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# Epidemiologic and genomic insights on *mcr-1*-harbouring *Salmonella* from diarrhoeal outpatients in Shanghai, China, 2006–2016

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ABSTRACT

*Background:* Colistin resistance mediated by *mcr-1*-harbouring plasmids is an emerging threat in Enterobacteriaceae, like *Salmonella*. Based on its major contribution to the diarrhoea burden, the epidemic state and threat of *mcr-1*-harbouring *Salmonella* in community-acquired infections should be estimated.
*Methods:* This retrospective study analysed the *mcr-1* gene incidence in *Salmonella* strains collected from a surveillance on diarrhoeal outpatients in Shanghai Municipality, China, 2006–2016. Molecular characteristics of the *mcr-1*-positive strains and their plasmids were determined by genome sequencing. The transfer abilities of these plasmids were measured with various conjugation strains, species, and serotypes.
*Findings:* Among the 12,053 *Salmonella* isolates, 37 *mcr-1*-harbouring strains, in which 35 were serovar Typhimurium, were detected first in 2012 and with increasing frequency after 2015. Most patients infected with *mcr-1*-harbouring strains were aged <5 years. All strains, including fluoroquinolone-resistant and/or extended-spectrum β-lactamase-producing strains, were multi-drug resistant. *S.* Typhimurium had higher *mcr-1* plasmid acquisition ability compared with other common serovars. Phylogeny based on the genomes combined with complete plasmid sequences revealed some clusters, suggesting the presence of *mcr-1*-harbouring *Salmonella* outbreaks in the community. Most *mcr-1*-positive strains were clustered together with the pork strains, strongly suggesting pork consumption as a main infection source.
*Interpretation:* The *mcr-1*-harbouring *Salmonella* prevalence in community-acquired diarrhoea displays a rapid increase trend, and the ESBL-*mcr-1*-harbouring *Salmonella* poses a threat for children. These findings highlight the necessary and significance of prohibiting colistin use in animals and continuous monitoring of *mcr-1*-harbouring *Salmonella*.


## 1. Introduction

Bacterial drug resistance is an ongoing severe public health problem. This issue concerns not only the bacteria causing nosocomial infections but also the pathogenic and non-pathogenic bacteria that spread in the community. Antibiotics use in hospitals and farms has caused the increasing emergence of multi-drug resistant (MDR, defined as resistant to more than three kinds of antibiotics) and pan-resistant strains, while global trade and travel contribute to their worldwide spread [1]. Additionally, there are fundamental driving forces that aggravate the spread of antibiotic resistance, including the competitive advantage for antibiotic-resistant bacteria under antibiotic pressure and

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**Research in context**

*Evidence before this study*

We searched PubMed with the terms "prevalence and *mcr-1* and *salmonella*" for reports published up to January 20, 2019, with no language restrictions. We found 18 results of relevance to this study, but none of these publications reported the prevalence and dissemination of *mcr-1*-positive *Salmonella* based on systematic clinical monitoring. The most relevant report detailed the molecular characteristics of *mcr-1*-positive *Salmonella* strains isolated from humans in China; however, it focused on a microbiological analysis of the isolates rather than on the prevalence and dissemination of these strains. Furthermore, that study lacked a systemic epidemiological surveillance and reported only two complete plasmid sequences. Thus, our search results indicated that no reports assessing the prevalence of *mcr-1*-positive *Salmonella* strains isolated from outpatients have been published previously.

*Added value of this study*

This study covering the population of a city for 11 consecutive years is the largest-scale study based on laboratory surveillance of community-acquired *Salmonella* infections. Our surveillance data allowed us to estimate the prevalence rate of *mcr-1*-harbouring *Salmonella* strains and revealed that the *mcr-1*-harbouring *Salmonella* in this study predominantly belonged to serovar Typhimurium and were mainly isolated from patients aged <5 years old. In addition to short-read sequencing, long-read sequencing with the MinION and PacBio RSII platforms was used to obtain the complete sequences of all 37 *mcr-1*-carrying plasmids, which enhanced the genome comparisons of the mega-plasmids. Data based on genome-scale comparisons indicate the presence of *mcr-1*-harbouring *Salmonella* outbreaks in the community and suggest that pork consumption is likely the contamination source for the epidemic *mcr-1*-positive *S.* Typhimurium strains.

*Implications of all the available evidence*

The present study revealed the low but rapidly increasing prevalence of *mcr-1*-harbouring *Salmonella* in community-acquired diarrhoea cases, highlighting *mcr-1*-harbouring *Salmonella* as an emerging threat in enteric infection and food safety. Additionally, this work provides a baseline for the prevalence of *mcr-1*-harbouring *Salmonella* before colistin withdrawal in China. To assess the effect of prohibiting the use of colistin as a feed additive in China, surveillance on diarrhoeal outpatients should be continued. Additionally, our results show that the spread of *mcr-1*-harbouring MDR *Salmonella* poses a threat for young children. Therefore, the risk factors of such infection patterns, as well as the transmission mode(s) and contamination source(s) should be determined through further epidemiological investigations.

the active horizontal transfer of antibiotic resistance gene-harbouring plasmids among bacteria [2]. In 2016, a plasmid encoding *mcr-1*-mediated polymyxin resistance in Enterobacteriaceae was newly recognized; its existence presents a severe threat in the global confrontation with antibiotic resistance [3]. MCR-1, coded by the *mcr-1* gene, is predicted to be an integral membrane protein with the catalytic activity of phosphoethanolamine transferases [4] that modifies the chemical structure of lipid A via the addition of phosphoethanolamine, resulting in a reduction in the binding affinity of lipopolysaccharide (LPS) to colistin [3–5]. Polymyxins are used as the last resort antibiotics for clinical infections caused by MDR Gram-negative bacteria, especially carbapenem-resistant Enterobacteriaceae [6]; however, colistin use in the farming sector has promoted the selection and transmission of *mcr-1* and *mcr*-like gene-mediated drug-resistant strains [7]. *mcr-1*-positive plasmids can also carry other antibiotic resistance genes, notably ESBL genes; this can generate resistance to multiple drugs and contribute to the spread of MDR bacteria in human populations [8].

Among the reported bacteria with *mcr-1*-mediated colistin resistance, most were isolated from animal sources, whereas only a few were isolated from hospital patients with nosocomial infections [9,10]; this uneven distribution is likely due to the asymmetric polymyxin use in these two sectors. Healthy carriers of these bacteria have also been reported [11]. Epidemiological investigations found that most *mcr-1*-harbouring plasmids appeared to be restricted to two Enterobacteriaceae species (*Escherichia coli* and *Klebsiella pneumoniae*). However, *mcr-1*-harbouring *Salmonella* have been detected from animals, food, and patients in Europe [12], as well as from swine, poultry, and carriers in China [11,13].

*Salmonella* are a common concern in food safety, as their frequent transmission from agricultural animals and food to humans causes numerous gastroenteritis cases, and these pathogens are responsible for >600,000 deaths annually [14]. Surveillance in the United States showed that non-typhoid *Salmonella* infections have been the leading cause of death among foodborne bacterial pathogens, with the highest incidence among children aged <5 years old (69.5 infections per 100,000 children) [15,16]. Furthermore, MDR *Salmonella*, which are important agents in the transmission of antibiotic resistance genes, have become a severe problem in the animal breeding sector as well as a medical threat to people [17,18].

