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Italian nationwide survey on *Pseudomonas aeruginosa* from invasive infections: activity of ceftolozane/tazobactam and comparators, and molecular epidemiology of carbapenemase producers

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Objectives: *Pseudomonas aeruginosa* is a major cause of severe healthcare-associated infections and often shows MDR phenotypes. Ceftolozane/tazobactam is a new cephalosporin/β-lactamase inhibitor combination with potent activity against *P. aeruginosa*. This survey was carried out to evaluate the susceptibility of *P. aeruginosa*, circulating in Italy, to ceftolozane/tazobactam and comparators and to investigate the molecular epidemiology of carbapenemase-producing strains.

Methods: Consecutive non-replicate *P. aeruginosa* clinical isolates (935) from bloodstream infections and lower respiratory tract infections were collected from 20 centres distributed across Italy from September 2013 to November 2014. Antimicrobial susceptibility testing was performed by broth microdilution and results were interpreted according to the EUCAST breakpoints. Isolates resistant to ceftolozane/tazobactam were investigated for carbapenemase genes by PCR, and for carbapenemase activity by spectrophotometric assay. WGS using an Illumina platform was performed on carbapenemase-producing isolates.

Results: Ceftolozane/tazobactam was the most active molecule, retaining activity against 90.9% of *P. aeruginosa* isolates, followed by amikacin (88.0% susceptibility) and colistin (84.7% susceptibility). Overall, 48 isolates (5.1%) were positive for carbapenemase genes, including bla_{VIM} ($n = 32$), bla_{IMP} ($n = 12$) and bla_{GES-5} ($n = 4$), while the remaining ceftolozane/tazobactam-resistant isolates tested negative for carbapenemase production. Carbapenemase producers belonged to 10 different STs, with ST175 ($n = 12$) and ST621 ($n = 11$) being the most common lineages. Genome analysis revealed different trajectories of spread for the different carbapenemase genes.

Conclusions: Ceftolozane/tazobactam exhibited potent *in vitro* activity against *P. aeruginosa* causing invasive infections in Italy. Carbapenemase production was the most common mechanism of resistance to ceftolozane/tazobactam.

Introduction

Pseudomonas aeruginosa is a common cause of severe healthcare-associated infections, including pneumonia and bloodstream infections (BSIs).[1] *P. aeruginosa* is intrinsically resistant to a variety of antibiotics, and is prone to acquire multiple resistance mechanisms to anti-*Pseudomonas* agents owing to chromosomal mutations or horizontal transfer of exogenous resistance genes.[1,2] Acquisition of multiple resistance mechanisms is common, leading to MDR and even XDR phenotypes, drastically reducing the number of antimicrobial agents available for clinical use.[3–6]

Concerning β-lactams, acquired resistance can be due to several mechanisms, including upregulation of the MexAB-OprM and MexXY efflux systems, reduced outer membrane permeability due to loss of OprD porin, penicillin-binding protein alterations, overproduction

of the endogenous AmpC-type β-lactamase, and production of acquired β-lactamases.[2,6–13] Indeed, several acquired β-lactamases of different classes have been reported in clinical isolates of *P. aeruginosa*, including class A serine-β-lactamases of narrow (e.g. PSE and TEM-1) or extended spectrum (e.g. PER, VEB, CTX-M, GES, KPC), class B metallo-β-lactamases (e.g. IMP, VIM, AIM, FIM, NDM and SPM), and class D serine-β-lactamases of narrow (e.g. OXA-3) or extended spectrum (e.g. OXA-10, OXA-11, OXA-28), or even with weak carbapenemase activity.[2,3,14,15]

Ceftolozane is a new cephalosporin with potent *in vitro* activity against *P. aeruginosa* that is not affected by most of the β-lactam resistance mechanisms present in this species, including production of the endogenous AmpC-type β-lactamase, upregulation of the MexAB and MexXY efflux systems and reduction of outer membrane permeability by OprD loss, which variably affect all other anti-*Pseudomonas* β-lactams.[16] The combination with the β-lactamase inhibitor tazobactam extends ceftolozane's spectrum of activity against many Enterobacteriaceae producing ESBLs.[17,18]

In this study we carried out a nationwide Italian survey on *P. aeruginosa* causing invasive bloodstream and lower respiratory tract infections, to investigate the antimicrobial susceptibility profiles for ceftolozane/tazobactam and comparators, and the molecular epidemiology of carbapenemase producers.

