Antibiotic-resistance genes in the bacteriophage DNA fraction of human fecal samples

Pablo Quirós[1], Marta Colomer-Lluch[1], Alexandre Martínez-Castillo[1], Elisenda Miró[2], Marc Argente[2], Juan Jofre[1], Ferran Navarro[2] and Maite Muniesa[1]*

[1] Department of Microbiology, University of Barcelona, Diagonal 643, Annex, Floor 0, E-08028 Barcelona, Spain. [2] Servei de Microbiologia, Hospital de la Santa Creu i Sant Pau, Institut d'Investigació Biomèdica Sant Pau, Sant Quintí 89, 08041 Barcelona, Spain.

Running Head: antibiotic-resistance genes in phages from human feces

Key words: antibiotic resistance, bacteriophage, feces, human, horizontal genetic transfer

***corresponding author**

Maite Muniesa

Phone: (+34) 93 403 9386

Fax: (+34) 93 403 9047

e-mail: mmuniesa@ub.edu

Abstract

A group of antibiotic-resistance genes (ARGs) (*bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *mecA*, *armA, qnrA* and *qnrS*) were analyzed by real-time qPCR in bacteriophage DNA isolated from feces of 80 healthy humans. Seventy-seven percent of samples in phage DNA were positive for one or more ARGs. *bla*$_{TEM}$, *qnrA* and, *bla*$_{CTX-M-1}$ were the most abundant and *armA, qnrS* and *mecA* were less prevalent. Free bacteriophages carrying ARGs may contribute to the mobilization of ARGs in intra- and extra-intestinal environments.

Antibiotic resistance could be obtained by spontaneous mutations or acquired by the incorporation of antibiotic-resistance genes (ARGs) (1). ARGs spread between cells using genetic platforms known as mobile genetic elements (MGEs). The most commonly studied MGEs are plasmids, transposons, integrons and, more recently, bacteriophages (2).

Bacteriophages or phage related elements carry ARGs in Gram-positive (3-6) and in Gram-negative bacteria (7-10). Recently, some studies have suggested that the role of phages carrying ARGs in the environment is much more important than previously thought (2,11-13). Abundant ARGs have been reported in the bacteriophage DNA fraction of fecally-contaminated water (14-16) and metagenomic analyses indicate that there are abundant ARGs in viral DNA (17). As a result of their higher incidence in clinical settings, much effort has been devoted to the study of plasmids, integrons and transposons. However, there is little information on phages carrying ARGs in clinical settings.

This study analyzes a group of ARGs in phage DNA isolated from stool samples. The ARGs studied include two groups of beta-lactamase genes from Gram-negative bacteria (bla_{TEM} and $bla_{CTX\text{-}M\text{-}1\text{-}group}$); *mecA,* responsible for resistance to methicillin in *Staphylococcus* spp.; *armA,* a gene which confers high-level resistance to aminoglycosides in Gram-negative bacteria and *qnrA* and *qnrS,* plasmid-mediated genes that provide some degree of reduced quinolone susceptibility.

The study was performed on 80 human fecal samples from 46 females and 34 males aged from 6 months to 102 years who attended the Sant Pau Hospital (Barcelona) during a six months period. Stool samples were processed according to conventional protocols for isolation of enteropathogenic bacteria, rotavirus, adenovirus and were microscopically examined for protozoa. Only samples that were negative for these pathogens were

included in the study. None of the patients selected was involved in a food-borne outbreak or showed any severe gastrointestinal pathology. To our knowledge, none of the patients were following antibiotic treatment during the time of the study, although previous antibiotic treatments could not be excluded.

Fecal samples were homogenized 1:5 (*w:v*) in PBS by magnetic stirring for 15 minutes. Fifty-ml of the homogenate was centrifuged at 3,000*xg* and the phage lysate was purified and concentrated as described previously (15, 16). Phage suspensions were treated with DNase (100 U/ml) to eliminate free DNA outside the phage particles. To confirm total removal of non-encapsidated DNA, eubacterial 16S rDNA and the different ARGs (Table S1) were evaluated in the sample after DNase treatment and before its desencapsidation.

Phage DNA was extracted from the suspension as previously described (16, 18). Total DNA (including Gram-positive and Gram-negative bacterial and viral DNA) was extracted from 200 µl of the homogenate using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, USA), following the manufacturer's instructions.

