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Clinical and microbiological features of ceftolozane/tazobactam-resistant *Pseudomonas aeruginosa* isolates in a university hospital in central Italy

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ABSTRACT

Objectives: Ceftolozane/tazobactam (C/T) is a novel cephalosporin and β-lactamase inhibitor combination with great activity against *Pseudomonas aeruginosa*. To assess *P. aeruginosa* susceptibility to C/T, a surveillance study was conducted from October 2018 to March 2019 at the University Hospital 'Ospedali Riuniti' in Ancona, Italy.

Methods: Minimum inhibitory concentrations (MICs) to C/T were determined by Etest strip. Resistant isolates were characterized by phenotypic (broth microdilution antimicrobial susceptibility testing and modified Carbapenem Inactivation Method [mCIM]) and genotypic (Polymerase Chain Reaction [PCR], Pulsed Field Gel Electrophoresis [PFGE], and whole-genome sequencing [WGS]) methods. Clinical variables of patients infected by C/T-resistant *P. aeruginosa* were collected from medical records.

Results: Fifteen of 317 *P. aeruginosa* collected showed resistance to C/T (4.7%). Ten strains demonstrated carbapenemase activity by mCIM method, and PCR confirmed that eight strains harbored a *blaVIM* gene while the other two were positive for *blaIMP*. Additionally, three isolates carried acquired extended spectrum β-lactamase genes (two isolates carried *blaPER* and one carried *blaGES*). Eight strains were strictly related by PFGE and WGS analysis confirmed that they belonged to sequence type (ST)111. The other STs found were ST175 (two isolates), ST235 (two isolates), ST70 (one isolate), ST621 (one isolate), and the new ST3354 (one isolate). Most patients had received previous antibiotic therapies, carried invasive devices, and experienced prolonged hospitalization.

Conclusion: This study demonstrated the presence of C/T-resistant *P. aeruginosa* isolates in a regional hospital carrying a number of resistance mechanisms acquired by different high-risk clones.

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1. Introduction

Pseudomonas aeruginosa is the main species involved in infection in patients with cystic fibrosis and one of the leading causes of hospital infections [1]. Included amongst ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.)

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pathogens, the combination of *P. aeruginosa's* intrinsic and acquired resistance traits led to its diffusion and also to the success of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* high-risk clones [2]. MDR or XDR *P. aeruginosa* infections have increased in Europe, reaching rates between 15% and 30% of the total infections sustained by this species, depending on geographical area [2]. Moreover, outbreaks of MDR *P. aeruginosa* are frequently reported, highlighting the widespread nature of this pathogen and raising concern [3,4].

To treat infections sustained by MDR *P. aeruginosa*, a combination including a new cephalosporin (ceftolozane) and a well-known β-lactamase inhibitor (tazobactam) has been recently developed [5]. Ceftolozane/tazobactam (C/T) was approved in 2015 by the European Medicine Agency for the treatment of complicated Intra-Abdominal Infection (cIAI), acute pyelonephritis, complicated Urinary-Tract Infection (cUTI), and Hospital-Associated Pneumonia (HAP), and has demonstrated great activity against MDR and XDR *P. aeruginosa* [6–9].

Despite the recent introduction of this combination in clinical settings, C/T-resistant *P. aeruginosa* isolates have been reported and characterized in several studies. Different resistance mechanisms have been identified, including: (i) mutations in the *ampC* gene and in its regulator *ampR*, leading to an overexpression or modification of the pseudomonas-derived cephalosporinase, PDC [10–12] (ii) the acquisition of some extended spectrum β-lactamases (ESBLs), such as GES, VEB, BEL, or PER enzymes [13] (iii) the acquisition of metallo-β-lactamases (MBLs) and oxacillinase, in particular VIM, IMP, and some OXA [6,7,12,14]. In Italy, recent studies demonstrated the presence of *P. aeruginosa* isolates resistant to C/T, showing an overall C/T resistance of about 10% [7,8].

Considering the importance of this new antibiotic and the high percentage of resistant isolates found in our country, we performed surveillance on a collection of *P. aeruginosa* isolated from the clinical laboratory of 'Ospedali Riuniti', Ancona, Italy, to investigate susceptibility to C/T. We characterized the resistant isolates and studied both clinical and epidemiological features as well as antibiotic resistance mechanisms.

2. Materials and methods

2.1. Hospital settings and patient data

The setting was a 980-bed University Hospital in Central Italy. Data regarding demographic characteristics and clinical risk factors were collected from the patients' medical records.

2.2. Strains

Pseudomonas aeruginosa strains were recovered from the Clinical Microbiology Laboratory of the 'Ospedali Riuniti' of Ancona, Italy from October 2018 to March 2019. Strains were identified by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF/MS). All strains included in the study were collected from clinical specimens without other exclusion criteria. Only one isolate per patient(s) was considered.

2.3. Determination of MICs

MICs of C/T were determined using MIC test strips (Liofilchem, Roseto Degli Abruzzi, IT) according to the manufacturer's recommendations and confirmed by broth microdilution method following EUCAST guidelines (www.eucast.org). MICs interpretation was based on EUCAST Clinical Breakpoints - bacteria document v12.0. *Escherichia coli* ATCC 25922 was used as quality control. MICs of other antibiotics were determined using the Vitek II system (bioMèrieux, Marcy L'Etoile, France), but colistin and ceftazidime/avibactam were tested by broth microdilution method (https://www.eucast.org/ast_of_bacteria/mic_determination) and cefiderocol was tested by broth microdilution method using an iron depleted Mueller-Hinton broth (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Guidance_documents/Cefiderocol_MIC_testing_EUCAST_guidance_document_201217.pdf).

