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Rapid Detection of β-Lactamase-Hydrolyzing Extended-Spectrum Cephalosporins in *Enterobacteriaceae* by Use of the New Chromogenic βLacta Test

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The chromogenic βLacta test developed for the rapid detection of β-lactamase-hydrolyzing extended-spectrum cephalosporins in *Enterobacteriaceae* revealed good performance with extended-spectrum β-lactamase (ESBL) producers (97.5% true-positive results). However, false-negative results occurred with chromosomal AmpC hyperproducers and plasmid AmpC producers, whereas uninterpretable results were mostly due to VIM-1 carbapenemase producers and possibly low levels of expressed ESBLs.

Detection of *Enterobacteriaceae* resistant to broad-spectrum cephalosporins, mostly due to production of extended-spectrum β-lactamases (ESBL), plasmid AmpC β-lactamases, and/or carbapenemases, has become a challenge in clinical microbiology laboratories because of important clinical consequences for infection control purposes and guidance of antimicrobial therapy (1–3). Methods routinely used to detect these organisms are primarily based on susceptibility testing results, either MICs or disk diffusion inhibition zones, as well as on ancillary testing using disk synergy tests with different β-lactamase inhibitors or MIC-gradient strips combining β-lactams and β-lactamase inhibitors (4). Molecular methods based on PCR or microarray hybridization techniques have been also developed (5, 6). In addition, mass spectrometry-based protocols using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and in-house colorimetric tests have been developed to detect the production of ESBLs in less than 4 h (7, 8). Increased interest in rapid colorimetric assays has been observed, because of their easy implementation in the routine workflow of clinical laboratories (8).

The βLacta test (Bio-Rad, Marnes la Coquette, France) is a new chromogenic method based on the use of a yellow substrate (HMRZ-86) that turns to red when hydrolyzed by ESBLs, AmpC β-lactamases, and most carbapenemases (9–11). According to the manufacturer, reading of the results can be performed visually in less than 15 min. In the present study, we assessed the performance of the βLacta test for rapid detection of β-lactamase-hydrolyzing extended-spectrum cephalosporins in two groups of *Enterobacteriaceae* clinical isolates. The first group (Table 1) consisted of 338 contemporary clinical isolates collected prospectively (in January to March 2012), and the second group (Table 2) included 106 clinical isolates with β-lactamase-mediated resistance mechanisms that were characterized at the molecular level and affected broad-spectrum cephalosporins (12–15). All isolates were recovered at the Ramón y Cajal University Hospital and were identified using both a MicroScan system (Siemens, West Sacramento, CA) and MALDI-TOF MS (Bruker Daltonics, Germany). In addition, the *Escherichia coli* ATCC 35218 strain (TEM-1 producer) was used as a negative control, whereas the *Klebsiella pneumoniae* ATCC 700603 strain (SHV-18 producer) was used as a positive control. Susceptibility testing of β-lactam antibiotics, including broad-spectrum cephalosporins (cefotaxime, ceftazidime, and cefepime) and carbapenems (imipenem and ertapenem), was performed using the MicroScan system (Siemens). EUCAST breakpoint criteria were used to define susceptible, intermediate, and resistant categories (16). β-Lactam MICs as well as in-house and commercial (Rosco-Diagnostica A/S, Taastrup, Denmark) ancillary test results (double-disk diffusion synergy techniques) using β-lactamase inhibitors (clavulanic acid, EDTA, dipicolinic acid, cloxacillin, and boronic acid) were used to infer phenotypes and resistance mechanisms affecting broad-spectrum cephalosporins (17). Isolates were classified according to these results.

Molecular characterization of *bla* genes was performed as described previously (12–15), for both contemporary clinical isolates (first group) with discrepant results in the βLacta test and isolates with well-characterized resistance mechanisms (second group). The βLacta test was performed according to the manufacturer's instructions. Briefly, several colonies from 18- to 20-h cultures were picked from blood agar medium, to yield a full 1-μl loop, and then were mixed with the βLacta test reagents (R1 and R2, one drop each) in plastic microtubes. The mixtures were left at room temperature, and color changes, when present, were read and interpreted after 2 and 15 min by following the manufacturer's instructions. Color changes were interpreted as follows: (i) red or purple, positive; (ii) no change (yellow), negative; (iii) orange, uninterpretable. The accuracy of the βLacta test was determined by using susceptibility test results and considering either

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TABLE 1 Contemporary clinical *Enterobacteriaceae* isolates ($n = 338$)

