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Bacteriophages Carrying Antibiotic Resistance Genes in Fecal Waste from Cattle, Pigs, and Poultry[▽]

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This study evaluates the occurrence of bacteriophages carrying antibiotic resistance genes in animal environments. bla_{TEM}, $bla_{\text{CTX-M}}$ (clusters 1 and 9), and $mecA$ were quantified by quantitative PCR in 71 phage DNA samples from pigs, poultry, and cattle fecal wastes. Densities of 3 to 4 $\log_{10}$ gene copies (GC) of bla_{TEM}, 2 to 3 $\log_{10}$ GC of $bla_{\text{CTX-M}}$, and 1 to 3 $\log_{10}$ GC of $mecA$ per milliliter or gram of sample were detected, suggesting that bacteriophages can be environmental vectors for the horizontal transfer of antibiotic resistance genes.

Antibiotics are widely used to protect human health and to increase the growth rate of animals in livestock husbandry. The use and abuse of antibiotics in humans and animals have exerted selective pressure on bacterial communities, resulting in the emergence of resistances (1, 22). There are concerns about the potential impact of antibiotic residues in the aquatic environment, where many antibiotics are discharged (26, 36).

In addition to the antibiotics synthesized for therapy, many antibiotics are produced by environmental microorganisms (15, 34). These organisms host antibiotic resistance genes (ARGs) that protect them from the antibiotics they produce (16). Environmental bacteria that do not produce antibiotics themselves also carry ARGs conserved as a consequence of the selective pressure of antibiotics in certain environments (26, 36) or used for different purposes (14). Therefore, when bacteria reach clinical settings, these ARGs from environmental origins are challenged with high concentrations of antibiotics and antibacterial resistance evolves and emerges under the strong selective pressure that occurs during the treatment of infections (26).

Many ARGs are acquired by bacteria through conjugative transfer by mobile elements (plasmids or integrative and conjugative elements), by transformation by naked DNA, or by transduction by bacteriophages (36). Compared with other genetic vectors, less is known about the contribution of phages to antibiotic resistance transfer. Recent reports (1, 7, 10) suggest that the horizontal transfer of ARGs by phages is much more widespread than previously believed and that the environment plays a crucial role in it (7).

This study was focused on bla_{TEM} and $bla_{\text{CTX-M}}$, which encode β-lactamases that are widespread among Gram-negative pathogens (11), and $mecA$, which encodes penicillin-binding protein 2a (PBP2a), associated with methicillin resistance in staphylococci (32). Quantification of these genes by quantitative PCR (qPCR) was done in the viral DNA fraction of animal fecal wastes. Assuming that phages are the major part of the viral fraction in most environments (17), we sought to highlight the potential role of phages in the spread of ARGs in animal settings.

This study was conducted with archived fecal wastes collected from several slaughterhouses and farms in Spain. We analyzed 8 cattle slurries, 9 wastewater samples from abattoirs slaughtering pigs and 16 from poultry slaughter, 10 wastewater samples containing mixed fecal wastes of poultry, ducks, rabbits, and domestic dogs and cats, and 28 fecal samples aseptically collected from cowpats in summer pastures in the Pyrenees mountains. The fecal contamination in the samples was established by enumerating fecal coliforms and *Escherichia coli* (9). Somatic coliphages, proposed as fecal viral indicators, allowed evaluation of the levels of bacteriophages (2, 5) The samples showed levels of bacterial and viral indicators that were relatively homogeneous (Table 1) and similar to those previously reported (5, 23).

Phage DNA was purified from the samples as described previously (10, 23). Samples were treated with DNase (100 units/ml) to rule out the possibility of nonphage DNA contamination. For this, an aliquot taken after DNase treatment and before disencapsidation was evaluated using conventional PCR of eubacterial 16S ribosomal DNA (rDNA) (Table 2) and using qPCR.

