Detection of Resistance to Beta-Lactamase Inhibitors in Strains with CTX-M Beta-Lactamases: a Multicenter External Proficiency Study Using a Well-Defined Collection of *Escherichia coli* Strains

Aida Ripoll,^a Juan-Carlos Galán,^{a,b} Cristina Rodríguez,^c Nuria Tormo,^d Concepción Gimeno,^d Fernando Baquero,^{a,b} Luis Martínez-Martínez,^{c,e,f} Rafael Cantón,^{a,f} SEIMC Quality Control Study Group

Servicio de Microbiología, Hospital Ramón y Cajal and Instituto de Investigación Sanitaria (IRYCIS), Madrid, Spain^a; CIBER en Epidemiología y Salud Pública (CIBERESP) Instituto de Salud Carlos III, Madrid, Spain^b; Servicio de Microbiología, Hospital Universitario Marqués de Valdecilla-IFIMAV,^c and Departamento de Biología Molecular, Universidad de Cantabria,^e Santander, Spain; Servicio de Microbiología, Hospital General de Valencia, Valencia, Spain^d; Spanish Network for the Research in Infectious Diseases (REIPI), Instituto de Salud Carlos III, Madrid, Spain^f

Under the auspices of the Spanish Society for Infectious Diseases and Clinical Microbiology Quality Control program, 14 *Escherichia coli* strains masked as blood culture isolates were sent to 68 clinical microbiology laboratories for antimicrobial susceptibility testing to β -lactam antibiotics. This collection included three control strains (*E. coli* ATCC 25922, an IRT-2 producer, and a CMY-2 producer), six isogenic strains with or without the OmpF porin and expressing CTX-M β -lactamases (CTX-M-1, CTX-M-15, and CTX-M-14), one strain carrying a double mechanism for β -lactam resistance (i.e., carrying CTX-M-15 and OXA-1 enzymes), and four strains carrying CTX-M variants with different levels of resistance to β -lactams and β -lactam- β -lactamase inhibitor (BLBLI) combinations. The main objective of the study was to ascertain how these variants with reduced susceptibilities to BLBLIs are identified in clinical microbiology laboratories. CTX-M variants with high resistance to BLBLIs were mainly identified as inhibitor-resistant TEM (IRT) enzymes (68.0%); however, isogenic CTX-M mutant strains with reduced susceptibilities to BLBLIs and cephalosporins were mainly associated with extended-spectrum β -lactamase production alone (51 to 80%) or in combination with other mechanisms (14 to 31%). Concerning all β -lactams tested, the overall interpretative discrepancy rate was 11.5%, of which 38.1% were the consequence of postreading changes in the clinical categories when a resistance mechanism was inferred. Therefore, failure to recognize these complex phenotypes might contribute to an explanation of their apparent absence in the clinical setting and might lead to inadequate drug treatment selection. A proposal for improving recognition is to adhere strictly to the current CLSI or EUCAST guidelines for detecting reduced susceptibility to BLBLI combinations, without any interpretative modification.

β -Lactamase production is the most important resistant mechanism to β -lactam antibiotics in Gram-negative bacteria (1). CTX-M extended-spectrum β -lactamases (ESBLs) are becoming one of the largest groups of β -lactamases, with a spectacular increase in the number of variants along the last years (147 variants; see www.lahey.org/Studies/other.asp). Isolates expressing these enzymes often confer resistance not only to oxyimino-cephalosporins but also to other antimicrobial agents, thus representing an important problem in therapeutics and public health (2–4).

Single mutations in CTX-M β -lactamases that increase the spectrum of action against cefotaxime and ceftazidime have been selected in nature and under laboratory conditions. Although CTX-M mutants with single amino acid changes (such as S130G, S237G, and K234R) conferring reduced susceptibility to β -lactam- β -lactamase inhibitor (BLBLI) combinations have been easily obtained under *in vitro* conditions (5), these variants have not yet been described in the clinical setting (6). This might be due to the limited exposure of the mutants to BLBLI combinations, as these agents are not usually recommended in infections due to ESBL-producing strains, and carbapenems remain the drugs of choice in empirical or oriented therapy (7). Treatment with BLBLI combinations is still controversial; however, recent studies suggest that BLBLI combinations might provide similar results to carbapenems in the treatment of infections caused by ESBL-producing organisms (8). Therefore, an expected increase in the consumption of BLBLI combinations might lead to the emergence and

spread of strains with CTX-M variants with reduced susceptibilities to these compounds.

