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Short Communication

First reported genome of an *mcr-9*-mediated colistin-resistant *Salmonella* Typhimurium isolate from Brazilian livestock

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ABSTRACT

Objectives: To investigate the genetic context of colistin resistance in an*mcr-9*-harbouring *Salmonella* Typhimurium ST19 strain from swine in Brazil.
Methods: Minimum inhibitory concentrations (MIC) to colistin were determined by broth microdilution. Whole-genome sequencing was performed on an Illumina MiSeq system, followed by de novo genome assembly using SPAdes 1.13.1. The draft genome sequence was annotated in Prokka using KBase online server. Downstream analyses for resistome and plasmid detection were performed using online tools available at the Center for Genomic Epidemiology. The strain was typed in silico using MLST 2.0. Phylogenetic analysis involving 24 other genomes of*Salmonella* Typhimurium ST19 and *mcr-9*-harbouring *Salmonella* Typhimurium isolated from humans, livestock and foodstuff in different regions was also performed.
Results: Assembly of the draft genome resulted in 5245 protein-coding sequences, 14 rRNAs, 83 tRNAs and a GC content of 51.81%. The strain was identified as*Salmonella* Typhimurium ST19 harbouring a 265.5-kb pN1566-2 plasmid carrying genes encoding resistance to colistin (*mcr-9.1*), aminoglycosides (*aadA1*), tetracycline [*tet*(C)] and sulfonamides (*sul1*). Our findings indicate that the *Salmonella* Typhimurium ST19 strain in this study showed low genetic variability compared with *Salmonella* Typhimurium ST19 isolated from swine and poultry in Brazil, and was less related to those reported in other countries.
Conclusions: This is the first reported genome of a phenotypically colistin-resistant*Salmonella* Typhimurium harbouring the *mcr-9* variant in Brazilian livestock. This genome will aid global investigations on epidemiological and evolutionary aspects of plasmid-mediated colistin resistance and the role of colistin-resistant *Salmonella* Typhimurium ST19 lineage as a zoonotic pathogen.

1. Introduction

Salmonella enterica serovar Typhimurium is a zoonotic food-borne pathogen posing a serious threat to public health globally, especially for the elderly, children and immunocompromised individuals [1]. Polymyxin has been used as a last-resort drug to treat diseases caused by carbapenem-resistant Enterobacteriaceae in humans [2]. However, polymyxin resistance mediated by plasmids harbouring the *mcr-1* gene or its variants has emerged among Enterobacteriaceae from food animals [1], which has been attributed to the extensive use of colistin (polymyxin E) in animal production for performance-enhancing and prophylactic purposes. Since its first identification, several variants of the *mcr-1* gene have been reported in different Enterobacteriaceae. Recently, a new *mcr* homologue (*mcr-9*) was identified in a bla_{SHV-12}-harbouring *Salmonella* Typhimurium isolate from a human patient [3]. The aim of this study was to investigate the genetic context of colistin resistance in an *mcr-9*-harbouring *Salmonella* Typhimurium ST19 strain from livestock in Brazil.

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2. Materials and methods

Between 2000 and 2017, a total of 277 isolates of *S. enterica* from pigs and pork in southern Brazil were subjected to determination of the minimum inhibitory concentration (MIC) of colistin by the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). A *Salmonella* Typhimurium strain with a colistin MIC > 8 μg/mL was subjected to whole-genome sequencing. Total DNA was extracted using a commercial kit (DNA Power Soil Kit; QIAGEN, Germany) and was quantified by fluorometry (Qubit™; Life Technologies, USA). A genomic library was prepared using a Nextera XT Kit (Illumina Inc., USA). Fragment sizes were evaluated using a capillary electrophoresis system (Fragment Analyzer; Agilent, USA), and paired-end sequencing was performed on an Illumina MiSeq system (Illumina Inc.) using a 600-cycle (2 × 300 bp) v3 kit. Reads were quality checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimmed using Trimmomatic 0.38 for removal of low-quality reads (Phred<20) and Illumina sequencing adapters. Surviving reads were assembled by de novo approach using SPAdes v.1.13.1 [4]. The generated contigs were submitted for automatic annotation in Prokka v.1.12 available at the KBase online server (https://kbase.us/). Downstream bioinformatic analyses were performed by means of online tools and databases available at the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org) to investigate the presence of antimicrobial resistance genes (ResFinder 3.2), plasmids (PlasmidFinder 2.0) and *Salmonella* pathogenicity island (SPIFinder 1.0) as well as for multilocus sequence typing (MLST 2.0). A search for virulence factors was performed by means of VFanalyzer against the Virulence Factor Database (VFDB) [5]. All analyses were performing using default parameters.