Standard and qPCR procedures for bla_{TEM}, $bla_{\text{CTX-M-1-group}}$ and *mecA* were performed as previously described (16). *armA* qPCR assay was designed using the sequence of *armA* in plasmid pMUR050 (NC_007682.3) from an *E. coli* pig isolate (19). pMUR050 was also used to generate standard curves (16). The *armA* qPCR assay has an average efficiency of 98.4% and a detection limit of 2.74 gene copies (GC). The *qnrA* qPCR assay detects seven variants (*qnrA*1-7) and the *qnrS* qPCR assay detects six variants (*qnrS*1-6) (20). The 565-bp fragment of *qnrA* was obtained by from *E. coli*, strain 266 and the 425-bp fragment of *qnrS* from the environmental *Enterobacter cloacae* strain 565 isolated from sewage. Both fragments were cloned in pGEM®-T-Easy Vector (Promega, Barcelona,

Spain) to generate the standard curves (16). The *qnrA* qPCR assay showed 98.2% efficiency and a detection limit of 3.1 GC/μl and *qnrS* assay showed 99.4% efficiency and a detection limit of 8.3 GC/μl. All qPCR assays (Table S1) were performed under standard conditions (15, 16). To screen for PCR inhibition, dilutions of the standard of each gene were spiked with the DNA isolated from the samples, and the results were compared to the true number of GC of the target genes in the standards. No inhibition of the PCR by the samples was detected. Sequencing was performed as previously described (16).

When analyzing the ARGs in the total DNA, all samples were positive for one or more ARGs (Fig. 1A). When using purified phage DNA, 22.5% of the samples were negative for all ARGs and 77.5% harbored one or more ARGs. All the ARGs analyzed were present in our samples and the distribution of the ARGs in phage DNA was found to follow the same order of prevalence as in total DNA (Fig. 1A). No correlation was found between the patient's age or gender and the presence of ARGs in total or phage DNA.

When quantifying the different ARGs found per gram of fecal sample (Fig. 1B), the highest values were seen in total DNA with differences in $\log_{10}$ units between total and phage DNA ranging from 1.2 for *armA* to 2.8 for *mecA*. In phage DNA, bla_{TEM} showed the highest prevalence and abundance with values as high as 6.8 $\log_{10}$GC/g. The second most prevalent gene was *qnrA.* $bla_{CTX\text{-}M\text{-}group1}$ was the third most prevalent gene, although the densities were lower than the two previous ARGs. *armA*, showed a low prevalence of only five positive samples, but remarkably high densities (up to 6 $\log_{10}$ units). There were a small number of positive samples for *qnrS* and *mecA* in phage DNA, which did not allow us to draw conclusions regarding their abundance.

111 The prevalence of the genes in this study corroborates the descriptions found in the
112 literature. *bla*$_{TEM}$ is probably the most prevalent ARG worldwide (21, 22) and in phage DNA
113 in wastewater (15, 16). *bla*$_{CTX\text{-}M\text{-}1\text{-}group}$, includes the *bla*$_{CTX\text{-}M\text{-}15}$, which over the past decade
114 has become one of the most prevalent extended-spectrum beta-lactamases (23). The
115 horizontally transferable *qnrA* and *qnrS* genes (24, 25) are widely distributed in our region
116 and clinically relevant (20), particularly *qnrA* that was the first quinolone-resistance gene
117 described and it is the most commonly found (26, 27). *armA* is also highly prevalent in
118 *Enterobacteriaceae* and it is spreading worldwide (19, 28, 29). *mecA* was not prevalent in
119 this study, may be because *Staphylococcus* spp. is not commonly found in the intestinal
120 tract. The previous detection of *mecA* in phages from sewage (16) could be attributable to
121 non-fecal origin.
122
123 The qPCR assays produce a short amplicon, and to better confirm the identity of the ARGs
124 detected in phage DNA by sequencing, we amplified longer fragments by conventional
125 PCR. Sequences were performed with forward and reverse primers and by duplicate. The
126 consensus of all sequences generated fragments of different size of *bla*$_{TEM,}$ *bla*$_{CTX\text{-}M\text{-}group1}$,
127 *qnrA* and *qnrS* that matched 100% previously described sequences of the corresponding
128 ARGs from different bacterial genera available in GenBank database
129 (http://www.ncbi.nlm.nih.gov/genbank/index.html) (Table 1). *ArmA* was not amplified by
130 conventional PCR in the few samples that showed positive for this gene. The specific
131 variant of the ARG sequenced was not determined because of the length and location of
132 some of these fragments and because the limited amount of DNA obtained from the
133 samples did not allow the amplification of the complete ARGs.
134

The high prevalence of ARGs in phage DNA isolated from fecally polluted environments (14-16) indicates that phages could play a role in the mobilization of ARGs. The question we address here is whether the origin of these phages could be free phage particles excreted in feces, free phages present in those environments or phages induced from bacteria (allochthonous or autochthonous) occurring in those environments. The results of the present study clearly indicate that free phages encoding ARGs are directly excreted from healthy individuals via feces. The phage particles could be infectious or not to a given host, but as previously shown, the genes harbored by the phages are functional and able to confer resistance to a given antibiotic (16). This would make it likely that a phage harboring ARGs infects a new host and transfers the ARG that could be incorporated into the host genome by recombination.

