International Journal of Antimicrobial Agents 53 (2019) 669–673

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Sequential emergence of colistin and rifampicin resistance in an OXA-72- producing outbreak strain of *Acinetobacter baumannii*

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ARTICLE INFO

Article history:
Received 27 September 2018
Accepted 21 January 2019

Editor: Jean-Marc Rolain

Keywords:
Acinetobacter baumannii
Outbreak
Colistin resistance
Rifampicin resistance
Carbapenemase

ABSTRACT

Objectives: This study reported a hospital outbreak due to an extensively drug-resistant (XDR) OXA-72-producing strain of *Acinetobacter baumannii* (*A. baumannii*).
Methods and Results: The isolates were found to be genotypically indistinguishable by whole-genome multiple locus sequence typing, and to belong to the international clonal complex CC2. One of these isolates sequentially developed a high resistance to colistin and rifampicin under treatment, as a result of mutations in genes *pmrB* and *rpoB*, respectively. The bla_{OXA-72} gene was localised on a 10-kb transferable plasmid, named pAB-STR-1, whose sequence is nearly identical to that of another plasmid previously found in Lithuanian strains, pAB120.
Conclusion: This report highlighted the need to carefully monitor the emergence of colistin and rifampicin resistance in patients treated for infections with multidrug-resistant *A. baumannii*.

1. Introduction

Carbapenem resistance in *Acinetobacter baumannii* (*A. baumannii*) is essentially linked to the expression of class D carbapenemases belonging to either the OXA-23, OXA-24/40 or OXA-58 group. The OXA-23 group largely predominates in several countries such as France [1]. However, a single amino-acid variant of OXA-24, named OXA-72, is increasingly being reported in Europe, Asia and South America [2]. Treatment of carbapenem-resistant *A. baumannii* infections with colistin, a polymyxin considered as the ultimate option against multidrug-resistant Gram-negative species, may unfortunately lead to the selection of colistin-resistant mutants. In *A. baumannii*, such a resistance relies on two main mechanisms [3]. The first consists of enzymatic addition of phosphoethanolamine to the lipid A of lipopolysaccharide (LPS) as a result of mutations occurring in the genes encoding a two-component regulatory system, PmrAB. The second one, mainly selected in *in vitro* experiments, relates to the complete loss of LPS by inactivation of lipid A biosynthesis genes [3]. The European Centre for Disease Prevention and Control reported that 4% of invasive *A. baumannii* strains were resistant to polymyxins in Europe in 2016, of which the majority (70.7%) were from Greece and Italy [4]. However, if the small number of isolates tested and the different laboratory methodologies used to characterise resistant organisms are considered, this rate may not be representative of Europe. In countries such as France, colistin-resistant *A. baumannii* strains remain scarce.

The present study analysed four clonally-related OXA-72-producing *A. baumannii* strains, of which two developed a resistance to last-resort antibiotics colistin and rifampicin under treatment.

2. Materials and methods

2.1. Bacterial isolates and drug susceptibility testing

The four MDR-AB strains were identified at the species level by using matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper CA system, Brucker Daltonics, Billerica MA, USA). Bacterial susceptibility to

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https://doi.org/10.1016/j.ijantimicag.2019.01.012
0924-8579/

antibiotics was assessed by the diffusion method in Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) with disks of minocycline and trimethoprim-sulfamethoxazole (Bio-Rad), and with Etests of ampicillin-sulbactam, tigecycline and rifampicin (BioMérieux, Marcy l'Etoile, France). Minimal inhibitory concentrations (MICs) of other β-lactams, aminoglycosides and colistin were determined by the broth microdilution method and with customised Sensititre™ plates (Thermo Fisher Scientific, Villebon-sur-Yvette, France). Colistin MICs were also determined with MIC strips from Liofilchem, Italy. The results were interpreted according to the Clinical and Laboratories Standards Institute breakpoints, as updated in 2018 [5].

