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Antibiotic resistance genes in phage particles isolated from human faeces and induced from clinical bacterial isolates

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ABSTRACT

Phage particles have emerged as elements with the potential to mobilise antibiotic resistance genes (ARGs) in different environments, including the intestinal habitat. This study aimed to determine the occurrence of ARGs in phage particles present in faecal matter and induced from strains isolated from faeces. Nine ARGs (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$, $bla_{OXA\text{-}48}$, *qnrA*, *qnrS*, *mecA*, *sul1* and *armA*) were quantified by qPCR in the phage DNA fractions of 150 faecal samples obtained from healthy individuals who had not received antibiotic treatment or travelled abroad in the 3 months prior to sample collection. On the suspicion that the detected particles originated from bacterial flora, 82 *Escherichia coli* and *Klebsiella pneumoniae* isolates possessing at least one identified ARG (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$, *armA*, *qnrA*, *qnrS* and *sul1*) were isolated and their capacity to produce phage particles carrying these ARGs following induction was evaluated. Of 150 samples, 72.7% were positive for at least one ARG, with bla_{TEM} and $bla_{CTX\text{-}M\text{-}9\ group}$ being the most prevalent and abundant. Of the 82 isolates, 51 (62%) showed an increase in the number of copies of the respective ARG in the phage fraction following induction, with bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$ and *sul1* being the most abundant. Phages induced from the isolates were further purified and visualised using microscopy and their DNA showed ARG levels of up to 10^{10} gene copies/mL. This study highlights the abundance of phage particles harbouring ARGs and indicates that bacterial strains in the intestinal habitat could be source of these particles.

1. Introduction

Bacteriophages, or bacterial viruses, are the most abundant biological entities on Earth and one of the keys to the evolution and potential control of bacterial populations [1]. Knowledge of phages has been essential for the progress of molecular biology and they have been used as models for studying different biological processes. In recent decades, phages have acquired increasing relevance in molecular biology owing to new insights into their presence in many bacterial genomes [1,2], their role in horizontal gene transfer [3], the phage–bacterium relationship, and bacterial defence mechanisms against phage infection [4].

Meanwhile, bacterial resistance to antibiotics continues to increase and is severely undermining our ability to control infectious diseases. The World Health Organization (WHO) has identified antibiotic resistance as one of the most challenging problems in public health care on a global scale (http://apps.who.int/gb/ebwha/pdf_files/WHA68-REC1/A68_R1_REC1-en.pdf). The causes of this increase in resistance are believed to include overuse and inconsistent application of antibiotics in humans, together with the use of antibiotics in animal husbandry [5,6]. The scientific community and governments have reacted by calling for better control of antibiotic usage both in humans and livestock. Researchers are trying to find new generations of antibiotics to treat infections by resistant strains, but more research into the mechanisms of resistance would also be advisable. This is the purpose of the multidisciplinary 'One Health' approach, which aims to encourage the collaborative efforts of multiple disciplines working locally, nationally and globally.

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Antibiotic resistance may be intrinsic or conferred either by mutation or by acquiring antibiotic resistance genes (ARGs) through mobile genetic elements [5]. Clinical studies point to conjugation and transformation as the most likely mechanisms of transfer [7]. However, little attention has been paid to other mechanisms, such as transduction mediated by phages or phage-derived particles, which have only recently emerged as potentially relevant [8–10]. Bacteriophages basically consist of one nucleic acid molecule (the phage genome) surrounded by a protein coating, the capsid. This packaging of the nucleic acid confers protection and hence an extracellular persistence that cannot be found for naked DNA or RNA. Therefore, capsid protection could be important in cases where there is no close cell-to-cell contact [11].

In preliminary studies, the presence of ARGs was determined in total and phage DNA in faeces of individuals without any enteric disease [12]. That work is extended here by analysis of more ARGs in the phage fraction of a new collection of faecal samples from 150 healthy individuals free of contact with clinical settings and who had not received antibiotic therapy in the previous 3 months before sample collection. The ARGs studied consisted of four β-lactamases (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$ and $bla_{OXA\text{-}48}$) [13], two quinolone resistance genes (*qnrA* and *qnrS*) [14], the *mecA* gene that confers resistance to methicillin in *Staphylococcus aureus* [15], the emerging *armA* gene that confers resistance to aminoglycosides [16], and *sul1*, the most widespread gene conferring resistance to sulfonamides [17]. Another aim of this study was to gain an insight into the origin of the phage particles, suspected to be derived from bacterial flora. Accordingly, bacterial strains (*Escherichia coli* and *Klebsiella pneumoniae*) isolated from faeces that possessed a given ARG were treated to induce phage particles carrying this ARG.

2. Materials and methods

2.1. Faecal samples

This study was performed with 150 human faecal samples from individuals living in the city of Barcelona (Catalonia, North East Spain) collected over a period of 6 months (February–August 2016). All individuals were healthy, not related to clinical environments or involved in a foodborne outbreak, and not showing any gastrointestinal pathology or known infection. None of the subjects had consumed antibiotics or travelled to foreign countries in the 3 months before sampling. All samples were completely anonymised. Besides ensuring the above criteria were fulfilled, no individual data were collected except for age. The samples were destroyed immediately after the study, which was approved by the Clinical Ethics Committee of Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). Informed consent was obtained for all individuals.

2.2. Bacterial strains

The *E. coli* strains used as controls for the different ARGs are listed in Table 1. Faecal samples were cultured on chromogenic agar (chromID® CPS® Elite; bioMérieux, Marcy-l'Étoile, France). Following 24 h of incubation at 37 °C, all isolates growing on the plates compatible with *E. coli* or *Klebsiella* were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (MALDI Autoflex II™/TOF/TOF; Bruker Daltonik GmbH, Bremen, Germany). Antimicrobial susceptibility testing was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [20].

A total of 70 *E. coli* and 12 *K. pneumoniae* isolates were selected on the basis of the presence of one of the target ARGs (one isolate per individual) to determine the presence of phage particles containing the ARGs.

