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 $10^1$ to $10^2$ 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).
Table 1: Preparation of the SuperCAZ/AVI medium

| Compound                        | Stock solution | Quantity or vol to add | Final concn |
|---------------------------------|----------------|------------------------|-------------|
| CHROMagar Orientation medium    | 13.2 g         | 3.3%                   |             |
| Distilled water                 | 400 ml         |                        |             |
| Cefazidime pentahydrate         | 6 mg/ml in PBS (pH 7.2) | 400 µl | 6 µg/ml |
| Avibactam sodium hydrate        | 4 mg/ml in water | 400 µl | 4 µg/ml |
| ZnSO₄·7H₂O                      | 70 mg/ml in water | 400 µl | 70 µg/ml |
| Daptomycin                      | 10 mg/ml in water | 400 µl | 10 µg/ml |
| Amphotericin B                  | 5 mg/ml in 10% D- (+)-glucose | 400 µl | 5 µg/ml |

*PBS, phosphate-buffered saline.

**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 \( \geq 8 \mu g/ml \) for Enterobacterales and P. aeruginosa with a fixed concentration of AVI (4 µg/ml) (11). The optimal final concentration of CAZ was 6 µg/ml with a fixed concentration of AVI at 4 µg/ml. Since Gram-positive bacteria, such as Enterococcus, Streptococcus, and Staphylococcus strains, may grow on CHROMagar Orientation medium, daptomycin (code 46137500; Acros Organics) (which can be replaced by vancomycin) was added as an anti-Gram-positive molecule at a final concentration of 10 µg/ml. Amphotericin B (code 45590050; Acros Organics) was also added as an antifungal at a final concentration of 5 µg/ml. In addition, ZnSO₄ (70 µg/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 \(-20^\circ\)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], \( \leq 8 \mu g/ml \); resistance [R], \( >8 \mu g/ml \)) (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-µl 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 µl 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 \( 1 \times 10^3 \) 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 \( 1 \times 10^3 \) CFU/ml (1 \times 10⁰ to 1 \times 10² CFU/ml) (Table 2). In contrast, growth of
| Category and strain | Species            | MIC of CZAa (mg/liter) | CZA susceptibility or resistanceb | Resistance determinant(s) | Lower limit of detection (CFU/ml)c in: | Culture | Stoolsd |
|---------------------|--------------------|-------------------------|----------------------------------|---------------------------|----------------------------------------|---------|---------|
| **Enterobacterales** |                    |                         |                                  |                           |                                        |         |         |
| R1433               | Enterobacter cloacae| France                  | 0.19                             | S                         | Wild type                             | >10⁸    | >10⁷    |
| R254                | Klebsiella pneumoniae| France              | 0.064                           | S                         | Porin deficiency, SHV, AmpC           | 10⁸     | 10⁸     |
| R1233               | Escherichia coli    | France                  | 0.5                              | S                         | ACC-1                                  | >10⁸    | >10⁷    |
| R1241               | Klebsiella pneumoniae| USA                   | 1.5                              | S                         | ACT-1                                  | 10⁸     | 10⁸     |
| R2077               | Escherichia coli    | Switzerland            | 0.5                              | S                         | ACC-1                                  | 10⁸     | 10⁸     |
| R1291               | Escherichia coli    | USA                     | 0.032                           | S                         | OXA-1                                  | >10⁸    | >10⁷    |
| R1335               | Escherichia coli    | France                  | 0.064                           | S                         | TEM-1                                  | 10⁸     | 10⁸     |
| R941                | Enterobacter cloacae| Switzerland            | 1.5                              | S                         | TEM-1                                  | >10⁸    | >10⁷    |
| R1906               | Escherichia coli    | France                  | 0.75                             | S                         | SHV-12                                 | >10⁸    | >10⁷    |
| R2180               | Enterobacter cloacae| France                  | 2.0                              | S                         | GES-5                                  | >10⁸    | >10⁷    |
| N23                 | Escherichia coli    | Switzerland            | 0.032                           | S                         | CTX-M-15                               | 10⁸     | >10⁷    |
| N41                 | Escherichia coli    | Switzerland            | 0.064                           | S                         | CTX-M-9                                | 10⁸     | >10⁷    |
| N71                 | Escherichia coli    | Switzerland            | 0.125                           | S                         | CTX-M-15                               | >10⁸    | >10⁷    |
| R1039               | Escherichia coli    | Switzerland            | 0.032                           | S                         | CTX-M-15                               | >10⁸    | >10⁷    |
| R1104               | Klebsiella pneumoniae| Thailand            | 0.25                             | S                         | VEB-1, OXA-10, TEM-1                   | >10⁸    | >10⁷    |
| R1103               | Klebsiella pneumoniae| Thailand            | 0.75                             | S                         | VEB-1                                  | >10⁸    | >10⁷    |
| R144                | Escherichia coli    | France                  | 0.75                             | S                         | VEB-1                                  | >10⁸    | >10⁷    |
| R1105               | Klebsiella pneumoniae| Thailand            | 0.25                             | S                         | VEB-1                                  | >10⁸    | >10⁷    |
| R2658               | Escherichia coli    | France                  | 0.125                           | S                         | VEB-1, TEM-1, OXA-10                   | >10⁸    | >10⁷    |
| R3659               | Escherichia coli    | USA                     | 0.5                              | S                         | KPC-2 (E. coli DH10B/pBRL322 b(la_KPC-2) | >10⁸    | >10⁷    |
