



First Clinical Case of *In Vivo* Acquisition of DHA-1 Plasmid-Mediated AmpC in a *Salmonella enterica* subsp. *enterica* Isolate

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ABSTRACT A pan-susceptible *Salmonella enterica* serovar Worthington isolate was detected in the stool of a man returning from Sri Lanka. Under ceftriaxone treatment, a third-generation cephalosporin (3GC)-resistant *Salmonella* Worthington was isolated after 8 days. Molecular analyses indicated that the two isolates were identical. However, the latter strain acquired a *bla*_{DHA-1}-carrying IncFII plasmid probably from a *Citrobacter amalonaticus* isolate colonizing the gut. This is the first report of *in vivo* acquisition of plasmid-mediated resistance to 3GCs in *S. enterica*.

KEYWORDS AmpC, DHA, Worthington, gut, plasmid-mediated resistance, treatment

Salmonella enterica isolates are important pathogens responsible for gastroenteritis but may also cause invasive infections where third-generation cephalosporins (3GCs) and fluoroquinolones are the treatments of choice (1). However, the rapid emergence of 3GC-resistant (3GC-R) strains in human and nonhuman settings presents a public health concern (2, 3). Most of these isolates produce extended-spectrum β -lactamases (ESBLs) or the plasmid-mediated AmpC (pAmpC) CMY-2 (4–7), whereas the DHA-1 pAmpC is rarely reported (8–12).

In this scenario, we note that cases of infection due to *Salmonella enterica* isolates where the organism undergoes *in vivo* acquisition of *bla*_{ESBL} or *bla*_{pAmpC} via mobile genetic elements (MGEs) from other species have not yet been reported. Here, we describe a clinical case in which this phenomenon was observed and define the characteristics of the recovered isolates.

In November 2018, a 77-year-old man presented with fever and respiratory symptoms 5 days after returning from a 2-month trip to Sri Lanka. His personal history included an IgG4 cholangiopathy, requiring immunosuppressive drugs, and hepatocellular carcinoma. Ceftriaxone was started empirically after sampling. Blood, sputum, and urine cultures did not reveal bacterial growth. PCR from a nasopharyngeal swab detected influenza B. Antimicrobial treatment was stopped after 6 days, and the patient was discharged. Two days before discharge, he passed loose stools revealing a pan-susceptible *Salmonella* spp. (strain 7101.67) in culture.

Five days after discharge, the patient was readmitted because of fatigue, nausea, persistent respiratory symptoms, and intermittent diarrhea. Another *Salmonella* spp. (strain 7102.58) grew in stool cultures. Ceftriaxone was restarted but, on detection of resistance toward 3GCs, it was switched to meropenem for 3 days and then streamlined to cefepime for 10 days (see Text S1 and Fig. S1 in the supplemental material for a full description of the clinical case).

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TABLE 1 Antimicrobial susceptibility tests for *Salmonella*, *E. coli* J53 transconjugant, and *C. amalonaticus* strains

Antibiotic	MIC ^a (μg/ml) for:				
	<i>S. Worthington</i> 7101.67	<i>S. Worthington</i> 7102.58 (collected 8 days later)	<i>E. coli</i> J53	<i>E. coli</i> J53 transconjugant (donor 7102.58)	<i>C. amalonaticus</i> 4 ^c
Ampicillin	≤4, S	≥32, R	≤4, S	≥32, R	≥32, R
Piperacillin-tazobactam	≤2, S	64, R	≤2, S	≤2, S	8, S
Ticarcillin-clavulanate	≤8, S	≥256, R	≤8, S	128, R	≥256, R
Cephalothin	≤4, NA	≥32, NA	≤4, NA	≥32, NA	≥32, NA
Cefoxitin	≤2, NA	≥128, NA	≤2, NA	≥128, NA	≥128, NA
Ceftriaxone	≤0.5, S	32, R	≤0.5, S	2, I	8, R
Cefotaxime	≤0.125, S	≥128, R	≤0.125, S	16, R	32, R
Cefotaxime-clavulanate	≤0.0625, NA	≥128, NA	≤0.0625, NA	8, NA	64, NA
Ceftazidime	≤0.125, NA	≥256, R	≤0.125, S	32, R	128, R
Cefotaxime-clavulanate	0.25, NA	≥256, NA	≤0.0625, NA	16, NA	≥256, NA
Cefepime	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S
Aztreonam	≤1, S	≥32, R	≤1, S	4, R	16, R
Imipenem	≤0.25, S	≤0.25, S	≤0.25, S	≤0.25, S	≤0.25, S
Meropenem	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S
Doripenem	≤0.0625, S	≤0.0625, S	≤0.0625, S	≤0.0625, S	≤0.0625, S
Ertapenem	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S
Gentamicin	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S
Tobramycin	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	4, I
Amikacin	≤2, S	≤2, S	≤2, S	≤2, S	≤2, S
Ciprofloxacin	≤0.125, NI	0.5, NI	≤0.125, S	≤0.125, S	1, R
Levofloxacin	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S
Azithromycin ^b	8, NA	≥256, NA	-	-	-
Doxycycline	≤1, NA	≤1, NA	≤1, NA	≤1, NA	4, NA
Minocycline	≤1, NA	≤1, NA	≤1, NA	≤1, NA	4, NA
Tigecycline	≤0.125, NA	≤0.125, NA	≤0.125, S	≤0.125, S	1, R
Co-trimoxazole	≤0.25, S	1, S	≤0.25, S	≤0.25, S	≥8, R
Colistin	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S