Materials and methods

Bacterial isolates

Twenty different centres distributed across Italy (Figure S1, available as Supplementary data at *JAC* Online) were asked to collect, during the period from September 2013 to November 2014, consecutive non-duplicate clinical isolates of *P. aeruginosa* from cases of BSIs and hospital-acquired (HAP) or ventilator-associated pneumonia (VAP).[19] For HAP/VAP, only bronchoalveolar lavage samples with a colony count $\geq 1\times10^4$ cfu/mL were considered significant.[20] Isolates from cystic fibrosis patients were excluded.

Identification of all collected isolates referred by the participating centres was confirmed using MALDI-TOF (Vitek-MS, bioMérieux, Marcy L'Etoile France).

Susceptibility testing

The activity of ceftolozane/tazobactam and comparators (cefepime, ceftazidime, piperacillin/tazobactam, meropenem, imipenem, ciprofloxacin, amikacin and colistin) was tested against the collection of isolates by broth microdilution[21] using lyophilized custom plates (Thermofisher Scientific). Results were interpreted as susceptible, intermediate or resistant according to the EUCAST breakpoints (EUCAST breakpoint tables version 7.1, 2017, www.eucast.org).

Analysis of carbapenemase genes and carbapenemase production

All isolates resistant to ceftolozane/tazobactam were screened by PCR, using protocols and conditions previously described[22] for the most common carbapenemase genes reported in *P. aeruginosa* (bla_{VIM} and bla_{IMP}). Carbapenemase activity was tested by spectrophotometry, using imipenem as substrate as described previously,[23] on crude extracts of ceftolozane/tazobactam-resistant isolates that tested negative for carbapenemase genes, and of ceftolozane/tazobactam-susceptible isolates with a carbapenem (imipenem or meropenem) MIC $\geq$64 mg/L.

WGS of carbapenemase-positive isolates and resistome analysis

Bacterial DNA of all isolates positive for carbapenemase genes or carbapenemase production was extracted using the phenol:chloroform method.[24] Genomic DNA was subjected to WGS with a MiSeq platform (Illumina, Inc., San Diego, CA), using a 2×250 bp or 2×300 bp paired-end approach, and reads were assembled using SPAdes.[25] Raw coverage of the assembled genomes, calculated using a total genome length of 6.3 Mbp corresponding to that of *P. aeruginosa* reference strain PAO1 (Accession: NC_002516),[26] ranged between 40× and 272×, with an average value of 114×. A mean of 155 contigs per strain was obtained, with an average N50 of 326 Kbp. Resistance gene content was investigated using the ResFinder tool available at the Center for Genomic Epidemiology at https://cge.cbs.dtu.dk/services/ResFinder/. Comparative analysis of the bla_{AmpC} sequences was carried out using the BLASTN Tool, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Clonal relatedness of carbapenemase-positive *P. aeruginosa*

Clonal relatedness was investigated by determination of the MLST profile obtained by the MLST 1.8 software (available at https://cge.cbs.dtu.dk/services/MLST/) using the assembled whole genome sequences as input data. Fine-tuning analysis of clonal relatedness was obtained with the CSI phylogeny 1.4 tool, available at https://cge.cbs.dtu.dk/services/CSIPhylogeny/, using default parameters except for the minimum distance between SNPs option, which was disabled. The phylogeny was inferred based on the concatenated alignment of the high-quality SNPs,[27] which were used to generate a phylogenetic tree. The overall phylogenetic tree was constructed using *P. aeruginosa* PAO1 genome (Accession: NC_002516) as reference, and draft assembled genomes as input data, after removing contigs <300 bp. The mean percentage of reference genome covered by all isolates was 89.9%. Genetic distance within strains belonging in the same sequence type was computed using as reference the internal strain with the oldest collection date, and as input data the raw reads of related strains. Phylogenetic trees were visualized and modified by FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Nucleotide sequence accession numbers

Draft genome sequences were deposited in the INSDC databases under accession numbers from NFFC00000000 to NFGX00000000.

