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Antimicrobial Resistance Genes in Animal Manure, Manure-Amended and Nonanthropogenically Impacted Soils in Spain

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Abstract

Environmental dissemination of antimicrobial resistance genes may occur through agricultural residues, such as animal manure. We studied the resistome of 16 pool samples of animal manure (pig slurry [n = 8] and poultry manure [n = 8]), and 16 soil samples (manure-amended [n = 8] and nonmanure-amended [n = 8]). All samples were collected in central Spain. Detection was based on 18 selected antimicrobial resistance genes (ARGs). The most commonly detected genes in animal manure were *sul*1 (16/16), *sul*2 (16/16), *tet*(*A*) (16/16), *aadA* (16/16), *tet*(*B*) (15/16), and *str* (15/16). Genes bla_{TEM} (7/8), *mecA* (6/8), *vanA* (5/8) and *qnrB* (4/8) were more frequently detected in chicken manure, whereas pig slurry samples presented higher levels of *tet*(*C*) (8/8) and *tet*(*M*) (8/8). Out of the four genes selected for their clinical relevance, three—bla_{CTX-M}, *vanA*, and *mecA*—were detected in animal manure. The bla_{CTX-M} (1/8) and *vanA* (5/8) genes were only identified in chicken manure. To our knowledge, this is the first report of direct detection of *mecA* gene in poultry manure and pig slurry. Eleven out of 18 ARGs were detected in amended soil, while only genes *sul*2 (3/8) and *str* (2/8) were found in nonanthropogenically impacted soils (NAIS), supporting the hypothesis that ARGs may serve as indicators of "anthropogenic impact" on the environment.

Keywords

Antibiotic Resistance, bla_{CTX-M}, *mecA*, Pig Slurry, *vanA*

1. Introduction

Antimicrobial resistance is a rising health threat worldwide, for both humans

and domestic animals [1]. An important resistance mechanism is the acquisition of antimicrobial resistance genes (ARGs) through spontaneous mutations or incorporation of ARGs from other bacteria [2]. ARGs are considered environmental pollutants and could be disseminated by horizontal gene transfer between bacterial species via mobile genetic elements, e.g. plasmids, integrons, transposons and transduction by bacteriophages [3] [4] [5]. These mobilizable genes, the "mobilome", include a collection of all genes that could contribute to a phenotype of antimicrobial resistance, the "resistome" [5].

Most antibiotics are developed from natural bioactive compounds produced by soil fungi or bacteria, and are an integral part of their ecological systems [6]. However, the composition of ARGs in soil has been under constant change since the antibiotic era [7] due to the use of antibiotics as therapeutic treatments and livestock feeding supplements, such as growth promoters. Although the use of growth promoters in animal production was banned in the member states of the European Union, including Spain, since 2006 [8], this practice is still active in many other countries. A significant amount of the antibiotics used in veterinary medicine (between 30% and 90%) is excreted essentially unchanged [9]. As a result, antibiotic residues and ARGs may be disseminated in the environment through agricultural residues, such as animal manure, which can be used as agricultural fertilizer [10] [11]. The occurrence and potential environmental impact of antimicrobial resistant bacteria and ARGs in the environment are still poorly understood [12].

We evaluated the presence of selected ARGs in two types of commonly used agricultural amendments in Spain; pig slurry and poultry manure, and established the impact of such practices by comparing the presence of ARGs in pig slurry-amended and nonanthropogenically impacted soils (NAIS) in the country.

2. Materials and Methods

2.1. Samples

A total of 32 samples were collected in the central area of Spain from April 2012 to April 2014; 16 samples of manure from different broiler poultry and swine farms (poultry manure [n = 8] and pig slurry [n = 8]), and 16 samples of soil (agricultural soil periodically amended with pig slurry [n = 8] and soil from the same region not amended in the last five years or more [n = 8]). Each of the 32 samples consisted of six subsamples, pooled from each collection site. Manure samples were collected from storage tanks. Soil samples were collected at 5 - 10 cm deep. Amended soil samples were collected at least 15 days after amendment with pig slurry, the most commonly used amendment in the studied area. Each sample of nonamended soil was collected approximately 10 km apart from the rest. We compared the presence of antibiotic resistance in poultry *vs.* pig slurry manure and amended soil *vs.* NAIS soils. The sample size was determined attending to logistics reasons, selecting those broiler poultry and swine farms that

allowed the sampling operations.

