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Short Communication

Comparison of methods to analyse susceptibility of German MDR/XDR *Pseudomonas aeruginosa* to ceftazidime/avibactam

Frieder Schaumburg[a,*], Stefan Bletz[b], Alexander Mellmann[a,b], Karsten Becker[a], Evgeny A. Idelevich[a]

[a] Institute of Medical Microbiology, University Hospital Münster, Münster, Germany
[b] Institute for Hygiene, University Hospital Münster, Münster, Germany

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ABSTRACT

Ceftazidime/avibactam (CZA) is a new β-lactam/β-lactamase inhibitor combination with promising properties as avibactam can inhibit a broad range of β-lactamases (e.g. bla_{KPC}, bla_{OXA-48}). The objectives of this study were: (i) to assess CZA susceptibility rates; (ii) to compare gradient and disk diffusion tests with broth microdilution (BMD) for CZA susceptibility testing; and (iii) to study the clonal structure and antimicrobial resistance genes in multi-drug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa*. Isolates (n=192) from routine diagnostics (Germany, 2013–2018) were tested by BMD reference method, gradient diffusion test (Etest, bioMérieux and MIC Test Strip, Liofilchem) and disk diffusion test (MAST and Oxoid). All isolates were whole-genome sequenced to screen for metallo-β-lactamases and to assess the clonal structure using core-genome multi-locus sequence typing. In total, 64.1% of isolates (n=123) were susceptible to CZA (minimum inhibitory concentration required to inhibit the growth of 50% of organisms 8 mg/L, minimum inhibitory concentration required to inhibit the growth of 90% of organisms >256 mg/L, range 0.5–>256 mg/L). Susceptibility rates were higher in MDR (85.0%) than in XDR (49.1%) *P. aeruginosa*. Among commercial susceptibility testing methods, Etest showed highest accuracy in comparison to BMD (essential agreement 94.8%, categorical agreement 94.3%). CZA-resistant isolates (n=69) mainly belonged to ST235 (n=29, bla_{IMP}-positive). In conclusion, CZA is a promising treatment option for infections caused by MDR *P. aeruginosa*. CZA-resistant *P. aeruginosa* mainly belong to the pandemic ST235 high-risk clone. Etest can be considered as an alternative to BMD.

1. Introduction

The increasing antimicrobial resistance rates among *Pseudomonas aeruginosa* are currently one of the biggest challenges in healthcare systems worldwide [1]. New antimicrobial agents, particularly those with new mechanisms of action, are therefore important to maintain treatment options in infections with drug-resistant *P. aeruginosa*.

The most common underlying mechanisms in multi-drug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* are alterations in porin channels, efflux pumps, target modifications and β-lactamases (e.g. AmpC, carbapenemases) [2,3]. The new non-β-lactam β-lactamase inhibitor avibactam is a promising addendum to existing antimicrobial agents, as it has good activity against AmpC and some carbapenemases (KPC, OXA-48), but remains inactive against metallo-β-lactamases (MBL, e.g. NDM, IMP) and some OXA-carbapenemases [2]. The US Food and Drug Administration and European Medicines Agency have approved ceftazidime-avibactam (CZA) for the treatment of complicated abdominal and urinary tract infections, as well as hospital-acquired and ventilator-associated bacterial pneumonia. To appraise the clinical value of CZA, resistance rates of CZA and the distribution of resistance genes are indispensable. However, the plethora of CZA resistance data in *P. aeruginosa* from North America contrasts with the sparse in-vitro data from Europe [4]. Therefore, the objectives of this study were: (i) to assess resistance rates of MDR and XDR *P. aeruginosa* to CZA; (ii) to compare the accuracy of disk and gradient diffusion CZA susceptibility testing with broth microdilution (BMD); and (iii) to analyse resistance genes and clonal structure (i.e. genotypes) of CZA-resistant *P. aeruginosa*.

* Corresponding author. Address: Institute of Medical Microbiology, Domagkstraße 10, 48149 Münster, Germany. Tel.: +49 (0)251 8352752.
E-mail address: frieder.schaumburg@ukmuenster.de (F. Schaumburg).

