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CASE REPORT

Whole genome sequence analysis of NDM-1, CMY-4, and SHV-12 coproducing *Salmonella enterica* serovar Typhimurium isolated from a case of fatal burn wound infection

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Abstract: *Salmonella* species are frequently associated with gastrointestinal infections such as diarrhea. However, extraintestinal *Salmonella* infections, including burn infections, have been described. Here, we report the first case of a carbapenem-resistant and metallo-β-lactamase (New Delhi metallo-β-lactamase), extended-spectrum β-lactamase (SHV-12), and AmpC β-lactamase (CMY-4) coproducing *Salmonella* Typhimurium isolated from a fatal case of burn wound infection. The publication highlights the necessity for the rational use of antibiotics (particularly the rational use of last-resort antibiotics such as carbapenems) in hospitals and burn units, as well as the need for systematic screening of *Salmonella* spp. (including *Salmonella enterica* serovar Typhimurium) for resistance to carbapenem antibiotics.

Keywords: *Salmonella*, metallo-β-lactamase, extended-spectrum and AmpC β-lactamase, coproduction, whole genome sequencing, fatal burn wound

Introduction

Salmonella species are commonly associated with gastrointestinal infections, although extraintestinal *Salmonella* infections involving sepsis, liver abscesses, surgical wounds, joint infections, and infected burn wounds have been described.[1] Particularly important with respect to the treatment of wounds from severe burns is the application of immediate specialized and effective care, which includes antimicrobial therapy, to minimize morbidity and mortality.[2] Both intestinal and extraintestinal *Salmonella* infections are often treated with cephalosporins and quinolones, although the bacterium is becoming increasingly resistant to these antibiotics. On the other hand, carbapenem resistance in *Salmonella* is rarely reported[3] – even though resistance to carbapenem antibiotics continues to be a major public health concern in other pathogens like *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[4] Importantly, carbapenem antibiotics are considered "last-resort" antibiotics for use in the treatment of infections caused by multidrug-resistant isolates of bacteria.[5] Associated with carbapenem resistance are several carbapenemase enzymes, including New Delhi metallo-β-lactamase (NDM-1) and its variants, which hydrolyze a wide range of β-lactam antibiotics.[6] Other nonhydrolyzing mechanisms associated with carbapenem resistance in pathogenic bacteria include enhanced removal of antibiotic via an increase in bacterial efflux pump activity and mutations in bacterial cell envelope porin genes, which hinder the uptake of carbapenem antibiotics into the bacterial cell.[7]

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Case report

In this report, the authors report on the first case of a *Salmonella enterica* serovar Typhimurium isolate that is carbapenem-resistant, NDM-1-positive, extended-spectrum β-lactamase (SHV-12)-positive, and AmpC β-lactamase (CMY-4)-producing. This strain was isolated from a fatal case following a burn wound infection. To the best of our knowledge, this is the first reported case of a fatal burn wound infection associated with a NDM-1 carbapenem-resistant (multidrug-resistant) *S. enterica* serovar Typhimurium.

A 17-year-old male patient with 70% burns was admitted to a private nursing home in Kolkata, India, in 2016. All methods were carried out in accordance with relevant guidelines and regulations. The microbial isolate mentioned in this case report was cultured from sample collected during routine care and diagnostic investigations. The single patient was not specifically sampled for this study, and the subject from whom it was isolated deceased after collection of first sample. Culture of the burn wound at the Drs Tribedi and Roy Diagnostic Laboratory, Kolkata, revealed the presence of *Salmonella*. Stool and blood samples were not available for laboratory diagnosis as the patient had unfortunately passed away by the time the laboratory report was received by the private nursing home. The cultured *Salmonella* spp. isolate was initially identified biochemically using bioMérieux VITEK® 2 compact system (bioMérieux, Inc., Durham, NC, USA), followed by confirmation using specific antisera (Murex Biotech, Dartford, England). Serovar determination was performed at the Central Research Institute, Kasauli, India, according to the Kauffmann–White scheme.

Antibiotic susceptibility testing by disc diffusion (antibiotic discs purchased from HiMedia Laboratories Ltd, Mumbai, India) and the bioMérieux Vitek® 2 compact system (as per CLSI, 2014) revealed that the isolate was only susceptible to colistin, tigecycline, and polymyxin B. This isolate was resistant to the following antibiotics: ampicillin, cefoxitin, ceftriaxone, cefotaxime, ceftazidime, cefepime, aztreonam, ciprofloxacin, nalidixic acid, levofloxacin, minocycline (tetracycline), cefoperazone–sulbactam, piperacillin–tazobactam, meropenem, imipenem, chloramphenicol, gentamicin, amikacin, tobramycin, and chloramphenicol. Minimum inhibitory concentrations (MICs) of various antibiotics against *Salmonella* Typhimurium as determined by the bioMérieux Vitek® 2 compact system are listed in Table 1.