For phylogenetic analysis, a rooted phylogenetic tree was built based on a concatenated nucleotide sequence alignment of 24 selected genomes (Supplementary Table S1), including the genomes *of Salmonella* Typhimurium ST19 ($n = 16$) and *mcr-9*-harbouring *Salmonella* Typhimurium other than ST19 ($n = 8$). These ST19 strains were associated with human salmonellosis ($n = 7$) or recovered from animals or foodstuff ($n = 16$) in different countries. The complete genomes of *Salmonella* Typhimurium strain TW-Stm6 (**NZ_CP019649.1**) and *Salmonella* Typhimurium LT2 were used as reference genomes. Sequences were aligned in MUSCLE [6] and phylogenetic distances were determined by the maximum likelihood method [7] according to the Tamura–Nei model [8], using complete deletion of the gaps and 1000 bootstrap replicates. *Citrobacter amalonaticus* (GenBank accession no. **CP014015.2**) was used as an outgroup (Fig. 1).

3. Results and discussion

A total of 1 221 271 reads (280 892 330 bp) averaging 230 bp in length were used for genome assembly, generating 192 contigs with N_{50} and N_{75} values of 90 710 bp and 49 431 bp, respectively. An ~55-fold coverage draft genome with 5 084 062 bp and 51.8% G + C content was obtained. A total of 5245 protein-coding sequences, 14 rRNA (5S, 16S and 23S) and 83 tRNA genes were identified. Supplementary Fig. S1 shows a graphical representation of the annotated genome.

The genomic features of the *Salmonella* Typhimurium ST19 strain are shown in Table 1. The strain was identified by MLST as ST19. It harboured plasmid replicon types IncHI2 and IncHI2A, and a 265 560-bp pN1566-2 plasmid carrying genes encoding resistance to colistin (*mcr-9.1*), aminoglycosides (*aadA1*), tetracycline [*tet*(C)] and sulfonamides (*sul1*). The plasmid also carried the

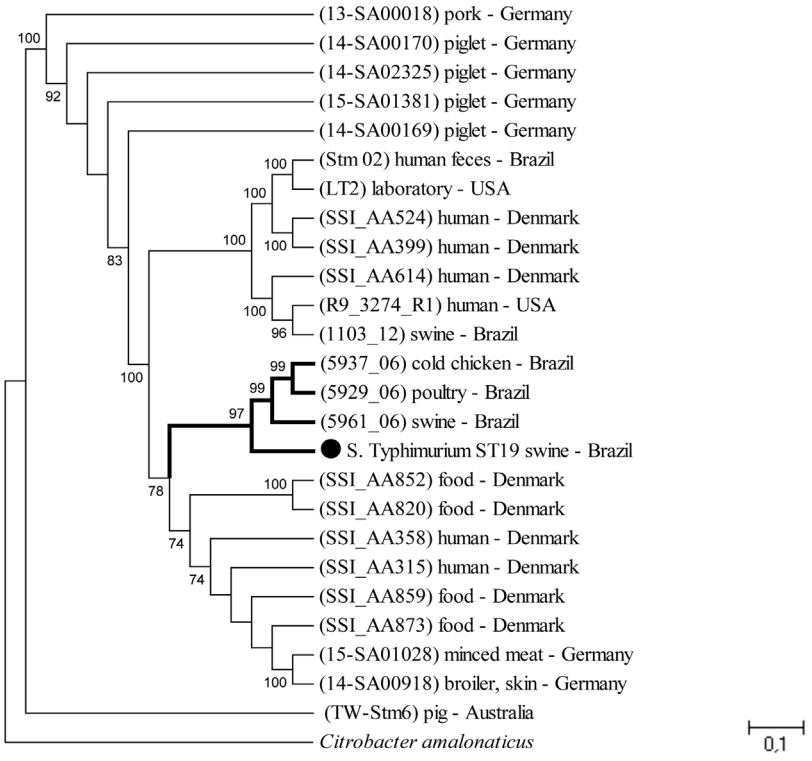

Fig. 1. Rooted phylogenetic tree of the *Salmonella enterica* serovar Typhimurium sequence type 19 (ST19) strain in this study (indicated with a filled circle) and 24 other *Salmonella* Typhimurium ST19 and *mcr-9*-carrying *Salmonella* Typhimurium from human clinical cases and foodstuff. Distances were determined by the maximum likelihood method using complete deletion of the gaps and 1000 bootstrap replicates. Bootstrap values below 70% are not shown. The scale bar indicates 0.1 changes. *Citrobacter amalonaticus* (GenBank accession no. **CP014015.2**) was used as an outgroup, and the complete genome of *Salmonella* Typhimurium strain TW-Stm6 (**NZ_CP019649.1**) was used as the reference genome.

