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Lytic bacteriophages against multidrug-resistant *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* isolates from orthopaedic implant-associated infections

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ABSTRACT

Orthopaedic implant-associated infections are a devastating complication of orthopaedic surgery with a significant impact on patients and healthcare systems. The aims of this work were to describe the patterns of antimicrobial resistance, pathogenicity and virulence of clinical bacterial isolates from orthopaedic implant-associated infections and to further isolate and characterise bacteriophages that are efficient in controlling these bacteria. *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli* isolated from orthopaedic infections showed multiresistance patterns to the most frequently used antibiotics in clinical settings. The presence of mobile genetic elements (*mecA*, Tn*916*/Tn*1545* and *intI1*) and virulence determinants (*icaB, cna, hlb, cylLs, cylM, agg, gelE, fsr* and *fimA*) highlighted the pathogenicity of these isolates. Moreover, the isolates belonged to clonal complexes associated with the acquisition of pathogenicity islands and antimicrobial resistance genes by recombination and horizontal gene transfer. Bacteriophages vB_SauM_LM12, vB_EfaS_LM99 and vB_EcoM_JB75 were characterised and their ability to infect clinical isolates of *S. aureus, E. faecalis* and *E. coli*, respectively, was assessed. Morphological and genomic analyses revealed that vB_EfaS_LM99 and vB_EcoM_JB75 belong to the Siphoviridae and Myoviridae families, respectively, and no genes associated with lysogeny were found. The bacteriophages showed low latent periods, high burst sizes, broad host ranges and tolerance to several environmental conditions. Moreover, they showed high efficiency and specificity to infect and reduce clinical bacteria, including methicillin-resistant *S. aureus* and vancomycin-resistant enterococci. Therefore, the results obtained suggest that the bacteriophages used in this work are a promising approach to control these pathogens involved in orthopaedic implant-associated infections.

1. Introduction

Orthopaedic implant-associated infections are a devastating complication of orthopaedic surgery with a significant impact on patient quality of life and healthcare systems [1]. The most commonly isolated bacteria from these infections are *Staphylococcus aureus* (33–43%), *Staphylococcus epidermidis* (18–40%) and *Enterococcus* spp. (2.5–15%, mainly *Enterococcus faecalis*). However, Gram-negative bacilli, including *Escherichia coli* and *Pseudomonas aeruginosa*, are less frequent causes of implant-associated infection (4–7%) [2–4].

The emergence of antimicrobial-resistant bacteria and their ability to produce virulence factors have contributed to enhancing the pathogenicity and severity of orthopaedic implant-associated infections [3,4–7]. According to the World Health Organization (WHO), these bacteria, namely methicillin-resistant

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Table 1
Antimicrobial resistance profile and genetic characterisation of bacteria isolated from orthopaedic implant-associated infections provided by the Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE (Vila Nova de Gaia, Portugal).

Species	Strain	Source	Patient sex/age (years)	Antimicrobial resistance[a]	Genetic characterisation			
Enterobacter cloacae	2107408	Thigh bone	F/76	STR/CIP/FOX/CEF/AMX/SUL/MFX/TMP				
Klebsiella pneumoniae	2133201	Shoulder	M/39	AMX/NEO				
Acinetobacter lwoffii	2170401	Hip prosthesis	M/74	FEP				
Pseudomonas aeruginosa	209960	Lumbar arthrodesis	F/69	ATM/GEN/NET				
					Type	VFs	ST	CC
Staphylococcus aureus	2117045	Synovial fluid	F/72	ERY/FOX/CIP/GEN/AMP	MRSA	*icaB–cna–hlb*	239	8
	2093367	Knee	F/60	ERY/FOX/CIP/AMP	MRSA	*cna*	22	22
	2104780	Ankle	F/75	ERY/FOX/CIP/AMP	MRSA	*icaB–cna–hlb*	22	22
	2117741	Knee	M/70	ERY/FOX/CIP/GEN/AMP	MRSA	*icaB–cna–hlb*	22	22
	2106876	Hip prosthesis	F/53	GEN/AMP	MSSA	*icaB–hlb*	72	8
	2179342	Synovial fluid	M/42	GEN/AMK/AMP	MSSA	*icaB–cna–hlb*	72	8
					Transposon	VFs	ST	CC
Enterococcus faecalis	2099610	Lumbar arthrodesis	F/69	QDA/CIP/VAN	N/D	*cylLs*	117	21
	2105322	Septic arthritis	M/77	ERY/QDA/CIP/VAN	N/D	*agg–gelE–fsr–cylLs*	117	21
	2104780	Ankle	F/75	ERY/QDA/CIP/TET/NOR/VAN/TEC	Tn*916*/Tn*1545*	*agg–fsr–cylLs*	6	2
	2133201	Shoulder	M/39	ERY/QDA/CIP/TET/NOR/VAN/TEC	Tn*916*/Tn*1545*	*agg–cylM–fsr–cylLs*	16	58
	2084972	Soft foot bones	F/79	ERY/QDA/CIP/TET/NOR/VAN/TEC	Tn*916*/Tn*1545*	*agg–cylLs*	6	2
	2093926	Knee	M/76	ERY/QDA/CIP/VAN	N/D	*agg–fsr–cylLs*	117	21
	8105329	Knee prosthesis	M/75	ERY/QDA/VAN	N/D	*agg–gelE–fsr–cylLs*	117	21
					Phylogenetic group	VFs	Integron	
Escherichia coli	2129975	Iliac crest	M/86	CIP/CEF/MFX/TMP/NEO/NAL/OFX	B1	*fimA*	*intl1*	
	2154120	Hip prosthesis	M/47	CEF/TMP/NEO/NAL/OFX	A	*fimA*	N/D	

VF, virulence factor; ST, sequence type; CC, clonal complex; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; N/D, not detected.
[a] AMK, amikacin; AMP, ampicillin; AMX, amoxicillin; ATM, aztreonam; CEF, cefalotin; CIP, ciprofloxacin; ERY, erythromycin; FEP, cefepime; FOX, cefoxitin; GEN, gentamicin; MFX, moxifloxacin; NAL, nalidixic acid; NEO, neomycin; NET, netilmicin; NOR, norfloxacin; OFX, ofloxacin; QDA, quinupristin/dalfopristin; STR, streptomycin; SUL, sulfonamides; TEC, teicoplanin; TET, tetracycline; TMP, trimethoprim; VAN, vancomycin.