^aMICs were obtained with microdilution Sensititre panels (GNX2F and ESB1F) and interpreted according to EUCAST 2019 criteria (16). R, resistant; I, intermediate; S, susceptible; NI, not interpretable; NA, not available; -, not tested.

^bThe azithromycin MIC value was obtained implementing the Etest (bioMérieux) method. EUCAST 2019 does not provide interpretative criteria, but strains with MICs of ≤16 μg/ml are defined as wild type (16).

^cMICs for six *C. amalonaticus* strains were available (see Fig. S5 in the supplemental material). Because all of them had the same phenotype, we show strain 4 as a representative (this strain also underwent WGS).

In the routine clinical laboratory, stools were enriched in selenite broth and then plated on xylose lysine deoxycholate, MacConkey II, Rambach, and brilliant green agar plates (Oxoid). Bacterial identification (ID) was achieved at the genus level using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker). Species, subspecies, and serovars were determined using the White-Kauffmann-Le Minor scheme (13). Antimicrobial susceptibility tests (ASTs) were performed using the disk-diffusion method for all morphologically different colonies (14). Production of ESBLs was further investigated using the double-disk synergy test (DDST) (15), and MICs were obtained implementing both GNX2F and ESB1F Sensititre microdilution panels (ThermoFisher Scientific) and interpreted according to EUCAST criteria (16).

Based on the MIC values, *Salmonella* strain 7101.67 was confirmed as pan-susceptible, whereas *Salmonella* strain 7102.58 isolate was resistant to azithromycin, β-lactam–β-lactamase inhibitor combinations, and 3GCs but not to cefepime (Table 1). For *Salmonella* strain 7102.58, DDST results were also suspicious for the production of an inducible AmpC (see Fig. S2 in the supplemental material) (11). Both isolates were identified as *Salmonella enterica* subsp. *enterica* Worthington (13). In Western countries, this serovar is rarely detected in human or nonhuman settings (4, 5, 17). Specific data regarding Sri Lanka are scarce (18), but we emphasize that *Salmonella* Worthington has a high prevalence in India, where it is responsible for outbreaks in both hospital and community settings (19–21).

Presence of ESBL, pAmpC, and carbapenemase *bla* genes was rapidly investigated using the CT103XL microarray (Check-Points), which indicated that *Salmonella* strain 7101.67 did not possess *bla* genes, whereas *Salmonella* strain 7102.58 carried *bla*_{DHA-1}

(22). Moreover, analysis of clonality using repetitive extragenic palindromic PCR (rep-PCR) showed that the two strains had identical band patterns (see Fig. S3 in the supplemental material) (23, 24). These findings supported the hypothesis that the first isolate acquired an MGE harboring *bla*_{DHA-1}. To confirm this hypothesis, conjugation experiments were performed at 37°C with the *Escherichia coli* J53 recipient (rifampin resistant) and selection on MacConkey plates containing ampicillin and rifampin (both 50 µg/ml) (25). As a result, transconjugants possessing *bla*_{DHA-1} were obtained at a frequency of 5.2×10^{-6} (Table 1).

Several ESBL- or CMY-2-producing *Salmonella* Worthington strains have been isolated from humans (India) and food animals (United States) (4, 26), but those expressing DHA-1 had not been detected. To date, this inducible pAmpC has been reported only in *Salmonella* serovars Thompson, Enteritidis, Indiana, and Anatum (9, 10, 12, 27, 28).

