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Evaluation of a diagnostic flow chart for detection and confirmation of extended spectrum β-lactamases (ESBL) in *Enterobacteriaceae*

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Abstract

This study aimed to develop a modular, diagnostic algorithm for extended spectrum β-lactamase (ESBL) detection in *Enterobacteriaceae*. Clinical *Enterobacteriaceae* strains (n = 2518) were screened for ESBL production using Clinical and Laboratory Standards Institute (CLSI) breakpoints for third-generation cephalosporins and by synergy image detection (clavulanic acid/extended-spectrum cephalosporins). Isolates screening positive for ESBL (n = 242, 108 by critical CLSI diameters alone, five by double disk synergy test (DDST) alone, and 129 by both critical diameters and DDST) and 138 ESBL screening negative isolates (control group) were investigated by molecular methods considered to be the reference standard (multiplex CTX-M type PCR, TEM and SHV type sequence characterization). One hundred and twenty-four out of 242 *Enterobacteriaceae* isolates screening positive for ESBL were confirmed to be ESBL positive by the reference standard, the majority of them in *E. coli*, *K. pneumoniae* and *E. cloacae* (94, 17 and nine isolates, respectively). Prevalence of ESBL production ranged from <1% for *P. mirabilis* to 4.7%, 5.1% and 6.6%, for *K. pneumoniae*, *E. cloacae* and *E. coli*, respectively. Combining CLSI ceftriaxone and cefpodoxime critical ESBL diameters was found to be the most sensitive phenotypic screening method (sensitivity 99.2%). Combining critical diameters of cefpodoxime and ceftriaxone with DDST for cefpodoxime resulted in a sensitivity of 100%. For phenotypic confirmation, combining the CLSI recommended combined disk test (CDT) for ceftazidime and cefotaxime amended with a cefepime CDT was highly sensitive (100%) and specific (97.5%). With respect to the studied population, the diagnostic ESBL algorithm developed would have resulted in sensitivity and specificity of 100%. The corresponding flow chart is simple, easy to use, inexpensive and applicable in the routine diagnostic laboratory.

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Introduction

Increasing prevalence of multidrug-resistant Gram-negative bacteria has continuously been reported over the past years, in particular *Enterobacteriaceae* producing extended spectrum β-lactamases (ESBLs). ESBLs have the ability to hydrolyse penicillins, first- second- and third-generation cephalosporins and aztreonam (but not cephamycins or carbapenems), and their activity is decreased by inhibitors such as clavulanic acid [1]. ESBL-producing organisms may be responsible for life-threatening infections, leading to increased morbidity, mortality and healthcare-associated costs [2–5].

Klebsiella pneumoniae and *Escherichia coli* are the most frequently isolated ESBL-producing strains worldwide; however, ESBLs have been identified in other members of the *Enterobacteriaceae* family [6]. Most ESBLs can be classified in TEM, SHV and CTX-M types [7]. To date, over 100 SHV- and 150 TEM-ESBL types and approximately 90 CTX-M variants (clustered in five groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) have been identified (for actual update see http://www.lahey.org/studies). In recent years CTX-M type β-lactamase-producing *E. coli* have emerged and now represent the most prevalent ESBL type in Europe and North America [8,9].

Until 2009 the Clinical and Laboratory Standards Institute (CLSI) recommended adjusting the results of *in vitro* drug susceptibility testing for penicillins, cephalosporins, cefepime and monobactams and reporting these compounds as resistant to ESBL-producing isolates of *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca* and *Proteus mirabilis* [10]. In 2010, the CLSI released new cephalosporin susceptibility breakpoints for *Enterobacteriaceae*. Subsequent adjustment of *in vitro* susceptibility test results for β-lactams due to ESBL production is no longer recommended, but screening for ESBL-producing isolates is still considered useful and should be continued for epidemiological reasons [11]. The new CLSI strategy is widely in agreement with the guidelines of the European Committee for Antimicrobial Susceptibility Testing (EUCAST), which considers screening for ESBL-producing isolates mandatory for epidemiological purposes [12].

Different phenotypic tests for ESBL detection have been evaluated and described in the literature. However, little information is available on the performance parameters of standardized approaches combining single methods to identify ESBL-producing *Enterobacteriaceae* spp. [13–15]. The aim of this study was to develop and prospectively evaluate a standardized, inexpensive and simple approach that is able to detect ESBL-producing *Enterobacteriaceae* isolates in a routine clinical laboratory.

Methods

Clinical isolates

In this prospective study all non-duplicate clinical *Enterobacteriaceae* isolates (n = 2518) isolated over a period of 7 months from October 2009 until April 2010 were systematically screened for ESBL production. Only isolates that were considered clinically relevant were included. Table 1 shows the study design, the numbers of studied clinical isolates and the overview of ESBL detection results. Two hundred and forty-two of the 2518 clinical isolates were selected for further characterization on the basis of (i) positive CLSI screening cut-off values for ESBL for at least one third-generation cephalosporin tested, and/or (ii) observation of a synergy zone between amoxicillin-clavulanic acid and cefpodoxime and/or ceftazidime, and/or ceftriaxone and/or cefotaxime. Of the 242 ESBL suspicious isolates, 108 were selected by critical CLSI diameters alone, five by double disk synergy test (DDST) alone, and 129 by both critical diameters and DDST. Ninety-one of the 242 ESBL suspicious isolates additionally produced an AmpC enzyme.