Results

Antimicrobial susceptibility of *P. aeruginosa* clinical isolates

A total of 935 non-replicate *P. aeruginosa* clinical isolates (382 from BSI and 553 from HAP/VAP) were collected from 20 centres distributed across Italy (Figure S1) in the period September 2013–November 2014. The number of collected isolates ranged from 16 to 138 between different centres (Table 1).

Overall, 60% of the isolates were resistant to at least one antipseudomonal agent among β-lactams (ceftolozane/tazobactam, cefepime, ceftazidime, piperacillin/tazobactam, meropenem and imipenem), ciprofloxacin, colistin and amikacin, whereas 37.2% exhibited an MDR phenotype (i.e. resistant to at least one tested antibiotic in three or more antimicrobial classes).[28] Six isolates were resistant to all tested drugs.

Ceftolozane/tazobactam was the most active agent (90.9% susceptibility), followed by amikacin (88.0% susceptibility) and colistin (84.7% susceptibility, considering the most recent EUCAST susceptibility breakpoint of 2 mg/L). Lower susceptibility rates were observed with the other agents (Table 2).

The ceftolozane/tazobactam MIC_{50} and MIC_{90} values (1 and 4 mg/L, respectively) were overall lower than those of other anti-pseudomonal β-lactams, with median and modal MIC values of 1 mg/L (Tables 2 and 3). Ceftolozane/tazobactam retained activity against the majority (59.6%) of the 183 isolates that were non-susceptible to all other β-lactams (19.6% of the total collection), and against almost half (46.8%) of the 64 isolates that were resistant to all other agents except colistin (6.8% of the total collection) (Table 3). Ceftolozane/tazobactam was also active against 9/15 isolates that were non-susceptible to all other tested agents. Among the ceftolozane/tazobactam-resistant isolates, 82.3%, 44.7%, 15.3% and 8.2% were susceptible to colistin, amikacin, ciprofloxacin and carbapenems, respectively.

Some differences were observed in the antimicrobial susceptibility pattern comparing isolates from BSI and from HAP/VAP, with the former overall less resistant to antibiotics (Table S1).

Table 1. Numbers and proportion of ceftolozane/tazobactam-resistant (CTZ-R) strains and of carbapenemase producers at different centres

Centre code	Total isolates collected	No. of CTZ-R isolates (%)	No. of carbapenemase producers (%)	Proportion of carbapenemase producers among CTZ-R (%)
1	89	7 (7.9)	5 (5.6)	71.4
2	49	4 (8.2)	3 (6.1)	75.0
3	28	0	0	—
4	16	2 (12.5)	0	—
5	21	0	0	—
6	80	4 (5.0)	4 (5.0)	100.0
7	48	4 (8.3)	0	—
8	50	1 (2)	0	—
9	50	12 (24)	9 (18.0)	75.0
10	138	13 (9.4)	4 (2.9)	30.8
11	24	4 (16.7)	0	—
12	34	0	0	—
13	50	8 (16.0)	6 (12.0)	75.0
14	48	6 (12.5)	4 (8.3)	66.7
15	54	4 (7.4)	2 (3.7)	50.0
16	22	5 (22.7)	3 (13.6)	66.7
17	50	7 (14)	7 (14.0)	100
18	18	2 (11.1)	1 (5.6)	50
19	26	0	0	—
20	40	2 (7.5)	0	—
Total	935	85 (9.1)	48 (5.1)	56.5

Carbapenemase determinants among the P. aeruginosa isolates and molecular epidemiology of carbapenemase producers