S. aureus (MRSA) and vancomycin-resistant enterococci (VRE), are classified as a serious threat to public health owing to limited therapeutic options [8].

To address this situation, the use of bacteriophages (phages) has been extensively studied as an alternative therapeutic strategy [9]. Phages are bacterial viruses that specifically infect bacteria, hijacking their machinery, replicating intracellularly and finally lysing the host bacterium [7,10]. In vitro and in vivo studies have shown that for several situations, phage therapy is more specific, accurate and without adverse effects/local tissue toxicity compared with antibiotic therapy [7,10,11]. Characteristics such as bioavailability at the site of infection and migratory ability to other infection sites make this therapy more attractive.

Therefore, phage therapy could be a promising alternative strategy to treat orthopaedic implant-associated infections. The purposes of this work were primarily to describe the patterns of antimicrobial resistance, pathogenicity and virulence of bacteria isolated from orthopaedic implant-associated infections and further to isolate and characterise phages that are efficient in controlling these pathogens.

2. Materials and methods

2.1. Characterisation of bacterial isolates

Bacterial isolates related to orthopaedic implant-associated infections were provided by Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE (CHVNG) (Vila Nova de Gaia, Portugal) (Table 1). Over a 5-month period, 18 samples from osteoarticular infections were collected from patients at CHVNG. Biological samples were collected enabling the isolation and identification of 19 bacterial isolates using VITEK®2 Compact Bacterial Identification and Monitoring System (bioMérieux Inc., Durham, NC) following standardised protocols implemented at CHVNG. The study was approved by the hospital ethics committee, and patient records were anonymised prior to analysis.

2.1.1. Antimicrobial resistance and genomic characterisation

Clinical isolates were subjected to antimicrobial susceptibility testing by the disk diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) guidelines [12,13]. The antimicrobial classes, antibiotic concentration and zone diameter breakpoint for each bacterial species were defined according to EUCAST and CLSI recommendations following the disk diffusion antimicrobial susceptibility testing method.

Genomic DNA of each isolate was extracted using specific methods: for *S. aureus*, lysostaphin/proteinase K/Tris–HCl [14]; for *E. faecalis*, InstaGene™ matrix [15]; and for *E. coli*, boiling [15].

MRSA identification was performed by amplification of the *mecA* and *nucC* genes [16]. Detection of *tndX* and *int* genes in *E. faecalis* isolates was performed to demonstrate the presence of Tn*5397*-like and Tn*916*/Tn*1545*-like transposons, respectively [15]. The *intI1* and *intI2* genes, encoding class 1 and 2 integrases, respectively, were amplified in *E. coli* isolates [15].

For *E. coli*, the phylogenetic group (A, B1, B2 and D) was identified by amplification of the *chuA* and *yjaA* genes as well as DNA fragment TspE4.C2 [15,17,18].

Screening of virulence genes for *S. aureus* (*cna, eta, etb, tst, hlb, icaA, icaB* and *icaC*), *E. faecalis* (*ace, agg, gelE, esp, hyl, fsr* and *cylL$_L$L$_S$ABM*) and *E. coli* (*fimA, papG*III, *stx, cnf1* and *papC*) was performed by PCR [14–18].

Positive and negative controls were used from the bacterial collection of the Medical Microbiology Laboratory of the Universidade de Trás-os-Montes e Alto Douro (UTAD) (Vila Real, Portugal). A list of the primers used is provided in the Supplementary material SS1.

2.1.2. Multilocus sequence typing (MLST)

Staphylococcus aureus and *E. faecalis* isolates were characterised by MLST. Internal fragments of seven housekeeping genes of *S. aureus* (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) and *E. faecalis* (*gdh, gyd, pstS, gki, aroE, xpt* and *yiqL*) were amplified using the primers listed in Supplementary material SS1 and were sequenced. The obtained sequences were analysed by https://pubmlst.org/general.shtml and eBURST V3 to assign a specific sequence type (ST) and clonal com-

plex (CC). Positive and negative controls were used from the bacterial collection of the Medical Microbiology Laboratory at UTAD.

2.2. Bacteriophage isolation/production and characterisation

A previously isolated phage from the Bacteriophage Biotechnology Group of the Centre for Biological Engineering (BBiG/CEB–Universidade do Minho, Braga, Portugal) was used to infect *S. aureus* isolates [19]. Specific new phages towards *E. faecalis* and *E. coli* strains were isolated from a wastewater treatment plant at Frossos (Braga, Portugal). A sample enrichment method was performed to isolate phages [20]. Briefly, centrifuged effluent was mixed with double-strength trypticase soy broth and exponentially grown *E. faecalis* and *E. coli* strains, respectively. The solution was then incubated at 37 °C and 120 rpm for 24 h and was further centrifuged and the supernatant was filtered through a PES 0.22 μM filter. Spot assays were performed against bacterial lawns to test for the presence of phages. Inhibition haloes were further purified and plaque picking was repeated until single-plaque morphology was observed.