Conventional PCRs for ARGs were performed as described previously (10) (Table 2), using environmental *E. coli* strains and a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) as controls for bla_{TEM}, $bla_{\text{CTX-M}}$, and $mecA$, respectively. TaqMan qPCR assays (Table 2) were used for quantification of ARGs using standards as previously described (10).

A real-time qPCR oligonucleotide set for $bla_{\text{CTX-M}}$ cluster 1 (10) and a new set for $bla_{\text{CTX-M}}$ cluster 9, developed in this study, were used to detect CTX-M in phage DNA isolated from animal wastes. The new qPCR oligonucleotides for $bla_{\text{CTX-M}}$ cluster 9 detect the most abundant variants of the cluster (CTX-M-9, 13, 14, 16 to 19, 21, and 27) (6) and showed a detection limit of 13 gene copies (GC) (threshold cycle of 31).

$bla_{\text{CTX-M}}$ clusters 1 and 9 were detected in phage DNA (Fig. 1A and B) without significant differences ($P > 0.05$, analysis of variance [ANOVA]) between the occurrence of the clusters. The densities of cluster 1 were slightly higher in swine samples,

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TABLE 1. Fecal coliforms and *E. coli* as bacterial indicators and somatic coliphages as viral indicators detected in animal waste and fecal samples

Sample type	No. of samples	No. [CFU/ml or CFU/g (SD)] of:		No. [PFU/ml or PFU/g (SD)] of somatic coliphages
		Fecal coliforms	*E. coli*	
Cattle slurry	8	1.7×10^5 (1.5×10^5)	3.3×10^4 (8.2×10^3)	1.1×10^4 (1.4×10^4)
Pig wastewater	9	1.4×10^6 (1.4×10^6)	4.6×10^5 (1.1×10^5)	7.6×10^5 (1.1×10^6)
Poultry wastewater	16	9.4×10^5 (3.6×10^5)	7.8×10^4 (2.2×10^4)	1.8×10^4 (1.8×10^4)
Mixed slurry[a]	10	3.2×10^5 (1.0×10^6)	2.2×10^4 (6.5×10^4)	8.5×10^3 (1.2×10^4)
Cowpats	28	7.9×10^3 (3.2×10^3)	3.9×10^3 (7.2×10^3)	4.0×10^3 (4.2×10^3)

[a] Slurries were from a farm with fecal loads from diverse animal origins.

while poultry samples showed a significantly ($P < 0.05$) higher prevalence of cluster 9. CTX-M-1 was previously detected in phage DNA from municipal sewage of the same area (10), although at lower densities than in animal wastes. CTX-M is currently the most prevalent β-lactamase family in many countries (11). CTX-M cluster 1 is the most prevalent in pig isolates in Spain (13), and within this cluster, CTX-M-15 is the most widely distributed (12, 27). CTX-M cluster 9 is the most prevalent in poultry in Spain (13, 27), and within it, CTX-M-9 and CTX-M-14 are the most frequent in animal isolates (11). Both CTX-M-15 and CTX-M-9 have been linked to *E. coli* O25b: H4, a serious human pathogen worldwide (12).

The qPCR oligonucleotide set for *bla*$_{TEM}$ (Table 2) (24) showed positive results in all samples and higher densities ($P < 0.05$) than were found for the other ARGs (Fig. 1C). Poultry waste carried the highest number of copies, and pig waste the lowest. *bla*$_{TEM}$ is the most prevalent β-lactamase in *E. coli* isolates from food of animal origin and from healthy livestock (8). This prevalence is consistent with the high densities of *bla*$_{TEM}$ detected in phage DNA in this study and in municipal sewage (10).

The qPCR for *mecA* (33) showed lower densities than qPCR for the other ARGs (Fig. 1D) and registered more negative samples. This was expected because this qPCR oligonucleotide set detects a specific gene instead of a family (TEM or CTX-M) spread among numerous bacterial genera. Swine and poultry showed a significantly ($P < 0.05$) higher prevalence than the other sources (Fig. 1D). *mecA* has been detected in isolates from domestic animals (20, 21, 25, 31) and in phage DNA from municipal sewage (10). The presence of *mecA* in animals has been associated with antimicrobial usage, contact with humans, and farm hygiene. Transmission is from humans to domestic animals (31) or from animals to farmers (20).