Table 1
Strains used in this study as controls for antibiotic resistance genes (ARGs).

Strain	ARG	Reference
Escherichia coli C600	bla_{TEM} in pGEM vector	Promega
E. coli	$bla_{CTX\text{-}M\text{-}1\ group}$	[9]
E. coli	$bla_{CTX\text{-}M\text{-}9\ group}$	[18]
MRSA	*mecA*	[9]
E. coli strain 226	*qnrA*	[14]
Enterobacter cloacae strain 565	*qnrS*	[14]
E. coli	*armA* in plasmid pMUR050	[12]
Klebsiella pneumoniae	$bla_{OXA\text{-}48}$	This study
E. coli J53 R388	*sul1*	[19]

MRSA, methicillin-resistant *Staphylococcus aureus*.

2.3. Partial purification of phage DNA

Faecal samples were homogenised 1:5 (w:v) in phosphate-buffered saline by shaking for 15 min. Then, 50 mL of the homogenate was centrifuged at 3000 × *g* and the supernatant was filtered through a low-protein-binding 0.22-μm pore size membrane filter (Millex-GP; Millipore, Bedford, MA). The suspensions were treated with chloroform, were centrifuged at 16 000 × *g* for 10 min and the supernatant was treated with DNAse (100 U/mL; Sigma-Aldrich, Madrid, Spain) for 1 h at 37 °C. DNAse was heat-inactivated at 75 °C for 5 min. To rule out the presence of non-packaged DNA, an aliquot of the sample was taken after DNase treatment and before its desencapsidation. Using this control sample, the absence of free 16S rDNA was established as well as the absence of the ARGs studied by quantitative PCR (qPCR), confirming total removal of non-encapsidated DNA [12,21]. Different controls were performed to verify the stability and appropriate inactivation of the DNase [9]. Packaged DNA was extracted by proteinase K digestion and was purified and quantified [9,12].

2.4. Purification of bacteriophages from clinical isolates

Cultures (20 mL) of each clinical isolate in Luria–Bertani broth were grown to mid-exponential phase [optical density at 600 nm (OD_{600}) of 0.3]. Each culture was aliquoted in two 10 mL tubes and one aliquot was treated with mitomycin C (mitC) (final concentration 0.5 μg/mL) to induce phage particles. Both tubes were incubated for 6 h at 37 °C by shaking in the dark and the absorbance of the culture following induction was monitored by comparing the OD_{600} values of the mitC-treated and non-treated cultures. Phages in the supernatant lysate from both aliquot cultures were partially purified as described above.

2.5. Standard PCR and qPCR procedures

PCR was performed with a GeneAmp® PCR 2700 system (Applied Biosystems, Barcelona, Spain). ARGs from the control strains were amplified by conventional PCR, were cloned in a pGEM®-T Easy Vector (Promega, Barcelona, Spain) to generate the constructs and were verified by sequencing (Table 2). The constructs were used to generate the standard curves.

Real-time qPCR assays for bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$, *mecA*, *armA*, *qnrA*, *qnrS* and *sul1* were performed as described previously [9,12,14,18,19,22,23]. The $bla_{OXA\text{-}48}$ gene qPCR assay (Table 2) was designed using Primer Express® Software v.3.0 (Applied Biosystems). The gene was amplified using specific primers (Table 2) from the sequence of $bla_{OXA\text{-}48}$ harboured in *K. pneumoniae* clinical isolate HSP172. The amplified $bla_{OXA\text{-}48}$ was sequenced and cloned in a pGEM®-T Easy Vector. The construct was confirmed by sequencing and was used to generate the standard curves [9]. The qPCR assay for $bla_{OXA\text{-}48}$ showed 99.8% efficiency and a limit of quantification

Table 2
Oligonucleotides used in this study.

Target gene	Reaction	Oligonucleotide	Sequence	Amplimer (bp)	LOQ of qPCR (GC)	Reference
bla_{TEM}	PCR	UP	CTCACCCAGAAACGCTGGTG	569		[22]
		LP	ATCCGCCTCCATCCAGTCTA			
	qPCR	UP	CACTATTCTCAGAATGACTTGGT	85	7.6	[22]
		LP	TGCATAATTCTCTTACTGTCATG			
		*Taq*Man TEM	FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ			
$bla_{CTX-M-1group}$	PCR	UP	ACGTTAAACACCGCCATTCC	356		[9]
		LP	TCGGTGACGATTTTAGCCGC			
	qPCR	UP	ACCAACGATATCGCGGTGAT	101	8.4	[9]
		LP	ACATCGCGACGGCTTTCT			
		*Taq*Man CTX-M-1	FAM-TCGTGCGCCGCTG-MGBNFQ			
$bla_{CTX-M-9group}$	PCR	UP	ACGCTGAATACCGCCATT	352		[18]
		LP	CGATGATTCTCGCCGCTG			
	qPCR	UP	ACCAATGATATTGCGGTGAT	85	13	[18]
		LP	CTGCGTTCTGTTGCGGCT			
		*Taq*Man CTX-M-9	FAM-TCGTGCGCCGCTG-MGBNFQ			
bla_{OXA-48}	PCR	UP	CGTTATGCGTGTATTAGCCTTAT	790		This study
		LP	TTTTTCCTGTTTGAGCACTTCTTT			
	qPCR	UP	CGGTAGCAAAGGAATGGCAA	133	18.2	This study
		LP	TGGTTCGCCCGTTTAAGATT			
		*Taq*Man OXA-48	FAM-CGTAGTTGTGCTCTGGA-MGBNFQ			
sul1	PCR	UP	TTCATGGGCAAAAGCTTGATG	965		[19]
		LP	GGCCGGAAGGTGAATGCTA			
	qPCR	UP	CCGTTGGCCTTCCTGTAAAG	67	5.9	[19]
		LP	TTGCCGATCGCGTGAAGT			
		*Taq*Man *sul1*	FAM-CGAGCCTTGCGGCGG-MGBNFQ			
mecA	PCR	UP	GATAGCAGTTATATTTCTA	434		[9]
		LP	ATACTTAGTTCTTTAGCGAT			
	qPCR	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	10.4	[23]
		LP	TGGTCTTTCTGCATTCCTGGA			
		*Taq*Man *mecA*	FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-MGBNFQ			
qnrA	PCR	UP	ACGCCAGGATTTGAGTGAC	565		[14]
		LP	CCAGGCACAGATCTTGAC			
	qPCR	UP	AGGATTGCAGTTTCATTGAAAGC	138	8.6	[14]
		LP	TGAACTCTATGCCAAAGCAGTTG			
		*Taq*Man *qnrA*	FAM-TATGCCGATCTGCGCGA-MGBNFQ			
qnrS	PCR	UP	AAGTGATCTCACCTTCACCGCTTG	425		[14]
		LP	TTAAGTCTGACTCTTTCAGTGATG			
	qPCR	UP	CGACGTGCTAACTTGCGTGA	118	8.3	[14]
		LP	GGCATTGTTGGAAACTTGCA			
		*Taq*Man *qnrS*	FAM-AGTTCATTGAACAGGGTGA-MGBNFQ			
armA	qPCR	UP	GAAAGAGTCGCAACATTAAATGACTT	93	8.4	[12]
		LP	GATTGAAGCCACAACCAAAATCT			
		*Taq*Man *armA*	FAM-TCAAACATGTCTCATCTATT-MGBNFQ			
pGEM	PCR	pGEM7up	TGTAATACGACTCACTAT			Promega