To assess sensitivity of the initial screening procedure a negative control group (138 negative in the ESBL screening) was selected, in which species distribution resembled that of the 2518 study isolates (representative sample). The 138 control isolates showed: (i) negative ESBL CLSI screening cut-off values for all third-generation cephalosporins tested; and (ii) no visible synergy zone between a β-lactamase inhibitor disk and a third-generation cephalosporin disk. To develop and evaluate the ESBL detection algorithm the 380 isolates (242 positive isolates screening positive for ESBL and 138 negative control isolates) were characterized for ESBL production by a combination of phenotypic screening and confirmation methods, with genetic methods (CTX-M-Multiplex PCR, TEM and SHV sequencing) serving as the reference standard (Table 1).

Susceptibility testing

For susceptibility testing the disk diffusion method according to Kirby-Bauer was used. Antibiotic disks (Becton Dickinson, Franklin Lakes, NJ, USA) were selected and results were interpreted according to the guidelines of the CLSI [11]. The following ESBL CLSI screening cut-off values were used for third-generation cephalosporins: cefpodoxime (10 μg/disk) $\leq$ 17 mm, ceftazidime (30 μg/disk) $\leq$ 22 mm, cefotaxime (30 μg/disk) $\leq$ 27 mm, and ceftriaxone (30 μg/disk) $\leq$ 25 mm. Susceptibility testing was carried out on Mueller-Hinton agar (bioMérieux, Marcy L'Etoile, France) using McFarland 0.5 from overnight cultures followed by incubation at 35°C for 16–18 h.

AmpC-β-lactamase (AmpC) detection

All isolates included in this study were systematically characterized for the production of an AmpC type β-lactamase as described [16]. In brief, a multiplex PCR was used for detection of plasmid-mediated *ampC* β-lactamase genes, which detects the six plasmid-mediated *ampC* families. The *ampC* promoter mutation analysis of *E. coli* isolates was carried out as described previously [17].

Double disk synergy test (DDST)

The double disk synergy test was conducted as described [14]. Synergy images were recorded between amoxicillin-clavulanic acid disks (20/10 μg/disk) and cefpodoxime, ceftazidime, ceftriaxone or cefotaxime disks. Antibiotic disks were placed 27 mm apart, centre-to-centre. β-lactam inhibitor-mediated enhancement of the third-generation cephalosporin inhibition zone was interpreted as synergy positive.

Combined disk test (CDT)

The CLSI recommended combined disk test (CDT) was performed with ceftazidime and cefotaxime disks with and without clavulanic acid [10]. Additionally, disks containing 30 μg cefepime and 30 μg cefepime/10 μg clavulanic acid were produced for this study (Liofilchem, Roseto degli Abruzzi, Italy)

TABLE 1. Study layout and numbers of isolates

***Enterobacteriaceae* isolates** ***n* = 2518**											
Critical ESBL diameters (CLSI) positive for CPD and/or CAZ and/or CTX and/or CRO and/or Synergy CAZ/AMC and/or CDP/AMC and/or synergy CTX/TZP and/or FEP/TZP											
ESBL screening negative ***n* = 2276**						**Putative ESBL producers**[a] ***n* = 242**					
ESBL negative control group (representative sample) ***n* = 138**											
AmpC negative ***n* = 113**		**AmpC positive** ***n* = 19**		***K. oxytoca*** ***n* = 6**		**AmpC negative** ***n* = 134**		**AmpC positive** ***n* = 91**		***K. oxytoca*** ***n* = 17**	
Negative CDT: CAZ and/or CTX and/or FEP *n* = 113	Positive CDT: CAZ and/or CTX and/or FEP *n* = 0	Negative CDT: CAZ and/or CTX and/or FEP *n* = 19	Positive CDT: CAZ and/or CTX and/or FEP *n* = 0	Negative CDT: CAZ *n* = 6	Positive CDT: CAZ *n* = 0	Negative CDT: CAZ and/or CTX and/or FEP *n* = 25	Positive CDT: CAZ and/or CTX and/or FEP *n* = 109	Negative CDT: CAZ and/or CTX and/or FEP *n* = 76	Positive CDT: CAZ and/or CTX and/or FEP *n* = 13 Borderline *n* = 2	Negative CDT: CAZ *n* = 16	Positive CDT: CAZ *n* = 1
				Negative CDT: CTX and/or FEP *n* = 6	Positive CDT: CTX and/or FEP *n* = 0					Negative CDT: CTX and/or FEP *n* = 14	Positive CDT: CTX and/or FEP *n* = 3
ESBL genetic analysis (CTX-M multiplex PCR and SHV and TEM sequence characterization) ***n* = 380**											
ESBL negative *n* = 113	ESBL positive *n* = 0	ESBL negative *n* = 19	ESBL positive *n* = 0	ESBL negative *n* = 6	ESBL positive *n* = 0	ESBL negative *n* = 25	ESBL[b] positive *n* = 109	ESBL negative *n* = 78	ESBL positive *n* =13	ESBL negative *n* = 15	ESBL positive *n* = 2

CPD, cefpodoxime; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CAZ CLA, ceftazidime/clavulanic acid; CTX CLA, cefotaxime/clavulanic acid; FEP CLA, cefepime/clavulanic acid; CDT, combined disk test.

[a]Of 242 putative ESBL producers, 108 were identified by critical CLSI diameters alone (10 confirmed ESBL), five by double disk synergy test (DDST) alone (one confirmed ESBL) and 129 by both critical diameters and DDST (113 confirmed ESBL).

