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Complete genetic analysis of a *Salmonella enterica* serovar Indiana isolate accompanying four plasmids carrying *mcr-1*, ESBL and other resistance genes in China

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ABSTRACT

One *mcr-1*-carrying *Salmonella enterica* serovar Indiana strain D90, was identified from 1320 *Salmonella enterica* isolates from poultry slaughterhouse in 2012 in China. The objective of this study was to verify the transferability of the *mcr-1* gene and also completely characterize the sequence of the strain at the whole-genome level. Broth matting assays were carried out to detect the transferability and whole-genome sequencing (WGS) of *S. enterica* serovar Indiana D90 was performed using the PacBio RS II system. Open reading frames were assigned using Rapid Annotation using Subsystem Technology (RAST) and analysed by BLASTn and BLASTp. *Salmonella* Pathogenisity Islands (SPIs) were annotated by SPIFinder platform. The complete genome sequence of *S. enterica* serovar Indiana D90 contained a circular 4,779,514-bp chromosome and four plasmids. Genome analysis and sequencing revealed that 24 multi-drug resistance (MDR) genes were located on plasmids. The largest plasmid pD90-1, was found to be of an IncHI2/HI2A/Q1/N type that encoded a $bla_{CTX\text{-}M\text{-}65}$ gene along with 20 additional antimicrobial resistance genes. A 60.5-kbp IncI2 plasmid pD90-2 contained a *nikA-nikB-mcr-1* genetic structure, that can be successfully transferred to *E. coli* and *S. enterica* serovar Typhimurium at low transfer rates. Interestingly, comparative sequence analysis revealed the plasmids pD90-1 and pD90-2 showed considerable nucleotide similarity to pHNSHP45-2 and pHNSHP45, respectively. Moreover, the genome and the plasmid pD90-2 also showed high similarity to one carbapenem resistant *S. enterica* serovar Indiana strain, C629 and its plasmid pC629, respectively. This is the first report of the complete nucleotide sequence of one *mcr-1*-carrying MDR *S. enterica* serovar Indiana strain.

1. Introduction

Salmonella enterica is the leading cause of global bacterial food poisoning outbreaks and is associated with increased morbidity and mortality (Kirk et al., 2015). *Salmonella enterica* serovar Indiana, is one of the major serovars of *S. enterica* in China and is responsible for economic losses to the livestock and poultry industries. Meanwhile, *S. enterica* serovar Indiana has gone from an infrequently reported serovar to one of the most common, especially in livestock and raw meat in China (Gong et al., 2016a). The relevance of *S. enterica* serovar Indiana is also marked by its capability to acquire resistance determinants, to various drug classes, especially those against third-generation cephalosporins, tetracyclines, (fluoro)quinolones, folate pathway inhibitors, phenicols, penicillines, monobactams and nitrofurans (Michael and Schwarz, 2016).

The plasmid-encoded polymyxin resistance gene, *mcr-1*, has been recently reported worldwide following its initial identification in *Enterobacteriaceae* from the environment, animals, and humans in China (Liu et al., 2016). Subsequently, the *mcr-1* gene, has been reported worldwide in *Escherichia coli*, *Klebsiella pneumonia* and *Salmonella*

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Table 1
Table showing the characteristic features associated with the transferability of the *mcr-1*-carrying plasmid pD90-2 to the plasmid-free *E. coli* 26R 793 and *S. enterica* serovar Typhimurium ST4/74 recipients.

Bacterial isolate	Size of plasmid(s) (kbp)	Inc type(s)	MIC of colistin (μg/mL)	*mcr-1*	Conjugative frequency
S. enterica serovar Indiana D90	222-, 60-, 38-, 17-	HI2, HI2A, N, Q1, I2, N, X1	2	+	–
E. coli 26R 793	–	–	0.03	–	–
E. coli 26R 793-(T)	60-	I2	4	+	3.2×10^{-6}
S. enterica serovar Typhimurium ST4/74	–	–	1	–	–
S. enterica serovar Typhimurium ST4/74-(T)	60-	I2	8	+	2.6×10^{-5}

(T): the corresponding transconjugants.

species (Castanheira et al., 2016; Doumith et al., 2016). Furthermore, this resistance trait has been co-transferred with the assistance of conjugative plasmids along with other antimicrobial resistant genes, such as ESBLs, metallo-β-lactamases (MBLs), and KPCs, a development that now constitutes a serious public risk for humans (Falgenhauer et al., 2016). The *mcr-1* gene has so far been reported to be associated with various plasmid replicon types, such as IncI2, IncHI1, IncHI2, IncP, IncFIB, and IncX4 among others. Among these, IncHI2 and IncX4 were the most common replicon types detected in *Salmonella* species (Yi et al., 2017).