The significant prevalence of phages in human feces has been shown by recent metagenomics studies (30-32). Among these, many remark on the high number of sequences of ARGs in the virome fraction of the human gut (17, 33). A recent report indicates that the number of ARGs in the “phageome” is significant and that the ARG content in the phage DNA fraction of the gut microbiome increases after antibiotic treatment (34). Specific phages could carry ARGs of Gram-positive (4-6) and Gram-negative bacteria (7-10). Although these reports do not indicate the nature of the phage particles, some authors suggest they could have been generated by means of generalized transducing phages that can mobilize chromosomal genes and plasmids (4, 8, 35).

As phages harboring ARGs are excreted in human feces from healthy individuals (or animals) (14-16), there must be many of these phages circulating in the population. These phages probably exist in some food and water, but they will not normally be detected by regular quality controls. They could be ingested as free particles and cause conversion of

susceptible hosts within the gut that could be later selected by the presence of antimicrobial agents. At present, phages seem to be suitable vehicles for the mobilization and transmission of ARGs, and probably many other genes, in both intra- and extra-intestinal environments.

Acknowledgements

Authors thank B. González-Zorn for the construct containing the *armA* gene used for the qPCR standards. This study was partially supported by the Spanish Ministry of Education and Science (AGL2012-30880), the Generalitat de Catalunya (2009SGR1043), by a project of the RecerCaixa program (La Caixa), by the Fundación Ramon Areces and, was partially supported by Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008).Marta Colomer-Lluch has a grant FI from the Generalitat de Catalunya,

References

1. **Lupo A, Coyne S, Berendonk TU**. 2012. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Front. Microbiol. **3**:18.

2. **Zhang XX, Zhang T, Fang HH.** 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. **82**:397-414.

3. **Ubukata K, Konno M, Fujii R**. 1975. Transduction of drug resistance to tetracycline, chloramphenicol, macrolides, lincomycin and clindamycin with phages induced from *Streptococcus pyogenes*. J. Antibiot. (Tokyo). **28:**681-688.

4. **Schuch R, Fischetti VA**. 2006. Detailed genomic analysis of the beta and gamma phages infecting *Bacillus anthracis*: implications for evolution of environmental fitness and antibiotic resistance. J.Bacteriol. **188:**3037-3051.

187 5. **Mazaheri Nezhad Fard R, Barton MD, Heuzenroeder MW.** 2011. Bacteriophage-
188 mediated transduction of antibiotic resistance in Enterococci. Lett. Appl. Microbiol. **52:**559-
189 564.
190 6. **Varga M, Kuntová L, Pantůček R, Mašlaňová I, Růžičková V, Doškař J.** 2012.
191 Efficient transfer of antibiotic resistance plasmids by transduction within methicillin-
192 resistant *Staphylococcus aureus* USA300 clone. FEMS Microbiol. Lett. **332:**146-152.
193 7. **Blahová J, Hupková M, Krcméry V, *Schäfer V.*** 1992. Imipenem and cefotaxime
194 resistance: transduction by wild-type phages in hospital strains of *Pseudomonas*
195 *aeruginosa*. J. Chemother. **4:**335-337.
196 8. **Willi K, Sandmeier H, Kulik EM, Meyer J.** 1997. Transduction of antibiotic resistance
197 markers among *Actinobacillus actinomycetemcomitans* strains by temperate
198 bacteriophages Aa phi 23. Cell. Mol. Life Sci. **53:**904-910.
199 9. **Schmieger H, Schicklmaier P**. 1999. Transduction of multiple drug resistance of
200 *Salmonella enterica* serovar typhimurium DT104. FEMS Microbiol.Lett. **170:**251-256.
201 10. **Oliver A, Coque TM, Alonso D, Valverde A, Baquero F, Cantón R.** CTX-M-10
202 linked to a phage-related element is widely disseminated among *Enterobacteriaceae* in a
203 Spanish hospital. Antimicrob. Agents Chemother. 49(4):1567-1571.
204 11. **American Society of Microbiology**. 2009. Antibiotic Resistance: An Ecological
205 Perspective on an Old Problem. Washington, DC, USA.
206 12. **Cangelosi GA, Freitag NE, Buckley MR**. 2004. From outside to Inside.
207 Environmental Microorganisms as Human Pathogens. Washington, DC: American
208 Academy of Microbiology Report. American Society of Microbiology, USA.
209 13. **Brabban AD, Hite E, Callaway TR**. 2005. Evolution of foodborne pathogens via
210 temperate bacteriophage-mediated gene transfer. Foodborne Pathog. Dis. **2**: 287-303.
211 14. **Muniesa M, García A, Miró E, Mirelis B, Prats G, Jofre J, Navarro F.** 2004.
212 Bacteriophages and diffusion of beta-lactamase genes. Emerg. Infect. Dis. **10**:1134-1137.