Microorganism (no. of isolates)	Phenotype or β-lactamase	No. with βLACTA test result of:		
		Positive	Negative	Uninterpretable
Escherichia coli (231)	Wild type		65	1
	Penicillinase production		77	
	Penicillinase hyperproduction		31	
	ESBL[a]	50		3[b]
	ESBL + plasmid AmpC	1		
	Plasmid AmpC		3[c]	
Klebsiella spp. (67)	Wild type (penicillinase)		41	
	Penicillinase hyperproduction		6	
	ESBL	18		
	ESBL + plasmid AmpC	1		
	Carbapenemase			1[d]
Proteus mirabilis (12)	Wild type		7	
	Penicillinase		5	
Enterobacter spp. (13)	Wild-type (inducible AmpC)		11	
	AmpC hyperproduction		1[c]	
	ESBL	1		
Serratia marcescens (6)	Wild type (inducible AmpC)		3	
	AmpC hyperproduction	3		
Morganella morganii (4)	Wild type (inducible AmpC)		2	
	AmpC hyperproduction		1[c]	
	ESBL	1		
Citrobacter freundii (2)	Wild type (inducible AmpC)		1	
	AmpC hyperproduction	1		
Providencia stuartii (3)	Wild type (inducible AmpC)		2	
	AmpC hyperproduction		1[c]	
Total		76	257	5

[a] ESBL, extended-spectrum β-lactamase.
[b] SHV-12 ($n = 2$) and SHV-2 ($n = 1$).
[c] False-negative results.
[d] VIM-1 producer.

a resistance or intermediate result for ceftazidime, cefotaxime, or cefepime as the reference value. Sensitivity, specificity, and likelihood ratios were determined. Confidence intervals (CIs) were estimated by Taylor's method.

Considering all routine clinical isolates and excluding isolates with uninterpretable results (Table 1), 96.7% (327/338 isolates) gave expected results (251 isolates yielded true-negative results and 76 true-positive results), in accordance with the inferred phenotype. However, six isolates (1.8%) gave unexpected negative (false-negative) results, including 3 *E. coli* isolates expressing CMY-2 plasmid AmpC β-lactamase and one isolate each of *Enterobacter cloacae*, *Providencia stuartii*, and *Morganella morganii* expressing a hyperproduced AmpC phenotype (see Table S1 in the supplemental material). Moreover, 5 isolates (1.5%) had uninterpretable results, including one β-lactam-susceptible *E. coli* isolate, three ESBL-producing *E. coli* isolates expressing either SHV-2 ($n = 1$) or SHV-12 ($n = 2$), and one VIM-1-producing *K. pneumoniae* isolate. The MIC values, resistance phenotypes, and β-lactamase types of these strains are included in Table S2 in the supplemental material. Interpretive βLacta test results for the routine clinical isolates, considering both the resistance phenotype and the resistance mechanism as gold standards, were as follows: sensitivity, 92.7% (95% CI, 88.8 to 97.3%); specificity, 100% (95% CI, 98.5 to 100.0%); positive predictive value, 100% (95% CI, 95.3 to 100.0%); negative predictive value, 97.7% (95% CI, 94.9 to 99.2%). The negative likelihood ratio was 0.07.

These results prompted us to investigate the performance of the βLacta test with a collection of isolates with well-characterized resistance mechanisms. Within this collection, including isolates expressing ESBLs, plasmid AmpC β-lactamases, or carbapenemases (Table 2), 97.03% (98/101 isolates) yielded expected results (0 true-negative results and 98 true-positive results), 5 isolates yielded uninterpretable results (3 *E. coli* isolates and 2 *K. pneumoniae* isolates expressing VIM-1), and 3 isolates yielded false-negative results (3 CMY-2-producing *E. coli* isolates).

These results confirmed that the βLacta test is useful for the detection of ESBL-producing organisms (97.5% of all ESBL producers demonstrated true-positive results) but not AmpC producers (either plasmid-mediated or chromosomally mediated). This was also noted in a multicenter evaluation performed in

TABLE 2 *Enterobacteriaceae* isolates ($n = 106$) with characterized β-lactamases

Microorganism (no. of isolates)	Phenotype or β-lactamase	No. with βLACTA test result of:		
		Positive	Negative	Uninterpretable
Escherichia coli (20)	ESBL[a] (1 CTX-M-9, 4 CTX-M-14, 5 CTX-M-15)	10		
	KPC (1 KPC-1, 1 KPC-2)	2		
	VIM-1			3
	VIM-1 + SHV-12	1		
	CMY-2		3[b]	
	OXA-48 + VIM-1 + CTX-M-15	1		
Klebsiella spp. (65)	ESBL (2 TEM-4, 8 SHV-12, 1 CTX-M-9, 2 CTX-M-14, 3 CTX-M-10, 6 CTX-M-15)	22		
	KPC (6 KPC-2, 13 KPC-3)	19		
	KPC-3 + VIM-1	3		
	VIM-1	7		2
	VIM-1 + SHV-12 + TEM-1	2		
	OXA-48	3		
	OXA-48 + CTX-M-15	3		
	OXA-48 + VIM-1 + CTX-M-15	4		
Enterobacter spp. (18)	ESBL (3 CTX-M-1, 1 SHV-2)	4		
	KPC (1 KPC-2, 1 KPC-3)	2		
	VIM-1	10		
	OXA-48	1		
	OXA-48 + CTX-M-15	1		
Serratia marcescens (1)	VIM-1	1		
Citrobacter freundii (2)	VIM-1	2		
Total		98	3	5

[a] ESBL, extended-spectrum β-lactamase.
[b] False-negative results.

