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Evaluation of a New Selective Chromogenic Agar Medium for Detection of Extended-Spectrum β-Lactamase-Producing *Enterobacteriaceae*▽

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A novel chromogenic agar medium (ESBL-Bx; bioMérieux, Marcy l'Etoile, France) was compared to MacConkey agar supplemented with 2 mg ceftazidime/liter (MCKC) for the selective isolation and presumptive identification of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* directly from clinical samples. Of a total of 644 clinical specimens (including 551 fecal samples), 496 yielded no growth and 148 yielded growth on one or both media. Overall, 44 ESBL-producing *Enterobacteriaceae* strains (*Escherichia coli* [*n* = 17], *Enterobacter aerogenes* [*n* = 17], *Klebsiella* spp. [*n* = 5], and *Citrobacter freundii* [*n* = 5]) were isolated from 37 specimens by a combination of both methods after 18 to 24 h of incubation. The sensitivities were 97.7 and 84.1% for ESBL-Bx and MCKC, respectively, with 43 ESBL-positive strains isolated as colored colonies from 36 specimens on ESBL-Bx versus 37 ESBL-positive organisms isolated from 32 specimens on MCKC. The specificities by specimens were 89 and 91% for ESBL-Bx and MCKC, respectively. On either one of the two media, natural AmpC-hyperproducing *Enterobacter* spp. (*n* = 25) and *Citrobacter* spp. (*n* = 14) were the most common false positives as well as non-ESBL-producing *Klebsiella oxytoca* (*n* = 18) on ESBL-Bx and *Morganella morganii* (*n* = 10) on MCKC. We conclude that ESBL-Bx is a sensitive and specific medium for the isolation of ESBL-producing *Enterobacteriaceae* from clinical samples. The main advantages of ESBL-Bx over MCKC reside in its chromogenic character and its sensitivity and selectivity, which enabled the recovery and presumptive identification of most ESBL-producing *Enterobacteriaceae* within 24 h and reduced by 27% the need for unnecessary identification and confirmation of ESBL testing when disregarding all colorless colonies growing on this medium.

Microbial resistance through extended-spectrum β-lactamase (ESBL) was first reported in the early 1980s in Europe and subsequently in the United States soon after the introduction of third-generation cephalosporins in clinical practice (12). Today, this resistance mechanism has emerged globally, and ESBL-producing *Enterobacteriaceae* are recognized worldwide as nosocomial pathogens of major importance (19, 28). Many clinical microbiology laboratories have problems with the detection of ESBL-mediated resistance, and the recent emergence and spread of novel types of community-acquired ESBLs, such as the CTX-M enzymes (2, 4), have created additional challenges that further complicate the detection of this resistance mechanism (1, 13). Several phenotypic tests have been recommended for screening and confirmation of ESBLs, but these are usually performed on isolated organisms following culture and antibiotic susceptibility testing (7, 24).

The failure to detect ESBL-mediated resistance has led to treatment failure (17, 26) and contributed to uncontrolled spread of ESBL-producing organisms (18). On the other hand, laboratory-based detection of patients infected or colonized by ESBL-producing organisms by surveillance cultures has proven useful to control and terminate nosocomial outbreaks (16, 19, 21).

Various selective media have been proposed to assess the carriage of ESBL producers in stools. Examples of such media include Drigalski agar supplemented with cefotaxime (27), MacConkey agar supplemented with ceftazidime (20), and nutrient agar supplemented with ceftazidime, vancomycin, and amphotericin B (21). In recent years, chromogenic media were initially developed for the detection and presumptive identification of urinary tract pathogens (6, 11) as well as for the improved isolation of *Staphylococcus aureus* from clinical specimens (5, 10). Recently, selective antibiotic-containing chromogenic media have been made available for the rapid detection of methicillin-resistant *S. aureus* (9, 23). One of the great advantages of such chromogenic selective media is that they allow the rapid and reliable screening of methicillin-resistant *S. aureus* colonization directly from contaminated clinical specimens (14).

The purpose of this study was to evaluate the sensitivity and specificity of a novel prototype of selective chromogenic agar medium (ESBL-Bx; bioMérieux, Marcy l'Etoile, France) that enables the detection and presumptive identification of ESBL-producing *Enterobacteriaceae* directly from clinical specimens.