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2. Materials and methods

2.1. Bacterial isolates

Isolates derived from routine diagnostics at University Hospital Münster [2013–2018, see Table S1 (online supplementary material) for more details]. All isolates are tested routinely using Vitek 2 (bioMérieux, Marcy l'Étoile, France). All isolates that are resistant to at least three out of four bactericidal antimicrobial classes (i.e. piperacillin, ceftazidime/cefepime, meropenem/imipenem and ciprofloxacin) are classed as MDR according to German health regulations [5]. These isolates were retested against a standard set of anti-pseudomonal agents using BMD (Micronaut-S 96-well microtitre plates, Merlin, Bornheim-Hersel, Germany). They were included in the final analysis if they met the criteria for MDR or XDR *P. aeruginosa* according to the US Centers for Disease Control and Prevention/European Centres for Disease Prevention and Control (CDC/ECDC) [3]. Isolates from patients with cystic fibrosis (bias due to frequent and often prolonged antimicrobial treatment), duplicate isolates from one patient and outbreak isolates were excluded.

2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of CZA were determined by the BMD reference method using cation-adjusted Mueller–Hinton broth (BD Diagnostics, Heidelberg, Germany), as recommended by ISO 20776-1 and the Clinical and Laboratory Standards Institute (CLSI) guidelines [6,7]. Ceftazidime was purchased from Sigma-Aldrich (Taufkirchen, Germany), and avibactam was provided by Pfizer Inc. (Peapack, NJ, USA). Ceftazidime was tested in double dilution concentrations from 0.016 to 256 mg/L, while the concentration of avibactam was fixed at 4 mg/L, according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/clinical_breakpoints/) and CLSI [8].

The gradient diffusion method (0.016–256 mg/L) was performed using commercially available assays from two manufacturers: MIC Test Strips (Liofilchem, Roseto degli Abruzzi, Italy) and Etest (bioMérieux). The testing was carried out on Mueller-Hinton agar (BD Mueller Hinton II, BD Diagnostics) according to the instructions of the respective manufacturer. The results were read after 18±2 h of incubation at 35±1°C in ambient air. The MIC results were rounded-up to the next double dilution step for comparison with BMD. Disk diffusion was performed on Mueller-Hinton agar according to EUCAST using 10/4 µg CZA disks from two manufacturers: MAST Diagnostica (Reinfeld, Germany) and Oxoid (Wesel, Germany). All tests were performed in triplicate and median values were calculated for analysis. CZA MICs and inhibition zone diameters were interpreted applying EUCAST clinical breakpoints for *P. aeruginosa* (susceptible: ≤8/4 mg/L or ≥17 mm, respectively; resistant: >8/4 mg/L or <17 mm, respectively).

P. aeruginosa 27853 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control (QC) strains, and were within the QC range throughout the study.

2.3. Whole-genome sequencing

Whole-genome sequencing was undertaken for all isolates to assess the clonal structure of MDR and XDR *P. aeruginosa*, as described recently [9]. Briefly, sequencing was done on an Illumina MiSeq or NextSeq sequencing platform (Illumina Inc., San Diego, CA, USA). De-novo assembly of reads using Velvet Version 1.1.04 and core-genome multi-locus sequence typing (MLST) was done with SeqSphere+ Version 5.1.9 (Ridom GmbH, Münster, Germany) [10]. A minimum spanning tree was constructed based on a gene-by-gene comparison as implemented in SeqSphere+. Conventional MLST sequence types (ST) were deduced in silico. Raw reads are deposited at the European Nucleotide Archive (Accession No. PRJEB30639). Genomes were screened for MBL and OXA carbapenemases (i.e. bla_{AIM}, bla_{CAR}, bla_{CAU}, bla_{DIM}, bla_{FEZ}, bla_{FIM}, bla_{GIM}, bla_{GOB}, bla_{HMB-1}, bla_{KHM-1}, bla_{L1}, bla_{OXA}, bla_{POM}, bla_{SIM}, bla_{SPM}, bla_{THIN-B} andbla_{VIM}). If MBLs or bla_{OXA} variants associated with poor inhibition by avibactam (e.g. bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}) were not detected, these genomes of CZA-resistant isolates were also screened using ResFinder 3.1 [11,12].

2.4. Statistical analysis

Susceptibility rates were calculated for all methods used, as well as MIC required to inhibit the growth of 50% of organisms (MIC_{50}), MIC required to inhibit the growth of 90% of organisms (MIC_{90}) and MIC ranges for BMD and gradient diffusion tests. Rates of very major errors (false-susceptible result) and major errors (false-resistant result), as well as categorical agreement (CA; results within the same category) were calculated for gradient and disk diffusion tests according to ISO 20776-2 [13]. Additionally, essential agreement (EA; MIC difference within one double dilution step) was calculated for gradient diffusion tests.

