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Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Human Fecal Samples

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A group of antibiotic resistance genes (ARGs) (*bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *mecA*, *armA*, *qnrA*, and *qnrS*) were analyzed by real-time quantitative PCR (qPCR) in bacteriophage DNA isolated from feces from 80 healthy humans. Seventy-seven percent of the samples were positive in phage DNA 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 extraintestinal environments.

Antibiotic resistance may be obtained by spontaneous mutations or acquired by the incorporation of antibiotic resistance genes (ARGs) (1). ARGs spread between cells by 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 Gram-negative (7–10) bacteria. 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*$_{CTX-M-1\ group}$); *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 using 80 human fecal samples from 46 females and 34 males from 6 months to 102 years of age who visited the Sant Pau Hospital (Barcelona, Spain) during a 6-month period. Stool samples were processed according to conventional protocols for the isolation of enteropathogenic bacteria, rotavirus, and 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 receiving antibiotic treatment during the time of the study, although previous antibiotic treatments could not be excluded.

Fecal samples were homogenized to a 1:5 (wt/vol) dilution in phosphate-buffered saline (PBS) by magnetic stirring for 15 min. Fifty milliliters of the homogenate was centrifuged at 3,000 ×g, 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 nonencapsidated DNA, eubacterial 16S rRNA genes and the different ARGs (see Table S1 in the supplemental material) were evaluated in the sample after DNase treatment and before its disencapsidation.

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 by use of a QIAamp DNA stool minikit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's instructions.

Standard and quantitative PCR (qPCR) procedures for *bla*$_{TEM}$, *bla*$_{CTX-M-1\ group}$, and *mecA* were performed as previously described (16). The *armA* qPCR assay was designed using the sequence of *armA* in plasmid pMUR050 (NC_007682.3) from an *Escherichia 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 to 7), and the *qnrS* qPCR assay detects six variants (*qnrS* 1 to 6) (20). The 565-bp fragment of *qnrA* was obtained from *E. coli* strain 266, and the 425-bp fragment of *qnrS* was obtained from the environmental strain *Enterobacter cloacae* 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 the *qnrS* assay showed 99.4% efficiency and a detection limit of 8.3 GC/μl. All qPCR assays (see Table S1 in the supplemental material) were performed under standard conditions (15, 16). To

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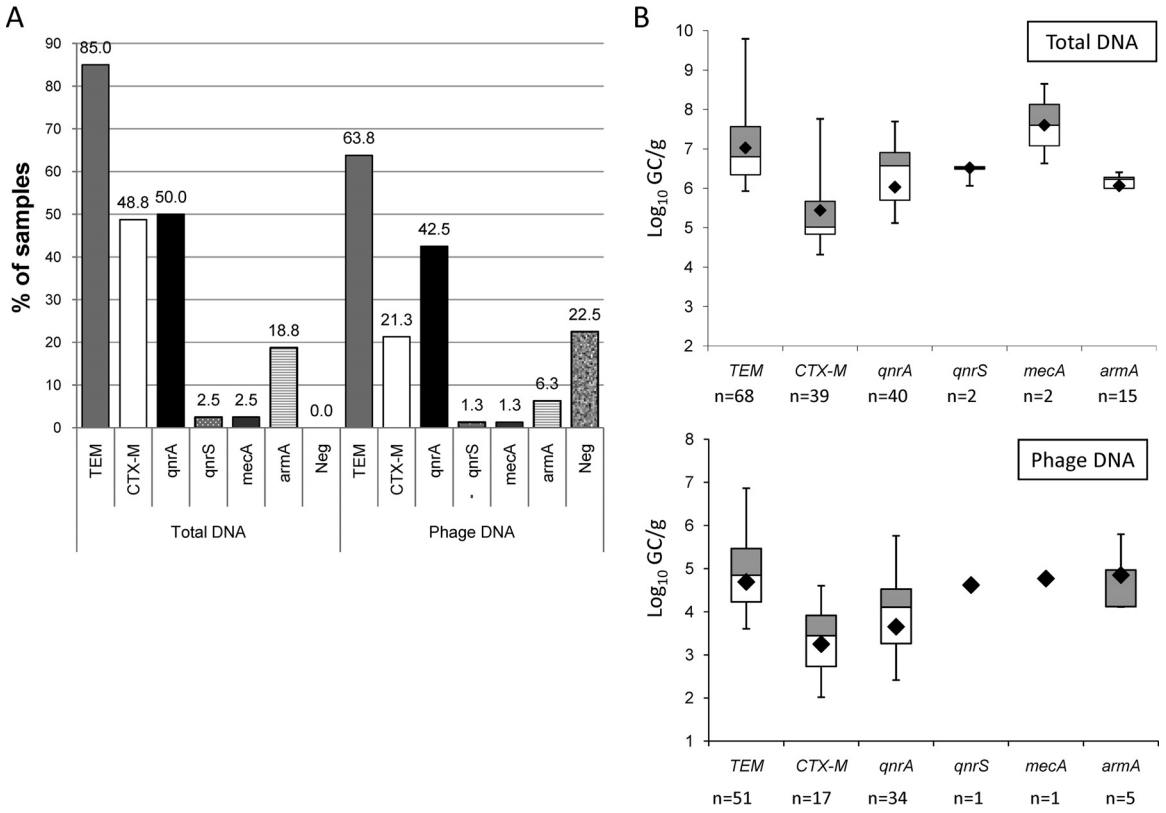