Modified Hodge test for the detection of carbapenemase was negative. However, metallo-β-lactamase (MBL) activity was detected using the double disc synergy test using imipenem and imipenem-EDTA discs (HiMedia Laboratories Ltd). But, repeated disc diffusion testing of the *Salmonella* isolate after 1 week of storage indicated that the isolate had become susceptible to meropenem, which suggested the potential involvement of bacterial outer membrane proteins (porins and efflux pumps) in the carbapenem-resistant phenotype.[8]

The isolate was positive for bla_{NDM} by multiplex PCR targeting bla_{NDM}, bla_{OXA-48}, bla_{VIM}, bla_{IMP}, and bla_{KPC}.[18] PCRs targeting five bla_{CTX-M} groups and AmpC β lactamase genes were also performed. The isolate was negative for all bla_{CTX-M} groups, but positive for the CIT Group type of AmpC β-lactamases (primer sequences [Synergy Scientific Services, Chennai, India] as per Table 2[19] and Pérez-Pérez and Hanson[20]).

Plasmid-based replicon typing indicated that the isolate possessed an FIB replicon type plasmid. In order to investigate the possibility of horizontal transfer of plasmid(s) bearing bla_{NDM} and AmpC β-lactamase CIT, a conjugation assay was performed using the clinical *Salmonella* Typhimurium isolate as donor and a sodium azide resistant *E. coli* J53 as

Table 1 MICs of antibiotics against *Salmonella* Typhimurium as determined by the bioMérieux Vitek® 2 compact system

Sl no	Antibiotic	MIC (μg/mL) interpretive criteria for resistance[a]	MIC (μg/mL) of antibiotics against the test isolate	Interpretation
1	Piperacillin–tazobactam	≥128/4[b]	≥128/4[b]	Resistant
2	Ceftazidime	≥16	≥64	Resistant
3	Cefepime	≥16	≥64	Resistant
4	Aztreonam	≥16	≥64	Resistant
5	Imipenem	≥4	≥16	Resistant
6	Meropenem	≥4	≥16	Resistant
7	Amikacin	≥64	≥64	Resistant
8	Gentamicin	≥16	≥16	Resistant
9	Ciprofloxacin	≥1	≥4	Resistant
10	Levofloxacin	≥2	≥8	Resistant

Notes: [a]MIC interpretive criteria for resistance to antibiotics based on CLSI 2014 guidelines. [b]Denotes values for 2 antibiotics together.
Abbreviations: MIC, minimum inhibitory concentration; Sl no, serial number.

recipient bacteria. The transconjugants were found to be resistant to cefotaxime and cefoxitin, but sensitive to imipenem. The transconjugates showed a 4 log decrease in MIC to cefotaxime, but were still classified as cefotaxime resistant, indicating that resistance to cefotaxime was not only plasmid mediated, but may also be due to additional mechanisms such as efflux pumps or loss of outer membrane porins. However, an efflux pump inhibition assay,[9] with cefotaxime and reserpine as the efflux pump inhibitor, indicated a lack of efflux pump-mediated resistance to cefotaxime.

As there existed the possibility of multiple genetic resistance mechanisms in the clinical *Salmonella* Typhimurium isolate, we analyzed the isolate by whole genome sequencing. Whole genome sequencing of the isolate was performed by BGI Co., Ltd. (Shenzhen, People's Republic of China) and Illumina (100×/sample, Hiseq 4000, PE100, San Diego, CA, USA). The "Phylogenomic Estimation with Progressive Refinement" program (https://github.com/enordber/pepr.git) was used to generate a phylogenomic tree from in silico translated amino acid sequences using a maximum likelihood algorithm. The program identifies the common orthologues among genomes, filters for horizontally transferred genes, aligns and concatenates sequences, and finally generates a phylogenomic tree. Subtrees with low bootstrap values are refined by subsequent steps of addition local shared genes. The genome was annotated using the Rapid Annotation Using Subsystem Technology program, a service present in the bioinformatics resource center of the Pathosystems Resource Integration Center.[10] Pathosystems Resource Integration Center's "Specialty Genes Search" tool integrates different databases to identify virulence factors (VFDB, Victors and PATRIC VF), antibiotic resistance (ARDB and CARD), drug targets (DrugBank and TTD) and human homologs (Proteins from the Reference Human Genome at NCBI RefSeq). The tool was used to identify antibiotic resistance genes in the genome of our *Salmonella* Typhimurium isolate, which included the β-lactamase-encoding genes NDM-1, CMY-4, SHV-12, OXA-9, and TEM1b. Several efflux pumps and outer membrane proteins associated with conferring resistance to antimicrobial drugs were identified. These comprised a type I secretion outer membrane protein, the TolC precursor, AcrA protein A and AcrB protein B, as well as a multidrug and toxin extrusion family efflux pump (Supplementary materials S1 and S2).