[b]From one *E. coli* strain a TEM gene was PCR amplified, the sequence electropherogram of which showed double peaks corresponding with the co-presence of a TEM wt and aTEM ESBL tvpe (E104K and G238S) in this isolate. Therefore this isolate was scored ESBL positive.

TABLE 2. ESBLs detected in *Enterobacteriaceae* clinical isolates

	Isolates (*n*, %)	ESBL screening positives (*n*, % of isolates)	Confirmed ESBLs (*n*, % of isolates)	CTX-M types (*N*, % of all ESBL)			SHV ESBL type (*n*, % of all ESBL)	TEM ESBL type (*n*, % of all ESBL)
				Group 1	Group 8	Group 9		
All species	2518 (100.0)	242 (9.5)	124 (4.9)	86 (69.4)	1 (0.8)	23 (18.5)	11 (8.9)	3 (2.4)
Escherichia coli	1435 (57.0)	136 (9.5)	94 (6.6)	66	1	17	8	2
Klebsiella pneumoniae	360 (14.2)	31 (8.6)	17 (4.7)	14	0	2	1	0
Enterobacter cloacae	178 (7.1)	34 (19.1)	9 (5.1)	6	0	3	0	0
Proteus mirabilis	131 (5.2)	2 (1.5)	1 (0.8)	0	0	0	0	1
Klebsiella oxytoca	99 (3.9)	17 (17.2)	2 (2.0)	0	0	1	1	0
Citrobacter. spp.	1 (0.1)	1 (100.0)	1 (100.0)	0	0	0	1	0
Others[a]	314 (12.5)	14 (4.5)	0					

Numbers are given for all *Enterobacteriaceae* species that were isolated and tested for antibiotic susceptibility during the study period. Isolates that were considered part of normal or colonization flora or rarely isolated species (<0.1% of all isolates in the study period) were not included.
[a]Others comprised *Citrobacter koseri* (74 isolates), *Proteus vulgaris* (26 isolates), *Enterobacter aerogenes* (52 isolates), *Citrobacter freundii* (54 isolates), *Morganella morganii* (33 isolates), *Serratia marcescens* (51 isolates), *Hafnia alvei* (20 isolates) and *Salmonella enterica* (4 isolates).

because this combination was not available from Becton Dickinson. AmpC non-producing strains were inoculated on Mueller-Hinton agar using McFarland 0.5 followed by incubation at 35°C for 16–18 h. For AmpC-producing isolates CDT was compared on Mueller-Hinton agar and Mueller-Hinton agar containing 250 mg/L of cloxacillin (AES CHEMUNEX, Bruz Cedex, France). A difference in the inhibition zone diameters of ≥5 mm for any third-generation cephalosporin/clavulanic acid combination vs. the corresponding third-generation cephalosporin alone was considered indicative of ESBL production.

TEM and SHV ESBL identification

DNA was extracted from colonies grown on sheep blood agar medium using the InstaGene Matrix (Bio-Rad, Reinach, Switzerland) following the manufacturer's instructions. Molecular TEM and SHV ESBL detection was carried out as described elsewhere [18]. TEM and SHV β-lactamase sequences were compared with *E. coli* AF427133.1 TEM-1 and *E. coli* AF148850 SHV-1 using the publicly available database at http://www.lahey.org/studies. Each sequence electropherogram was screened for the presence of double peak signals, which would indicate the presence of multiple SHV or TEM variants.

TABLE 3. Distribution of ESBL-producing *Enterobacteriaceae* isolates by sample origin

Sample origin	*Enterobacteriaceae* isolates (*n*, %)	ESBL isolates (*n*, %)
Urine	1002 (39.8)	64 (51.6)
Wound	332 (13.2)	12 (9.7)
Respiratory tract[a]	264 (10.5)	15 (12.1)
Groin	81 (3.2)	16 (12.9)
Blood culture	209 (8.3)	2 (1.6)
Vagina	40 (1.6)	2 (1.6)
Miscellaneous	546 (21.7)	4 (3.2)
Unknown[b]	43 (1.7)	9 (7.3)
Total	2518 (100)	124 (100)

[a]Respiratory material includes sputum, tracheal secretion and bronchoalveolar lavage fluid.
[b]Unknown origin represents swabs without localization sent to the laboratory for ESBL screening.

CTX-M β-lactamase gene identification

For the detection of CTX-M β-lactamase genes a multiplex PCR was performed as described by Pitout *et al.* [8]. This multiplex PCR detects the CTX-M group 1, CTX-M group 2, CTX-M group 8 (former group III) and CTX-M group 9 (former group IV) genes. In the case of *K. oxytoca*, the CTX-M primers for group 8 (former group III) also target similar DNA sequences in the chromosomal K1 gene. To discriminate K1 from CTX-M group 8 amplification, CTX-M group 8 (former group III) genes in *K. oxytoca* were confirmed in a second step by amplification of a 666 bp fragment using primers 'group 8 forward' (5′-TCGCGTTAAGCGGATGATGC-3′) and 'group 8/25 reverse' (5′-AACCCACGATGTGGGTAGC-3′) as published by Woodford *et al.* [19].

Interpretation

Molecular methods were considered to be the reference standard for calculation of performance parameters.