LOQ, limit of quantification (determined with the standard curve used in this study for each qPCR assay); qPCR, quantitative PCR; UP, upper primer; LP, lower primer; FAM, 6-carboxyflouorescein reporter; MGBNFQ, minor groove-binding non-fluorescent quencher.

of 18.2 gene copies/μL (threshold cycle of 32.4), similar to the other genes.

Primers and *Taq*Man™ hydrolysis probes (Table 2) were used under standard conditions in a StepOne™ Real-Time PCR system (Applied Biosystems) [9]. To further screen for PCR inhibition, dilutions of known gene copy concentrations of the *mecA* standard were spiked with DNA isolated from the samples and the results were compared with the expected concentration. No inhibition of the PCR by the samples was detected. All of the samples were run in duplicate.

2.6. Phage purification by caesium chloride (CsCl) density gradients

Eight isolates showing good induction and a high level of ARGs in phage DNA were selected for purification by CsCl density gradients [24] and electron microscopy observations. The easily visible grey bands corresponding to bacteriophages [24,25] were collected and dialysed. Phage DNA was extracted from the particles in the band and was used to quantify the ARGs. Phage particles forming a band were visualised by electron microscopy in a JEOL 1010 transmission electron microscope (JEOL Inc., Peabody, MA) operating at 80 kV [26].

2.7. Statistical analysis

Computation of data and statistical tests was performed using SPSS Statistics v.17.0 (SPSS Inc., Chicago, IL). A tolerance interval with 90% confidence in 90% of the population (considering collected isolates as the population) was used to determine which isolates were considered positive for induction following mitC treatment (using OD_{600} and ARGs gene copy data).

3. Results and discussion

3.1. Antibiotic resistance genes in phage particles isolated from faeces

Stool samples from healthy individuals in the Barcelona area were selected as described in Section 2.1. It was verified that the subjects had no contact with a clinical environment and had not received any antibiotic treatment or travelled abroad in the 3 months before sampling. The age of the individuals ranged from 1.3–85 years.

From the 150 samples, 109 (72.7%) of the phage suspensions obtained from the stools were positive for at least one ARG: 29 samples (19.3%) were positive for one ARG, 40 samples (26.7%) for two ARGs,

