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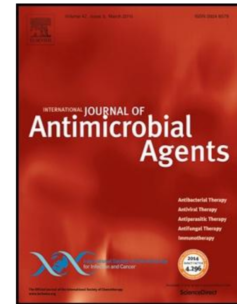

Title: Antibiotic resistance genes in phage particles isolated from human feces and induced from clinical bacterial isolates

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1 **Antibiotic resistance genes in phage particles isolated from human feces and**
2 **induced from clinical bacterial isolates**

3

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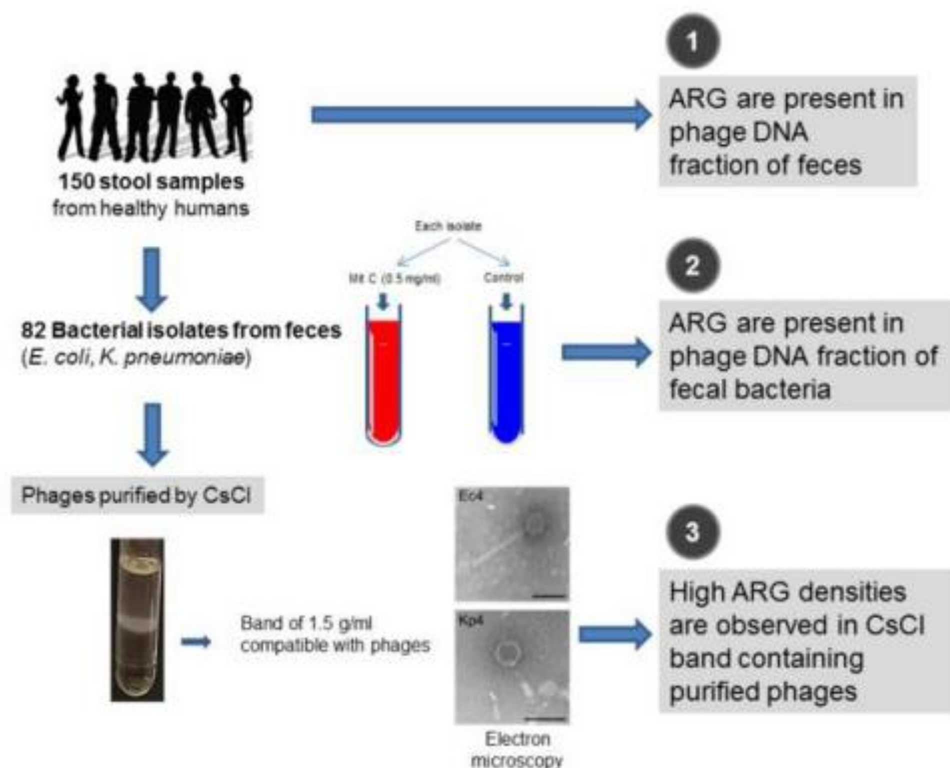
26 **Graphical Abstract**



27

28 **Highlights**

29 • 72% of the stools of healthy individuals showed at least one ARG in phage
30 DNA
31 • All ARG studied (*bla* genes, *qnr* genes, *mecA*, *sul1* and *armA*) were detected
32 • *bla*$_{TEM}$ was the most prevalent and abundant ARG in the phage DNA
33 fraction of feces
34 • ARGs were detected in phage DNA obtained from bacteria isolated from
35 feces
36 • Phage suspensions confirmed by electron microscope contained high ARG
37 densities

38

39 **Abstract**

40 Phage particles have emerged as elements with the potential to mobilize antibiotic
41 resistance genes (ARGs) in different environments, including the intestinal habitat. The
42 aim of this study was to determine the occurrence of ARGs in phage particles present in
43 fecal matter and induced from strains isolated from feces. Nine ARGs (*bla*$_{TEM}$, *bla*$_{CTX-M-}$

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44 $_1$-group, *bla*$_{CTX-M-9}$-group, *bla*$_{OXA-48}$, *qnrA*, *qnrS*, *mecA*, *sul1* and *armA*) were quantified
45 by qPCR in the phage DNA fractions of 150 fecal samples obtained from healthy
46 individuals. These subjects had not received antibiotic treatment or travelled abroad in
47 the three months prior to the sample collection. On the suspicion that the detected
48 particles originated from bacterial flora, 82 *Escherichia coli* and *Klebsiella pneumoniae*
49 isolates possessing at least one identified ARG (*bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group, *bla*$_{CTX-M-9}$-
50 group, *armA*, *qnrA*, *qnrS*, and *sul1*) were isolated and their capacity to produce phage
51 particles carrying these ARGs after induction was evaluated. Seventy-two percent of
52 samples were positive for at least one ARG, with *bla*$_{TEM}$ and *bla*$_{CTX-M-9}$-group being the
53 most prevalent and abundant. Fifty-one isolates (62%) showed an increase in the
54 number of copies of the respective ARG in the phage fraction after induction, with
55 *bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group, *bla*$_{CTX-M-9}$-group and *sul1* being the most abundant. Phages
56 induced from the isolates were further purified and visualized using microscopy and
57 their DNA showed ARG levels of up to 10^{10} gene copies/ml. This study highlights the
58 abundance of phage particles harboring ARGs and indicates that bacterial strains in the
59 intestinal habitat could be sources of these particles.

60

61 **Key words:** antibiotic resistance, bacteriophage, feces, *Escherichia coli*, *Klebsiella*
62 *pneumoniae*, horizontal genetic transfer, transduction.

63

64 1. Introduction

65

66 Bacteriophages, or bacterial viruses, are the most abundant biological entities on
67 Earth and one of the keys to the evolution and potential control of bacterial populations
68 [1]. Knowledge of phages has been essential for the progress of molecular biology and

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69 they have been used as models for studying different biological processes. In recent
70 decades, phages have acquired increasing relevance in molecular biology due to new
71 insights into their presence in many bacterial genomes [1,2], their role in horizontal
72 gene transfer [3], the phage-bacterium relation and bacterial defense mechanisms
73 against phage infection [4].

74

75 Meanwhile, bacterial resistance to antibiotics continues to increase and is severely
76 undermining our ability to control infectious diseases. The World Health Organization
77 (WHO) has identified antibiotic resistance as one of the most challenging problems in
78 public health care on a global scale (available at
79 http://apps.who.int/gb/ebwha/pdf_files/WHA68-REC1/A68_R1_REC1-en.pdf). The
80 causes of this increase in resistance are believed to include overuse and inconsistent
81 application of antibiotics in humans, together with the use of antibiotics in animal
82 husbandry [5,6]. The scientific community and governments have reacted by calling for
83 a better control of antibiotic usage in both humans and livestock. Researchers are trying
84 to find new generations of antibiotics to treat infections by resistant strains, but more
85 research into the mechanisms of resistance would also be advisable. This is the purpose
86 of the multidisciplinary "One-Health" approach [8], which aims to encourage the
87 collaborative efforts of multiple disciplines working locally, nationally, and globally.