2.2. Real-Time PCR Detection of ARGs

High Pure Template DNA (Roche Diagnostics, Mannheim, Germany) and E.Z.N.A® soil DNA kit (Promega, Madison, WI, USA) were used to extract DNA from poultry manure and pig slurry samples, and soil samples, respectively. The presence of 16S rRNA gene and 18 selected ARGs was tested using real-time PCR (rtPCR); 18 of them based on SYBR® Green and one based on a TaqMan® probe (**Table 1**), all performed in an Eco Illumina thermocycler (Illumina, San Diego, CA, USA).

To study different profiles, we selected genes related to some of the initially most commonly used antibiotics (e.g., *catA*1, *catA*2, *tet*(*A*), *tet*(*B*), *tet*(*C*), *tet*(*M*), *tet*(*Q*), *sul*1, *sul*2, *str* and *aadA*), the currently most commonly used antibiotics in poultry and swine production (*qnrB*, *qnrS*, bla_{TEM}), and selected antibiotics of clinical relevance (*armA*, bla_{CTX-M}, *mecA* and *vanA*, respectively responsible to aminoglycoside-, extended-spectrum β-lactamase-, methicillin- in *Staphylococcus aureus* and vancomycin-resistances). It was not possible to discriminate between *strA*–*strB* genes or the different *aadA* alleles. Gut microbiome samples previously tested as positive for the selected genes were selected as positive controls. Unfortunately, information regarding the antibiotics used in the studied animal farms was confidential.

The 16S rRNA gene (present in all bacteria) was used to check the samples' viability. A sample was considered validated when a 1/10 dilution of the purified DNA showed a cycle threshold less than 25. In addition, this gene was used to quantify the presence of the selected ARGs in different samples, establishing the relative concentration of the *sul*2 gene in comparison with the 16S rRNA gene, as follows: number of copies of *sul*2 gene per reaction/number of copies of 16S rDNA gene. The *sul*2 gene was selected to perform the relative quantification of its presence because it was one of the two ARGs detected in NAIS. Standard controls for the 16S rRNA gene and the *sul*2 gene were established based on previously tested samples. Purified DNA fragments were cloned into a plasmid vector (pGEM®-T, Promega, Madison, WI, USA) and purified for a second time (Wizard® Plus SV Minipreps DNA Purification System, Promega, Madison, WI, USA). Plasmid colonies were subcultured in LB medium supplemented with ampicillin. Total DNA was measured by a spectrophotometer. The estimated number of copies was obtained by dividing the absorbance value at 260 nm by the plasmid molecular weight (including the specific insert). Subsequently, ten-fold dilutions of quantified plasmids were evaluated by rtPCR to establish the detection limits of *sul*2 and 16S rRNA genes.

2.3. Statistical Analysis

Chi-square test was used to compare the occurrence of ARGs between samples of poultry manure and pig slurry, and pig slurry-amended soils and NAIS. Relative

Table 1. Primers used for real time PCR detection of selected ARGs.

