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ArmA Methyltransferase in a Monophasic *Salmonella enterica* Isolate from Food[▽]

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The 16S rRNA methyltransferase ArmA is a worldwide emerging determinant that confers high-level resistance to most clinically relevant aminoglycosides. We report here the identification and characterization of a multidrug-resistant *Salmonella enterica* subspecies I.4,12:i:− isolate recovered from chicken meat sampled in a supermarket on February 2009 in La Reunion, a French island in the Indian Ocean. Susceptibility testing showed an unusually high-level resistance to gentamicin, as well as to ampicillin, expanded-spectrum cephalosporins and amoxicillin-clavulanate. Molecular analysis of the 16S rRNA methyltransferases revealed presence of the *armA* gene, together with $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{CTX-M-3}}$. All of these genes could be transferred *en bloc* through conjugation into *Escherichia coli* at a frequency of 10^{-5} CFU/donor. Replicon typing and S1 pulsed-field gel electrophoresis revealed that the *armA* gene was borne on an ~150-kb broad-host-range IncP plasmid, pB1010. To elucidate how *armA* had integrated in pB1010, a PCR mapping strategy was developed for Tn*1548*, the genetic platform for *armA*. The gene was embedded in a Tn*1548*-like structure, albeit with a deletion of the macrolide resistance genes, and an IS*26* was inserted within the *mel* gene. To our knowledge, this is the first report of ArmA methyltransferase in food, showing a novel route of transmission for this resistance determinant. Further surveillance in food-borne bacteria will be crucial to determine the role of food in the spread of 16S rRNA methyltransferase genes worldwide.

Aminoglycosides are used for the treatment of a wide range of infections due to both Gram-negative and Gram-positive bacteria. Resistance to these antimicrobials is usually due to the production of aminoglycoside-modifying enzymes, which are able to acetylate, phosphorylate, or adenylate the antibiotic molecule (8). Since 2003, the 16S rRNA methyltransferases have emerged in Gram-negative pathogens as an acquired aminoglycoside resistance mechanism that confers high-level resistance to most clinically relevant aminoglycosides (13, 17). Seven different 16S rRNA methyltransferase genes have been described thus far: *armA, rmtA, rmtB, rmtC, rmtD, rmtE*, and *npmA* (9, 10, 35). *armA* and *rmtB* are the most prevalent among *Enterobacteriaceae*. Except for the identification of *armA* and *rmtB* in animal isolates from Spain and China (5, 11, 17) and *rmtC* in *Salmonella enterica* from food in the United Kingdom (23), all methyltransferase genes described to date were identified in human clinical samples (36). Although *rmtC* has been reported in the bacterial chromosome integrated through the IS*Ecp*1 mobile element (23), in most cases these genes have been related to large conjugative plasmids in association with extended-spectrum β-lactamases (ESBLs) or, more recently, carbapenemases such as KPC-2 (25) or NDM-1 (29). Although the genetic plasticity of these conjugative elements is high, *armA* has been always identified in the transposable element Tn*1548* (14, 16).

In order to determine whether food animal products are a source of 16S rRNA methyltransferases, we studied the presence of these genes in bacteria isolated from food in La Reunion Island. We report here a multidrug-resistant *Salmonella enterica* isolate, 09CEB904SAL, recovered from food and producing the ArmA methyltransferase. The isolate was identified as *S. enterica* I.4,12:i:−, a monophasic variant of *S. enterica* serovar Typhimurium. Molecular characterization of 09CEB904SAL revealed the presence of *Salmonella* genomic island 1 (SGI1) (2, 19, 27) and of the virulence plasmid *S.* Typhimurium pSLT (31). In addition, three β-lactamase genes—$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{CTX-M-3}}$—were detected, the latter borne on a broad-host-range IncP conjugative plasmid together with the *armA* methyltransferase gene. To our knowledge, this is the first report of ArmA methyltransferase from food. This reflects a novel transmission route for *armA* and confirms presence of 16S rRNA methyltransferases in the East of Africa.