To estimate the trend and threat of colistin resistance mediated by plasmids in community-acquired *Salmonella* infections, a population- or outpatient-based continuous epidemiological surveillance is necessary. In Shanghai Municipality in China, a laboratory surveillance project on *Salmonella* infection has been underway since 2006. Here, we conducted a retrospective study to determine the prevalence of *mcr-1*-positive *Salmonella* in community-acquired infections and to ascertain the molecular characteristics of the epidemic *mcr-1*-harbouring strains.

## 2. Materials and methods

### *2.1. Clinic-based salmonellosis surveillance on diarrhoeal outpatients in Shanghai and Salmonella strain identification*

A clinic-based *Salmonella* infection surveillance of outpatients with diarrhoea was started in Shanghai city in 2006. Shanghai is a municipality in China that had a population of 19,640,000 in 2006 and of 24,200,000 in 2016 (National Bureau of Statistics of China, http://www.stats.gov.cn/tjsj/ndsj/2017/indexch.htm). From April 2006 to June 2010, four public health laboratories in four District Centers for the Disease Control and Prevention (CDC) and 12 sentinel clinical laboratories from 12 general hospitals participated in the project. In July 2010, the number of district public health laboratories was expanded to eight, and the number of sentinel clinical laboratories was increased to a total of 24 (22 general hospitals and two paediatric hospitals) (Supplementary Fig. 1). These hospitals and CDC laboratories covered 12 of the 16 Shanghai districts.

In this *Salmonella* surveillance, diarrhoea was defined as ≥3 abnormally loose stools in the previous 24 h. Faecal samples were collected from an average of one out of four diarrhoeal outpatients and cultured for *Salmonella* spp. isolation in accordance with the World Health Organization recommended protocol of *Salmonella* isolation from stool. From April 2006 to December 2016, a total of 538,852 diarrhoeal outpatients visited the sentinel hospitals; from these outpatients, 134,868 non-duplicate faeces samples were collected, and 12,053 *Salmonella* spp. strains were isolated. All these strains were serotyped by slide agglutination with commercial

antiserum (S&A Reagents Laboratory, Thailand), and 108 serotypes were identified. All strains were preserved in lysogeny broth with 30% glycerol and stored at −80 °C. Basic clinical data were collected on the patients from whom the *mcr-1*-harbouring strains were isolated, including their age, sex, and date of *Salmonella* isolation.

### 2.2. mcr-1 gene screening

All *Salmonella* strains were recovered from the strain pool and isolated with CHROMagar *Salmonella* agar (CHROMagar Company, Paris, France). Suspected isolates were identified with the Vitek 2 system (BioMerieux, Lyon, France). The genomic DNA from each of the 12,053 strains was extracted by boiling and freeze-thawing processes, and the resulting supernatant was recovered for use as the PCR template. The *mcr-1* gene was detected using real-time PCR assays.

### 2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on the *mcr-1*-positive isolates for 23 antimicrobial agents (ampicillin (AMP), ampicillin/sulbactam 2:1 ratio (A/S2), tetracycline (TET), nalidixic acid (NAL), ciprofloxacin (CIP), azithromycin (AZI), chloramphenicol (CHL), cefazolin (FAZ), cefoxitin (FOX), ceftazidime (TAZ), cefotaxime (FOT), gentamicin (GEN), trimethoprim/sulfamethoxazole (SXT), cefazolin (CZO), cefuroxime (CXM), cefotaxime (CTX), cefotaxime/clavulanic acid (CTC), ceftazidime (CAZ), ceftazidime/clavulanic acid (CCV), aztreonam (AZT), cefepime (FEP), imipenem (IMI), and polymyxin B (PB)) using the reference broth microdilution method with custom plates (PRCDCN2, Thermo, USA) [19]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used to assess the results. EUCAST defines colistin resistance as >2 μg/mL for Enterobacteriaceae [20].

### 2.4. Genome and plasmid sequencing

DNA was extracted from each of the *mcr-1*-positive strains using the Wizard Genomic DNA Extraction Kit (Promega, Madison, USA) and was sequenced using the HiSeq sequencer (Illumina). The plasmid sequences were determined using PacBio RSII (Pacific Biosciences) and MinION (Oxford Nanopore, Oxford, United Kingdom). Plasmid DNA was purified from 100 mL of liquid culture of the strain using the Qiafilter Plasmid max kit (Qiagen) as per the protocol for low-copy plasmids. For the PacBio RSII platform, a 10-kb DNA library was constructed and sequenced using single-molecule real-time sequencing technology. For the MinION platform, the library was prepared using the ONT 1D ligation sequencing kit (SQK-LSK108) with the native barcoding expansion kit (EXP-NBD103). Porechop (https://github.com/rrwick/Porechop) was used to trim off the adaptors from the raw nanopore sequencing reads as well as to split them into different samples. The short reads of <2 kb in length were filtered out for further analysis. We assembled each genome using a combination of short- and long-reads using the SPAdes and Unicycler hybrid assembler [21].

The complete sequences of all 37 detected plasmids have been deposited in GenBank (MH522409–MH522426 and MK477602–MK477620).

### 2.5. Molecular typing and drug-resistance gene analysis

Determination of the Multi-Locus Sequence Type (MLST), plasmid replicons, and drug-resistance gene content was performed in silico using online tools (http://www.genomicepidemiology.org/).

### 2.6. Phylogenetic analysis of the genomic sequences

SNP calling was performed using Snippy 3.1, and recombination variants were excluded using ClonalFrameML 1.0 [22]. Phylogenetic trees were constructed by the maximum likelihood method with RAxML (http://phylobench.vital-it.ch/raxml-bb/index.php) based on the recombination-free SNPs. The nucleotide sequences of the common gene regions from each of the selected plasmids were obtained by using cd-hit and blast+; the core genes were then aligned and merged.

To reveal the possible source for the epidemic of *mcr-1*-carrying S. Typhimurium strains, the 35 *mcr-1*-harbouring *S.* Typhimurium strains isolated from humans in this study, another 10 *mcr-1*-positive *S.* Typhimurium strains from swine obtained by our team, and 55 *mcr-1*-negative *S.* Typhimurium genomic sequences retrieved from GenBank (including 19 from poultry, 11 from swine, and 25 from humans) were included in the phylogenetic analysis.

### 2.7. Conjugation and transformation analysis

To test the host range and the transferability of each plasmid, conjugation experiments were performed using *S.* Typhi CT18, *Escherichia coli* J53 $Azi^R$ (met pro; azide-resistant), and *Klebsiella pneumoniae* BJ1988 as recipients. Transfer of the colistin-resistance determinant by conjugation was assayed on LB agar plates (Oxoid) with an initial donor/recipient ratio of 1:1, using *E. coli* J53 as the recipient. After incubation at 37 °C for 4 h or 24 h, transconjugants were selected on LB agar supplemented with colistin (2 μg/mL) and sodium azide (100 μg/mL). When using *S.* Typhi CT18 or *K. pneumoniae* BJ1988 as recipients, the transformants were selected on LB agar with colistin (2 μg/mL) and streptomycin (5000 μg/mL). The transfer frequency is expressed as the number of transconjugants per total recipients. Positive transconjugants were confirmed by real-time PCR targeting the *mcr-1* gene. The transfer frequency data were analysed by a multiple comparison with Kruskal-Wallis using the agricolae package in R language. The default alpha parameter is 0.05. Post hoc tests used the criterium for Fisher's least significant difference. The adjustment methods included the Bonferroni correction. The factors used in our analysis were: recipient type (*E. coli* J53, *S.* Typhi CT18, and *K. pneumoniae* BJ1988 strains were tested), replicon sequence type of the *mcr-1*-positive plasmids (IncI2, IncHI2, and IncX4 plasmids were tested), and transfer time (experiments were conducted for 4 h or 24 h).

