antimicrobial agents.

1.3.4.1 Semi-automated systems in non-inducible Enterobacteriaceae

Several studies evaluating the performance of only one semi-automated ESBL detection system have been published [23][27][45][46][47]. Because of differences in study design (e.g. in ESBL types, species, reference methods,....), it is impossible to compare the performance of the two systems based on these results. Up to now, only one study performed a direct comparison of Phoenix and Vitek2 ESBL detection tests for detection of 38 distinct TEM-, SHV- and CTX-M ESBLs in *E. coli* and *Klebsiella* spp [48]. Sensitivity rates of 96% for Phoenix and 89% for Vitek2 were reported. Their specificity rates were 81% and 85% respectively. Activation of two additional rules (rules 325 and 1437) in the Phoenix system that are intended to enhance ESBL detection (SN=99%), reduced the specificity to 58%. The reduced specificity resulted mostly from the rules interpreting high-level AmpC production as evidence of an ESBL.

1.3.4.2 Semi-automated systems in AmpC coproducers

In the study of Wiegand et al. [23] 90% of the ESBL's among *Enterobacter* spp. and *C. freundii* isolates were detected by the Phoenix. But the authors report a rather low specificity (33.3%) with numerous false ESBL-positive results.
The Vitek2 ESBL detection test, on the other hand, has been reported as a confirmation test with a significantly lower sensitivity than other phenotypic ESBL confirmation methods in *Enterobacter* spp. [27].

1.3.4.3 Recommendations of guidelines

The use of semi-automated systems for ESBL detection is beyond the scope of the applied guidelines. Only the NVMM [4] guideline notes that the use of Vitek2 ESBL confirmation test is not recommended due to the limited amount and diverse published performance data.

1.3.5 Cica-Beta test

The Cica-Beta test confirmation method (Kanto Chemical, Japan) is a technically simple and fast method to detect ESBLs as well as overexpressed AmpC and metallo-β-lactamases (MBL). It is based on hydrolysis of a chromogenic cephalosporin, HMRZ-86, on paper strips. Four strips are available: a control strip without inhibitor to detect hydrolysis of extended-spectrum cephalosporins, another with CLA to detect ESBL, a third with boronic acid to detect overproduction of AmpC and finally one strip with sodium mercapto-acetic acid to detect metallo-β-lactamases (Figure 6). The four tests can be performed within 15 minutes using 7 colonies isolated on solid media, reducing ESBL detection time with one day compared to other phenotypic confirmation methods.

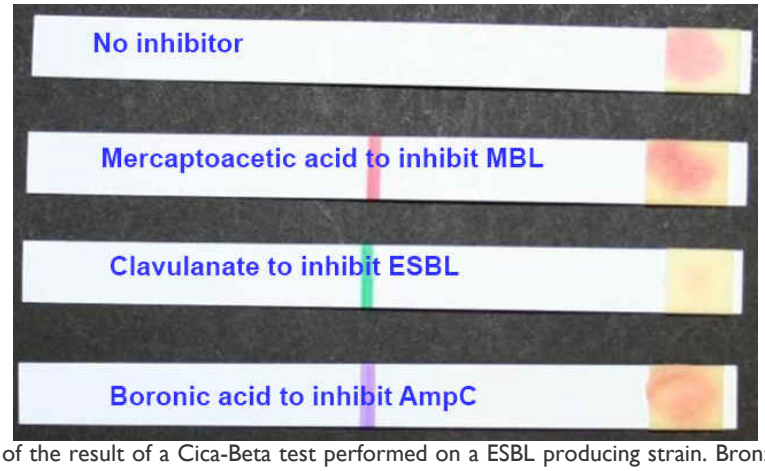

Figure 6: An example of the result of a Cica-Beta test performed on a ESBL producing strain. Bron: Livermore D. ß-lactam susceptibility tests in Enterobacteriaceae Report by mechanism. Presented at the ECCMID 2010 in Vienna.

Some recent articles concerning the performance of the Cica-Beta test in a wide variety of species with diverse ESBL types (*E. coli, Klebsiella spp., P. mirabilis, Salmonella typhimurium, Enterobacter spp., M. morganii, S. marcescens, Citrobacter koseri, P. aeruginosa, A. baumanii* producing TEM-, SHV-, CTX-M-, VEB- and PER- ESBL types), report sensitivity rates of 74% [27], 85% [49], 95% [50] and specificity rates of 80% [27], 92% [49] and 84% [50]. The lowest sensitivity rate in the study of Garrec at al. [27] was probably mainly caused by 14% of indeterminate results. Note that, in the study of Lavigne et al. [50], all false positive results were due to derepressed AmpC production, whereas in the study of Livermore et al. [49] this never occurred.

1.3.6 Comparison of phenotypic ESBL confirmation assays

Many studies comparing some of the existing ESBL confirmation assays have been published [23][26][27][33][35][36]. Garrec et al. [27] compared nine phenotypic methods for detection of ESBL production by Enterobacteriaceae: (1)Vitek2, DDST with 20mm (2) and 30mm (3) spacing on a Mueller-Hinton agar, (4) DDST with 30mm spacing on cloxacillin-containing MH agar, CDDST on MH agar (5) and on cloxacillin-containing MH- agar (6), ESBL Etests® on MH agar (7) and on cloxacillin-containing MH agar (8) and (9) the Cica-Beta test. Up to now, this study is obviously the most complete comparison of different ESBL confirmation methods. Moreover, this study is the first to determine which indicator antimicrobial agent(s) combination results in the highest sensitivity rate for the DDST, the CDDST and the Etests.

Within the group of non-inducible Enterobacteriaceae, the best performance for each method was reached by using:

- DDST (30mm spacing) combining CTX with FEP (SN=97%, SP=93%)
- DDST (20 mm spacing) with FEP alone (SN=100%, SP=93%)
- CDDST combining CTX with FEP (SN=100%, SP=86%)
- PM/PML Etest® strip (SN=97%, SP=79%)
- Vitek 2 N052 + extended card (SN=92%, SP=79%)
- Cica-Beta test (SN=74%, SP=57%)

Note that the specificity rates of Vitek2 and PM/PML Etest® strip reached only 79%.

Within the group of inducible Enterobacteriaceae, the best performance for each method was reached by using:

- DDST (30mm spacing) with ATM alone (SN=93%, SP=100%)
- DDST (20mm spacing) with FEP alone (SN=100%, SP=100%)
- CDDST with CTX, CAZ or FEP on a cloxacillin-containing agar (SN=100%, SP=not available)
- PM/PML® Etest strip on a cloxacillin-containing agar (SN=100%, SP=not available)
- Vitek 2 N017 + extended card (SN=31%, SP=56%)
- Cica-Beta test (SN=77%, SP=88%)

Overall, it must be noted that the Cica-Beta test has a significantly lower sensitivity compared to the other methods. Also, the Vitek2 had a significantly lower sensitivity, but only in inducible Enterobacteriaceae. Other differences in the comparison did not reach the level of statistical significance.

Previous results were only studied in *E. coli*, *Klebsiella spp.*, *P. mirabilis* and *Enterobacter* spp. producing the most prevalent ESBL-types. Comparable studies including other species in which ESBL production have already been described (*P. aeruginosa*, *A. baumanii*, *Salmonella* spp.,...) and isolates producing rare ESBL-types are of interest.

2. Derepressed chromosomal or plasmid mediated AmpC-β-lactamases

Currently, the consulted guidelines only propose recommendations concerning screening of chromosomal or plasmid mediated AmpC ß-lactamases [2][3][4]. Recommendations on confirmation are lacking.

2.1 Screening for AmpC in surveillance samples

Up to now, there are no chromogenic media for the to selectively screening for derepressed chromosomal or plasmid-mediated AmpC producers in surveillance samples available.

2.2 Screening for AmpC among Gram-negative isolates

Derepressed chromosomal or plasmid-mediated AmpC-β-lactamases are usually resistant to almost all penicillins, cephalosporins (except cefepime and cefpirome) and aztreonam. AmpC producers can be distinguished from ESBLs by the fact that they confer resistance to cefamycines (cefoxitin, cefotetan, cefmetazole).