The apparent absence of CTX-M variants with reduced susceptibilities to BLBLI combinations in the clinical setting might derive from the difficulties in the recognition of these phenotypes with conventional susceptibility testing methods or confusion with other phenotypes. We hypothesize that CTX-M variants with reduced susceptibilities to BLBLI combinations might naturally occur but might be undetectable or misidentified in routine clinical laboratories and are probably assigned as strains harboring previously known resistance mechanisms, such as inhibitor-resistant TEMs (IRTs) or complex mutant TEMs (CMTs), or even as ESBL producers susceptible to BLBLI combinations. To docu-

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Address correspondence to Juan-Carlos Galán, juancarlos.galan@salud.madrid.org.

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TABLE 1 MIC reference values and interpretative category according to CLSI and EUCAST criteria^a

Strain	Porin status/ β -lactamase	Phenotype according to MIC (μ g/ml) (resistance category ^b) for indicated antibiotic														
		AMP	AMC ^c	TIC	TZP ^d	CEF ^e	CFZ ^e	CXM	FOX	CTX	CAZ	ATM	FEP	IPM	MEM	ETP
CCSRC01	OmpF ⁺ /CTX-M-1	>256 (R/R)	16 (U/R)	>256 (R/R)	2 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	4 (S/-)	>256 (R/R)	4 (S/U)	64 (R/R)	16 (U/R)	0.5 (S/S)	0.06 (S/S)	0.06 (S/S)
CCSRC02	OmpF ⁺ /CTX-M-1	>256 (R/R)	32 (R/R)	>256 (R/R)	2 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	8 (S/-)	>256 (R/R)	16 (R/R)	256 (R/R)	128 (R/R)	0.5 (S/S)	0.12 (S/S)	0.06 (S/S)
CCSRC03	OmpF ⁺ /CTX-M-1-S130G	>256 (R/R)	128 (R/R)	>256 (R/R)	128 (R/R)	>256 (R/-)	>256 (R/-)	16 (U/R)	8 (S/-)	0.125 (S/S)	1 (S/S)	0.5 (S/S)	0.25 (S/S)	0.5 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC04	OmpF ⁺ /CTX-M-1-S130G	>256 (R/R)	128 (R/R)	>256 (R/R)	2 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	8 (S/-)	>256 (R/R)	16 (R/R)	256 (R/R)	32 (R/R)	0.5 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC05	OmpF ⁺ /CTX-M-15	>256 (R/R)	8 (S/S)	>256 (R/R)	2 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	4 (S/-)	128 (R/R)	4 (S/U)	16 (R/R)	32 (R/R)	0.5 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC06	OmpF ⁺ /CTX-M-15	>256 (R/R)	16 (U/R)	>256 (R/R)	4 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	8 (S/-)	256 (R/R)	16 (R/R)	32 (R/R)	32 (R/R)	0.5 (S/S)	0.125 (S/S)	0.125 (S/S)
CCSRC07	OmpF ⁺ /CTX-M-15-S130G	>256 (R/R)	128 (R/R)	>256 (R/R)	64 (U/R)	>256 (R/-)	>256 (R/-)	16 (U/R)	8 (S/-)	0.125 (S/S)	1 (S/S)	1 (S/S)	1 (S/S)	0.25 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC08	OmpF ⁺ /CTX-M-15 + OXA-1	>256 (R/R)	128 (R/R)	>256 (R/R)	64 (U/R)	>256 (R/-)	>256 (R/-)	>128 (R/R)	8 (S/-)	128 (R/R)	32 (R/R)	64 (R/R)	32 (R/R)	0.5 (S/S)	$\leq$ 0.03 (S/S)	0.125 (S/S)
CCSRC09	OmpF ⁺ /CTX-M-14	>256 (R/R)	32 (R/R)	>256 (R/R)	2 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	4 (S/-)	>256 (R/R)	4 (S/U)	16 (R/R)	128 (R/R)	0.5 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC10	OmpF ⁺ /CTX-M-14	>256 (R/R)	32 (R/R)	>256 (R/R)	4 (S/S)	>256 (R/-)	>256 (R/-)	>128 (R/R)	8 (S/-)	>256 (R/R)	16 (R/R)	256 (R/R)	32 (R/R)	0.5 (S/S)	$\leq$ 0.03 (S/S)	0.5 (S/S)
CCSRC11	OmpF ⁺ /CTX-M-14-K234R	>256 (R/R)	64 (R/R)	>256 (R/R)	2 (S/S)	>256 (R/-)	>256 (R/-)	64 (R/R)	8 (S/-)	1 (S/S)	8 (R/R)	8 (R/R)	8 (R/R)	0.5 (S/S)	$\leq$ 0.03 (S/S)	0.5 (S/S)
CCSRC12	ATCC 25922	4 (S/S)	4 (S/S)	4 (S/S)	2 (S/S)	8 (S/-)	2 (S/-)	4 (S/S)	4 (S/-)	0.06 (S/S)	0.25 (S/S)	0.25 (S/S)	0.25 (S/S)	$\leq$ 0.01 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC13	IRT-2	>256 (R/R)	32 (R/R)	>256 (R/R)	8 (S/S)	16 (U/-)	2 (S/-)	16 (U/R)	4 (S/-)	0.06 (S/S)	0.25 (S/S)	$\leq$ 0.01 (S/S)	1 (S/S)	0.12 (S/S)	$\leq$ 0.03 (S/S)	$\leq$ 0.03 (S/S)
CCSRC14	CMY-2	>256 (R/R)	64 (R/R)	>256 (R/R)	8 (S/S)	>256 (R/-)	>256 (R/-)	128 (R/R)	>128 (R/-)	16 (R/R)	32 (R/R)	8 (U/R)	1 (S/S)	0.25 (S/S)	0.06 (S/S)	0.5 (S/S)