| R89                 | Klebsiella pneumoniae| France              | 0.047                           | S                         | KPC-2                                  | 10⁸     | >10⁷    |
| R3521               | Klebsiella pneumoniae| Switzerland       | 1.5                              | S                         | KPC-2                                  | 10⁸     | >10⁷    |
| R3668               | Escherichia coli    | USA                     | 0.064                           | S                         | KPC-2 (E. coli DH10B/pBRL322 b(la_KPC-2) | >10⁸    | >10⁷    |
| R82                 | Escherichia coli    | France                  | 0.047                           | S                         | KPC-2                                  | >10⁸    | >10⁷    |
| R91                 | Klebsiella pneumoniae| France              | 0.75                             | S                         | KPC-2                                  | >10⁸    | >10⁷    |
| R94                 | Klebsiella pneumoniae| France              | 2.0                              | S                         | KPC-2                                  | >10⁸    | >10⁷    |
| R3485               | Klebsiella pneumoniae| Switzerland       | 1.0                              | S                         | KPC-2                                  | 10⁸     | >10⁷    |
| R3486               | Klebsiella pneumoniae| Switzerland       | 1.0                              | S                         | KPC-2                                  | 10⁸     | >10⁷    |
| R3488               | Klebsiella pneumoniae| Switzerland       | 1.0                              | S                         | KPC-2                                  | 10⁸     | >10⁷    |
| R3522               | Klebsiella pneumoniae| Switzerland       | 1.5                              | S                         | KPC-2                                  | >10⁸    | >10⁷    |
| R132                | Klebsiella pneumoniae| France              | 1.0                              | S                         | KPC-2                                  | >10⁸    | >10⁷    |
| R297                | Klebsiella pneumoniae| France              | 0.25                             | S                         | KPC-2, OXA-1                           | >10⁸    | >10⁷    |
| R100                | Klebsiella pneumoniae| France              | 1.5                              | S                         | KPC-11                                 | 10⁸     | >10⁷    |
| R22                 | Escherichia coli    | France                  | 0.094                           | S                         | OXA-48                                 | >10⁸    | >10⁷    |
| R740                | Escherichia coli    | The Netherlands        | 1.0                              | S                         | OXA-48                                 | >10⁸    | >10⁷    |
| R19                 | Klebsiella pneumoniae| France              | 0.5                              | S                         | OXA-48                                 | >10⁸    | >10⁷    |
| R23                 | Klebsiella pneumoniae| France              | 0.5                              | S                         | OXA-48                                 | 10⁸     | 10²     |
| N59                 | Escherichia coli    | Switzerland            | 0.023                           | S                         | OXA-181                                | >10⁸    | >10⁷    |
| R131                | Klebsiella pneumoniae| France              | 1.5                              | S                         | OXA-181                                | 10⁸     | >10⁷    |
| R3338               | Klebsiella pneumoniae| USA                   | 24.0                             | R                         | CMY-4, VIM-1                           | 10⁸     | 10¹     |
| R169                | Klebsiella pneumoniae| USA                   | 24.0                             | R                         | VIM-19                                 | 10¹     | 10¹     |
| N284                | Enterobacter cloacae| Switzerland            | 48.0                             | R                         | VIM-1                                  | 10¹     | 10¹     |
| R48                 | Klebsiella pneumoniae| France              | >256.0                           | R                         | VIM-1                                  | 10¹     | 10¹     |
| R61                 | Escherichia coli    | France                  | 24.0                             | R                         | VIM-1, SHV-12                         | 10²     | 10¹     |
| R63                 | Klebsiella pneumoniae| France              | 24.0                             | R                         | VIM-19                                 | 10¹     | 10¹     |
| N6                  | Escherichia coli    | Switzerland            | >256.0                           | R                         | NDM-5                                  | 10¹     | 10¹     |
| R464                | Escherichia coli    | France                  | >256.0                           | R                         | NDM-4, OXA-1                           | 10¹     | 10¹     |
| R466                | Escherichia coli    | France                  | >256.0                           | R                         | NDM-4, OXA-1, CTX-M-15                 | 10²     | 10¹     |
| R3778               | Klebsiella pneumoniae| Spain                 | 48.0                             | R                         | KPC-3 D179Y                            | 10¹     | 10¹     |
| R3780               | Klebsiella pneumoniae| Spain                 | >256.0                           | R                         | KPC-3 G168N E169H                      | 10¹     | 10¹     |
| R3781               | Klebsiella pneumoniae| Spain                 | 64.0                             | R                         | KPC-3 E169P L172T                     | 10¹     | 10¹     |
| R3776               | Klebsiella pneumoniae| Spain                 | 96.0                             | R                         | KPC-3 D179Y                            | 10¹     | 10¹     |
| R3777               | Klebsiella pneumoniae| Spain                 | >256.0                           | R                         | KPC-3 D179Y A172T                     | 10¹     | 10¹     |
| N435                | Klebsiella pneumoniae| Switzerland          | >256.0                           | R                         | KPC-41                                 | 10¹     | 10¹     |
| N859                | Klebsiella pneumoniae| Switzerland          | >256.0                           | R                         | KPC-50                                 | 10¹     | 10¹     |