FIG 1 (A) Proportion of each ARG studied among 80 samples in total and phage DNA. In total DNA, values are expressed for 80 positive samples. In total DNA, all samples were positive for at least one ARG, while in phage DNA, 18 samples were negative for all ARGs (Neg). (B) Box plot chart with the averaged 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 (horizontal black bar), lower quartile, and minimum values. A black diamond indicates the mean value. The gray boxes in the box plot chart include samples showing values within the 75th percentile, and white boxes include samples showing values within the 25th percentile. n, number of positive samples for each ARG.

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 the ARGs in the total DNA were analyzed, all samples were positive for one or more ARGs (Fig. 1A). When purified phage DNA was used, 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 the different ARGs found were quantified 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*. $bla_{\mathrm{CTX\text{-}M\ group1}}$ was the third most prevalent gene, although the densities were lower than those of 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 samples positive for *qnrS* and *mecA* in phage DNA, which did not allow us to draw conclusions regarding their abundance.

The prevalence of the genes in this study corroborates the descriptions found in the literature. bla_{TEM} is probably the most prevalent ARG worldwide (21, 22) and in phage DNA in wastewater (15, 16). $bla_{\mathrm{CTX\text{-}M\text{-}1\ group}}$ includes $bla_{\mathrm{CTX\text{-}M\text{-}15}}$, which over the past decade has become one of the most prevalent extended-spectrum beta-lactamase genes (23). The horizontally transferable *qnrA* and *qnrS* genes (24, 25) are widely distributed in our region and clinically relevant (20), particularly *qnrA*, which was the first quinolone resistance gene described and the most commonly found (26, 27). *armA* is also highly prevalent in *Enterobacteriaceae*, and it is spreading worldwide (19, 28, 29). *mecA* was not prevalent in this study, perhaps because *Staphylococcus* spp. are not commonly found in the intestinal tract. The previous detection of *mecA* in phages from sewage (16) may be attributable to a nonfecal origin.

The qPCR assays produce a short amplicon, and to better confirm the identity of the ARGs detected in phage DNA by sequencing, we amplified longer fragments by conventional PCR. Sequencing was performed with forward and reverse primers and in duplicate. The consensus of all sequences generated fragments of different sizes for bla_{TEM}, $bla_{\mathrm{CTX\text{-}M\ group1}}$, *qnrA*, and *qnrS* that matched 100% previously described sequences of the corresponding ARGs from different bacterial genera available in the GenBank

TABLE 1 Sequence homology of some of the ARGs amplified from phage DNA of fecal samples[a]

Fragment size (bp)	ARG assay result	First sequence homologue	GenBank accession no.
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	*mecA*	*mecA*	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

[a] 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 the *armA* gene with the samples that showed positive for this ARG.

database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) (Table 1). *armA* was not amplified by conventional PCR in the few samples that tested positive for this gene. The specific variant of the ARG sequenced was not determined because of the length and location of some of these fragments and because the limited amount of DNA obtained from the samples did not allow the amplification of the complete ARGs.

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 (7–10) bacteria. Although these reports do not indicate the nature of the phage particles, some authors suggest that 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 extraintestinal environments.

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