^a The CLSI and EUCAST categories were obtained from reference 26 and the EUCAST website (see http://www.eucast.org/clinical_breakpoints/), respectively.^b R, resistant; I, intermediate; S, susceptible.^c Amoxicillin-clavulanic acid, 2:1 concentration.^d Piperacillin-tazobactam, 4μg/ml of tazobactam.^e —, absence of EUCAST criteria.

ment the difficulty in detecting IR-CTX-M or CM-CTX-M variants (adapting here the expression used for TEM nomenclature) (9) and to ascertain how these variants, if present, can be identified in clinical practice, we performed a multicenter antimicrobial susceptibility testing (AST) study in Spain using a well-characterized collection of *Escherichia coli* strains showing a combination of transferable and mutational mechanisms affecting β -lactams, including BLBLI combinations. The participating laboratories were also requested to provide MICs and to infer the resistance mechanisms of the strains. The influence of using CLSI versus EUCAST breakpoints was also analyzed.

MATERIALS AND METHODS

Bacterial strains. Fourteen *E. coli* strains included in three different batches were studied. Batch I included strains expressing natural CTX-M β -lactamases: three *E. coli* MKW505 strains and their isogenic MH621 OmpF⁺ variants harboring pBGS18-*bla*_{CTX-M-1}, pBGS18-*bla*_{CTX-M-15}, and pBGS18-*bla*_{CTX-M-14} plasmids (10, 11). Batch II included strains expressing CTX-M variants with decreased susceptibilities or resistances to BLBLI combinations: four *E. coli* MH621 strains harboring pBGS18-*bla*_{CTX-M-1-S130G}, pBGS18-*bla*_{CTX-M-1-S237G}, pBGS18-*bla*_{CTX-M-15-S130G}, and pBGS18-*bla*_{CTX-M-14-K234R} plasmids. In addition, this batch included an *E. coli* MH621 strain simultaneously expressing CTX-M-15 and OXA-1 β -lactamases. Finally, batch III included three control strains with well-established phenotypes: the susceptible *E. coli* ATCC 25922, a clinical *E. coli* strain with an IRT-2 β -lactamase (12) and a clinical *E. coli* strain expressing a CMY-2 enzyme, an AmpC plasmid-mediated β -lactamase (13).

Antibiotic susceptibility testing was performed in duplicate by two reference microbiology laboratories (Ramón y Cajal University Hospital, Madrid, Spain, and Marqués de Valdecilla University Hospital, Santander, Spain), by using standard disk diffusion, a diffusion gradient test (Etest, bioMérieux, France), and broth microdilution methods according to CLSI guidelines (14, 15). The MIC values for all strains were coincident in the laboratories (a few numbers of discrepancies were solved after replicating tests in both centers). The strains were categorized as susceptible, intermediate, or resistant using both the CLSI and EUCAST criteria (see reference 16 and http://www.eucast.org/clinical_breakpoints/). Fifteen β -lactam antibiotics were evaluated: ampicillin (AMP), amoxicillin-clavulanic acid (AMC), ticarcillin (TIC), piperacillin-tazobactam (TZP), cephalothin (CEF), cefazolin (CFZ), cefuroxime (CXM), ceftazidime (FOX), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), aztreonam (ATM), imipenem (IPM), meropenem (MEM), and ertapenem (ETP). Table 1 includes a description of all strains used and their susceptibility profiles.