Since carbapenemase production is known to be one of the resistance mechanisms to ceftolozane/tazobactam, we considered ceftolozane/tazobactam resistance as a marker of possible carbapenemase production, and first screened all the ceftolozane/tazobactam-resistant isolates for the presence of bla_{IMP} and bla_{VIM} genes, which are the most common carbapenemase determinants in *P. aeruginosa*.[8,29] Overall, 32 and 12 isolates were positive for bla_{VIM} or bla_{IMP} genes, respectively. The remaining 41 isolates were screened for carbapenemase activity by reference spectrophotometric assay, and four of them exhibited weak but measurable carbapenemase activity (specific activity ranged from 6.5 to 8.4 nmol/min/mg), while 37 did not express any detectable carbapenemase activity. No carbapenemase activity was detected in the few strains ($n = 9$) susceptible to ceftolozane/tazobactam that exhibited high carbapenem MICs (≥64 mg/L).

The genomes of the 48 carbapenemase-producing isolates were completely sequenced. Among isolates carrying a bla_{VIM} gene, bla_{VIM-2} was the most frequent variant ($n = 17$, 53% of the 32 isolates) followed by bla_{VIM-1} ($n = 15$). bla_{IMP} genes were the second most common carbapenemase determinant ($n = 12$, 25%), with 11 bla_{IMP-13} and one bla_{IMP-19}. The four bla_{IMP} and bla_{VIM}-negative isolates showing weak carbapenemase activity carried a bla_{GES-5} gene. No other known carbapenemase genes were detected in these isolates.

All the metallo-β-lactamase producers (of either IMP- or VIM-type) exhibited ceftolozane/tazobactam MICs ≥64 mg/L and, in most cases, MICs were >128 mg/L. All of the GES-5 producers showed ceftolozane/tazobactam MICs of 8 mg/L (Table 3).

Carbapenemase producers were isolated in 11 of 20 centres, with proportions ranging from 2.9% to 18% in those centres (Table 1).

Analysis of the bla_{AmpC} genes in the sequenced isolates revealed none of the mutations previously associated with increased ceftolozane/tazobactam MICs.[30–32] All bla_{AmpC} genes

Table 2. MIC_{50} and MIC_{90} (mg/L) of ceftolozane/tazobactam and comparators for the collected isolates (935 in total)

	CTZ	FEP	CAZ	TZP	MEM	IPM	CIP	CST	AMK
MIC_{50}	1	4	4	16	1	2	0.25	2	4
MIC_{90}	4	32	64	>128	32	32	>16	4	16
%S	90.9	71.1	70.4	59.9	65.3	65.7	65.1	84.7	88.0

CTZ, ceftolozane/tazobactam (tazobactam at fixed concentration of 4 mg/L); FEP, cefepime; CAZ, ceftazidime; TZP, piperacillin/tazobactam (tazobactam at fixed concentration of 4 mg/L); MEM, meropenem; IPM, imipenem; CIP, ciprofloxacin; CST, colistin; AMK, amikacin; %S, percentage of susceptible isolates.

Table 3. Distribution of ceftolozane/tazobactam MICs against *P. aeruginosa* from Italy. *P. aeruginosa* isolates have been sorted based on different resistance patterns and carbapenemase types

	Number of isolates (%) with an MIC (mg/L) of ceftolozane/tazobactam:										
P. aeruginosa (no. of tested isolates)	0.25	0.5	1	2	4	8	16	32	64	128	>128
All *P. aeruginosa* ($n = 935$)	6 (0.6)	245 (26.2)	383 (41.0)	158 (16.9)	58 (6.2)	14 (1.5)	4 (0.4)	7 (0.7)	8 (0.9)	19 (2.0)	33 (3.5)
Sorted by phenotype											
CST-susceptible only (64)	0 (–)	0 (–)	1 (1.6)	24 (37.5)	5 (7.8)	3 (4.7)	1 (1.6)	3 (4.7)	4 (6.3)	9 (14.1)	14 (21.9)
CST and AMK susceptible only (70)	0 (–)	0 (–)	3 (4.3)	28 (40.0)	18 (25.7)	4 (5.7)	1 (1.4)	2 (2.9)	0 (–)	5 (7.1)	9 (12.9)
CST-resistant isolates (143)	3 (2.1)	34 (23.8)	53 (37.0)	28 (19.6)	10 (7.0)	1 (0.7)	1 (0.7)	0 (–)	2 (1.4)	3 (2.1)	8 (5.6)
resistant to other β-lactams (183)	0 (–)	0 (–)	6 (3.3)	71 (38.8)	32 (17.5)	7 (3.8)	3 (1.6)	6 (3.3)	6 (3.3)	19 (10.4)	33 (18.0)
Sorted by resistance mechanism											
no carbapenemase expression (887)	6 (0.7)	245 (27.6)	383 (43.2)	158 (17.8)	58 (6.5)	10 (1.2)	4 (0.4)	7 (0.8)	7 (0.8)	8 (0.9)	1 (0.1)
VIM- or IMP-type (44)									1 (2.3)	11 (25)	32 (72.7)
GES-5 (4)						4 (100)					