To test the ability of the different *Salmonella* serotype strains to obtain a *mcr-1*-positive plasmid, conjugation experiments using four *S.* Derby, four *S.* Enteritidis, and five *S.* Typhimurium strains as recipients and using two *E. coli* J53 carrying the *mcr-1*-positive IncI2 plasmid, two *E. coli* J53 carrying the *mcr-1*-positive IncHI2 plasmid, and one *E. coli* J53 carrying the *mcr-1*-positive IncX4 plasmid as donors were performed. In total, 65 independent conjugative experiments were conducted. The resulting transformants were selected on LB agar with colistin (4 μg/mL) and streptomycin (1000 μg/mL). Positive transconjugants were confirmed by real-time PCR. The transfer frequency is expressed as the number of transconjugants per total recipients. The transfer frequency data were analysed as described above. The factors used in our analysis were: recipient strain (four *S.* Derby, four *S.* Enteritidis, and five *S.* Typhimurium strains were tested) and replicon sequence type of the donor *mcr-1*-positive plasmid (IncI2, IncHI2, and IncX4 *mcr-1*-positive plasmids were tested).

## 3. Results

*mcr-1*-harbouring *Salmonella* emerged after 2012 and were mainly isolated from the youngest age group of outpatients.

*Salmonella* infections were surveyed yearly from 2006 in diarrhoeal outpatients in Shanghai. Following the expansion of the surveillance laboratories in June 2010, the number of strains isolated in 2011 increased (Fig. 1). Over the course of the study, 12,053 *Salmonella* strains were collected, and 37 of these were found to harbour the *mcr-1* gene. The minimal inhibitory concentration (MIC) for polymyxin B of each of these strains was 4–8 μg/mL. The first isolation of a *mcr-1*-positive strain occurred in 2012, and increasing isolation rates were observed

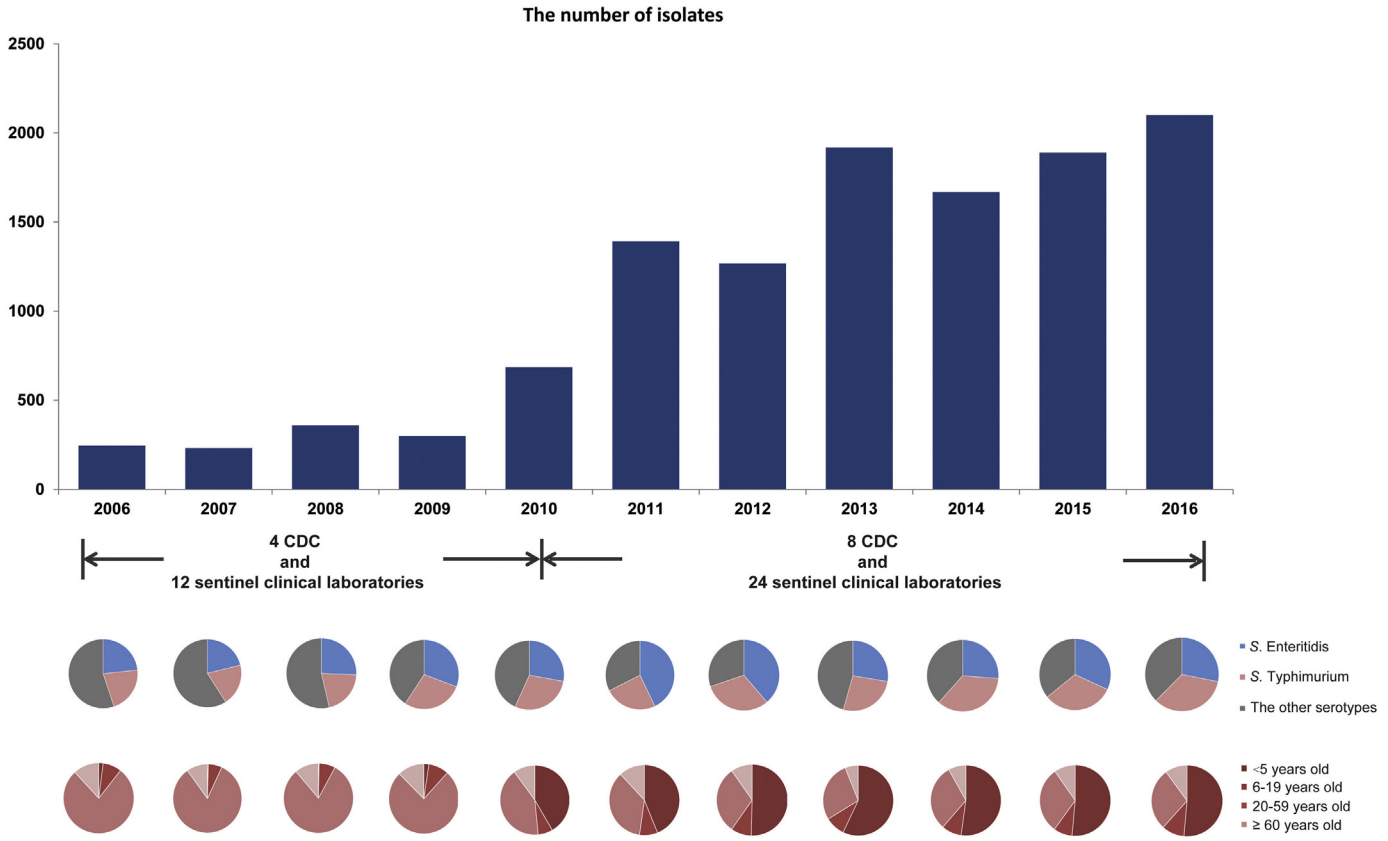



**Fig. 1.** Epidemiological description of the *Salmonella* isolates used in this study. From top to bottom: (1) the amount of isolates in each year from 2006 to 2016; (2) the serotype composition ratios of *S.* Typhimurium, *S.* Enteritidis, and the group of other serotypes in each year; (3) the composition ratios of each age group in each year.

in 2015 and 2016 (Table 1, Fig. 2a). After the initial appearance of *mcr-1*-positive *Salmonella* in 2012, its prevalence rate through 2016 in Shanghai was 4.2/1000 (37/8842) (Table 1, Fig. 2a).

Among the 37 patients infected with *mcr-1*-positive *Salmonella*, 33 (89%) were aged <5 years old (Table 1, Supplementary Fig. 2). The detection rates of *mcr-1* strains, both across all *Salmonella* serotypes and in only serotype Typhimurium, were much lower in the older age groups (Fig. 2b and c).

### 3.1. Serotypes of the mcr-1-harbouring Salmonella strains had a highly skewed distribution

Among the 37 *mcr-1*-positive *Salmonella* strains, three different serotypes were identified, but they showed a highly skewed distribution: *S.* Typhimurium (35 strains), *S.* Enteritidis (one strain), and *S.* Wandsworth (one strain) (Table 1). The *mcr-1*-positive rate of *S.* Typhimurium (1.24%) was significantly higher than that of *S.* Enteritidis (0.04%) ($p < .001$, chi-squared test) (Fig. 2d), despite similar percentages of the *Salmonella* strains isolated post-2012 belonging to each of these two serotypes (31.9% for *S.* Typhimurium and 30.0% for *S.* Enteritidis).