Categorical variables (i.e. resistance) were compared with chi-squared test, and odds ratios (OR) and 95% confidence intervals (CI) were calculated (α=0.05). Changes in resistance rates over time were assessed with chi-squared test for trend in proportions (without continuity correction).

The MIC agreement between BMD and gradient diffusion test was assessed with a Bland–Altman analysis. All analyses were performed using R Version 3.4.2 (epiDisplay).

3. Results

3.1. Resistance rates

In total, 192 isolates met the criteria of MDR (n=80) or XDR (n=112) *P. aeruginosa*. A few derived from colonization (n=24), but the majority caused infections (n=168) and were isolated from superficial swabs (n=44), respiratory tract (n=36), urine (n=19), blood culture (n=16), tissue (n=14) or others (n=39). At the time of sampling, the patients were being treated in normal care units (n=86), intensive care units (n=70), bone marrow transplant units (n=20), outpatient departments (n=10), intermediate care units (n=4) or emergency departments (n=2).

In total, 35.9% (n=69) *P. aeruginosa* were resistant to CZA according to BMD (Table 1). MIC_{50}, MIC_{90} and MIC range were 8, >256 and 0.5–>256 mg/L, respectively (Table 1). CZA resistance rates were significantly lower in MDR isolates than XDR isolates (15% vs. 50.9%, OR=0.2, 95% CI 0.1–0.4, P<0.005).

CZA resistance rates decreased significantly over time from 43% (2013), 53% (2015), 37% (2016), 19% (2017) to 12% (2018, P-value

Table 1
Susceptibility of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa*

Isolates (n)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range	Susceptibility rate (n)
MDR (80)	4	16	0.5–64	85.0% (68)
XDR (112)	16	>256	1–>256	49.1% (55)
Total (192)	8	>256	0.5–>256	64.1% (123)

MIC_{50}, minimum inhibitory concentration required to inhibit the growth of 50% of organisms; MIC_{90}, minimum inhibitory concentration required to inhibit the growth of 90% of organisms.
According to broth microdilution.

Table 2
Comparison of ceftazidime/avibactam susceptibility testing methods in multi-drug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* (n=192)

	Susceptibility [% (n)]	MIC (mg/L)			Performance (%, n)			
		50%	90%	Range	EA	CA	VME[a]	ME[b]
BMD	64.1% (123)	8	>256	0.5–>256	-	-	-	-
Gradient diffusion (Etest)	63.5% (122)	8	>256	0.75–>256	94.8% (182)	94.3% (181)	7.2% (5)	4.9% (6)
Gradient diffusion (MIC Test Strip)	52.1% (100)	8	>256	0.75–>256	89.1% (171)	85.9% (165)	2.9% (2)	20.3% (25)
Disk diffusion (MAST)	44.8% (86)	-	-	-	-	79.7% (153)	1.4% (1)	30.9% (38)
Disk diffusion (Oxoid)	53.1% (102)	-	-	-	-	88.0% (169)	1.4% (1)	17.9% (22)

BMD, broth microdilution; EA, essential agreement; CA, categorical agreement; VME, very major errors; ME, major errors.
[a] Using number of isolates that are resistant according to BMD as denominator (calculation of VME rate according to ISO 20776-2).
[b] Using number of isolates that are susceptible according to BMD as denominator (calculation of ME rate according to ISO 20776-2).

for trend=0.007). Among CZA-resistant isolates, the proportion of MDR/XDR (n of MDR isolates/n of XDR isolates) changed between 2013 (5/17), 2015 (2/16), 2016 (4/16), 2017 (0/7) and 2018 (1/1).

3.2. Comparison of susceptibility testing methods

The gradient diffusion tests of the two manufacturers uniformly categorized a lower proportion of isolates as susceptible (63.5% and 52.1%, respectively) compared with BMD (64.1%, Table 2). EA and CA were ≥90% in a single gradient diffusion test (Etest: EA 94.8%, CA 94.3%, Table 2). The MIC Test Strip showed CA of 85.9%, which was mainly due to a high proportion of false-resistant results (20.3% major error rate, Table 2). Both gradient tests had good agreement with BMD at a CZA concentration ≤48 mg/L, and differed markedly from BMD results at higher CZA concentrations (Bland–Altman analysis, Fig. S1, see online supplementary material).