AMK, amikacin; CST, colistin.

corresponded to previously described variants, and their distribution was overall associated with the clonal lineage (Table S2).

Population structure of carbapenemase producers

Genome sequence data were used to investigate the population structure of carbapenemase producers circulating in Italy. Overall, 10 different STs were represented among carbapenemase producers, including ST111, ST175, ST179, ST233, ST235, ST260, ST308, ST395, ST532 and ST621 (Figure 1). ST175 and ST621 were the most prevalent ($n = 12$ and $n = 11$ isolates, respectively), followed by ST235 and ST111 ($n = 7$ and $n = 6$ isolates, respectively).

The ST621 isolates were closely related to each other (SNP variation: 36–643, mean 197, median 134) and were invariably associated with the bla_{IMP-13} gene and detected in five different centres (Figure 2a). In contrast, isolates of ST175 exhibited a somewhat higher diversity (SNP variation: 0–1265, mean 315, median 212). The bla_{VIM-2}-positive isolates were all highly related to each other and from a single centre, whereas the bla_{IMP-19}-positive isolate was the most divergent (Figure 2b). An even higher diversity was observed among isolates of ST235 (SNP variation: 10–2242, mean 1162, median 1196), with two different clusters of which one included the bla_{GES-5}-positive isolates and the other the bla_{VIM-1}-positive isolates (Figure 2c). ST111 also exhibited a higher diversity (SNP variation: 41–2501, mean 927, median 889), with at least two clusters associated with different bla_{VIM}-type alleles (Figure 2d).

Discussion

P. aeruginosa remains a major cause of severe healthcare-associated infections worldwide and, among Gram-negative nosocomial pathogens, has been among the first to exhibit MDR and XDR phenotypes, owing to its ability to accumulate different resistance mechanisms.

In this survey, we investigated the antimicrobial susceptibility profiles of *P. aeruginosa* causing BSI and HAP/VAP in Italy, a country among the most affected in Europe by the problem of antibiotic resistance.[33] Resistance rates >28% were observed with most anti-*Pseudomonas* agents, including cefepime, ceftazidime, piperacillin/tazobactam and carbapenems, while resistance rates <12% were only observed with amikacin and ceftolozane/tazobactam, a new β-lactam/β-lactamase inhibitor combination with potent anti-*Pseudomonas* activity. Even colistin showed a notable rate of resistance according to the recently updated EUCAST breakpoints (susceptibility breakpoint lowered from 4 to 2 mg/L). These data represent a step-change from the current clinical scenario. In fact, colistin is usually considered the prototype last-resort anti-pseudomonal agent, as it is active against almost 100% of isolates.