Phages were produced as previously described with some modifications [21]. Briefly, phage solutions were spread on lawns of their respective host strains (*S. aureus* 12, *E. faecalis* 99 and *E. coli* 2129975) using a paper strip and were incubated overnight at 37 °C. After full lysis, salt magnesium buffer was added to each plate and the plates were incubated at 4 °C and 120 rpm for 24 h. Subsequently, both liquid and top agar were collected and centrifuged and the supernatant was filtered. Chloroform was added to the filtered solution and the samples were stored at 4 °C for further use.

Lytic spectra and efficiency of plating (EOP) were determined according to Kvachadze et al. [22]. In brief, phage suspensions were serially diluted and were placed over original or target host bacteria and the presence of a clear zone of lysis was examined following incubation at 37 °C for 16–18 h,. The relative EOP was calculated as the ratio of the phage titre (PFU/mL) obtained in each isolate and that obtained in the propagating host. Three independent experiments were performed in duplicate.

2.2.1. Thermal and pH stability tests

To assess thermostability, phage solutions were incubated at different temperatures (–20 °C to 60 °C) for 24 h. To assess pH stability, phage suspensions were prepared at different pH values (pH 1–13) and were incubated at 4 °C for 24 h. In both cases, following incubation phages were titrated using the double-layer agar plate method to determine surviving phages. Three independent experiments were performed in duplicate.

2.2.2. Bacteriophage morphology

Phages were sedimented by centrifugation and the pellet was washed in tap water by repeating the centrifugation step [23]. Phages were deposited on copper grids with a carbon-coated Formvar film grid, were stained with 2% uranyl acetate (pH 4) and were examined using a JEOL JEM transmission electron microscope (JEOL, Tokyo, Japan).

2.2.3. One-step growth curves

One-step growth curves were performed as previously described [21]. Briefly, host bacteria were grown to exponential phase and were then harvested and re-suspended in fresh medium. Respective phage solutions were added to exponential-phase cultures of host bacteria at a multiplicity of infection (MOI) of 0.01 and were allowed to adsorb for 5 min at 37 °C. The mixtures were centrifuged and the pellets were then re-suspended in fresh medium broth. Samples were taken at 10-min intervals and phage titration was performed by the double-layer agar plating method. Three independent experiments were performed in duplicate.

2.2.4. Genome sequencing analysis

Escherichia phage vB_EcoM_JB75 (JB75) and *Enterococcus* phage vB_EfaS_LM99 (LM99) genomic DNA was extracted essentially as previously described [24]. In brief, purified phages were treated with 0.016% (v/v) L1 buffer at 37 °C for 2 h. The enzymes were further thermally inactivated for 30 min at 65 °C. Then, phage proteins were digested with 50 μg/mL proteinase K, 20 mM ethylene diamine tetra-acetic acid (EDTA) and 1% sodium dodecyl sulfate (SDS) at 56 °C for 18 h. This was followed by phenol, phenol:chloroform (1:1, v/v) and chloroform extractions. DNA was precipitated with ice-cold absolute ethanol and 3 M sodium acetate (pH 4.6) and was then centrifuged. Pellets were washed in 70% ice-cold ethanol and were further air-dried and re-suspended in nuclease-free water. Phage genomes were sequenced using an Illumina HiSeq system (Illumina Inc., San Diego, CA) with individual libraries of two non-homologous phages pooled together in equal amounts. Libraries were constructed using the KAPA DNA Library Preparation Kit Illumina (KAPA Biosystems, San Diego, CA) with the KAPA HiFi preparation protocol and were sequenced using 100-bp paired-end mode. The quality of the produced data was determined by Phred quality score at each cycle. Reads were demultiplexed and de novo assembled into a single contig with average coverage above 100 × using CLC Genomics Workbench v.7.0 (CLC Bio, Aarhus, Denmark) and were manually inspected.

Phage genomes were first annotated using myRAST algorithm and were further manually inspected for potential alternative start codons or for the presence of non-annotated coding sequences (CDSs) using Geneious 9.1.4 (Biomatters Ltd., Auckland, New Zealand). Functions of the gene products were searched with BLASTp (coverage >80%; *E*-value $\leq 10^{-5}$) and Pfam programs (*E*-value $\leq 10^{-5}$). The presence of transmembrane domains was checked using TMHMM and Phobius, and membrane proteins were annotated when both tools were in concordance. Protein parameters (molecular weight and isoelectric point) were determined using ExPASy Compute pI/Mw tool. Moreover, transfer RNAs (tRNAs) were scanned using tRNAscan-SE and ARAGORN. Promoter regions were determined using PromoterHunter from the phiSITE database and were further checked manually. ARNold was used to predict rho-independent terminators, and the energy was calculated using Mfold.

2.3. Activity of bacteriophages against clinical bacteria

The activity of phages against the bacterial isolates from orthopaedic implant-associated infections was evaluated. Bacterial cultures were grown to exponential phase and were re-suspended in fresh medium. Phage solutions were added to bacterial cultures of each respective bacterium at a MOI of 10. These suspensions were incubated at 37 °C at 120 rpm for 2, 6 and 24 h. The number of cultivable cells was determined using the microdrop method. Three independent experiments were performed in duplicate.

2.4. Statistical analysis

Experimental data were analysed using IBM SPSS Statistics v.22.0 (IBM Corp., Armonk, NY). Results were reported as the mean ± standard deviation. One-way analysis of variance (ANOVA) followed by post-hoc Turkey HSD multiple comparison test was used to determine significant differences ($P < 0.05$).

3. Results

3.1. Antimicrobial resistance and genomic characterisation

A total of 19 clinical bacteria were isolated from orthopaedic implant-associated infections, showing resistance to several

antibiotics (Table 1). Among these, 15 isolates were resistant to at least one antibiotic in three or more antimicrobial classes (Table 1), thus showing a multidrug-resistant (MDR) profile [25]. For further analysis, only isolates with a MDR profile were taken into consideration for further genomic characterisation as well as isolation and characterisation of phages against these target bacteria. This option was based on the therapeutic limitations for treating infections caused by MDR bacteria, with phage therapy being a possible solution.

