A Selective Culture Medium for Screening Ceftazidime-Avibactam Resistance in *Enterobacterales* and *Pseudomonas aeruginosa*

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ABSTRACT The SuperCAZ/AVI medium was developed for screening ceftazidime-avibactam (CZA) resistance among Gram-negative bacteria (*Enterobacterales* and *Pseudomonas aeruginosa*). It was evaluated using 50 CZA-susceptible and 42 CZA-resistant Gram-negative isolates. Its sensitivity and specificity of detection were 100%. Excellent performance of the medium was also observed by testing spiked stools, with the lower limit of detection ranging from 10¹ to 10² CFU/ml. This screening medium provides the opportunity to detect CZA-resistant isolates regardless of their resistance mechanisms.

KEYWORDS ceftazidime, avibactam, screening, *Enterobacterales*, *Pseudomonas aeruginosa*

The emergence and spread of β -lactam resistance, especially resistance to carbapenems, are currently of great concern worldwide, particularly in *Enterobacterales* and *Pseudomonas aeruginosa* (1). Among the recently developed agents active against multidrug-resistant Gram-negative pathogens, a novel drug combination has been launched, namely, ceftazidime-avibactam (CZA) (2). Avibactam (AVI) is a non- β -lactam- β -lactamase inhibitor that inhibits the activities of Ambler class A, class C, and some class D β -lactamases, including carbapenemases (e.g., KPC, OXA-48) (3, 4). However, acquired resistance to CZA is increasingly reported and is mostly related to amino acid substitutions in the active sites of the respective β -lactamases. Many studies have identified KPC variants in *Klebsiella pneumoniae*, such as KPC-31, KPC-35, KPC-41, and KPC-50, all conferring resistance to CZA (5–8). Those KPC variants confer acquired resistance to CZA on the corresponding producers mainly as a consequence of decreased inhibitory activity of AVI against those enzymes, but also due to higher hydrolytic efficiency toward ceftazidime (CAZ). In addition, resistance to CZA in Gram-negative bacteria may be related to the production of Ambler class B enzymes (metallo- β -lactamases [MBL]), such as NDM, VIM, and IMP, or of several non-OXA-48-like class D β -lactamases, such as OXA-28 or OXA-32, whose hydrolytic activity includes CAZ but which are not inhibited by AVI (1). Furthermore, CZA resistance may be related to overproduction of efflux pumps and/or porin defects (9). Taking into account the increasing use of the CZA combination and consequently the increasing isolation of CZA-resistant Gram-negative bacteria, we have developed a selective culture medium for screening CZA-resistant isolates among Gram-negative species (*Enterobacterales*, *P. aeruginosa*).

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TABLE 1 Preparation of the SuperCAZ/AVI medium

Compound	Stock solution ^a	Quantity or vol to add ^b	Final concn
CHROMagar Orientation medium		13.2 g	3.3%
Distilled water		400 ml	
Ceftazidime pentahydrate	6 mg/ml in PBS (pH 7.2)	400 μ l	6 μ g/ml
Avibactam sodium hydrate	4 mg/ml in water	400 μ l	4 μ g/ml
ZnSO ₄ ·7H ₂ O	70 mg/ml in water	400 μ l	70 μ g/ml
Daptomycin	10 mg/ml in water	400 μ l	10 μ g/ml
Amphotericin B	5 mg/ml in 10% D-(+)-glucose	400 μ l	5 μ g/ml

^aPBS, phosphate-buffered saline.^bThe volume of 400 ml of SuperCAZ/AVI medium was for 20 plates.