Compared with data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported for the same period,[34] we found an overall higher level of resistance to the tested molecules, except aminoglycosides (Table 2). These differences could be partially explained by the fact that the EARS-Net system only reports data for bloodstream isolates, which also in our study showed lower resistance rates when compared with isolates from respiratory samples (Table S1). Moreover, the EARS-Net system reports data for aminoglycosides in an aggregated form (gentamicin and/or tobramycin and/or amikacin), which could explain the lower aminoglycoside resistance rates observed in our study, where only amikacin (i.e. the most active anti-*Pseudomonas* aminoglycoside) was tested.[2]

In this scenario ceftolozane/tazobactam could play a central role. In fact, in our surveillance, it was the most active anti-*Pseudomonas* agent, with $MIC_{50/90}$ values lower than those of all other β-lactams and with higher activity than colistin and amikacin. Moreover, ceftolozane/tazobactam was active against approximately half of the isolates resistant to all other β-lactams or resistant to all other agents except colistin. Our susceptibility results are concordant with results from other recent surveys, where ceftolozane/tazobactam susceptibility rates ranging from 84%–99% were reported for *P. aeruginosa* clinical isolates.[17,18,35–44]

In this study, ceftolozane/tazobactam resistance was used as a marker of potential carbapenemase production. In fact, more than half of the ceftolozane/tazobactam-resistant isolates were carbapenemase producers, confirming that acquisition of