In a recent publication, we reported the emergence and diversity of 133 *S. enterica* serovar Indiana isolates exhibiting concurrent resistance to ciprofloxacin and cefotaxime, cultured independently from 1320 *Salmonella enterica* isolates from poultry slaughterhouses in 2012 in China (Bai et al., 2016). Among these isolates, one isolate, entitled as *S. enterica* serovar Indiana D90, was detected as *mcr-1*-positive. In this study, we verified the transferability of the *mcr-1* gene and also determined the complete nucleotide sequence of *S. enterica* serovar Indiana D90.

2. Methods and materials

2.1. Identification of the mcr-1 strain

Salmonella enterica serovar Indiana D90 was isolated from a whole chicken carcasse collected in a poultry slaughterhouse in 2012 in Henan, China, and identified by using an API 20E test (bioMérieux, Beijing, China) and amplification of the *invA* gene by PCR (Bai et al., 2016; Malorny et al., 2003). PCR and subsequent amplicon sequencing was performed to confirm the presence of the *mcr-1* gene in this isolate using primers as previously reported (Liu et al., 2016).

2.2. Plasmid conjugal transfer and plasmid characterization

The *S. enterica* serovar Indiana D90 isolate was analysed for its ability to transfer the colistin resistance phenotype using broth matting to the plasmid-free recipients, *E. coli* 26R 793 and *S. enterica* serovar Typhimurium ST4/74 respectively. Transfer of *mcr-1* to transconjugants was confirmed by PCR (Liu et al., 2016). Plasmid DNA profiles of both the donor and transconjugants were carried out using the S1-nuclease digestion pulsed-field gel electrophoresis (S1-PFGE) molecular subtyping method (Wang et al., 2013).

2.3. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of colistin were determined for both the *S. enterica* serovar Indiana D90 isolate and its transconjugants using the broth dilution tests and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) document M100-S25 (CLSI, 2016). In addition, *S. enterica* serovar Indiana D90 was also tested for its susceptibility to a panel of antimicrobial compounds by disk diffusion, following recommendations of the CLSI (CLSI, 2016). Reference strain *E. coli* ATCC™ 25922 was served as a quality control.

2.4. Whole-genome sequencing and annotation

To assess the genomic background of *S. enterica* serovar Indiana D90, whole-genome sequencing (WGS) was performed using a PacBio RS II system (Pacific Biosciences, Menlo Park, CA, USA). Annotation of the genomes was performed using RAST (http://rast.nmpdr.org), BLASTn and BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) programs. The ORF Finder program (http://www.ncbi.nlm.nih.gov/orffinder) was also used to identify features. Standard method was used to annotate the *Salmonella* Pathogenisity Islands (SPIs) by SPIFinder platform (http://cge.cbs.dtu.dk/services/SPIFinder).

2.5. Nucleotide accession numbers

Sequences were deposited in GenBank under accession numbers: *S. enterica* serovar Indiana D90 genome (CP022450), plasmids pD90-1 (CP022451), pD90-2 (CP022452), pD90-3 (CP022453) and pD90-4 (CP022454).

3. Results and discussion

3.1. Horizontal transfer of mcr-1 genes and associated determinants

Four plasmids were identified from *S. enterica* serovar Indiana D90 by S1-PFGE and only one plasmid could be transferred to the recipients under laboratory conditions. Plasmid profile analysis of all transconjugants showed that the plasmid (approximately 60-kbp) of IncI2-type was transferable (Table 1). MIC values for all the transconjugants were recorded at 4- or 8-μg/mL using broth dilution tests showing 133- or 8-fold increases in the MICs of colistin when compared with the plasmid-free recipients (Table 1).

Conjugation assays revealed that *mcr-1* was transferable from *S. enterica* serovar Indiana D90 to the recipients *E. coli* 26R 793 and *S. enterica* serovar Thyphimurium ST4/74, with conjugation frequencies of 3.2×10^{-6} and 2.6×10^{-5} per recipient, respectively. No other antimicrobial resistance phenotypes were co-transferred with *mcr-1* into the transconjugants. However, the transfer frequency of the IncI2-type plasmid carrying the *mcr-1* gene reported in the original study was surprisingly high, ranging from 10^{-1} to 10^{-3} between *E. coli* strains (Liu et al., 2016). In this study these results showed that transfer rates of the *mcr-1* gene from *S. enterica* serovar Indiana to *E. coli*, and from *S. enterica* serovar Indiana to *S. enterica* serovar Typhimurium, had frequencies that were lower. Nonetheless, it confirmed that the *mcr-1*-carrying IncI2-type plasmids can be transferred between different genera of *Enterobacteriaceae*.

3.2. General features of the S. enterica serovar indiana D90 genome

The complete genome sequence of *S. enterica* serovar Indiana D90 contained a circular 4,779,514-bp chromosome with G+C content of 52.0%. There were 4628 predicted genes in the chromosome, including 573 subsystems, and 108 of RNAs. A *mcr-1* gene and three β-lactamase genes ($bla_{\text{CTX-M-65}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1B}}$) were identified in *S. enterica* serovar Indiana D90. Furthermore, the isolate also carried 20 other