Gene	Sequence (5'-3')	Function	Annealing temperature (˚C)	Amplicon size (bp)	Reference
aadA	F: GCAGCGCAATGACATTCTTG	F	60	282	[35]
	R: ATCCTTCGGCGCGATTTTG	R			
armA	ATTCTGCCTATCCTAATTGG	F	55	315	[36]
	ACCTATACTTTATCGTCGTC	R			
bla_{CTX-M}	TTTGCGAT GTGCAGTACCAGTAA	F	60	591	[37]
	CGATATCGTTGGTGGTGCCATA	R			
bla_{TEM}	AAAGATGCTGAAGATCA	F	44	425	[38]
	TTTGGTATGGCTTCATTC	R			
*catA*1	GGTGATATGGGATAGTGTT	F	60	349	[39]
	CCATCACATACTGCATGATG	R			
*catA*2	GATTGACCTGAATACCTGGAA	F	60	567	
	CCATCACATACTGCATGATG	R			
mecA	CATTGATCGCAACGTTCAATTT	F	60	99	[40]
	TGGTCTTTCTGCATTCCTGGA	R			
	TGGAAGTTAGATTGGGATCATAGCGTCAT	probe[a]			
qnrB	GGMATHGAAATTCGCCACTG	F	62	263	[41]
	TTYGCBGYYCGCCAGTCGAA	R			
qnrS	GACGTGCTAACTTGCGTGAT	F	62	118	
	TGGCATTGTTGGAAACTTG	R			
tet(*A*)	GCGCTNTATGCGTTGATGCA	F	62	387	[42]
	ACAGCCCGTCAGGAAATT	R			
tet(*B*)	TACGTGAATTTATTGCTTCGG	F	60	206	[43]
	ATACAGCATCCAAAGCGCAC	R			
tet(*C*)	CTTGAGAGCCTTCAACCCAG	F	66	418	[44]
	ATGGTCGTCATCTACCTGCC	R			
tet(*M*)	ACAGAAAGCTTATTATATAAC	F	60	171	
	TGGCGTGTCTATGATGTTCAC	R			
tet(*Q*)	AGAATCTGCTGTTTGCCAGTG	F	63	169	[45]
	CGGAGTGTCAATGATATTGCA	R			
str	AATGAGTTTTGGAGTGTCTCAACGTA	F	60	147	[46]
	AATCAAAACCCCTATTAAAGCCAAT	R			
*sul*1	CGCACCGGAAACATCGCTGCAC	F	63	163	[47]
	TGAAGTTCCGCCGCAAGGCTCG	R			
*sul*2	TCCGGTGGAGGCCGGTATCTGG	F	63	191	
	CGGGAATGCCATCTGCCTTGAG	R			
vanA	GAAATCAACCATGTTGATGTAGCA	F	61	572	[48]
	TTTGCCGTTTCCTGTATCCGT	R			
16S rDNA	ATGGCTGTCGTCAGCT	F	62	352	[49]
	ACGGGCGGTGTGTAC	R			

[a]TaqMan probes: 5' (6FAM); 3' (nonfluorescent quencher). F = Forward, R = Reverse.

quantification (number of copies of ARG/number of copies of 16S rDNA) and the mean number of genes per sample among all four categories were evaluated by the Mann-Whitney U test. All statistical studies were performed with the aid of SPSS 15.0 software. Statistical significance for all tests was defined as $P < 0.05$.

3. Results and Discussion

Results are summarized in Table 2. Out of the 18 ARGs analyzed in this study, 16 were detected. The presence of *armA* and *catA*2 was below the detection limits.

3.1. Poultry Manure *vs.* Pig Slurry

Statistically significant differences were detected between poultry manure and pig slurry in relation to the mean number of ARGs present in the samples ($P = 0.013$); *mecA* ($P = 0.012$), *vanA* ($P = 0.007$), *qnrB* ($P = 0.021$), and bla_{TEM} ($P = 0.000$) genes were found more frequently in poultry manure, while *tet*(*C*) ($P =$

Table 2. Selected ARGs tested by real time PCR, relative amount of *sul*2 gene, and mean number of genes per sample.

Gene	Poultry manure	Pig slurry	Amended soil	NAIS
aadA	8/8	8/8	4/8	0/8
armA	0/8	0/8	0/8	0/8
$bla_{\text{CTX-M}}$	1/8	0/8	0/8	0/8
bla_{TEM}	7/8	0/8	3/8	0/8
*catA*1	3/8	1/8	0/8	0/8
*catA*2	0/8	0/8	0/8	0/8
mecA	6/8	1/8	0/8	0/8
qnrB	4/8	0/8	0/8	0/8
qnrS	6/8	3/8	3/8	0/8
str	8/8	7/8	4/8	2/8
*sul*1	8/8	8/8	5/8	0/8
*sul*2	8/8	8/8	8/8	3/8
*sul*2 (relative[a])	6.2×10^{-3} (1.2×10^{-6} - 2.6×10^{-2})	5.1×10^{-2} (1.4×10^{-2} - 8.8×10^{-2})	9.8×10^{-4} (1.7×10^{-4} - 2.0×10^{-3})	0.3×10^{-4} (0.8×10^{-6} - 0.7×10^{-4})
tet(*A*)	8/8	8/8	3/8	0/8
tet(*B*)	7/8	8/8	3/8	0/8
tet(*C*)	3/8	8/8	3/8	0/8
tet(*M*)	4/8	8/8	1/8	0/8
tet(*Q*)	7/8	7/8	5/8	0/8
vanA	5/8	0/8	0/8	0/8
Mean number of genes/sample	11.6 (9 - 14)	9.4 (8 - 11)	5.2 (1 - 10)	0.6 (0 - 2)