(Continued on next page)
the CZA-susceptible isolates was possible only when an inoculum of \( >10^3 \) CFU/ml was used (the lower limit of detection was above the cutoff value of \( 10^3 \) 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 \( \mu l \) of serial 10-fold bacterial dilutions to 900 \( \mu l \) 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 \( \mu l \)) of the spiked stool suspension were inoculated onto the SuperCAZ/AVI medium. Aliquots (100 \( \mu l \)) 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 \( 10^1 \) to \( 10^2 \) 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

### 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) in: | Culture | Stools\(^c\) |
|---------------------|--------------------------------|--------|-----------------------------|----------------------------------------|-------------------------------|--------------------------------------|---------|-------------|
| R3671               | Escherichia coli               | USA    | >128                        | R                                      | KPC-2 (E. coli DH10B/pBR322 KPC-2 D179M) | \( 10^2 \) | \( 10^2 \) |
| R3779               | Klebsiella pneumoniae          | Spain  | 128                         | R                                      | KPC-3 D179Y                   | \( 10^2 \) | \( 10^2 \) |
| R72                 | Escherichia coli               | France | 128                         | R                                      | IMP-1                         | \( 10^1 \) | \( 10^1 \) |
| R73                 | Klebsiella pneumoniae          | France | >256                        | R                                      | IMP-1                         | \( 10^1 \) | \( 10^1 \) |