MATERIALS AND METHODS

The SuperCAZ/AVI medium. In the design of our medium (named the SuperCAZ/AVI medium), the necessity of preventing contamination by Gram-positive bacteria and fungi was considered. Based on our experience in the development of screening media (10), the optimal screening medium was based on the CHROMagar Orientation medium (reference RT412; CHROMagar, Paris, France), which is commonly used as a differential medium for the isolation and differentiation of common urinary tract pathogens. The CZA resistance breakpoint is defined as $>8 \mu\text{g/ml}$ for *Enterobacterales* and *P. aeruginosa* with a fixed concentration of AVI (4 $\mu\text{g/ml}$) (11). The optimal final concentration of CAZ was 6 $\mu\text{g/ml}$ with a fixed concentration of AVI at 4 $\mu\text{g/ml}$. Since Gram-positive bacteria, such as *Enterococcus*, *Streptococcus*, and *Staphylococcus* strains, may grow on CHROMagar Orientation medium, daptomycin (code 461375000; Acros Organics) (which can be replaced by vancomycin) was added as an anti-Gram-positive molecule at a final concentration of 10 $\mu\text{g/ml}$. Amphotericin B (code 45590050; Acros Organics) was also added as an antifungal at a final concentration of 5 $\mu\text{g/ml}$. In addition, ZnSO₄ (70 $\mu\text{g/ml}$) was added to enhance the activity of MBL producers (10). The stock solutions of CAZ, AVI, daptomycin, and amphotericin B were prepared as shown in Table 1 and may be kept at -20°C for 1 year. For the preparation of the SuperCAZ/AVI medium, the diluted powder of CHROMagar Orientation medium was autoclaved at 121°C for 15 min. After the medium was cooled for 1 h at 56°C , the antibiotic stock solutions were added (Table 1). The SuperCAZ/AVI plates were stored at 4°C and were protected from direct light exposure before use, for as long as 1 week.

Susceptibility testing. The MICs of CZA were determined using Etest strips (bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates at 37°C , and the results were interpreted according to the latest EUCAST breakpoints for *Enterobacterales* and *P. aeruginosa* (i.e., susceptibility [S], $\leq 8 \mu\text{g/ml}$; resistance [R], $>8 \mu\text{g/ml}$) (Table 2) (10).

RESULTS

A total of 92 isolates of worldwide origin were included in this study to evaluate the performance of the SuperCAZ/AVI medium. The β -lactamase contents of all strains were characterized at the molecular level by PCR and sequencing or, for some isolates, by whole-genome sequencing (Table 2). A total of 50 strains were susceptible to CZA (40 *Enterobacterales*, including *Enterobacter cloacae*, *K. pneumoniae*, and *Escherichia coli*, and 10 *P. aeruginosa* strains), and 42 were resistant to CZA (20 *Enterobacterales*, including *E. cloacae*, *K. pneumoniae*, and *E. coli*, and 22 *P. aeruginosa* strains) (Table 2).

Starting with an optical density of a 0.5 McFarland standard (an inoculum of $\sim 1.5 \times 10^8$ CFU/ml), serial 10-fold dilutions were made in 0.85% saline solution, and 100- μ l aliquots of each dilution were plated onto the SuperCAZ/AVI medium. To quantify the viable bacteria in each dilution step, tryptic soy agar plates were inoculated concomitantly with 100 μ l of each suspension and were incubated overnight at 37°C . Viable colonies were counted the following day. When no growth was observed after 18 h, incubation was extended up to 48 h in order to definitely assess the negativity of the culture. The lower limit of detection for the strains tested was determined using the SuperCAZ/AVI medium.

The sensitivity and specificity cutoff values for the detection of CZA-resistant *Enterobacterales* and *P. aeruginosa* were set at 1×10^3 CFU/ml, i.e., the CZA-resistant isolates recovered on SuperCAZ/AVI medium plates at $<1 \times 10^3$ CFU/ml were considered positive, while the CZA-susceptible isolates grown using an inoculum of $\geq 1 \times 10^3$ CFU/ml were considered negative (10). All the CZA-resistant isolates could be recovered within 24 h on SuperCAZ/AVI medium plates by using an inoculum below the cutoff value of 1×10^3 CFU/ml (1×10^1 to 1×10^2 CFU/ml) (Table 2). In contrast, growth of