Results

Screening and confirmation of *Enterobacteriaceae* isolates for ESBL production

One hundred and twenty-four initial isolates screening positive for ESBL were confirmed as ESBL producers by phenotypic and molecular methods (Table 2). The majority of ESBL producers were found in *E. coli*, *K. pneumoniae* and *E. cloacae* (94, 17 and 9 isolates, respectively). Prevalence of ESBL production ranged from <1% for *P. mirabilis* to 4.7%, 5.1% and 6.6% for *K. pneumonia*, *E. cloacae* and *E. coli*, respectively. The mean prevalence of ESBL production in the 2518 *Enterobacte-*

TABLE 4. Performance parameters of methods for detection of ESBL production in *Enterobacteriaceae* calculated for the complete study population

Method/Method combination	Isolates (*n*)	TP (*n*)	FP (*n*)	TN (*n*)	FN (*n*)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Critical diameters									
CPD	2518	119	86	2308	5	96.0	96.4	58.0	99.8
CAZ	2518	90	67	2327	34	72.6	97.2	57.3	98.6
CTX	2518	121	93	2301	3	97.6	96.1	56.5	99.9
CRO	2518	122	83	2311	2	98.4	96.5	59.5	99.9
CRO and CPD	2518	123	99	2295	1	99.2	95.9	55.4	99.9
CRO and CAZ	2518	122	89	2305	2	98.4	96.3	57.8	99.9
CRO and CTX	2518	122	99	2295	2	98.4	95.9	55.2	99.9
CPD and CTX	2518	122	104	2290	2	98.4	95.7	54.0	99.9
CPD and CAZ	2518	121	91	2297	3	97.6	96.2	57.1	99.9
CTX and CAZ	2518	122	97	2303	2	98.4	96.0	55.7	99.9
CRO and CPD and CTX	2518	123	110	2284	1	99.2	95.4	52.8	99.9
DDST									
Synergy AMC CPD	2518	109	16	2378	15	87.9	99.3	87.2	99.4
Synergy AMC CAZ	2518	105	10	2384	19	84.7	99.6	91.3	99.2
Synergy AMC CTX	2518	109	12	2385	12	90.1	99.5	90.1	99.5
Synergy AMC CRO	2518	115	6	2389	8	93.5	99.7	95.0	99.7
Synergy AMC CPD and/or CAZ	2518	113	18	2376	11	91.1	99.2	86.3	99.5
Critical diameters plus DDST									
CRO and CPD plus synergy AMC CPD	2518	123	108	2287	0	100.0	95.5	53.2	100.0
CDT									
CAZ CLA/CAZ	2518	102	2	2392	21	82.9	99.9	98.1	99.1
CTX CLA/CTX	2518	122	1	2393	1	99.2	100.0	99.2	99.9
FEP CLA/FEP	2518	112	1	2393	11	91.1	100.0	99.1	99.5
CAZ CTX FEP plus/minus CLA combined	2518	123	3	2391	0	100.0	99.9	97.6	100.0
ESBL algorithm	2518	123	0	2394	0	100.0	100.0	100.0	100.0

CPD, cefpodoxime; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; AMC, amoxicillin-clavulanic acid; CAZ CLA vs. CAZ, ceftazidime-clavulanic acid; CTX CLA vs. CTX, cefotaxime-clavulanic acid; FEP CLA vs. FEP, cefepime-clavulanic acid; TP, true positive; TN, true negative; FP, false positive; FN, false negative; PPV, positive predictive value; NPV, negative predictive value; DDST, double disk synergy test; CDT, double disk synergy test. A combination of methods was rated positive if at least one parameter was positive.

TABLE 5. Performance parameters of methods for detection of ESBL production in *Enterobacteriaceae* for ESBL screening positive isolates

Method/Method combination	Isolates (*n*)	TP (*n*)	FP (*n*)	TN (*n*)	FN (*n*)	Sensitivity (%)	Specificity (%)
Critical diameters							
CPD	242	119	86	32	5	96.0	27.1
CAZ	242	90	67	51	34	72.6	43.2
CTX	242	121	93	25	3	97.6	21.2
CRO	242	122	83	35	2	98.4	29.7
CRO and CPD	242	123	99	19	1	99.2	16.1
CRO and CAZ	242	122	89	29	2	98.4	24.6
CRO and CTX	242	122	99	19	2	98.4	16.1
CPD and CTX	242	122	104	14	2	98.4	11.9
CPD and CAZ	242	121	91	27	3	97.6	22.9
CTX and CAZ	242	122	97	21	2	98.4	17.8
CRO and CPD and CTX	242	123	110	8	1	99.2	6.8
DDST							
Synergy AMC CPD	242	109	16	102	15	87.9	86.4
Synergy AMC CAZ	242	105	10	108	19	84.7	91.5
Synergy AMC CTX	242	109	12	109	12	90.1	90.1
Synergy AMC CRO	242	115	6	113	8	93.5	95.0
Synergy AMC CPD and/or CAZ	242	113	18	100	11	91.1	84.7
Critical diameters plus DDST							
CRO and CPD plus synergy AMC CPD	242	123	108	11	0	100.0	9.2
CDT							
CAZ CLA/CAZ	242	102	2	116	21	82.9	98.3
CTX CLA/CTX	242	122	1	117	1	99.2	99.2
FEP CLA/FEP	242	112	1	117	11	91.1	99.2
CAZ CTX FEP plus/minus CLA combined	242	123	3	115	0	100.0	97.5
ESBL algorithm	242	123	0	118	0	100.0	100.0

CPD, cefpodoxime; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; AMC, amoxicillin-clavulanic acid; CAZ CLA vs. CAZ, ceftazidime-clavulanic acid; CTX CLA vs. CTX, cefotaxime-clavulanic acid; FEP CLA vs. FEP, cefepime-clavulanic acid; TP, true positive; TN, true negative; FP, false positive; FN, false negative; DDST, double disk synergy test; CDT, double disk synergy test. A combination of methods was rated positive if at least one parameter was positive.

riaceae isolates was 4.9% (Table 2). The highest proportion of false-positive ESBL screening tests was observed with *E. cloacae* and *K. oxytoca* isolates (Table 2).