Among six *S. aureus* isolates, four were MRSA and two isolates were methicillin-susceptible *S. aureus* (MSSA) (Table 1). All *S. aureus* isolates were shown to contain virulence determinants including polysaccharide intercellular adhesion gene (*icaB*), collagen-binding adhesin gene (*cna*) and haemolytic toxin β-haemolysin gene (*hlb*) (Table 1).

All seven *E. faecalis* isolates were vancomycin-resistant, three of which were also resistant to teicoplanin. The Tn*916*/Tn*1545* transposon was found in the latter isolates. Moreover, cytolysins (*cylLs* and *cylM*), aggregation protein (*agg*), gelatinase (*gelE*) and pheromone gelatinase biosynthesis-activating pheromone (*fsr*) virulence genes were found in the *E. faecalis* isolates (Table 1).

Lastly, the two *E. coli* isolates were classified into phylogenetic groups A and B1. The type 1 fimbriae gene (*fimA*) was detected in both isolates. Remarkably, the *E. coli* isolate classified into phylogenetic group B1 possessed the class 1 integron gene *intl1* (Table 1).

3.1.1. Multilocus sequence typing

The *S. aureus* isolates were divided into three STs, namely ST239, ST72 and ST22 (Table 1). The MRSA isolates belonged to ST239 and ST22, whilst the MSSA isolates belonged to ST72. According to eBURST V3 analysis, *S. aureus* isolates belonged to CC8 (ST239 and ST72) and CC22 (ST22), (Table 1; Supplementary Fig. S1). Isolates assigned to ST239 and ST72 (Table 1) belonged to the same cluster, having closely related genotypes (Supplementary Fig. S1). CC8 had a primary founder ST5, whilst CC22 had as the primary founder ST22.

Regarding *E. faecalis*, the isolates were divided into three different STs belonging to three CCs: ST117 belonging to CC21; ST6 belonging to CC2; and ST16 belonging to CC58 (Table 1; Supplementary Fig. S1). CC21 had a primary founder ST21, whilst CC2 and CC58 had as the primary founders ST6 and ST16, respectively.

3.2. Bacteriophage isolation/production and characterisation

The phages used in this study were named vB_SauM_LM12 (LM12), vB_EfaS_LM99 (LM99), vB_EfaS_LM00I (LM00I), vB_EfaS_LM00II (LM00II), vB_EcoM_JB75 (JB75) and vB_EcoM_JB75I (JB75I) according to the recommendations of Kropinski et al. [26]. One phage (LM12) was used to infect *S. aureus* isolates (Table 2), whilst three phages (LM99, LM00I and LM00II) and two phages (JB75 and JB75I) were isolated to infect *E. faecalis* and *E. coli* isolates, respectively (Table 2). Phages LM12, LM99 and JB75 were able to lyse 91%, 64% and 55% of all *S. aureus, E. faecalis* and *E. coli* tested, respectively (Table 2). These phages were selected for further characterisation owing to their broad spectra of activity.

The EOP was determined in bacterial isolates from orthopaedic implant-associated infections (Table 2). Phage LM12 was able to infect all *S. aureus* isolates, with a high EOP in five of six *S. aureus* isolates (Table 2). Phage LM99 was able to infect one *E. faecalis* isolate with high lytic efficiency and to promote lysis from without in another two isolates (Table 2). Furthermore, phage JB75 infected only one *E. coli* isolate with high EOP.

3.2.1. Thermal and pH stability tests

The thermal stability of phage LM12 was assessed and a 100% survival rate was observed at 4 °C and 18 °C, whilst at 37 °C and 42 °C the survival rates were 98% and 94%, respectively (Fig. 1a). This phage was able to survive at –20 °C (42% survival rate), whereas it was killed at 60 °C (Fig. 1a). Regarding the thermal stability of phage LM99, a 100% survival rate was observed for a wide range of temperatures from –20 °C to 42 °C (Fig. 1a). Furthermore, the survival rate at 60 °C was approximately 39%. Phage JB75 was shown to be more sensitive to temperature, being stable from 4–42 °C. The survival rate was 75% at –20 °C, whilst no survival was detected at 60 °C (Fig. 1a).

Regarding pH stability, the three phages were highly stable over a wide range of pH values (Fig. 1b). Phage LM12 showed a loss of stability at pH 10 (70% survival rate), whilst the stability of phage LM99 was affected at pH 4 and pH 12 (78% and 67% survival rates, respectively) (Fig. 1b). The phages were completely inactivated at extreme pH values (pH 1, 2 and 13) (Fig. 1b).

3.2.2. Phage morphology

Phage LM99 particles had an icosahedral head of 63 nm in diameter and a non-contractile tail of 212 nm in length and 9 nm in width (Fig. 1c), proving that it belongs to the Siphoviridae family. Phage JB75 revealed an isometric head of 73 nm diameter with a contractile tail 96 nm long and 24 nm wide, which is a morphology indicative of the Myoviridae family (Fig. 1c).

Table 2
Lytic spectrum and efficiency of plating (EOP) of phages against *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli* strains.