TABLE 2 MICs of CZA for the strains tested and detection limits of the SuperCAZ/AVI screening medium

Category and strain	Species	Origin	MIC of CZA ^a (mg/liter)	CZA susceptibility or resistance ^b	Resistance determinant(s)	Lower limit of detection (CFU/ ml) ^c in:	
						Culture	Stools ^c
<i>Enterobacterales</i>							
R1433	<i>Enterobacter cloacae</i>	France	0.19	S	Wild type	>10 ⁸	>10 ⁷
R254	<i>Klebsiella pneumoniae</i>	France	0.064	S	Porin deficiency, SHV, AmpC	10 ⁶	10 ⁶
R1233	<i>Escherichia coli</i>	France	0.5	S	ACC-1	>10 ⁸	>10 ⁷
R1241	<i>Klebsiella pneumoniae</i>	USA	1.5	S	ACT-1	10 ⁶	10 ⁶
R2077	<i>Escherichia coli</i>	Switzerland	0.5	S	ACC-1	10 ⁷	10 ⁷
R1291	<i>Escherichia coli</i>	USA	0.032	S	OXA-1	>10 ⁸	>10 ⁷
R1335	<i>Escherichia coli</i>	France	0.064	S	TEM-1	10 ⁸	≥10 ⁷
R941	<i>Enterobacter cloacae</i>	Switzerland	1.5	S	TEM-1	10 ⁷	10 ⁷
R1906	<i>Escherichia coli</i>	France	0.75	S	SHV-12	>10 ⁸	>10 ⁷
R2180	<i>Enterobacter cloacae</i>	France	2	S	GES-5	>10 ⁸	>10 ⁷
N23	<i>Escherichia coli</i>	Switzerland	0.032	S	CTX-M-15	10 ⁸	>10 ⁷
N41	<i>Escherichia coli</i>	Switzerland	0.064	S	CTX-M-9	10 ⁸	≥10 ⁷
N44	<i>Escherichia coli</i>	France	0.125	S	CTX-M-15	>10 ⁸	>10 ⁷
N71	<i>Escherichia coli</i>	Switzerland	0.032	S	CTX-M-15	>10 ⁸	>10 ⁷
R1039	<i>Escherichia coli</i>	Vietnam	0.25	S	VEB-1, OXA-10, TEM-1	>10 ⁸	>10 ⁷
R1104	<i>Klebsiella pneumoniae</i>	Thailand	0.75	S	VEB-1	>10 ⁸	>10 ⁷
R1103	<i>Klebsiella pneumoniae</i>	Thailand	0.5	S	VEB-1	>10 ⁷	>10 ⁷
R144	<i>Escherichia coli</i>	France	0.75	S	VEB-1	>10 ⁸	>10 ⁷
R1105	<i>Klebsiella pneumoniae</i>	Thailand	0.25	S	VEB-1	>10 ⁸	>10 ⁸
R2658	<i>Escherichia coli</i>	France	0.125	S	VEB-1, TEM-1, OXA-10	>10 ⁸	>10 ⁷
R3659	<i>Escherichia coli</i>	USA	0.5	S	KPC-2 (<i>E. coli</i> DH10B/ pBR322 <i>bla</i> _{KPC-2})	>10 ⁸	>10 ⁸
R99	<i>Klebsiella pneumoniae</i>	France	0.5	S	KPC-2	10 ⁷	10 ⁵
R3521	<i>Klebsiella pneumoniae</i>	Switzerland	1.5	S	KPC-2	10 ⁵	10 ⁶
R3668	<i>Escherichia coli</i>	USA	0.064	S	KPC-2 [<i>E. coli</i> DH10B/ pBC SK(+) <i>bla</i> _{KPC-2}]	>10 ⁸	>10 ⁸
R82	<i>Escherichia coli</i>	France	0.047	S	KPC-2	>10 ⁸	>10 ⁷