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MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility testing. 09CEB904SAL, an *S. enterica* isolate, was recovered from chicken meat sampled during a control by the retailer in a supermarket on February 2009 in La Reunion, a French island in the Indian Ocean (22). The isolate was serotyped on the basis of the somatic O antigen and phase 1 and phase 2 flagellar H antigens, as specified by the White-Kauffmann-Le Minor scheme (18) using agglutination tests with antisera

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TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)	Position[a] or amplicon size (bp)	Source or reference
Tn1F	GGCACTGTTGCAAATAGTCGG	10326–10346	This study
Tn1R	TTGCTGCTTGGATGCCCGAGG	11981–12001	This study
Tn2F	CCGGGTGACGCACACCGTGGA	11784–11804	This study
Tn2R	TCATTTACCAACTGACTTGAT	12863–12883	This study
Tn3F	TTATTCGCTTTGTGAAAGGCG	12837–12857	This study
Tn3R	TCCAGACGGCCACATTGGAGG	14817–14837	This study
Tn4F	TCAACGACCTCCTCCAATGTG	14807–14827	This study
Tn4R	ACCGCATGGGTTGTGGCATCC	16787–16807	This study
Tn5F	GGATGCCACAACCCATGCGGT	16787–16807	This study
Tn5R	ACAATAAGATTGTTGTACTTT	18813–18833	This study
Tn6F	TATGGGCAGGGCGAAGCGCTA	18792–18812	This study
Tn6R	TTTGAGTAAACTACTCTTTCC	20773–20793	This study
Tn7F	CGTATTGGTCTTGTGGGTGAT	20743–20763	This study
Tn7R	GTGAAATCTGCCCATAGAACA	22722–22742	This study
Tn8F	GGGAAAATGATTTGAAAATTA	22589–22609	This study
Tn8R	CTGCTATTGTGCCTTTAATTA	24589–24609	This study
Tn9F	TTGTTCTTCTAACCTAGTAAT	24572–24592	This study
Tn9R	CACTGTTGCAAAGTTAGCGAT	25584–25604	This study
armAF	CAAATGGATAAGAATGATGTT	774	13
armAR	TTATTTCTGAAATCCACT	774	13
rmtA.F	ATGAGCTTTGACGATGCCCTA	756	This study
rmtA.R	TCACTTATTCCTTTTTATCATG	756	This study
rmtB.F	ATGAACATCAACGATGCCCT	769	36
rmtB.R	CCTTCTGATTGGCTTATCCA	769	36
rmtC.F	CGAAGAAGTAACAGCCAAAG	711	10
rmtC.R	ATCCCAACATCTCTCCCACT	711	10
rmtD.F	CGGCACGCGATTGGGAAGC	401	10
rmtD.R	CGGAAACGATGCGACGAT	401	10
rmtE.F	ATGAATATTGATGAAATGGTTGC	818	9
rmtE.R	TGATTGATTTCCTCCGTTTTTG	818	9
U7-L12	ACACCTTGAGCAGGGCAAAG	SGI1 left junction	2
LJ-R1	AGTTCTAAAGGTTCGTAGTCG	SGI1 left junction	2
104-RJ	TGACGAGCTGAAGCGAATTG	SGI1 right junction	2
C9L2	AGCAAGTGTGCGTAATTT	SGI1 right junction	2
L1	GGCATCCAAGCAGCAAG	5′CS of class 1 integron	26
R1	AAGCAGACTTGACCTGA	3′CS of class 1 integron	26

[a] That is, the position in plasmid pMUR050 (AY522431).

(obtained from Bio-Rad [Marnes-la-Coquette, France] and AES [Bruz, France]) according to an in-house method certified by the French Accreditation Committee (accreditation no. 1-0245). The absence of flagellar phase 2 antigens was confirmed following phase inversion method as recommended by EFSA (12). *Escherichia coli* K802N was used as the recipient strain for plasmid conjugation experiments. Antimicrobial susceptibility tests were first performed by disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (6). MICs were performed, and results were interpreted according to CLSI guidelines (7).

DNA analysis and manipulation. ESBL genes were detected by using Check KPC ESBL (Check-Points B.V., Wageningen, The Netherlands), a microarray using Clondiag technology (Jena, Germany) capable of detecting bla_{KPC} and groups of bla_{CTX-M} and discriminate ESBLs from non-ESBLs for bla_{TEM} and bla_{SHV}.