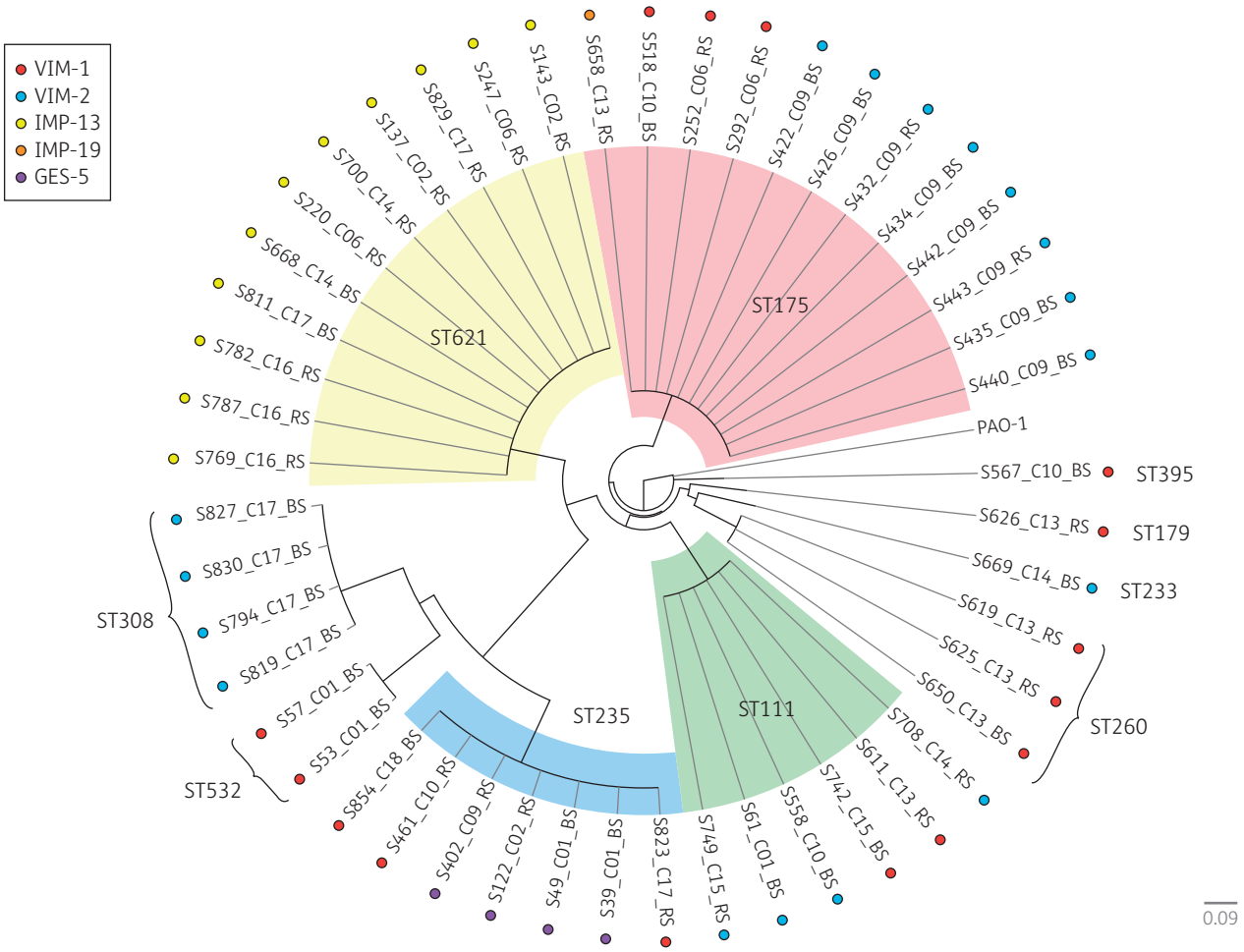

Figure 1. Phylogenetic tree of all carbapenemase-producing *P. aeruginosa* ($n = 48$). For each isolate, the sample code (S), the centre code (C) and the source (BS, blood sample; RS, respiratory sample) are reported. Filled circles of different colours identify the different types of carbapenemase. Sequence type (ST) is also shown, and branches including the most-represented STs are shown in different colours. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

carbapenemase genes is a relevant mechanism of resistance to ceftolozane/tazobactam. However, the presence of other ceftolozane/tazobactam resistance mechanisms was detected in a substantial number of isolates, and will be the subject of future investigation. Since most isolates (96.9%) with a ceftolozane/tazobactam MIC >128 mg/L were carbapenemase producers (Table 3), an MIC value >128 mg/L was highly predictive of carbapenemase production. Interestingly, 8.3% of the carbapenemase producers were positive for a bla_{GES-5} gene (to the best of our knowledge, this is the first description of GES-5-producing *P. aeruginosa* isolates from Italy). GES-5 β-lactamase production was not previously associated with ceftolozane/tazobactam resistance, but a paper by Giske *et al.*[45] described GES-2 producers as resistant to ceftolozane/tazobactam. This information could be relevant to the design of panels for molecular detection of resistance genes for rapid prediction of β-lactam resistance among Gram-negative pathogens.

Italy was among the first European countries to report carbapenemase-producing *P. aeruginosa* strains, as early as 1997.[23] In a survey on metallo-β-lactamase-producing Gram-negative organisms, carried out in 2004, the overall proportion of metallo-β-lactamase-positive *P. aeruginosa* was 1.3%.[46] In this study, the overall proportion of carbapenemase-producing *P. aeruginosa* was 5.1%, with almost 90% accounted for by metallo-β-lactamase producers, suggesting an increasing trend for metallo-β-lactamase producers in Italy. Since in this study ceftolozane/tazobactam resistance was used as a criterion to suspect potential carbapenemase production, some carbapenemase producers might have been missed if they remained susceptible to ceftolozane/tazobactam (owing to very low-level carbapenemase expression or to the production of an as yet unknown carbapenemase that does not hydrolyse ceftolozane/tazobactam). Therefore, the observed prevalence may even be an underestimate. In surveys carried out in Spain and Russia in the 2008–10 period, the proportion of carbapenemase producers varied between 6.9% and 28.7%, respectively.[47,48]

In this study, VIM-type enzymes were the most common carbapenemase, followed by IMP-types. These data are in accordance with the 2004 survey, even if in that survey VIM-1