CTX-M type genes were predominantly detected among ESBL strains (88.7% of all ESBL), with CTX-M group 1 as the most frequently identified subtype (69.4%). The second most

frequently isolated subtype was CTX-M group 9 (18.5%); SHV and TEM ESBL types were observed in 8.9% and 2.4% of all ESBL-producing isolates, respectively (Table 2).

Enterobacteriaceae species were isolated from different clinical materials (Table 3). The majority of ESBL isolates was isolated from urine (51.6%), groin (12.9%), respiratory tract (12.1%) and wound (9.7%) specimens.

All 138 isolates of the negative control group (initial ESBL screening negative) were confirmed ESBL negative by molecular methods. This result was extrapolated to all 2276 isolates negative in initial screening for ESBL and performance parameters of all tests and the algorithm including positive predictive value (PPV) and negative predictive value (NPV) were recalculated (Table 4).

Comparison of primary screening markers for ESBL production

Considering critical diameters as the primary screening marker for ESBL detection, ceftriaxone showed the highest sensitivity (98.4%). One ESBL-producing isolate was detected exclusively by the critical diameter of cefpodoxime; this isolate was also positive with the cefpodoxime and ceftazidime DDST. Ceftazidime diameters alone showed the lowest sensitivity for ESBL detection (72.6%). The most sensitive combination was ceftriaxone with cefpodoxime (sensitivity 99.2%); adding cefotaxime to these two substances did not result in a further increase of sensitivity (Table 5). One ESBL-positive isolate was exclusively detected by the DDST with cefpodoxime and/or ceftazidime compared with both critical diameters and the other DDSTs.

Combining critical diameters for ceftriaxone and cefpodoxime with the cefpodoxime DDST resulted in a sensitivity of 100% for the potentially ESBL-producing clinical strains used in this study (Table 5). Of 16 false-positive cefpodoxime DDST, one was observed in *E. cloacae*, one in *K. pneumoniae*, six in *E. coli* and eight were seen with *K. oxytoca*. Of 10 false-positive ceftazidime DDST, four occurred with *E. coli*, four with *K. oxytoca*, and two with *K. pneumoniae*. Of 12 false-positive cefotaxime DDST, five were observed with *E. coli* and seven were seen in *K. oxytoca*. Of six false-positive ceftriaxone DDST, two were seen in *E. coli* and four in *K. oxytoca*. Two of the *E. coli* isolates listed above showed false-positive DDSTs for all screening agents (cefpodoxime, ceftazidime, cefotaxime and cefepime). One of these isolates produced a CIT type AmpC. Of the 124 confirmed ESBL producers, 10 were detected by critical CLSI diameters alone, one by the DDST alone, and 113 by both methods.

Evaluation of confirmation assays for ESBL production

The CLSI recommended CDTs with ceftazidime and/or cefotaxime and clavulanic acid as inhibitors were compared as phenotypic confirmation tests using molecular methods as the reference standard. Additionally, a cefepime CDT with clavulanic acid as inhibitor was evaluated. All third-generation cephalosporin-clavulanic acid combinations showed a specificity of >98.3%. For the ceftazidime CDT a sensitivity of 82.9% was observed; cefotaxime and cefepime CDTs showed a sensitivity of 99.2% and 91.1%, respectively (Table 5). Four ESBL-positive isolates were detected by the cefotaxime CDT alone; one ESBL was detected by the cefepime CDT alone. In contrast, the ceftazidime CDT alone did not identify any ESBL-producing isolates. If all CDTs were combined, sensitivity and specificity were 100% and 97.5%, respectively. Extrapolated to the 2518 study isolates, the sensitivity, specificity, PPV and NPV of all CDTs combined were 100%, 99.9%, 97.6% and 100%, respectively (Table 4).

Using 91 AmpC-positive ESBL screening positive isolates (Table 1), the three CDTs were compared on Mueller-Hinton agar and Mueller-Hinton agar supplemented with cloxacillin. A significantly increased overall sensitivity of the

TABLE 6. Comparison of performance parameters of combined disk tests in AmpC β-lactamase-positive putative ESBL-producing isolates[a] on Mueller-Hinton agar plates with and without supplementation of cloxacillin

Method	Isolates (*n*)	TP (*n*)	FP (*n*)	TN (*n*)	FN (*n*)	Sensitivity (%)	Specificity (%)
MH with cloxacillin							
CAZ CLA/CAZ	91	10	2	76	3	76.9	97.4
CTX CLA/CTX	91	13	0	78	0	100	100
FEP CLA/FEP	91	13	0	78	0	100	100
CAZ CTX FEP plus/minus CLA combined	91	13	2	76	0	100	97.4
MH without cloxacillin							
CAZ CLA/CAZ	91	8	2	76	5	61.5	97.4
CTX CLA/CTX	91	10	0	78	3	76.9	100
FEP CLA/FEP	91	11	0	78	2	84.6	100
CAZ CTX FEP plus/minus CLA combined	91	11	2	76	2	84.6	97.4

CAZ CLA vs. CAZ, ceftazidime-clavulanic acid; CTX CLA vs. CTX, cefotaxime-clavulanic acid; FEP CLA vs. FEP, cefepime-clavulanic acid; TP, true positive; TN, true negative; FP, false positive; FN, false negative; DDST, double disk synergy test; CDT, double disk synergy test. A combination of methods was rated positive if at least one parameter was positive.

