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Comparison of three biochemical tests for rapid detection of extended-spectrum β-lactamase-producing *Enterobacteriaceae*

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Enterobacterial isolates producing clavulanic-inhibited extended-spectrum β-lactamases (ESBLs) are increasingly spreading in the community and are often responsible for nosocomial infections. Rapid biochemical tests have been developed recently for their detection. Three tests, namely the Rapid ESBL NDP test, the β-Lacta test and the Rapid ESBL Screen have been evaluated with a collection of 108 well-characterized strains including wild-type strains, strains producing ESBLs, overexpressed cephalosporinases, and carbapenemases. The ESBL NDP test and the Rapid ESBL Screen (a copy of the ESBL NDP test) are aimed to detect ESBL producers while the ß-Lacta test is aimed to detect not only ESBL producers but also cephalosporinase and carbapenemase producers. The sensitivity and specificity of detection of ESBL producers (n = 60) were 95% and 100% for the Rapid ESBL NDP test, 80% and 87% (after 30 min) and 92% and 83% (after 2 h) for the Rapid ESBL Screen, and 91% and 96% for the ß-Lacta test. Variable and time-consuming detection (up to 2h) of ESBLs by the Rapid ESBL Screen and concomittant and variable detection of producers of AmpC and several type of carbapenemases correspond to significant shortcomings for using the Rapid Screen ESBL and ß-Lacta tests, respectively.

Introduction

Acquired resistance to broad-spectrum cephalosporins in *Enterobacteriaceae* is mainly due to production of clavulanic-acid inhibited extended-spectrum β-lactamases (ESBLs), which have extensively disseminated worldwide (1). Along with carbapanemase producers, ESBL producers represent the most important resistance trait in *Enterobacteriaceae* in 2015. The European Antimicrobial Resistance Surveillance System network (EARS-Net) including thirty European countries has reported in 2013 the prevalence rates of non-susceptibility to broad-spectrum cephalosporins among invasive enterobacterial isolates (http://www.ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2013.pdf). The proportion of *Escherichia coli* resistant to broad-spectrum cephalosporins ranged from 5% to 39.6% depending on the country. In *Klebsiella pneumoniae*, the percentage of resistance to broad-spectrum cephalosporins showed a significantly increase from 22.8% in 2012 to 30% in 2013, encompassing 85 to 100% of ESBL producers. In the US, the percentage of healthcare-associated infections caused by broad-spectrum cephalosporin-resistant *Enterobacteriaceae* has

been estimated to be 14% and 23% for *E. coli* and *Klebsiella* spp., respectively (http://www.cdc.gov/drugresistance/threat-report-2013). Those ESBL-producing *Enterobacteriaceae* are identified either as a source of hospital or community-acquired infections (2).

Rapid detection of ESBL producers is therefore crucial in order to prevent their dissemination, and for guiding the treatments of infected patients. Several phenotypic techniques are based on the inhibition of the ESBL activity by clavulanic acid or tazobactam. Those techniques require a preliminary growth step of 24-48 hours (3). Molecular detection of ESBL encoding genes is interesting but remains costly, requires expertise, and does not detect all genes encoding enzymes exhibiting an ESBL activity (3-5). Other techniques such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (6) are being developed but they do require additional material and a significant degree of expertise.

Recently, two rapid diagnostic tests have been developed to identify enterobacterial strains being resistant to broad-spectrum cephalosporins. The Rapid ESBL NDP test (7) which is based on the detection of hydrolysis of the cefotaxime β-lactam ring revealing the production of a broad-spectrum ß-lactamase, coupled with a tube containing

tazobactam signing the likely ESBL production. Another test is the β-Lacta test, based on the cleavage of a chromogenic cephalosporin HMRZ-86 (8, 9) (Bio-Rad, Marnes-la-Coquette, France). It is claimed that this test detects any activity that may lead to the hydrolysis of broad-spectrum cephalosporins, such as ESBLs, overproduction of cephalosporinases, and carbapenemases of the KPC and metallo-ß-lactamase types, without distinction between those mechanisms of resistance. Finally a commercial adaptation of the Rapid ESBL NDP test (not validated by the inventors of the original Rapid ESBL NDP test) named Rapid ESBL Screen Kit 98022 (Rosco-Diagnostica A/S, Taastrup, Denmark) has been developed.

Several authors have reported variable results using those three biochemical tests but using different collections of strains which resistance mechanisms were in some cases even not identified. The aim of this study was to compare those three tests using the exact same collection of strains possessing well-characterized resistance mechanisms.