**Pseudomonas aeruginosa**

| R1553               | Pseudomonas aeruginosa         | France | 1.5                         | S                                      | None (wild type)              | \( >10^6 \) | \( >10^7 \) |
| R2267               | Pseudomonas aeruginosa         | France | 0.75                        | S                                      | None (wild type)              | \( >10^6 \) | \( >10^7 \) |
| N382                | Pseudomonas aeruginosa         | Switzerland | 0.38 | S | None (wild type) | \( >10^6 \) | \( >10^7 \) |
| N339                | Pseudomonas aeruginosa         | Switzerland | 0.5  | S | None (wild type) | \( >10^6 \) | \( >10^7 \) |
| N146                | Pseudomonas aeruginosa         | Switzerland | 4   | S | GES-5            | \( 10^6 \) | \( 10^7 \) |
| N254                | Pseudomonas aeruginosa         | Switzerland | 1   | S | None (wild type) | \( >10^6 \) | \( >10^7 \) |
| N214                | Pseudomonas aeruginosa         | Switzerland | 0.5  | S | None (wild type) | \( >10^6 \) | \( >10^7 \) |
| R1187               | Pseudomonas aeruginosa         | Belgium | 4   | S | BEL-2            | \( 10^6 \) | \( 10^7 \) |
| R1188               | Pseudomonas aeruginosa         | Brazil  | 2   | S | CTX-M-2          | \( >10^6 \) | \( >10^7 \) |
| R3451               | Pseudomonas aeruginosa         | France  | 1   | S | GES-6            | \( 10^6 \) | \( 10^7 \) |
| R3680               | Pseudomonas aeruginosa         | USA     | 24  | R | Unknown mechanism | \( 10^6 \) | \( 10^7 \) |
| R3681               | Pseudomonas aeruginosa         | USA     | 32  | R | Unknown mechanism | \( 10^6 \) | \( 10^7 \) |
| R3682               | Pseudomonas aeruginosa         | USA     | 64  | R | Unknown mechanism | \( 10^6 \) | \( 10^7 \) |
| R3683               | Pseudomonas aeruginosa         | USA     | >256 | R | Unknown mechanism | \( 10^6 \) | \( 10^7 \) |
| R1308               | Pseudomonas aeruginosa         | France  | >256 | R | OXA-28           | \( 10^6 \) | \( 10^7 \) |
| R1311               | Pseudomonas aeruginosa         | France  | 12  | R | OXA-32           | \( 10^6 \) | \( 10^7 \) |
| R609                | Pseudomonas aeruginosa         | Turkey  | 64  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R50                 | Pseudomonas aeruginosa         | France  | 24  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R51                 | Pseudomonas aeruginosa         | France  | >256 | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R52                 | Pseudomonas aeruginosa         | France  | 16  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R54                 | Pseudomonas aeruginosa         | France  | >256 | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R598                | Pseudomonas aeruginosa         | France  | 24  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R599                | Pseudomonas aeruginosa         | France  | 16  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R600                | Pseudomonas aeruginosa         | Japan    | 16  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R604                | Pseudomonas aeruginosa         | The Netherlands | 12  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R608                | Pseudomonas aeruginosa         | France  | 16  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| R610                | Pseudomonas aeruginosa         | France  | 32  | R | VIM-2            | \( 10^6 \) | \( 10^7 \) |
| N885                | Pseudomonas aeruginosa         | Switzerland | >256 | R | NDM-1           | \( 10^6 \) | \( 10^7 \) |
| N520                | Pseudomonas aeruginosa         | Switzerland | >256 | R | NDM-1           | \( 10^6 \) | \( 10^7 \) |
| N521                | Pseudomonas aeruginosa         | Switzerland | >256 | R | NDM-1           | \( 10^6 \) | \( 10^7 \) |
| R186                | Pseudomonas aeruginosa         | France  | 16  | R | NDM-6           | \( 10^6 \) | \( 10^7 \) |
| R2760               | Pseudomonas aeruginosa         | France  | >256 | R | NDM-1           | \( 10^6 \) | \( 10^7 \) |

\(^a\)CZA, ceftazidime-avibactam. MICs of CZA were determined using Etest.

\(^b\)R, resistant; S, susceptible.

\(^c\)Underlined CFU counts are considered negative results (cutoff values were set at \( >10^7 \) CFU/ml).
at $10^8$ 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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