Species	Strain	Lytic spectrum[a]				EOP[b]
		Phage				Phage
		LM12				LM12
Staphylococcus aureus	12	+				High
	ATCC 25923	+				
	ATCC 49230	H				
	ATCC 6538	–				
	ATCC 33591	H				
	2117045[c]	+				High
	2093367[c]	+				High
	2104780[c]	+				High
	2117741[c]	H				LFW
	2106876[c]	+				High
	2179342[c]	+				High
		LM09	LM99	LM00I	LM00II	LM99
Enterococcus faecalis	I899	+	+	+	+	High
	I900	+	+	+	+	
	I436	+	+	–	–	
	I980	H	+	+	+	
	2099610[c]	N/D	–	N/D	N/D	N/D
	2105322[c]	N/D	–	N/D	N/D	N/D
	2104780[c]	N/D	H	N/D	N/D	LFW
	2133201[c]	H	+	H	H	High
	2084972[c]	N/D	H	N/D	N/D	LFW
	2093926[c]	N/D	–	N/D	N/D	N/D
	8105329[c]	N/D	–	N/D	N/D	N/D
	JB75			JB75I		JB75
Escherichia coli	30	+		H		
	31	+		H		
	32	H		–		
	33	+		H		
	34	–		–		
	35	–		–		
	U923366	+		+		
	U924005	–		–		
	U923087	–		–		
	2129975[c]	+		+		High
	2154120[c]	–		–		N/D

LFW, lysis from without; N/D, not determined.
[a] +, distinct clear plaques; –, plaques not formed; H, hazy plaques.
[b] The EOP was recorded as high, low and LFW, representing >10, 0.1–1 and <0.1%, respectively.
[c] Bacteria isolated from orthopaedic implant-associated infections.

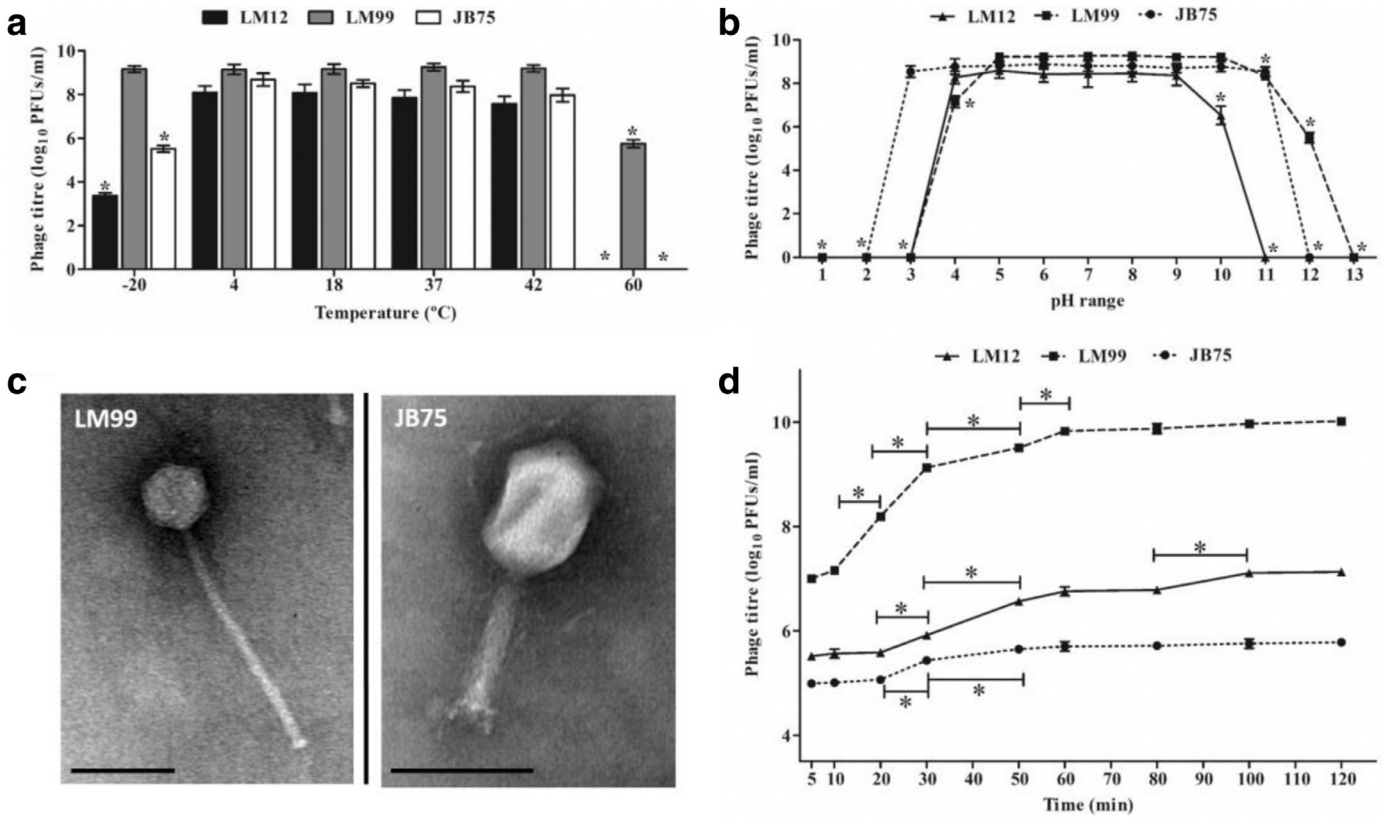

Fig. 1. (a,b) Stability of phages LM12, LM99 and JB75 at different temperatures (a) and pH values (b). (c) Morphology of phages LM99 and JB75 observed by transmission electron microscopy with uranyl acetate (2%) staining. Bar = 100 nm. (d) Curves for one-step growth of phages LM12, LM99 and JB75. * Statistically significant difference ($P < 0.05$).

3.2.3. One-step growth curves

The latent and rise periods for phage LM12 were 20 min and 30 min, respectively. The burst size was 52 PFU/infected cell (Fig. 1d). Regarding phage LM99, the latent and rise periods were 10 min and 20 min, respectively, and the burst size was 107 PFU/infected cell (Fig. 1d). Regarding phage JB75, the latent and rise periods were 20 min and 30 min, respectively, and the burst size was 82 PFU/infected cell (Fig. 1d).