15. **Colomer-Lluch M, Imamovic L, Jofre J, Muniesa M.** 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. Antimicrob. Agents Chemother. **55**: 4908-4911.

16. **Colomer-Lluch M, Jofre J, Muniesa M.** 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS One.* **6**:e17549.

17. **Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, Wu GD, Lewis JD, Bushman FD**. 2011.The human gut virome: inter-individual variation and dynamic response to diet. Genome Res. **21:**1616-1625.

18. **Sambrook J, Russell DW**. 2001. Molecular cloning: a laboratory manual, 3rd ed. New York: Cold Spring Harbor Laboratory, USA.

19. **González-Zorn B, Catalan A, Escudero JA, Domínguez L, Teshager T, Porrero C, Moreno MA.** 2005. Genetic basis for dissemination of *armA*. J. Antimicrob. Chemother. **56:** 583-585.

20. **Lavilla S, González-López JJ, Sabaté M,García-Fernández A, Larrosa MN, Bartolomé RM, Carattoli A, Prats G.** 2008. Prevalence of *qnr* genes among extended-spectrum beta-lactamase-producing enterobacterial isolates in Barcelona, Spain. J. Antimicrob. Chemother. **61:** 291-295.

21. **Rodríguez-Baño J, Alcalá JC, Cisneros JM, Grill F, Oliver A, Horcajada JP, Tórtola T, Mirelis B, Navarro G, Cuenca M, Esteve M, Peña C, Llanos AC, Cantón R, Pascual A.** 2008. Community infections caused by extended-spectrum β-lactamase-producing *Escherichia coli*. Arch. Intern. Med. **168**:1897-1902.

22. **Díaz MA, Hernández-Bello JR, Rodríguez-Baño J, Martínez-Martínez L, Calvo J, Blanco J, Pascual A; Spanish Group for Nosocomial Infections (GEIH).** 2010. The Diversity of *Escherichia coli* Producing Extended-Spectrum β-lactamases in Spain: Second Nationwide Study. J. Clin. Microbiol. **48**:2840-2845.

238 23. **Cantón R, Coque TM.** 2006. The CTX-M beta-lactamase pandemic. Curr. Opin.
239 Microbiol. **9:**466-475.
240 24. **Rodríguez-Martínez JM, Cano ME, Velasco C, Martínez-Martínez L, Pascual A.**
241 2011.Plasmid-mediated quinolone resistance: an update. J. Infect. Chemoter. **17**:149-182.
242 25. **Robicsek A, Jacoby GA, Hooper DC.** 2006. The worldwide emergence of plasmid-
243 mediated quinolone resistance. Lancet Infect. Dis. **6**:629-640.
244 26. **Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, Hooper DC.** 2006. *qnr*
245 prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States.
246 Antimicrob. Agents Chemother. **50:**2872-2874.
247 27. **Nordmann P, Poirel L.** 2005. Emergence of plasmid-mediated resistance to
248 quinolones in *Enterobacteriaceae*. J. Antimicrob. Chemother. **56:**463-469.
249 28. **Galimand M, Sabtcheva S, Courvalin P, Lambert T.** 2005. Worldwide disseminated
250 *armA* aminoglycoside resistance methylase gene is borne by composite transposon
251 Tn1548. Antimicrob. Agents Chemother. **49:**2949-2953.
252 29. **Granier SA, Hidalgo L, San Millan AEscudero JA, Gutierrez B, Brisabois A,**
253 **Gonzalez-Zorn B.** 2011. ArmA methyltransferase in a monophasic *Salmonella enterica*
254 isolate from food. Antimicrob. Agents Chemother. **55:**5262-5266.
255 30. **Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P, Rohwer F**.
256 2003. Metagenomic analyses of an uncultured viral community from human feces. J.
257 Bacteriol. **185:** 6220-6223.
258 31. **Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI.** 2010.
259 Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature. **466:**334-
260 338.
261 32. **Stern A, Mick E, Tirosh I, Sagy O, Sorek R**. 2012. CRISPR targeting reveals a
262 reservoir of common phages associated with the human gut microbiome. Genome Res.
263 **22:**1985-1994.