CA of the disk diffusion test was 79.7% (MAST) and 88.0% (Oxoid). The poor agreement was mainly due to high rates of false-resistant CZA test results (ME 30.9% and 17.9%, respectively; Table 2, Fig. 1).

When tests were compared for MDR (excluding XDR isolates), CA was ≥90% for both gradient diffusion tests (Etest 95.0%, MIC Test Strip 90.0%) and one disk diffusion test (Oxoid 91.3%; Table S2, see online supplementary material). In contrast, when analysing XDR *P. aeruginosa* separately, only one gradient diffusion test had CA ≥90% (Etest 93.8%; Table S3, see online supplementary material).

3.3. Clonal structure and resistance genes

The clonal structure of MDR and XDR *P. aeruginosa* was diverse comprising 85 different STs. The most common STs in MDR isolates were ST395 (n=9), 274 (n=5) and 298 (n=5) (other n=61). XDR isolates mainly belonged to ST235 (n=32), ST395 (n=7) and ST273 (n=6) (other n=67). In terms of ST abundance, MDR *P. aeruginosa* were more diverse than XDR isolates (Simpson's index of diversity 0.98 vs. 0.91). Among CZA-resistant isolates (n=69), ST235 (n=29, all XDR) was predominant, followed by ST17 (n=4) and ST111 (n=2) (other n=34).

One cluster of CZA-resistant isolates uniquely consisting of ST395 *P. aeruginosa* was detected (Fig. 2). Apart from this cluster, CZA-resistant isolates were scattered throughout the whole population. Of the 69 CZA-resistant isolates, the likely underlying mechanisms of CZA resistance were $bla_{\text{IMP-31}}$ (n=27), $bla_{\text{VIM-2}}$ (n=4), $bla_{\text{VIM-1}}$ (n=2) and $bla_{\text{IMP-13}}$ (n=1). In 35 isolates, no MBL or bla_{OXA}