### 3.2. All mcr-1-harbouring Salmonella strains were multi-antibiotic resistant and most carried MDR plasmids

Antimicrobial resistance testing was performed on the 37 *mcr-1*-positive strains. Among them, 30 strains were MDR, and seven were extensively drug-resistant (XDR, defined as sensitive to less than three kinds of antibiotics) (Table 1). Additionally, 29 of these strains were ESBL-producing strains, 32 of them were resistant to fluoroquinolone, and 24 of them were resistant to both cephalosporins and fluoroquinolone; thus, these strains showed severe resistance to the antibiotics commonly used in the treatment of salmonellosis patients.

We sequenced the genomes of the *mcr-1*-positive strains and their *mcr-1*-harbouring plasmids, which yielded 37 complete *mcr-1* plasmid sequences (Table 1). Based on these complete plasmid sequences, the co-existence of various resistance genes in the plasmids and the similarity among the different plasmids could be accurately determined. Among the 37 plasmids, the replicon types IncI2, IncHI2, and IncX4 were detected in 8, 24, and 5 plasmids, respectively (Table 1, Fig. 3). The earliest isolation of a strain carrying the IncHI2 type *mcr-1* plasmid occurred in 2014; the detection of strains carrying this plasmid increased rapidly after the summer of 2015, and these strains were commonly isolated in 2015 and 2016 (Table 1, Fig. 3, 4).

The antibiotic resistance genes in the sequences of these *mcr-1*-harbouring plasmids were further identified. Twenty-four out of the 25 IncHI2 plasmids carried 4–21 resistance genes other than *mcr-1*. One of the eight IncI2 plasmids carried the $bla_{\text{CTX-M-55}}$ gene, whereas none of the IncX4 plasmids were found to harbour other antibiotic resistance genes (Supplementary Table 1, Fig. 3). Searching the assembled genome contigs of these 37 strains revealed that 35 carried ESBL genes, including $bla_{\text{CTX-M-3/14/24/55}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{CMY-2}}$, $bla_{\text{DHA-1}}$, and $bla_{\text{OXA-1}}$. Therefore, among these strains, 23 were confirmed to harbour *mcr-1* and ESBL genes ($bla_{\text{CTX-M-14/55}}$ and $bla_{\text{OXA-1}}$) on the same plasmid; this group of plasmids harbouring genes for both *mcr-1* and ESBL was comprised of one IncI2 and 22 IncHI2 plasmids (Fig. 3, Supplementary Table 1). Additionally, four plasmids simultaneously harboured *mcr-1* and *qnrS2*.

### 3.3. All the mcr-1 plasmids were transferable, and S. Typhimurium acquired the mcr-1 plasmids more easily compared with other common serotypes

To evaluate the transferability of the *mcr-1*-harbouring plasmids obtained in this study, conjugation experiments were performed. Transconjugants were obtained from each of the mixtures of the

**Table 1**
Brief information about the 37 *mcr-1*-harbouring *Salmonella* strains identified in this study and their *mcr-1* plasmids.

| Strains | Sampling date | Sex of patients | Age of patients | Serotypes | MIC of polymyxin B | Phenotype Resistance to third and fourth-generation cephalosporins[a] | Phenotype Resistance to ciprofloxacin[b] | Levels of antibiotic resistance[c] | Strategies of sequencing | Replicon sequence type of *mcr-1*-positive plasmids |
|---|---|---|---|---|---|---|---|---|---|---|
| SH12G402 | 2012/6/7 | female | 1 year | Enterica | 8 | CAZ, CTX, FEP | R | MDR | PacBio RSII | IncI2 |
| SH12G950 | 2012/7/28 | male | 10 months | Typhimurium | 8 | CTX | R | MDR | MinION +HiSeq | IncI2 |
| SH13G1582 | 2013/7/31 | male | 2 years | Wandsworth | 8 | CTX | R | MDR | PacBio RSII | IncI2 |
| SH13G841 | 2013/10/20 | male | 9 months | Typhimurium | 8 | CAZ, CTX, FEP | S | MDR | HiSeq+Sanger | IncI2 |
| SH14G1670 | 2014/6/1 | female | 2 years | Typhimurium | 4 | CTX, FEP | R | XDR | PacBio RSII | IncHI2 |
| SH15G1169 | 2015/8/26 | male | 34 years | Typhimurium | 8 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH15G1219 | 2015/9/7 | male | 1 year | Typhimurium | 4 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH15G1397 | 2015/12/10 | male | 8 years | Typhimurium | 4 | CAZ, CTX, FEP | S | XDR | MinION +HiSeq | IncX4 |
| SH15G1428 | 2015/9/7 | female | 1 year | Typhimurium | 8 | CTX, FEP | R | XDR | MinION +HiSeq | IncHI2 |
| SH15G1450 | 2015/9/7 | female | 3 years | Typhimurium | 8 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH15G1452 | 2015/10/13 | male | 1 year | Typhimurium | 4 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH15G1493 | 2015/9/28 | male | 4 years | Typhimurium | 8 | CAZ, CTX | R | MDR | MinION +HiSeq | IncI2 |
| SH15G1527 | 2015/10/19 | male | 10 months | Typhimurium | 8 | S | S | MDR | PacBio RSII | IncI2 |
| SH15G1531 | 2015/10/27 | male | 8 months | Typhimurium | 8 | S | S | MDR | HiSeq+sanger | IncI2 |
| SH15G1571 | 2015/11/14 | female | 10 months | Typhimurium | 8 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH15G1598 | 2015/12/28 | female | 2 years | Typhimurium | 8 | S | R | MDR | MinION +HiSeq | IncHI2 |
| SH15G2167 | 2015/7/15 | female | 9 months | Typhimurium | 8 | S | R | MDR | MinION +HiSeq | IncX4 |
| SH15G2169 | 2015/7/15 | male | 2 years | Typhimurium | 8 | S | R | MDR | MinION +HiSeq | IncX4 |
| SH15G2194 | 2015/10/28 | female | 1 year | Typhimurium | 4 | CAZ, CTX | R | XDR | MinION +HiSeq | IncHI2 |
| SH16G0600 | 2016/5/10 | male | 3 months | Typhimurium | 4 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G0612 | 2016/6/12 | male | 9 months | Typhimurium | 8 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G0648 | 2016/6/17 | male | 1 year | Typhimurium | 4 | CTX | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G1369 | 2016/6/26 | female | 32 years | Typhimurium | 4 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G1394 | 2016/8/1 | female | 2 years | Typhimurium | 4 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G1508 | 2016/7/18 | male | 8 months | Typhimurium | 8 | S | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G1509 | 2016/8/16 | male | 8 years | Typhimurium | 8 | CTX, FEP | S | MDR | MinION +HiSeq | IncX4 |
| SH16G2456 | 2016/6/19 | male | 1 year | Typhimurium | 4 | CTX, FEP | R | XDR | MinION +HiSeq | IncHI2 |
| SH16G2457 | 2016/8/17 | female | 1 year | Typhimurium | 4 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH16G2543 | 2016/8/14 | male | 2 years | Typhimurium | 4 | S | S | MDR | MinION +HiSeq | IncX4 |
| SH16G4466 | 2016/6/21 | female | 9 months | Typhimurium | 4 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G4498 | 2016/8/23 | male | 10 months | Typhimurium | 4 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH16G4511 | 2016/7/4 | male | 1 year | Typhimurium | 8 | CTX, FEP | S | XDR | MinION +HiSeq | IncHI2 |
| SH16G4525 | 2016/8/25 | male | 1 year | Typhimurium | 8 | CTX, FEP | R | MDR | MinION +HiSeq | IncHI2 |
| SH16G4909 | 2016/8/10 | male | 3 years | Typhimurium | 8 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G4918 | 2016/8/16 | female | 9 months | Typhimurium | 4 | CTX, FEP | S | MDR | MinION +HiSeq | IncHI2 |
| SH16G4926 | 2016/8/27 | male | 7 months | Typhimurium | 8 | CTX, FEP | S | XDR | MinION +HiSeq | IncHI2 |
| SH16G4928 | 2016/8/31 | male | 1 year | Typhimurium | 4 | CTX | S | MDR | MinION +HiSeq | IncI2 |

[a] Resistance to the third- and fourth-generation cephalosporins, including ceftazidime (CAZ), cefotaxime (CTX), or cefepime (FEP), in the antimicrobial susceptibility testing.
[b] R: resistance to ciprofloxacin; S: sensitivity to ciprofloxacin.
[c] MDR: multidrug-resistant strains, defined as resistant to more than three kinds of antibiotics; XDR: extensively drug-resistant strains, defined as sensitive to less than three kinds of antibiotics.