2.2. Plasmid characterisation and mating-out assays

Plasmid deoxyribonucleic acid (DNA) was extracted according to the method by Kieser et al. [6]. The four plasmids harboured by *Escherichia coli* NCTC 50192 (154-kb, 66-kb, 48-kb, and 7-kb, respectively) served as size markers to calibrate agarose gels of electrophoresis. Mating-out assays were performed between clinical isolates and azide-resistant *A. baumannii* recipient strain BM4547 [7]. Mean plasmid transfer rate and standard deviation were calculated from three independent conjugation experiments, as previously described [7].

2.3. Molecular biology, whole DNA sequencing and biostatistics methods

Carbapenem resistance gene $bla_{\text{OXA-72}}$ was identified by polymerase chain reaction (PCR)-sequencing, as reported elsewhere [8]. Total bacterial DNA was extracted from overnight cultures by using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), was then quantified with a NanoDrop™ spectrophotometer (Ozyme, Montigny-le-Bretonneux, France), and finally sequenced by Microsynth AG (Balgach, Switzerland) on an Illumina NextSeq sequencer with v2 chemistry, using 2×150-bp paired-end reads. The DNA libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA). Genomes of the *A. baumannii* isolates were *de novo* assembled by using CLC Genomics Workbench 10.1.1 (Qiagen). Contigs were downloaded to the Patric server (https://www.patricbrc.org) for sequence annotation. Total data sequences were submitted to ResFinder 3.0 server (http://www.genomicepidemiology.org/) to identify antibiotic resistance genes. Assembled genomic DNAs were submitted to single nucleotide polymorphism (SNP) analysis and whole genome multiple locus sequence typing analysis (wgMLST) with BioNumerics 7.6.3. Each retained SNP position had a minimum 10X coverage with a frequency >90%, and was covered at least once in both forward and reverse directions. A 12-bp minimum distance between SNP positions was also retained. The sequence type (ST) of the isolates was determined according to the Oxford and Pasteur MLST schemes (http://pubmlst.org/abaumannii/), by using the online MLST 1.8 tool (https://cge.cbs.dtu.dk/services/MLST/). AbaR genomic islands were analysed by PCR with the primers listed in Table S1 (supplemental material).

2.4. Nucleotide accession numbers

Genomic DNA of *A. baumannii* isolates was submitted to the GenBank database under accession numbers RAHS00000000 (isolate 1), RAHR00000000 (isolate 2), RAHQ00000000 (isolate 3) and SETH00000000 (isolate 4).

Table 1
Antimicrobial susceptibility of multidrug-resistant OXA-72-producing *A. baumannii* isolates, France, September 2014–March 2015 (n = 4).

Antibiotic	MIC [mg/L]			
	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Ampicillin-sulbactam**[a]	4	6	6	4
Ticarcillin-clavulanic acid[b]	**>256**	**>256**	**>256**	**>256**
Piperacillin-tazobactam[c]	**256**	**256**	**256**	**256**
Ceftazidime	**128**	**256**	**128**	**128**
Cefepime	**64**	**128**	**64**	**64**
Imipenem	**>32**	**>32**	**>32**	**>32**
Meropenem	**>32**	**>32**	**>32**	**>32**
Ciprofloxacin	**>32**	**>32**	**>32**	**>32**
Gentamicin	**>64**	**>64**	**64**	**64**
Amikacin	**64**	**64**	**32**	**64**
Tobramycin	**>32**	**>32**	**32**	**>32**
Tigecycline**	*2*	*2*	*2*	*2*
Rifampicin**	*4*	*4*	*>256*	*>256*
Colistin* (MIC strip)	0.75	1	1	0.75
Colistin (broth dilution)	1	1	**16**	**32**

MIC values in italic do not allow characterisation of strains as S-I-R, as no breakpoints have been established by the Clinical and Laboratories Standards Institute for the corresponding antibiotics.
Values in boldface correspond to non-susceptible strains, as defined by the Clinical and Laboratories Standards Institute. Other values refer to susceptible strains.
* MICs were determined with MIC strips (Liofilchem), ** MICs were determined with Etests (BioMérieux). MICs were determined by broth microdilution for other antibiotics
[a] MIC values of ampicillin with a fixed ampicillin to sulbactam ratio of 2:1
[b] MIC values of ticarcillin with a fixed concentration of clavulanate equal to 2 mg/L
[c] MIC values of piperacillin with a fixed concentration of tazobactam equal to 4 mg/L