88

89 Antibiotic resistance may be intrinsic, or conferred either by mutation or by
90 acquiring antibiotic resistance genes (ARGs) through mobile genetic elements (MGEs)
91 [5]. Clinical studies point to conjugation and transformation as the most likely
92 mechanisms of transfer [7]. However, little attention has been paid to other
93 mechanisms, such as transduction mediated by phages or phage-derived particles, which

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94 have only recently emerged as potentially relevant [8–10]. Bacteriophages basically
95 consist of one nucleic acid molecule (the phage genome) surrounded by a protein
96 coating, the capsid. This packaging of the nucleic acid confers protection and hence an
97 extracellular persistence that cannot be found in naked DNA or RNA. Therefore, capsid
98 protection could be important in cases where there is no close cell-to-cell contact [11].

99

100 In preliminary studies the presence of ARGs was determined in total and phage
101 DNA in feces of individuals without any enteric disease [12]. That work is extended
102 here by the analysis of more ARGs in the phage fraction of a new collection of fecal
103 samples from 150 healthy individuals free of contact with clinical settings and who had
104 not received antibiotic therapy in the previous three months. The ARGs studied
105 consisted of four β-lactamases (bla_{TEM}, $bla_{CTX-M-1}$-group, $bla_{CTX-M-9}$-group, bla_{OXA-48})
106 [13], two quinolone resistance genes (*qnrA* and *qnrS*)[14], the *mecA* gene that confers
107 resistance to methicillin in *Staphylococcus aureus* [15], the emerging *armA* gene that
108 confers resistance to aminoglycosides [16] and *sul1*, the most extended gene conferring
109 resistance to sulfonamides [17]. Another aim of the study was to gain insight into the
110 origin of the phage particles, suspected to be derived from bacterial flora. Accordingly,
111 bacterial strains (*Escherichia coli* and *Klebsiella pneumoniae*) isolated from the feces
112 that possessed a given ARG were treated to induce phage particles carrying this ARG.

113

114 **2. Materials and methods**

115

116 *2.1. Fecal samples*

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118 This study was performed with 150 human fecal samples of individuals living in
119 the city of Barcelona (Catalonia, North East Spain), collected over a period of six
120 months (from February to August 2016). All individuals were healthy, not related with
121 clinical environments nor involved in a food-borne outbreak or showing any gastro-
122 intestinal pathology or known infection. None of the subjects had consumed antibiotics
123 or travelled to foreign countries in the three months before the sampling. All samples
124 were completely anonymized. Besides ensuring the above criteria were fulfilled, no
125 individual data were collected except for age. The samples were destroyed immediately
126 after the study, which was approved by the Clinical Ethics Committee (12/065/1350).
127 Informed consent was obtained for all individuals.

128

129 *2.2. Bacterial strains*

130

131 The *E. coli* strains used as controls for the different ARGs are listed in Table 1.
132 Fecal samples were cultured on chromogenic agar chromID® CPS® Elite (bioMérieux,
133 Marcy-l'Étoile, France). After 24 h of incubation at 37ºC, all the isolates growing on the
134 plates compatible with *E. coli* or *Klebsiella* were identified by matrix-assisted laser
135 desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (MALDI
136 Autoflex IITM / TOF/TOF, Bruker, Daltonik GmbH, Germany). Antimicrobial
137 susceptibility testing was performed according to the guidelines of the Clinical and
138 Laboratory Standards Institute [18].
139 Seventy *E. coli* and 12 *K. pneumoniae* isolates were selected on the basis of the
140 presence of one of the target ARGs (one isolate per individual) to determine the
141 presence of phage particles containing the ARGs.

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143 *2.3. Partial purification of phage DNA*

144

145 Fecal samples were homogenized 1:5 (*w:v*) in phosphate buffered saline (PBS)
146 by shaking for 15 minutes. Then, a final volume of 50 ml of the homogenate was
147 centrifuged at 3,000 *g* and the supernatant was filtered through low protein-binding 0.22
148 µm pore-size membrane filters (Millex-GP, Millipore, Bedford, MA). The suspensions
149 were treated with chloroform, centrifuged at 16,000 *g* for 10 minutes and the
150 supernatants were treated with DNAse (100 units/ml; Sigma-Aldrich, Spain) for 1 hour
151 at 37ºC. DNAse was heat-inactivated at 75ºC for 5 minutes. To rule out the presence of
152 non-packaged DNA, an aliquot of the sample was taken after DNase treatment and
153 before its desencapsidation. Using this control sample, the absence of free 16SrDNA
154 was established as well as the absence of the ARGs studied by qPCR, confirming total
155 removal of non-encapsidated DNA [12,19]. Different controls were performed to verify
156 the stability and appropriate inactivation of the DNase [9]. Packaged DNA was
157 extracted by proteinase K digestion, purified and quantified [9,12].

158

159 *2.4. Purification of bacteriophages from clinical isolates*

160

161 Twenty ml cultures of each clinical isolate in Luria Beltrani (LB) broth were
162 grown to the mid-exponential phase (optical density $(OD)_{600}$ of 0.3). Each culture was
163 aliquoted in two 10 ml tubes and one aliquot was treated with mitomycin C (mitC)
164 (final concentration 0.5 µg/ml) to induce phage particles. Both tubes were incubated for
165 6h at 37ºC by shaking in the dark and the absorbance of the culture after induction was
166 monitored by comparing the OD of the mitC-treated and non-treated cultures. Phages in

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167 the supernatant lysate from both aliquot cultures were partially purified as described
168 above.
169
170 *2.5. Standard PCR and qPCR procedures*
171
172 PCRs were performed with a GeneAmp® PCR 2700 system (Applied
173 Biosystems, Barcelona, Spain). ARGs from the control strains were amplified by
174 conventional PCR, cloned in pGEM-T Easy vectors (pGEM®-T-Easy Vector, Promega,
175 Barcelona, Spain) to generate the constructs and verified by sequencing (Table 2). The
176 constructs were used to generate the standard curves.
177
178 Real-time qPCR assays for bla_{TEM}, $bla_{\mathrm{CTX\text{-}M\text{-}1}}$-group, $bla_{\mathrm{CTX\text{-}M\text{-}9}}$-group ,*mecA,*
179 *armA, qnrA* group*, qnrS* group and *sul1* were performed as previously described
180 [9,12,20,21]. The $bla_{\mathrm{OXA\text{-}48}}$ gene qPCR assay (Table 2) was designed with the Primer
181 Express Software version 3.0 (Applied Biosystems). The gene was amplified using
182 specific primers (Table 2) from the sequence of $bla_{\mathrm{OXA\text{-}48}}$ harbored in the *K.*
183 *pneumoniae* clinical isolate HSP172. The amplified $bla_{\mathrm{OXA\text{-}48}}$ was sequenced and cloned
184 in pGEM-T Easy. The construct was confirmed by sequencing and used to generate the
185 standard curves [9]. The qPCR assay for $bla_{\mathrm{OXA\text{-}48}}$ showed a 99.8% efficiency and a
186 quantification limit of 18.2 gene copies /µL (threshold cycle of 32.4), similar to the
187 other genes.
188
189 Primers and Taqman hydrolysis probes (Table 2) were used under standard
190 conditions in a StepOne Real-Time PCR system [9]. To further screen for PCR
191 inhibition, dilutions of known gene copy concentration of the *mecA* standard were