MATERIALS AND METHODS

Specimens. A total of 644 clinical samples, including 561 stool, 63 lower respiratory tract (sputum, bronchial, or endotracheal aspirates), and 20 miscellaneous samples (wound swabs or ear-nose-throat specimens), were referred to our department for the screening of ESBL-producing organisms. The specimens originated from 460 patients who had been hospitalized in various wards (geriatric unit, general medicine, oncohematology, and general surgery departments) for more than 48 h.

Inoculation of media and incubation. Each specimen was homogenized in 1 ml of sterile physiological saline (0.85%), and 50-μl aliquots of the resulting suspension were inoculated on MacConkey agar (Oxoid, Basingstoke, United Kingdom) supplemented with 2 mg/liter ceftazidime (MCKC) and onto ESBL-Bx. In

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the first stage of the study, a subset of 365 clinical samples was also plated in parallel onto MacConkey agar in order to qualitatively assess the commensal flora as well as the selectivity of the two selective media (ESBL-Bx and MCKC). The chromogenic ESBL-Bx was obtained from the manufacturer as a prepared plate medium, and MCKC was prepared from a dehydrated medium according to the manufacturer's instructions. All media were incubated in air at 37°C for 18 to 24 h. For a subset of 279 specimens, the incubation of cultures was prolonged for 48 h.

Identification of ESBL-producing isolates. All culture plates were interpreted independently by two laboratory staff members. The density of growth was scored semiquantitatively (<10 colonies; +1; +2; +3; +4 according to the number of quadrants of the agar plates on which growth was observed). The type of coloration (pink-burgundy, blue-green, orange to brown) or the colorless aspect of each of the colonies growing on ESBL-Bx was also recorded. Any colored colonies on ESBL-Bx were regarded as presumptive ESBL producers. All types of colonies presenting different morphological aspects and growing on ESBL-Bx or on MCKC after 18 to 24 h were systematically subcultured onto Columbia blood agar (BD, Erembodegem, Belgium) and identified and tested for susceptibility by VITEK 2 using GN cards and AST-N020 cards (bioMérieux, Marcy l'Etoile, France). The confirmation of ESBL-producing isolates was performed by combined double disks (30-μg ceftazidime and 30-μg cefotaxime disks alone and the same antimicrobials with 10 μg clavulanic acid) according to CLSI guidelines (7). Genotypic characterization of resistance mechanisms was determined by isoelectric focusing and by PCR assays targeting bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA}, and bla_{ampC} genes with amplicon sequencing according to previously published methods (8, 22). Isolates were considered ESBL positive when the sequence of the bla_{TEM} and/or bla_{SHV} gene amplification products matched previously identified ESBLs (3) and/or when multiplex PCR was positive for $bla_{CTX\text{-}M}$-carrying genes belonging to the three major groups (CTX-M-1, CTX-M-2, and CTX-M-9) of this class of ESBL enzymes (2, 4). The constitutive overexpression of cephalosporinases was diagnosed by a multiplex PCR assay targeting known chromosomal- and plasmid-mediated AmpC β-lactamase genes according to previously published methods (22, 25). The evaluation of the performances of the two media (sensitivity and specificity), based on the total number of samples positive for ESBL-producing organisms as well as on total number of organisms detected, was assessed by statistical analysis using nonparametric McNemar and binomial tests or 95% confidence intervals (CI) for each performance.

TABLE 1. Identification and distribution of bacterial isolates recovered from 644 clinical samples on two different selective media

Group/species	No. of isolates recovered on:		
	ESBL-Bx	MCKC	Both media
Escherichia coli	28	24	34
KESC group[a]	79	59	87
Klebsiella oxytoca	20	4	20
Klebsiella pneumoniae	3	4	4
Enterobacter aerogenes	26	20	27
Enterobacter cloacae	14	12	15
Citrobacter freundii	14	17	18
Citrobacter koseri	1	0	1
Hafnia alvei	1	2	2
PMP group[b]	4	10	14
Morganella morganii	2	10	12
Proteus vulgaris	2	0	2
Total no. of *Enterobacteriaceae*	111	93	135
Others	41	30	45
Pseudomonas aeruginosa	27	21	31
Stenotrophomonas maltophilia	12	8	12
Acinetobacter baumannii	1	1	1
Enterococcus spp.[c]	1	0	1

[a] KESC, *Klebsiella-Enterobacter-Serratia-Citrobacter* group (also includes *Hafnia alvei*).
[b] PMP, *Proteus-Morganella-Providencia* group.
[c] Glycopeptide-resistant *Enterococcus faecium* (*vanA*).