2.4. Detection of carbapenemases and β-lactamase genes

Phenotypic detection of carbapenemases was performed using the modified carbapenem-inactivation method (mCIM) [15]. Real Time PCR was used to identify β-lactamase genes involved in C/T resistance. Primer pairs used to detect bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA-48}, bla_{IMP}, and bla_{GES} were described previously [16], while bla_{PER} and bla_{VEB} were assessed with custom primers (Supplementary Table S1).

2.5. Molecular typing

Molecular relatedness of C/T-resistant isolates was evaluated by SpeI-PFGE, as described previously [17]. Isolates were classified following criteria established by Tenover et al. [18]. One strain for each PFGE pattern (10 isolates in total) was subsequently subjected to WGS. The phylogenetic relatedness of sequenced isolates was evaluated with CSI Phylogeny 1.4 (available at https://cge.cbs.dtu.dk/services/CSIPhylogeny/), using default parameters, publicly available genomes of *P. aeruginosa* ST111 from the NCBI-NIH database, and the *P. aeruginosa* PAO1 genome (accession number NC_002516.2) as a reference.

2.6. WGS and genome analysis

WGS was performed using an Illumina platform (2 × 150bp) on 10 isolates. Reads were assembled using SPAdes software [19]. Resistance genes and ORFs were annotated with ResFinder v3.2 (https://cge.cbs.dtu/services/ResFinder) and RAST v2.0 (https://rast.theseed.org). Sequence type (ST) was determined using the *P. aeruginosa* MLST Database (https://pubmlst.org/paeruginosa/). NCBI Blast was used to search β-lactamase genes and mutations in *ampC, ampD, ampR*, and *dacB* genes, using *P. aeruginosa* PAO1 (accession number NC_002516.2) as a reference. WGS data has been deposited in the NCBI database (BioProject number PRJNA715368).

3. Results

3.1. C/T screening and characterization of resistant strains

A total of 317 *P. aeruginosa* were collected during the study period. Resistance rates to antibiotics were 31.4% for piperacillin/tazobactam, 17.4% for meropenem, 24.4% for cefepime, and 12.1% for amikacin. C/T demonstrated greater activity in comparison with other antibiotics; indeed, 302 isolates were susceptible to C/T, while 15 (4.7%) showed a C/T MIC ≥8mg/L.

The susceptibility patterns of resistant strains are reported in Table 1. Besides C/T, all the strains were resistant to carbapenems and showed MDR phenotypes. Colistin was the most active antibiotic; only one isolate (PACT-11) was resistant to this molecule.

Ten of 15 C/T-resistant strains showed carbapenemase activity according to mCIM. Consistently, Real Time PCR assays revealed the presence of carbapenemase-encoding genes: eight isolates harbored bla_{VIM}, while the other two were positive for bla_{IMP}. Other carbapenemase genes were not found amongst the remaining isolates, but three carried acquired extended spectrum β-lactamase genes (two bla_{PER} and one bla_{GES}). Two strains were negative for all tested genes (Table 1).

Table 1
Antimicrobial susceptibility profiles and resistance markers of ceftolozane/tazobactam-resistant *Pseudomonas aeruginosa* strains isolated at 'Ospedali Riuniti' of Ancona, Italy

Strain	MIC (mg/L)[a]												*bla* gene PCR						
	C/T	MEM	IPM	ATM	TZP	CAZ	CZA	FEP	FDC	COL	CIP	AMK	PFGE	mCIM	VIM	KPC	IMP	NDM	OXA-48	GES	PER
PACT-1	>64/4(R)	32(R)	128(R)	4(I)	>64/4(R)	>64(R)	>64/4(R)	>16(R)	0.5(S)	1(S)	≥4(R)	8(S)	A	+	-	-	+	-	-	-	-
PACT-2	32/4(R)	64(R)	>128(R)	16(I)	16/4(I)	8(I)	8/4(S)	8(I)	0.5(S)	1(S)	≥4(R)	>16(R)	B1	+	+	-	-	-	-	-	-
PACT-3	64/4(R)	32(R)	>128(R)	16(I)	16/4(I)	16(R)	8/4(S)	8(I)	0.25(S)	1(S)	≥4(R)	>16(R)	B2	+	+	-	-	-	-	-	-
PACT-4	64/4(R)	8(I)	32(R)	16(I)	32/4(R)	>64(R)	8/4(S)	>16(R)	0.25(S)	1(S)	≥4(R)	≤4(S)	C	-	-	-	-	-	-	+	-
PACT-5	64/4(R)	32(R)	>128(R)	16(I)	16/4(I)	16(R)	16/4(R)	8(I)	0.25(S)	2(S)	≥4(R)	16(S)	B3	+	+	-	-	-	-	-	-
PACT-6	64/4(R)	32(R)	>128(R)	16(I)	16/4(I)	16(R)	16/4(R)	16(R)	0.25(S)	2(S)	≥4(R)	16(S)	B3	+	+	-	-	-	-	-	-
PACT-7	64/4(R)	32(R)	128(R)	16(I)	16/4(I)	16(R)	16/4(R)	16(R)	0.25(S)	2(S)	≥4(R)	16(S)	B3	+	+	-	-	-	-	-	-
PACT-8	64/4(R)	32(R)	>128(R)	16(I)	16/4(I)	16(R)	16/4(R)	16(R)	0.25(S)	1(S)	≥4(R)	>16(R)	B2	+	+	-	-	-	-	-	-
PACT-9	64/4(R)	32(R)	>128(R)	16(I)	16/4(I)	16(R)	16/4(R)	16(R)	0.25(S)	2(S)	≥4(R)	16(S)	B3	+	+	-	-	-	-	-	-
PACT-10	16/4(R)	8(I)	64(R)	>128(R)	>128/4(R)	>64(R)	32/4(R)	>16(R)	0.5(S)	1(S)	0.25(I)	≤4(S)	D	-	-	-	-	-	-	-	-
PACT-11	16/4(R)	16(R)	1(I)	32(R)	64/4(R)	>64(R)	8/4(S)	>16(R)	0.25(S)	>4(R)	≥4(R)	>16(R)	E	-	-	-	-	-	-	-	-
PACT-12	>64/4(R)	>64(R)	4(I)	>128(R)	64/4(R)	>64(R)	16/4(R)	>16(R)	0.25(S)	1(S)	≥4(R)	16(S)	F	-	-	-	-	-	-	-	+
PACT-13	64/4(R)	32(R)	128(R)	16(I)	16/4(I)	16(R)	32/4(R)	16(R)	0.25(S)	2(S)	≥4(R)	16(S)	B3	+	+	-	-	-	-	-	-
PACT-14	>64/4(R)	8(I)	64(R)	0.5(I)	4/4(I)	>64(R)	64/4(R)	>16(R)	8(R)	≤0.5(S)	≥4(R)	>16(R)	G	+	-	-	+	-	-	-	-
PACT-15	>64/4(R)	32(R)	8(R)	>128(R)	32/4(R)	>64(R)	8/4(S)	>16(R)	0.5(S)	4(S)	≥4(R)	16(S)	H	-	-	-	-	-	-	-	+