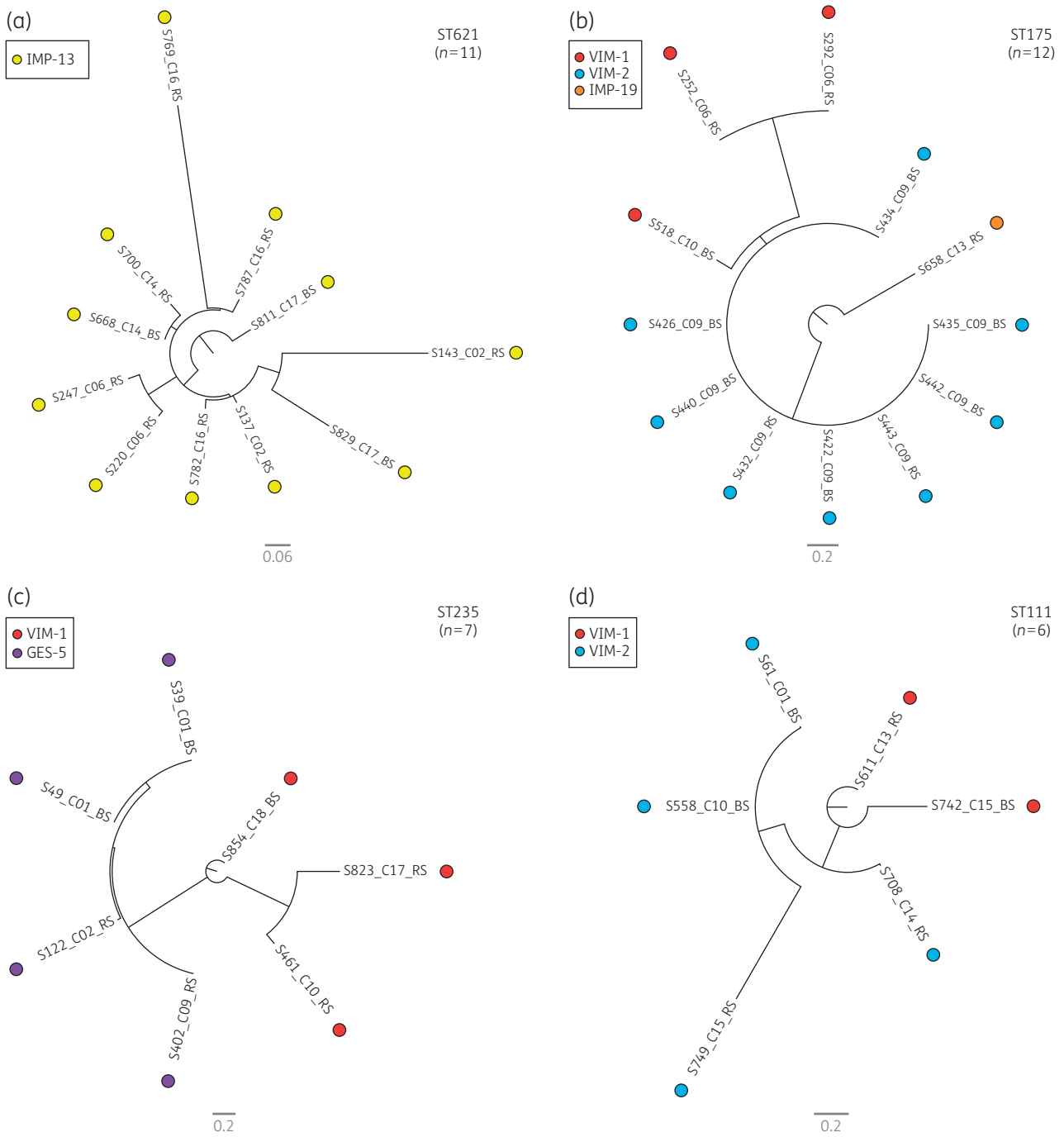

Figure 2. Phylogenetic relatedness between each of the four most represented sequence types: (a) ST621; (b) ST175; (c) ST235; and (d) ST111. For each isolate, the sample code (S), the centre code (C) and the source (BS, blood sample; RS, respiratory sample) are reported. Filled circles of different colours identify the different types of carbapenemase. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

enzymes were detected more frequently than VIM-2 enzymes.[46] The dissemination of such enzymes was mainly related to expansion of successful clones in single-centre outbreaks (e.g. ST175-VIM-2), although a multicentre spread of some resistant clones (e.g. ST235-GES-5) was also observed.

A limitation of this study was that the isolate collection was not very recent (2013–14). However, the present data could be considered as a baseline for later surveillance studies on the activity of anti-*Pseudomonas* molecules and carbapenemase production in *P. aeruginosa* from Italy.

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Transparency declarations

None to declare.

Disclaimer

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Supplementary data

Figure S1 and Tables S1 and S2 appear as Supplementary data at *JAC* Online.

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