For both *Salmonella* isolates, whole-genome sequencing (WGS) was performed using NovaSeq 6000 (Illumina) and MinION (Oxford Nanopore) (6, 25). Annotation was achieved using the NCBI Prokaryotic Genome Annotation Pipeline. Genomes were analyzed by employing the tools of the Center for Genomic Epidemiology (www.genomicepidemiology.org/). Results indicated that *Salmonella* isolate 7101.67 carried *aac(6')-Ia* in the chromosome and *qnrB19* on a 2.5-kb Col440I plasmid; four additional plasmids (not typeable) without antimicrobial resistance genes (ARGs) were also present. Conversely, *Salmonella* isolate 7102.58 possessed an additional 82-kb IncFII plasmid (named p7102_58-6) harboring *qnrB4*, *sul1*, *dfpA17*, *mph(A)*, and *bla*_{DHA-1} ARGs. Both *Salmonella* strains were of sequence type (ST) 592, and they were genetically identical, as confirmed by cgMLST analysis (cgST 161578; see Fig. S4 in the supplemental material).

Worldwide, *bla*_{DHA-1} is detected mostly in *Klebsiella pneumoniae* and *E. coli*, and it is harbored by plasmids of different sizes and incompatibility groups (8, 29). In Switzerland, *bla*_{DHA-1} has been associated with plasmids R, FIIk, F, and HIB (30–32). To our knowledge, only two IncFII plasmids carrying *bla*_{DHA-1} were previously reported: one (82 kb) in *E. coli* from the United Kingdom (GenBank accession no. [MK048477](https://www.ncbi.nlm.nih.gov/nuccore/MK048477)) that was almost identical to p7102_58-6 and another one (111 kb) in an ST11 *K. pneumoniae* isolate from Malaysia (GenBank accession no. [KY751925](https://www.ncbi.nlm.nih.gov/nuccore/KY751925)) (33). In all of these IncFII plasmids, the *bla*_{DHA-1} [along with *qnrB4*, *sul1*, and *mph(A)*] was part of a common large module (16.5 kb) that was similar to others already deposited and carried by different Inc group plasmids (Fig. 1). Such an element is included between two IS26, comprises a phage shock protein operon, and has likely been acquired through a transposition process (7).

In the effort to detect the natural *bla*_{DHA-1} donor carried at the gut level, stools (~100 µg) were enriched overnight in Luria-Bertani broth supplemented with cefoxitin 12 µg/ml and vancomycin 1.5 µg/ml. A total of 100 µl was plated on ChromID ESBL (bioMérieux) and incubated overnight. Thirty resistant colonies underwent PCR to detect *bla*_{DHA-1} (34); those testing positive were characterized (ID, AST, and WGS) as described above.

Unfortunately, patient stools collected during the hospitalizations (November/December 2018) were not available for further analyses. Only in April 2019 we could analyze such a sample, and a *bla*_{DHA-1}-positive *Citrobacter amalonaticus* (strain 4) was detected (see Fig. S5 in the supplemental material; Table 1). No further *bla*_{DHA-1}-positive species (e.g., *E. coli* or *Klebsiella* spp.) were found, with the exception of the *bla*_{DHA-1}-positive *Salmonella* Worthington, which was still isolated by routine culture methods. WGS analysis indicated that *C. amalonaticus* strain 4 did not harbor ARGs in the chromosome, whereas *bla*_{DHA-1} was carried on an 82-kb IncFII plasmid identical to p7102_58-6 (Fig. 1).

To our knowledge, this is the first report of *in vivo* acquisition of plasmid-mediated resistance to 3GCs in a clinical isolate of *S. enterica*. Our best hypothesis is that under ceftriaxone selective pressure, the initial pan-susceptible *Salmonella* strain acquired the *bla*_{DHA-1}-IncFII plasmid via conjugation. This MGE was carried by the *C. amalonaticus* isolate colonizing the intestinal tract and likely acquired in Sri Lanka together with the initial *Salmonella* Worthington. Our findings also emphasize that travel to the Indian