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192 spiked with the DNA isolated from the samples, and results were compared to the
193 expected concentration. No inhibition of the PCR by the samples was detected. All the
194 samples were run in duplicate.
195
196 *2.6. Phage purification by CsCl density gradients.*
197
198 Eight isolates showing good induction and a high level of ARGs in phage DNA
199 were selected for purification by cesium chloride (CsCl) density gradients [22] and
200 electron microscopy observations. The easily visible grey bands corresponding to
201 bacteriophages [22,23] were collected and dialyzed. Phage DNA was extracted from the
202 particles in the band and used to quantify the ARGs. Phage particles forming a band
203 were visualized by electron microscopy in a JEOL 1010 transmission electron
204 microscope (JEOL Inc. Peabody, MA USA) operating at 80 kV [24].
205
206 *2.7. Statistical analysis*
207
208 Computation of data and statistical tests was performed using the Statistical
209 Package for Social Science software (SPSS). A tolerance interval with 90% confidence
210 in 90% of the population (considering collected isolates as the population) was used to
211 determine which isolates were considered positive for induction after mitC treatment
212 (using OD_{600} and ARGs gene copy data).
213
214
215 **3. Results and Discussion**

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216

217 *3.1. ARGs in phage particles isolated from feces*

218

219 Stool samples from healthy individuals in the Barcelona area were selected as
220 described in Materials and methods. It was verified that the subjects had no contact with
221 a clinical environment and had not received any antibiotic treatment or travelled abroad
222 in the three months before sampling. The age of the individuals ranged from 1.3 to 85
223 years.

224

225 About 72% of the phage suspensions obtained from the stools were positive for
226 at least one ARG. From these, 29 samples (19.3%) were positive for one ARG; 40
227 samples (26.7%) for 2 ARGs; 24 samples (16.0%) for 3 ARGs; 14 samples (9.3%) for 4
228 and 2 samples (1.3%) for 5 ARGs. Among the detected ARGs, bla_{TEM} was the most
229 prevalent, followed by $bla_{CTX-M-9}$-group, *sul1* and *qnrA* (Figure 1A). bla_{TEM} was also the
230 most abundant (Fig. 1B), reaching maximum densities of 10^6 gene copies/g, although
231 the samples showed a great heterogeneity in the number of particles carrying bla_{TEM}
232 gene copies. Next in abundance were both bla_{CTX-M} groups 1 and 9. Although not
233 among the most prevalent ARGs, *mecA* and *qnrS* showed an average abundance of 3.1
234 and 2.4 log_{10} gene copies, respectively. The average number of particles bearing bla_{OXA-}
235 $_{48}$, *qnrA* and *armA* was close to 2 log_{10} gene copies /g (although some samples showed
236 higher densities of *qnrA*), while *sul1*, despite its high prevalence, was one of the least
237 abundant genes (Fig. 1B).

238 Analyzing the distribution of ARGs among subjects in different age segments
239 (<20, 20-40, 40-60 or >60) revealed a higher prevalence of *sul1* and $bla_{CTX-M-1}$-group in
240 samples from older subjects, which also showed a lower level of $bla_{CTX-M-9}$-group, as

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241 the most remarkable observations (Fig. S1A). Individuals aged 20-60 years gave a
242 higher percentage of samples without any ARGs, although this group also provided the
243 most samples. The trend line (Fig. S1B) did not show any correlation with different age
244 groups..

245

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

254

255 *3.2. Phage particles induced from clinical bacterial isolates*

256

257 The nature of phage particles carrying ARGs detected in feces is unknown. They
258 could be free particles in the gut incorporated by ingestion of food or water, or particles
259 produced by bacterial strains present in the microbiota. To evaluate this second
260 possibility, 70 *E. coli* and 12 *K. pneumoniae* isolates from fecal samples of different
261 individuals in this study (82 individuals) were selected on the basis of the presence of
262 one or more targeted ARGs (Table 3). The isolates allowed us to analyze phage
263 particles carrying bla_{TEM}, $bla_{\text{CTX-M-1}}$-group, $bla_{\text{CTX-M-9}}$-group, *armA, qnrA, qnrS* and *sul1*.
264 Phage particles were induced from the isolates using mitC at a subinhibitory
265 concentration, a commonly used method [14]. The *Enterobacteriaceae* group does not

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266 harbor *mecA,* therefore this gene was not included in this part of the study. The absence
267 of isolates with *bla*$_{OXA-48}$ could be expected considering that the isolation performed
268 was not specific for its detection, and in addition this gene should not be prevalent in
269 healthy carriers [25].
270
271 The effect of mitC on the isolates was determined by monitoring the reduction of
272 the OD_{600} of the treated aliquot of each culture in comparison with the untreated aliquot
273 after 6 hours of incubation at 37ºC. The OD reduction is interpreted as activation of the
274 phage lytic cycle or a similar mechanism causing cell lysis. Each isolate was mitC-
275 treated at least in duplicate, and although the OD reached differed slightly between
276 replicates, the differences between the control and the treated aliquot of the culture were
277 consistent between replicates. To statistically support which samples showed induction,
278 we considered a tolerance interval of 90% confidence in 90% of the population.
279 Therefore, we excluded isolates falling outside the tolerance range, i.e. those showing
280 an OD_{600} reduction of less than 0.2 points, which indicates a lack of cell lysis. Only
281 seven of the 82 isolates were considered non-inducible (isolates marked with an asterisk
282 in Fig. 2). In contrast, 54 isolates showed an OD_{600} decrease of > 0.5 points (Fig. 2) and
283 these were suspected of harboring prophages or phage-derived particles causing the
284 lysis of the host strain after induction.
285
286 *3.3. ARGs in phage particles induced from clinical isolates*
287
288 *bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group, *bla*$_{CTX-M-9}$-group, *armA*, *qnrA*, *qnrS*, and *sul1* were
289 quantified in the phage DNA in the culture supernatant of 82 isolates treated or not with
290 mitC. ARG values were usually higher in the mitC-treated culture, attributed to the

