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Short Communication

Emergence of multidrug resistance in locally-acquired human infections with *Salmonella* Typhimurium in Australia owing to a new clade harbouring $bla_{CTX\text{-}M\text{-}9}$

Sarah J. Sparham [a,b,1], Jason C. Kwong [a,b,c,d,1], Mary Valcanis [a], Marion Easton [e], Darren J. Trott [f], Torsten Seemann [a,c,g], Timothy P. Stinear [c,d], Benjamin P. Howden [a,b,c,d,*]

[a] *Microbiological Diagnostic Unit Public Health Laboratory, Melbourne, VIC, Australia*
[b] *Infectious Diseases Department, Austin Health, Heidelberg, VIC, Australia*
[c] *Doherty Applied Microbial Genomics, Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Parkville, VIC, Australia*
[d] *Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia*
[e] *Department of Health and Human Services, Victorian Government, Australia*
[f] *Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA, Australia*
[g] *Melbourne Bioinformatics, The University of Melbourne, Carlton, VIC, Australia*

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ABSTRACT

Antimicrobial resistance in non-typhoidal *Salmonella* is a critical problem globally, with the emergence of resistance to third-generation cephalosporins (3GCs) a particular concern. The aim of this study was to use whole-genome sequencing (WGS) to characterise recently identified human and non-human isolates of 3GC-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium from Australia. The Illumina NextSeq sequencing platform was used to determine the genome sequences of 78 *S.* Typhimurium definitive type 44 isolated in Australia between 1992 and 2016, including 31 3GC-resistant isolates. Phylogenetic and bioinformatics analyses were subsequently performed using a number of in silico tools. We report the emergence of 3GC resistance in locally-acquired Australian *S.* Typhimurium for the first time. Phenotypically resistant isolates of human and animal origin were geographically restricted and were found by WGS all to be closely related and to carry $bla_{CTX\text{-}M\text{-}9}$. Dairy cattle were the suspected source based on geographical clustering of animal isolates, which were predominantly bovine in origin. In conclusion, locally-acquired human cases of *S.* Typhimurium carrying $bla_{CTX\text{-}M\text{-}9}$ were identified that appear to be of bovine origin, raising concerns regarding the human impact of off-label use of ceftiofur in cattle.

1. Introduction

Salmonellosis is a major cause of bacterial gastroenteritis worldwide. Whilst this is a largely self-limiting disease in immunocompetent hosts, it occasionally causes invasive diseases requiring treatment with antibiotics. *Salmonella* spp. are the second most common cause of acute bacterial gastroenteritis in Australia and the leading cause of death from this condition [1]. Approximately one-half of human cases of non-typhoidal salmonellosis are due to *Salmonella enterica* subsp. *enterica* serovar Typhimurium [2].

Australian rates of antimicrobial resistance in locally-acquired *S.* Typhimurium have historically been low compared with published rates overseas [3,4]. Of note, virulent multidrug-resistant (MDR) strains, such as *S.* Typhimurium definitive type 104 (DT104), which has been prominent internationally including in Europe, have not previously been found in Australia. No *S.* Typhimurium isolates resistant to third-generation cephalosporins (3GC) in the state of Victoria have been reported in the literature prior to this study. Since 1992, a subset of *S.* Typhimurium DT44 isolates has been noted to be MDR, with raised minimum inhibitory concentrations (MICs) to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides, tetracycline and trimethoprim [5]. As far as we are aware, multidrug resistance in this strain has not previously been reported outside of Australia. Here we report the additional development of cefotaxime (CTX) resistance in MDR *S. Typhimurium* DT44 as detected by routine antimicrobial susceptibility testing performed for epidemiological purposes.

* Corresponding author. Microbiological Diagnostic Unit, 792 Elizabeth St., Melbourne, VIC 3000, Australia. Fax: +61 3 8344 7833.
E-mail address: bhowden@unimelb.edu.au (B.P. Howden).
[1] These two authors contributed equally to this work.

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2. Materials and methods

2.1. Isolates

In the state of Victoria, Australia, *Salmonella* isolates of human, veterinary, food and environmental origin were voluntarily submitted to the national *Salmonella* Reference Laboratory at the Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL) (Melbourne, VIC, Australia). Information on isolate source and geospatial data are recorded in the National Enteric Pathogen Surveillance System (NEPSS) at MDU PHL. Upon receipt, submitted isolates underwent serotyping, phage typing and antimicrobial susceptibility testing and were stored long-term at room temperature in sealed vials containing buffered *Salmonella* Maintenance Medium. All CTX-resistant *S.* Typhimurium DT44 submitted to MDU since their first detection in 2012 ($n = 31$) were included in the study. A total of 47 CTX-susceptible isolates were randomly selected from among 202 *S.* Typhimurium DT44 obtained between 1992 and 2016 for which susceptibility was known and were included for comparison.

2.2. Phenotypic antimicrobial susceptibility testing

All isolates were tested for susceptibility to ampicillin, CTX, meropenem, ciprofloxacin, trimethoprim/sulfamethoxazole, streptomycin, spectinomycin and gentamicin using agar dilution methods as previously described [6]. CTX-resistant isolates also underwent automated susceptibility testing by the VITEK®2 system (bioMérieux, Marcy-l'Étoile, France) and double-disk synergy testing using amoxicillin/clavulanic acid with aztreonam, CTX and cefepime. Cefoxitin disks were also placed on the same nutrient agar plate as a screen for the presence of an AmpC β-lactamase.