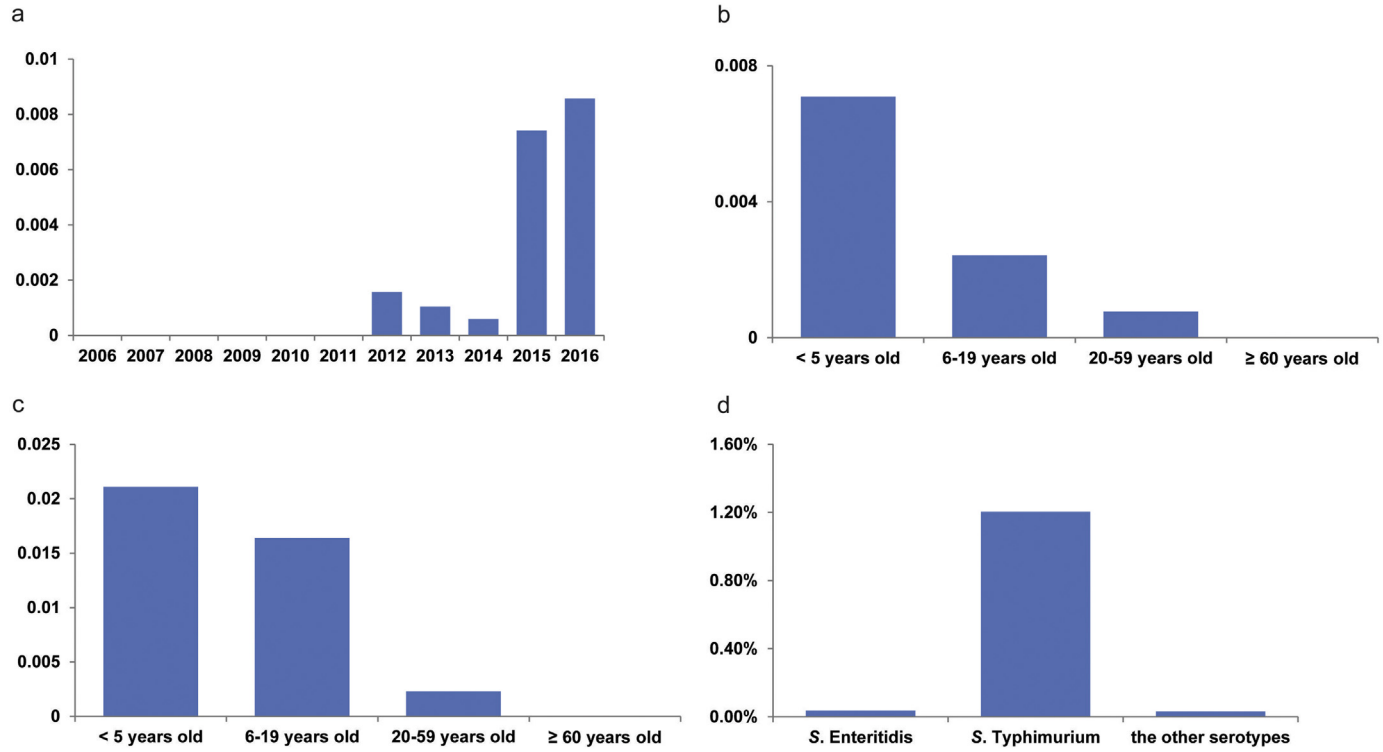



**Fig. 2.** The rate of *mcr-1*-harbouring isolates grouped by different factors. (a–b) The rates of *mcr-1*-harbouring isolates among the total *Salmonella* sp. strains in different years (a) and in different age groups (b). (c) The rates of *mcr-1*-harbouring *S.* Typhimurium strains among just the *S.* Typhimurium isolates in different age groups. (d) The rates of *mcr-1*-harbouring isolates among the total *Salmonella* sp. strains in different serotypes.

recipient *E. coli* J53 with one of the 37 *mcr-1*-positive strains. All the tested transconjugants showed 32- to 64-fold higher polymyxin B MICs compared with that of *E. coli* J53. The transfer frequency of the *mcr-1*-positive IncX4 plasmid, which is the main *mcr-1* gene carrier in *E. coli*, was higher than those of the *mcr-1*-positive IncI2 and IncHI2 plasmids ($p < .05$, Kruskal-Wallis test; Fig. 5a). All 37 *mcr-1*-harbouring plasmids were also capable of transferring from the *S.* Typhimurium strains to *S.* Typhi CT18 and *K. pneumoniae* BJ1988, and the transfer frequencies of both the IncX4 and IncI2 plasmids were higher than those of the IncHI2 plasmids ($p < .01$, Kruskal-Wallis test; Fig. 5b and c). Furthermore, when *mcr-1* plasmids were transferred from *E. coli* to *Salmonella* spp., the conjugation rates for the *S.* Typhimurium strains were up to seven or ten times higher than those of *S.* Derby and *S.* Enteritidis, respectively ($p < .05$,

