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Seven *Klebsiella pneumoniae* isolates from dogs and cats in Spain were found to be highly resistant to aminoglycosides, and ArmA methyltransferase was responsible for this phenotype. All isolates were typed by multilocus sequence typing (MLST) as ST11, a human epidemic clone reported worldwide and associated with, among others, OXA-48 and NDM carbapenemases. In the seven strains, *armA* was borne by an IncR plasmid, pB1025, of 50 kb. The isolates were found to coproduce DHA-1 and SHV-11 β-lactamases, as well as the QnrB4 resistance determinant. This first report of the ArmA methyltransferase in pets illustrates their importance as a reservoir for human multidrug-resistant *K. pneumoniae*.

Aminoglycosides are widely used for the treatment of various bacterial infections due to Gram-positive and Gram-negative bacteria. Resistance to these antibiotics is frequently mediated by modifying enzymes that are able to acetylate, phosphorylate or adenylate the antibiotic molecule (1). Recently, 16S rRNA methyltransferases (ArmA, RmtA to -F, and NmpA) have been described as a new high-level aminoglycoside resistance mechanism among Gram-negative pathogenic bacteria (2, 3, 4). Since 2003, these methyltransferases have been reported worldwide, usually from human clinical isolates, except for ArmA and RmtB, reported from chickens (5) and pigs (6, 7, 8), and ArmA and RmtC, reported from food isolates (9, 10). There was no report of a 16S rRNA methyltransferase in bacteria isolated from house pets until 2011, when RmtB was found in several *Enterobacteriaceae* collected from pets in China (11).

The aim of this study was to find the genetic determinant responsible for the high-level resistance to clinically important aminoglycosides, such as gentamicin and amikacin, in seven *Klebsiella pneumoniae* strains isolated from pets (dogs and cats) at the Faculty of Veterinary Medicine in Madrid, Spain. Strains were collected throughout 2008, 2009, and 2010 from different animals with diverse diseases, all referred from the same veterinary surgery (Table 1). Pulsed-field gel electrophoresis (PFGE) typing was performed with all isolates (9) and showed a high genetic relatedness between them. *K. pneumoniae* multilocus sequence typing (MLST) was performed by using the primers described by Diancourt et al. (12) except for a different rpoB-forward primer (5′-TCTGACCCGTGAGCGCGCAGGCT). Allelic profiles and sequence types (STs) were verified at http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html. A unique allelic profile was obtained with the seven isolates, corresponding to ST11. ST11 is an epidemic clone of *K. pneumoniae* that has been isolated from humans worldwide, and it is associated with the spread of resistance determinants such as OXA-48 or NDM (13, 14, 15). The MICs, determined and interpreted according to Clinical and Laboratory Standards Institute guidelines (16, 17), showed high-level resistance to all 4,6-disubstituted 2-deoxystreptamine aminoglycosides, as well as to ampicillin, ceftazidime, sulfamethoxazole, tetracycline, chloramphenicol, and fluoroquinolones (Table 2).

Screening of 16S rRNA methyltransferase-encoding genes was performed by PCR and sequencing (9), revealing the presence of the *armA* gene in the seven isolates. Transfer of this gene into a laboratory *Escherichia coli* INVF′ strain was carried out by transformation using plasmid DNA extraction (Plasmid Midi Kit; Qiagen, Inc., Chatworth, CA) and demonstrated that *armA* was borne by a plasmid in all the *K. pneumoniae* strains. In order to elucidate β-lactam and quinolone resistance determinants, multiplex PCR assays for TEM, SHV, CMY, DHA, and Qnr genes were performed with wild-type and transformant bacteria (18, 19). bla_{SHV-11} was identified in the seven *K. pneumoniae* isolates, although it was absent in the corresponding transformants. A bla_{DHA} gene was amplified in the seven wild-type isolates and their transformants, and it was confirmed by sequencing to be bla_{DHA-1}. The bla_{DHA-1} gene was coharbored by the same plasmid as *armA* in the seven *K. pneumoniae* isolates. A *qnrB4* gene was detected in the wild-type strains, and it was found with *armA* and bla_{DHA-1} in the same plasmid in five strains (BB1097, BB1099, BB1101, BB1102, and BB1103) (Table

TABLE 1 Features of the seven *K. pneumoniae* isolates investigated in this study