To detect AmpC production in Gram-negative isolates EUCAST [2] and NVMM [4] recommend to use a reduced cefoxitin (FOX) susceptibility [1] (zone diameter < 19mm [2] or MIC ≥ 16 µg/ml [4]) as indicator.

In *plasmid-mediated* AmpC producers this seems to be a non-specific indicator, as many false positive results due to ESBLs have been reported [51][52]. Furthermore, this method is not able to detect AAC-1 AmpC-producing strains as they are FOX susceptible. Changing decreased FOX susceptibility to full resistance might increase the specificity. However, this has not yet been evaluated.

In *derepressed* AmpC producers, Kim et al. [53] showed that FOX resistance (≥ 32 µg/ml [1]) could be used instead of reduced FOX susceptibility as indicator without a loss in sensitivity. Anyhow, FOX resistance is still a non-specific indicator as 50% of the inducible AmpC strains were cefoxitin resistant [53][54]. Decreased susceptibility to cefotetan (zone diameter ≤15mm or MIC ≥32 µg/ml [1]) is only reported in a few inducible AmpC producers. And therefore, it is a more specific indicator to screen for derepressed AmpC in Gram-negative isolates preserving a ≥94% sensitivity rate.

As reduced cefoxitin or cefotetan susceptibility can also be caused by the production of a metallo-β-lactamase, an ESBL or a porin loss confirmation testing is necessary. Different confirmation approaches will be discussed in the following section.

2.3 AmpC confirmation

2.3.1 Modified indirect three-dimensional

In the modified indirect three-dimensional test for AmpC detection a 30µg FOX disk is placed on a MH-agar inoculated with a 0,5 McF suspension of a susceptible *E. coli* ATCC 25922 strain. A slit beginning 3 to 5 mm from the edge of the disk is then cut in the agar in an out- or inward radial direction. Finally the slit, or a small circular well made on the slit, is filled with a suspension of test organism or their enzyme extract. Enhanced growth of the surface organism where the slit intersects the zone of inhibition is considered as a positive test result (Figure 7) [52][55][56]. This test is reliable to confirm isolates that harbor the AmpC enzyme. However, it is complex and labor-intensive. A variation of this technique, as described by Nasim and colleagues, is reported as simpler and able to test multiple samples per plate. However, this method still requires multiple freeze-thawing steps to prepare the enzyme extract which is not feasible for most routine clinical laboratories [57].

2.3.2 Inhibitor based confirmation tests

Another approach for AmpC detection is the use of an inhibitor, analogous to the use of clavulanic acid in a confirmatory test for class A ESBLs. Novel ß-lactamase inhibitors LN-2-128, Ro 48-1220 and Syn 2190 have been evaluated for this purpose. The best results were detected using the combination of Syn 2190 and cefotetan (SN= 91%, SP=100%). Unfortunately, these inhibitors are not yet commercially available [52][65].
The use of commercially available inhibitors, boronic acid and cloxacillin, has been described in different inhibitor based AmpC confirmation methods: double disk synergy tests, disk potentiation tests, Etests® and chromogenic Cica-Beta test. Currently, large studies evaluating their performance to detect *derepressed* AmpC producers are lacking. Therefore, only data published on the performance of these methods to detect *plasmid-mediated* AmpC producers will be discussed.

It must be notified that inhibitor based AmpC confirmation tests are unable to distinguish plasmid-mediated from derepressed chromosomal AmpC genes [58][59][60]. In addition, KPC carbapenemases cause resistance to cephamycins and can result in false-positive BA-based AmpC confirmation results. Strains that test positive therein with subsequent decreased susceptibility to carbapenems should be investigated for possible KPC production in a reference laboratory.

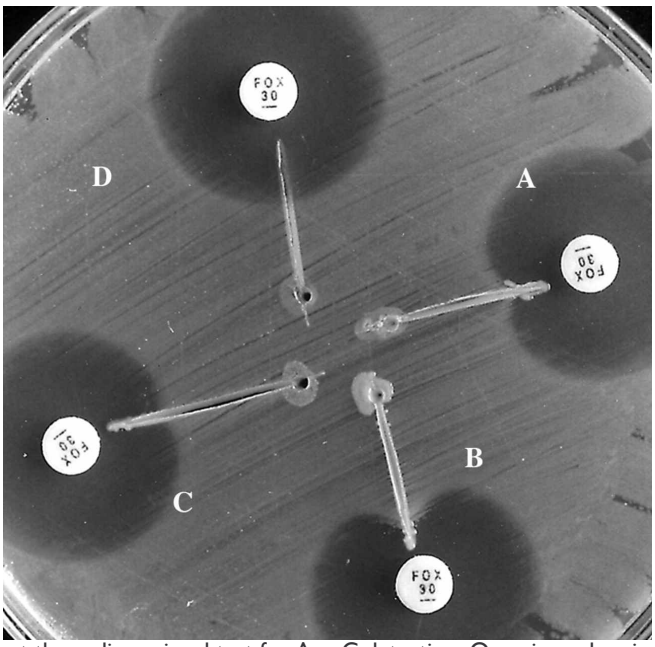

Figure 7. The modified indirect three-dimensional test for AmpC detection. Organisms showing clear distortion in the zone of inhibition are considered as AmpC producers (Test and control strains A and B). Strain C showing minimal distortion is considered indeterminate. Strain D is the negative control [56].

2.3.3 Double disk synergy test

The double disk synergy test (DDST) is based on the detection of a synergy between a cephalosporin and boronic acid (BA) or cloxacillin (CLOX) containing disks in AmpC producing strains (Figure 8). Thre are a small number of studies evaluating this AmpC confirmation method [61][62][63]. Merilis et al. showed that a DDST using cloxacillin to detect an AmpC is not appropriate in *E. Coli* isolates because of false positive results due to natural AmpC ß-lactamase production [61]. On the other hand, by using BA as inhibitor, all plasmid-mediated class C ß-lactamases produced by *E. coli* and *K. pneumoniae* isolates (ACT-1, CMY-2, CMY-8, CMY-9, CMY-19, FOX-5,MOX-1, DHA-1,LAT-1, HKY28) were detected. The authors observed no false positives in the negative control group (*K. pneumoniae* and *E. coli* strains producing class A ESBLs or a non-KPC carbapenamases) indicating a high specificity [62]. Rosco provides a commercially available DDST. This test is based on synergy between CAZ or FOX and cloxacillin and synergy between CAZ+CA or CTX+CA and BA. Currently, it has only been evaluated in a small population of plasmid-mediated AmpC producing Enterobateriaceae. By

interpreting 2 or more positive synergy tests as indicative of an AmpC ß-lactamase, a sensitivity and specificity of 100% was observed [63].

2.3.3.1 Disk potentiation test

The disk potentiation test (DPT) for AmpC confirmation is comparable to the CDDST for ESBL confirmation described in section 1.3.2, with the difference that BA or CLOX are used as inhibitors.
Yagi et al. found that a DPT with (1) BA (300µg) as inhibitor, (2) CTX and CAZ as indicator antimicrobial agents and (3) a 5-mm cut-off value reliably detected all plasmid-mediated AmpC varieties present in *E. coli* and *K. pneumoniae* strains (ACT-1, CMY-2, CMY-8, CMY-9, CMY-19, FOX-5,MOX-1, DHA-1,LAT-1, HKY2) [62]. Even two AmpC producing isolates coproducing an ESBL were detected. No false positive results due to ESBL or non-KPC carbapenamase producing *E. coli* or *K. pneumoniae* strains were observed. Using only CAZ as indicator, an ATC-1 producing *K. pneumoniae* strain was missed. This was confirmed by other studies including different types of plasmid-mediated AmpC (ACC-1, FOX-1, FOX-3, FOX-4, MIR-1,MOX-2) and ESBL or carbapenamase (e.g. KPC-3) in the negative control group [16].
Alternative BA acid based disk potentiation tests, differing from the one described by Yagi et al. [62] by type of indicator cephalosporin or BA concentration, also showed acceptable performance data in their set of tested strains [51][52][64][66][67]. Tenover et al.[51] observed no significant performance difference between BA solutions prepared in distilled water or DMSO. The latter being a toxic chemical agent requiring special handling.
Studies evaluating the reliability of a DPT using CLOX to detect different plasmid-mediated AmpC-types are limited [67].