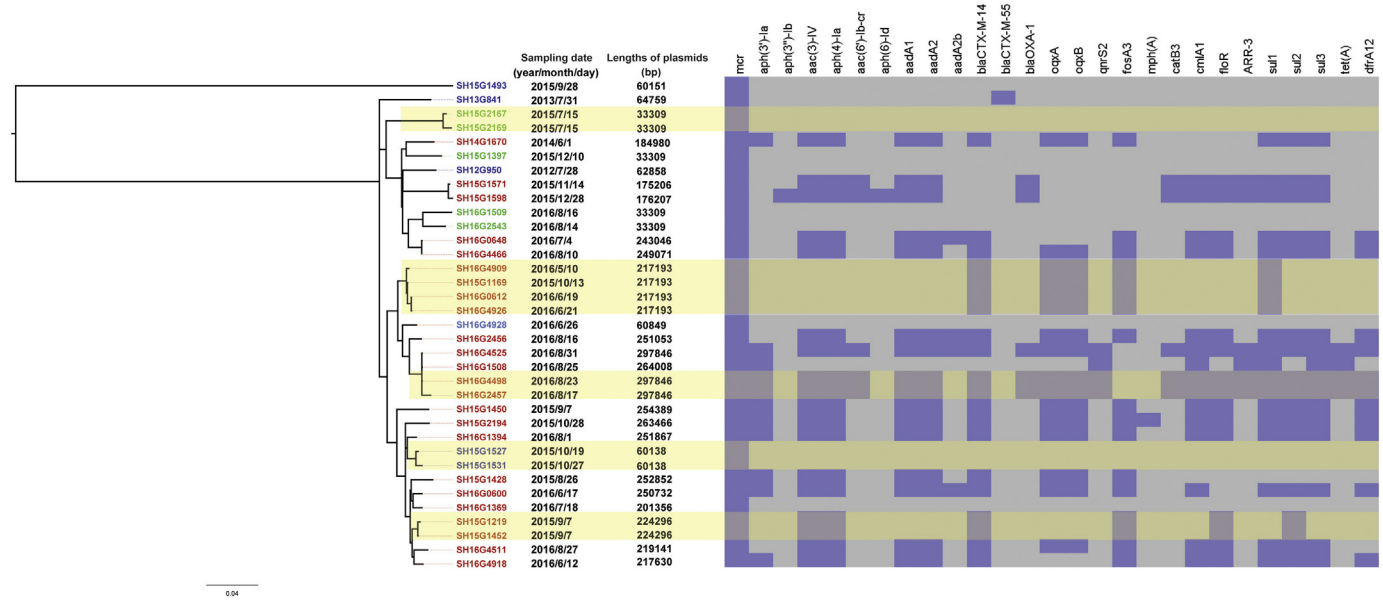



**Fig. 3.** Phylogenetic analysis of the *mcr-1*-harbouring *S.* Typhimurium isolates obtained in this study. From left to right: (1) Maximum likelihood (ML) tree of *mcr-1*-harbouring *S.* Typhimurium isolates. The font colour of the labels in the tree represents the replicon sequence type of the *mcr-1*-harbouring plasmids (blue: IncI2, red: IncHI2, green: IncX4); (2) The sampling dates of the outpatients; (3) The length of the *mcr-1*-harbouring plasmids; (4) A heatmap of the antimicrobial resistance genes on the *mcr-1*-harbouring plasmids. The yellowish shade indicates close genomic clonal clusters.

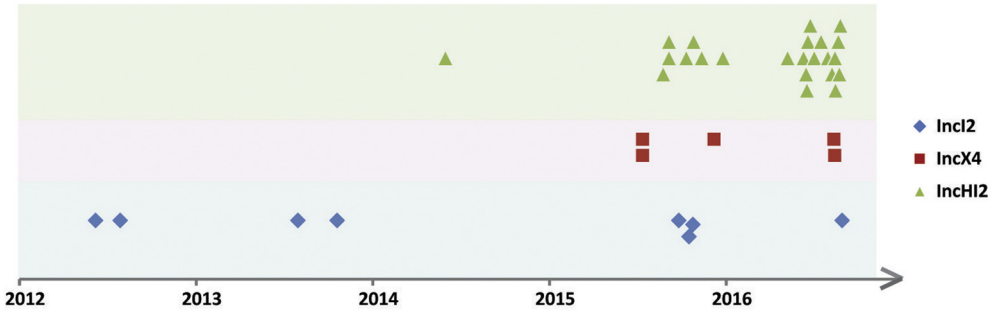



**Fig. 4.** Distribution of the replicon sequence types of *mcr-1*-harbouring plasmids, based on the sampling dates.

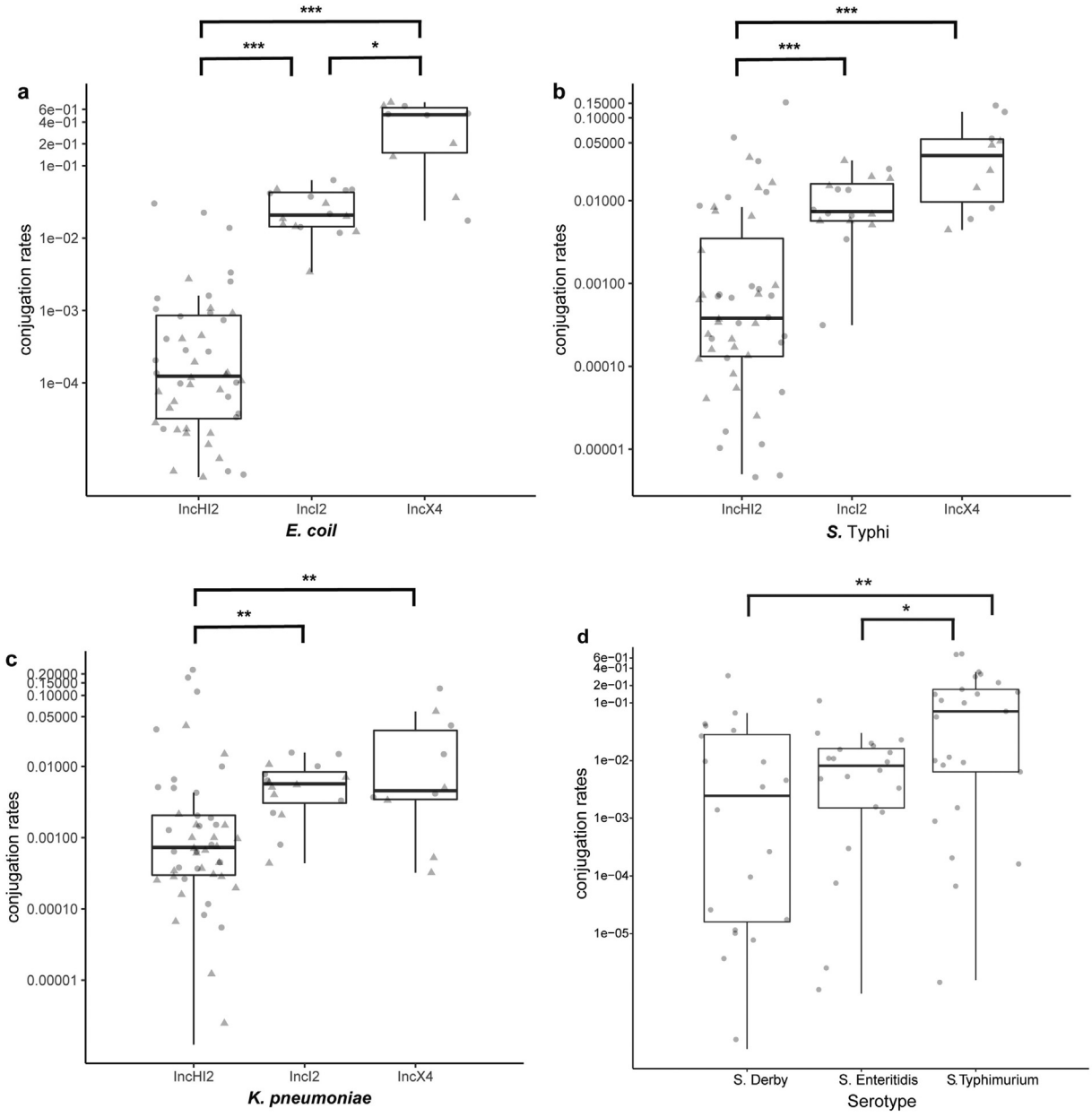



**Fig. 5.** The conjugation rates of the *mcr-1*-harbouring plasmids isolated in this study. (a–c) The 37 *mcr-1*-harbouring *Salmonella* strains were used as the donors, and *E. coli* J53 strain (a), *S.* Typhi CT18 strain (b), or *K. pneumoniae* BJ1988 strain (c) was used as the recipient. (d) Five *E. coli* J53 strains (two carrying the *mcr-1* IncHI2 plasmid, two carrying the *mcr-1* IncI2 plasmid, and one carrying the *mcr-1* IncX4 plasmid) were used as the donors, and four *S.* Derby strains, four *S.* Enteritidis strains, and five *S.* Typhimurium strains were used as the recipients. The grey triangles and circles are the $\log_{10}$-transformation of the transfer frequency data after incubation at 37 °C for 4 h and 24 h, respectively.