Isolate no.	Source	Date of isolation	Sample	Plasmid bearing *armA*	Other resistance gene(s) in plasmid
BB1097	Cat	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1098	Dog	2009	Urine	pB1025-1	bla_{DHA-1}
BB1099	Dog	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1100	Dog	2008	Urine	pB1025-1	bla_{DHA-1}
BB1101	Cat	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1102	Dog	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1103	Dog	2010	Abscess	pB1025	bla_{DHA-1}, *qnrB4*

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TABLE 2 MICs for *K. pneumoniae* isolates and transformants

Strain[a]	MIC (mg/liter)[b]														
	AMK	AMP	CAZ	CHL	CIP	CST	CTX	FFN	GEN	KAN	NAL	SXT	STR	TET	TMP
K. pneumoniae BB1097	>512	>32	16	>64	>8	≤2	>4	16	512	>128	>64	>1024	32	>64	>32
E. coli BB1104	>512	16	2	>64	0.015	≤2	2	≤2	512	>128	≤4	≤8	>128	64	≤0.5
K. pneumoniae BB1098	>512	>32	4	>64	>8	≤2	1	16	512	>128	>64	>1024	32	>64	>32
E. coli BB1105	>512	16	2	≤2	≤0.008	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	32	≤0.5
K. pneumoniae BB1099	>512	>32	16	>64	>8	≤2	>4	16	>512	>128	>64	>1,024	16	2	>32
E. coli BB1106	>512	16	2	≤2	0.015	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	≤1	≤0.5
K. pneumoniae BB1100	>512	>32	8	>64	>8	≤2	2	16	>512	>128	>64	>1,024	32	2	>32
E. coli BB1107	>512	16	2	≤2	≤0.008	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	≤1	≤0.5
K. pneumoniae BB1101	>512	>32	4	>64	>8	≤2	1	16	>512	>128	>64	>1024	32	>64	>32
E. coli BB1108	>512	32	2	≤2	0.015	≤2	≤0.5	≤2	>512	>128	≤4	≤8	>128	64	≤0.5
K. pneumoniae BB1102	>512	>32	8	>64	>8	≤2	>4	8	>512	>128	>64	>,1024	32	>64	>32
E. coli BB1109	>512	16	1	≤2	0.015	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	64	≤0.5
K. pneumoniae BB1103	>512	>32	8	>64	>8	≤2	2	16	512	>128	>64	>1,024	32	>64	>32
E. coli BB1110	>512	16	2	≤2	0.015	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	64	≤0.5
E. coli INVF'	1	2	≤0.25	≤2	≤0.008	≤2	≤0.5	≤2	≤0.5	≤4	≤4	≤8	>128	≤1	≤0.5

[a] The rows show each *K. pneumoniae* strain isolated in this study followed by its respective transformant bearing a plasmid with *armA*.
[b] AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FFN, florfenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; SXT, trimethoprim-sulfamethoxazole; STR, Streptomycin; TET, tetracycline; TMP, trimethoprim.

1). Interestingly, a report of a multidrug resistance plasmid from *K. pneumoniae* in China carrying a 25-kb region with *armA*, bla_{DHA-1}, and *qnrB4*, pKP048, was recently published (GenBank accession number FJ628167) (20), and an identical region was subsequently detected in several *K. pneumoniae* plasmids from Taiwan (21). With the aim of ascertaining whether our isolates bore the pKP048 plasmid or a derivative thereof, three PCR products of ca. 3.2, 1.1, and 1.2 kb were developed by designing 3 pairs of primers along the genetic environment of *armA*, bla_{DHA-1}, and *qnrB4* in pKP048. The five strains with *armA*, bla_{DHA-1}, and *qnrB4* in the same plasmid (Table 1) carried these genes on a genetic structure identical to that present in pKP048 (Fig. 1). However, pKP048 is an IncF plasmid of 150 kb in size, whereas S1 nuclease digestion (Promega, Madison, WI) and PFGE of the wild-type strains and transformants showed that these genes were borne by a plasmid of approximately 50 kb in the seven strains (data not shown). This plasmid was named pB1025, and it was confirmed to belong to the IncR family using a PCR-based replicon typing kit