2.3.3.2 Etests® AmpC detection strips

Etest® strips, with a gradient of cefotetan (CN/CNI) or cefoxitin (FX/FXI) on one half and on the other half the same antibiotic combined with a constant concentration of cloxacillin, have been evaluated mostly for plasmid-mediated AmpC detection [63][68]. Either a reduction in cephamycin MIC of at least three dilutions, a deformation of the ellipse of inhibition or a "phantom zone" should be interpreted as positive. With more than 200, mainly plasmid mediated, AmpC producing strains tested, an overall sensitivity and specificity of 91 and 93% for CN/CNI was reported [69]. Combining Etest® CN/CNI with FX/FXI did not increase the sensitivity significantly (CN/CNI= 94%, combined= 96%).

2.3.3.3 Cica-Beta test

Currently, there are no data available on the performance of the Cica Beta-test for the detection of *plasmid-mediated* AmpC production. Although only evaluated with 25 *derepressed* AmpC producers, this test seems unreliable for derepressed AmpC confirmation (SN=78%) [49].

2.3.3.4 Comparison of phenotypic AmpC confirmation assays

The few studies comparing the described AmpC confirmation methods, are limited by the amount of strains and AmpC-types researched [70][71].

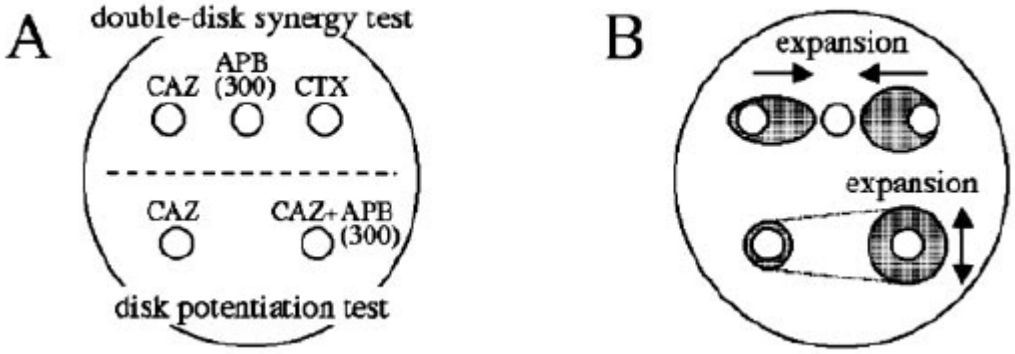

Figure 8. A) Scheme of disk arrangement for two inhibitor based AmpC detection tests. The upper three disks are for DDST, and the lower two are for the disk potentiation test. CAZ= ceftazidime, CTX= cefotaxime, APB 300=3-aminophenyl-boronic acid,30µg. (B) Typical observations of the growth-inhibitory zones among class C ß-lactamase producers. The growth-inhibitory zones are expanded toward the APB disk in DDST. In the disk potentiating test, the diameter of the growth-inhibitory zone is expanded around the disk containing both CAZ and APB compared with that around the disk containing solely CAZ. *Source:* [62].

3. Carbapenemases

Currently, guidelines for phenotypic screening and confirmation of carbapenemases exist only for Enterobacteriaceae [1] [72]. In these guidelines recommendations related to confirmation are restricted to class A (e.g. KPC) and class B (metallo-beta-lactamases e.g. VIM) carbapenemases. For non-fermenting bacilli, the literature is restricted to articles evaluating different metallo-beta-lactamase confirmation tests.

3.1 Screening for carbapenemases in surveillance samples

Recently a CHROMagar™ KPC (Hy-Labs, Rehovot, Israel) for direct screening and presumptive identification of carbapenemases in surveillance samples, has become commercially available. This chromogenic solid medium is supplemented with agents that inhibit the growth of carbapenem-sensitive bacteria. Enterobacteriaceae colonies with reduced carbapenem susceptibility appear after a 24h incubation period with different colours according to their specific enzymatic properties. This chromogenic medium has shown to be a useful screening medium for KPC and VIM carbapenemase-producing Enterobacteriaceae [73][74].
Further studies are required to establish the reliability of this medium to detect other carbapenemases.

3.2 Screening for carbapenemases in Enterobacteriaceae isolates

There are many reasons why screening for carbapenemases in Enterobacteriaceae isolates based on carbapenem susceptibility testing can be troublesome:

(i) in carbapenemase-producing microorganisms the carbepenem MIC can exhibit considerable variation depending on
   a. the type and the expression of the carbapenemase enzyme,
   b. the bacterial species,
   c. the presence of other resistance mechanisms, such as ESBL and AmpC,
   d. the presence of reduced permeability and/or efflux pumps.

(ii) reduced carbapenem MICs may also arise from other factors, such as high-level expression of AmpC or CTX-M ESBLs in combination with porin alteration or outer membrane defects.

There are some important differences among the recommendations on this subject by the CLSI [1] and the Dutch Working Party on the Detection of Highly Resistant Microorganisms (DWP) [72] (Table 5) as the settings of the applied carbapenem screening breakpoints are based on different principles. CLSI breakpoints are largely derived from tests of US Enterobacteriaceae isolates and provide a high level of sensitivity (>90%) and specificity (>90%) in detecting KPC-type carbapenemases. The sensitivity and specificity of the CLSI screening approach for the detection of low-level metallo-β-lactamase or OXA-type carbapenemase production are unknown. The setting of the screening breakpoints by the DWP are guided by the following principles:

(i) the breakpoint MIC should be higher than the highest MIC of the wild-type population (http://www.eucast.org), as the specificity of the screening test may otherwise become too low

(ii) the MIC breakpoint should be lower than the lowest carbapenem MICs described in the literature for strains shown to have a carbapenemase gene (cfr. Table 6).

A study of Pasteran et al. [75] revealed a significantly higher sensitivity rate for class A carbapenemase detection in Enterobacteriaceae when the meropenem (MEM) MIC screening breakpoint was set at ≥0,5 µg/mL (100%) instead of ≥2 µg/mL (75%). Using a breakpoint of ≥0,5 µg/mL, only sporadic carbapenemases with meropenem MICs < 0,5 µg/mL will not be detected [76]. When using meropenem disks (10µg), a meropenem zone diameter breakpoint set at ≤23mm seemed to be more sensitive than CLSI's meropenem zone diameter breakpoint (≤21mm) for carbapenemase detection. However, this zone diameter breakpoint is slightly less sensitive than the MIC screening breakpoint of ≥0,5 µg/mL (84% vs. 100%) [75]. Increasing the zone diameter breakpoint to 27mm resulted in a 100% sensitivity [75]. However, this breakpoint would result in an unacceptably high level of false-positive isolates based on the published meropenem zone diameter distributions [1].
Both guidelines do not recommend the IPM screen test for carbapenemase detection in *Proteus* spp., *Serratia* spp., *Providencia* spp. and *Morganella* spp. because (1) these genera often have elevated MICs to imipenem (IPM) by mechanisms other than production of carbapenemases and (2) the usefulness of an IPM screen test for carbapenemases detection in these genera has not yet been established.
In both guidelines the IPM screeing breakpoint for *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp. and *Citrobacter* spp. is set at ≥ 2,0 µg/mL, even though this was shown to have a low sensitivity (+/- 78%) [75][78][79]. On the other hand, reducing the breakpoint to ≥ 1,0 µg/mL increases the sensitivity [75] but reduces specificity, as the imipenem MIC distribution of the wild-type population is up to 1 µg/mL (Table 6). CLSI does not recommend the use of an imipenem disk (10µg) for carbapenemase detection, because its assumed poor performance, which is not supported by data. As an IPM zone diameter screening breakpoint ≤21 mm was shown to detect all different class A carbapenemases and VIM metallo-beta-lactamases [75][77], the imipenem disk screen test is recommended by DWP.