SGI1 was detected by amplifying the right and left junction and the class1 integron gene cassette as previously described (1, 26). The bla_{CMY-2} gene was detected as previously described (28). PCRs for all of the methylase genes were performed with the primers listed in Table 1. Plasmid DNA extraction was performed using a Plasmid Midi kit (Qiagen, Inc., Chatworth, CA). Tn*1548* mapping was carried out using plasmid extractions as a template and by performing overlapping PCRs with nine pairs of designed primers along the transposon (Table 1 and Fig. 2). The PCR products were purified and subsequently sequenced with their corresponding primers.

Conjugation experiments. Conjugation experiments were carried out in brain heart infusion (BHI) broth with 09CEB904SAL as the donor and *E. coli* K802N (which is resistant to nalidixic acid) as the recipient. Both strains were grown separately in BHI with moderate shaking until the donor strain reached an optical density at 600 nm of 0.9. Then, 1 ml of the donor culture, 1 ml of the recipient culture, and 1 ml of fresh BHI were mixed in a sterile flask, followed by incubation for 2 to 3 h at 37°C without shaking. Transconjugants were selected on BHI agar plates with nalidixic acid (50 μg/ml) and either ampicillin or gentamicin (50 μg/ml) to select for plasmid-encoded aminoglycosides and β-lactam resistance. Putative transconjugants were confirmed not to be nalidixic acid-resistant mutant donors by antimicrobial susceptibility testing and subsequent comparison with the donor and recipient resistance phenotype. *armA* PCR and S1 pulsed-field gel electrophoresis (S1-PFGE) were performed with the transconjugants. The conjugation frequency for each plasmid was calculated as the percentage of grown transconjugants bearing each plasmid versus the total conjugation frequency of the donors. To assess the possibility for the conjugation of more than one plasmid, 100 colonies grown using ampicillin-selected conjugation were replicated onto selective plates of gentamicin and amoxicillin-clavulanate in order to determine transconjugants with a plasmid bearing *armA,* bla_{CMY-2}, or both genes.

Plasmid analysis. In order to determine plasmid size, agarose gel plugs of total DNA were prepared according to the PulseNet PFGE protocol for the subtyping of *E. coli* O157:H7, *Salmonella* spp., and *Shigella* spp. (30). These plugs were digested with S1 nuclease (Promega, Madison, WI) according to the manufacturer's instructions. Plugs were loaded into a 1% agarose gel. PFGE was performed as previously described (32) with the following modifications: a running time of 21 h, a temperature of 14°C, a field strength of 6 V/cm, an included angle of 120°, an initial pulse time of 2.2 s, and a final pulse time of 63.8 s. The gels were stained with Sybr Safe (Invitrogen, Paisley, United Kingdom) for 20 min, destained in MilliQ water, and photographed under UV light. Lambda-ladder PFGE marker (New England Biolabs, Ipswich, MA) was used for molecular weight determinations. A PCR method based on five multiplex and three simplex PCRs was performed in order to determine the plasmid incompatibility group (4).

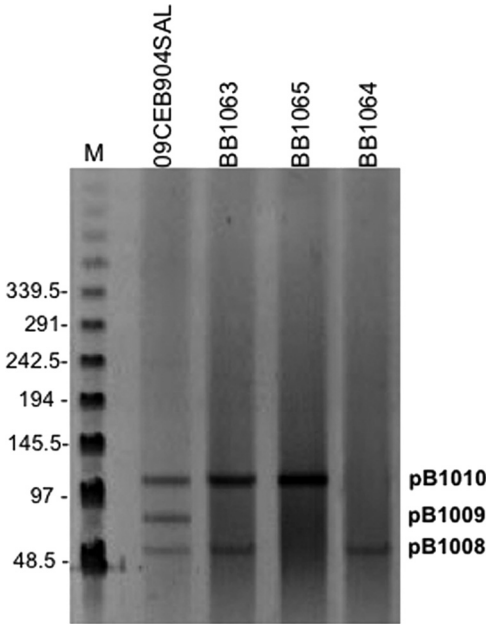

FIG. 1. Plasmid profile of the strains used in the present study. M, lambda ladder molecular marker. The numbers on the left show molecular weights in kilobase pairs. *Salmonella enterica* 09CEB904SAL and transconjugants BB1063, BB1065, and BB1064 are indicated in lanes 2 to 5, respectively. Each band represents one plasmid. *S. enterica* 09CEB904SAL bears pB1008, pB1009, and pB1010. The image reveals three types of transconjugants, one with pB1008 alone, one with pB1010 alone, and one with both pB1008 and pB1010.