[a]Isolates consist of 54 isolates with known chromosomal *ampC*, 24 isolates harbouring a plasmidic *ampC* (22 CIT-type and 2 DHA-type AmpC) and 13 *E. coli* isolates with promoter/attenuator mutations resulting in *ampC* overexpression; see also Table 2.

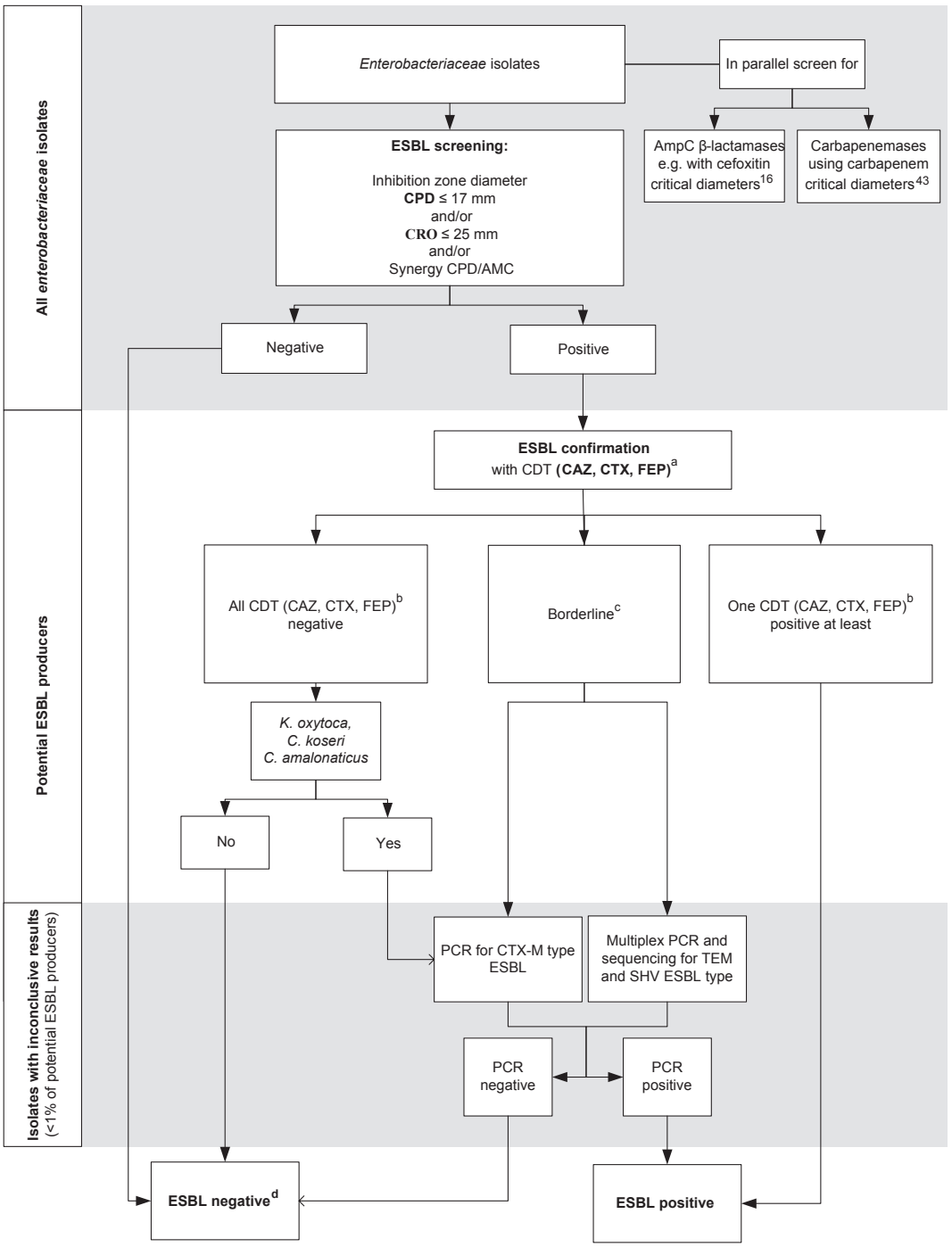

FIG. 1. Diagnostic approach combining screening and confirmation of ESBL production in *Enterobacteriaceae*. Proposed diagnostic algorithm for detection and confirmation of ESBL. CPD, cefpodoxime; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; AMC, amoxicillin-clavulanic acid; CDT, combined disk test. (a) For isolates with positive AmpC screening or species with known chromosomal AmpC, the use of Mueller-Hinton agar with cloxacillin is recommended. (b) For *Klebsiella oxytoca*, *Citrobacter koseri* and *Citrobacter amalonaticus* use CAZ for CDT confirmation testing only. (c) Results for two isolates in this study were considered borderline or inconclusive, because only one of the three CDT tests was marginally positive. For both isolates ESBL genes were not detected by PCR methods. No general recommendations can be deduced because only two isolates were considered borderline. The borderline category was created to allow clinical microbiologists to subject indifferent or questionable results to further analyses. (d) Isolates that are ESBL screening positive regarding critical diameters of third-generation cephalosporins, but negative with the CDT, should be checked for the presence of AmpC enzymes and/or carbapenemases.