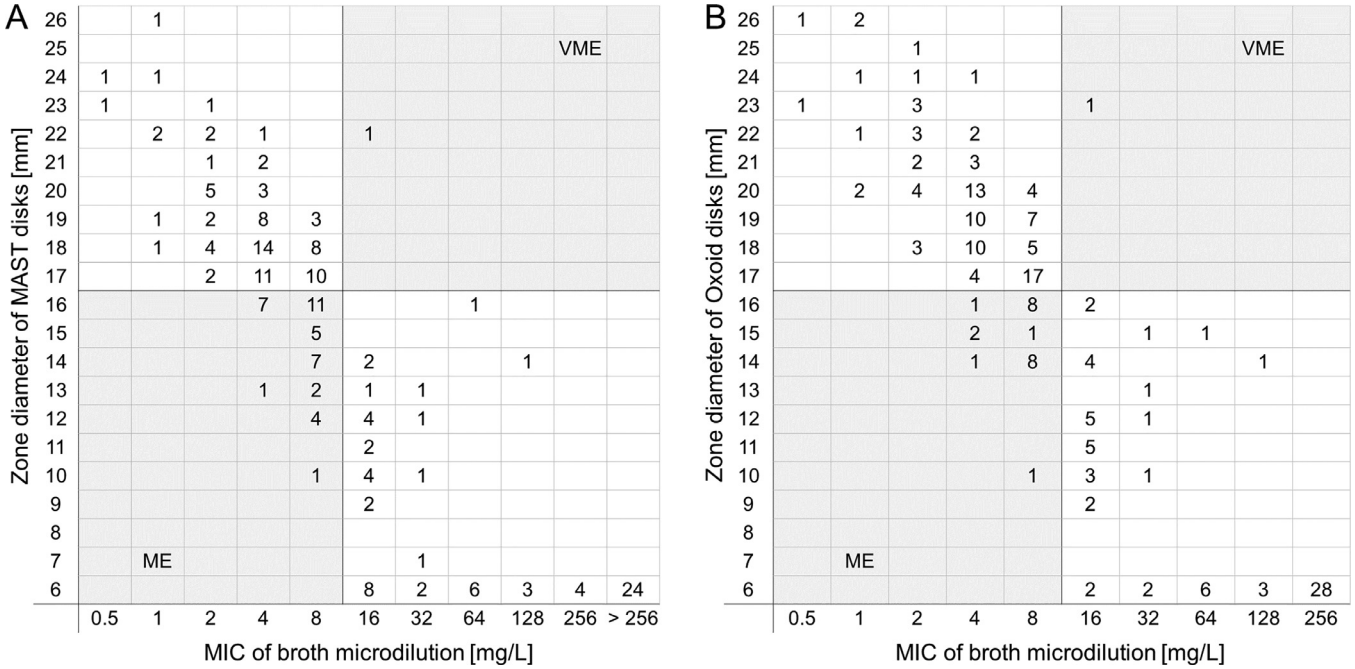

Fig. 1. Comparison of ceftazidime/avibactam (CZA) susceptibility testing with disk diffusion and broth microdilution (BMD). The minimum inhibitory concentration (MIC) according to BMD (x-axes) and the zone diameter of CZA disks (10/4 µg) of two manufacturers (A: MAST, B: Oxoid, y-axes) are shown. The numbers in the cells correspond to the number of isolates to which the respective test result applies. Black lines indicate clinical breakpoints for disk diffusion (horizontal line) and BMD (vertical line). Shaded areas indicate major errors (ME, false resistant) and very major errors (VME, false susceptible) according to BMD.