Using the CLSI carbapenem breakpoints (MIC ≥ 2,0 µg/mL) for carbapenemase detection, ertapenem (ETP) has proved to be the most sensitive for the detection of bla_{KPC}-positive organisms [80][81][82][83]. However, for the detection of different class A carbapenemases, this CLSI ertapenem breakpoint seemed unreliable (SN=75%).
In the guideline of the DWP ertapenem is not advised as indicator as it has a lower specificity than imipenem and meropenem [75][81]. This is caused by the fact that isolates with AmpC/ESBL and decreased permeability have higher MICs for ETP than for IPM or MEM.
It must be notified that the CLSI only recommends a carbapenemase confirmation test when a positive screening test is combined with resistance to at least one subclass III cephalosporin.

Indicator antimicrobial agent(s)	**Cut-off values for DD (mm)**	**Cut-off values for MIC (µg/mL)**	**Guidelines**
Meropenem (MEM)	≤ 21 (10µg)	≥ 2	CLSI [1]
	≤ 23 (10µg)	≥ 0,5	Dutch Working Party [72]
Imipenem (IPM)	NA*	≥ 2[a]	CLSI [1]
	NA*,[b] ≤ 21[c] (10µg)	NA*,[b] ≥ 2[c]	Dutch Working Party [72]
Ertapenem (ETP)	≤ 21[d] (10µg)	≥ 2	CLSI [1]
	NA*	NA*	Dutch Working Party [72]

Table 5. An overview of the carbapenem screening breakpoints recommended by either the CLSI [1] and the Dutch Working Party on the Detection of Highly Resistant Microorganisms [72]. *NA=not available. [a] not evalueted in *Proteus* spp., *Providencia* spp., and *Morganella* spp. [b] for *Proteus* spp., *Serratia* spp., *Providencia* spp. and *Morganella morganii.* [c] for *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp. and *Citrobacter* spp.. [d]concerning ETP, it is important to consider individual colonies within the inhibition zone as they can suggest the presence of a carbapenemase.

Carbapenem susceptibility testing can be done by one of the following standardized methods:
(1) MIC determination by broth microdilution technique, a semi-automatic system or an Etest®
(2) inhibition zone diameter determination by disk diffusion.

Studies show that the use of a correct inoculum is important in carbapenem susceptibility testing with a broth microdilution method or a semi-automatic system as a moderate decrease in the inoculum may lead to inaccurate carbapenem susceptibility results [72][80][84][85]. In addition, determining the MIC of carbapenemase-positive strains by Etest® can be complicated because mutant colonies with a higher MIC than the dominant population may be found in the inhibition ellipse (Figure 9). These colonies should be included when interpreting the Etest® in accordance with the manufacturer's instructions [72][80].

Species	Meropenem		Imipenem			Ertapenem
	Escherichia coli, *Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp.	*Citrobacter* spp., *Serratia* spp., *Proteus mirabilis*, *Morganella morganii*, *Providencia* spp.	*E. coli*	*Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp., *Citrobacter* spp.	*Serratia* spp., *P. mirabilis*, *M. morganii*, *Providencia* spp.	*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp., *Serratia* spp., *P. mirabilis*, *M. morganii*, *Providencia* spp.
Carbapenemase screening breakpoint	≥0.5[a]	≥0.5[a]	≥2[b]	≥2[b]	N/A	≥0.5[c]
Epidemiological cut-off wild-type	S ≤0.125	S ≤0.25	S ≤0.5	S ≤1	S ≤4 (*Serratia* S ≤2)	S ≤0.064
EUCAST breakpoint	S ≤2	S ≤2	S ≤2	S ≤2	S ≤2	S ≤0.5
CLSI breakpoint	S ≤1	S ≤1	S ≤1	S ≤1	S ≤1	S ≤1

Table 6. Carbapenem screening breakpoints of the DWP, EUCASTs clinical breakpoints and epidemiological cut-off values (µg/mL). *Source*: [72].

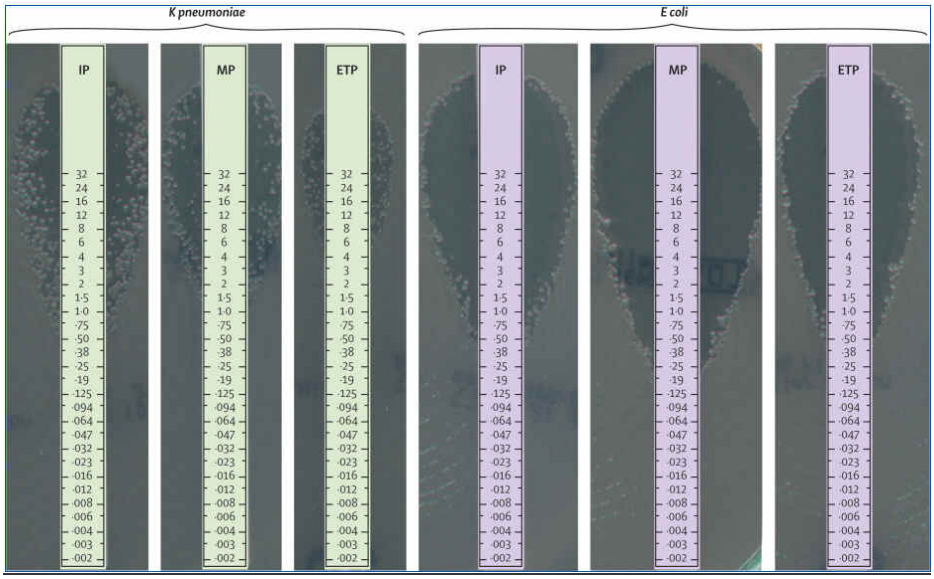

Figure 9. Carbapenem susceptibility testing using carbapenem Etest® strips to detect carbapenemase producing *K pneumoniae* and *E coli*. The presence of scattered colonies makes it difficult to read the defined endpoint. The numbers on the strips represent the MIC (μg/mL) for the antibiotic in question. IP=imipenem. MP=meropenem. ETP=ertapenem. *Source*: [80].

3.3 Carbapenemase confirmation

3.3.1 Class A carbapenemase confirmation in Enterobacteriaceae

The next section will discuss two different phenotypic methods for class A carbapenemase confirmation in Enterobacteriaceae which can be employed in routine clinical laboratories.

3.3.1.1 Modified Hodge test

The modified Hodge test (MHT) (Figure 10) has been recommended by the CLSI [1] for the confirmation of carbapenemase production in Enterobacteriaceae. This test has a high sensitivity (93-95%) for detection of class A carbapenemase-producing strains [75][79][86]. Apart from being technically demanding and time consuming, disadvantages of this test include possible interpretation difficulties and the inability to distinguish between different classes of carbapenemases [87][88]. Furthermore, high false-positive rates have been reported. These are mainly caused by CTX-M type ESBLs and, to a lesser extent, AmpC hyperproduction and ESBL production coupled with porin loss or porin mutations [75][86][87][89][90][91]. Recently, Pasteran et al. developed a modified boronic acid- and oxacillin-based MHT (Figure 11) for class A carbapenemase confirmation. By this modification the amount of false-positive results was significantly reduced and class A carbapenemases were succesfully distinguished from other class carbapenemases (SP=100%) while a sensitivity rate of 92% was preserved [86]. Only for some GES-producing strains a false negative result was obtained.

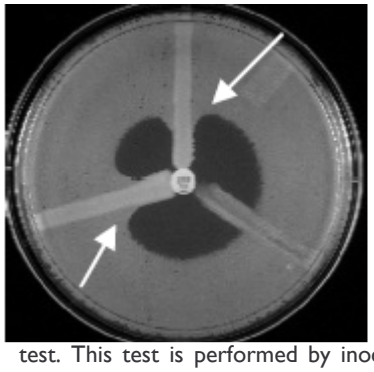

Figure 10. Modified Hodge or Cloverleaf test. This test is performed by inoculating a Mueller-Hinton agar with a 1:10 dilution of a 0,5McF suspension of *E. coli* ATCC 25922 as described in the routine disk diffusion CLSI procedure. Then the plate is allowed to dry 3 to 10 minutes. Next, one or two carbapenem disk are placed on the plate. Subsequently, by using a 10μl loop, 3-5 colonies of the test or QC organisms, grown overnight on an agar plate, are inoculated onto the plate in a straight line from the edge of the disk. The presence of growth of the indicator strain toward the carbapenem disks (arrows) should be interpreted as a positive result. *Source*: [1].