3.2.4. Genomic sequencing analysis

The complete genomic sequences of phages LM99 and JB75 were deposited in GenBank under the accession nos. **MH355583** and **MH355584**, respectively. Genome analysis revealed that both phages are virulent, not encoding any genes associated with lysogeny. However, phage LM99 encodes a gene homologous to a putative toxin gene (gp17 – haemolysin) and a metallo-β-lactamase gene. In silico analysis showed that phage JB75 does not encode any known virulence-associated or toxin proteins.

The genome of phage LM99 consists of a linear double-stranded DNA of 40 203 bp with a G+C% content of 30.5% (Fig. 2a). LM99 encodes 64 CDSs with an average length of 573 bp, tightly packed occupying 91% of its genome. Twenty-five of the predicted CDSs have an assigned function (39%) and two are unique (Supplementary Table S1). No tRNA genes were detected. The majority (97%) of the CDSs possess methionine as start codon, whilst CTG and GTG are the start codons of only one CDS each. Furthermore, 14 promoters and 6 rho-independent terminators were predicted. BLASTN search revealed high homology with enterococci siphoviruses vB_EfaS_AL3, LY0322, SHEF5, SHEF2, PMBT2, SANTOR1 and EfaCPT1. Interestingly, these phages are not inserted in any genus.

The genome of phage JB75 consists of a linear double-stranded DNA of 167 208 bp with a G+C% content of 35% (Fig. 2b). This phage encodes 277 putative CDSs with an average length of 546 bp, also very tightly packed occupying approximately 94% of its genome. No unique proteins were detected and it was possible to predict a function for 134 CDSs (48%) (Supplementary Table S2). Unsurprisingly, 262 CDSs have methionine as start codon (95%), whilst 2 start with CTG, 6 with GTG and 7 with TTG. Ten tRNA genes were predicted (tRNA-Arg, tRNA-Asn, tRNA-Gln, tRNA-Gly, tRNA-Leu, tRNA-Met, tRNA-Pro, tRNA-Ser, tRNA-Thr and tRNA-Tyr). Moreover, 13 promoters and 26 rho-independent terminators were predicted. Homology searches revealed that JB75 has very high homologies with several *E. coli* myoviruses, namely YUEEL01, vB_EcoM-fHoEco02 and vB_EcoM-fFiEco06. These phages are inserted in the T4 virus genus.

3.3. Activity of bacteriophages against clinical bacteria

The antimicrobial activity of the phages was assessed against bacterial isolates with high EOP values (Table 2). In the control group (without phage), *S. aureus, E. faecalis* and *E. coli* bacterial counts increased continuously (Fig. 3, solid lines). However, when phages LM12, LM99 and JB75 were applied, significant reductions were observed in the tested isolates (Fig. 3, dotted lines). Noticeably, the phage effect varied according to the bacterial strain tested.

Phage LM12 showed high antimicrobial activity for the four *S. aureus* isolates tested (Fig. 3a). Despite the slight increase in bacterial counts observed at 6 h and 24 h, bacterial counts were significantly lower compared with the controls ($P < 0.05$). Phage LM12 reduced the bacterial density of *S. aureus* 2093367 by 91%, 97% and 99% at 2, 6 and 24 h, respectively. Reductions of approximately