French and Belgian hospitals, in which poor performance of the βLacta test with AmpC producers was observed (11). Unlike in our study, none of ESBL producers in the multicenter evaluation gave negative results, a situation that occurred with 3 *E. coli* isolates (two expressing SHV-12 and one expressing SHV-2) in our work. Extended-spectrum cephalosporins were variably affected, with MICs ranging from ≤1 to >16 mg/liter (see Table S2 in the supplemental material). Variable expression of ESBLs was described several years ago, particularly with SHV variants, affecting the hydrolysis of cephalosporins and MIC values (18). This could also eventually affect the hydrolysis of HMRZ-86, a fact that has been shown with nitrocefin and *in vitro* variants obtained by mutagenesis of bla_{SHV-1} (19).

On the other hand, results for carbapenemase producers ($n = 67$) were mainly positive (92.5% [62/67 isolates]), including results for producers of OXA-48, an enzyme with minor hydrolytic activity against extended-spectrum cephalosporins (20). Uninterpretable results (7.5% [5/67 isolates]) were specifically associated with VIM-1 producers. The variable expression of resistance phenotypes in these isolates might affect extended-spectrum and carbapenem MICs, as we demonstrated previously (13).

The isolates with unexpected negative (yellow) or uninterpretable (orange) results were tested under different conditions in order to enhance enzymatic activity to favor a positive result (red). For this purpose, and to enhance β-lactamase release, a bacterial suspension of each tested isolate was frozen and thawed twice and then incubated with the R1 and R2 reagents at room temperature and at 37°C, in separate tubes. Cells disrupted by sonication were also tested. In all cases, the results remained unchanged (data not shown).

In summary, the chromogenic βLacta test was demonstrated to be a rapid and reliable assay for the detection of ESBL-producing *Enterobacteriaceae* and presumably KPC and class D (OXA-48) carbapenemase producers. However, results were negative for AmpC β-lactamase producers, including both chromosomal AmpC hyperproducers and plasmid AmpC producers. Moreover, the percentage of uninterpretable results was low, mostly due to VIM-1 carbapenemases and possibly low levels of expressed ESBLs. Use of the chromogenic βLacta test might provide useful early guidance for detection of ESBLs in *Enterobacteriaceae* in routine clinical microbiology laboratories.