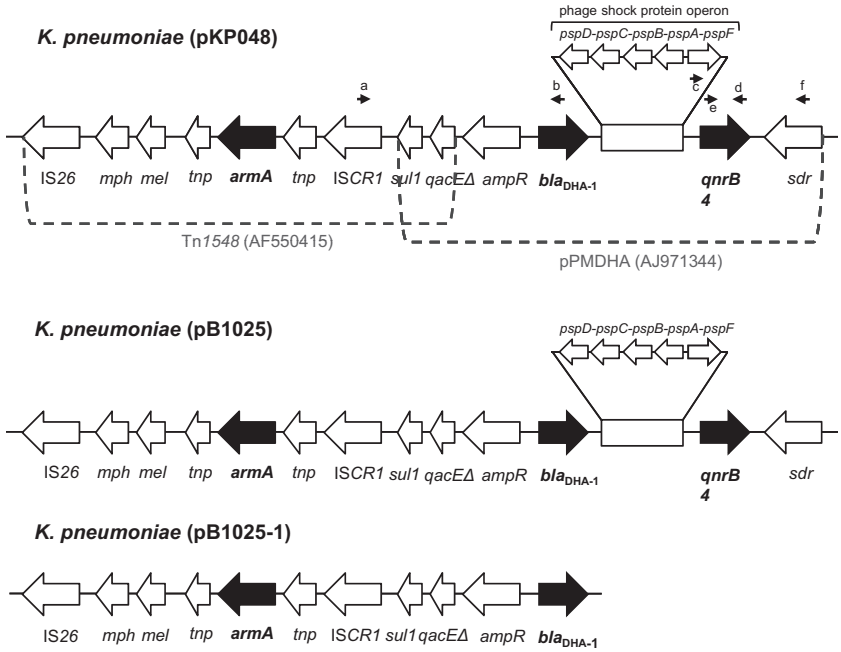

FIG 1 Illustration of the 25-kb genetic structure where *armA*, *bla*DHA-1, and *qnrB4* are embedded in pKP048 and comparison with plasmids pB1025 and pB1025-1. pB1025 has the same genetic structure as pKP048 despite being of a different size and belonging to another Inc family, whereas in pB1025-1, with the same size and Inc group as pB1025, *qnrB4* is not located on this genetic structure. The primers are indicated with small arrows: a, 5′ TCCAGACGGCCACATT GGAGG; b, 5′ TCAAATAGTGTATTTTCAGTG; c, 5′ CGATATCATGTTAATGGCTGA; d, 5′ GACGCCTTGCAAATCAACCCCG; e, 5′ CAGGTTCACCGGT GAAAAAGTT; f, 5′ ATCGCTGGCGAAGCAACTGGC.

(Diatheva, Fano, Italy). Interestingly, IncR plasmids have often been associated with human isolates, but to the best of our knowledge they have not been reported from pet animals to date. In strains BB1098 and BB1100, *armA* and bla_{DHA-1} are embedded in the same genetic structure as in the other five strains (Fig. 1). This genetic structure also takes part of an IncR plasmid of 50 kb, most likely a pB1025 derivative that was named pB1025-1, since *qnrB4* has a different environment and seems to be borne by a different plasmid. Attempts to conjugate pB1025 and pB1025-1 into a recipient laboratory strain as previously described (9) were unsuccessful.

This study describes the occurrence of the ArmA methyltransferase in an ST11 clone of *K. pneumoniae* isolated from pets in Spain, in association with the resistance genes bla_{DHA-1}, bla_{SHV-11}, and *qnrB4*. This is the first time that *armA* has been detected in bacteria from pet animals. To the best of our knowledge, this is also the first report of an IncR plasmid in bacteria isolated from pets. Interestingly, several reports from clinical settings point out that *K. pneumoniae* ST11 is a pathogenic clone adapted to humans and usually produces emerging resistance mechanisms. Thus, this finding is of utmost clinical relevance due to the relationship of pet animals with humans, as it poses a new reservoir for the dissemination of both the ST11 epidemic clone and these resistance genes. Further monitoring of emerging resistance genes in bacteria isolated from pets is essential to minimize their spread between humans and animals.