2.3. Genome sequencing and analysis

All isolates (CTX-resistant and -susceptible) underwent DNA extraction and preparation for sequencing using Nextera XT (Illumina Inc., San Diego, CA) libraries and protocols. Whole-genome sequencing (WGS) was performed on an Illumina NextSeq 500 platform (target mean coverage >50 ×; quality score >Q30) with 2 × 150-bp paired-end chemistry. Sequencing reads were processed using the Nullarbor v.1.20 pipeline (https://github.com/tseemann/nullarbor) as recently described [7], including read alignment, single nucleotide polymorphism (SNP) calling, de novo draft genome assembly and annotation, multilocus sequence typing (MLST) assignment, resistance gene detection, and pangenome comparison. A maximum likelihood phylogeny was inferred in RAxML v.8.2.8 [8] from an alignment of core genome SNPs using the genome for strain DT104 (GenBank accession no. **GCA_000493675.1**), the most phylogenetically related closed reference genome to our isolates at the time of analysis. The phylogeny was rooted using DT104 (ST19 by MLST) as an outgroup and was confirmed using ST34 *S.* Typhimurium and ST50 *S. enterica* serovar Saintpaul genomes as outgroups. All major nodes had >70% bootstrap support from 1000 replicate trees. Raw sequencing data were uploaded to the European Nucleotide Archive under study accession no. **PRJEB15708**.

3. Results

3.1. Phenotypic antimicrobial susceptibility testing

In total, 31 CTX-resistant isolates (13 human isolates and 18 animal isolates) were identified from 2012–2016, each with a ceftriaxone MIC of ≥64 μg/mL reported by VITEK®2. Positive double-disk synergy testing and cefoxitin susceptibility was consistent with extended-spectrum β-lactamase (ESBL) production and was suggestive of a CTX-M-type β-lactamase. Isolates retained susceptibility to the fluoroquinolones ciprofloxacin and norfloxacin. All 31 CTX-resistant isolates were confirmed to be DT44 by phage typing.

3.2. Genomic analysis

WGS revealed the presence of the β-lactamase-encoding gene $bla_{\text{CTX-M-9}}$ in all resistant isolates, with generally excellent correlation between the WGS resistome and phenotypic resistance. Analysis of draft assembled contigs around $bla_{\text{CTX-M-9}}$ showed co-localisation with the aminoglycoside resistance genes *aadB* and *aadA2*, the quaternary ammonium compound resistance gene *qacEΔ1*, and the sulphonamide resistance gene *sul1* on a class 1 In*60*-like integron. The contig sequence had 100% BLAST identity to a previously described In*60* variant on an IncHI2 plasmid in a *S. enterica* serotype Bovismorbificans isolate from Portugal [9]. Further analysis of genomic context was limited by the fragmented assemblies from short-read data, although IncHI2 replicons were detected in the genomes of all CTX-resistant isolates. Other resistance genes almost universally detected included *strA*, *strB*, *dfrA5*, *tet*(A), *tet*(B) and $bla_{\text{TEM-1B}}$, whilst *aph(3′)-Ia* was predominantly found in the genomes of CTX-resistant isolates.

Phylogenetic comparison demonstrated that all 3GC-resistant isolates both of human and non-human origin were closely related (≤15 SNPs difference) and formed a distinct clade from the CTX-susceptible *S.* Typhimurium DT44 lineage (Fig. 1).

3.3. Epidemiology

On review of the geographic location of the isolate sources, resistant isolates of bovine origin clustered in a dairy-producing region of Victoria, whereas human and CTX-susceptible bovine isolates sources were scattered across the state (Fig. 2). The close relatedness of isolates suggests that they may have arisen from a point source that seeds both human and animal populations intermittently.

4. Discussion

Although CTX resistance in international *S.* Typhimurium isolates has been described [10], resistance in Australian isolates has only rarely been reported and has been predominantly due to plasmid-mediated bla_{AmpC} genes [3,11,12]. Here we report the emergence of CTX-M-producing *S.* Typhimurium in Australia, with human and non-human (predominantly bovine) samples being closely related based on WGS. It is hypothesised that cattle from a dairy-producing region of Victoria are the original source given the concentration of isolates from this area and the bovine predilection of this strain, and further investigation into potential sources of the cluster of human infections is ongoing. Analysis of the resistome showed a strong association between the presence of $bla_{\text{CTX-M-9}}$ and CTX resistance as expected. There was also correlation between the CTX-resistant isolates and spectinomycin resistance, conferred by the *aadA2* gene encoding an aminoglycoside-modifying enzyme, supporting co-transmission and co-localisation of this resistance gene with $bla_{\text{CTX-M-9}}$. However, despite the genetic presence of *aadB*, which encodes the gentamicin resistance enzyme AAD(2″), isolates remained susceptible to gentamicin, suggesting it was not expressed. These findings are consistent with previous reports of the *aadB–aadA2* gene cassette in this In*60*-like class I integron, differentiating this In*60* variant from other In*60* integrons that more commonly have a *dfrA16–aadA2* cassette [9].

In Australia, the 3GC ceftiofur is registered for use in cattle, although it is limited in its use in bovines to individual cases of respiratory illness. However, given the nil withholding period in milk, anecdotal reports suggest that off-label use for bacterial foot rot in adult dairy cattle and calf scours does occur in Australia, although these indications are against current prudent use guidelines [13].