Study design. The study was designed as a nationwide multicenter proficiency study, under the scientific auspices of the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC) External Quality Control Program. In October 2012, the 14 strains forming part of this quality survey (see “Bacterial strains,” above) were sent to all the participant laboratories masked as blood culture isolates. For this purpose, the isolates were inoculated onto nutrient agar swabs and distributed to 68 participating clinical microbiology laboratories in public hospitals in Spain. Before shipping to the participating laboratories, all genotypes (i.e., those with *bla*_{CTX-M}, *bla*_{IRT-2}, and *bla*_{CMY-2} genes) were confirmed with PCRs and sequencing. The swabs were labeled with a number (CCSRC01 to CCSRC14) and the bacterial species name (*E. coli*). Before inoculation onto the swabs, strains CCSRC01 to CCSRC11 were selected on Luria-Bertani agar (LB) supplemented with 50 μg/ml of kanamycin in order to ensure the presence of the constructed plasmids, derived from pBGS18 (i.e., Kan^r) and harboring *bla*_{CTX-M} genes (17). The instructions given to the participating laboratories indicated that the strains should be studied for susceptibility testing by the routine method used in each laboratory. The participating laboratories were requested to

TABLE 2 Distributions of categorical error rates by antimicrobial agent tested

Antibiotic ^a	No. of organism-antibiotic combinations tested	No. of strains classified as:		Discrepancies in the interpretative category		Results by error type:					
						mE		ME		VME	
		S	R	n	%	n	% ^b	n	% ^c	n	% ^d
AMP	800	58	742	2	0.2	1	0.1	0	0.0	1	0.1
AMC	864	110	556	207	24.0	159	18.4	12	10.9	36	6.5
TIC	153	9	140	4	2.6	1	0.7	0	0.0	3	2.1
TZP	822	492	85	221	26.9	92	11.2	125	25.4	6	7.1
CEF	594	42	488	57	9.6	51	9.0	1	2.4	5	0.8
CFZ	479	398	62	19	4.0	10	2.1	6	1.5	3	4.8
CXM	839	61	603	145	17.0	102	12.2	0	0.0	43	7.1
FOX	836	678	61	97	11.6	8	1.0	89	13.1	0	0.0
CTX	854	247	543	64	7.5	9	1.1	50	20.2	5	0.9
CAZ	858	337	345	175	20.0	75	8.7	96	28.5	4	1.2
ATM	674	194	423	53	7.9	35	5.2	8	4.1	10	2.4
FEP	861	316	373	173	20.9	63	7.3	92	29.1	18	4.8
IPM	864	858	0	6	0.7	3	0.3	3	0.3	NA ^e	NA
MEM	357	357	0	0	0.0	0	0.0	0	0.0	NA	NA
ETP	834	824	0	10	1.2	3	0.4	7	0.8	NA	NA
Total	10,689	4,981	4,421	1,233	11.5	610	5.7	489	9.8	134	3.0

^a The nomenclature of the antibiotics is described in the text.

^b Percentages of mEs were calculated considering the total number of susceptibility testing determinations for each antibiotic as the denominator.

^c Percentages of MEs were calculated considering the number of susceptible strains for each antibiotic as the denominator.

^d Percentages of VMEs were determined using the number of resistant strains for each antibiotic as the denominator.

^e NA, not applicable.

complete an electronic form for each strain, including (i) the routine tests for AST (i.e., the type of automatic or semiautomatic system or manual method(s) used), (ii) the quantitative susceptibility results (inhibition zone diameters or MIC values), (iii) the breakpoint criteria used (CLSI, EUCAST, or other), (iv) the clinical categorization (susceptible, intermediate, or resistant) as would be reported to clinicians, (v) any additional test used to modify the clinical category or to confirm the obtained AST phenotype, and (vi) the inferred antibiotic resistance mechanism.

Evaluation of the results. Discrepancies between the AST results and reference values were classified as very major (VME), major (ME), or minor (mE) errors (18). VMEs occurred when the results obtained by the participating laboratory were in the susceptible category and the reference value was assigned to the resistant category (i.e., a false-susceptible result). Conversely, MEs occurred when the results obtained by the participating laboratory were in the resistant category and they were in the susceptible category with the reference procedure (i.e., a false-resistant result). Finally, mEs occurred when the results obtained by the participating laboratory were susceptible or resistant and intermediate by the reference laboratories, or when the results obtained by the participant were intermediate and were susceptible or resistant by the reference laboratories. Also, the modification of clinical categories that might be reported to the clinicians when a resistance mechanism was identified was considered. The error calculations were performed for each laboratory according to the breakpoint used, CLSI or EUCAST.