NOTE: Minimum inhibitory concentration values were interpreted following EUCAST Clinical Breakpoint Tables v12.0 as R, resistant; I, susceptible, increased exposure; S, susceptible, standard dosing regimen.
AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; C/T, ceftolozane/tazobactam; CZA, ceftazidime/avibactam; IPM, imipenem; FDC, cefiderocol; FEP, cefepime; mCIM, modified carbapenem inhibition method; MEM, meropenem; PFGE, pulsed field gel electrophoresis; TZP, piperacillin-tazobactam.

3.2. Molecular typing and genome analysis of C/T-resistant isolates

Characterization by SpeI-PFGE revealed the presence of 10 different pulsotypes (Table 1). Of note, three pulsotypes were strictly related and included eight of 15 isolates, all carrying bla_{VIM}. In silico MLST and WGS analysis performed on three isolates selected as representatives of the most frequent PFGE pattern revealed that they belonged to ST111 and were characterized by a high clonal relatedness (Fig. 1), exhibiting 7 to 9 separating SNPs (mean: 5; median: 7; Supplementary Table S2). Besides bla_{VIM-2}, the ST111 isolates carried OXA-395 and PDC-3, but no mutations involved in C/T resistance were found (Table 2). Evaluation of clonal relatedness including other ST111-bla_{VIM} isolates from Italy[7] showed that PACT-2, PACT-3, and PACT-5 constituted a distinct cluster (Fig. 2); the most closely related strain was S749_C15_RS (a *blaVIM-2*–carrying isolate), exhibiting 31 to 33 separating SNPs (mean and median: 32; Supplementary Table S3).

The seven remaining pulsotypes (Table 1) included strains belonging to five different STs: ST175 (n = 2), ST235 (n = 2), ST170 (n = 1), and ST621 (n = 1); all were identified as hospital clones. ST3354 (n = 1), a new ST not related to common high-risks clones, was also identified. Despite different PFGE patterns, phylogenic analysis showed that the ST175 and ST235 isolates clustered in two branches but showed greater genetic diversity compared with the ST111-bla_{VIM} clones (107 SNPs amongst the ST175 strains and 4215 amongst the ST235 strains; Supplementary Table S3).

Concerning β-lactams, ST175 strains carried different resistance determinants: (i) in addition to bla_{IMP-19}, PACT-1 carried ESBL gene bla_{PSE-1}; PACT-4 only carried bla_{GES-1}, despite belonging to the same ST (ii) both strains harbored bla_{OXA-50} and bla_{PDC-1}, showing no mutations previously associated with C/T resistance (Table 2).

Despite the great difference in SNPs, the two members of ST235, PACT-12 and PACT-15, were characterized by an identical β-lactamase content; both carried bla_{PER-1}, bla_{OXA-2}, $bla_{OXA-448}$, and bla_{PDC-35} (Table 2).

The remaining isolates were a ST621 isolate (PACT-14), carrying bla_{IMP-13}, bla_{OXA-50}, and bla_{PDC-3}, and ST170 and ST3354 strains (PACT-10 and PACT-11, respectively), negative for MBL and ESBL genes but harboring a different variant of the resident oxacillinase gene ($bla_{OXA-396}$) in comparison with the other strains. In addition, PACT-11 showed mutations in PBP3 (G63D and R504H) and PBP4 (G117S) (Table 2).

Alongside acquired and/or chromosome-borne β-lactamases, several mutations in AmpR and AmpD were found (Supplementary Table S4). AmpR was mutated (G283E, E287G, 3288Q, A290V, V291L, A293S, R294E, G295A, and Δ296) in ST111, ST235, and ST621 isolates, and all strains showed a G148A substitution in AmpD. Further mutations in AmpD were found in five of 10 isolates.

Analysis of the resistome showed that the C/T-resistant *P. aeruginosa* isolates carried a heterogeneous content of antibiotic resistance genes (Supplementary Table S4). Common identified resistance genes included *crpP* (quinolone resistance, nine of 10 isolates), *sul1* (sulfonamide resistance, eight of 10 isolates), and *catB7* (chloramphenicol resistance, nine of 10 isolates).