combined three CDTs was observed when using cloxacillin containing agar (sensitivity 100% and 84.6% for Mueller-Hinton agar with and without cloxacillin, respectively; see Table 6). Overall specificity was equal for both media types (97.4%). Two of the 13 ESBL-positive isolates in AmpC producers would have been missed by all CDTs using conventional Mueller-Hinton agar, while CDTs on Mueller-Hinton agar with cloxacillin detected all 13 ESBLs.

Two isolates were considered as 'borderline results' with the CDT combination, because merely one CDT showed a diameter difference of ≤7 mm. In these two isolates TEM-, SHV- and CTX-M type ESBLs could not be detected by molecular methods (Table 1).

In total, 23 *K. oxytoca* isolates were analysed: 17 were putative ESBL producers, six served as a negative control group (Table 1). Of the 17 isolates positive in the ESBL screening, one isolate was correctly identified as SHV type ESBL by the ceftazidime, cefotaxime and cefepime CDTs. A second *K. oxytoca* isolate with a negative result in the ceftazidime CDT and positive results in the cefotaxime and cefepime CDTs was identified as CTX-M group 1 type ESBL by PCR (false-negative ceftazidime CDT). In a third *K. oxytoca* isolate with positive results in the cefotaxime and cefepime CDTs but a negative result in the ceftazidime CDT the presence of an ESBL could not be confirmed by molecular methods (false-positive cefotaxime and cefepime CDTs). The remaining 20 isolates showed congruent results between phenotypic and genotypic tests. All 23 *K. oxytoca* isolates would have been correctly assigned as ESBL positive (two isolates) and ESBL negative (21 isolates) by the proposed flow chart (see below).

Proposed flow chart for ESBL detection in *Enterobacteriaceae*

Evaluating the results of the various screening and confirmation assays we aimed to use this information to propose a flow chart for reliable and standardized ESBL detection in *Enterobacteriaceae*. The proposed procedure is based on: (i) cefpodoxime and ceftriaxone diameters combined with the clavulanic acid DDST as the primary screening markers; (ii) CDTs using ceftazidime, cefotaxime and cefepime combined as phenotypic confirmation tests; (iii) ceftazidime CDT for *K. oxytoca* in combination with a CTX-M type PCR added in case of a negative result; and (iv) molecular methods added in the case of borderline results with CDTs. For ESBL detection the sensitivity and specificity of the phenotypic algorithm alone would have been 100% and 97.5%, respectively, for the isolates investigated in this study (Fig. 1 and Table 5). Adding molecular methods (required for resolution of borderline phenotypic confirmation tests) would increase specificity to 100%. Extrapolated to the 2518 study isolates (both initial ESBL screening positives and negatives) the ESBL detection algorithm would have displayed a sensitivity, specificity, PPV and NPV each of 100% (see Table 4).

Discussion

Detection of ESBL is potentially critical to ensure adequate therapy and to guide epidemiological and hospital hygiene measures [13,20,21]. Laboratory methods for screening and confirmation of ESBL should be accurate, simple and rapid. During the past decades several phenotypic and molecular tests have been evaluated for ESBL detection [13,14,22], though a comprehensive algorithm is lacking. Molecular methods have been suggested as screening methods for ESBL [23–25]. However, in routine clinical diagnostic laboratories implementation of molecular methods is often hampered by the complexity of these assays, personnel needs and associated costs. Hence, an integrative approach combining phenotypic and molecular methods seems to be best suited for optimal and cost-efficient ESBL detection. This study aimed to develop an integrated phenotypic and molecular algorithm for the detection of ESBL production in *Enterobacteriaceae*. Automated phenotypic methods were not considered in our study because these methods: (i) reportedly show weaknesses in sensitivity and specificity; and (ii) include *E. coli* and *Klebsiella* spp. in their expert systems only [14,26–31].

Detection of ESBL is based on a combination of sensitive screening and confirmation assays. First, we evaluated the use of third-generation cephalosporin critical diameters and DDSTs as primary ESBL screening tests. Ceftriaxone critical diameters demonstrated highest sensitivity (98.4%) as a single substance (Table 5). One ESBL isolate, however, was detected exclusively by cefpodoxime. The combination of ceftriaxone and cefpodoxime was the most sensitive screening parameter (99.2%). Addition of cefotaxime as a third substance did not improve sensitivity, but resulted in a decrease of specificity (see Table 5). We do not consider ceftazidime as a primary screening marker on its own because sensitivity was low (72.6%) and no ESBL-producing isolate was exclusively detected by ceftazidime. This finding is most probably related to the higher hydrolytic activity of CTX-M type enzymes on cefotaxime compared with ceftazidime [32]. Sensitivity of ceftazidime in a population with an increased proportion of SHV-type ESBLs may possibly be higher. However, predominance of CTX-M type ESBLs in our study population closely resembles the epidemiological situation in Europe and North America [33–35]. Our results are in agreement with other studies, which showed: (i) cefpodox-

ime critical diameters to be highly sensitive for ESBL detection, but poorly specific; and (ii) combinations of third-generation cephalosporin critical diameters to result in a higher specificity [31,36]. Critical diameters of third-generation cephalosporins alone or in combination, however, were not able to detect all ESBLs, but combining critical diameters of cefpodoxime and ceftriaxone with DDST for cefpodoxime resulted in a sensitivity of 100% for ESBL screening (Table 5). Thus, a combination of three disks was required for initial and sensitive screening: cefpodoxime, ceftriaxone and amoxicillin-clavulanic acid. In particular, for AmpC β-lactamase-producing species, such as *E. cloacae*, our results parallel the data reported previously [14,22].