RESULTS

Participation rates, susceptibility testing methods, and breakpoints. Sixty-eight clinical microbiology laboratories from different regions of Spain agreed to take part in this study, but only 64 (94.1%) finally provided results. Automatic or semiautomatic systems were used by 62/64 centers; among them, 10 centers reported data using ≥ 2 systems (16.1%), 12 laboratories (19.3%) used one automatic system in combination with a manual method, and only 2 centers (3.1%) used exclusively manual methods for AST. Therefore, the total number of results received using different

automatic systems was 77 among 64 participant laboratories. The MicroScan WalkAway (Dade MicroScan, Inc., West Sacramento, CA, USA) was the most extensively used equipment (37/77 [48.0%]), followed by the Vitek 2 (bioMérieux, Inc., Durham, NC, USA) (20/77 [26.0%]), Wider (Francisco Soria de Melguizo, Madrid, Spain) (14/77 [18.1%]), and Phoenix (BD Biosciences, Sparks, MD, USA) (6/77 [7.8%]). Many centers performed complementary tests to infer resistance phenotypes (i.e., ESBL production) or to confirm AST values: 51.6% (33/64) of the centers performed a disk diffusion method and 18.8% (12/64) used a gradient test (Etest strips used in all cases).

CLSI interpretative criteria were used in most laboratories (43/64 [67.2%]), EUCAST criteria were used in 25.0% (16/64) of the centers, 6.2% (4/64) combined CLSI and EUCAST criteria, and one center (1.6%) used other criteria (19).

Proficiency results in susceptibility testing. The reference MIC values obtained by a broth microdilution assay are shown in Table 1. Comparing these reference values with data from the participants, the overall interpretative discrepancy rate was 11.5% (Table 2), with categorical errors ranging from 3.0% to 9.8% for VMEs and MEs, respectively. The highest percentages of VMEs (false susceptibility) were observed for TZP (7.1%), CXM (7.1%), and AMC (6.5%). Contrary to VMEs, higher categorical MEs (false resistance) were observed, with FEP (29.1%), CAZ (28.5%), and TZP (25.4%) being the most affected antimicrobials.

Globally, the automatic and semiautomatic systems were responsible for 61.9% (724/1,169) of all the detected errors, mainly due to mEs (484/724) (see Table S1 in supplemental material). On the other hand, errors as a consequence of postreading changes in the interpretation of the clinical categories reached 38.1% (445/1,169). These errors involved 80% of all participants and were mostly categorized as MEs (347/445 [77.9%]). Among the MEs,

TABLE 3 Distributions of categorical error rates by tested strain and interpretive criteria (CLSI or EUCAST)

Strain	Porin status/ β -lactamase carriage	Criteria	No. of data points ^a	Total errors ^b		Results by error type ^c					
						mE		ME		VME	
				<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
CCSRC01	OmpF ⁺ /CTX-M-1	CLSI	596	119	20.0	71	11.9	48	8.1	0	0.0
		EUCAST	199	28	14.1	18	9.0	7	3.5	3	1.5
CCSRC02	OmpF ⁻ /CTX-M-1	CLSI	572	47	8.2	34	5.9	13	2.3	0	0.0
		EUCAST	196	8	4.1	0	0.0	7	3.6	1	0.5
CCSRC03	OmpF ⁻ /CTX-M-1 _{S130G}	CLSI	578	78	13.5	51	8.8	21	3.6	6	1.0
		EUCAST	195	23	11.8	4	2.1	6	3.1	13	6.7
CCSRC04	OmpF ⁻ /CTX-M-1 _{S237G}	CLSI	588	29	4.9	11	1.9	17	2.9	1	0.2
		EUCAST	194	9	4.6	0	0.0	8	4.1	1	0.5
CCSRC05	OmpF ⁺ /CTX-M-15	CLSI	574	62	10.8	8	1.4	52	9.1	2	0.3
		EUCAST	194	45	23.2	19	9.8	26	13.4	0	0.0
CCSRC06	OmpF ⁻ /CTX-M-15	CLSI	543	58	10.7	42	7.7	13	2.4	3	0.6
		EUCAST	193	18	9.3	1	0.5	5	2.6	12	6.2
CCSRC07	OmpF ⁻ /CTX-M-15 _{S130G}	CLSI	572	88	15.4	77	13.5	10	1.7	1	0.2
		EUCAST	182	21	11.5	1	0.5	6	3.3	14	7.7
CCSRC08	OmpF ⁻ /CTX-M-15 + OXA-1	CLSI	586	65	11.1	47	8.0	11	1.9	7	1.2
		EUCAST	193	10	5.2	1	0.5	3	1.6	6	3.1
CCSRC09	OmpF ⁺ /CTX-M-14	CLSI	582	90	15.5	31	5.3	48	8.2	11	1.9
		EUCAST	183	24	13.1	16	8.7	6	3.3	2	1.1
CCSRC10	OmpF ⁻ /CTX-M-14	CLSI	528	53	10.0	28	5.3	13	2.5	12	2.3
		EUCAST	159	11	6.9	1	0.6	9	5.7	1	0.6
CCSRC11	OmpF ⁻ /CTX-M-14 _{K234R}	CLSI	559	129	23.1	23	4.1	99	17.7	7	1.3
		EUCAST	193	48	24.9	19	9.8	20	10.4	9	4.7
CCSRC12	ATCC 25922	CLSI	584	2	0.3	0	0.0	2	0.3	0	0.0
		EUCAST	192	2	1.0	1	0.5	1	0.5	0	0.0
CCSRC13	IRT-2	CLSI	585	93	15.9	72	12.3	19	3.2	2	0.3
		EUCAST	193	19	9.8	1	0.5	3	1.6	15	7.8
CCSRC14	CMY-2	CLSI	582	38	6.5	27	4.6	8	1.4	3	0.5
		EUCAST	194	14	7.2	6	3.1	6	3.1	2	1.0
Total		CLSI	8,028	951	11.8	522	6.5	374	4.7	55	0.7
		EUCAST	2,661	282	10.6	88	3.3	115	4.3	79	3.0
		All	10,689	1,233	11.5	610	5.7	489	4.6	134	1.3