R91	<i>Klebsiella pneumoniae</i>	France	0.75	S	KPC-2	>10 ⁸	>10 ⁷
R94	<i>Klebsiella pneumoniae</i>	France	2	S	KPC-2	>10 ⁸	≥10 ⁷
R3485	<i>Klebsiella pneumoniae</i>	Switzerland	1	S	KPC-2	10 ⁶	10 ⁶
R3486	<i>Klebsiella pneumoniae</i>	Switzerland	1	S	KPC-2	10 ⁸	>10 ⁷
R3488	<i>Klebsiella pneumoniae</i>	Switzerland	1	S	KPC-2	10 ⁷	>10 ⁷
R3522	<i>Klebsiella pneumoniae</i>	Switzerland	1.5	S	KPC-2	>10 ⁸	>10 ⁷
R132	<i>Klebsiella pneumoniae</i>	France	1	S	KPC-2	>10 ⁸	>10 ⁷
R297	<i>Klebsiella pneumoniae</i>	France	0.25	S	KPC-2, OXA-1	>10 ⁸	>10 ⁷
R100	<i>Klebsiella pneumoniae</i>	France	1.5	S	KPC-11	10 ⁸	>10 ⁷
R22	<i>Escherichia coli</i>	France	0.094	S	OXA-48	>10 ⁸	>10 ⁷
R740	<i>Escherichia coli</i>	The Netherlands	1	S	OXA-48	>10 ⁸	>10 ⁷
R19	<i>Klebsiella pneumoniae</i>	France	0.5	S	OXA-48	10 ⁷	10 ⁶
R23	<i>Klebsiella pneumoniae</i>	France	0.5	S	OXA-48	10 ⁷	10 ⁷
N59	<i>Escherichia coli</i>	Switzerland	0.023	S	OXA-181	>10 ⁸	>10 ⁷
R131	<i>Klebsiella pneumoniae</i>	France	1.5	S	OXA-181	10 ⁸	>10 ⁷
R3338	<i>Klebsiella pneumoniae</i>	USA	24	R	CMY-4, VIM-1	10 ²	10 ¹
R169	<i>Klebsiella pneumoniae</i>	USA	24	R	VIM-19	10 ¹	10 ¹
N284	<i>Enterobacter cloacae</i>	Switzerland	48	R	VIM-1	10 ¹	10 ¹
R48	<i>Klebsiella pneumoniae</i>	France	>256	R	VIM-1	10 ¹	10 ¹
R61	<i>Escherichia coli</i>	France	24	R	VIM-1, SHV-12	10 ²	10 ¹
R63	<i>Klebsiella pneumoniae</i>	France	24	R	VIM-19	10 ¹	10 ¹
N6	<i>Escherichia coli</i>	Switzerland	>256	R	NDM-5	10 ²	10 ²
R464	<i>Escherichia coli</i>	France	>256	R	NDM-4, OXA-1	10 ¹	10 ¹
R466	<i>Escherichia coli</i>	France	>256	R	NDM-4, OXA-1, CTX-M-15	10 ²	10 ¹
R3778	<i>Klebsiella pneumoniae</i>	Spain	48	R	KPC-3 D179Y	10 ¹	10 ¹
R3780	<i>Klebsiella pneumoniae</i>	Spain	>256	R	KPC-3 G168N E169H	10 ¹	10 ¹
R3781	<i>Klebsiella pneumoniae</i>	Spain	64	R	KPC-3 E169P L172T	10 ¹	10 ¹
R3776	<i>Klebsiella pneumoniae</i>	Spain	96	R	KPC-3 D179Y	10 ¹	10 ¹
R3777	<i>Klebsiella pneumoniae</i>	Spain	>256	R	KPC-3 D179Y A172T	10 ¹	10 ¹
N435	<i>Klebsiella pneumoniae</i>	Switzerland	>256	R	KPC-41	10 ¹	10 ¹
N859	<i>Klebsiella pneumoniae</i>	Switzerland	>256	R	KPC-50	10 ¹	10 ¹