Materials and Methods

Strain collection. A total of 108 clinical enterobacterial isolates were included in this study. They were from worldwide origin and had been recovered from different

types of clinical specimens. This collection included isolates producing the main ESBL types (CTX-M, TEM, SHV, PER, GES) (n = 60), and also isolates showing resistance to broad-spectrum cephalosporins due to other mechanisms such as plasmid-encoded AmpC-type β-lactamases or overproduced chromosomal AmpC. Some strains were also tested as negative controls including strains producing β-lactamases with a narrow spectrum activity and wild-type strains (n = 24). Some carbapenemase producers (n = 10) were also included in the study. All isolates had been previously characterized at the molecular level to identify the different mechanisms responsible for resistance to β-lactam antibiotics. In addition, they were all clonally-unrelated according to pulsed-field gel electrophoresis (data not shown). They were cultured onto Mueller-Hinton agar plates and incubated for 16 to 24 hours at 37ºC. Tests were interpreted blindly by three persons from our lab.

Rapid ESBL NDP test. The Rapid ESBL NDP test was performed as described (7). A single full 10-µl calibrated loop of bacterial colonies studied was suspended in the lysis buffer (B-PERII, Bacterial Protein Extraction Reagent, Pierce/Thermo Scientific, Villebon-sur-Yvette, France) and disposed in three 1.5-ml different Eppendorf tubes (A, B, C). Ten µl of a concentrated tazobactam solution (40 µg/ml)

were added to tube C. Then, 100 µl of the revealing solution containing pH indicator (phenol red) were added in tube A, and 100 µl of the same solution supplemented with cefotaxime (6 µg/ml) were added in tubes B and C. The three tubes were incubated at 37ºC for 20 min. Results were considered negative when all tubes were red and thus interpreted as non-ESBL strains. When tube B was yellow/orange and both tubes A and C were red, the test was considered as positive (ESBL-producing isolate). When tube A turned to yellow/red, the test was considered as non non-interpretable regardless of any color change for tubes B and C.

β-Lacta test. The β-Lacta test (Bio-Rad, Marnes la Coquette, France) was performed according to the manufacturer instructions. A single 1-µl loop of bacterial colonies studied was put into a microtube with a drop of reagent R1 and with a drop of reagent R2. Micro-tubes were left at ambient temperature and reading of the results was performed visually within 15 min. No change in color was considered as a negative result (no hydrolysis of HMRZ-86), color change to purple-red was considered as positive and color change to orange was considered as non interpretable.

Rapid ESBL Screen Kit 98022. The Rapid ESBL Screen Kit 98022 was performed according to the manufacturer indications (Rosco Diagnostica, Axonlab AG,

Baden, Switzerland). Several 1-µl loops of each strain were added to 150 µl of lysis buffer (B-PERII), incubated at room temperature for 30 min and then 50 µl of this suspension was diluted in a tube with 100 µl of a 0.9% sodium chloride solution. A tablet of cefotaxime + indicator was added in the tube. The same process was repeated using the tablet cefotaxime + tazobactam + indicator. Tubes were incubated at 37º C from 20 min until 2 hours and results interpreted as follows: (i) cefotaxime + indicator turning yellow and cefotaxime + tazobactam + indicator remaining red the test was considered positive for ESBL; (ii) cefotaxime + indicator turning yellow and cefotaxime + tazobactam + indicator also turning yellow, the test was considered negative for ESBL production but likely positive for another type of ß-lactamase; (iii) if both tubes remaining red, the test was considered negative for ESBL production.

Sensitivities and specificities were calculated for each test. A non-interpretable result was included as a negative result.

Results

Evaluation of the Rapid ESBL NDP test. By using the Rapid ESBL NDP test with all CTX-M producers, all tubes turned from red to yellow in presence of

cefotaxime and remained red in presence of cefotaxime and tazobactam; therefore sensitivity of the test was 100% for detection of ESBL production on that collection of ESBL producers (Table 1). The global sensitivity of the test for detection of ESBL production was 95% (57/60). As expected, the test remained negative with all wild-type strains and for all strains expressing β-lactamases with a narrow spectrum of activity (Table 2).

β-Lacta test. Using the β-Lacta test, all except two CTX-M producers (CTX-M-15-producing *Proteus mirabilis*, CTX-M-37-producing *E. coli*) and two non ESBL producers (TEM-12-positive *E. coli* and TEM-24-positive *E. coli*) were found positive. In addition, a yellow-to-orange color change was observed for three isolates, thus corresponding to non interpretable results. Therefore, sensitivity of detection of CTX-M producers was of 91% (Table 1). The sensitivity of detection of non-CTX-M ESBL producers was lower (84%), and the overall sensitivity for any type of ESBL was of 88%. Noticeably, no color change was observed when wild-type and narrow-spectrum β-lactamase-producing isolates were tested (Table 2).