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291 generation of phage particles by the treatment. Isolates Ec7, Ec22, Ec37, Ec60 Ec70,
292 Kp2, Kp3, which did not show a reduction in the OD_{600} measurements after mitC
293 treatment (Fig. 2), accordingly did not show an increase in gene copies/ml of the
294 corresponding ARGs in phage DNA after induction (Fig. 3). *armA* is not included
295 because no differences between the induced culture and the control were detected.
296
297 After two independent induction experiments, 51 isolates (62.2%) showed an
298 increase in the ARG copy number in the phage fraction. We selected as inducible those
299 isolates with an increase of more than 0.2 $\log_{10}$ gene copies, on the basis of a tolerance
300 interval of 90% confidence in 90% of the population. In fact, for all 51 isolates that
301 showed an increase in the ARG copy number the difference was equal or greater than
302 0.5 $\log_{10}$. Thirty-one strains (37.8%) did not show an increase in the gene copy values
303 ($\leq 0.2 \log_{10}$ units). In some cases (*qnrA* and *qnrS*) (Fig. 3), particles containing an ARG
304 were only observed after induction, probably because the number of ARG-particles in
305 the untreated culture was too low and below the limit of quantification of our qPCR
306 assays. In contrast, the occurrence of ARG-particles in the uninduced culture is
307 attributed to basal, spontaneous generation of phage particles, widely reported in phages
308 [26] and phage-related particles, such as gene transfer agents (GTA)[27]. Moreover,
309 some isolates showed higher gene copy densities in the control than in the induced
310 culture, although the differences were not significant ($P > 0.05$) (Fig. 3). These results
311 could be attributed to a reduction in cell number caused by activation of the lytic cycle
312 of other prophages in the isolate chromosome, which are very commonly found in *E.*
313 *coli* or *Klebsiella spp.* [28]. Another possibility is that the treatment with mitC reduced
314 the growth rate of the isolate, thereby diminishing the number of particles produced per
315 cell.

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316

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

323

324 *3.4. Observation of phage particles carrying ARGs*

325

326 Observation of fecal samples and the supernatant of some of the mitC-treated
327 bacterial cultures revealed tailed phage particles, corresponding to the *Myoviridae* and
328 *Siphoviridae* morphological types (like those shown in Fig. 5). Not all samples allowed
329 visualization of phage particles, either because they corresponded to samples where no
330 integral capsids were present, or very probably because they were present in
331 concentrations below those required for visualization in the electron microscope (*c.a.*
332 10^8 particles/ml) [24].

333

334 The phage particles were obtained from induced cultures of 4 *E. coli* (Ec4, Ec11,
335 Ec30, Ec69) and 4 *K. pneumoniae* (Kp4, Kp8, Kp9, Kp11) isolates selected on the basis
336 of high induction rates and an increase in the gene copies of one or more ARG after
337 mitC treatment (Fig. 3). Particles were further purified by CsCl gradients and the
338 resulting grey band corresponded to a density of 1.5 g/ml, which is in accordance with
339 what is expected for phage particles [23] (Fig. 5A). The band was recovered and used to

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340 confirm the presence of phage particles. After chloroform and DNAse treatment, the
341 DNA from the phage capsids was extracted and the ARG quantified.
342
343 The eight strains showed the presence of phage particles of the *Myoviridae* and
344 *Siphoviridae* morphological types (Fig. 5B). Both groups have been reported as the
345 most abundant infecting *E. coli* and *Bacteroides fragilis* in fecally polluted water
346 samples [29,30] and stool samples [31]. Analysis of fecal viromes also indicates that
347 tailed, doubled-stranded DNA viruses of the order Caudovirales, which include
348 *Siphoviridae*, *Myoviridae*, and *Podoviridae*, are the most abundant types in feces [32].
349
350 A minimal amount of 10^7 particles/ml of sample is required for electron
351 microscopy observation, therefore the phages observed are assumed to be the most
352 abundant in these samples. Accordingly, the packaged DNA extracted from the CsCl
353 density gradient bands containing the phages showed densities of gene copies/ml > 10^7
354 for at least one of the ARGs (Fig. 5C) and some ARGs showed densities of up to 10^{10}
355 gene copies/ml. It can be assumed that at least a fraction of the phage particles
356 visualized by microscopy would carry one of the ARGs in densities in accordance with
357 the particles observed by microscopy and at the same order of magnitude as shown in
358 Fig. 3.
359
360 The mobilome [33] includes all the mobile genetic elements (MGEs) in a
361 genome, while the resistome [34] refers to all the ARGs and their precursors in a
362 bacterial genome. The two concepts are closely linked, because in general, ARGs found
363 intrinsically in certain bacteria are mobilized to recipient cells by a range of MGEs, and
364 their spread is the main cause of the alarming emergence of antibiotic-resistant bacteria

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365 worldwide [6]. However, the scope of the elements that comprise the mobilome has not
366 yet been definitively defined. The role of plasmids in ARG transfer in clinical settings
367 has been widely reported [7,35], but it is now suspected that other elements, such as
368 phages [9,10,19] or phage-derived particles [11], could also be involved.
369
370 The intestinal habitat is a densely populated environment where phages play a
371 determinant role, either in regulation of intestinal populations, thereby influencing
372 human welfare, or as MGEs of genes related to pathogenicity [2,12,36]. It has been
373 suggested [11] that phages are efficient genetic vehicles due to the protection conferred
374 by the protein capsid in extracellular environments.
375
376 Some phage genomes are spontaneously induced from resistant strains by
377 environmental conditions [14,26], resulting in transcription and production of new
378 phage particles which then infect and lysogenize other uninfected host cells. Other
379 elements that can be considered as phage-related, because of their evident similarities
380 with phages, are induced in a similar way: this is the case of GTA [37].
381
382 The particles produced by the bacterial isolates in this study seemed to be
383 resident in our isolates as prophages and were induced by the mitC treatment. The
384 presence of ARGs in these particles opens up two possibilities. The first is that these are
385 prophages with the ARG inserted in their genome. We would then expect to be able to
386 isolate these ARG-harboring phages and plausibly to transduce the gene in relatively
387 high frequencies. This was not the case here: our transduction attempts were not
388 successful, in line with previous attempts using phage particles isolated from fecally

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389 polluted samples [9]. Moreover, some sequencing studies [9,12], as in the present work
390 (data not presented), have shown a lack of phage genes flanking the targeted ARGs.
391
392 The second possibility is that after induction, prophage genes in the bacterial
393 isolates have packaged bacterial DNA (including the ARG) in a sort of generalized
394 transduction or GTA-like particle. These would then be detectable by the methods used
395 and show an increase after induction, but with an absence of phage DNA genes, which
396 is more in accord with our observations. These ARG-containing phage particles would
397 theoretically be capable of attaching to new host cells and injecting their DNA [38]. In
398 fact, quite a number of phages reportedly involved in ARG transfer seem to be derived
399 from generalized transduction [39–41]. In terms of their genome, these cannot be
400 considered phages, because they contain only bacterial and not phage DNA. In line with
401 these assumptions, an interesting recent study revealed that bacterial DNA, including
402 ARGs, found in viromes was rarely encoded in phage genomes [42]. Once the
403 possibility of bacterial DNA contamination is discarded (although not completely ruled
404 out), and considering ARGs as those genes that confer real resistance, the most
405 plausible explanation for the presence of ARGs in the studied phage particles is that
406 bacterial DNA is mobilized through generalized transduction or related mechanisms
407 [42].
408
409

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424
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426

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553 **Figure 1.- ARGs in the phage fraction of human fecal samples**. A) Percentage of
554 positive samples for each ARG. B) Abundance of each ARG. Box plot of the average
555 values ((log_{10} gene copies)/g feces) of all ARGs in the positive samples. In the box plot,
556 the cross-pieces of each box represent (from top to bottom) the maximum, upper-
557 quartile, median (black bar), lower-quartile and minimum values. The black diamond
558 shows the mean value. The upper boxes in the box plot include samples showing values
559 within the 75th percentile and lower white box samples show values within the 25th
560 percentile.