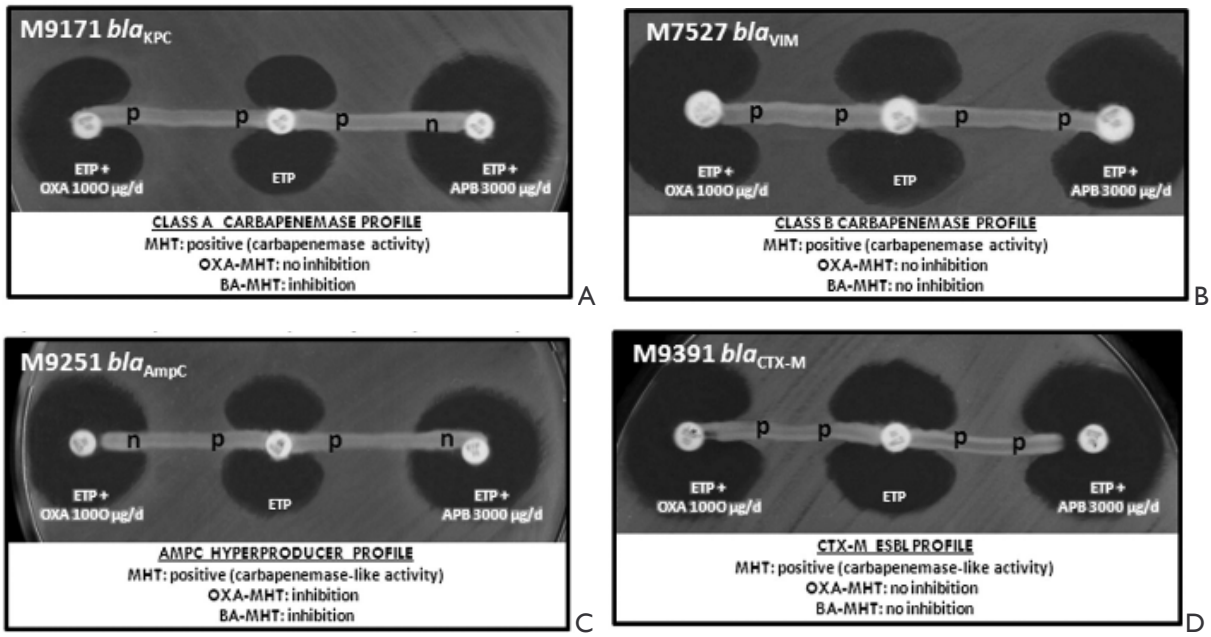

Figure 11. Modified boronic acid- and oxacillin-based MHT. A) This pattern, inhibition by 3-amino-phenyl boronic acid (APB) and refractory action with OXA, corresponds unequivocally to class A carbapenemases. B+D) This pattern, no inhibition by OXA and APB, corresponds unequivocally to non-class A carbapenemases. As shown in figure B, this pattern can correspond to MBL producer. C) This pattern, inhibition by both APB and OXA, corresponds unequivocally to AmpC hyperproducing strains. *Source*: [86].

3.3.1.2 Boronic acid inhibitor based confirmation

Boronic acid inhibitor based confirmation is based on the *in vitro* inhibition of class A carbapenemases by boronic acid (BA). In routine clinical laboratories, this class A carbapenemase confirmation method can be carried out using either double-disk synergy tests (DDST) or combined double-disks synergy tests (CDDST).

Double-disk synergy tests

In the double disk synergy tests for class A carbapenemase confirmation one or more carbapenem containing (10µg) disks are placed at a well determined distance from a BA containing disk. The test is considered positive when there is an extension of the inhibition zone around a carbapenem disk towards the BA disk. Currently, only one study evaluated this technique in different class A carbapenemase producers using IPM, MEM and ETP 10µg disks and a BA (300µg) disk [75]. Of the different distances between the BA disk and the carbapenem disk that were evaluated, the best results were obtained when disks were placed 20mm apart (center-to-center). In this setting, an IPM disk combined with a BA disk proved the most sensitive combination of the evaluated DDSTs (SN=96%, SP=92%). However, IPM-BA CDDST, also evaluated in this study, performed even better (SN=100%, SP=100%).

Double disk synergy testing is not recommended by the DWP as its sensitivity depends on optimal disk spacing.

Combined double- disk synergy tests

The combined double disk synergy test (CDDST) for class A carbapenemase confirmation is based on the comparison of the inhibition zones of carbapenem containing disk (IPM, ETP,MEM, (10µg)) with and without BA. A class A carbapenemase is present when the zone of the carbapenem containing disk is enlarged in the presence of BA.

To date, BA-based CDDSTs have mainly been evaluated on their efficiency to detect KPC-possessing *K. pneumoniae* strains. Studies evaluating the performance of home prepared BA-based CDDSTs differ in type of BA-derivative, amount of applied BA and/or cut-off value of zone diameter differences between disks with a carbapenem plus APB (3-amino-phenyl boronic acid) and the carbapenem alone (Table 7). The best performing carbapenem-BA combination of each study was able to detect all tested class A carbapenemases and reliable distinguish class A carbapenemases from ESBLs, plasmid mediated AmpC and MBLs. Only Giske et al. evaluated the specificity of BA-based CDDSTs in isolates containing a class D oxacillinases or a plasmid-mediated/derepressed AmpC β-lactamase combined with porin loss and reported false positive results due to these enzymes. The false positive results caused by AmpC plasmid-mediated/derepressed AmpC β-lactamase combined with porin loss are due to the fact that AmpCs are also inhibited by BA. Isolates with combined plasmid-mediated/derepresed AmpC production can however be distinguished from KPC producers by showing cloxacillin synergy.

Rosco (Rosco Diagnostica A/S, Taastrup, Denmark) provides a commercially available BA-based CDDST (KPC+MBL Confirm ID kit) with MEM as indicator and APB as inhitor. In the study of Giske et al. [91] this kit was evaluated for the detection of KPC production in *K. pneumoniae,* showing a 100% sensitivity and 98%

specificity rate which was comparable to a home-prepared MEM+APB 600µg CDDST. Note that the APB-based assays failed to detect the KPC-producing *Klebsiella* isolates in the case of co-production of VIM enzyme. This can be attributed to a masking effect of the coproduced VIM-1[93]. The authors suggest that these isolates can possibly be identified by simultaneous addition of a MBL-inhibitor (e.g.dipicolinic acid, DPA) and class A carbapenemase inhibitor (APB) to carbapenem disks [91].

Tested carbapenemase producing strains	Type of BA derivative	Added amount BA (µg)	Cutoff value (mm)	Bestperforming carbapenem-BA combination: SN/SP (%)	References
KPC producing *K. pneumoniae*	APB	600	5	MEM*: 100/98**	Giske CG et al., Clin Microbiol Infect. 2011 [91].
KPC producing *K. pneumoniae*	PBA	400	5	MEM: 100/100 IPM:100/100	Tsakaris A et al., J Clin Microbiol. 2009 [88].
KPC producing *K. pneumoniae & E. coli*	APB	300	5	MEM: 100/100	Doi Y et al., J Clin Microbiol. 2008 [82].
KPC, GES, Sme, IMI, and NMC-A producing *K. pneumoniae, E. coli, C. freundii, Salmonella spp., E. cloacae*	APB	300	4	IPM:100/100	Pasteran F et al., J Clin Microbiol. 2009 [75].

Table 7. Overview of the performance of BA-based CDDST to detect class A carbapenemases in the literature. * Only meropenem was evaluated in this study. **SP% if the additional criterion of a negative cloxacillin result was included. APB=3-amino-phenyl boronic acid , PBA= phenyl boronic acid.

BA-based CDDSTs for the confirmation of class A carbapenemases as recommended by the Dutch Working Party on the Detection of Highly Resistant microorganisms are shown in Table 8. These recommendations are based on advises of EUCAST experts and the ESCMID Study Group for Antibiotic Resistance Surveillance (ESGARS).