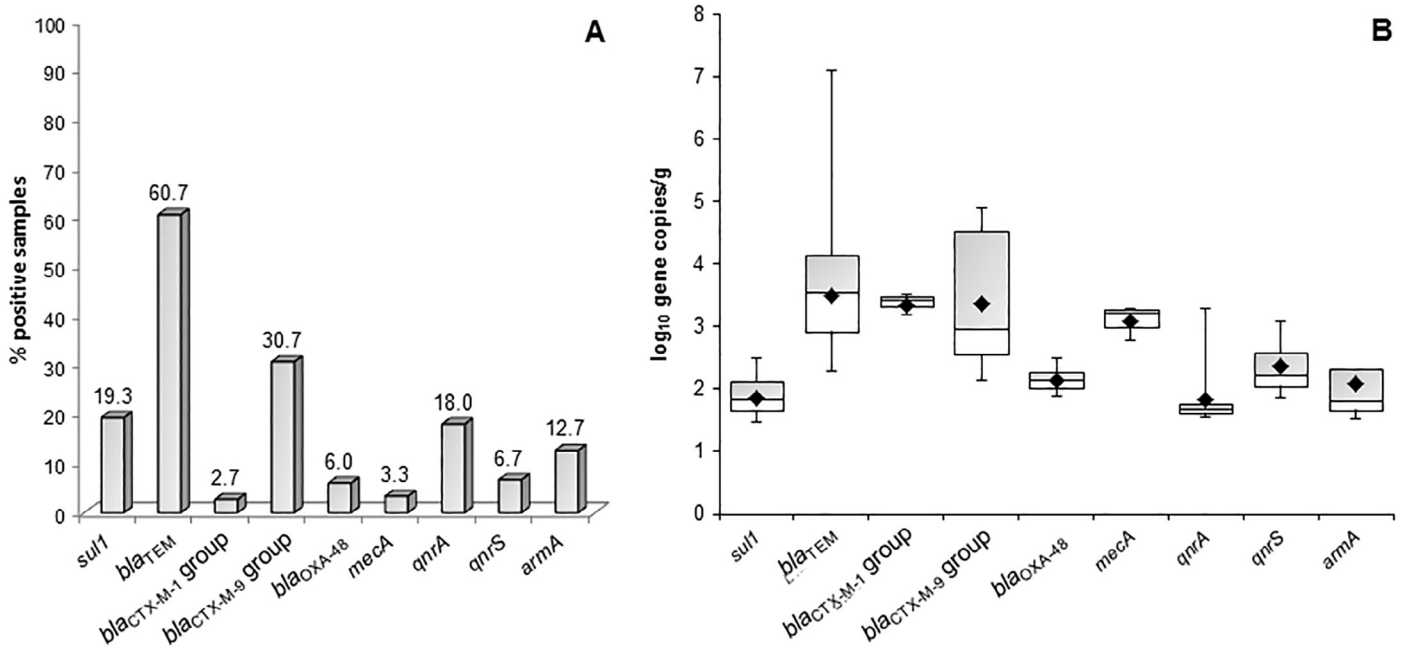


Fig. 1. Antibiotic resistance genes (ARGs) in the phage fraction of human faecal samples. (A) Percentage of positive samples for each ARG. (B) Abundance of each ARG. Box plot of the average values (log_{10} gene copies/g faeces) of all ARGs in the positive samples. In the box plot, the cross-pieces of each box represent (from top to bottom) the maximum, upper quartile, median (black bar), lower quartile and minimum values. The black diamond shows the mean value. The upper grey boxes in the box plot include samples showing values within the 75th percentile and the lower white boxes represent samples showing values within the 25th percentile.

24 samples (16.0%) for three ARGs, 14 samples (9.3%) for four ARGs and 2 samples (1.3%) for five ARGs. Among the detected ARGs, bla_{TEM} was the most prevalent, followed by $bla_{CTX\text{-}M\text{-}9\ group}$, *sul1* and *qnrA* (Fig. 1A). bla_{TEM} was also the most abundant (Fig. 1B), reaching maximum densities of 10^6 gene copies/g, although the samples showed great heterogeneity in the number of particles carrying bla_{TEM} gene copies. Next in abundance were both $bla_{CTX\text{-}M\ groups\ 1\ and\ 9}$. Although not among the most prevalent ARGs, *mecA* and *qnrS* showed an average abundance of 3.1 and 2.4 log_{10} gene copies/g, respectively. The average number of particles bearing $bla_{OXA\text{-}48}$, *qnrA* and *armA* was close to 2 log_{10} gene copies/g (although some samples showed higher densities of *qnrA*), whilst *sul1*, despite its high prevalence, was one of the least abundant genes (Fig. 1B).

Analysing the distribution of ARGs among subjects in different age divisions (<20, 20–39, 40–60 or >60 years) revealed a higher prevalence of *sul1* and $bla_{CTX\text{-}M\text{-}1\ group}$ in samples from older subjects, which also showed a lower level of $bla_{CTX\text{-}M\text{-}9\ group}$, as the most remarkable observations (Supplementary Fig. S1A). Individuals aged 20–60 years gave a higher percentage of samples without any ARGs, although this group also provided the most samples. The trend line (Supplementary Fig. S1B) did not show any correlation with different age groups.

The ARG prevalence in the phage fraction is in agreement with previous data obtained with a different set of faecal samples [12]. In the former study, correlations between age and the number of ARGs in phage DNA were also not observed [12]. In that study, 22.5% of samples were negative compared with ca. 28% here, and the proportions of each ARG were slightly higher in abundance. The most notable difference between the studies is that whereas both cohorts of individuals were not affected by gastrointestinal disease, in the previous study prior administration of antibiotics or travel abroad was not monitored.

3.2. Phage particles induced from clinical bacterial isolates

The nature of phage particles carrying ARGs detected in faeces is unknown. They could be free particles in the gut incorporated by ingestion of food or water, or particles produced by bacterial strains present in the microbiota. To evaluate this second possibility, 70 *E. coli* and 12 *K. pneumoniae* isolates from faecal samples of different individuals in this study (82 individuals) were selected on the basis of the presence of one or more targeted ARGs (Table 3). The isolates allowed us to analyse phage particles carrying bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$, *armA*, *qnrA*, *qnrS* and *sul1*. Phage particles were induced from the isolates using mitC at a subinhibitory concentration, a commonly used method [14]. The Enterobacteriaceae group does not harbour *mecA*, therefore this gene was not included in this part of the study. The absence of isolates with $bla_{OXA\text{-}48}$ could be expected considering that the isolation performed was not specific for its detection, and in addition this gene should not be prevalent in healthy carriers [27].

The effect of mitC on the isolates was determined by monitoring the reduction in OD_{600} of the treated aliquot of each culture in comparison with the untreated aliquot following 6 h of incubation at 37 °C. The reduction in OD_{600} is interpreted as activation of the phage lytic cycle or a similar mechanism causing cell lysis. Each isolate was treated with mitC at least in duplicate, and although the OD_{600} differed slightly between replicates, the differences between the control and the treated aliquot of the culture were consistent between replicates. To statistically support which samples showed induction, we considered a tolerance interval of 90% confidence in 90% of the population. Therefore, isolates falling outside the tolerance range, i.e. those showing an OD_{600} reduction of <0.2 points, which indicates a lack of cell lysis, were excluded. Only 7 of the 82 isolates were considered non-inducible (isolates marked with an asterisk in Fig. 2). In contrast, 54 isolates showed an OD_{600} decrease of >0.5 points (Fig. 2) and these were suspected of harbouring prophages or phage-derived particles causing lysis of the host strain after induction.

3.3. Antibiotic resistance genes in phage particles induced from clinical isolates

The genes bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$, *armA*, *qnrA*, *qnrS* and *sul1* were quantified in phage DNA in the culture supernatant of 82

Table 3
List of isolates from faeces and determination of the presence of various antibiotic resistance genes (ARGs).