^a AST susceptibility testing determinations with each criterion.^b Total errors, including all antibiotics tested.^c mE, ME, and VME percentages were calculated with the number of determinations for each strain as the denominator.

the most affected strains were those expressing CTX-M-15 and CTX-M-14_{K234R} in an OmpF⁻ background (strains CCSRC06 and CCSRC11, respectively) (Table 3), with CCSRC11 being the strain with the highest error rates for BLBLI combinations (see Table S2 in the supplemental material). Moreover, considering all antibiotics tested, VMEs were mainly present in strains expressing CTX-M-15_{S130G} (CCSRC03) and CTX-M-1_{S130G} (CCSRC07) variants (Table 3). However, the error rates for BLBLI combinations were highest in CCSRC06 (CTX-M-15) and CCSRC08 (CTX-M-15 plus OXA-1) strains (see Table S2 in the supplemental material).

The centers that used the CLSI criteria showed slightly higher error rates (11.8%) than centers that used the EUCAST criteria (10.6%), mainly due to mEs (6.5% and 3.3%, respectively), but this difference was not statistically significant (Table 3). Nevertheless, VMEs were proportionally more frequent in centers using the EUCAST criteria (0.7% versus 3.0% in centers using CLSI and EUCAST criteria, respectively).

Ability of the centers to infer the resistance mechanisms. The results for the control strains were as follows: (i) ATCC 25922 *E. coli* (CCSRC12) was recognized as a susceptible strain in all cases

FIG 1 Resistance mechanisms inferred by participating laboratories. Shown are control strains (A), wild-type ESB-CTX-M-harboring strains (B), and CTX-M variants with reduced susceptibility to BLBLIs (C). Lowercase letters represent the other resistance mechanisms: a, TEM-1/2, OXA-1/IRT, TEM hyperexpression, and porin alteration; b, chromosomal AmpC/TEM hyperexpression. In the CTX-M variant-harboring strains, centers reported ESB phenotypes despite showing BLBLI resistance.

(100%), (ii) plasmid AmpC-producer strain (CCSRC14) was correctly identified in 90.5% (57/63) of the centers, while 7.9% (5/63) of the centers inferred the simultaneous presence of a chromosomal AmpC β -lactamase and ESB, and one center did not suggest any resistance mechanisms, and (iii) the IRT-2 (CCSRC13) strain was correctly identified in 54.0% (34/63) of the laboratories, although 20.6% (13/63) suggested penicillinase hyperproduction, and other centers, representing 25.4% (16/63), did not report any resistance mechanisms. Figure 1 details the resistance mechanisms reported by the participating centers.

Globally, the wild-type CTX-M- β -lactamase producers (CCSRC01, CCSRC02, CCSRC05, CCSRC06, CCSRC09, and CCSRC10) were correctly detected in 91.8% (56/63, 60/62, 61/62, 57/59, 50/62, and 51/57, respectively) of the centers reporting data. In 5.5% of these combinations, the presence of an ESB was presumed to be associated with penicillinase hyperproduction and/or membrane porin alteration. One center reported a CMT phenotype in the case of the CCSRC10 strain (an *E. coli* *OmpF*⁻ strain expressing CTX-M-14). Additionally, wrong genotypes were suggested in six cases (1.6%) (two carbapenemases and four ESB negative). On <1% of occasions (3/365), a laboratory did not include any comments on resistance mechanism(s).