3.3. Clinical data

Patients' data (Supplementary Table 3, Supplementary Table S5) indicated that most were admitted to intensive or sub-intensive care wards, especially those infected with the ST111-bla_{VIM} clone. Half of the patients developed pneumonia. Interestingly, previous antibiotic therapy with β-lactams was common, accounting for 80% of cases, although C/T was used in only two patients. Other diffused variables included the presence of devices (central venous

Table 2
Epidemiological and genetic background of ceftolozane/tazobactam-resistant *Pseudomonas aeruginosa* isolates subjected to whole-genome sequencing in this study

Strain	PFGE	ST	β-lactams resistance				PBP3 mutations	PBP4 mutations
			MBL	ESBL	class D	PDC		
PACT-1	A	ST175	IMP-19	-	OXA-50	PDC-1	WT	WT
PACT-2	B1	ST111	VIM-2	-	OXA-395	PDC-3	WT	WT
PACT-3	B2	ST111	VIM-2	-	OXA-395	PDC-3	WT	WT
PACT-4	C	ST175	-	GES-1	OXA-50	PDC-1	WT	WT
PACT-5	B3	ST111	VIM-2	-	OXA-395	PDC-3	WT	WT
PACT-10	D	ST170	-	-	OXA-396	PDC-3	WT	WT
PACT-11	E	ST3354	-	-	OXA-396	PDC-208	G63D; R504H	G117S
PACT-12	F	ST235	-	PER-1	OXA-2; OXA-488	PDC-35	WT	WT
PACT-14	G	ST621	IMP-13	-	OXA-50	PDC-3	WT	WT
PACT-15	H	ST235	-	PER-1	OXA-2; OXA-488	PDC-35	WT	WT

ESBL, extended spectrum β-lactamase; MBL, metallo-β–lactamase; OXA, oxacillinase; PDC, *Pseudomonas aeruginosa*-derived cephalosporinase; PFGE, pulsed field gel electrophoresis; ST, sequence type.

Table 3
Clinical variables of patients infected with ceftolozane/tazobactam-resistant *Pseudomonas aeruginosa* strains

Variables	*P. aeruginosa* C/T-R (n = 15)		*P. aeruginosa* C/T-R ST111 (n = 8)	
	N	%	n	%
Mean age (Median)	65.4		72.2	
Ward				
Intensive and Subintensive care unit	9	60.0	7	87.5
Cystic fibrosis	2	13.3	0	0
Other[a]	4	26.7	1	12.5
Chronic comorbidities				
Gastrointestinal	2	13.3	1	12.5
Neurological	9	60.0	5	62.5
Cardiological	10	66.7	7	87.5
Cystic Fibrosis	2	13.3	0	0
COPD	3	20.0	2	25.0
Other[b]	7	46.7	4	50.0
Acute comorbidities				
Pneumonia	8	53.3	4	50.0
Stroke	3	20.0	3	37.5
Sepsi	2	13.3	2	25.0
Other[c]	3	20.0	3	37.5
Antibiotic therapy[d]				
C/T	2	13.3	2	20.0
β-lactams (including Carbapenems)	12 (8)	80.0 (53.3)	6 (4)	75.0 (50.0)
Colistin	2	13.3	1	12.5
Aminoglycosides	3	20.0	0	0
Other[e]	3	20.0	2	25.0
Not available	3	20.0	2	25.0
Invasive procedures[f]	6	40.0	3	37.5
Devices	12	80.0	7	87.5
Surgery[g]	4	26.7	3	37.5
Immunosuppressive or steroid therapy[h]	5	33.3	3	37.5
Days of hospitalisation >3	14	93.3	7	87.5
Survival[i]	8	53.3	2	25.0

C/T, ceftolozane/tazobactam; MDR, multi-drug resistant; XDR, extensively-drug resistant; cIAI, complicated intra-abdominal infections; cUTI, complicated urinary tract infection; HAP, hospital-acquired pneumonia; ESBL, extended spectrum β-lactamase; MBL, metallo-β-lactamase; MIC, minimum inhibitory concentration; mCIM, modified carbapenem-inactivation method; PFGE, pulsed field gel electrophoresis; WGS, whole genome sequencing; ST, sequence type; MLST, multilocus sequence typing; ICU, intensive care unit.

[a] Other wards included infectious disease (n = 1), long term care (n = 2), and 1 outpatient.
[b] Other comorbidities included diabetes mellitus, chronic kidney failure, tumor, and hypothyroidism.
[c] Other comorbidities included pressure ulcer, obstructive jaundice, and clostridium colitis.
[d] During the 12 months preceding positive culture.
[e] Other antibiotic therapies included macrolides, tetracyclines, quinolones, and glycopeptides.
[f] During the 72 hours preceding the isolation of *P. aeruginosa*. They included percutaneous endoscopic gastrostomy, mechanical ventilation, surgical drainage, and tracheotomy.
[g] During the three months preceding the isolation of *P. aeruginosa*.
[h] During the 30 days preceding the isolation of *P. aeruginosa*.
[i] Outcome was referred to 30 days after the isolation of *P. aeruginosa*.