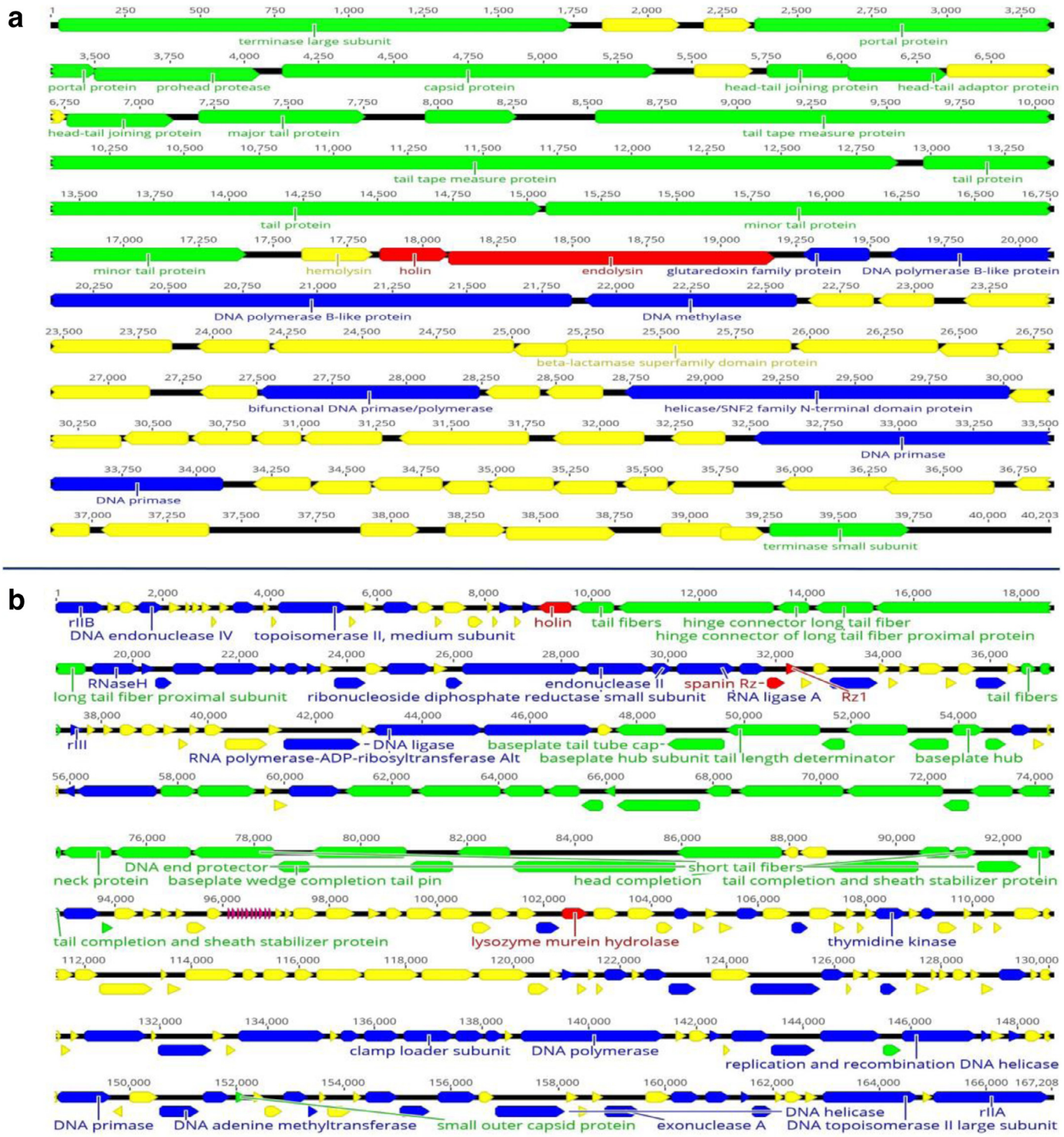

Fig. 2. Genome overview of (a) phage LM99 and (b) phage JB75. (a) The genome map of phage LM99 predicted 64 CDSs and (b) the genome map of phage JB75 predicted 277 CDSs. The CDSs are numbered and coloured according to their predicted function: yellow, hypothetical protein; blue, DNA replication and transcription gene; green, DNA packaging and phage morphogenesis gene; and red, cells lysis gene. Above the genomes, the nucleotide position (in kb) is given. The figure was created using Geneious 9.1.4. CDS, coding sequence.

96%, 95% and 93% at 2, 6 and 24 h, respectively, were observed in *S. aureus* 2104780, *S. aureus* 2106876 and *S. aureus* (Fig. 3a). Regarding *S. aureus* 2117045 and *S. aureus* 2117741, the antimicrobial effect of LM12 decreased over time, showing a maximum effect at 2 h with a 77% reduction (Supplementary Fig. S2).

Phage LM99 demonstrated significant antimicrobial activity against *E. faecalis* 2133201 (Fig. 3b). This phage was able to significantly reduce *E. faecalis* 2133201 density by 99% at 2, 6 and 24 h (Fig. 3b). The other *E. faecalis* isolates were not significantly affected by the presence of phage LM99 (Supplementary Fig. S2).

Regarding *E. coli*, phage JB75 demonstrated a significant effect on *E. coli* 2129975 planktonic cells (Fig. 3c). The highest reduction was achieved at 6 h of incubation with a 96% reduction. At 2 h and