Different results were obtained in the four strains lacking the *OmpF* porin and harboring CTX-M variants with mutations conferring decreased susceptibilities to BLBLI combinations. Strains with a pure inhibitor-resistant phenotype (IR-CTX-M), corre-

sponding to S130G mutants (CTX-M-1_{S130G} [CCSRC03] and CTX-M-15_{S130G} [CCSRC07]) were identified as inhibitor-resistant β -lactamase producers (mostly IRT) in 67.7% (44/62 and 40/62, for CCSRC03 and CCSRC07, respectively) of the centers reporting data. Wrong phenotypes were reported in 9.7% of these strains (six incorrect phenotypes in each one), which on 11 occasions were attributed to an ESB phenotype and in one center was inferred as representing CMT production (CCSRC03 strain).

In strains with a complex mutant phenotype (CM-CTX-M), which included two types of mutants (CTX-M-1_{S237G} [CCSRC04] and CTX-M-14_{K234R} [CCSRC11]), none of the centers suggested this phenotype, despite the AMC and TZP MICs being in the resistant category on many occasions. The ESB phenotype was the most commonly inferred. The strain harboring CTX-M-1_{S237G} was mostly identified as an ESB producer (79.4% [50/63]). An association of ESBs with other resistance mechanisms (such as chromosomal AmpC β -lactamase and penicillinase hyperproduction) was reported in 14.3% (9/63) of the centers. A wrong phenotype was identified in 1.6% (1/63) of the centers, and 4.8% (3/63) did not report any resistance mechanisms. The strain harboring CTX-M-14_{K234R} was identified in 51.6% (32/62) of the centers as expressing an ESB phenotype. In 30.6% (19/62) of the centers, ESB production was suspected in combination with other resistance mechanisms (as in the previous cases). Wrong phenotypes were suggested on seven occasions (in 5 cases, a penicillinase was reported, and in 2 cases, the resistance phenotype

was not detected). No comments were included for 6.4% (4/62) of the centers.

In the strain with a double enzymatic mechanism (i.e., an ESBL phenotype as a consequence of harboring a CTX-M-15 enzyme and BLBLI phenotype due to the presence of the OXA-1 enzyme [CCSRC08 strain]), an adequate identification was observed in only 27.0% (17 out of 63) of the centers, which reported an association of an ESBL with other resistance mechanisms (chromosomal AmpC β -lactamase or penicillinase hyperproduction, such as OXA or TEM). However, most of the times (73.0% [46/63]), an ESBL producer was identified despite displayed resistance to AMC.

DISCUSSION

The increasing complexity, spread, and clinical implications of antimicrobial resistance mechanisms deserve expert knowledge and make the pursuit of further ASTs one of the most important and specialized tasks in clinical microbiology laboratories (20). In ASTs, proficiency studies are useful for improving expertise in the detection of resistance mechanisms and consequently for making rational decisions regarding antimicrobial use. This work was motivated by a previous study in which CTX-M variants with resistances or reduced susceptibilities to BLBLI combinations were readily obtained under *in vitro* conditions (5). It was shown that single mutations in the *bla*_{CTX-M} genes gave rise to CTX-M variants with IR-CTX-M (carrying the S130G mutation) or CM-CTX-M phenotypes (carrying the S237G or K234R mutation), like other β -lactamase types, such as IRTs (9), or inhibitor-resistant (IR)-SHV (21, 22). However, IR-CTX-M and CM-CTX-M enzymes have not yet emerged or been recognized in the clinical setting. The absence of selection of these CTX-M variants in nature might be explained by the coexistence of several mechanisms conferring BLBLI resistance, such as (i) the simultaneous presence of CTX-M enzymes and loss of porin channels, which are easily selectable under natural conditions (9), (ii) an association among CTX-M β -lactamases and other enzymatic mechanisms of BLBLI resistance, such as CTX-M-15 and OXA-1 (2, 23), and (iii) other mechanisms, such as the hyperproduction of AmpC-type β -lactamases, which might evolve in the populations exposed to BLBLIs (6). National surveillance programs directed toward characterizing the mechanisms of resistance to BLBLIs did not reveal the presence of CTX-M variants involved in the inhibitor-resistant phenotype, suggesting that these CTX-M variants, if they exist, must be in a very low proportion (6, 24). The lack of detection of these CTX-M variants in the clinical setting does not necessarily mean they are really absent, due to the predominant interpretation of similar phenotypes that are attributed to IRTs and IR-SHVs (25). It is of note that in some national surveillance programs, nearly 5% of AMC-resistant *E. coli* isolates had unknown resistance mechanisms (26). Moreover, the complex effects, such as antagonistic pleiotropy, observed in IR-CTX-M enzymes, significantly reduce the oxyimino-cephalosporin MICs and result in unexpected phenotypes in which the possible mechanism(s) of resistance is even more difficult to infer (6, 27, 28).