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REFERENCES

1. **Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O.** 2013. Antibiotic resistance: the need for global solutions. Lancet Infect. Dis. **13:**1057–1098. http://dx.doi.org/10.1016/S1473-3099(13)70318-9.
2. **Rice LB.** 2009. The clinical consequences of antimicrobial resistance. Curr. Opin. Microbiol. **12:**476–481. http://dx.doi.org/10.1016/j.mib.2009.08.001.
3. **Livermore DM, Andrews JM, Hawkey PM, Ho PL, Keness Y, Doi Y, Paterson D, Woodford N.** 2012. Are susceptibility tests enough, or should laboratories still seek ESBLs and carbapenemases directly? J. Antimicrob. Chemother. **67:**1569-1577. http://dx.doi.org/10.1093/jac/dks088.
4. **Thomson KS.** 2010. Extended-spectrum-β-lactamase, AmpC, and carbapenemase issues. J. Clin. Microbiol. **48:**1019–1025. http://dx.doi.org/10.1128/JCM.00219-10.
5. **Naas T, Cuzon G, Truong H, Bernabeu S, Nordmann P.** 2010. Evaluation of a DNA microarray, the Check-Points ESBL/KPC array, for rapid detection of TEM, SHV, and CTX-M extended-spectrum β-lactamases and KPC carbapenemases. Antimicrob. Agents Chemother. **54:**3086–3092. http://dx.doi.org/10.1128/AAC.01298-09.
6. **Ellem J, Partridge SR, Iredell JR.** 2011. Efficient direct extended-spectrum β-lactamase detection by multiplex real-time PCR: accurate assignment of phenotype by use of a limited set of genetic markers. J. Clin. Microbiol. **49:**3074–3077. http://dx.doi.org/10.1128/JCM.02647-10.
7. **Sparbier K, Schubert S, Weller U, Boogen C, Kostrzewa M.** 2012. Matrix-assisted laser desorption ionization–time of flight mass spectrometry-based functional assay for rapid detection of resistance against β-lactam antibiotics. J. Clin. Microbiol. **50:**927–937. http://dx.doi.org/10.1128/JCM.05737-11.
8. **Nordmann P, Dortet L, Poirel L.** 2012. Rapid detection of extended-spectrum-β-lactamase-producing *Enterobacteriaceae*. J. Clin. Microbiol. **50:**3016–3022. http://dx.doi.org/10.1128/JCM.00859-12.
9. **Hanaki H, Kubo R, Nakano T, Kurihara M, Sunagawa K.** 2004. Characterization of HMRZ-86: a novel chromogenic cephalosporin for the detection of extended-spectrum β-lactamases. J. Antimicrob. Chemother. **53:**888–889. http://dx.doi.org/10.1093/jac/dkh166.
10. **Hanaki H, Koide Y, Yamazaki H, Kubo R, Nakano T, Atsuda K, Sunakawa K.** 2007. Substrate specificity of HMRZ-86 for β-lactamases, including extended-spectrum β-lactamases (ESBLs). J. Infect. Chemother. **13:**390–395. http://dx.doi.org/10.1007/s10156-007-0563-2.
11. **Renvoisé A, Decré D, Amarsy-Guerle R, Huang TD, Jost C, Podglajen I, Raskine L, Genel N, Bogaerts P, Jarlier V, Arlet G.** 2013. Evaluation of the βLacta test, a rapid test detecting resistance to third-generation cephalosporins in clinical strains of *Enterobacteriaceae*. J. Clin. Microbiol. **51:**4012–4017. http://dx.doi.org/10.1128/JCM.01936-13.
12. **Morosini MI, García-Castillo M, Coque TM, Valverde A, Novais A, Loza E, Baquero F, Cantón R.** 2006. Antibiotic coresistance in extended-spectrum-β-lactamase-producing *Enterobacteriaceae* and in vitro activity of tigecycline. Antimicrob. Agents Chemother. **50:**2695–2699. http://dx.doi.org/10.1128/AAC.00155-06.
13. **Tato M, Coque TM, Ruíz-Garbajosa P, Pintado V, Cobo J, Sader HS, Jones RN, Baquero F, Cantón R.** 2007. Complex clonal and plasmid epidemiology in the first outbreak of *Enterobacteriaceae* infection involving VIM-1 metallo-β-lactamase in Spain: toward endemicity? Clin. Infect. Dis. **45:**1171–1178. http://dx.doi.org/10.1086/522288.
14. **Curiao T, Morosini MI, Ruiz-Garbajosa P, Robustillo A, Baquero F, Coque TM, Cantón R.** 2010. Emergence of bla_{KPC-3}-Tn*4401a* associated with a pKPN3/4-like plasmid within ST384 and ST388 *Klebsiella pneumoniae* clones in Spain. J. Antimicrob. Chemother. **65:**1608–1614. http://dx.doi.org/10.1093/jac/dkq174.
15. **Ruiz-Garbajosa P, Curiao T, Tato M, Gijón D, Pintado V, Valverde A, Baquero F, Morosini MI, Coque TM, Cantón R.** 2013. Multiclonal dispersal of KPC genes following the emergence of non-ST258 KPC-producing *Klebsiella pneumoniae* clones in Madrid, Spain. J. Antimicrob. Chemother. **68:**2487–2492. http://dx.doi.org/10.1093/jac/dkt237.
16. **European Committee on Antimicrobial Susceptibility Testing.** 2013. Breakpoint tables for interpretation of MICs and zone diameters, version 3.1. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf.
17. **Giske CG, Gezelius L, Samuelsen Ø, Warner M, Sundsfjord A, Woodford N.** 2011. A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin. Microbiol. Infect. **17:**552–556. http://dx.doi.org/10.1111/j.1469-0691.2010.03294.x.
18. **Bush K.** 1989. Excitement in the β-lactamase arena. J. Antimicrob. Chemother. **24:**831–836. http://dx.doi.org/10.1093/jac/24.6.831.
19. **Li M, Conklin BC, Taracila MA, Hutton RA, Skalweit MJ.** 2012. Substitutions at position 105 in SHV family β-lactamases decrease catalytic efficiency and cause inhibitor resistance. Antimicrob. Agents Chemother. **56:**5678–5686. http://dx.doi.org/10.1128/AAC.00711-12.
20. **Poirel L, Potron A, Nordmann P.** 2012. OXA-48-like carbapenemases: the phantom menace. J. Antimicrob. Chemother. **67:**1597–1606. http://dx.doi.org/10.1093/jac/dks121.