[a]Calculated as follows: number of copies of ARG per reaction/number of copies of 16S rDNA gene.

0.007) and *tet*(*M*) ($P = 0.021$) ARGs were more frequently found in pig than in poultry slurry.

The most commonly detected ARGs in animal manure samples were *tet*(*A*) (16/16), *sul*1 (16/16), *sul*2 (16/16), *aadA* (16/16), *tet*(*B*) (15/16) and *str* (15/16), all related to some of the initially most commonly used antibiotics. Efflux pump genes—*tet*(*A*) and *tet*(*B*)—were present in almost all the animal manure samples (8/8 and 8/8 in pig slurry samples and 8/8 and 7/8 in poultry manure, respectively). The ribosomal protection protein gene *tet*(*M*) and the tetracycline efflux pump gene *tet*(*C*) were more frequently found in pig than in poultry manure, whereas *tet*(*Q*) was more frequent in both poultry manure and pig slurry samples. Based on our findings, pig slurry may be considered a major source of *tet* genes, followed by poultry manure. Our findings partially agree with Cheng *et al.* [13], who reported *tet* and *sul* genes as the most frequently detected ARGs in animal manure.

Genes able to confer resistance to streptomycin-*str* and *aadA*- [14] and spectinomycin-*aadA*- [15] were detected in poultry manure and pig slurry. The presence of *str* genes and *aadA* in *E. coli* from poultry manure and pig slurry was previously reported [16].

The gene *catA*1 was present in four out of 16 samples of animal origin (poultry manure [3/8] and pig slurry [1/8]). None of the samples presented the *catA*2 gene. Interestingly, the use of chloramphenicol in food-producing animals has been banned in the EU since 1994 [17]. The presence of *catA*1 in the absence of chloramphenicol residues to induce selective pressure has been attributed to its relationship with other plasmid-mediated ARGs [18]. Bacterial isolation was not performed in this study; therefore, we were not able to determine if different ARGs were present in the same isolate. However, we found that all *cat* positive samples were also positive for *sul*1 and *sul*2, which is in agreement with previously published reports [19].

Regarding quinolone resistance genes, *qnrS* was present in pig slurry samples (3/8) and highly prevalent in poultry manure (6/8), while *qnrB* was only detected in poultry manure samples (4/8). The presence of *qnr* genes in poultry products is well documented, but our prevalence is considerably higher than those previously published: 3.9% and 0.9% for *qnrB*; 5.1% and 8.11% for *qnrS,* respectively [20] [21], probably due to our methodology, more sensitive than previously culture-based studies [20] [21]. Thus, the real prevalence of *qnr* genes could be higher than expected.

Beta-lactamase genes bla_{TEM} and $bla_{\text{CTX-M}}$, were detected in poultry manure—7/8 and 1/8, respectively—but not in pig slurry. These genes are related to extended-spectrum beta-lactamases (ESBLs), important Enterobacteriaceae resistance mechanisms against cephalosporins [22]. CTX-M-type resistance genes have replaced other families, such as TEM and SHV, emerging all over the world [23]. The presence of $bla_{\text{CTX-M}}$ gene in *E. coli* isolated from poultry manure has been previously reported [16]. All pig slurry samples were negative, although

other studies have isolated *E. coli* with bla_{TEM} and bla_{CTX} from pig slurry [16].

Vancomycin resistance gene (*vanA*) was only detected in poultry manure (5/8). Vancomycin-resistant enterococci have been related to the use of avoparcin, which was the first growth promoter used in food-producing animals banned by the European Union, in 1997 [24] [25]. After an initial decline, the presence of *vanA* gene seems to persist in poultry [26] [27], and based on our results, is still widely present in poultry manure.