The ß-Lacta test is aimed to detect also cephalosporinase overproducers, and carbapenemases of the KPC type together with metallo-ß-lactamases. Here, only four

out of fourteen AmpC overproducers were detected, and only four out of eight carbapenemase producers (with OXA-48 producers supposed to be not detected) (Table 2). Therefore the sensitivity of detection was low in both those cases.

Rapid ESBL Screen Kit 98022 (Rosco test). Test results were interpreted after thirty minutes of incubation and until two hours according to manufacturer instructions. After 30 min, no color change was observed for six CTX-M and seven non-CTX-M ESBL producers, respectively (Tables 1 and 2), leading to a sensitivity of 83% and 72%, respectively, and a global sensitivity for ESBL detection of 80%. All wild-type and narrow-spectrum β-lactamase-producing strains gave a negative result (Table 2). However, when tubes were incubated up to 2 hours, sensitivity for detection of CTX-M and non-CTX-M ESBL producers reached values of 94% and 88%, respectively (92% of global sensitivity for ESBL detection), but the specifity was lowered (three wild-type or narrow-spectrum β-lactamase-producing strains gave a false-positive result) (Tables 1 and 2). The specificities of the test after 30 minutes and 2 hours were of 87% and 83%, respectively (Table 3).

Discussion

This study showed that the sensitivity of detection of the three tests was good for all CTX-M producers, although the Rapid ESBL NDP test was the only test being able to flag at 100% the CTX-M producers. The global sensitivity of any kind of ESBL producer varied from 74% for the Rapid ESBL Screen test (reading after 30 min) to 92% for the Rapid ESBL NDP test. Partial lack of detection of ESBL-producing isolates being not CTX-M producers explains some discrepancies published regarding the potencies of the tests (7, 8, 10-12). The Rapid ESBL NDP test and the Rapid ESBL Screen Kit 98022 test are not designed to detect plasmid-mediated and chromosomal overproducers of AmpC. The ß-Lacta test which is aimed to detect those AmpC overproducers failed to detect those strains in 10/14 of the cases. Similar failure of detection has been reported by Morosini et al. (12). The overall specificity of the ß-Lacta test to detect ESBL producers was much lower than that of the Rapid ESBL NDP test since it also partially detects AmpC producers and several types of carbapenemase producers. Indeed, the ß-Lacta test is also aimed to detect production of carbapenemases of the KPC and metallo-enzyme types. Data obtained through this comparative study showed that KPC producers are well detected but not metallo-ß-lactamase producers.

Incomplete detection of AmpC or carbapenemase producers might therefore be a source of confusion when using the ß-Lacta test.

From a technical point of view, it must be highlighted that the best results for detection of ESBL producers by using the Rapid ESBL Screen were obtained after 2h of incubation. This incubation time does not make the test as rapid as the ESBL NDP test or the ß-Lacta test, which results are obtained within 20 min. In addition, prolonged incubation of the Rapid ESBL Screen test generated false-positive results. It is likely that the weak positive results obtained several times after the first reading time (30 min) may be associated to weak dissolution of the tablets. Weak positive results were sometimes difficult to interpret at the first time-point (30 min) by using the Rapid ESBL Screen test probably because of the poor dissolution of the tablet. Regarding the β-Lacta test, interpreting any color change as a positive result might also increase the sensitivity but conversely the specifity might be affected.

Noticeably, both the β-Lacta and the Rapid ESBL Screen Kit 98022 tests do not include any internal control well that would be free of antibiotic. We believe that this is a major shortcoming when comparing with the Rapid ESBL NDP, since such control allows a

better appreciation of the color change (especially for weak positive strains) and allows to detect possible false-positive results due to non-specific reactions (10).

As opposed to the Rapid ESBL NDP test that does not misidentify a KPC producer as an ESBL producer, the ß-Lacta test cannot differentiate between KPC producers and ESBL producers. This feature may be important in countries with high prevalence rates of KPC producers such as the US, Canada, Colombia, Italy, and Israel.

As a conclusion, and even though all tests evaluated here overall performed well for the detection of ESBL producers, higher perfomances are obtained with the Rapid ESBL NDP test. One main disadvantage of the ß-Lacta test is a lack of specificity with AmpC and carbapenemase producers. The Rapid ESBL Screen test, which is actually a copy of the Rapid ESBL NDP test, has much poorer performances. Also, it requires additional delay (2 h versus 20 min) for reading that may be considered as significant for patient management and antibiotic stewardship. Finally, we believe that use of rapid biochemical tests for detection of ESBL producers from clinical sites (13, 14) will be an alternative to molecular techniques since they are easy to be implemented, are affordable and may detect any kind of ESBLs.