RESULTS

Four hundred ninety-six specimens (77%) yielded no growth on any media, and 148 (23%) yielded growth on at least one of the selective media after 18 to 24 h of incubation. The growth of various gram-negative organisms was observed on MacConkey agar without antibiotic for 283 out of 365 specimens (77.5%) that were found culture negative on ESBL-Bx and on MCKC, hence demonstrating the good inhibitory activity of these two selective media against the commensal flora.

Overall, 135 *Enterobacteriaceae* isolates were recovered on 112 specimens (17.4%) by at least one of the two media; 111 of them were detected from 96 samples on ESBL-Bx versus 93 from 83 samples on MCKC. *Enterobacter* spp. ($n = 42$), *Escherichia coli* ($n = 34$), and *Klebsiella* spp. ($n = 24$) were the predominant isolates in this study. *Klebsiella* spp., *Enterobacter* spp., *Serratia marcescens* (KES group) and *Citrobacter* spp. isolates were more frequently isolated on ESBL-Bx medium than on MCKC, while *Proteus* spp. and *Morganella* spp. (PMP group) were more often recovered on MCKC (Table 1). All *Enterobacteriaceae* isolates recovered on the chromogenic ESBL-Bx medium produced the expected colony colors (blue-green with or without purple halo for the KES group and *Citrobacter* spp., pink-burgundy for *E. coli*, and beige to brown with a diffusible orange halo for the PMP group). Thirty-seven (33.0%) of the 112 specimens which grew *Enterobacteriaceae* isolates yielded one or several ESBL-producing strains. ESBL-producing organisms were detected on ESBL-Bx in 36 specimens (sensitivity, 97.3%) and on MCKC in 32 specimens (sensitivity, 86.5%) ($P = 0.09$; not significant). Of the ESBL-positive organisms detected, 43 out of 44 were recovered on ESBL-Bx (sensitivity, 97.7%) and 37 were recovered on MCKC (sensitivity, 84.1%) ($P = 0.03$). Two or three different ESBL-producing isolates were simultaneously detected from six specimens on ESBL-Bx and from four on MCKC.

Overall, TEM-derived enzymes were the most frequently encountered ESBLs (in 33 isolates), especially TEM-24, which was present in all *Enterobacter aerogenes* strains but also in several other *Enterobacteriaceae* species (Table 2). Various CTX-M enzymes (either alone or in association with other enzymes) were found in 10 isolates, predominantly in *E. coli* (7 isolates), while SHV-derived ESBLs were the least commonly found (4 isolates) (Table 2). There were no marked differences in the recovery rates on the two media according to the types of ESBL enzymes produced by the organisms; however, three out of four *E. coli* isolates detected solely on ESBL-Bx medium produced a CTX-M-type ESBL.

A comparison of the density of growth showed identical scores on both media for 19 ESBL-producing isolates, while a higher number of colonies was found on ESBL-Bx for 14 isolates and on MCKC for 11 isolates. Of the seven isolates that grew on only ESBL-Bx (four *E. coli* and three *E. aerogenes* isolates), five were detected in small numbers, with less than 10 colonies in three cases and growth in the first quadrant of the plate in two isolates. Likewise, the single ESBL-producing isolate (*E. aerogenes*) that was detected by MCKC grew only two colonies on the plate. The prolongation of incubation did not increase the sensitivity of detection of ESBL-producing organisms on either of the two media and only marginally

TABLE 2. Characterization of ESBLs from *Enterobacteriaceae* isolates growing on either of the two selective media