No. of isolates	Species	Strain	ARGs present
9	*Escherichia coli*	Ec1–3, Ec6–7, Ec11, Ec13–15	bla_{TEM}, *sul1*
3	*E. coli*	Ec5, Ec16–17	bla_{TEM}
1	*E. coli*	Ec4	bla_{TEM}, *qnrA*, *qnrS*, *sul1*
5	*E. coli*	Ec18, Ec20 Ec26–28	$bla_{CTX\text{-}M\text{-}1\ group}$
2	*E. coli*	Ec19, Ec21	$bla_{CTX\text{-}M\text{-}1\ group}$, *sul1*
1	*E. coli*	Ec25	$bla_{CTX\text{-}M\text{-}1\ group}$, $bla_{CTX\text{-}M\text{-}9\ group}$
1	*E. coli*	Ec23	$bla_{CTX\text{-}M\text{-}1\ group}$, *qnrA*, *qnrS*
1	*E. coli*	Ec24	$bla_{CTX\text{-}M\text{-}1\ group}$, *armA*
1	*E. coli*	Ec22	$bla_{CTX\text{-}M\text{-}1\ group}$, *qnrA*, *qnrS*, *sul1*
4	*E. coli*	Ec29–32	$bla_{CTX\text{-}M\text{-}9\ group}$
3	*E. coli*	Ec34, Ec36–37	*qnrA*, *qnrS*
5	*E. coli*	Ec33, Ec61–64	*qnrA*, *qnrS*, *sul1*
34	*E. coli*	Ec38–60, Ec65–75	*sul1*
6	*Klebsiella pneumoniae*	Kp1–3, Kp5–7	bla_{TEM}
1	*K. pneumoniae*	Kp4	bla_{TEM}, *sul1*
3	*K. pneumoniae*	Kp8–9, Kp11	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$, *sul1*
1	*K. pneumoniae*	Kp10	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1\ group}$
1	*K. pneumoniae*	Kp12	$bla_{CTX\text{-}M\text{-}1\ group}$
Total = 82			24 bla_{TEM}, 16 $bla_{CTX\text{-}M\text{-}1\ group}$, 5 $bla_{CTX\text{-}M\text{-}9\ group}$, 1 *armA*, 11 *qnrA*, 11 *qnrS*, 56 *sul1*

isolates treated or not with mitC. ARG values were usually higher in the mitC-treated culture, attributed to the generation of phage particles by the treatment. Isolates Ec7, Ec22, Ec37, Ec60 Ec70, Kp2 and Kp3, which did not show a reduction in the OD_{600} measurements after mitC treatment (Fig. 2), accordingly did not show an increase in gene copies/mL of the corresponding ARGs in phage DNA after induction (Fig. 3). *armA* is not included because no differences between the induced culture and the control were detected.

After two independent induction experiments, 51 isolates (62%) showed an increase in the ARG copy number in the phage fraction. Those isolates with an increase of >0.2 log_{10} gene copies, on the basis of a tolerance interval of 90% confidence in 90% of the

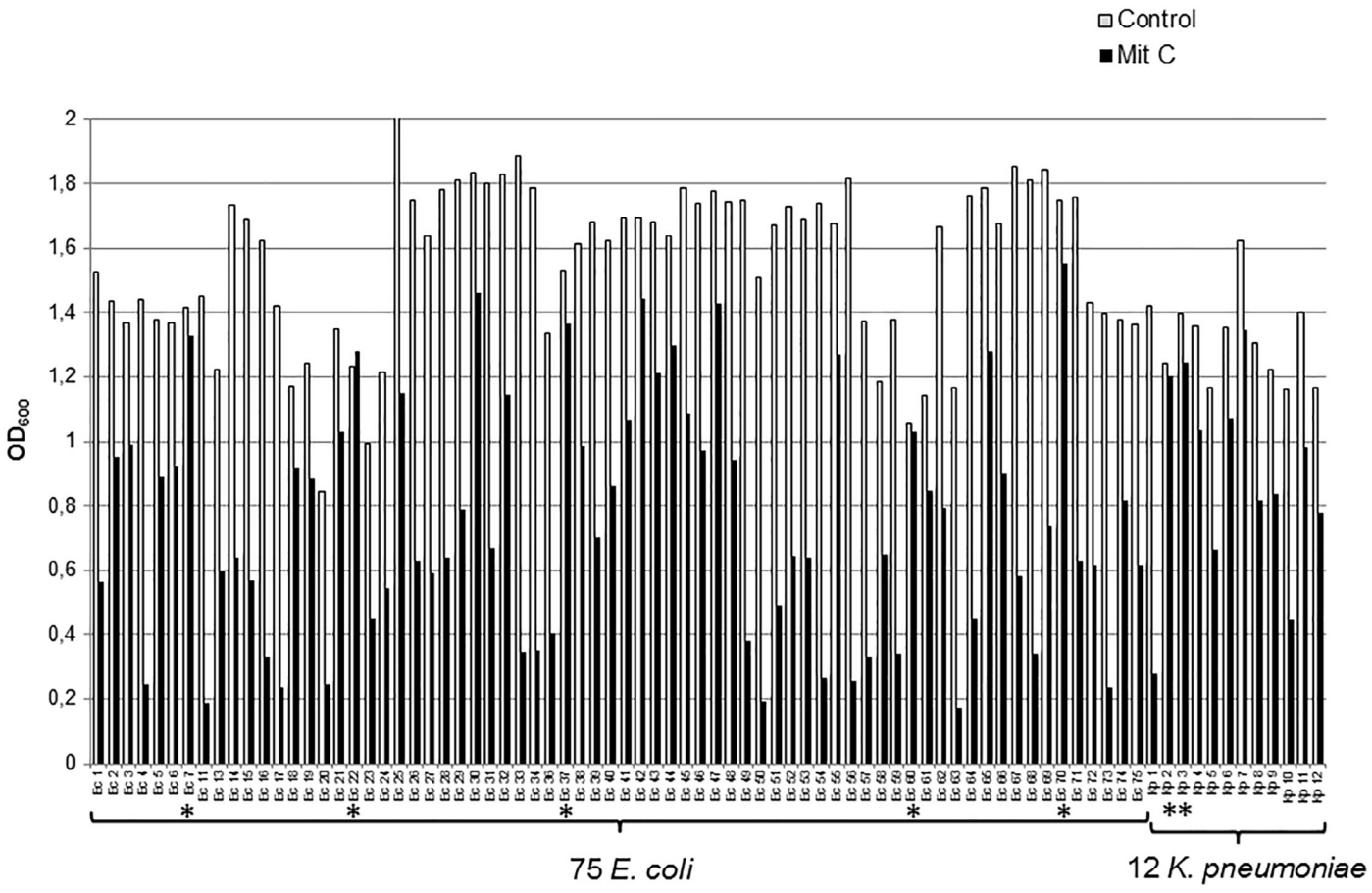


Fig. 2. Induction of phage particles from bacterial isolates by mitomycin C treatment. OD_{600} measurements for cultures of *Escherichia coli* (Ec) and *Klebsiella pneumoniae* (Kp) isolates with (mitC) or without (control) mitomycin C (0.5 μg/mL) treatment. * Asterisks indicate non-inducible strains. OD_{600}, optical density at 600 nm.