The high prevalence of *mecA* gene, linked to methicillin resistant *Staphylococcus aureus* (MRSA), was noteworthy in chicken manure samples (6/8). This gene was also detected in one sample of pig slurry (1/8). To our knowledge, this is the first report of *mecA* gene direct detection in poultry manure and pig slurry. Colomer-Lluch *et al.* found the *mecA* gene in fecal waste samples from slaughterhouses, suggesting its transport by bacteriophages and linking it to bacteria affecting avian and swine species [28].

3.2. Amended Soil *vs.* NAIS

There were statistically significant differences between amended and no-amended soils regarding the presence of *sul*1, *sul*2, *tet*(*Q*), and *aadA* ($P = 0.007$, $P = 0.007$, $P = 0.007$ and $P = 0.021$, respectively); the relative concentration of *sul*2 ($P = 0.014$), and the mean number of ARGs per sample ($P = 0.009$). The presence of ARGs was higher in amended soils, as well as the relative concentration of *sul*2, showing that these genes are probably derived from human activities, as observed by Pruden *et al.* [29].

Eleven different ARGs were present in at least one manure amended soil sample: all *sul* and *tet* genes, bla_{TEM}, *qnrS*, *aadA* and *str* genes. On the other hand, only *sul*2 and *str* genes were found in NAIS (3/8 and 2/8, respectively). No soil samples presented the genes selected by their clinical relevance (*armA*, $bla_{CTX\text{-}M}$, *mecA*, and *vanA*). Our results differ from previous studies that detected several ARGs in both natural and anthropogenically impacted environments [29] [30].

Gene bla_{TEM} (3/8) and *qnrS* (3/8), related to antibiotics frequently found in swine production, were detected in pig slurry amended soil, but *qnrB* gene was not detected in this study. The presence of bla_{TEM} in this type of soil was observed by Binh *et al.* [31], while *qnrS* gene has been previously detected in other anthopogenically impacted soils, like soils irrigated with wastewater [32].

We amplified the *str* gene in both amended soils and NAIS, and *aadA* only in amended soils. Both genes, *str* and *aadA*, have been detected in antic soil samples from the Siberian permafrost [33], previous to the antibiotic era, so these elements could be related to other factors beside the modern use of antibiotics.

The *sul*2 gene was found in amended and nonamended soils, but its relative concentration was significantly higher in amended soil samples. The relative concentration of *sul*2 gene in agricultural soils presented the same order of magnitude as previously published [34]. It is important to remark that this gene is considered the most sensible indicator of anthropogenic/agricultural impact

[29].

Our findings demonstrate that agricultural activities may contribute significantly to the environmental spread of *aadA*, *sul*1, *sul*2, and *tet*(*Q*) through the use of pig slurry to amend agricultural soils. Although not significant, the presence of other tet genes, bla_{TEM} and *qnrS* seem to be higher in those soils amended with pig slurry. In addition, amended soils presented higher mean ARGs number per sample than NAIS.

As a consequence, in despite of the impossibility to determine the individual bacterial ARGs due to the lack of culture and subsequent isolation, it is possible to obtain specific patterns of anthropogenic and nonanthropogenic environments based on ARG profiles and concentrations.

3.3. Conclusion

Animal waste carries ARGs, some of clinical relevance (*vanA*, *mecA*, and bla_{CTX-M}), which may be spread to the environment through the use of animal manure to amend agricultural soils. This is the first report of direct detection of *mecA* in poultry manure and pig slurry. Despite the limited sample size and number of ARGs we studied, our results show the impact of anthropogenic activities on the environment through the detection of several ARGs, e.g., *sul*1, bla_{TEM} and *qnrS*, in manure-amended agricultural soils, while the same ARGs were below the detection limit in NAIS. Our results support the hypothesis that the detection and quantification of ARGs may serve as indicators of the "anthropogenic impact" on the environment and propose the development of specific ARG profiles of these pollutants to compare different environmental scenarios. Further studies, focusing on the consequences of anthropogenic activities on microbial populations in different scenarios are needed to elucidate the real impact of ARGs.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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