Species (no. of isolates)	Classes of ESBL enzymes (no. of isolates)[a]		
	TEM	SHV	CTX-M
E. aerogenes (17)	TEM-24 (17)		
E. coli (17)	TEM-52 (5) TEM-24 (4) TEM-3 (1)	SHV-4* (1)	CTX-M-1 (1) CTX-M-2 (1) CTX-M-3 (1) CTX-M-5 (1) CTX-M-9* (1) CTX-M-15 (2)
C. freundii (5)	TEM 3 (2) TEM-24 (1)	SHV-4* (2)	CTX-M-9* (2)
K. pneumoniae (3)	TEM-24 (1)	SHV-5 (1)	CTX-M-9 (1)
K. oxytoca (2)	TEM-3 (1) TEM-24 (1)		

[a] Two ESBLs (CTX-M-9 and SHV-4) were present in three isolates (one *E. coli* and two *C. freundi* isolates) (represented by asterisks). The total numbers of TEM, SHV, and CTX-M isolates were 33, 4, and 10, respectively.

TABLE 3. Mechanisms of resistance of *Enterobacteriaceae* isolates growing on the two selective media

Bacterial species (no. of isolates)	Frequency of indicated resistance mechanisms on:					
	ESBL-Bx			MCKC		
	ESBL	Cephalosporinase	Others	ESBL	Cephalosporinase	Others
Escherichia coli (34)	17	5	6[a]	13	11	
Citrobacter freundii (17)	5	9		5	12	
Citrobacter koseri (1)			1			
Enterobacter cloacae (15)		14			12	
Enterobacter aerogenes (27)	16	10		14	6	
Hafnia alvei (2)		1			2	
Klebsiella oxytoca (20)	2		18[b]	2		2[b]
Klebsiella pneumoniae (4)	3			3		1[c]
Morganella morganii (10)		2			10	
Proteus vulgaris (2)			2			
Total	43	41	27	37	53	3

[a] Five isolates produced a TEM-derived penicillinase (PCR positive for the bla_{TEM} gene); four isolates had pIs of 5.4 and were susceptible to clavulanate (TEM-1); one isolate had a pI of 5.2 and was resistant to clavulanate (IRT-2); one *E. coli* isolate was PCR positive for the bla_{OXA} gene, with a pI of 6.3 (OXA-1).

[b] All isolates found as K-OXY-1 or K-OXY-2 penicillinase hyperproducers (pIs were 5.2 and 6.8 [OXY-2] and 7.1, 7.5, and 8.2 [OXY-1]).

[c] PCR positive for the bla_{SHV} gene; pI was 7.6 (SHV-1).

increased the growth of other gram-negative isolates, but it increased the turnaround time to diagnosis since colonies suspected to be ESBL organisms had to be subcultured and reisolated more often after 48 h of incubation.

The specificity assessed on 607 specimens that were found negative for ESBL-producing isolates by both media was 90.4% on ESBL-Bx (95% CI, 87.8 to 92.6%) and 91.8% on MCKC (95% CI, 89.3 to 93.7%) ($P = 0.28$). A total of 58 and of 50 specimens yielding the growth of non-ESBL-producing organisms presenting the typical colonial appearance and chromogenic character of *Enterobacteriaceae* species were found on ESBL-Bx and on MCKC, respectively. When considering the total number of bacterial isolates growing on the two selective media, the specificity was 50% (95% CI, 41.6 to 58.5%) on ESBL-Bx (68 out of 136 true negatives) versus 58.1% (95% CI, 49.5 to 66.2%) on MCKC (79 out of 126 true negatives) ($P = 0.18$; not significant). There was no difference in selectivity between the two media regarding the density of growth of the associated flora (data not shown).

Natural AmpC-hyperproducing *Enterobacteriaceae* isolates, in particular *Enterobacter* spp. ($n = 25$) and *Citrobacter* spp. ($n = 14$), accounted for most of the false-positive results on both media; non-ESBL-producing *E. coli* isolates overexpressing AmpC or with other resistance mechanisms were detected at similar frequencies on MCKC and on ESBL-Bx (Table 3). On the other hand, *Klebsiella oxytoca* hyperproducers of chromosomal K-1 penicillinase ($n = 18$) were recovered mostly on ESBL-Bx, while *Morganella morganii* isolates ($n = 10$) were predominantly found on MCKC. Besides, 44 non-*Enterobacteriaceae* isolates, mainly *Pseudomonas aeruginosa* ($n = 30$) and *Stenotrophomonas maltophilia* ($n = 12$), grew on 37 specimens on either of the two media.