561

562 **Figure 2.- Induction of phage particles from the bacterial isolates by mitomycin C**
563 **treatment**. OD_{600} measurements for cultures of the *E. coli* (Ec) and *K. pneumoniae* (Kp)
564 isolates with (mitC) or without (control) mitomycin C (0.5 µg/ml) treatment. An
565 asterisk indicates the non-inducible strains.

566

567 **Figure 3.- ARGs in the phage fraction of each bacterial isolate from human fecal**
568 **samples.** ARG densities (gene copies/ml) in phage DNA extracted from the cultures of
569 the *E. coli* (Ec) and *K. pneumoniae* (Kp) isolates with (mitC) or without (control)
570 mitomycin C (0.5 µg/ml) treatment. Results correspond to one independent induction
571 experiment. An asterisk indicates the non-inducible strains in Figure 2.

572

573 **Figure 4.- Average ARG densities in the phage fraction of bacterial isolates.**
574 Average number of ARG copies (log_{10} gene copies/ml) in phage DNA from isolates
575 showing significant ($P<0.05$) (*) increase in the number of ARG copies after mitC
576 treatment versus uninduced controls in all ARGs except *sul1*. In the box plot, the cross-
577 pieces of each box represent (from top to bottom) the maximum, upper-quartile, median

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578 (black bar), lower-quartile and minimum values. A black diamond shows the mean
579 value. The colored boxes in the box plot represent samples showing values within the
580 75th percentile; white boxes represent samples showing values within the 25th
581 percentile.

582

583 **Figure 5.- Visualization of phage particles from the induced cultures carrying**
584 **ARGs**. (A) Example of the grey band corresponding to a density of 1.5 g/ml in a tube of
585 CsCl density gradients prepared with the induced fraction of the isolate Ec4. (B).
586 Electron micrographs of phage particles purified from the eight *E. coli* and *K.*
587 *pneumoniae* induced isolates. (C) The qPCR results of the ARGs present in the phage
588 particles purified from the CsCl density bands and visualized by electron microscopy in
589 (B). Bar: 100nm.

590

591 **Figure S1.- Distribution of the ARGs in the phage DNA fraction of feces in**
592 **different ages.** A) Results for ARGs in phage DNA are presented in a stacked column
593 chart that compares the percentage of positive samples among the total number of
594 samples analyzed for each segment. B) Distribution of the number of ARGs in phage
595 DNA (0-5 ARGs) detected in each individual in relation to age. Dotted line represents
596 the trend line.

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599 **Table 1.** Strains used in this study as controls for ARGs

Strain	ARG	Reference
C600	*bla*$_{TEM}$ in pGEM vector	Promega
E. coli	*bla*$_{CTX-M-1-}$group	[9]
E. coli	*bla*$_{CTX-M-9-}$group	[21]
S. aureus MRSA	*mecA*	[9]
E. coli strain 226	*qnrA*	[14]
E. cloacae strain 565	*qnrS*	[14]
E. coli	*armA* in plasmid pMUR050	[12]
K. pneumoniae	*bla*$_{OXA-48}$	This study
E. coli J53 R388	*sul1*	[20]

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603 **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		[43]
		LP	ATCCGCCTCCATCCAGTCTA			
	qPCR	UP	CACTATTCTCAGAATGACTTGGT	85	7.6	[43]
		LP	TGCATAATTCTCTTACTGTCATG			
		TaqMan TEM	FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ			
bla$_{CTX-M-1}$-group	PCR	UP	ACGTTAAACACCGCCATTCC	356		[9]
		LP	TCGGTGACGATTTTAGCCGC			
	qPCR	UP	ACCAACGATATCGCGGTGAT	101	8.4	[9]
		LP	ACATCGCGACGGCTTTCT			
		TaqMan CTX-M-1	FAM–TCGTGCGCCGCTG- MGBNFQ			
bla$_{CTX-M-9}$-group	PCR	UP	ACGCTGAATACCGCCATT	352		[21]
		LP	CGATGATTCTCGCCGCTG			
	qPCR	UP	ACCAATGATATTGCGGTGAT	85	13	[21]
		LP	CTGCGTTCTGTTGCGGCT			
		TaqMan 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			
		TaqMan OXA-48	FAM-CGTAGTTGTGCTCTGGA - MGBNFQ			
sul1	PCR	UP	TTCATGGGCAAAAGCTTGATG	965		[20]
		LP	GGCCGGAAGGTGAATGCTA			
	qPCR	UP	CCGTTGGCCTTCCTGTAAAG	67	5.9	[20]
		LP	TTGCCGATCGCGTGAAGT			

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		TaqMan sul1	FAM-CGAGCCTTGCGGCGG-MGBNFQ			
mecA	PCR	UP	GATAGCAGTTATATTTCTA	434		[9]
		LP	ATACTTAGTTCTTTAGCGAT			
	qPCR	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	10.4	[44]
		LP	TGGTCTTTCTGCATTCCTGGA			
		TaqMan mecA	FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-MGBNFQ			
qnrA	PCR	UP	ACGCCAGGATTTGAGTGAC	565		[14]
		LP	CCAGGCACAGATCTTGAC			
	qPCR	UP	AGGATTGCAGTTTCATTGAAAGC	138	8.6	[14]
		LP	TGAACTCTATGCCAAAGCAGTTG			
		TaqMan qnrA	FAM-TATGCCGATCTGCGCGA- MGBNFQ			
qnrS	PCR	UP	AAGTGATCTCACCTTCACCGCTTG	425		[14]
		LP	TTAAGTCTGACTCTTTCAGTGATG			
	qPCR	UP	CGACGTGCTAACTTGCGTGA	118	8.3	[14]
		LP	GGCATTGTTGGAAACTTGCA			
		TaqMan qnrS	FAM-AGTTCATTGAACAGGGTGA-MGBNFQ			
armA	qPCR	UP	GAAAGAGTCGCAACATTAAATGACTT	93	8.4	[12]
		LP	GATTGAAGCCACAACCAAAATCT			
		TaqMan armA	FAM-TCAAACATGTCTCATCTATT-MGBNFQ			
pGEM	PCR	pGEM7up	TGTAATACGACTCACTAT			Promega

604 **PCR**: conventional PCR. **qPCR**: quantitative real time PCR. **UP**: upper primer, **LP**: lower primer, **FAM**: 6-carboxyflouorescein reporter,

605 **MGBNFQ**: Minor groove binding non-fluorescent quencher, **LOQ.** Limit of quantification determined with the standard curve used in this study

606 for each qPCR assay.