After determination of the most sensitive screening procedure, we evaluated the CDT as an inexpensive and simple phenotypic confirmation method. The Etest ESBL (bioMérieux) is widely used, but was not evaluated in this study for several reasons. The test: (i) shows low specificity for ESBL production in *Enterobacteriaceae* isolates other than *E. coli*; (ii) is significantly more expensive than the CDT; and (iii) was shown to be unreliable if used with isolates producing AmpC β-lactamases [14,37]. ESBLs may be missed by phenotypic confirmation tests if an AmpC enzyme is present. For example, in this study all false-negative results with the ceftazidime CDT occurred in *E. cloacae* isolates overexpressing AmpC [25]. Thus, two modifications of the CLSI recommended CDT were evaluated: (i) performing CDT for AmpC-producing isolates on Mueller-Hinton agar containing cloxacillin that suppresses AmpC activity; and (ii) addition of a cefepime CDT [38]. Our results for the CDT comparing Mueller-Hinton agar with and without cloxacillin show a significantly enhanced sensitivity for the cloxacillin-containing plates (see Table 6). Cefepime is poorly hydrolysed by AmpC enzymes. Addition of a cefepime CDT can, thus, improve the performance of the CLSI recommended cefotaxime and ceftazidime CDTs in the present epidemiological setting with ESBLs frequently present in species producing AmpC β-lactamases. None of the three CDTs applied alone resulted in a sensitivity of 100% (Table 5). In contrast, combining three CDT substances including cefepime proved to be highly sensitive and specific (sensitivity and specificity of 100% and 97.5%, respectively). If performance parameters were calculated for AmpC-producing isolates alone the cefepime CDT displayed sensitivity and specificity both of 100% using Mueller-Hinton agar with cloxacillin (Table 6). All false-negative results in AmpC-producing species listed in Table 6 were seen in *E. cloacae* isolates overexpressing AmpC using Mueller-Hinton agar without cloxacillin. Our results for the three CDTs are in agreement with data from other studies [14,39]. The ceftazidime CDT did not exclusively detect any ESBL-positive isolate. Omitting ceftazidime CDT as a confirmation test, however, cannot be recommended because ceftazidime is recommended for ESBL confirmation in *K. oxytoca* by EUCAST to avoid false-positive results with cefotaxime [40]. According to EUCAST rules, one ceftazidime CDT negative, cefotaxime and cefepime CDT positive *K. oxytoca* isolate overexpressing K1-β-lactamase was correctly identified as ESBL negative in our study. For *K. oxytoca* the ceftazidime CDT alone should, therefore, be considered as a phenotypic confirmation test to avoid false-positive results. If the ceftazidime CDT gives a negative result a CTX-M type PCR should be carried out for isolates screening positive for ESBL because a ceftazidime CDT negative isolate may have a CTX-M type ESBL (one isolate in this study, Table 1).

Combining three CDTs facilitated correct assignment of borderline CDT results to the ESBL positive or negative category. We considered results borderline or inconclusive if merely one of the three CDTs was marginally positive (Fig. 1) Usually, such isolates would be classified as ESBL positive because one positive CDT alone is considered indicative of ESBL [10]. The two isolates considered 'borderline' were subjected to molecular confirmation methods and were shown to be ESBL negative. To define and resolve 'borderline' criteria further studies with higher numbers of borderline CDT results are needed.

One possible concern is that the increasing prevalence of carbapenemases will interfere with this diagnostic algorithm. In the case of metallo-carbapenemase producers like NDM-1, CDTs with clavulanic acid would most likely give negative or inconclusive test results because those enzymes are characteristically not inhibited by clavulanic acid [41]. Carbapenemases of Ambler class A (e.g. *Klebsiella pneumoniae* carbapenemase, KPC) that are inhibited by clavulanic acid could produce false-positive CDTs. Isolates harbouring carbapenemases are, however, readily detected by the use of carbapenems as screening markers [42]. All 2518 isolates included in this study were categorized as susceptible to imipenem, meropenem and ertapenem according to CLSI 2009 guidelines [10]. All ESBL suspicious, but ESBL confirmation test negative, isolates in this study have been additionally checked for the presence of KPC by PCR and were found to be KPC negative (data not shown).

In summary, the proposed diagnostic flow chart (Fig. 1) would have resulted in a sensitivity and specificity of 100% for ESBL detection in the isolates tested. This algorithm is accurate, simple, easy to use, inexpensive and, therefore, applicable in the standard routine clinical microbiology laboratory. In the case of inconclusive results, which will rarely (<1%) occur, molecular methods are recommended. Such a diagnostic flow chart may be automatized in an open expert system as described by Winstanley *et al.* [43].

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Transparency Declarations

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