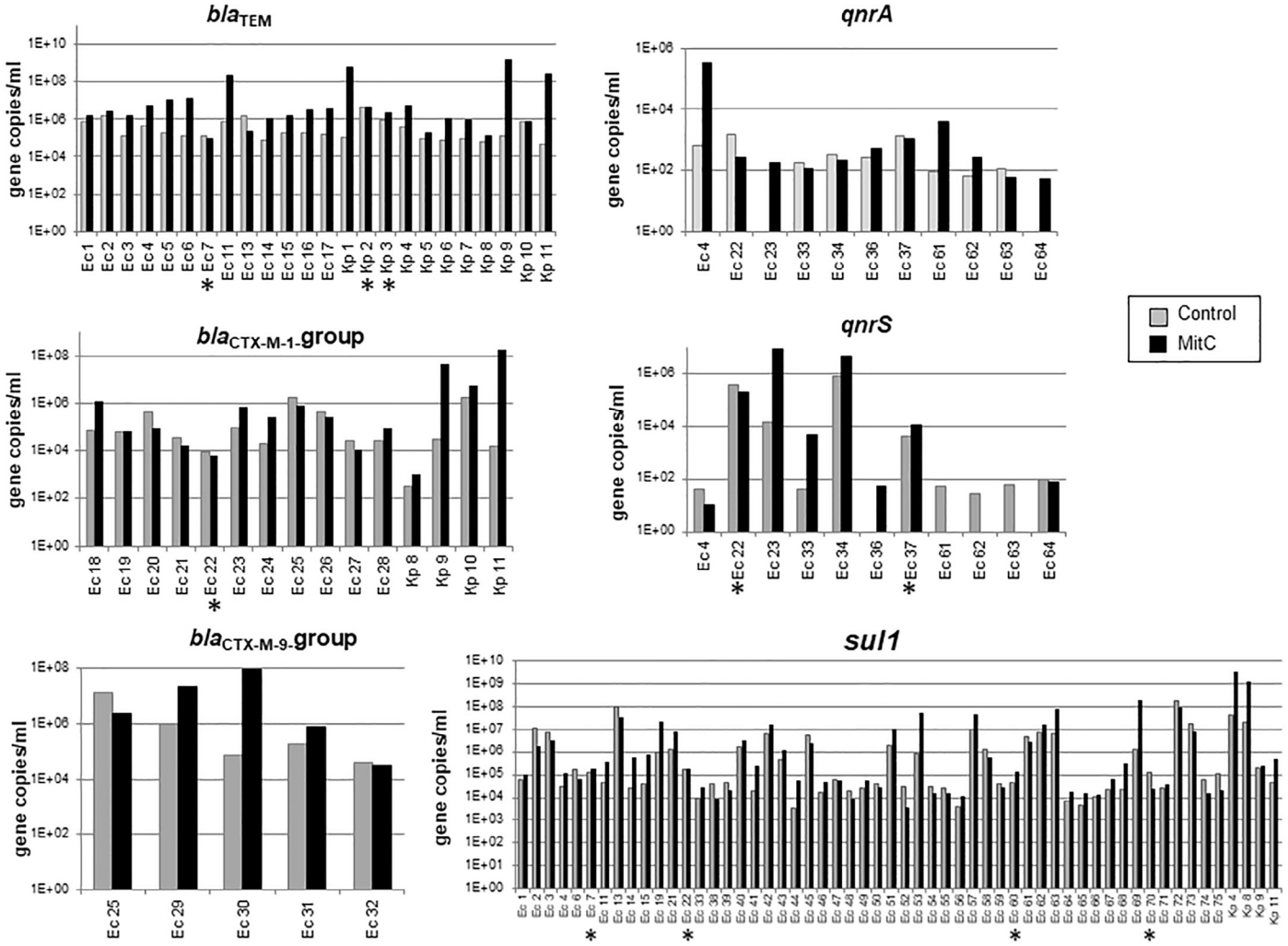


Fig. 3. Antibiotic resistance genes (ARGs) in the phage fraction of each bacterial isolate from human faecal samples. ARG densities (gene copies/mL) in phage DNA extracted from the cultures of *Escherichia coli* (Ec) and *Klebsiella pneumoniae* (Kp) isolates with (mitC) or without (control) mitomycin C (0.5 μg/mL) treatment. Results correspond to one independent induction experiment. * Asterisks indicate the non-inducible strains in Fig. 2.

population, were selected as inducible. In fact, for all 51 isolates that showed an increase in the ARG copy number, the difference was ≥0.5 $\log_{10}$. Of the 82 strains, 31 (38%) did not show an increase in the gene copy values (≤0.2 $\log_{10}$ units). In some cases (*qnrA* and *qnrS*) (Fig. 3), particles containing an ARG were only observed after induction, probably because the number of ARG particles in the untreated culture was too low and below the limit of quantification of the qPCR assays. In contrast, the occurrence of ARG particles in the uninduced culture is attributed to basal, spontaneous generation of phage particles, widely reported in phages [28] and phage-related particles, such as gene transfer agents (GTAs) [29]. Moreover, some isolates showed higher gene copy densities in the control than in the induced culture, although the differences were not significant ($P > 0.05$) (Fig. 3). These results could be attributed to a reduction in cell number caused by activation of the lytic cycle of other prophages in the isolate chromosome, which are very commonly found in *E. coli* and *Klebsiella* spp. [30]. Another possibility is that the treatment with mitC reduced the growth rate of the isolate, thereby diminishing the number of particles produced per cell.