In our external proficiency multicenter study, participant laboratories variably deduced the presence of an ESBL in strains harboring CTX-M_{S130G}, CTX-M_{K234R}, and CTX-M_{S237G} (8.9%, 51.6%, and 79.4%, respectively), in spite of their reduced susceptibilities to BLBLIs. A combination of an ESBL with another resistance mechanism was attributed to the resistance toward BLBLI

combinations and oxyimino-cephalosporins in CTX-M_{K234R}- and CTX-M_{S237G}-producing strains (30.7% and 14.3%, respectively). Surprisingly, the complex mutant phenotype (CM type) was only suspected in a strain carrying natural CTX-M-14 (CCSRC10) in an OmpF⁻ background.

Previously, CLSI or national breakpoints (19) were followed in Spain, but currently, the EUCAST criteria are increasingly being introduced (25% in our study compared with 14% in a recent published study [29] and 0% in previous ones [30, 31]). Globally, the error rates were slightly higher (but not significant) in centers using the CLSI criteria (11.8%) than in centers following the EUCAST criteria (10.3%), except for the case of VMEs (3.0% of discrepancies when using EUCAST criteria compared to 0.7% in centers that used CLSI criteria).

It is noteworthy that strains classified as susceptible and intermediate by the manual or automatic testing procedures were changed by microbiologists, probably as a precautionary principle, to the resistant category in 80% of the laboratories when a specific resistance mechanism was inferred, representing 38.1% of all categorical errors for the most affected antibiotics (see Table S1 in the supplemental material). Moreover, the majority (80%) of the microbiology laboratories performed changes in clinical category interpretations not currently recommended by either the CLSI or EUCAST committees. Years ago, changes in the interpretive categories were recommended when ESBLs were detected on the assumption that current breakpoints were inadequate (32). With the new CLSI and EUCAST criteria for *Enterobacteriaceae* (see reference 16 and http://www.eucast.org/clinical_breakpoints/), extended-spectrum cephalosporins should be reported as found and considered a valid therapeutic option when interpreted as susceptible, thus preventing the overuse of carbapenems. In addition, this recommendation allows for the detection of resistance without the necessity of delays in reporting AST results when performing confirmatory tests (32). In any case, an important factor is to report resistance or decreased susceptibility to BLBLI combinations when obtained in the laboratory, although the genotype and the corresponding resistance mechanisms are not entirely recognized (8). This problem was not exclusive to the CTX-M variant with reduced susceptibility to BLBLI combinations, but it was also found for the CTX-M-15-plus-OXA-1-producing strain, which was identified as an ESBL producer in 73.2% of the laboratories despite its resistances to AMC and TZP. Susceptibility reports mentioning both types of results should be submitted to clinicians, and the strains should be carefully collected for a more detailed microbiological examination of the involved resistance mechanisms.

In summary, the mechanisms explaining the phenotypes associated with CTX-M β -lactamase variants (IR-CTX-Ms or CM-CTX-Ms) conferring decreased susceptibility to BLBLI combinations remain widely unrecognized and are frequently misidentified as being caused by ESBLs and to a lesser extent as IRTs. The total or partial superposition of all these phenotypes might be responsible in part for the absence of the recognition of these CTX-M variants in the clinical setting. Additionally, the fact that the laboratories frequently do not report resistances to BLBLI combinations when found in ESBL producers might lead to a reduction in the number of isolates detected and consequently reported. Further studies to improve the detection of phenotypes resulting from inhibitor-resistant β -lactamases are needed, as our study demonstrates. A limitation of our study was the failure to include a control strain with a complex mutant phenotype

(CM-TEM) derived from a TEM β -lactamase in order to compare the interpretative results between the CM-CTX-M and CM-TEM producers. The recognition of possible emergent resistance mechanisms, such as IR-CTX-M and CM-CTX-M enzymes, should be immediately followed by proficiency exercises, as was the one carried out in this work, in order to ensure that interventions will be rapid and prevent their uncontrollable spread.

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