Carbapenemase	Method	Antibiotic	Concentration	Inoculum	Medium	Carbapenemase positive when:
Class A	Combination diffusion tablet (Rosco)	Meropenem + boronic acid	Meropenem 10 µg	0.5 McF	MHA	Rosco: an increase of ≥5 mm of the zone surrounding the combination of meropenem + boronic acid relative to the zone with meropenem only
	Combination diffusion disk (in-house)	Meropenem + boronic acid	Meropenem 10 µg, boronic acid 600 µg	0.5 McF	MHA	In-house disks: an increase of ≥4 mm of the zone surrounding the combination of meropenem + boronic acid relative to the zone with meropenem only
Class B	Combination diffusion tablet (Rosco)	Imipenem + EDTA[a]	Imipenem 10 µg, EDTA 750 µg	0.5 McF	MHA	Rosco: an increase of ≥7 mm of the zone surrounding the combination of imipenem + EDTA relative to the zone with imipenem only
	Combination diffusion disk (in-house)	Imipenem + EDTA[a]	Imipenem 10 µg, EDTA 0.5 M	0.5 McF	MHA	In-house disks: an increase of ≥5 mm of the zone surrounding the combination of imipenem + EDTA relative to the zone with imipenem only
	Combination diffusion tablet (Rosco)	Meropenem + DPA	Imipenem 10 µg	0.5 McF	MHA	Rosco: an increase of ≥5 mm of the zone surrounding the combination of imipenem + DPA relative to the zone with imipenem only
	MBL Etest[b]	Imipenem + EDTA [a]	N/A	0.5 McF	MHA	MIC ratio ≥8 or phantom effect or deformation of ellipse

Table 8. Recommendations of the Dutch Working Party on the Detection of Highly Resistant microorganisms to confirm class A and B carbapenemases in Enterobacteriaceae. *Source*:[72].

3.3.2 MBL-confirmation in Enterobacteriaceae, Pseudomonas and Acinetobacter spp.

Next, two approaches for the phenotypic confirmation of MBL in Enterobacteriaceae, *Pseudomonas spp.* and *Acinetobacter spp.* will be discussed.

3.3.2.1 Modified Hodge test

Although recommended by the CLSI, the MHT is not appropriate to confirm MBL production due to disadvantages described above [94][95]. Nor is the more specific modified boronic acid- and oxacillin-based MHT as it has limitations such as the inabilty to distinguish class B carbapenemases from ESBLs [86].

3.3.2.2 Inhibitor based confirmation

The mechanism of ß-lactam hydrolysis by MBL is dependent on interaction of the ß-lactams with zinc ions in the active site of the enzyme. Thus, chelating agents, such as ethylene diamine tetra-acetic acid (EDTA) and 2-mercaptopropionic acid (2-MPA) block MBL activity and can therefore be used in inhibitor based MBL confirmation tests. Four different approaches that can be used in routine clinical laboratories will be described and discussed next.

Double-disk synergy and combined double disk synergy tests

Double-disk synergy and combined double disk synergy tests for MBL confirmation differ only from class A carbapenemase confirmation tests in the type of inhibitor used (a chelating agent instead of BA).
These tests have been studied extensively. Unfortunately, many articles have major limitations, such as:

(i) the inclusion of only a small number of MBL-producing isolates, sometimes harboring the same type of enzyme [94][96][97][98],
(ii) the absence of molecular typing to exclude the influence of a single clone in the interpretation of their results [99][100],
(iii) a lack of tests evaluating the inhibitory effect of the chelating agents on bacterial growth and β-lactam hydrolysis by these chelating agents [94][97][101],
(iv) no inclusion of carbapenem susceptible MBL producing strains, which often cause false negative results in the DDSTs using a carbapenem as indicator [95][102][103],
(v) a lack of results stratified according to pathogen/species which is important, as highly varied performance data are achieved depending on the species used [94][97][104],
(vi) no inclusion of MBL strains coproducing an ESBL or derepressed/plasmid mediated AmpC which can mask the presence of the MBL.

Furthermore, the result of many studies can not be compared because they were performed under different experimental conditions (e.g. different amounts of added inhibitor, cut-off values of zone diameter differences). In addition, data on the reliability of these tests to detect MBLs in strains coproducing another type of carbapenemases, which can mask the presence of a MBL, are lacking.

Up to now, the most complete study [105] assessed the accuracy of a DDST and a CDDST to detect the major types of mobile MBLs produced by diverse bacterial genera with distinct imipenem susceptibility patterns (GIM, IMP, SIM, SPM and VIM). Both imipenem and ceftazidime were evaluated as substrates in combination with distinct inhibitors. Further, tests for evaluating the inhibitory effect of the chelating agents on bacterial growth and β-lactam hydrolysis were conducted. The results indicate that the choice of phenotypic method (CDDST or DDST) and their used indicator antimicrobial agents to detect MBL should be based on the genus of the tested isolate, regardless of the enzyme produced by such isolates, as well as on the local prevalence of MBL producers. A sensitivity and specificity of 100% was achieved by DDST using a 2-mercaptopropionic acid (5 µl of a 1,4 mM solution) disk placed 20mm apart from a CAZ (30µg) and IPM disk (10µg) among MBL producing *P. aeruginosa* and *Acinetobacter* species. The CDDST showed the same results for detecting MBL-producing Enterobacteriaceae by combining IPM (10µg) and EDTA (10µL from a 100mM solution), with a 5 mm-breakpoint increase in the size of the inhibition zone. Though the sensitivity of CDDSTs with CAZ and EDTA (8µL of a 100mM solution) or MPA (8µL of a 1,4mM solution) was marginally lower than the DDST as described above for the detection of MBL producing *P. aeruginosa*, it remains a reliable alternative (SN=96%, SP=100%) as its interpretation is less subjective. This study also revealed that none of the evaluated CDDSTs were reliable for MBL detection in *Acinetobacter* spp.. As an alternative to the DDST with MPA, a toxic chemical agent, a DDST with IPM and EDTA disks (5µl from a 100 mM solution) placed 10mm apart, could be considered (SN= 100%) for MBL detection in *Acinetobacter* spp.. However, it shows a very low specificity (33,3%). Unfortunately, the chelating agent DPA, described to be superior to EDTA was not included in this study [100][102][106].

Data on the performance of the commercially available Rosco tablets (Rosco Diagnostica A/S, Taastrup, Denmark) to MBL confirmation are limited [91][96]. One study [96] evaluated Rosco's CDDST using IPM and EDTA and Rosco's DDST with DPA as inhibitor and CAZ, MEM, IPM as indicator antibiotics to detect different types of MBLs in *Pseudomonas* and *Acinetobacter* spp.. The DDST could only detect 75% of the MBLs, whereas the CDDST test detected them all. However, the latter had many false positive results due to IPM resistant non-MBL producing *P. aeroginosa* strains and *A. baumannii* strains with an OXA-gene.
The other study evaluated Rosco's CDDST present in the KPC+MBL Confirm ID kit (including MEM and DPA) to detect VIM-1 producing *K. pneumoniae* and IMP-producing *K. pneumoniae* and *E. coli* isolates [91]. It showed the same 100% sensitivity rate as the home-prepared MEM+DPA 1000µg and MEM+ 730µg EDTA CDDST. It also revealed that the DPA based CDDSTs were more specific than the EDTA based ones, with only one false positive result in the Rosco's CCDST due to an OXA-48 ß-lactamase.
It is unknown if in the above mentioned studies carbapenem susceptible MBL producing strains, which can decrease overall sensitivity, were included.

Although performance data are limited, the DWP [72] recommends, based on the advise of EUCAST experts and the ESGARS, Rosco's combination disks or home-prepared combinations disks IPM+EDTA (10µL of a 0.5M solution) [94] for MBL detection in Enterobacteriaceae (Table 8).

MBL Etest® strip

The commercially available MBL Etest® strip (IP/IPE, bioMérieux), containing a double-sided seven-dilution range of IPM (4 to 256 µg/mL) on one end and IPM (1 to 64 µg/mL) in combination with a fixed concentration of EDTA (320µg/mL) on the other, should be interpreted in the same way as ESBL Etest® strips (cfr. supra).
This simple test is costly and highly insensitive at detecting MBL-carrying organisms with a IPM MIC <4 µg/ml. As a MIC <4 µg/ml leads to an indeterminate MBL Etest® result [79][94][101][104][107]. In the published reports,

its sensitivity to detect the most frequent reported plasmid-mediated MBL in isolates with an IPM MIC ≥ 16 µg/ml is 100%. Only false negative results have been reported in some chromosomal encoded MBL producers (BlaB/IND-1 producing *Chryseobacterium* spp. and L1 producing *S. maltophilia*) [101][108]. Concerning its specificity, false positive results have been described with carbapenem-resistant *A. baumannii* carrying bla_{OXA-23}, in *Chryseobacterium* spp. and *S. maltophilia* strains [79][107][108].