3. Results and discussion

3.1. Outbreak description

The index case (Patient 1) was a patient suffering from ischaemic heart disease. During a stay in Algeria in June 2014, the patient underwent surgery in Alger for leg cellulitis, and developed septic shock with renal failure. He was repatriated to France on 10 September 2014 and was admitted to the nephrology unit of a tertiary care hospital. Microbial analysis of a dialysis catheter yielded a multidrug-resistant *A. baumannii* (MDR-AB) only susceptible to ampicillin-sulbactam and colistin (Table 1). The following month, the patient was transferred to the intensive care unit of the same hospital, where he successively developed several septic shocks caused by different multidrug-resistant bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae*). He died in late November.

Patient 2 was admitted to the same unit on 31 October 2014 for cardiorespiratory arrest and pneumothorax. On 20 November, an arterial catheter and a respiratory specimen were found positive with an MDR-AB interpreted as a colonisation. The patient did not receive antibiotics and died from asystole on January 2015.

Patient 3, who suffered from acute leukaemia, was hospitalised in the same intensive care unit from 4–6 October 2014, and again from 5 December 2014 to 23 January 2015. She received several antibiotic regimens, including colistin from 9–30 December. Culture of a tracheal aspirate yielded an MDR-AB resistant to colistin but exhibiting a wild-type susceptibility to rifampicin on 25 December. This isolate was deemed to be non-clinically significant, and all antibiotics were discontinued on 30 December. However, the patient experienced septic shock from pulmonary origin on 5 January, caused by a colistin-resistant MDR-AB displaying the same drug susceptibility profile as previously. A new and successful treatment was initiated with colistin, tigecycline and rifampicin

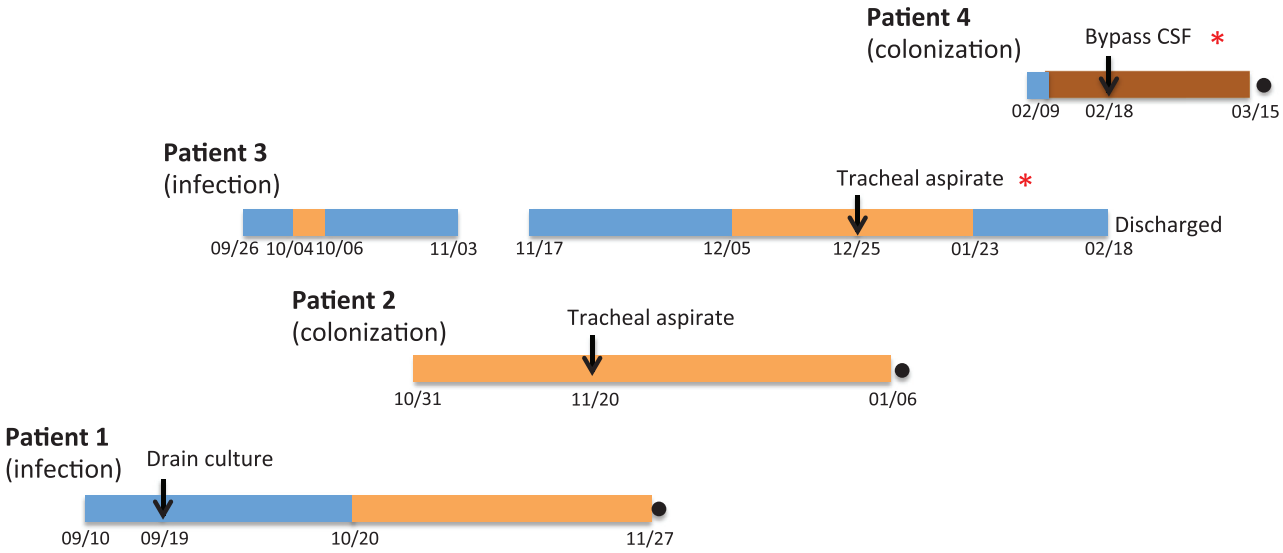

Figure 1. Timeline of patients infected/colonised with carbapenem-resistant OXA-72-producing *Acinetobacter baumannii*, September 2014–March 2015 ($n = 4$). , medical intensive care unit; , surgical intensive-care unit; , other wards; ↓ first *A. baumannii* isolate; *, colistin-resistant isolate; ●, patient's death; CSF: cerebrospinal fluid. Important dates (month/day) are indicated for each patient.