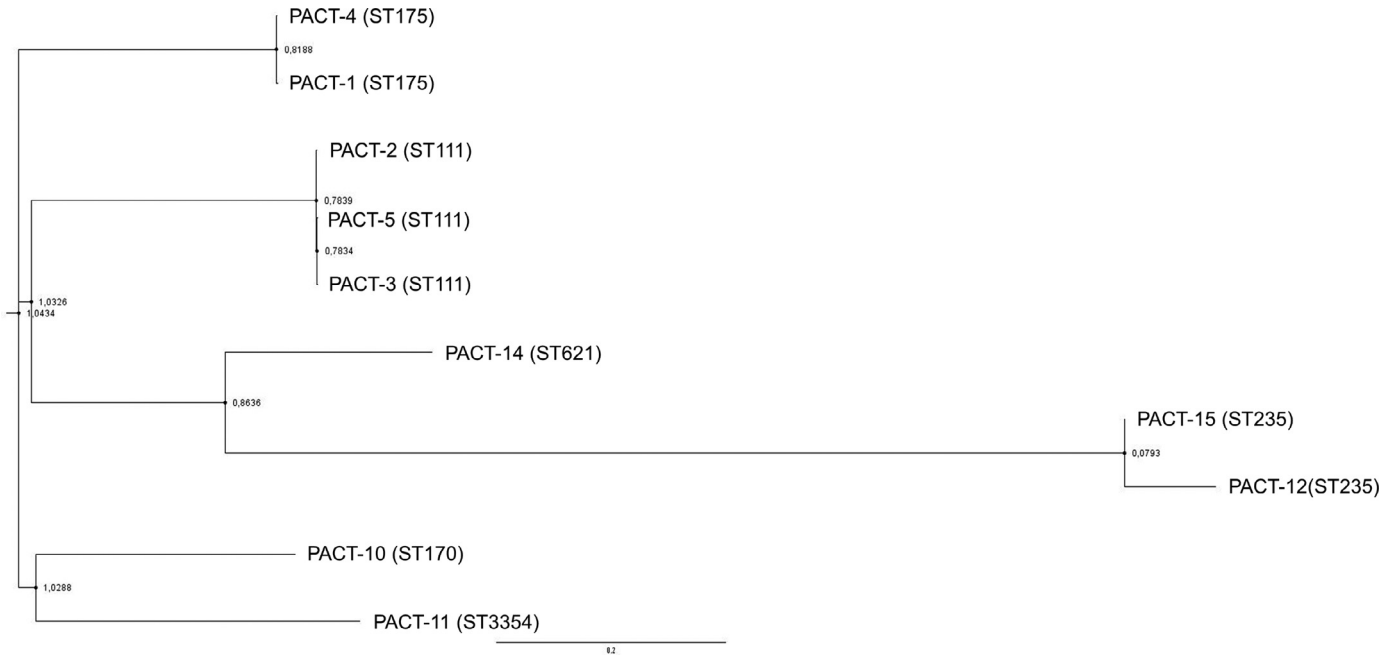

Fig. 1. Phylogenic tree of ceftolozane/tazobactam-resistant *Pseudomonas aeruginosa* strains subjected to whole-genome sequencing. Numbers next to the node represent the node ages.

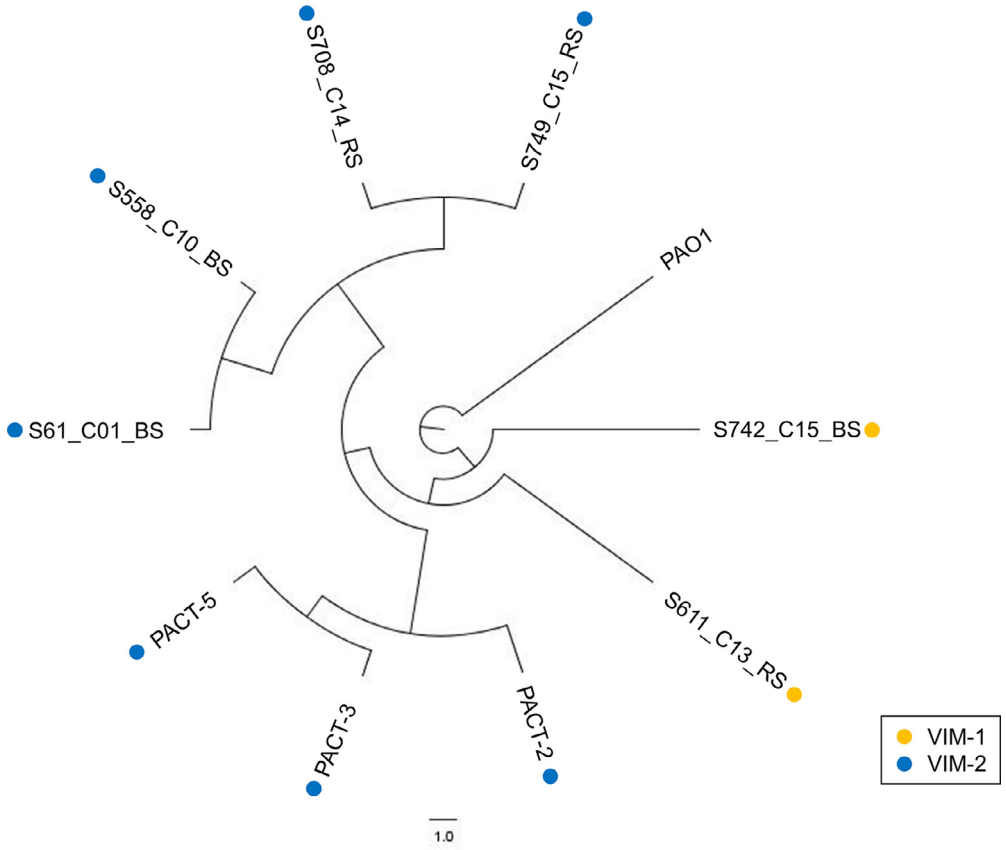

Fig. 2. Phylogenic tree of sequence type (ST)111 VIM-carrying ceftolozane/tazobactam-resistant *Pseudomonas aeruginosa* strains reported in Italy. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

catheters were the most used) in 80% of patients and prolonged hospitalization (more than three days).