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608 **Table3.** List of isolates from feces and determination of the presence of the different

609 ARGs

Nº of isolates	Specie	strain	ARGs present
9	*E. 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*$_{CTX-M-1}$-group
2	*E. coli*	Ec19, Ec21	*bla*$_{CTX-M-1}$-group, *sul1*
1	*E. coli*	Ec25	*bla*$_{CTX-M-1}$-group, *bla*$_{CTX-M-9}$-group
1	*E. coli*	Ec23	*bla*$_{CTX-M-1}$-group, *qnrA*, *qnrS*
1	*E. coli*	Ec24	*bla*$_{CTX-M-1}$-group, *armA*
1	*E. coli*	Ec22	*bla*$_{CTX-M-1}$-group, *qnrA, qnrS, sul1*
4	*E. coli*	Ec29-32	*bla*$_{CTX-M-9}$-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	*K. pneumoniae*	Kp1-3, Kp5-7	*bla*$_{TEM}$
1	*K. pneumoniae*	Kp4	*bla*$_{TEM}$, *sul1*
3	*K. pneumoniae*	Kp8-9, Kp11	*bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group, *sul1*
1	*K. pneumoniae*	Kp10	*bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group
1	*K. pneumoniae*	Kp12	*bla*$_{CTX-M-1}$-group
TOTAL	**82**		**24 *bla*$_{TEM}$, 16 *bla*$_{CTX-M-1}$-group, 5 *bla*$_{CTX-M-9}$-group, 1 *armA*, 11 *qnrA* , 11 *qnrS* , 56 *sul1***