Table 1
Genomic features of an mcr-9-carrying *Salmonella enterica* serovar Typhimurium ST19 (strain 42) recovered from swine in Brazil.

Genome size (Mb)	5.1
CDS	5245
rRNAs	14
tRNAs	83
MLST[a]	ST19
Plasmids[b]	IncHI2; IncHI2A, pN1566-2
Resistome[c]	
Aminoglycosides	*aac(6′)-Iaa*, *aadA1*
Colistin	*mcr-9*
Phenicols	*catA1*
Sulfonamides	*sul1*
Tetracycline	*tet*(C)
Trimethoprim	*dfrA8*
Virulence factors[d]	
Fimbrial adherence determinants	*csgA*, *csgB*, *csgC*, *csgD*, *csgE*, *csgF*, *csgG*, *bcfA*, *bcfB*, *bcfC*, *bcfD*, *bcfE*, *bcfF*, *bcfG*, *fimA*, *fimC*, *fimD*, *fimF*, *fimH*, *fimI*, *fimW*, *fimY*, *fimZ*, *lpfA*, *lpfB*, *lpfC*, *lpfD*, *lpfE*, *safB*, *safC*, *stbA*, *stbB*, *stbC*, *stbD*, *stbE*, *stcA*, *stcB*, *stcC*, *stcD*, *stdA*, *stdB*, *stdC*, *stfA*, *stfC*, *stfD*, *stfE*, *stfF*, *stfG sthA*, *sthB*, *sthC*, *sthD*, *sthE*, *stiA*, *sthB*, *sthC*, *sthH*, *stjB*, *stjC*
Macrophage-inducible genes	*mig-14*
Magnesium uptake	*mgtB*, *mgtC*
Non-fimbrial adherence determinants	*misL*, *ratB*, *shdA*, *sinH*
Regulation	*phoP*, *phoQ*
Secretion system	*hilA*, *hilD*, *iacP*, *iagB*, *invA*, *invB*, *invC*, *invE*, *invF*, *invG*, *invH*, *invI*, *invJ*, *orgA*, *orgB*, *orgC*, *prgH*, *prgI*, *prgJ*, *prgK*, *sicA*, *sicP*, *sipD*, *spaO*, *spaP*, *spaQ*, *spaR*, *spaS*, *sprB*, *ssaC*, *ssaD*, *ssaE*, *ssaG*, *ssaH*, *ssaI*, *ssaJ*, *ssaK*, *ssaL*, *ssaM*, *ssaN*, *ssaO*, *ssaP*, *ssaQ*, *ssaR*, *ssaT*, *ssaU*, *ssaV*, *sscA*, *sscB*, *sseB*, *sseC*, *sseD*, *sseE*, *ssrA*, *ssrB*, *slrP*, *avrA*, *sipA*, *sipB*, *sipC*, *sopA*, *sopB/sigD*, *sopD*, *sopE2*, *sptP*, *gogB*, *pipB2*, *pipB*, *sifA*, *sifB*, *sseF*, *sseG*, *sseI/srfH*, *sseJ*, *sseK1*, *sseK2*, *sseL*, *sspH2*
Stress adaptation	*sodC1*
Immune evasion	*gtrA*
Invasion	*ibeB*

CDS, coding sequences.
[a] MLST 2.0 (Multi-Locus Sequence Typing) (https://cge.cbs.dtu.dk/services/MLST/).
[b] PlasmidFinder 2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/).
[c] ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/).
[d] VFanalyzer (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer).

determinant *sdiA* encoding a resistance–nodulation–cell division (RND) antibiotic efflux pump conferring resistance to several antimicrobial classes. This plasmid was originally reported in *S. enterica* serovar Schwarzengrund strain WAPHL_SAL-A00527 (GenBank accession no. **SAMN02782579**). Fig. 2 presents the pN1555-2 plasmid map containing the 1655-bp *mcr-9* gene starting at position 160 292 in the forward DNA strand.

In Brazil, *mcr-1* has been reported to be mainly carried by IncX4 plasmids in *Salmonella* Typhimurium ST19 [9], whereas *mcr-9* has been globally reported mainly in IncHI2 plasmids as part of the core cassette structure *rcnR–rcnA–pcoE–pcoS*–IS*903*–*mcr-9*–*wbuC* [10]. The pN1555-2 plasmid reported in our study showed the *mcr-9* cassette *rcnA–pcoS–mcr-9*. Our findings support previous investigations reporting the *mcr-9* gene in plasmid types other than IncH12 [10]. However, the size of these plasmids usually ranges from 56–133 kb. To the best of our knowledge, the plasmid described in the present study (~265 kb) is the largest *mcr-9*-carrying plasmid reported in the literature.