All 40 non-*Enterobacteriaceae* isolates growing on ESBL-Bx produced colorless colonies and could rapidly be discarded without any further testing, while 30 nonfermenter isolates recovered on the MCKC plates produced lactose-negative colonies and had to be subcultured for further confirmation tests.

DISCUSSION

The detection of ESBL-mediated resistance in gram-negative bacilli is of paramount importance because of its clinical implications and the limited therapeutic options (19, 26, 28). Laboratory-based detection of patients infected or colonized with ESBL-producing organisms by active surveillance cultures is also important because it has proven useful to control and terminate prolonged nosocomial outbreaks (16, 19, 21, 29). Asymptomatic gastrointestinal tract colonization with ESBL producers without signs of overt infection may frequently occur in endemic or hyperendemic settings in hospitals, and such patients may represent an important reservoir of these organisms (20, 27). Moreover, patients who develop infections with ESBL-producing *Enterobacteriaceae* have been frequently documented as having prior gastrointestinal carriage with such organisms (15, 21). Methods to detect ESBL-producing organisms from clinical specimens should have high sensitivity and high specificity combined with a short time to the reporting of results. In order to identify ESBL-producing gram-negative bacilli from contaminated samples more easily and reliably, selective media should ideally achieve the identification of the organisms and detection of ESBL in one step. At the least, it should decrease the workload and reduce the need of unnecessary confirmations. Several selective media, such as MacConkey (20, 30) or Drigalski agar (16), have been proposed for the detection of fecal carriage of ESBLs. Most of the time, the media were homemade, freshly prepared, and supplemented with one or several antimicrobials, including cefotaxime and/or ceftazidime, at various concentrations in order to increase the selectivity and limit the growth of competing associated flora. As yet, no selective, ready-to-use commercial medium was available, and the absence of standardized screening media can make the process of isolating specific multidrug-resistant, gram-negative bacilli a relatively labor-intensive process. The prototype ESBL-Bx evaluated here is a novel chromogenic

selective medium intended for the screening of the carriage of ESBL-producing *Enterobacteriaceae*.

In this study, 97% of the ESBL-producing isolates were recovered by ESBL-Bx after 18 to 24 h of incubation from clinical specimens, mostly from fecal samples. Since the prolongation of incubation for 48 h was not found to increase the recovery rates of ESBL-positive organisms, but rather rendered the recovery sometimes more complex because of the growth of associated flora, it was decided to record only definitive results after 18 to 24 h.

On the whole, ESBL-Bx medium proved more sensitive than our conventional, homemade MCKC medium consisting of MacConkey medium supplemented with 2 mg/liter ceftazidime, though the difference did not reach statistical significance in this study.

It is difficult to speculate on the reasons for the higher recovery rates of ESBL producers on ESBL-Bx medium, as all the ingredients of the selective antibiotic supplement of this medium are not yet fully disclosed. Although the better performance of the ESBL-Bx agar could indeed relate to the choice and concentration of antibiotics in the medium, it could also be due to the fact that colonies, rather than the surrounding medium, are colored on chromogenic agar, allowing easier detection of the target organisms, even in mixed cultures and when present at low colony counts. In comparison to MCKC, ESBL-Bx plates yielded final results 18 to 24 h earlier in 6 of 36 (17%) specimens which grew ESBL-positive isolates on both media. Moreover, it would have allowed us to reduce the number of unnecessary confirmations by 28% (in 25 of 91 specimens which yielded growth on both media) since colorless colonies growing on ESBL-Bx were never found to be *Enterobacteriaceae* when subsequently identified to the species level.