(600 mg twice a day) for 3 weeks until discharge from the hospital on 18 February 2015.

Patient 4 was admitted to the emergency department on 9 February 2015 for cerebellar haematoma. She underwent surgery and was transferred to the surgical intensive care unit on 10 February. On 18 February, a bypass cerebrospinal fluid culture turned out to be positive with a colistin-resistant and rifampicin-resistant MDR-AB, which was considered as a colonisation. The patient died on March 2015 from multiple organ failure. Details on the patients' history are indicated in Figure 1.

3.2. Bacterial susceptibility to antibiotics

All the isolates exhibited a high level of resistance to penicillins, broad-spectrum cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones (MICs in Table 1), and contact-to-disk resistance to trimethoprim-sulfamethoxazole and minocycline (data not shown). On the other hand, all bacteria were susceptible to ampicillin-sulbactam, according to the Clinical and Laboratories Standards Institute breakpoints ($S \leq 8/4$ mg/L; $R \geq 32/16$ mg/L). In contrast to isolates 1 and 2, isolates 3 and 4 were resistant to colistin with the broth microdilution reference method but when tested with Etest strips displayed a false susceptibility to this antibiotic, which was consistent with previous reports of very major errors with ellipsomer tests of colistin (Table 1) [9]. Interestingly, a strong increase in rifampicin MICs was noted in isolates 3 and 4 (MIC > 256 mg/L) compared with isolates 1 and 2 (MIC = 4 mg/L) (Table 1). The four isolates were classified as XDR (extensively-drug resistant) with regards to the criteria defined by Magiorakos et al. [10].

3.3. Plasmid characterisation and mating-out assays

PCR targeting known carbapenemase genes revealed the presence of $bla_{\text{OXA-24-like}}$ and $bla_{\text{OXA-51-like}}$ determinants in the four isolates, which in parallel were found to contain a plasmid of ca. 10-kb by agarose gel electrophoresis. This plasmid, named pAB-STR-1, could be transferred along with the $bla_{\text{OXA-24-like}}$ gene to azide-resistant *A. baumannii* strain BM4547 (data not shown), with a mean transfer frequency of $3.1 \pm 2.6 \times 10^{-7}$.

3.4. Whole genome analysis

High throughput sequencing of the bacterial genomes yielded a mean number of contigs of 95 per strain, with a N50 of 91,992-bp. The size of in silico reconstructed genomes was 3.9 Mb, with an average GC% of 39, which is in accordance with *A. baumannii* species. Multiple locus sequence typing (MLST) of the four isolates indicated that they belonged to ST2 in reference to the 'Pasteur Institute scheme', and to ST348 according to the Oxford database. ST348 is a single locus variant and double locus variant of ST218 and ST208, respectively, all belonging to international clone 2 [11,12].

Analysis of whole genome sequencing (WGS) data revealed that the isolates possessed the naturally-occurring $bla_{\text{OXA-66}}$ gene and shared the same resistome (Table 2). Their resistance profile to β-lactams was the result of insertion of mobile element IS*Aba1* upstream of gene $bla_{\text{ADC-30}}$, an event that triggers overproduction of intrinsic cephalosporinase ADC, and of acquisition of carbapenemase OXA-72-encoding gene $bla_{\text{OXA-72}}$ (Table 2). Though mostly identified in international clone 2, gene $bla_{\text{OXA-72}}$ has also spread in distinct clonal lineages such as IC1 ST1, IC6 ST78 or ST25 [13-15]. Furthermore, WGS showed that the $bla_{\text{OXA-72}}$-carrying plasmid pAB-STR-1 was 10,879-bp in length and belonged to GR2 plasmid family. The pAB-STR-1 differs from two previously characterised plasmids: pAba70743_1 from Sweden (accession number NZ_CM009924.1) and pAB120 from Lithuania [16] by only 1 and 3 SNPs, respectively. Plasmid pAB120 was considered as the main vehicle of $bla_{\text{OXA-72}}$ gene in Lithuanian *A. baumannii* isolates and held responsible for the increasing incidence of carbapenem-resistant *A. baumannii* in this country [16]. Like pAB120, pAB-STR-1 carries two copies of $bla_{\text{OXA-72}}$, bracketed by XerC/XerD-like sites. Xer proteins are predicted to promote the diffusion of $bla_{\text{OXA-72}}$ among *Acinetobacter* isolates, by acting as a site-specific recombination system [16]. In addition to five aminoglycoside resistance genes (Table 2), the outbreak isolates in the current study contained fluoroquinolone-resistance associated mutations in DNA gyrase (Ser-81-Leu in GyrA subunit) and topoisomerase IV (Ser-84-Leu and Ser-467-Gly in subunit ParC; Val-237-Ala in subunit ParE) [2].