Average gene copy/mL values of those samples with an increase in the number of particles following mitC induction were boxplotted (Fig. 4) and the differences between control and mitC-treated samples were significant ($P < 0.05$) for all ARGs except *sul1*. Similar averaged densities were observed for all ARGs except *qnrA* and *qnrS*, which showed lower values. Some ARGs (*sul1*, bla_{TEM} and $bla_{CTX\text{-}M\text{-}1\ group}$) showed up to 10^9 gene copies/mL after induction (Fig. 4).

3.4. Observation of phage particles carrying antibiotic resistance genes

Observation of faecal samples and the supernatant of some of the mitC-treated bacterial cultures revealed tailed phage particles, corresponding to the Myoviridae and Siphoviridae morphological types (like those shown in Fig. 5). Not all samples allowed visualisation of phage particles, either because they corresponded to samples where no integral capsids were present, or very probably because they were present at concentrations below those required for visualisation in the electron microscope (ca. 10^8 particles/mL) [26].

Phage particles were obtained from induced cultures of four *E. coli* (Ec4, Ec11, Ec30 and Ec69) and four *K. pneumoniae* (Kp4, Kp8, Kp9 and Kp11) isolates selected on the basis of high induction rates and an increase in the gene copies of one or more ARGs following mitC treatment (Fig. 3). Particles were further purified by CsCl gradients and the resulting grey band corresponded to a density of

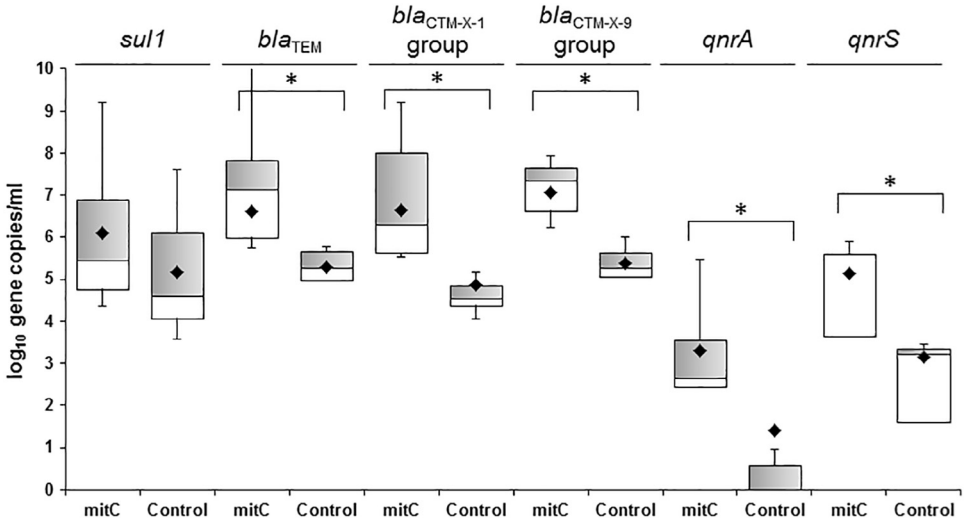
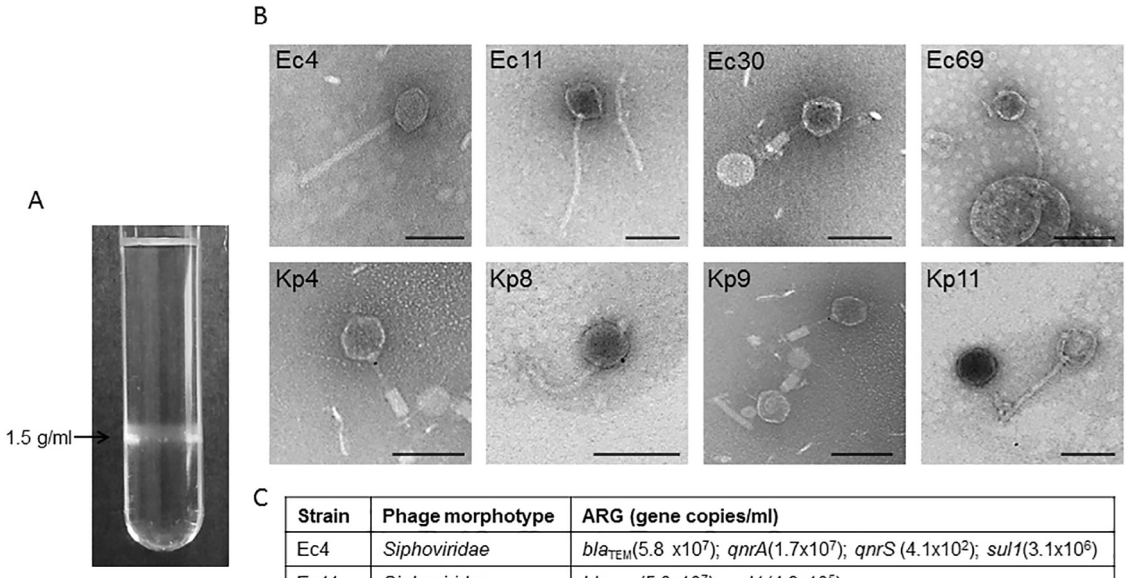


Fig. 4. Average antibiotic resistance gene (ARG) densities in the phage fraction of bacterial isolates. Average number of ARG copies (log_{10} gene copies/mL) in phage DNA from isolates showed a significant (* $P < 0.05$) increase in the number of ARG copies after mitomycin C (mitC) treatment versus uninduced controls in all ARGs except *sul1*. In the box plot, the cross-pieces of each box represent (from top to bottom) the maximum, upper quartile, median (black bar), lower quartile and minimum values. The black diamond shows the mean value. The upper grey boxes in the box plot represent samples showing values within the 75th percentile and the lower white boxes represent samples showing values within the 25th percentile.