The inclusion of cefpodoxime, rather than cefotaxime or ceftazidime, in ESBL-Bx may possibly explain the higher sensitivity of this medium over that of MCKC. Cefpodoxime has indeed been shown as the best general substrate to screen for all types of ESBLs presently found in clinical specimens (13), and ceftazidime alone can no longer be recommended with the advent of CTX-M ESBL enzymes which are usually susceptible to this antimicrobial agent (2, 3). Hence, the inclusion of ceftazidime as a selective agent in our routine MacConkey medium probably explains the low detection rate of CTX-M-type ESBL-producing isolates on MCKC in comparison to that on ESBL-Bx.

On the whole, 7% of the patients screened were found to be colonized or infected with ESBL-producing *Enterobacteriaceae* isolates. Although this figure may not reflect the actual prevalence of ESBL-producing isolates at our hospital due to some degree of selection in screening regarding the origin of specimens and localization of patients in different wards, the distribution of ESBL-producing organisms is similar to that found in routine clinical specimens in our institution (data not shown). *Enterobacter aerogenes* and *E. coli* each accounted for about 40% of all ESBL-producing isolates, while miscellaneous other *Enterobacteriaceae* species were found in a minor proportion.

Ten of 33 patients found to be colonized at one or several sites were already known as colonized or infected by ESBL-producing isolates. Interestingly, however, the screening of specimens on ESBL-Bx medium allowed the successful isolation of ESBL-producing isolates in 23 patients who were not previously known as being colonized. In five of these, the detection of ESBL in stool samples preceded the isolation of the same organism by 14 days to >3 months from the routine clinical specimens, while in two other patients, the detection of an ESBL producer in the screening samples was made at the same time as its isolation from routine specimens.

Concerning the specificity of the two media, one inherent difficulty resides in their inability to differentiate the presence of ESBL enzymes from some other resistance mechanisms, in particular, plasmid-mediated or chromosomally mediated AmpC hyperproduction. *Enterobacter* spp. and *Citrobacter* spp. accounted for the most frequent causes of false positives on both media. The presence of blue colonies of the KES group subsequently identified as *K. oxytoca* was also frequently recorded on ESBL-Bx (but not on MCKC). Since most of these isolates were chromosomal penicillinase hyperproducers rather than ESBL-positive organisms, it could be valuable to optimize the chromogenic substrates in order to improve the recognition of *K. oxytoca* isolates on this medium. Alternatively, the direct detection of indole production by green-blue-pigmented colonies may allow a rapid presumptive identification of *K. oxytoca* isolates among organisms belonging to the KES group.

Besides *Enterobacteriaceae* isolates, nonfermentative gram-negative bacilli, mainly *P. aeruginosa* and *S. maltophilia*, were occasionally recovered on both selective media. Since these isolates grew as colorless colonies on ESBL-Bx medium and did not spread on the agar, they did not interfere with the detection of ESBLs when they were present in mixed flora. Also, the nonfermenter isolates were easily recognized on the chromogenic medium by the pattern of the colonies and/or by rapid presumptive identification tests which could be performed directly on the colonies (i.e., positive oxidase character of *P. aeruginosa* strains). On MCKC, however, nonfermentative gram-negative bacilli presented as lactose-negative colonies and had to first be subcultured and identified by other biochemical reactions to exclude the presence of an ESBL-producing *Enterobacteriaceae* isolate.

As mentioned above, there is growing interest for the detection of ESBL to identify colonized patients and limit the spread of these organisms within hospitals.

As shown here, almost 80% of the specimens yielded growth of one or several gram-negative isolates on MacConkey agar. In comparison, selective media supplemented with antibiotics inhibited the growth of the competing associated flora in more than 75% of all tested samples, hence significantly reducing the workload for identification and susceptibility testing of the isolates. ESBL-Bx agar is one of the first ready-to-use chromogenic selective media specifically designed for the detection of multidrug-resistant, gram-negative bacilli from clinical specimens. This medium offers good selectivity and exploits the advantages of the chromogenic media, allowing easy discrimination of the different colonies by color, especially in specimens containing a resident associated flora. Our results show that ESBL-Bx appears as an excellent medium for the screening and presumptive identification of ESBL-producing *Enterobacteriaceae* directly from clinical samples and that it compares well with other conventional media used for this purpose. Further studies with a wider range of clinical specimens are required to confirm the utility of this medium also taking into

account the possible occurrence of wide variations in the epidemiology of ESBL-producing species between countries.

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