4. Discussion

P. aeruginosa is an increasing threat in hospitals and resistant isolates have become a common etiological cause of infection. Several papers have identified MDR or XDR strains in nosocomial settings: a recent study analyzing *P. aeruginosa* strains from Spanish, Greek, and Italian hospitals revealed that the percentage of MDR and XDR isolates reached 30% [20]. In this scenario, new antibiotics represent the last (and often the only) resort for the treatment of MDR and XDR *P. aeruginosa*. C/T was designed to overcome some of the resistance mechanisms developed by pseudomonas (such as porin loss), and is a combination of a new cephalosporin and a well-known β-lactamase inhibitor [5]. Surveillance studies denoted the efficacy of C/T against *P. aeruginosa* strains, with greater activity when compared with cephalosporin, carbapenem, and piperacillin/tazobactam; although susceptible isolates were the majority, with susceptibility rates ranging from 90.9% to 99.0%, resistant isolates were not so rare [6,7,21–24]. In our study, resistance to C/T reached 4.7% of the isolates collected. This value is consistent with the worldwide percentage, but slightly lower than the value detected by the Italian surveillance study on C/T-resistant pseudomonas (8.9%) [7]. This difference may be explained by the different selection criteria for the isolates: the national surveillance was limited to isolates from bloodstream or lower respiratory tract infections.

C/T does not retain activity against isolates expressing KPC, metallo-β–lactamases, or OXA-48–lactamases, but ceftolozane resists the hydrolysis mediated by AmpC β-lactamases and its activity is not affected by permeability defects [5]. Alongside already well-known resistance mechanisms against this new antibiotic, novel mechanisms rapidly emerged with the use of C/T. Cabot et al. demonstrated the development of AmpC mutations in laboratory conditions on *P. aeruginosa* treated with C/T [10]. Moreover, several papers reported C/T-resistant isolates collected from clinical settings; resistance mechanisms included *ampC* mutations [11,12,25] or acquired β-lactamases, such as GES, PER, or FOX [13,26]. In our hospital, resistance was mainly because of production of carbapenemases (VIM and IMP) and/or ESBLs (GES-1 and PER-1). No mutations of PDC conferring C/T resistance were detected, although several variants involved in resistance to β-lactams were identified. In two strains (PACT-10 and PACT-11), resistance seemed to be caused by different mechanisms because no carbapenemases or ESBLs were found. Although PACT-11 showed a R504H substitution in PBP3, a mutation previously identified in C/T-resistant *P. aeruginosa* [27], and a substitution in PBP4, a gene directly involved in *ampC* regulation and resistance to β-lactams [28], its resident bla_{OXA} gene could further affect the observed phenotype. Indeed, OXA variants have been associated with resistance mechanisms to C/T in *P. aeruginosa* [29], and OXA-396, an OXA-50 variant produced by both PACT-10 and PACT-11, could contribute to C/T resistance in these isolates. Although its role in ceftazidime-avibactam resistance was excluded [30], further investigations are needed to clarify the real contribution of this variant to C/T resistance.

Despite C/T being recently introduced in our hospital, our results demonstrate that resistance mechanisms are various and widespread in a small clinical setting, suggesting the importance of determination using MICs prior to the administration of antibiotics.

Regarding the epidemiology of the C/T-resistant isolates, WGS data showed that almost all strains belonged to STs related to nosocomial clones. Indeed, ST111, ST175, and ST235 are high-risk clones widespread worldwide, with different rates of prevalence depending on which countries are considered [31,32]. In our hospital, ST111 was the most diffused lineage, and the few SNPs observed amongst the ST111 isolates were strongly suggestive of a nosocomial cluster, as also demonstrated by their phylogenetic distance from other ST111-bla_{VIM} strains isolated in Italy [7]. Moreover, all the ST111 strains were isolated from an intensive or sub-intensive department, further confirming this hypothesis. Other studies have reported outbreaks in hospitals sustained by ST111 *P. aeruginosa* [33,34]. In addition, ST111 *P. aeruginosa* strains were strictly associated with genetic elements carrying *blaVIM*, as also reported in the literature [35]. Along with the ST111 clone, a variety of other clones resistant to C/T were responsible for infection in our hospital. Resistance mechanisms were variable and involved different acquired resistance genes. Interestingly, PACT-11 belonged to a new ST (ST3354), which has never been found to be associated with C/T resistance. Our findings suggest that C/T resistance is a complex phenomenon involving several different resistance mechanisms and clones (not only those associated with hospital settings).

Most patients infected with C/T-resistant *P. aeruginosa* carried medical devices and had a history of previous antibiotic therapy, mainly with β-lactams. It has been reported that β-lactam administration induces the development of resistance [36]. Besides previous antibiotic therapy, the prolonged hospitalization of our patients could have contributed to the selection of resistant strains, in particular ST111-bla_{VIM} clones.

Because of the limited number of cases, we did not perform statistical analysis; however, a comparison between all infections and those sustained by ST111 clones showed no noticeable differences excepting mortality. Only 25% of patients with ST111 infections survived, compared with 53.3% of those infected. This difference may be explained by differences in patient status, taking into account that ST111 infections mainly involved patients admitted to intensive or sub-intensive care units. The number of patients was too low to achieve significant conclusions regarding clinical variables, which deserve further investigation.

5. Conclusions

P. aeruginosa, with its broad resistance to antibiotics, is a public health concern. In this study, we determined the incidence of C/T-resistant *P. aeruginosa* isolates in a teaching hospital in central Italy. Our results confirmed the presence of C/T-resistant strains in a small setting and demonstrated the variability of resistance mechanisms to this new combination. Moreover, we identified a cluster responsible for infection in intensive care wards. Identification and molecular, as well as epidemiological, study of resistant isolates is necessary to prevent and control the spread of these bacterial species in hospital settings.

Declaration of Competing Interest

None declared

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Ethical approval

The present research was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. The Institutional Review Board of the Azienda Ospedaliero-Universitaria Ospeadali Riuniti Umberto I°-Lancisi-Salesi granted retrospective access to the data without need for individual informed consent because the data were analyzed anonymously.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.07.010.

References

[1] Moradali MF, Ghods S, Rehm BH. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol 2017;7:39. doi:10.3389/fcimb.2017.00039.

[2] Horcajada JP, Montero M, Oliver A, Sorlí L, Luque S, Gómez-Zorrilla S, et al. Epidemiology and treatment of multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa* infections. Clin Microbiol Rev 2019;32 e00031–19. doi:10.1128/CMR.00031-19.