Nucleotide sequence accession number. A partial sequence of the Tn*1548* variant observed in the present study has been deposited in GenBank under accession number HQ728525.

RESULTS AND DISCUSSION

Identification and characterization of *S. enterica* 09CEB904SAL. In February 2009, microbial analyses of food isolates in supermarkets from La Reunion Island identified a chicken meat sample with the presence of *S. enterica* 09CEB904SAL. This *Salmonella* strain was serotyped as *S. enterica* subspecies I.4,12:i:−. A complete antimicrobial profile showed that the bacterium possessed an unusual high-level multidrug resistance profile to ampicillin, amoxicillin-clavulanate, cephalothin, cefotaxime, ceftazidime, cefoxitin, streptomycin, kanamycin, gentamicin, sulfonamides, trimethoprim, tetracycline, and chloramphenicol (Table 2).

PCR for the SGI1 showed that this isolate bore the canonical right and left junctions from *S.* Typhimurium (Table 1). Integron cassette analysis identified two genes cassettes of 1,000 and 1,200 bp, which have been previously described in DT104 *S.* Typhimurium (2). PFGE analysis, performed as previously described (24), showed only 71% similarity with the subspecies I.4,12:i:− profile of the European epidemic clone STYMXB.0131 (24).

Identification and characterization of resistance determinants. Conjugation experiments were performed using 09CEB904SAL as the donor, *E. coli* K802N as the recipient, and gentamicin, ampicillin, and nalidixic acid as the selection markers. Analysis of the transconjugants revealed presence of two different phenotypes corresponding with two types of transconjugants: (i) transconjugant BB1063 expressing high-level resistance to gentamicin, ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, cefoxitin, and sulfonamides (Table 2) and (ii) transconjugant BB1064 showing only high-level resistance to ampicillin, amoxicillin-clavulanate, and ceftazidime but not to aminoglycosides or sulfonamides. In order to study the high-level resistance to aminoglycosides and beta-lactams, primers targeting all of the 16S rRNA methyltransferase genes, as well as the main ESBL genes, were used with plasmid extractions of the wild-type bacterium and transconjugants as a template (Table 1). KPC ESBL microarray and conventional PCR and sequencing (21) confirmed the presence of a $bla_{\text{CTX-M-3}}$ gene and a non-ESBL $bla_{\text{TEM-1}}$ gene. DNA fragments corresponding to *armA,* $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$, and group 1 $bla_{\text{CTX-M-3}}$ were amplified from the wild-type strain and sequenced. Concordantly, a non-ESBL $bla_{\text{TEM-1}}$ and a group 1 $bla_{\text{CTX-M-3}}$ were identified from the wild-type and transconjugant BB1063, whereas only $bla_{\text{CMY-2}}$ was amplified from transconjugant BB1064, suggesting the presence of two different plasmids in 09CEB904SAL. To the best of our knowledge, this is the first report of *armA* gene in an isolate from food origin. Previously, *armA* has been identified from several Gram-negative pathogens as one of the most prevalent 16S rRNA methyltransferase genes (20, 37). To date, most 16S rRNA methyltransferase-producing Gram-negative bacteria were recovered from human clinical samples. However, in the case of *armA*, it has also been found in porcine and chicken *E. coli* isolates from Spain and China, respectively (11, 17).