1.5 g/mL, which is in accordance with what is expected for phage particles [25] (Fig. 5A). The band was recovered and used to confirm the presence of phage particles. Following chloroform and DNAse treatment, the DNA from the phage capsids was extracted and the ARGs were quantified.

The eight strains showed the presence of phage particles of the Myoviridae and Siphoviridae morphological types (Fig. 5B). Both groups have been reported as the most abundant infecting *E. coli* and *Bacteroides fragilis* in faecally polluted water samples [31,32] and stool samples [33]. Analysis of faecal viromes also indicates that



C

Strain	Phage morphotype	ARG (gene copies/ml)
Ec4	*Siphoviridae*	bla_{TEM}(5.8 x10^7); *qnrA*(1.7x10^7); *qnrS* (4.1x10^2); *sul1*(3.1x10^6)
Ec11	*Siphoviridae*	bla_{TEM} (5.3x10^7); *sul1* (4.9x10^5)
Ec30	*Myoviridae*	$bla_{CTX-M-9}$ group (2.6x10^7)
Ec69	*Siphoviridae*	*sul1* (3.1x10^7)
Kp4	*Myoviridae*	bla_{TEM} (4.9x10^5); *sul1* (4.9x10^{10})
Kp8	*Siphoviridae*	bla_{TEM} (2.5x10^4); $bla_{CTX-M-1}$ group (2.6x10^4); *sul1* (5.1x10^8)
Kp9	*Myoviridae*	bla_{TEM} (4.0x10^{10}); $bla_{CTX-M-1}$ group (3.3x10^9); *sul1* (9.0x10^5)
Kp11	*Siphoviridae*	bla_{TEM} (5.0x10^8); $bla_{CTX-M-1}$ group (9.9x10^8); *sul1* (7.6x10^5)

Fig. 5. Visualisation of phage particles from the induced cultures carrying antibiotic resistance genes (ARGs). (A) Example of the grey band corresponding to a density of 1.5 g/mL in a tube of caesium chloride (CsCl) density gradients prepared with the induced fraction of isolate Ec4. (B). Electron micrographs of phage particles purified from the eight *Escherichia coli* (Ec) and *Klebsiella pneumoniae* (Kp) induced isolates. Bar = 100 nm. (C) Quantitative PCR (qPCR) results of the ARGs present in the phage particles purified from the CsCl density bands and visualised by electron microscopy in (B).

tailed, doubled-stranded DNA viruses of the order Caudovirales, which include Siphoviridae, Myoviridae and Podoviridae, are the most abundant types in faeces [34].

A minimal amount of 10^7 particles/mL of sample is required for electron microscopy observation, therefore the phages observed are assumed to be the most abundant in these samples. Accordingly, the packaged DNA extracted from the CsCl density gradient bands containing the phages showed densities of gene copies/mL of $>10^7$ for at least one of the ARGs (Fig. 5C) and some ARGs showed densities of up to 10^{10} gene copies/mL. It can be assumed that at least a fraction of the phage particles visualised by microscopy would carry one of the ARGs in densities in accordance with the particles observed by microscopy and at the same order of magnitude as shown in Fig. 3.

The mobilome [35] includes all the mobile genetic elements (MGEs) in a genome, whilst the resistome [36] refers to all ARGs and their precursors in a bacterial genome. The two concepts are closely linked because, in general, ARGs found intrinsically in certain bacteria are mobilised to recipient cells by a range of MGEs and their spread is the main cause of the alarming emergence of antibiotic-resistant bacteria worldwide [6]. However, the scope of the elements that comprise the mobilome has not yet been definitively defined. The role of plasmids in ARG transfer in clinical settings has been widely reported [7,37], but it is now suspected that other elements, such as phages [9,10,21] or phage-derived particles [11], could also be involved.

The intestinal habitat is a densely populated environment where phages play a determinant role, either in regulation of intestinal populations thereby influencing human welfare, or as MGEs of genes related to pathogenicity [2,12,38]. It has been suggested [11] that phages are efficient genetic vehicles owing to the protection conferred by the protein capsid in extracellular environments.

Some phage genomes are spontaneously induced from resistant strains by environmental conditions [14,28], resulting in transcription and production of new phage particles that then infect and lysogenise other uninfected host cells. Other elements that can be considered as phage-related, because of their evident similarities with phages, are induced in a similar way: this is the case for GTAs [39].

The particles produced by the bacterial isolates in this study appeared to be resident in the isolates as prophages and were induced by mitC treatment. The presence of ARGs in these particles opens up two possibilities. The first is that these are prophages with the ARG inserted in their genome. We would then expect to be able to isolate these ARG-harbouring phages and plausibly to transduce the gene in relatively high frequencies. This was not the case here: the transduction attempts were not successful, in line with previous attempts using phage particles isolated from faecally polluted samples [9]. Moreover, some sequencing studies [9,12], as in the present work (data not presented), have shown a lack of phage genes flanking the targeted ARGs.

The second possibility is that following induction, prophage genes in the bacterial isolates have packaged bacterial DNA (including the ARG) in a sort of generalised transduction or GTA-like particle. These would then be detectable by the methods used and show an increase after induction, but with an absence of phage DNA genes, which is more in accord with our observations. These ARG-containing phage particles would theoretically be capable of attaching to new host cells and injecting their DNA [40]. In fact, quite a number of phages reportedly involved in ARG transfer appear to be derived from generalised transduction [41–43]. In terms of their genome, these cannot be considered phages because they contain only bacterial and not phage DNA. In line with these assumptions, an interesting recent study revealed that bacterial DNA, including ARGs, found in viromes was rarely encoded in phage genomes [44]. Once the possibility of bacterial DNA contamination is discarded (although not completely ruled out), and considering ARGs as those genes that confer real resistance, the most plausible explanation for the presence of ARGs in the studied phage particles is that bacterial DNA is mobilised through generalised transduction or related mechanisms [44].

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2017.11.014.

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