Despite the fact that most carbapenemase producing Enterobacteriaceae have a MIC <4 µg/ml, which results in a false negative result, the MBL Etest® is one of the DWP's recommended MBL confirmation tests in Enterobacteriaceae (Table 8).

Cica-Beta test
Performance data on the Cica-Beta test (described above) to detect MBL, reveal that it is not appropriate for MBL confirmation as its sensitivity rates are too low (77%) [49].

CLINICAL BOTTOM LINE

Data concerning *direct* screening for MDR-GNB using selective media supplemented with antimicrobial agents are scarce. But, since there is a great interest in clinical laboratories for the use of chromogenic media, this review focussed on the value of these for selective isolation of Gram-negative bacilli with emerging ß-lactamases (ESBL's, plasmid-mediated or derepressed chromosomal AmpC's, carapenemases). Besides, different screening and confirmation assays to identify these enzymes among *Gram-negative isolates* were discussed. In this section, based on the comparison of these methods, recommendations will be made on which phenotypic assays to use for each type of emerging ß-lactamase.

ESBL

Screening for ESBL in surveillance samples

Recently chromogenic media designed for selective isolation and presumptive identification of ESBL producing Enterobacteriaceae from clinical screening samples have become commercially available. ChromID ESBL (bioMérieux) showed similar to better performance when compared to selective Gram-negative media supplemented with CTX and CAZ.
The performance of Brilliance ESBL (Oxoid) seems comparable to that of the ChromID ESBL. Based on these findings, Chromogenic ESBL agars seem to be an excellent screening tool for ESBL detection in surveillance samples. Moreover, if ESBL-screening in non-Enterobacteriaceae (colorless colonies on chromogenic agars) is disregarded based on low prevalence in Europe, the use of chromogenic media reduces the need for unnecessary identification and confirmation testing.

Screening for ESBL among Gram-negative isolates

Currently, uniform recommendations, on the choice of indicator antimicrobial agent(s) and cut-off value(s) for ESBL detection in Gram-negative bacteria, are lacking (Table 1). There is a consensus in guidelines and literature data that the use of CAZ, CTR or CTX as sole indicator antimicrobial agent is inappropriate for ESBL screening. CPD has proven to be the most sensitive molecule for ESBL screening, however screening with CAZ and CTR or CTX allows a better specificity.

ESBL confirmation

Currently, all ESBL confirmation tests are based on *in vitro* inhibition of ESBL by clavulanic acid. As coproduction of an AmpC or carbapenemases may cause false negative results in ESBL confirmation assays, adaptations are required if coproduction of these enzymes is suspected.

Although (adapted) double disk synergy testing is an inexpensive, well performing ESBL confirmation method, it is not preferred for use in routine clinical microbiology labs as the interpretation is difficult and subjective. Furthermore, compared to other techniques, due to the need for optimal disk spacing, it is technically more demanding and time consuming.
The Cica-Beta test is interesting for use in routine clinical laboratories as it is simple and reduces ESBL confirmation time with 24h.. However, due to variations in reported sensitivity rates (74-95%), more evaluation is required before it can be introduced in routine clinical laboratories.
Concerning the use of ESBL confirmation tests in semi-automated systems, Thomson et al. [48] showed that the Phoenix ESBL confirmation test is more sensitive (96%) than the Vitek2 system in *E. coli* and *Klebsiella* strains (89%). In inducible Enterobacteriaceae, neither Vitek2 nor Phoenix provides an optimal ESBL confirmation test [23][27]. For Vitek2, this is caused by the fact that the test is significant less sensitive for ESBL detection in *Enterobacter* spp. than other confirmation methods [27]. For Phoenix, the ESBL detection test showed a rather low specificity to detect ESBLs in *Enterobacter* and *Citrobacter* spp. (33.3%) [23].
Regarding ESBL Etest® strips, the manufacturer recommends to test always cefotaxime (CT/CTL) and ceftazidime (TZ/TZL) ESBL Etests®. Only when the result is inconclusive, the cefepime ESBL Etest® (PM/PML) is recommended. In contrast to these recommendations, a recent study [27] revealed that in non-inducible

Enterobacteriaceae the sensitivity of the CT/CTL Etest® is not significantly improved by concomitant testing of the TZ/TZL Etest®. Moreover, the highest sensitivity (97%) and specificity (79%) results were obtained by performing only the PM/PML Etest®. In AmpC producing strains both the CT/CTL and the TZ/TZL Etest® appear to be improper to detect ESBLs. Performing the PM/PML Etest® on a cloxacillin containing agar (which inhibits AmpC activity) has shown to be the most sensitive Etest® strategy to confirm ESBL production in AmpC coproducing strains [27]. Compared to combined double disk synergy testing, ESBL Etest® strips do not seem to improve ESBL detection in Enterobacteriaceae. Lower specificity rates have been reported for ESBL Etests® in *E. coli* and *Klebsiella* spp.. Other disadvantages of this technique are:

(i) failure to detect an ESBL when the MIC values for cephalosporines fall outside the range of MICs available on the test strips [18][109],
(ii) delicate interpretation that requires training. Laboratories fail to correctly interpret the inhibition ellipse in ± 30% of the cases [18][110],
(iii) high cost [18].

Based on this literature research, the combined double disk synergy test (CDDST) seems to be the preferred method for phenotypic ESBL confirmation in routine clinical laboratories. In the group of non-inducible Enterobacteriaceae, without coproduction of a plasmid mediated AmpC or carbapenemase, this approach is also recommended by CLSI, NVMM and BSAC. They all recommend to perform the CDDST using both ceftazidime and cefotaxime as indicator antimicrobial agent. Garrec et al. [27] however, revealed that in non-inducible Enterobacteriaceae the sensitivity of performing a CDDST with cefotaxime as the only indicator is not significantly improved by concomitant testing of ceftazidime. Moreover, the best performance (SN=100%, SP= 86%) for the detection of the most prevalent ESBLs was reached using cefotaxime and cefepime as indicator antimicrobial agents [27]. Further evaluation of this approach in Enterobacteriaceae species in which ESBL production is less frequently described (e.g. *Salmonella* spp., *Proteus* spp.) and in strains producing rare ESBL types is necessary. In ESBL producing strains that coproduce a chromosomal or plasmid mediated AmpC β-lactamases, the sensitivity of a CDDST with third generation cephalosporins as indicators is too low. This can be explained by the fact that AmpC enzymes can mask the synergy arising from inhibition of the ESBL by CA. The sensitivity of the CDDST to detect an ESBL in AmpC coproducers improves by using cefepime (stable against AmpC hydrolysis) and even more by also performing this on a cloxacillin-containing agar (200-250 µg/ml, cloxacillin inhibiting AmpC β-lactamases) [23][27][31]. However, further evaluation of combined double disk synergy testing on cloxacillin-containing agars in inducible non-*Enterobacter* spp. and strains producing a plasmid-mediated AmpC is still necessary. Currently, we prefer to perform the CDDST using antibiotic-containing disks (cefotaxime/ceftazidime with or without clavulanate) with 3-aminophenyl boronic acid (400µg, an AmpC inhibitor) added. This method has already shown to increase sensitivity and specificity of ESBL detection in different chromosomal Enterobacteriaceae as well as in plasmid-mediated AmpC producers (Table 4) [20][38][39][40].

Concerning ESBL detection in carbapenemase coproducing strains, too little data are available to make recommendations concerning ESBL detection. The CLSI confirmation method is unreliable for ESBL detection in KPC producing strains, as Tsakaris et al. reported a sensitivity of only 67%. On the other hand, all ESBLs could be detected in these strains when BA (400µg, inhibitor of KPC) was added to the CAZ, CTX, CAZ+CA, CTX+CA disks.