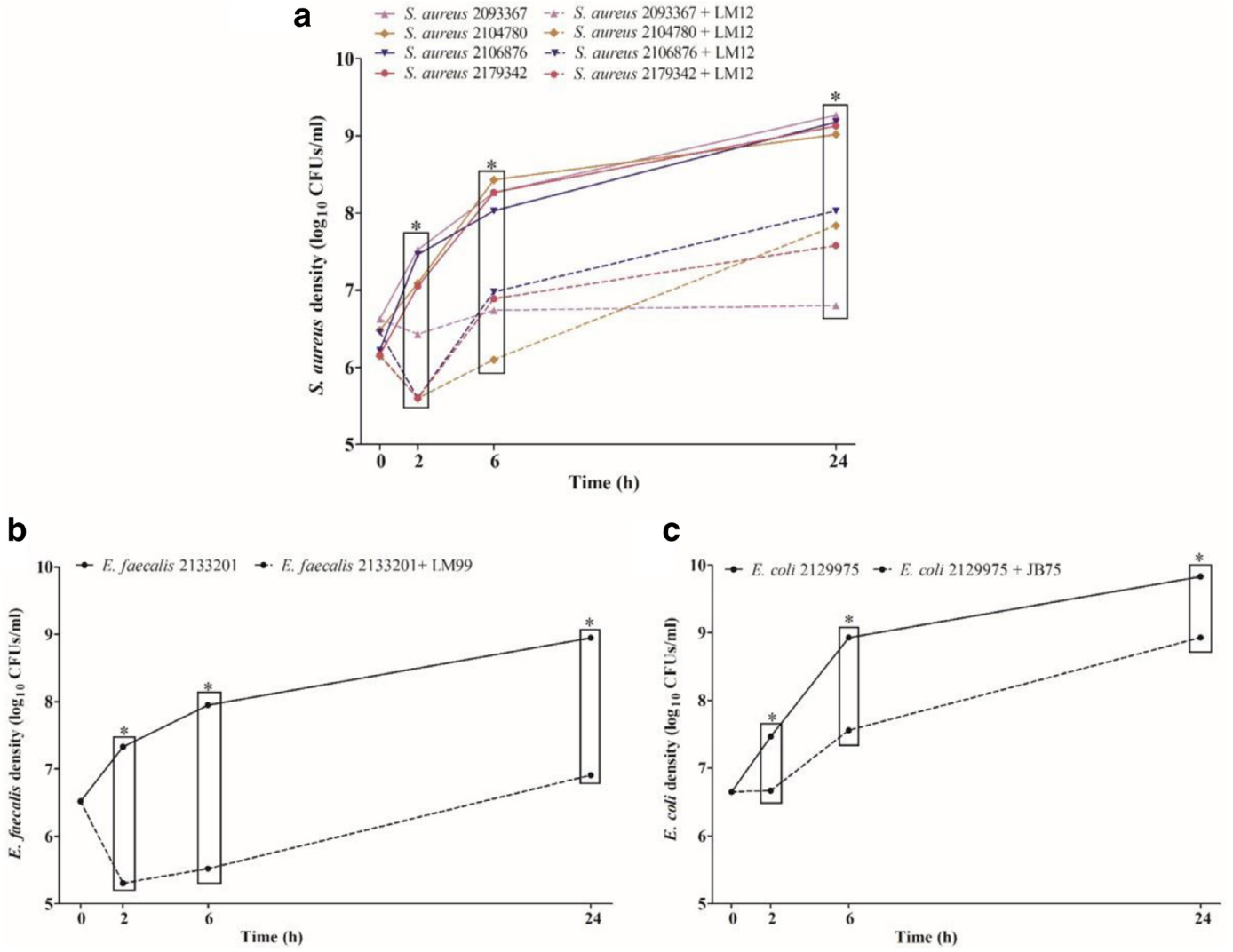

Fig. 3. Inhibitory effect of (a) phage LM12 on planktonic *Staphylococcus aureus*, (b) phage LM99 on planktonic *Enterococcus faecalis* and (c) phage JB75 on planktonic *Escherichia coli* isolated from orthopaedic implant-related infections. The solid lines represent the growth of bacteria without phages (control) and the dashed lines represent the growth of bacteria with phages. * Statistically significant difference ($P < 0.05$) between planktonic bacteria + phage and control densities for the same time of incubation.

24 h, the phage was able to reduce the bacterium by 84% and 87%, respectively (Fig. 3c).

4. Discussion

Orthopaedic implant-related infections remain one of the major complications of orthopaedic clinical activity, causing a significant impact on patients and healthcare systems. In addition, the presence of MDR bacteria, namely MRSA and VRE, increases concern about these type of infections owing to limited therapeutic options [2,3]. The pathogenicity of these infections is even greater when the isolates possess mobile genetic elements (*mecA* in *S. aureus*, Tn*916*/Tn*1545* in *E. faecalis* and *intI1* in *E. coli*) and virulence elements (such as *icaB, hlb* and *cna* in *S. aureus, agg, gelE, cylM, cylL* and *fsr* in *E. faecalis* and *fimA* in *E. coli*) [6,27–30]. These mobile genetic elements can mediate the transfer and integration of resistance and virulence determinants into new host DNA [30]. The virulence elements can contribute to bacterial binding to host matrix proteins and consequently bacterial adhesion to implants [6,27]. As observed by MLST and eBURST V3 analysis, both the phenotypic and genotypic profile obtained are in accordance with the findings of other authors [2,3,6,27,31], showing that these pathogens belong to lineages frequently involved in pandemic nosocomial infections [27,31]. Besides, intraspecies diversity between isolates was observed with different resistance and virulence patterns, increasing concern about the diagnosis and treatment of infections caused by these type of bacteria [6,31].

Considering the mentioned pathogenicity of bacterial strains and the poor bioavailability of antibiotics in bone tissue [32], there is an urgent need to develop novel therapeutic approaches to combat isolates involved in orthopaedic implant-associated infections. Phage therapy has long been shown to be a promising antibacterial strategy, mainly due to its high specificity and effectiveness in killing targeted pathogenic bacteria [7,10]. In the present study, three phages, namely LM12 (previously isolated), LM99 (isolated in this study) and JB75 (isolated in this study), were characterised in order to control bacterial pathogens previously isolated from orthopaedic implant-associated infections. The three phages presented outstanding characteristics such as broad bactericidal spectrum against the target pathogenic bacterium, short latent periods, large burst sizes and high stability to several environmental conditions. Furthermore, it is important to highlight the specificity of the phages used in this study. The characterised phages belong to the Caudovirales order, which are double-stranded DNA viruses, having as a major advantage their inherent combined bacteriostatic and bacteriolytic mode of action [33]. By

comparative genomics analysis, phage LM99 is not inserted in any genus, consequently a new genus should be created comprising LM99 and its closest relative. The homologies detected for phage JB75 and the more identic phages suggest the assignment of this phage to the T4 virus genus, which is one of the most well studied Enterobacteriaceae strictly lytic phages [34]. Furthermore, the phages are virulent and do not encode any genes associated with lysogeny. Genes homologous to a putative toxin gene (gp17 – haemolysin) and to a metallo-β-lactamase gene were detected in the genome of phage LM99. However, both genes are not homologous to genes found in bacteria, therefore their true function needs to be determined. All of these characteristics associated with the high efficiency of phages LM12, LM99 and JB75 in infecting bacteria, including MRSA and VRE isolated from orthopaedic implant-associated infections, make them potential candidates in therapeutic applications. Likewise, the three studied phages were efficient in reducing the number of culturable bacterial cells over time compared with controls. Phage therapy studies with animal models have shown that, under certain circumstances, phages may help in reducing the density of the infecting bacterial population to a level that may allow the host immune response to mount a successful defence and clear the infection [7]. Recent studies have shown the efficacy and accuracy of phage therapy in the treatment of wound infections, diabetic foot ulcers, acute kidney injury, ulcers and chronic otitis caused by clinical pathogens [35–39]. In line with these potential clinical applications and considering the data obtained in the present work, phages LM12, LM99 and JB75 could be suitable to treat orthopaedic implant-associated infections.

5. Conclusions

Bacteria isolated from orthopaedic implant-associated infections showed patterns of multidrug resistance, virulence and pathogenicity. Bacteriophage therapy offers a possible alternative to classic antibiotic treatment to reduce bacterial colonisation. The studied phages were efficient in controlling clinical bacteria, suggesting that phage therapy could be suitable to treat pathogenic bacteria involved in orthopaedic implant-associated infections.

Declaration of Competing Interest

None declared.

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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.2019.06.007.

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