Analysis of colistin-resistance mechanisms was performed. Compared with the two colistin-susceptible strains (isolates 1 and 2), the colistin-resistant isolates 3 and 4 harboured a single amino

Table 2
Antimicrobial resistance genes and in silico-deduced resistance phenotypes.

Antibiotic family	Resistance gene	% identity	Localisation	Enzyme		GenBank accession no.	Antimicrobial resistance[c]	
				Name	Function		In silico deduced	Observed
Aminoglycosides	*aacC1*	100	Chromosome	AAC(3)-Ia	Aminoglycoside acetyltransferase	NC_024997	GEN	GEN, TOB, AMK
	aacA16	100	Chromosome	AAC(6′)-Ia	Aminoglycoside acetyltransferase	NG_052380.1	TOB, AMK	GEN, TOB, AMK
	ant(3′′)-II	100	Chromosome	ANT(3′′)-II	Aminoglycoside adenyltransferase	ADX01798	STR, SPN	GEN, TOB, AMK
	strA	100	Chromosome	APH(3′′)-1b	Aminoglycoside phosphatase	M96392	TOB, AMK	GEN, TOB, AMK
	strB	100	Chromosome	APH(6)-1d	Aminoglycoside phosphatase	M96392	STR	GEN, TOB, AMK
β-Lactams	$bla_{\text{ADC-30}}$[a]	100	Chromosome	ADC-30	Cephalosporinase	EF016355	Penicillins and expanded-spectrum cephalosporins (if overexpressed)	All β-lactams
	$bla_{\text{OXA-66}}$	100	Chromosome	OXA-66	CHDL[b]	FJ360530	Penicillins and carbapenems (if overexpressed)	All β-lactams
	$bla_{\text{OXA-72}}$	100	Plasmid	OXA-72	CHDL[b]	GU199039	Penicillins and carbapenems	All β-lactams
Sulfonamide	*sul2*	100	Chromosome	Sul2	Alternate dihydropteroate synthetase	GQ421466	SUL	SUL
Tetracycline	*tetB*	100	Chromosome	TetB	Efflux pump	NG_048161	TET	TET

[a] IS*Aba1* upstream of $bla_{\text{ADC-30}}$
[b] CHDL, carbapenem-hydrolysing Ambler class D β-lactamase
[c] GEN, gentamicin; TOB, tobramycin; AMK, amikacin; STR, streptomycin, SPN, spectinomycin, SUL, sulfonamide; TET, tetracycline

acid substitution in PmrB (Ala226Thr), the histidine kinase sensor of two-component regulatory system PmrAB that controls enzymatic addition of phosphoethanolamine to the LPS in *A. baumannii*. Of interest, this substitution was very recently identified in an *in vitro*-selected colistin-resistant mutant [17]. In addition, an Ala226Val change at the same position was detected in a colistin-resistant (32 mg/L) blood isolate from the USA [18]. Alanine 226, which is close to the phosphorylation site (His228) of PmrB, thus might represent a hot spot for polymyxin-resistance associated mutations. On the other hand, this study confirmed that such mutations do not affect the capacity of *A. baumannii* to spread in healthcare facilities, as the same MDR-AB strain was recovered from patients (3 and 4) located in distinct wards (Figure 1).