Plasmid-mediated or chromosomal AmpC

Screening for AmpC in surveillance samples

Up to now, there are no chromogenic media for the to selectively screening for derepressed chromosomal or plasmid-mediated AmpC producers in surveillance samples available.

Screening for AmpC among Gram-negative isolates

Reduced cefoxitin susceptibility (zone diameter <19mm or MIC ≥16µg/ml), a sensitive but non-specific approach to screen for AmpC production in Gram-negative isolates, is recommended by EUCAST [2] and NVMM [4]. In *derepressed* AmpC producers, however, decreased susceptibility to cefotetan should be preferred as it has proven to be a more specific and still reliable indicator.

AmpC Confirmation

There are no recommendations concerning the confirmation of chromosomal or plasmid mediated AmpC ß-lactamases in the citated guidelines.

The modified indirect three-dimensional test and its variation are reliable AmpC confirmation tests but are not feasible for most routine clinical laboratories as they require multiple freeze-thawing steps.

The Cica-Beta test seems unreliable for *derepressed* AmpC confirmation and has not yet been evaluated for plasmid-mediated AmpC confirmation.

Due to a limited number of evaluations and practical disadvantages, the double-disk synergy test it not a preferred AmpC confirmation method for use in routine labs.

Etest® strips CN/CNI and FX/FXI, have mainly been evaluated in plasmid-mediated AmpC producing Enterobacteriaceae and have shown good performance (SN=91%, SP= 93%) [69]. Furthermore, Bolström et al.

reported no significant improvement of AmpC detection when the CN/CNI Etest® was combined with a FX/FXI Etest® [69]. This finding, however, needs to be further evaluated in derepressed AmpC producers.
Currently, different studies show that disk potentiation tests (DPT) with boronic acid as inhibitor, are reliable to confirm plasmid-mediated AmpC production in Enterobacteriaceae. However, these studies used various indicator cephalosporins and different amounts of inhibitor in their disks. Thus, studies determining the best performing indicator(s) and added amount of inhibitor, are of interest. Also, it is necessary to further evaluate the performance of AmpC confirmation methods in derepressed AmpC producers.

In addition, KPC carbapenemases cause resistance to cephamycins and can result in false-positive BA-based AmpC confirmation results. Strains that test positive therein with subsequent decreased susceptibility to carbapenems should be investigated for possible KPC production in a reference laboratory.

Carbapenemases

Screening for Carbapenemases in surveillance samples

Recently a CHROMagar™ KPC (Hy-Labs, Rehovot, Israel) for direct screening and presumptive identification of carbapenemases in surveillance samples, has become commercially available. This chromogenic medium has already shown to be a useful screening medium for KPC and VIM carbapenemase-producing Enterobacteriaceae [73]. Further studies are however still required to establish its reliability to detect other carbapenemases.

Screening for Carbapenemases among Enterobacteriaceae isolates

Screening for carbapenemases in Enterobacteriaceae isolates is based on carbapenem susceptibility testing.
There are some important differences among the recommendations on this subject by CLSI [1] and the Dutch Working Party on the Detection of Highly Resistant Microorganisms (DWP) [72] (Table 5) as the settings of the applied carbapenem screening breakpoints are based on different principles.
Based on this literature research, the best way to screen is by performing a meronem susceptibility test. Strains with a meropenem MIC value ≥ 0,5 µg/mL or an inhibition zone diameter ≤ 23mm, combined with resistance to at least one subclass III cephalosporin need to be further evaluated by a carbapenemase confirmation test.

Confirmation of carbapenemases

Class A carbapenemase confirmation in Enterobacteriaceae

Although recommended by the CLSI, the Modified Hodge test (MHT) is not appropriate to confirm class A carbapenemases in Enterobacteriaceae. This, mainly because of its low specificity and its inability to distinguish between different classes of carbapenemases [86][87][88][89][90][91]. A recently developed modification of this MHT no longer suffers from these disadvantages. However it is technically demanding, time consuming and in some cases hard to interpret.
DDST is not a preferred method to confirm class A carbapenemases in Enterobacteriaceae.
Firstly, because combined double disk synergy test have shown to perform better. Secondly, since its interpretation is difficult and subjective. Finally, it is not recommended by the DWP and the CLSI.
Concerning home-prepared boronic-acid based CDDST, several studies have evaluated varying configurations, all showing good performance (Table 7). Rosco's commercially available KPC+MBL Confirm ID kit demonstrates similar performance. Note, that the majority of these studies only included KPC-producing *Klebsiella* isolates. Furthermore, in these tests class D oxacillinases or a plasmid-mediated/derepresed AmpC β-lactamase combined with porin loss can cause false positives. Coproduction of a MBL, on the other hand, can cause false negatives. The BA-based CDDSTs to confirm class A carbapenemases as recommended by the Dutch Working Party on the Detection of Highly Resistant microorganisms are shown in Table 8.

MBL confirmation in Enterobacteriaceae, P. aeruginosa and Acinetobacter spp.

Currently, guidelines for phenotypic confirmation of metallo-beta lactamases exist only for Enterobacteriaceae.
Although recommended by the CLSI, the MHT is not appropriate to confirm MBL production due to disadvantages described above [94][95]. Nor is the more specific modified MHT as it has limitations such as the inabilty to distinguish class B carbapenemases from ESBLs [86].

Other MBL confirmation tests (DDST, CDDST, Cica-Beta test, MBL Etest® strip) are based on *in vitro* inhibition of the MBL activity by a chelating agent (e.g. EDTA, DPA, MPA).
Performance data on the Cica-Beta test, reveal that it is an inappropriate MBL confirmation test as its sensitivity is too low [49].
The MBL Etest® strip may only be used to confirm the presence of a MBL in strains with an IMP MIC > 4 µg/mL as it will otherwise lead to an indeterminate result [79][104][107]. Despite the fact that most carbapenemase producing Enterobacteriaceae have a MIC <4 µg/ml, the MBL Etest® is one of the DWP's recommended MBL confirmation tests in Enterobacteriaceae (Table 8).
The DDST and the CDDST have been studied extensively. Unfortunately, many articles have major limitations. Furthermore, data on the reliabilty to detect MBLs in strains coproducing another type of carbapenemases, which can mask the presence of a MBL, are lacking. Up to now, the most complete study [105] indicates that the choice of phenotypic method (CDDST or DDST) and the used indicator antimicrobial agents to detect MBL should be based on the genus of the tested isolate as well as on the local prevalence of MBL producers. Based on the fact that MBL confirmation tests need to be highly sensititive and taking into account that MPA is preferably

not used in routine clinical laboratories (due to its toxicity), the following MBL confirmation tests have shown to be convenient:

(i) in Enterobacteriaceae: CDDST with IPM as indicator disk (10µg), EDTA as added inhibitor (10µL from a 100mM solution) and a 5 mm-breakpoint increase as cut-off value (SN & SP=100%).
(ii) in *P. aeruginosa*: CDDSTs with CAZ (30µg), EDTA (8µL of a 100mM solution) and a 5 mm-breakpoint increase as cut-off value (SN=96%, SP=100%).
(iii) in *Acinetobacter* spp.: DDST with IPM (30µg) and EDTA disks (5µl from a 100 mM solution) placed 10mm apart (SN= 100%). As this has only a 33.3% specificity further confirmation by PCR testing is necessary.

Rosco (Rosco Diagnostica A/S, Taastrup, Denmark) combination disk test, consisting of dipicolinic acid (DPA) as inhibitor and CAZ, IPM as indicator antibiotics, is able to detect different types of MBLs in *Pseudomonas* and *Acinetobacter* spp.. However, it shows many false positive results [96]. Among MBL producing Enterobacteriaceae Rosco's KPC+MBL Confirm ID kit showed a 100% sensitivity rate, with only one false positive result due to an OXA-48 ß-lactamase [91]. It is unknown if in this latter study carbapenem susceptible MBL producing strains, which can decrease overall sensitivity, were included.

Althoug performance data are limited, the DWP [72] recommends Rosco's combination disks or home-prepared combination disks (Galani et al.) for MBL detection in Enterobacteriaceae (Table 8).

TO DO/ACTIONS

Rewriting the standard operating procedure (SOP) concerning screening for MDR Gram-negative bacilli in the Jessa Hospital, conform to the findings of this work.

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