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Figure 1

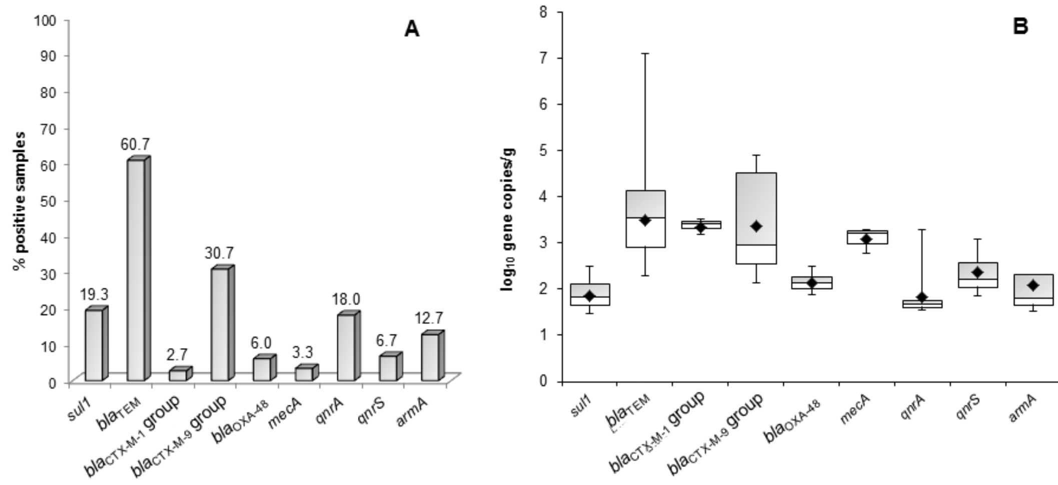

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Figure 2

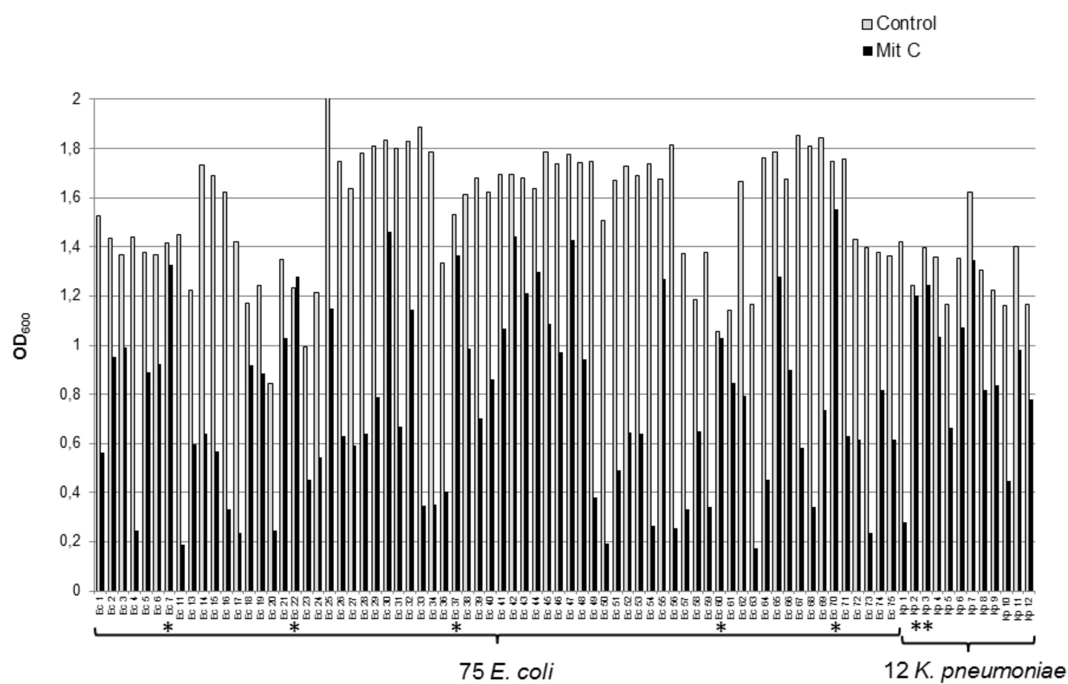

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Figure 3

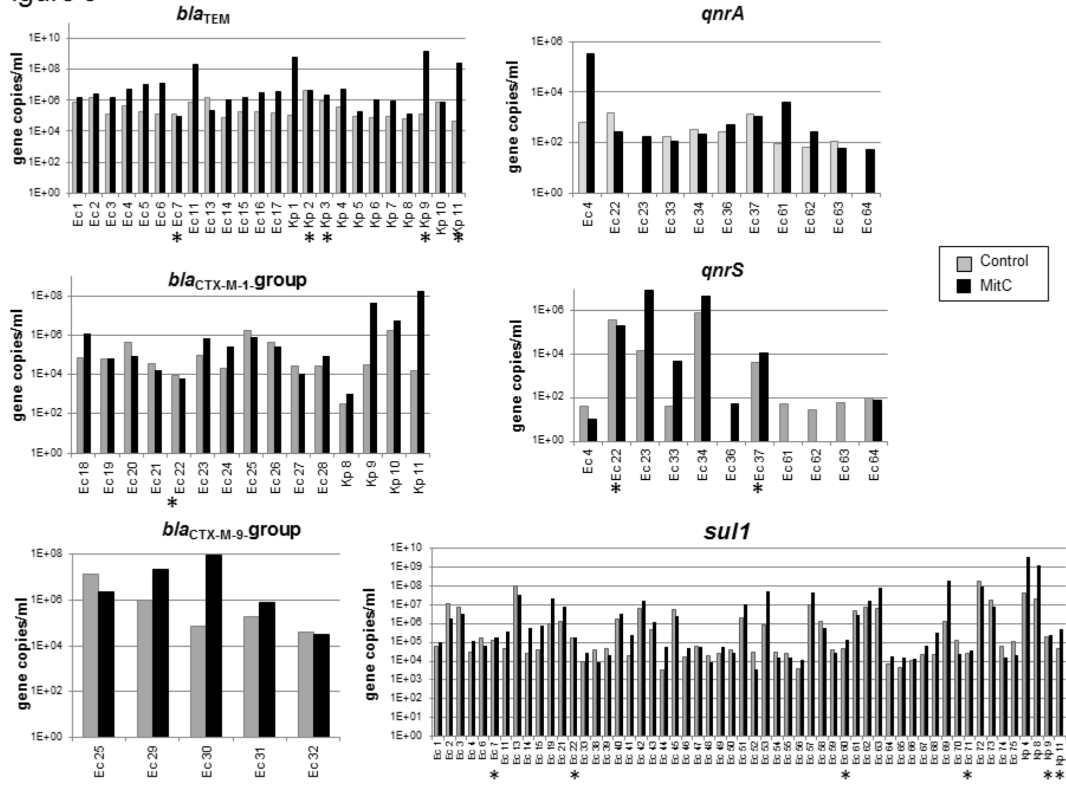

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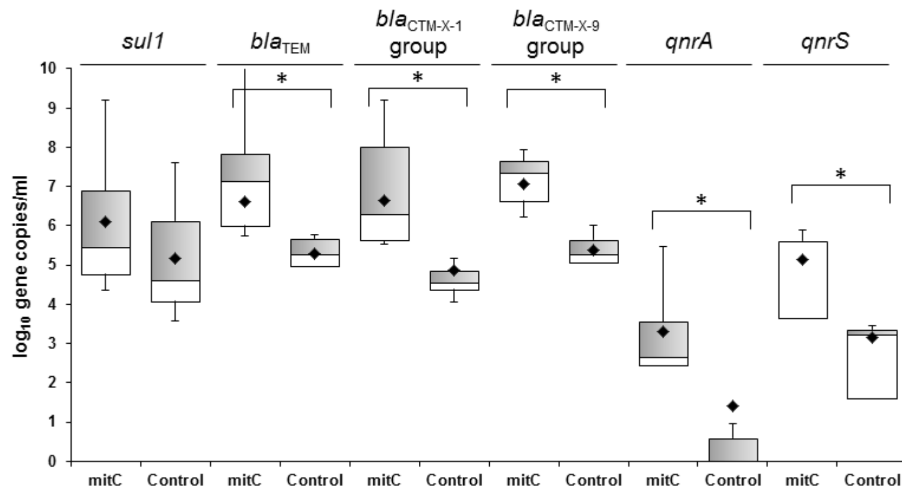
Figure 4



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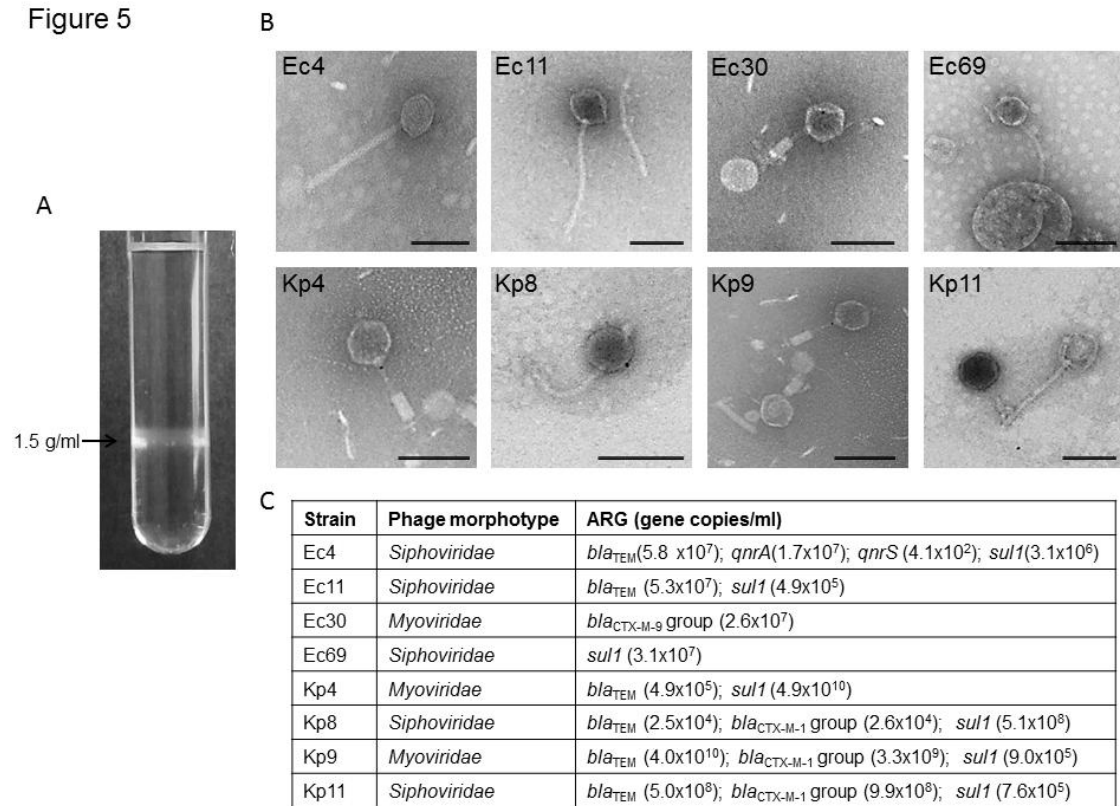