Indeed, SNP analysis revealed that the four isolates differed from each other by 1–3 core SNPs. Of these, two occurred in the RNA polymerase-encoding gene *rpoB* in isolate 3 (Ser518Arg substitution in β subunit of RNA polymerase, RpoB) and isolate 4 (Asp525Asn), respectively, leading to high rifampicin MICs for those isolates (Table 1). In Patient 3, resistance to rifampicin was acquired *in vivo* under rifampicin treatment, since sequencing of *rpoB* gene in a MDR-AB isolate recovered in December 2015 (before rifampicin treatment) showed a wild-type *rpoB* genotype (data not shown). Since isolates 3 and 4 exhibited the same PmrB substitution Ala226Thr, and given that only Patient 3 was treated with rifampicin, it is unclear which selection event enabled the emergence of the rifampicin-resistant mutant isolated from Patient 4. A trivial explanation would be that isolate 4 was not acquired by Patient 4 from Patient 3, but from another infected or colonised patient treated with rifampicin for staphylococcal infection. Mutations Ser518Arg and Asp525Asn are both located in the conserved domain I of RpoB, with position Asp525 being involved in direct binding to rifampicin [19]. Several amino acid variations at this position, including Asp525Asn, have been associated with rifampicin resistance in clinical strains of *A. baumannii* [20]. Compared with isolate 1, isolates 2, 3 and 4 displayed an SNP in gene *csuE* (Leu58Ser change in protein CsuE). This gene is part of the chaperone-usher pilus assembly system that produces pili necessary for biofilm formation. The *csu* operon is differentially expressed in colistin-resistant isolates [21]. However, further research is required to clarify the impact of this SNP on the resistance or the pathogenicity of *A. baumannii*. The four isolates were compared by whole-genome MLST. Over the 3414 *loci* examined, a 99.9% sequence identity was found between the strains, which is consistent with their clonal relationship.

The presence of AbaR genomic islands was investigated by analysing the *comM* gene, which is the hot-spot integration site of those genetic structures [2]. As the *comM* gene was truncated, the presence of one AbaR structure was strongly suggested. Seven contigs that matched AbaR25 were found in the isolate 1 draft genome. AbaR25 was detected in a ST2 isolate responsible for an outbreak in Latvia [22]. This genomic island was 46,469-bp long and carried the *sul2, strA* and *strB* genes, a truncated *tet-like* determinant, and carbapenemase OXA-23-encoding gene $bla_{\text{OXA-23}}$ within transposon Tn*2006*. The seven contigs identified in isolate 1 were ordered and linked using PCR (Table S1), which revealed the existence of a 41,663-bp resistance island identical to AbaR25, except that it lacked transposon Tn*2006*. This resistance island was detected in the four isolates.

4. Conclusion

The hospital outbreak involving clone ST2, OXA-72-producing *A. baumannii* strains was initiated by a patient repatriated from North Africa. Interestingly, this clone shared common genetic features with carbapenem-resistant *A. baumannii* isolates from Baltic countries. It was shown to carry the $bla_{\text{OXA-72}}$ gene on a GR2 plasmid previously identified in *A. baumannii* isolates from Lithuanian hospitals, and to possess an AbaR resistance island nearly identical to AbaR25 described in Latvia [16,22]. It is believed that only two outbreaks of colistin-resistant *A. baumannii* have been reported to date: the first one in Spain in 2009 and the second one in Italy in 2014 [23,24]. In France, national guidelines do not recommend screening of repatriated patients for MDR-AB. Considering the

ability of *A. baumannii* to persist in the hospital environment, to develop multidrug resistance and to cause nosocomial outbreaks, the issue of such a systematic screening needs to be reconsidered. While colistin and rifampicin repeatedly demonstrate synergistic interactions in vitro and in animal models of infection, even towards colistin-resistant *A. baumannii* strains, the therapeutic efficacy of this drug combination against MDR-AB is more mitigated (recently reviewed by Lee et al.) [25]. Because it abrogates synergism with colistin, rifampicin resistance can be a possible cause of clinical failure of colistin-rifampicin combined therapy, and should be monitored in sequential isolates from individual patients.

Funding

This study was financially supported by the Santé publique France Agency via the French Ministry of Health.

Competing Interests

None.

Ethical Approval

Not required.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.01.012.

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