The genome also carried genes conferring resistance to trimethoprim (*dfrA8*), aminoglycosides [*aac(6′)-Iaa*] and phenicols (*catA1*).

Interestingly, resistance determinants against broad-spectrum cephalosporins have not been detected in this genome, despite their common occurrence among *S. enterica* serovars (including Typhimurium) circulating in the food chain in Brazil [11]. Furthermore, no mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes have been detected. The results of the homology analysis performed by means of Basic Local Alignment Search Tool (BLAST) are shown in Supplementary Fig. S2.

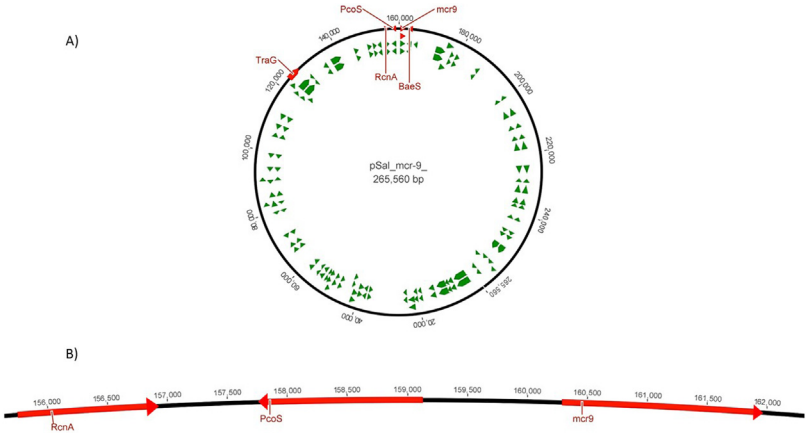

Fig. 2. Genomic representation of the pN1555-2 plasmid containing the 1655-bp *mcr-9* gene starting at position 160 292 in the forward DNA strand.

The *Salmonella* Typhimurium ST19 strain showed a large repertoire of virulence factors including fimbrial and non-fimbrial adherence determinants, macrophage-inducible genes, magnesium uptake, stress adaptation, immune evasion, secretion system and invasion (Table 1). These findings are in agreement with the different *Salmonella* pathogenicity islands (SPIs) found in the genome, including SPI-1, SPI-2, SPI-5, SPI-13, SPI-14 and C63PI (Supplementary Table S2).

Some of the virulence factors that we found in the *Salmonella* Typhimurium ST19 genome, such as effector proteins SodC1 and SopE2, are encoded by phages or phage remnants [12].

According to the phylogenetic tree (Fig. 1), the *Salmonella* Typhimurium ST19 strain in this study clustered with other *Salmonella* Typhimurium ST19 strains from chicken (5937_06), poultry (5929_06) and pigs (5961_06) in Brazil. Low genetic variability was observed among these strains, indicating no major evolutionary divergences. Therefore, our findings indicate that the *Salmonella* Typhimurium ST19 in this study is less related to those reported in other countries.

Salmonella Typhimurium ST19 is globally distributed and associated with gastroenteritis outbreaks in humans. Its capacity to survive and proliferate in vacuoles within macrophages and epithelial cells mediated by SPI-2 type III secretion system is well documented [13]. The *Salmonella* strain in the present study carried a plethora of virulence factors, consistent with the many SPI types harboured by the strain.

Our findings corroborate a previous investigation indicating that the *Salmonella* Typhimurium ST19 lineage in Brazil is particularly associated with swine and poultry reservoirs and seems to be distinct from ST19 causing human diseases worldwide [3,11,14–16]. In a recent study in Brazil, the *mcr-1* gene was found in 1.6% (8/450) of *S. enterica* isolates, the majority of which were ST19 and were recovered from pig carcasses [9]. Therefore, *Salmonella* Typhimurium ST19 seems to play an important role in the dissemination of *mcr* resistance determinants in the pork production chain.

4. Conclusion

This is the first report of a phenotypically *mcr-9*-mediated colistin-resistant *Salmonella* Typhimurium strain from livestock in Brazil. This finding reinforces the potential role of livestock as potential reservoirs of *mcr*-harbouring *Salmonella* Typhimurium ST19. This genome can aid global epidemiological studies addressing the emergence and spread of *mcr*-mediated colistin resistance, which is a global public-health problem.

Nucleotide sequence accession no

The draft genome has been deposited at GenBank under the accession no. **JABBJM000000000** (BioSample **SAMN14609621**).

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.09.012.

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