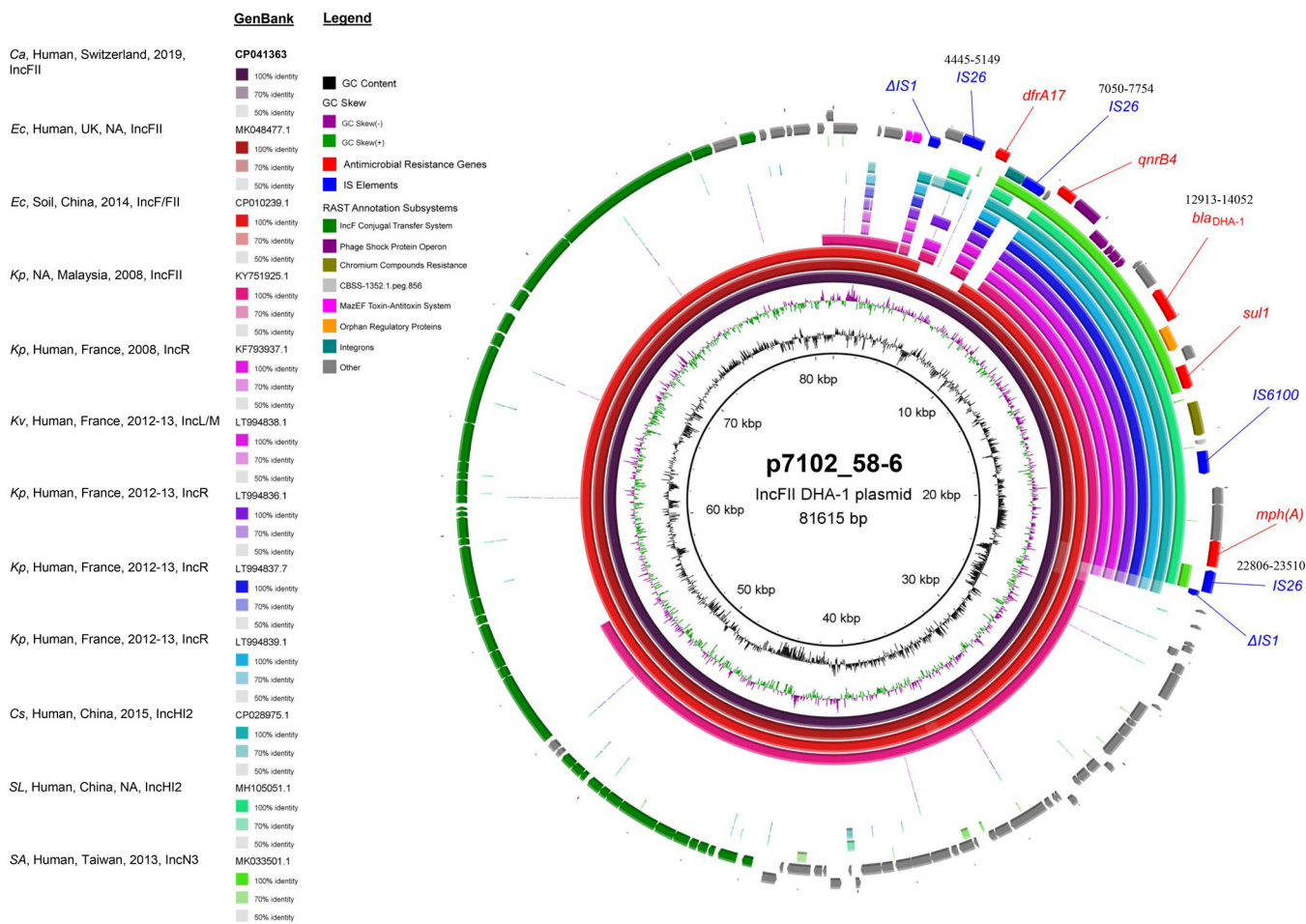


FIG 1 Map of the IncFII-DHA-1 plasmid carried by *Salmonella* Worthington strain 7102.58. From center to outer band: GC skew, GC content, BLAST hits from plasmid containing the same region, and annotated codons. For each plasmid, species, host, region, and year of isolation, along with Inc group, are indicated. Ca, *C. amalonaticus*; Ec, *E. coli*; Kp, *K. pneumoniae*; Kv, *Klebsiella variicola*; Cs, *Cronobacter sakazakii*; SL, *Salmonella* Lamita; SA, *Salmonella* Anatum; NA, not available. Image was created using BRIG software with blast option “-culling_limit 1.”

subcontinent represents a serious risk of importing unusual multidrug-resistant Gram-negative bacteria that may serve as sources of life-threatening resistance genes that can be transferred to important human pathogens.

Accession numbers. The following strains, together with their plasmids, were deposited in GenBank: *Salmonella* Worthington strain 7101.67 (GenBank accession no. CP039503–CP039508), *Salmonella* Worthington strain 7102.58 (GenBank accession no. CP039509–CP039515), and *C. amalonaticus* strain 4 (GenBank accession no. CP041362–CP041363).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00992-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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