Twenty-four amplicons of the ARGs, selected according to the highest GC densities, were generated by conventional PCR and sequenced (23). All amplicons were confirmed as to their identity, although for some β-lactamase genes, discrimination between allelic variants was not possible, since the sequences were partial.

Indirect evidence suggests that selective pressures have mobilized ARGs from their initial chromosomal location in bacteria (4, 32). Phages persist better in aquatic environments than their bacterial hosts (3, 18) and, due to their structural characteristics, better than free DNA (37). This higher survival and the abundance of phages carrying ARGs in animal and human wastewater (10, 28) support the notion that phages are

TABLE 2. Oligonucleotides used in this study

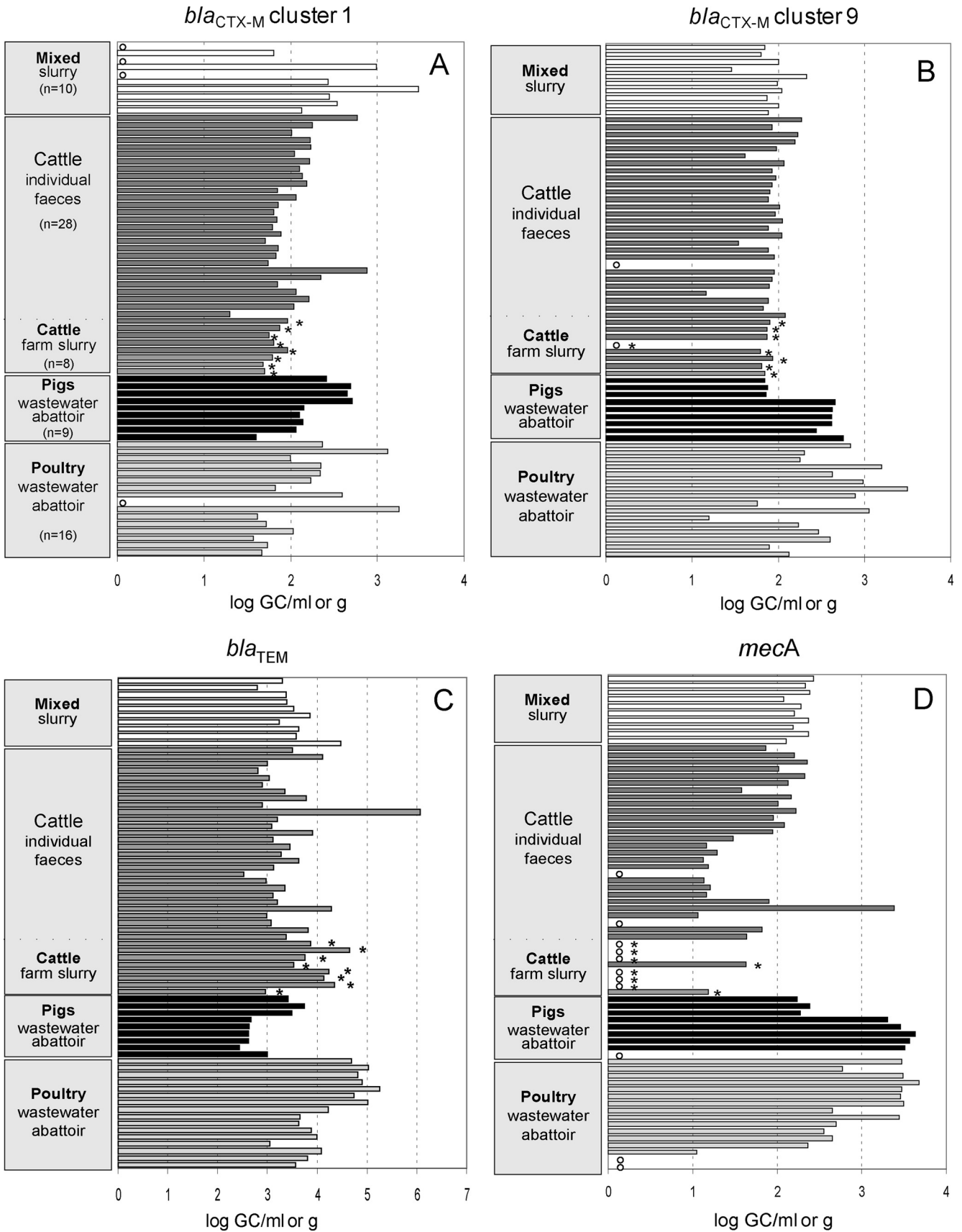| Target gene	Reaction	Oligonucleotide	Sequence	Amplimer size (bp)	Reference
bla$_{TEM}$	TEM PCR	UP	CTCACCCAGAAACGCTGGTG	569	10
		LP	ATCCGCCTCCATCCAGTCTA		
	TEM qPCR	UP	CACTATTCTCAGAATGACTTGGT	85	24
		LP	TGCATAATTCTCTTACTGTCATG		
		Probe	6FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ		
bla$_{CTX-M}$ cluster 1	CTX-M-1 PCR	UP	ACGTTAAACACCGCCATTCC	356	10
		LP	TCGGTGACGATTTTAGCCGC		
	CTX-M-1 qPCR	UP CTX-M	ACCAACGATATCGCGGTGAT	101	10
		LP CTX-M	ACATCGCGACGGCTTTCT		
		Probe	6FAM-TCGTGCGCCGCTG-MGBNFQ		
bla$_{CTX-M}$ cluster 9	CTX-M-9 PCR	UP	ACGCTGAATACCGCCATT	346	This study
		LP	CGATGATTCTCGCCGCTG		
	CTX-M-9 qPCR	UP CTX-M	ACCAATGATATTGCGGTGAT	85	This study
		LP CTX-M	CTGCGTTCTGTTGCGGCT		
		Probe	6FAM-TCGTGCGCCGCTG-MGBNFQ		
mecA	MecA PCR	UP	ATACTTAGTTCTTTAGCGAT	434	10
		LP	GATAGCAGTTATATTTCTA		