Plasmid analysis. As mentioned before, conjugation experiments suggested that 09CEB904SAL bore at least two transferable plasmids. S1-PFGE analyses revealed the presence of three plasmids of approximately 50, 80, and 110 kb that were named pB1008, pB1009, and pB1010, respectively (Fig. 1). The plasmids were classified according to a PCR-based replicon typing protocol (4). The 110-kb plasmid (pB1010) belonged to the IncP incompatibility group, whereas the 50-kb (pB1008) and 80-kb (pB1009) plasmids were not typeable using this methodology. Interestingly, this is the first time that the *armA* gene has been documented on an IncP plasmid, despite the fact that IncP plasmids are broad-host-range plasmids, and they have been widely described since the 1980s among several genus of *Proteobacteria* generally associated with antibiotic resistance determinants (33, 34). This finding suggests that further spread of *armA* to other strains could be enhanced from this genetic platform, since genes linked to IncP plasmids have a large host range, including *Pseudomonas* spp. or even Gram-positive bacteria. To precisely calculate the conjugation frequency of plasmid pB1010 bearing *armA,* $bla_{\text{TEM-1}}$, and $bla_{\text{CTX-M-3}}$ and plasmid pB1008 bearing $bla_{\text{CMY-2}}$, a conjugation experiment using ampicillin was performed. Transconjugants were replicated onto gentamicin and amoxicillin-clavulanate plates, since resistance to the latter is due to $bla_{\text{CMY-2}}$. Plasmid pB1010 was transferred into *E. coli* at a frequency of 9×10^{-4} (giving rise to transconjugant BB1065), whereas pB1008 was transferred at 4×10^{-4} per donor CFU. The two plasmids were cotransferred at a frequency of 10^{-5} per donor CFU. No resistance determinant was identified on plasmid pB1009, but the *spvC* gene, which is characteristic of pSLT, the virulence plasmid of *S.* Typhimurium, was detected by PCR (3)

