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 was detected in the stools of a man returning from Sri Lanka. Under ceftriaxone treatment, a third-generation cephalosporin (3GCs)-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* colonizing the gut. This is the first report of *in vivo* acquisition of plasmid-mediated resistance to 3GCs in *Salmonella enterica*.

Salmonella enterica are important pathogens responsible for gastroenteritis, but may also cause invasive infections where third-generation cephalosporins (3GC) and fluoroquinolones are the treatments of choice (1). However, the rapid emergence of 3GC-resistant (3GC-R) strains in both human and non-human settings is representing 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), while the DHA-1 pAmpC is rarely reported (8-12).

In this scenario, we note that cases of infection due to *Salmonella enterica* 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.

On November 2018, a 77-year old man presented with fever and respiratory symptoms five days after returning from a two-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 revealed bacterial growth. PCR from a nasopharyngeal swab detected Influenza B. Antimicrobial treatment was stopped after 6 days and the patient discharged. Two days prior to 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 stools culture. Ceftriaxone was restarted, but upon detection of resistance towards 3GCs switched to meropenem for 3 days, and then streamlined to cefepime for 10 days (Text S1 and Figure S1: full description of the clinical case).

In the routine clinical laboratory, stools were enriched in Selenite broth and then plated on XLD, MacConkey II, Rambach, and Brilliant Green agar plates (Oxoid). Bacterial identification (ID) was achieved at genus level using the MALDI-TOF MS (Bruker). Species, subspecies and serovar 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 ESBL(s) was further investigated using the double-disk synergy test (DDST) (15), while MICs were obtained implementing both GNX2F and ESB1F Sensititre microdilution panels (ThermoFisher Scientific) and interpreted according to the EUCAST criteria (16).

Based on the MIC values, *Salmonella* 7101.67 was confirmed as pan-susceptible, whereas isolate 7102.58 was resistant to azithromycin, β-lactam/β-lactamase inhibitor combinations and 3GCs, but not to cefepime (Table 1). For 7102.58, DDST results were also suspicious for the production of an inducible AmpC (Figure S2) (11). Both isolates were identified as *Salmonella enterica* subsp. *enterica* Worthington (13). In Western countries, this serovar is rarely detected in human and non-human 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) indicating that *Salmonella* 7101.67 did not possess *bla* genes, whereas 7102.58 carried the $bla_{\text{DHA-1}}$ (22). Moreover, analysis of clonality using the rep-PCR showed that the two strains had identical band patterns (Figure S3) (23, 24). These findings supported the hypothesis that the first isolate acquired a MGE harboring the $bla_{\text{DHA-1}}$. Therefore, to confirm such hypothesis conjugation experiments were performed at 37°C using the *Escherichia coli* J53 recipient (rifampicin-resistant) and selecting on MacConkey plates containing ampicillin and rifampicin (both 50 µg/ml) (25). As a result, transconjugants possessing $bla_{\text{DHA-1}}$ 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 the DHA-1 were not yet detected. To date, this inducible pAmpC was exceptionally 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 both NovaSeq 6000 (Illumina) and MinION (Oxford Nanopore) (6, 25). Annotation was achieved using the NCBI Prokaryotic Genome Annotation Pipeline. Genomes were analyzed employing the tools of the Center for Genomic Epidemiology (www.genomicepidemiology.org/). Results indicated that isolate 7101.67 carried *aac(6')-Ia* in the chromosome and *qnrB19* on a 2.5kb Col440I plasmid; four additional plasmids (not typable) without antimicrobial resistance genes (ARGs) were also present. Conversely, *Salmonella* 7102.58 possessed an additional 82kb IncFII plasmid (named p7102_58-6) harboring *qnrB4*, *sul1*, *dfrA17*, *mph(A)*, and bla_{DHA-1} ARGs (GenBank: CP039513). Both *Salmonella* strains were of ST592 and genetically identical as confirmed by cgMLST analysis (cgST 161578; Figure S4).

Worldwide, bla_{DHA-1} is mostly detected in *Klebsiella pneumoniae* and *E. coli* and it is harbored by plasmids of different size and incompatibility group (8, 29). In Switzerland, bla_{DHA-1} has been associated to plasmids R, FIIk, F, and HIB (30-32). To our knowledge, only two IncFII plasmids carrying bla_{DHA-1} were previously reported: one (82kb) in *E. coli* from UK (GenBank: MK048477) and almost identical to p7102_58-6, while another one (111kb) in ST11 *K. pneumoniae* from Malaysia (GenBank: 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 (16.5kb) very similar to others already deposited and carried by different Inc group plasmids (Figure 1). Such element is included between two IS*26*, comprises a phage shock protein operon, and has been likely acquired through a transposition process (7).

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

Unfortunately, patient's stools collected during the hospitalizations (November/December, 2018) were not available for further analyses. Only on April 2019 we could analyze such sample and a *bla*$_{DHA-1}$-positive *Citrobacter amalonaticus* (strain #4) was detected (Figure S5, 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 that was still isolated implementing routine culture methods. WGS analysis indicated that *C. amalonaticus* #4 did not harbor ARGs in the chromosome, while the *bla*$_{DHA-1}$ was carried on an 82kb IncFII plasmid identical to p7102_58-6 (Figure 1).

To our knowledge, this is the first report of *in vivo* acquisition of plasmid-mediated resistance to 3GCs in a clinical isolate of *Salmonella enterica*. Our best hypothesis is that, under ceftriaxone selective pressure, the initial pan-susceptible *Salmonella* strain acquired via conjugation the *bla*$_{DHA-1}$-IncFII plasmid. This MGE was carried by the *C. amalonaticus* colonizing the intestinal tract and likely acquired in Sri Lanka together with the initial *Salmonella* Worthington. Our finding also emphasize that traveling to the Indian sub-continent represents a serious risk to import unusual multidrug-resistant Gram-negatives that may serve as a source of life-threatening resistance genes that can be transferred to important human pathogens.

Accession numbers. *Salmonella* Worthington 7101.67 (GenBank: CP039503-CP039508), *Salmonella* Worthington 7102.58 (GenBank: CP039509-CP039515), and *Citrobacter amalonaticus* #4 (GenBank: CP041362-CP041363).

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Table 1. Antimicrobial susceptibility tests (ASTs) for the *Salmonella*, *E. coli* J53 transconjugant, and *C. amalonaticus* strains

Antibiotics	MIC, µg/ml				
	Salmonella **Worthington 7101.67**	***Salmonella*** **Worthington 7102.58 (collected 8 days later)**	***E. coli* J53**	***E. coli* J53 Transconjugant (donor 7102.58)**	***Citrobacter amalonaticus* #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
Cephalotin	≤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
Cotrimoxazole	≤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

Note. R, resistant; I, intermediate; S, susceptible; NI, not interpretable; NA, not available; -, not tested

[a] MICs were obtained with microdilution Sensititre panels (GNX2F and ESB1F) and interpreted according to the EUCAST 2019 criteria (16)

[b] The azithromycin MIC value was obtained implementing the Etest (bioMérieux) method. The EUCAST 2019 does not provide interpretative criteria, but strains with MIC≤16 µg/ml are defined as wild-type (16)

[c] MICs for six *C. amalonaticus* strains were available (see Figure S5). Since all of them had the same phenotype, here we show only strain #4 as representative (this strain also underwent WGS)

258 **LEGEND TO THE FIGURE 1**

259

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