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TABLE 1. Detection of extended-spectrum β-lactamase-producing isolates using different tests*

ESBL type	Species	No. of isolates	Rapid ESBL NDP test			β-Lacta test	ROSCO ESBL test 30 min.			ROSCO ESBL test 2 h.		
			CTX	CTX + TZB	IT[a]	HMRZ-86	CTX	CTX + TZB	IT[a]	CTX	CTX + TZB	IT[a]
CTX-M-1	*E. coli*	1	+	-	+	+	+	-	+	+	-	+
	P. mirabilis	1	+	-	+	+	+	-	+	+	-	+
	S. enterica	1	+	-	+	+	+	-	+	+	-	+
CTX-M-2	*E. coli*	1	+	-	+	+	+	-	+	+	-	+
	K. pneumoniae	1	+	-	+	+	+	-	+	+	-	+
	P. mirabilis	1	+	-	+	+	+	-	+	+	-	+
CTX-M-3	*E. aerogenes*	1	+	-	+	+	+	-	+	+	-	+
	E. coli	1	+	-	+	+	+	-	+	+	-	+
CTX-M-10	*E. coli*	1	+	-	+	NI	-	-	+	+	-	+
CTX-M-14	*E. coli*	2	+	-	+	+	+	-	+	+	-	+
	K. oxytoca	1	+	-	+	+	+	-	+	+	-	+
CTX-M-15	*C. freundii*	1	+	-	+	+	+	-	+	+	-	+
	E. coli	5	+	-	+	+	+	-	+	+	-	+
	E. coli	2	+	-	+	+	-	-	+	+	-	+
	E. coli	1	+	-	+	+	-	-	+	-	-	+
	K. pneumoniae	3	+	-	+	+	+	-	+	+	-	+
	M. morganii	1	+	-	+	+	+	-	+	+	-	+
	P. mirabilis	1	+	-	+	+	-	-	+	-	-	+
CTX-M-18	*E. coli*	2	+	-	+	+	+	-	+	+	-	+
	K. pneumoniae	1	+	-	+	+	+	-	+	+	-	+
CTX-M-19	*K. pneumoniae*	1	+	-	+	+	+	-	+	+	-	+
CTX-M-32	*E. coli*	3	+	-	+	+	+	-	+	+	-	+
CTX-M-37	*E. coli*	1	+	-	+	+	-	-	+	+	-	+
	K. pneumoniae	1	+	-	+	+	+	-	+	+	-	+
TEM-3	*E. coli*	1	+	-	+	+	-	-	+	-	-	+
TEM-12	*E. coli*	1	-	-	+	+	-	-	+	-	-	+

TEM-24	*E. coli*	1	-	-	-	-	-	-	-	-	-	-
TEM-52	*K. oxytoca*	1	+	-	+	+	+	-	+	+	-	+
	K. pneumoniae	5	+	-	+	+	+	-	+	+	-	+
SHV-2	*E. coli*	1	+	-	+	+	+	-	+	+	-	+
SHV-5	*K. pneumoniae*	1	-	-	-	+	-	-	-	+	-	+
SHV-9	*S. enterica*	1	+	-	+	+	-	-	-	+	-	+
SHV-12	*E. coli*	3	+	-	+	+	+	-	+	+	-	+
	E. coli	1	-	-	-	+	-	-	-	+	-	+
	E. aerogenes	2	+	-	+	+	+	-	+	+	-	+
GES-1	*K. pneumoniae*	1	+	-	+	NI	-	-	-	+	-	+
GES-5	*E. cloacae*	1	+	-	+	NI	+	-	+	+	-	+
PER-1	*E. cloacae*	3	+	-	+	+	+	-	+	+	-	+
PER-1	*P. mirabilis*	1	+	-	+	+	+	-	+	+	-	+
PER-1	*S. enterica*	1	+	-	+	+	+	-	+	+	-	+

+, color change from red to yellow/orange for the Rapid ESBL NDP test; -, no color change; +, hydrolysis of HMRZ-86 for the ß-Lacta test; NI, not interpretable (color change to orange)

*An overall positive Rapid ESBL NDP test corresponds to a positive result for CTX hydrolysis and a negative result when tazobactam is added.