Table 2 PCR primer sequences used to confirm the presence/absence of CTX-M ESBL-encoding resistance genes

Target gene	Primer sequence 5′–3′	Amplicon size (bp)
bla_{CTX-M} grp 1	F: AAAAATCACTGCGCCAGTTC R: AGCTTATTCATCGCCACGTT	415
bla_{CTX-M} grp 2	F: CGACGCTACCCCTGCTATT R: CCAGCGTCAGATTTTTCAGG	552
bla_{CTX-M} grp 8	F: TCGCGTTAAGCGGATGAT GC R: AACCCACGATGTGGGTAC	666
bla_{CTX-M} grp 9	F: CAAAGAGAGTGCAACGGA TG R:ATTGGAAAGCGTTCATCACC	205
bla_{CTX-M} grp 25	F: GCACGATGACATTCGGG R: AACCCACGATGTGGGTAGC	327

Note: Data adapted from Woodford et al.[19]
Abbreviations: ESBL, extended-spectrum β-lactamase; F, sense primer; R, antisense primer.

Discussion

Salmonella infections in burn patients are seldom reported except for an outbreak of *Salmonella senftenburg*.[11] β-lactamase production is the main mechanism associated with antibiotic resistance in *Enterobacteriaceae*, with ESBL and AmpC β-lactamase production being of particular importance. ESBL- and AmpC β-lactamase producers have not only been isolated from hospital settings, but are also disseminated in the environment, in animals, and in healthy humans.[12] The prevalence of global CTX-M-type ESBLs exceeded TEM- and SHV-type enzymes in the early 2000s and plasmid-mediated AmpC β-lactamases of CIT-type are also currently widespread, both being major causes of β-lactam resistance.[12]

The advent of carbapenemase producing bacteria worldwide, mainly NDM-1 and its variants in *Enterobacteriaceae*, has been a major public health concern. NDM-1 hydrolyzes even "last-resort" antibiotics that are used for treating infections caused by multidrug-resistant pathogens. That said, not only the expression of β-lactamases, but alteration in bacterial cell envelope porin expression, the production of penicillin-binding proteins and the upregulation of efflux pumps are also known mechanisms responsible for carbapenem resistance among *Enterobacteriaceae*.[6]

Carbapenem resistance in *Salmonella* spp. is rarely reported. There are range of mechanisms by which *Salmonella* spp. may acquire carbapenem resistance, including acquisition of carbapenemases such as KPC, NDM, IMP, VIM, and OXA.[13] Carbapenem resistance due to the production of extended-spectrum β-lactamases ESBLs or AmpC β-lactamases combined with porin loss has also been detected in nontyphoidal *Salmonella*.[13,14] Huang et al,[15] in 2013 isolated a single community-acquired NDM-1-producing *Salmonella* Stanley strain from a child with acute diarrhea and a diagnosis of bacterial enteritis, which subsequently resolved after treatment with intravenous azithromycin and latamoxef

(an oxacephem antibiotic usually grouped with the cephalosporins). This *Salmonella* Stanley strain was resistant to all β-lactam antimicrobial drugs tested, including cephalosporins and carbapenems, but susceptible to chloramphenicol, ciprofloxacin, tetracycline, and fosfomycin, and had azithromycin MICs of 4 μg/mL.[15] Irfan et al,[16] in 2015 reported two cases of infantile diarrhea caused by MDR, NDM-1 producing, *Salmonella* Agona from Pakistan. In the first case, a stool culture grew carbapenem-resistant *Salmonella* spp., which was susceptible only to azithromycin, fosfomycin, and colistin. Treatment with 250 mg oral fosfomycin (three times a day) improved the infant's condition.[16] NDM-1 and NDM-5 producing *S. enterica* have been reported from many countries including the People's Republic of China, Pakistan, and United Kingdom.[15–17]

Conclusion

In conclusion, we report the first case of a carbapenem-resistant and MBL (NDM-1), ESBL (SHV-12), and AmpC β-lactamase (CMY-4) coproducing *Salmonella* Typhimurium isolated from a fatal case of burn wound infection. The publication highlights the necessity for the rational use of antibiotics (particularly the rational use of last-resort antibiotics such as carbapenems) in hospitals and burn units, as well as the need for systematic screening of *Salmonella* spp. (including *S. enterica* serovar Typhimurium**)** for resistance to carbapenem antibiotics.

Informed consent

All methods were carried out in accordance with relevant guidelines and regulations. The microbial isolate mentioned in this case report is cultured from sample collected during routine care and diagnostic investigations. The single patient was not specifically sampled for this study and the subject from whom it was isolated was deceased after collecting first sample. Written informed consent was obtained from the patient's next of kin for the publication of this report.

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Author contributions

KB, PS, SR, and GAM were involved in conception and the design of the study. PS, PK, and KB were involved in performing the experiments. GAM, PS, and KB cowrote the manuscript, which was revised by JPH, PK, ARW, and SR. The whole genome sequencing data was analyzed mainly by ARW and interpreted by PS, GAM, and JPH. JPH prepared the respective supplementary table. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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