Strain	Phage morphotype	ARG (gene copies/ml)
Ec4	*Siphoviridae*	*bla_{TEM}*(5.8 x10^7); *qnrA*(1.7x10^7); *qnrS* (4.1x10^2); *sul1*(3.1x10^6)
Ec11	*Siphoviridae*	*bla_{TEM}* (5.3x10^7); *sul1* (4.9x10^5)
Ec30	*Myoviridae*	*$bla_{CTX-M-9}$* group (2.6x10^7)
Ec69	*Siphoviridae*	*sul1* (3.1x10^7)
Kp4	*Myoviridae*	*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)

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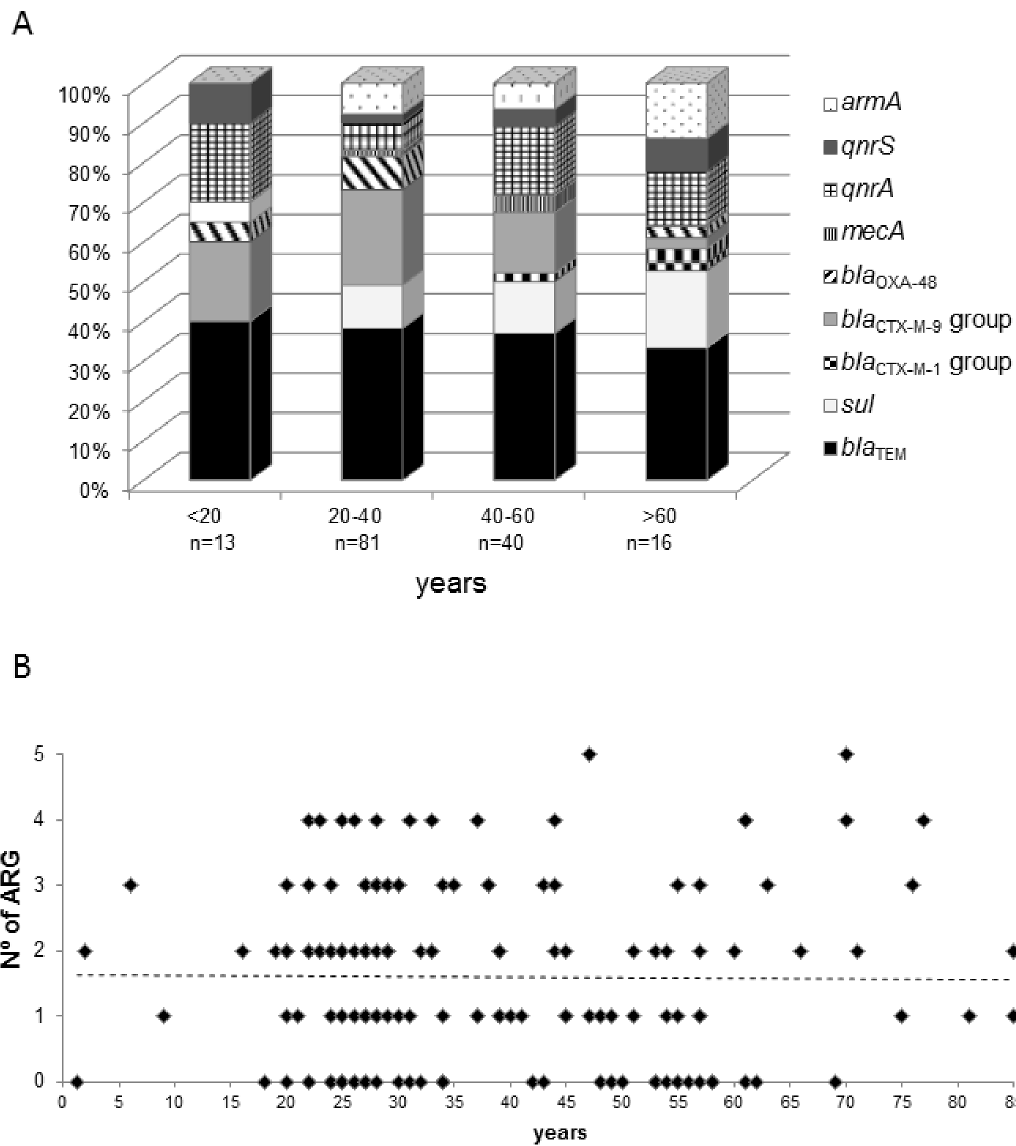

Figure S1

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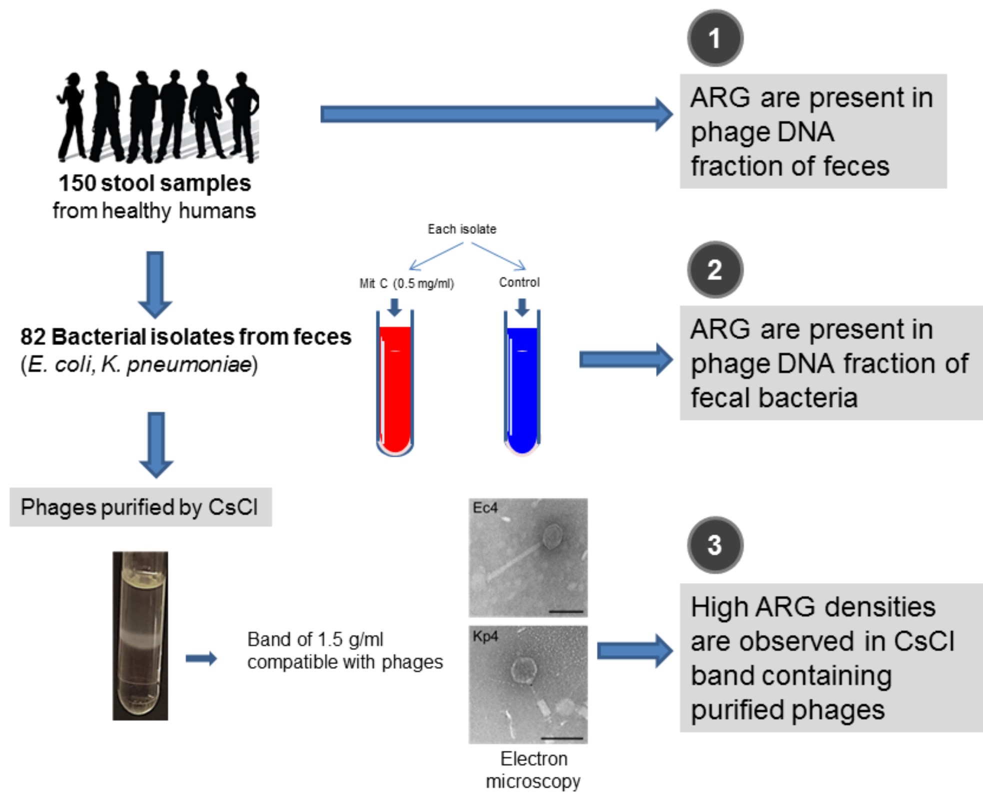

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