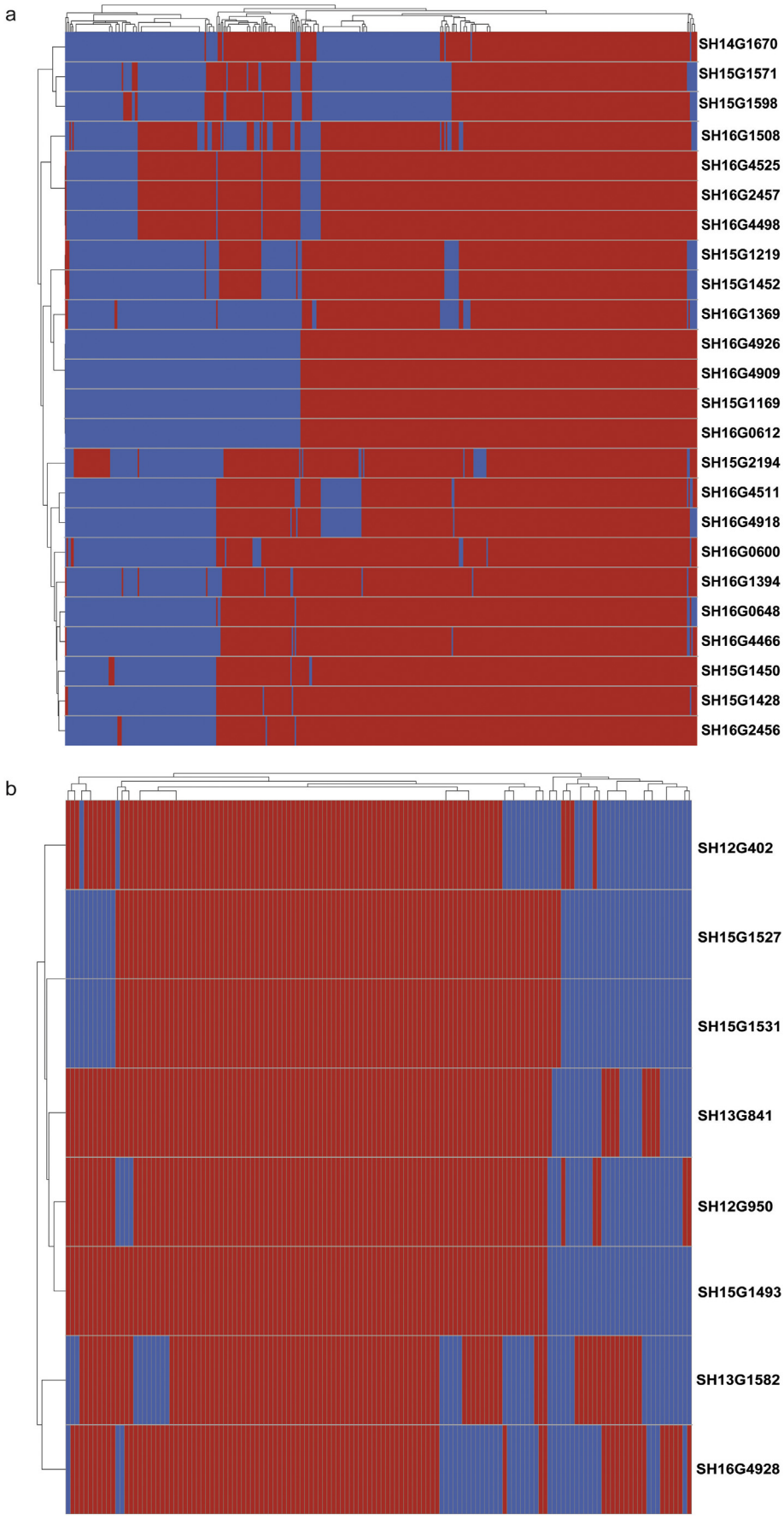
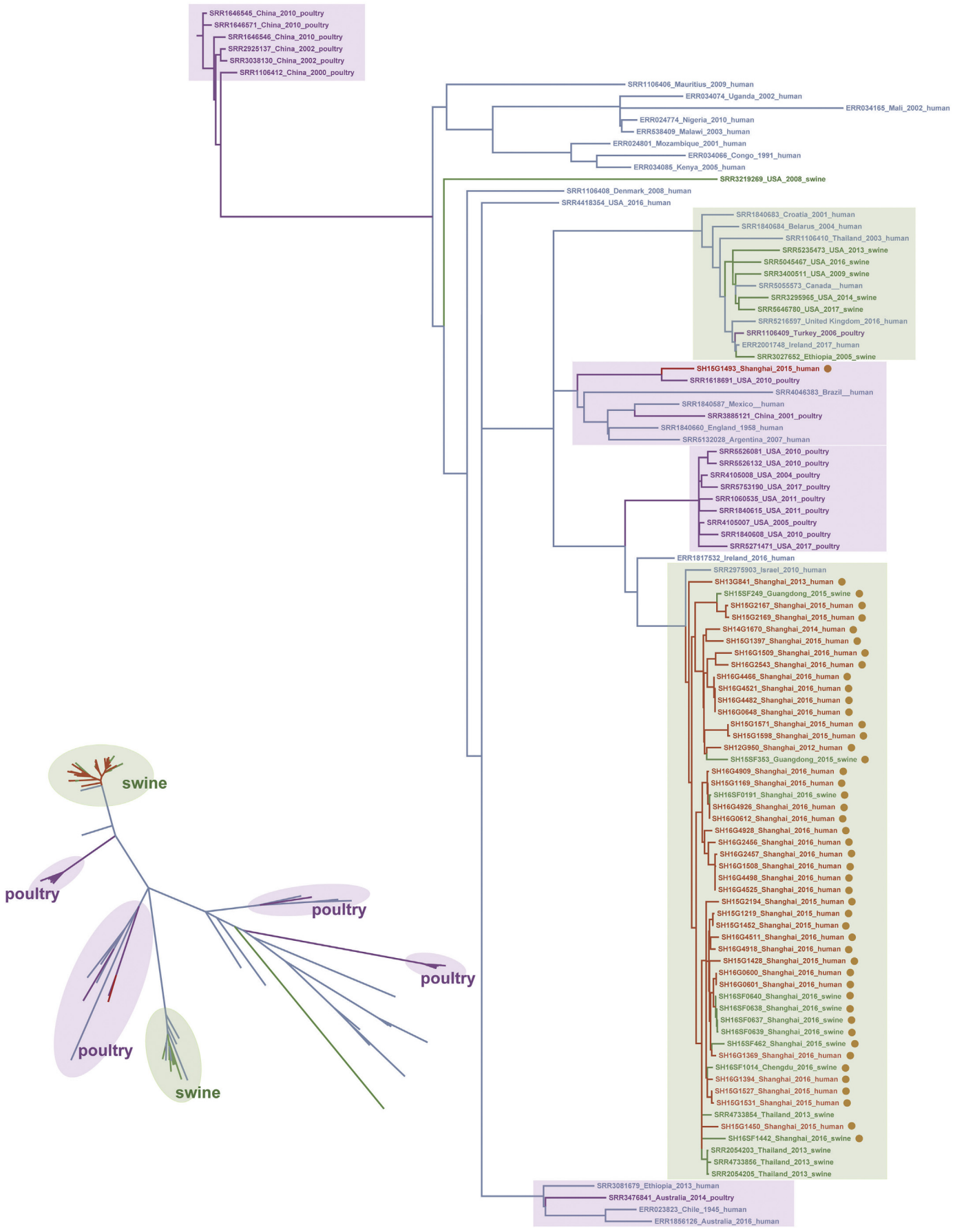



**Fig. 6.** Heatmap of *mcr-1*-harbouring plasmids based on the presence of coding sequences (cds). (a–b) Heatmap of *mcr-1*-harbouring IncHI2 plasmids (a) and *mcr-1*-harbouring IncI2 plasmids (b). Red represents the presence of similar cds.