(Continued on next page)

TABLE 2 (Continued)

Category and strain	Species	Origin	MIC of CZA ^a (mg/liter)	CZA susceptibility or resistance ^b	Resistance determinant(s)	Lower limit of detection (CFU/ ml) ^c in:	
						Culture	Stools ^c
R3671	<i>Escherichia coli</i>	USA	>128	R	KPC-2 (<i>E. coli</i> DH10B/ pBR322 KPC-2 D179M)	10 ²	10 ²
R3779	<i>Klebsiella pneumoniae</i>	Spain	128	R	KPC-3 D179Y	10 ²	10 ²
R72	<i>Escherichia coli</i>	France	128	R	IMP-1	10 ¹	10 ¹
R73	<i>Klebsiella pneumoniae</i>	France	>256	R	IMP-1	10 ¹	10 ¹
<i>Pseudomonas aeruginosa</i>							
R1553	<i>Pseudomonas aeruginosa</i>	France	1.5	S	None (wild type)	>10 ⁸	10 ⁷
R2267	<i>Pseudomonas aeruginosa</i>	France	0.75	S	None (wild type)	>10 ⁸	10 ⁷
N382	<i>Pseudomonas aeruginosa</i>	Switzerland	0.38	S	None (wild type)	10 ⁶	10 ⁷
N339	<i>Pseudomonas aeruginosa</i>	Switzerland	0.5	S	None (wild type)	>10 ⁸	10 ⁷
N146	<i>Pseudomonas aeruginosa</i>	Switzerland	4	S	GES-5	10 ⁴	10 ⁶
N254	<i>Pseudomonas aeruginosa</i>	Switzerland	1	S	None (wild type)	>10 ⁸	10 ⁸
N214	<i>Pseudomonas aeruginosa</i>	Switzerland	0.5	S	None (wild type)	>10 ⁸	10 ⁷
R1187	<i>Pseudomonas aeruginosa</i>	Belgium	4	S	BEL-2	10 ⁶	10 ⁵
R1188	<i>Pseudomonas aeruginosa</i>	Brazil	2	S	CTX-M-2	>10 ⁸	>10 ⁷
R3451	<i>Pseudomonas aeruginosa</i>	France	1	S	GES-6	10 ⁶	10 ⁷
R3680	<i>Pseudomonas aeruginosa</i>	USA	24	R	Unknown mechanism	10 ¹	10 ²
R3681	<i>Pseudomonas aeruginosa</i>	USA	32	R	Unknown mechanism	10 ¹	10 ¹
R3682	<i>Pseudomonas aeruginosa</i>	USA	64	R	Unknown mechanism	10 ¹	10 ¹
R3683	<i>Pseudomonas aeruginosa</i>	USA	>256	R	Unknown mechanism	10 ¹	10 ¹
R1308	<i>Pseudomonas aeruginosa</i>	France	>256	R	OXA-28	10 ¹	10 ²
R1311	<i>Pseudomonas aeruginosa</i>	France	12	R	OXA-32	10 ¹	10 ¹
R609	<i>Pseudomonas aeruginosa</i>	Turkey	64	R	VIM-2	10 ¹	10 ¹
R50	<i>Pseudomonas aeruginosa</i>	France	24	R	VIM-2	10 ²	10 ¹
R51	<i>Pseudomonas aeruginosa</i>	France	>256	R	VIM-2	10 ¹	10 ¹
R52	<i>Pseudomonas aeruginosa</i>	France	16	R	VIM-2	10 ¹	10 ¹
R54	<i>Pseudomonas aeruginosa</i>	France	>256	R	VIM-2	10 ²	10 ¹
R598	<i>Pseudomonas aeruginosa</i>	France	24	R	VIM-2	10 ¹	10 ¹
R599	<i>Pseudomonas aeruginosa</i>	France	16	R	VIM-2	10 ¹	10 ¹
R600	<i>Pseudomonas aeruginosa</i>	Japan	16	R	VIM-2	10 ²	10 ¹
R604	<i>Pseudomonas aeruginosa</i>	The Netherlands	12	R	VIM-2	10 ¹	10 ¹
R608	<i>Pseudomonas aeruginosa</i>	France	16	R	VIM-2	10 ²	10 ²
R610	<i>Pseudomonas aeruginosa</i>	France	32	R	VIM-2	10 ²	10 ²
N885	<i>Pseudomonas aeruginosa</i>	Switzerland	>256	R	NDM-1	10 ¹	10 ¹
N520	<i>Pseudomonas aeruginosa</i>	Switzerland	>256	R	NDM-1	10 ¹	10 ¹
N521	<i>Pseudomonas aeruginosa</i>	Switzerland	>256	R	NDM-1	10 ¹	10 ¹
R186	<i>Pseudomonas aeruginosa</i>	France	16	R	NDM-6	10 ²	10 ²
R2760	<i>Pseudomonas aeruginosa</i>	France	>256	R	NDM-1	10 ²	10 ¹