[a]IT; interpretation of the test

[b]The ß-Lacta test is aimed to detect the activity of all broad-spectrum cephalosporinases

Shaded symbols highlight the non-expected results

TABLE 2. Detection of non-ESBL-producing isolates using different tests*

Phenotype of β-lactam resistance	β-Lactamase	Species	No. of isolates	Rapid ESBL NDP test			β-Lacta test[b]	ROSCO ESBL test 30 min.			ROSCO ESBL test 2 h.		
				CTX	CTX + TZB	IT[a]	HMRZ-86	CTX	CTX + TZB	IT[a]	CTX	CTX + TZB	IT[a]
No resistance	Wild type	*E. coli*	9	-	-	-	-	-	-	-	-	-	-
		E. coli	1	-	-	-	-	-	-	-	+	-	+
Penicillinase	TEM-1	*E. coli*	3	-	-	-	-	-	-	-	-	-	-
	OXA-1	*E. coli*	2	-	-	-	-	-	-	-	+	-	+
	Wild type	*K. pneumoniae*	2	-	-	-	-	-	-	-	-	-	-
	IRT-2	*K. pneumoniae*	1	-	-	-	-	-	-	-	-	-	-
	PSE-1	*S. enterica*	1	-	-	-	-	-	-	-	-	-	-
Chromosome-encoded cephalosporinase	Wild type	*E. cloacae*	3	-	-	-	-	-	-	-	-	-	-
	Wild type	*M. morganii*	1	-	-	-	-	-	-	-	-	-	-
	Narrow AmpC	*E. coli*	1	-	-	-	-	-	-	-	-	-	-
Acquired cephalosporinase	DHA-1	*E. coli*	2	-	-	-	-	-	-	-	-	-	-
		K. pneumoniae	1	+	+	-	-	+	+	-	+	+	-
	DHA-2	*K. pneumoniae*	1	+	+	-	+	-	-	-	-	-	-
	ACC-1	*E. coli*	1	+	+	-	+	+	+	-	+	+	-
		P. mirabilis	1	+	+	-	+	+	+	-	+	+	-
	CMY-2	*E. coli*	1	+	+	-	-	+	+	-	+	+	-
		K. pneumoniae	1	-	-	-	-	+	+	-	+	+	-
		P. mirabilis	1	-	-	-	-	-	-	-	-	-	-
		P. vulgaris	1	-	-	-	-	-	-	-	-	-	-
	FOX-5	*K. pneumoniae*	1	-	-	-	+	-	-	-	-	-	-
Overexpressed chromosome-encoded cephalosporinase		*C. freundii*	1	+	+	-	NI	-	-	-	-	-	-
		E. coli	1	-	-	-	-	-	-	-	-	-	-
		E. cloacae	1	-	-	-	-	-	-	-	-	-	-
Carbapenemase producers	KPC-2	*E. coli*	1	+	+	-	+	+	-	+	+	-	+
		K. pneumoniae	1	+	+	-	+	+	-	+	+	-	+
		S. marcescens	1	+	+	-	+	+	-	+	+	-	+
	NDM-1	*E. cloacae*	1	+	+	-	+	+	+	-	+	+	-
		P. mirabilis	1	-	-	-	-	-	-	-	-	-	-
	NDM-4	*E. coli*	1	-	-	-	-	-	-	-	+	-	+
	OXA-48	*E. coli*	1	+	+	-	-	+	-	+	+	-	+
		K. pneumoniae	1	+	+	-	NI	+	-	+	+	-	+
	VIM-1	*E. coli*	1	+	+	-	+	+	-	+	+	-	+
		K. pneumoniae	1	+	+	-	+	+	+	-	+	+	-

+, color change from red to yellow/orange; -, no color change; H, hydrolysis of HMRZ-86; NH, no hydrolysis of HMRZ-86; NI, non interpretable (color change to orange)

*An overall positive Rapid ESBL NDP test corresponds to a positive result for CTX hydrolysis and a negative result when tazobactam is added.

[a]IT; interpretation of the test

[b]The ß-Lacta test is aimed to detect the activity of all broad-spectrum cephalosporinases

Shaded symbols highlight the non-expected results

TABLE 3. Diagnostic parameters of the different tests*

Diagnostic test parameters	Rapid ESBL NDP test	β-Lacta test	Rapid ESBL Screen Kit 30 min	Rapid ESBL Screen Kit 2 h
Sensitivity CTX-M type ESBL	100%	91.4%	82.8%	94.3%
Sensitivity non CTX-M type ESBL	88%	84%	72%	88.0%
Global sensitivity ESBL	95.0%	88.0%	80%	91.7%
Global specifity	100%	70.8%	87%	83%

*Values are calculated for specific detection of ESBL producers