	MecA qPCR	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	33
		LP	TGGTCTTTCTGCATTCCTGGA		
		Probe	FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-TAMRA		
	16S rDNA	UP	AAGAGTTTGATCCTGGCTCAG	1,503	29
		LP	TACGGCTACCTTGTTACGACTT		

FIG. 1. Number of detected copies (log GC/ml or log GC/g) of each ARG in DNA isolated from farm fecal wastes (mixed samples), cattle, pigs, and poultry. Shown are the number of detected copies of bla_{CTX-M} genes of cluster 1 (A), bla_{CTX-M} genes of cluster 9 (B), bla_{TEM} genes (C), and $mecA$ genes (D). White circles indicate those samples showing results that were negative or below the detection limit. Asterisks indicate cattle slurries from a farm, while the rest of the cattle samples were fecal samples from summer pastures.

vehicles for mobilization of the environmental pool of ARGs that contribute to the maintenance and emergence of new resistances.

Despite the recent efforts of many international health organizations (19, 20, 30, 35) that recommend a controlled use of antibiotics and to withdraw their use in animal husbandry, new resistances continue to emerge. This could suggest that the origin of resistances is not the antibiotic pressure but the ARGs present in the environmental pool. The results for cattle feces presented here support this hypothesis, since these animals graze on pasture outside the farms and, thus, are not exposed to antibiotics. The study of this environmental pool and of the mechanisms of ARG mobilization, such as bacteriophages, could provide an early warning system for future clinically relevant resistance mechanisms.

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