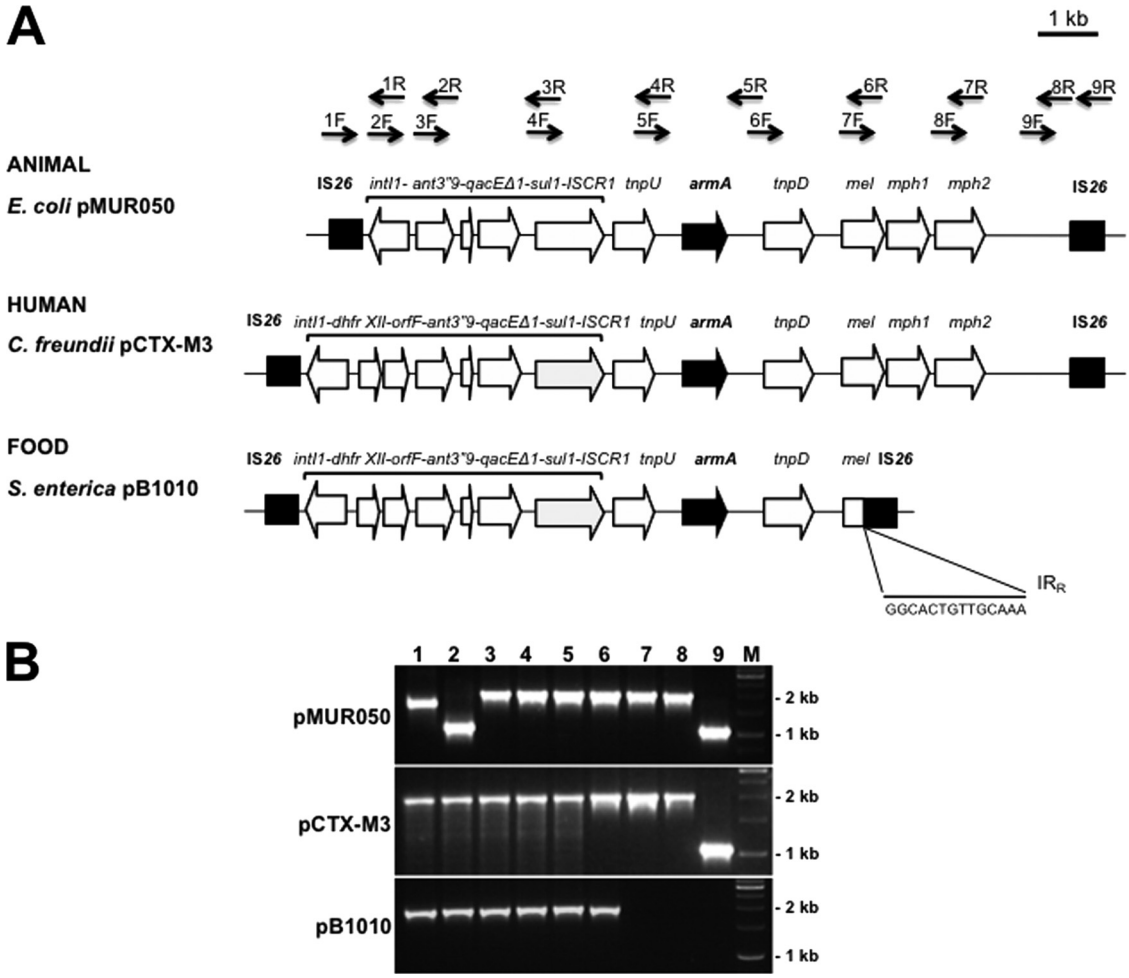

FIG. 2. (A) Genetic structure of Tn*1548* reported from an animal isolate (pMUR050), a human isolate (pCTX-M-3), and a food isolate of *Salmonella enterica* 09CEB904SAL (pB1010). Black arrows in the upper part of the panel represent pairs of primers designed for the mapping of Tn*1548*. (B) PCR fragments obtained in Tn*1548* mapping with plasmids from an animal isolate (pMUR050), a human isolate (pCTX-M-3), and a food isolate (pB1010). Lanes 1 to 9 represent the corresponding pairs of primers. M, molecular weight marker. The PCR results for lanes 7, 8, and 9 are negative in pB1010.

in 09CEB904SAL, with plasmids pB1008, pB1009, and pB1010, but not in the transconjugants (which lack pB1009), suggesting that pB1009 could be a pSLT derivative.

Mapping of Tn*1548*. Tn*1548* is the genetic platform that mobilizes *armA* between plasmids (14, 16). For this reason, a PCR mapping method for Tn*1548* was developed by designing nine pairs of primers that amplify overlapping PCR products. PCR fragments for the expected size were amplified from the control plasmids pCTX-M3 and pMUR050 (15, 17). In *Salmonella* 09CEB904SAL, Tn*1548* harbors the *dhfrXII* trimethoprim resistance cassette, like the strains bearing *armA* identified in clinical settings (13), whereas in the first *E. coli*

TABLE 2. MICs, resistance genes, and plasmids

Strain	MIC (mg/liter)[a]										Resistance gene(s)	Plasmid(s)
	AMC	AMP	CTX	CAZ	CIP	GEN	KAN	SXT	TET	TMP		
00CEB904SAL	>128	>32	>4	>16	0.06	>256	>128	>1,024	>64	>32	*armA*, $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$	pB1008, pB1009, pB1010
K802N	2	4	0.12	0.5	0.25	0.5	≤4	≤8	≤1	≤0.5	None	None
BB1065	4	>32	>4	>16	0.25	>256	>128	>1,024	64	>32	*armA*, $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1}}$	pB1010
BB1064	>128	>32	4	16	0.25	0.5	≤4	≤8	≤1	≤0.5	$bla_{\text{CMY-2}}$	pB1008
BB1063	>128	>32	>4	16	0.25	>256	>128	>1,024	>64	>32	*armA*, $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$	pB1008, pB1010

[a] AMC, amoxicillin-clavulanate; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

isolate described with *armA* and in animals, Tn*1548* lacked this cassette (16). Furthermore, in 09CEB904SAL, PCRs for the amplification of the downstream region of the transposon, corresponding to the pairs 7, 8, and 9 were negative. Suspecting the possibility of a deletion in that region, a long PCR with primers Tn6F and Tn9R was performed. An amplicon of ~3 kb was obtained, instead of the 7-kb amplicon that would correspond to the complete Tn*1548* element. Subsequent sequencing of this DNA fragment with the primers Tn6F and Tn9R revealed insertion of an IS*26* element with its IR_R region interrupting the macrolide resistance genes downstream *armA*. These data show that Tn*1548* is a dynamic element that is ultimately responsible for the spread of *armA*.

Overall, these results confirm the presence of *armA* in East Africa and imply a novel route of transmission for this emerging resistance determinant. Further surveillance in food-borne bacteria will be crucial to determine the role of food products in the spread of the 16S rRNA methyltransferase genes worldwide.

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