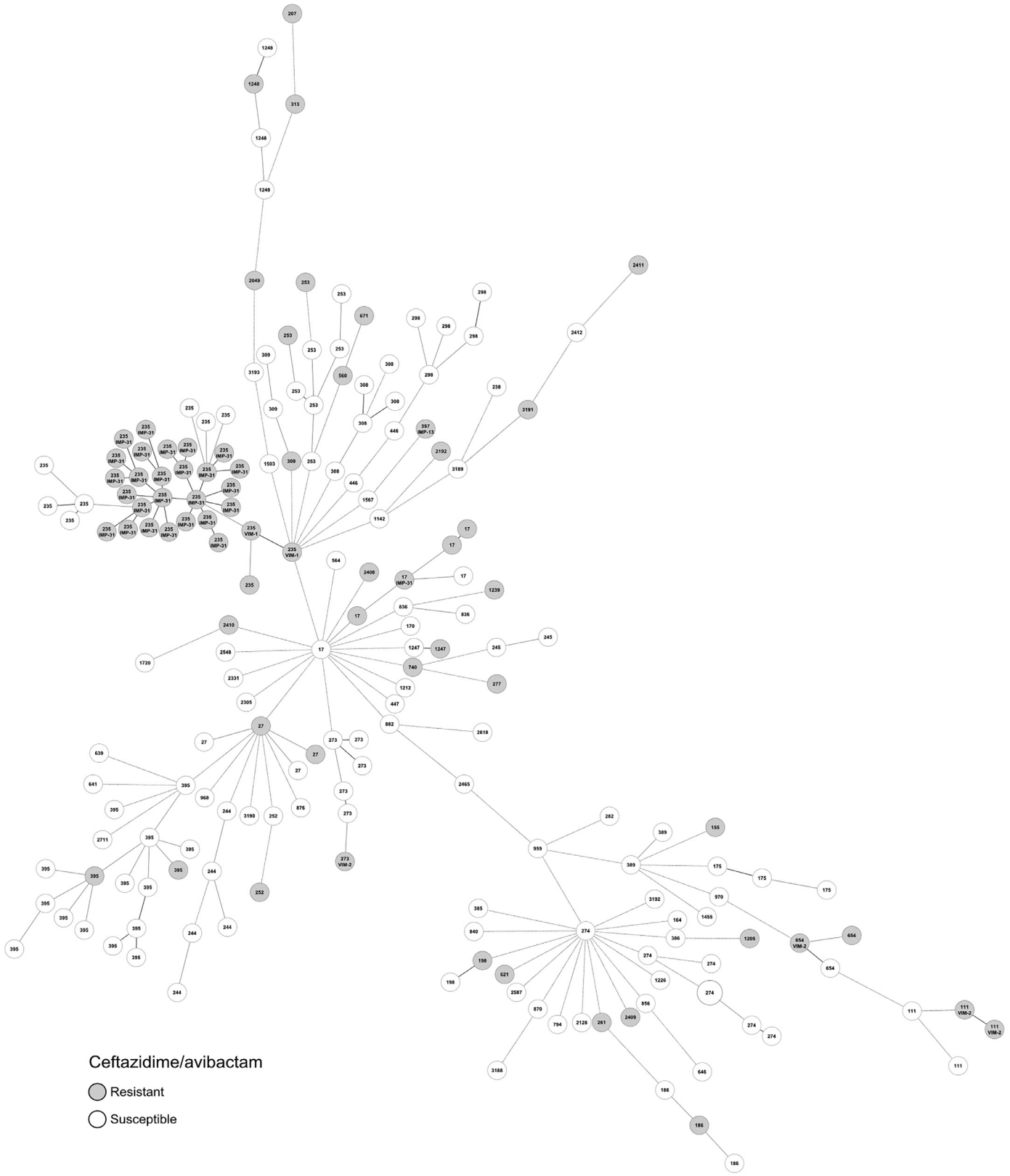

Fig. 2. Minimum spanning tree of multi-drug resistant and extensively drug-resistant *Pseudomonas aeruginosa*. The phylogenetic tree was constructed using the allelic profiles of the *P. aeruginosa* core-genome multi-locus sequence typing (MLST) scheme and the 'pairwise ignoring missing values' option as implemented in SeqSphere+. Each node is labelled with the MLST sequence type. The distance of the alleles between the nodes is displayed as a different line style (black ≤14; grey ≤100; dashed >100). Nodes of ceftazidime/avibactam-resistant isolates are shaded in grey.

variant was found that could explain resistance to CZA. Members of the bla_{OXA-50} family (i.e. $bla_{OXA-395}$, $bla_{OXA-396}$, $bla_{OXA-486}$, $bla_{OXA-488}$) were not considered as determinants of CZA resistance [14].

4. Discussion

This study analysed CZA susceptibility rates of MDR and XDR *P. aeruginosa* (n=192) spanning a sampling period of 6 years. The main findings were an overall CZA resistance rate of 35.9%. Among commercial susceptibility testing methods, Etest showed highest accuracy in comparison to BMD.

The comparability of resistance rates between studies is hampered by differing inclusion criteria, and only those studies that applied the same CDC/ECDC definition of MDR/XDR were included in the comparison. CZA susceptibility in *P. aeruginosa* from intensive care patients with pneumonia (USA, 2015–2017) was similar for MDR isolates (86.2%) but markedly higher for XDR isolates (77.8%) compared with the present study (Table 1) [15].

Two gradient diffusion tests and two disk diffusion tests were compared with BMD. Etest performed best, in agreement with a previous study which demonstrated good accuracy of this method for CZA testing [16]. Both disk diffusion methods did not meet acceptance criterion of ≥90% for CA (Table 2) [13]. The present results are consistent with recent studies comparing CZA Etest and disk diffusion tests with BMD using collections mainly consisting of *Klebsiella pneumoniae* and carbapenem-resistant *P. aeruginosa* [17,18]. In the study by Wenzler et al., no susceptibility test met the acceptance threshold for *P. aeruginosa* of ≥90% CA and EA [13,17]. Similar to the present study (Table 2), major drawbacks included a high rate of major errors (up to 80% in one disk diffusion test) [17].

Whole-genome sequencing revealed lower diversity of XDR *P. aeruginosa* compared with MDR *P. aeruginosa* (Fig. 2). This seems to be a common trait and might be related to the clonal expansion of single resistant lineages under antimicrobial pressure. Among XDR clones, ST235 *P. aeruginosa* is a high-risk clone due to the notorious acquisition of Class A, B (e.g. bla_{IMP}, bla_{VIM}) and D β-lactamase resistance genes [19].

MBLs (bla_{IMP}, bla_{VIM}) were detected in 34 of 69 CZA-resistant isolates (49.3%), but it was not possible to determine the underlying mechanism of CZA resistance in the remaining isolates. The likely mechanism of CZA resistance in these isolates is an upregulation of the MexAB/OprM efflux pump and bla_{ampC} [20].

Some limitations of this study need to be addressed. First, the expression levels of efflux pumps, porins and *ampC* were not studied. These functional assays would be useful to decipher the mechanism in one-half of CZA-resistant isolates. Second, no data exist regarding antimicrobial exposure of the *P. aeruginosa* isolates before they were cultured in the laboratory. This information would be helpful to explain CZA resistance in the absence of MBLs, as ceftazidime can lead to overexpression of MexAB/OprM [21].

5. Conclusion

CZA is a promising treatment option, particularly in infections caused by MDR *P. aeruginosa*. CZA-resistant *P. aeruginosa* mainly belong to the bla_{IMP}-positive ST235 high-risk clone. Of all methods tested, only Etest was acceptable as an alternative to BMD.

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Competing interests

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Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.05.001.

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