[3] Decraene V, Ghebrehewet S, Dardamissis E, Huyton R, Mortimer K, Wilkinson D, et al. An outbreak of multidrug-resistant *Pseudomonas aeruginosa* in a burns service in the north of England: challenges of infection prevention and control in a complex setting. J Hosp Infect 2018;100:e239–45. doi:10.1016/j.jhin.2018.07.012.

[4] Buhl M, Kästle C, Geyer A, Autenrieth IB, Peter S, Willmann M. Molecular evolution of extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains from patients and hospital environment in a prolonged outbreak. Front Microbiol 2019;10:1742. doi:10.3389/fmicb.2019.01742.

[5] van Duin D, Bonomo RA. Ceftazidime/avibactam and ceftolozane/tazobactam: second-generation β-lactam/β-lactamase inhibitor combinations. Clin Infect Dis 2016;63:234–41. doi:10.1093/cid/ciw243.

[6] Del Barrio-Tofiño E, Zamorano L, Cortes-Lara S, López-Causapé C, Sánchez-Diener I, Cabot G, et al. Spanish nationwide survey on *Pseudomonas aeruginosa* antimicrobial resistance mechanisms and epidemiology. J Antimicrob Chemother 2019;74:1825–35. doi:10.1093/jac/dkz147.

[7] Giani T, Arena F, Pollini S, D'Andrea MM, Henrici De Angelis L, Bassetti M, et al. Italian nationwide survey on *Pseudomonas aeruginosa* from invasive infections: activity of ceftolozane/tazobactam and comparators, and molecular epidemiology of carbapenemase producers. J Antimicrob Chemother 2018;73:664–71. doi:10.1093/jac/dkx453.

[8] Gherardi G, Linardos G, Pompilio A, Fiscarelli E, Di Bonaventura G. Evaluation of in vitro activity of ceftolozane-tazobactam compared to other antimicrobial agents against *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Diagn Microbiol Infect Dis 2019;94:297–303. doi:10.1016/j.diagmicrobio.2019.01.012.

[9] Shortridge D, Pfaller MA, Streit JM, Flamm RK. Antimicrobial activity of ceftolozane/tazobactam tested against contemporary (2015–2017) *Pseudomonas aeruginosa* isolates from a global surveillance programme. J Glob Antimicrob Resist 2020;21:60–4. doi:10.1016/j.jgar.2019.10.009.

[10] Cabot G, Bruchmann S, Mulet X, Zamorano L, Moyà B, Juan C, et al. *Pseudomonas aeruginosa* ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. Antimicrob Agents Chemother 2014;58:3091–9. doi:10.1128/AAC.02462-13.

[11] Berrazeg M, Jeannot K, Ntsogo Enguéné VY, Broutin I, Loeffert S, Fournier D, et al. Mutations in β-lactamase AmpC increase resistance of *Pseudomonas aeruginosa* isolates to antipseudomonal cephalosporins. Antimicrob Agents Chemother 2015;59:6248–55. doi:10.1128/AAC.00825-15.

[12] Fraile-Ribot PA, Cabot G, Mulet X, Periañez L, Martín-Pena ML, Juan C, et al. Mechanisms leading to in vivo ceftolozane/tazobactam resistance development during the treatment of infections caused by MDR *Pseudomonas aeruginosa*. J Antimicrob Chemother 2018;73:658–63. doi:10.1093/jac/dkx424.

[13] Ortiz de la Rosa JM, Nordmann P, Poirel L. ESBLs and resistance to ceftazidime/avibactam and ceftolozane/tazobactam combinations in *Escherichia coli* and *Pseudomonas aeruginosa*. J Antimicrob Chemother 2019;74:1934–9. doi:10.1093/jac/dkz149.

[14] Fraile-Ribot PA, Mulet X, Cabot G, Del Barrio-Tofiño E, Juan C, Pérez JL, et al. In vivo emergence of resistance to novel cephalosporin-β–lactamase inhibitor combinations through the duplication of amino acid D149 from OXA-2 β-lactamase (OXA-539) in sequence type 235 *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2017;61 e01117–17. doi:10.1128/AAC.01117-17.

[15] Pierce VM, Simner PJ, Lonsway DR, Roe-Carpenter DE, Johnson JK, Brasso WB, et al. Modified Carbapenem Inactivation Method for phenotypic detection of carbapenemase production among Enterobacteriaceae. J Clin Microbiol 2017;55:2321–33. doi:10.1128/JCM.00193-17.

[16] Coppi M, Antonelli A, Giani T, Spanu T, Liotti FM, Fontana C, et al. Multicenter evaluation of the RAPIDEC® CARBA NP test for rapid screening of carbapenemase-producing Enterobacteriaceae and Gram-negative nonfermenters from clinical specimens. Diagn Microbiol Infect Dis 2017;88:207–13. doi:10.1016/j.diagmicrobio.2017.04.009.

[17] Hu H, Manos J. Pulsed-field gel electrophoresis of *Pseudomonas aeruginosa*. Methods Mol Biol 2015;1301:157–70. doi:10.1007/978-1-4939-2599-5_14.

[18] Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–9. doi:10.1128/jcm.33.9.2233-2239.1995.

[19] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–77. doi:10.1089/cmb.2012.0021.

[20] Pérez A, Gato E, Pérez-Llarena J, Fernández-Cuenca F, Gude MJ, Oviaño M, et al. High incidence of MDR and XDR *Pseudomonas aeruginosa* isolates obtained from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. J Antimicrob Chemother 2019;74:1244–52. doi:10.1093/jac/dkz030.