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REFERENCES

1. **Davies J, Wright GD.** 1997. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol. **5:**234–240.
2. **Wachino J, Arakawa Y.** 2012. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. Drug Resist. Updat. **15:**133–148.
3. **Galimand M, Courvalin P, Lambert T.** 2012. RmtF, a new member of the aminoglycoside resistance 16S rRNA N7 G1405 methyltransferase family. Antimicrob. Agents Chemother. **56:**3960–3962.
4. **Gonzalez-Zorn B, Escudero JA.** 2012. Ecology of antimicrobial resistance: humans, animals, food and environment. Int. Microbiol. **15:**101–109.
5. **Du XD, Wu CM, Liu HB, Li XS, Beier RC, Xiao F, Qin SS, Huang SY, Shen JZ.** 2009. Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens. J. Antimicrob. Chemother. **64:**1328–1330.
6. **Chen L, Chen ZL, Liu JH, Zeng ZL, Ma JY, Jiang HX.** 2007. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. J. Antimicrob. Chemother. **59:**880–885.
7. **Yao Q, Zeng Z, Hou J, Deng Y, He L, Tian W, Zheng H, Chen Z, Liu JH.** 2011. Dissemination of the *rmtB* gene carried on IncF and IncN plasmids among *Enterobacteriaceae* in a pig farm and its environment. J. Antimicrob. Chemother. **66:**2475–2479.
8. **Gonzalez-Zorn B, Teshager T, Casas M, Porrero MC, Moreno MA, Courvalin P, Dominguez L.** 2005. *armA* and aminoglycoside resistance in *Escherichia coli*. Emerg. Infect. Dis. **11:**954–956.
9. **Granier SA, Hidalgo L, San Millan A, Escudero JA, Gutierrez B, Brisabois A, Gonzalez-Zorn B.** 2011. ArmA methyltransferase in a monophasic *Salmonella enterica* isolate from food. Antimicrob. Agents Chemother. **55:**5262–5266.
10. **Hopkins KL, Escudero JA, Hidalgo L, Gonzalez-Zorn B.** 2010. 16S rRNA methyltransferase RmtC in *Salmonella enterica* serovar Virchow. Emerg. Infect. Dis. **16:**712–715.
11. **Deng Y, He L, Chen S, Zheng H, Zeng Z, Liu Y, Sun Y, Ma J, Chen Z, Liu JH.** 2011. F33:A−:B− and F2:A−:B− plasmids mediate dissemination of *rmtB-bla*CTX-M-9 group genes and *rmtB-qepA* in *Enterobacteriaceae* isolates from pets in China. Antimicrob. Agents Chemother. **55:**4926–4929.
12. **Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S.** 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J. Clin. Microbiol. **43:**4178–4182.
13. **Oteo J, Hernández JM, Espasa M, Fleites A, Sáez D, Bautista V, Pérez-Vázquez M, Fernández-García MD, Delgado-Iribarren A, Sánchez-Romero I, García-Picazo L, Miguel MD, Solís S, Aznar E, Trujillo G, Mediavilla C, Fontanals D, Rojo S, Vindel A, Campos J.** 2013. Emergence of OXA-48-producing Klebsiella pneumoniae and the novel carbapenemases OXA-244 and OXA-245 in Spain. J. Antimicrob. Chemother. **68:**317–321.
14. **Giske CG, Fröding I, Hasan CM, Turlej-Rogacka A, Toleman M, Livermore D, Woodford N, Walsh TR.** 2012. Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of bla_{NDM-1} in India, Sweden, and the United Kingdom. Antimicrob. Agents Chemother. **56:**2735–2738.
15. **Lascols C, Peirano G, Hackel M, Laupland KB, Pitout JD.** 2013. Surveillance and molecular epidemiology of *Klebsiella pneumoniae* isolates that produce carbapenemases: first report of OXA-48-like enzymes in North America. Antimicrob. Agents Chemother. **57:**130–136.
16. **Clinical and Laboratory Standards Institute.** 2012. M2-A11: performance standards for antimicrobial disk susceptibility tests; approved standards, 11th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
17. **Clinical and Laboratory Standards Institute.** 2013. M100-S23: performance standards for antimicrobial susceptibility testing; 23rd informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
18. **Dallenne C, Da Costa A, Decré D, Favier C, Arlet G.** 2010. Development of a set of mutiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. J. Antimicrob. Chemother. **65:**490–495.
19. **Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P.** 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. **60:**394–397.
20. **Jiang Y, Yu D, Wei Z, Shen P, Zhou Z, Yu Y.** 2010. Complete nucleotide sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKP048, carrying *bla*KPC-2, *bla*DHA-1, *qnrB4*, and *armA*. Antimicrob. Agents Chemother. **54:**3967–3969.
21. **Lee CH, Liu JW, Li CC, Chien CC, Tang YF, Su LH.** 2011. Spread of ISCR1 elements containing bla_{DHA-1} and multiple antimicrobial resistance genes leading to increase of flomoxef resistance in extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **55:**4058–4063.