264 33. **Wang X, Kim Y, Ma Q, Hong SH, Pokusaeva K, Sturino JM, Wood TK.** 2010.
265 Cryptic prophages help bacteria cope with adverse environments. Nat. Commun **1**:147.
266 34. **Modi SR, Lee HH, Spina CS, Collins JJ.** 2013. Antibiotic treatment expands the
267 resistance reservoir and ecological network of the phage metagenome. 2013. Nature.
268 **499**:219-22.
269 35. **Schicklmaier P, Moser E, Wieland T, Rabsch W, Schmieger H.** 1998. A
270 comparative study on the frequency of prophages among natural isolates of *Salmonella*
271 and *Escherichia coli* with emphasis on generalized transducers. Antonie Van
272 Leeuwenhoek. **73:**49-54.
273
274

Table 1- Sequence homology of some of the ARGs amplified from phage DNA of fecal samples. The fragment size corresponds to the length of the consensus sequence generated with the forward and reverse sequences of the PCR amplimer, performed in duplicate, and is the fragment used to search for homologies. No PCR amplimer was obtained for *ArmA* gene with the samples that showed positive for this ARG.

Fragment size (bp)	ARG assay	First sequence homologue	Genbank Code
574	*bla*$_{TEM}$	*bla*$_{TEM-1}$	GQ470444.1
571	*bla*$_{TEM}$	*bla*$_{TEM-1}$	JN002397.1
			AY832935.1
580	*bla*$_{TEM}$	*bla*$_{TEM-116}$	NZ_ADUR01000022
			NZ_ADFT01000030
			AY265885.1
			JF327796.1
576	*bla*$_{TEM}$	*bla*$_{TEM-1}$	JN002397.1
			AY832935.1
386	*bla*$_{CTX-M}$	*bla*$_{CTX-M-15}$	JX129219.1
		bla$_{CTX-M-15-like}$	KC107824.1
380	*bla*$_{CTX-M}$	*bla*$_{CTX-M-33}$	AY238472.1
		bla$_{CTX-M-15-like}$	KC107824.1
		bla$_{CTX-M-114}$	GQ351346.1
360	*bla*$_{CTX-M}$	*bla*$_{CTX-M-15}$	NC_013122.1
359		*bla*$_{CTX-M-33}$	AY238472.1
		bla$_{CTX-M-15}$	JX129219.1
359	*bla*$_{CTX-M}$	*bla*$_{CTX-M-109}$	JF274248.1
		bla$_{CTX-M-33}$	AY238472.1
		bla$_{CTX-M-15}$	EF158301.1
355	*bla*$_{CTX-M}$	*bla*$_{CTX-M-15}$	EU979556.1
			HQ256746.1
		bla$_{CTX-M-15-like}$	JX268658.1
412	*mec*A	*mec*A	KC243783.1
437			JQ764731.1
466			HE978800.1
436			HE978798.1
			GU301100.1
			GU301101.1
452	*qnrA*	*qnrA1*	JN687470.1
			JF728153.1
			JF969163.1
			HQ184955.1
			GU324551.1
456	*qnrA*	*qnrA1*	JN687470.1
			JF728153.1
			JF969163.1
			GQ891753.1
			GU295955.1
352	*qnrS*	*qnrS2*	HE616910.2
			JN315883.1
			JF773350.1
			DQ485530.1

Figure legend

Figure 1- A: Proportion of each ARG studied among 80 samples in total and phage DNA. In total DNA, values were expressed for 80 positive samples. In total DNA, all samples were positive for at least one ARG, while in phage DNA 18 samples were negative for all ARGs (Neg). B: Box plot chart with the averaged values obtained from all ARGs in positive samples for total and phage DNA. Within the box plot chart, the cross-pieces of each box plot represent (from top to bottom) maximum, upper-quartile, median (black bar), lower-quartile and minimum values. The black diamond shows the mean value. The grey boxes in the box plot chart include samples showing values within the 75^{th} percentile and white box samples show values within the 25^{th} percentile. N indicates the number of positive samples for each ARG.