[21] Farrell DJ, Sader HS, Flamm RK, Jones RN. Ceftolozane/tazobactam activity tested against Gram-negative bacterial isolates from hospitalised patients with pneumonia in US and European medical centres (2012). Int J Antimicrob Agents 2014;43:533–9. doi:10.1016/j.ijantimicag.2014.01.032.

[22] Walkty A, Karlowsky JA, Adam H, Baxter M, Lagacé-Wiens P, Hoban DJ, et al. In vitro activity of ceftolozane-tazobactam against *Pseudomonas aeruginosa* isolates obtained from patients in Canadian hospitals in the CANWARD study, 2007 to 2012. Antimicrob Agents Chemother 2013;57:5707–9. doi:10.1128/AAC.01404-13.

[23] Farrell DJ, Flamm RK, Sader HS, Jones RN. Antimicrobial activity of ceftolozane-tazobactam tested against Enterobacteriaceae and *Pseudomonas aeruginosa* with various resistance patterns isolated in U.S. Hospitals (2011–2012). Antimicrob Agents Chemother 2013;57:6305–10. doi:10.1128/AAC.01802-13.

[24] Kresken M, Körber-Irrgang B, Pfennigwerth N, Gatermann SG, Seifert H, et al. Dissemination of carbapenem-resistant *Pseudomonas aeruginosa* isolates and their susceptibilities to ceftolozane-tazobactam in Germany. Int J Antimicrob Agents 2020;55:105959. doi:10.1016/j.ijantimicag.2020.105959.

[25] MacVane SH, Pandey R, Steed LL, Kreiswirth BN, Chen L. Emergence of ceftolozane-tazobactam-resistant *Pseudomonas aeruginosa* during treatment is mediated by a single AmpC structural mutation. Antimicrob Agents Chemother 2017;61 e01183–17. doi:10.1128/AAC.01183-17.

[26] Fraile-Ribot PA, Del Rosario-Quintana C, López-Causapé C, Gomis-Font MA, Ojeda-Vargas M, Oliver A. Emergence of resistance to novel β-lactam–β-lactamase inhibitor combinations due to horizontally acquired AmpC (FOX-4) in *Pseudomonas aeruginosa* sequence type 308. Antimicrob Agents Chemother 2019;64:e02112–19. doi:10.1128/AAC.02112-19.

[27] López-Causapé C, Cabot G, Del Barrio-Tofiño E, Oliver A. The versatile mutational resistome of *Pseudomonas aeruginosa*. Front Microbiol 2018;9:685. doi:10.3389/fmicb.2018.00685.

[28] Zamorano L, Moyá B, Juan C, Oliver A. Differential beta-lactam resistance response driven by ampD or dacB (PBP4) inactivation in genetically diverse *Pseudomonas aeruginosa* strains. J Antimicrob Chemother 2010;65:1540–2. doi:10.1093/jac/dkq142.

[29] Arca-Suárez J, Lasarte-Monterrubio C, Rodiño-Janeiro BK, Cabot G, Vázquez-Ucha JC, Rodríguez-Iglesias M, et al. Molecular mechanisms driving the in vivo development of OXA-10–mediated resistance to ceftolozane/tazobactam and ceftazidime/avibactam during treatment of XDR *Pseudomonas aeruginosa* infections. J Antimicrob Chemother 2021;76:91–100. doi:10.1093/jac/dkaa396.

[30] Schaumburg F, Bletz S, Mellmann A, Becker K, Idelevich EA. Comparison of methods to analyse susceptibility of German MDR/XDR *Pseudomonas aeruginosa* to ceftazidime/avibactam. Int J Antimicrob Agents 2019;54:255–60. doi:10.1016/j.ijantimicag.2019.05.001.

[31] Del Barrio-Tofiño E, López-Causapé C, Oliver A. *Pseudomonas aeruginosa* epidemic high-risk clones and their association with horizontally-acquired β-lactamases: 2020 update. Int J Antimicrob Agents 2020;56:106196. doi:10.1016/j.ijantimicag.2020.106196.

[32] Hernández-García M, García-Castillo M, García-Fernández S, Melo-Cristino J, Pinto MF, Gonçalves E, et al. Distinct epidemiology and resistance mechanisms affecting ceftolozane/tazobactam in *Pseudomonas aeruginosa* isolates recovered from ICU patients in Spain and Portugal depicted by WGS. J Antimicrob Chemother 2021;76:370–9. doi:10.1093/jac/dkaa430.

[33] Witney AA, Gould KA, Pope CF, Bolt F, Stoker NG, Cubbon MD, et al. Genome sequencing and characterization of an extensively drug-resistant sequence type 111 serotype O12 hospital outbreak strain of *Pseudomonas aeruginosa*. Clin Microbiol Infect 2014;20:O609–18. doi:10.1111/1469-0691.12528.

[34] Wendel AF, Kolbe-Busch S, Ressina S, Schulze-Röbbecke R, Kindgen-Milles D, Lorenz C, et al. Detection and termination of an extended low-frequency hospital outbreak of GIM-1–producing *Pseudomonas aeruginosa* ST111 in Germany. Am J Infect Control 2015;43:635–9. doi:10.1016/j.ajic.2015.02.024.

[35] Botelho J, Grosso F, Quinteira S, Brilhante M, Ramos H, Peixe L. Two decades of *blaVIM*-2–producing *Pseudomonas aeruginosa* dissemination: an interplay between mobile genetic elements and successful clones. J Antimicrob Chemother 2018;73:873–82. doi:10.1093/jac/dkx517.

[36] Carsenti-Etesse H, Cavallo JD, Roger PM, Ziha-Zarifi I, Plesiat P, Garrabé E, et al. Effect of beta-lactam antibiotics on the in vitro development of resistance in *Pseudomonas aeruginosa*. Clin Microbiol Infect 2001;7:144–51. doi:10.1046/j.1469-0691.2001.00225.x.