^aCZA, ceftazidime-avibactam. MICs of CZA were determined using Etest.^bR, resistant; S, susceptible.^cUnderlined CFU counts are considered negative results (cutoff values were set at >10³ CFU/ml).

the CZA-susceptible isolates was possible only when an inoculum of >10³ CFU/ml was used (the lower limit of detection was above the cutoff value of 10³ CFU/ml), giving rise to 100% sensitivity and specificity.

Spiked stools were also tested with the same representative collection of CZA-resistant and -susceptible Gram-negative bacteria ($n = 92$) using this selective culture medium. Spiked fecal samples were made by adding 100 μ l of serial 10-fold bacterial dilutions to 900 μ l of a stool suspension. Stool suspensions were obtained by suspending 6 g of freshly pooled feces from healthy volunteers in 60 ml of distilled water as described previously (10). Aliquots (100 μ l) of the spiked stool suspension were inoculated onto the SuperCAZ/AVI medium. Aliquots (100 μ l) of stool suspensions with no bacteria added were plated onto the SuperCAZ/AVI medium as negative controls. The lower limit of detection was below the cutoff value for all CZA-resistant strains with which stools were spiked, ranging from 10¹ to 10² CFU/ml, whereas the lower limit of detection for the CZA-susceptible strains was above the cutoff value, at $\geq 10^6$ CFU/ml (Table 2). Sensitivity and specificity were determined using the same cutoff value, set

at 10^3 CFU/ml (10). Again, the sensitivity and specificity of the SuperCAZ/AVI medium for isolating CZA-resistant isolates were both 100%.

To assess the storage stability of the SuperCAZ/AVI medium, *Candida albicans* and *Staphylococcus aureus* strains, as well as the CZA-susceptible *E. coli* ATCC 25955 reference strain, were subcultured daily onto the SuperCAZ/AVI medium from a single batch of medium stored at 4°C. No growth was observed consistently for at least a 7-day period.

DISCUSSION

The SuperCAZ/AVI medium constitutes an adequate screening medium for the detection of CZA-resistant bacteria regardless of their resistance mechanisms. This SuperCAZ/AVI medium may be used for the screening of patients potentially colonized with CZA-resistant strains in order to rapidly implement infection control measures aimed at limiting their spread. This medium is also adequate for epidemiological surveys aiming to evaluate the prevalence of CZA-resistant Gram-negative bacteria in a given population. Further clinical evaluation of the proposed medium in daily clinical practice is needed. It may be useful for rapid identification of outbreaks of CZA-resistant strains, such as those reported in the United States (12) and Italy (13).

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