SRR1646545_China_2010_poultry
SRR1646571_China_2010_poultry
SRR1646546_China_2010_poultry
SRR2925137_China_2002_poultry
SRR3038130_China_2002_poultry
SRR1106412_China_2000_poultry
SRR1106406_Mauritius_2009_human
ERR034074_Uganda_2002_human
ERR034165_Mali_2002_human
ERR024774_Nigeria_2010_human
ERR538409_Malawi_2003_human
ERR024801_Mozambique_2001_human
ERR034066_Congo_1991_human
ERR034085_Kenya_2005_human
SRR3219269_USA_2008_swine
SRR1106408_Denmark_2008_human
SRR4418354_USA_2016_human
SRR1840683_Croatia_2001_human
SRR1840684_Belarus_2004_human
SRR1106410_Thailand_2003_human
SRR5235473_USA_2013_swine
SRR5045467_USA_2016_swine
SRR3400511_USA_2009_swine
SRR5055573_Canada__human
SRR3295965_USA_2014_swine
SRR5646780_USA_2017_swine
SRR5216597_United Kingdom_2016_human
SRR1106409_Turkey_2006_poultry
ERR2001748_Ireland_2017_human
SRR3027652_Ethiopia_2005_swine
SH15G1493_Shanghai_2015_human
SRR1618691_USA_2010_poultry
SRR4046383_Brazil__human
SRR1840587_Mexico__human
SRR3885121_China_2001_poultry
SRR1840660_England_1958_human
SRR5132028_Argentina_2007_human
SRR5526081_USA_2010_poultry
SRR5526132_USA_2010_poultry
SRR4105008_USA_2004_poultry
SRR5753190_USA_2017_poultry
SRR1060535_USA_2011_poultry
SRR1840615_USA_2011_poultry
SRR4105007_USA_2005_poultry
SRR1840608_USA_2010_poultry
SRR5271471_USA_2017_poultry
ERR1817532_Ireland_2016_human
SRR2975903_Israel_2010_human
SH13G841_Shanghai_2013_human
SH15SF249_Guangdong_2015_swine
SH15G2167_Shanghai_2015_human
SH15G2169_Shanghai_2015_human
SH14G1670_Shanghai_2014_human
SH15G1397_Shanghai_2015_human
SH16G1509_Shanghai_2016_human
SH16G2543_Shanghai_2016_human
SH16G4466_Shanghai_2016_human
SH16G4521_Shanghai_2016_human
SH16G4482_Shanghai_2016_human
SH16G0648_Shanghai_2016_human
SH15G1571_Shanghai_2015_human
SH15G1598_Shanghai_2015_human
SH12G950_Shanghai_2012_human
SH15SF353_Guangdong_2015_swine
SH16G4909_Shanghai_2016_human
SH15G1169_Shanghai_2015_human
SH16SF0191_Shanghai_2016_swine
SH16G4926_Shanghai_2016_human
SH16G0612_Shanghai_2016_human
SH16G4928_Shanghai_2016_human
SH16G2456_Shanghai_2016_human
SH16G2457_Shanghai_2016_human
SH16G1508_Shanghai_2016_human
SH16G4498_Shanghai_2016_human
SH16G4525_Shanghai_2016_human
SH15G2194_Shanghai_2015_human
SH15G1219_Shanghai_2015_human
SH15G1452_Shanghai_2015_human
SH16G4511_Shanghai_2016_human
SH16G4918_Shanghai_2016_human
SH15G1428_Shanghai_2015_human
SH16G0600_Shanghai_2016_human
SH16G0601_Shanghai_2016_human
SH16SF0640_Shanghai_2016_swine
SH16SF0638_Shanghai_2016_swine
SH16SF0637_Shanghai_2016_swine
SH16SF0639_Shanghai_2016_swine
SH15SF462_Shanghai_2015_swine
SH16G1369_Shanghai_2016_human
SH16SF1014_Chengdu_2016_swine
SH16G1394_Shanghai_2016_human
SH15G1527_Shanghai_2015_human
SH15G1531_Shanghai_2015_human
SRR4733854_Thailand_2013_swine
SH15G1450_Shanghai_2015_human
SH16SF1442_Shanghai_2016_swine
SRR2054203_Thailand_2013_swine
SRR4733856_Thailand_2013_swine
SRR2054205_Thailand_2013_swine
SRR3081679_Ethiopia_2013_human
SRR3476841_Australia_2014_poultry
ERR023823_Chile_1945_human
ERR1856126_Australia_2016_human
swine
poultry
poultry
poultry
poultry
swine

Kruskal-Wallis test; Fig. 5d), suggesting that *S.* Typhimurium is more efficient at acquiring *mcr-1*-harbouring plasmids.

### 3.4. Several of the mcr-1-carrying S. Typhimurium strains formed close genomic clonal clusters

To evaluate the genomic clonality and similarity of the 35 *mcr-1*-harbouring *S.* Typhimurium strains, their phylogenetic relationships were analysed based on 1231 recombination-free SNPs; these are presented in Fig. 3 as a maximum likelihood tree. Our analysis shows that the ST19 strain SH15G1493 formed a single branch 546 SNPs away from the ST34 strains. The other 34 ST34 strains comprised several different but closely related phylogenetic branches. At least five clonal clusters of strains were identified: SH15G2167/SH15G2169, SH16G4909/SH15G1169/SH16G0612/SH16G4926, SH16G4498/SH16G2457, SH15G1527/SH15G1531, and SH15G1219/SH15G1452 (Fig. 3). In each of these clusters, a limited number of core SNPs (1 to 10) existed in the chromosome genomes, which were each separated from the nearest neighbour isolate by >29 core SNPs. Additionally, the *mcr-1*-carrying plasmids within each cluster have the same lengths, the same antimicrobial resistance gene composition patterns, a similar presence of coding sequences, and a limited number of variations (0 to 4 SNPs) in the common genes of the plasmids (Fig. 3, 6), strongly supporting a close genetic relationship among the plasmids in each cluster. The intervals between the clinical visits of the corresponding patients were within 40 days for the *mcr-1*-positive isolates in each cluster, except for SH15G1169 in its cluster. This result could suggest that the strains within each cluster may have had a common source, and the corresponding cases likely had epidemiological associations.

### 3.5. Genomic comparisons reveal a probable swine source for the epidemic of mcr-1carrying S. Typhimurium strains

*S.* Typhimurium is a very common pathogen that can infect humans via the meat products of poultry and livestock. We selected *S.* Typhimurium strains from human, swine, and poultry sources (including 45 *mcr-1*-harbouring strains and 55 *mcr-1*-negative strains) (Supplementary Table 2) and performed a phylogenetic comparison based on the genomic sequences. In total, 7091 recombination-free SNPs were obtained. Among the 35 *mcr-1*-harbouring *S.* Typhimurium strains isolated from patients in our study, only one strain, SH15G1493, was clustered with the poultry-source strains, whereas the other 34 strains were clustered in the branch containing swine-source *S.* Typhimurium strains, particularly those from Thailand and China (Fig. 7). These data suggest the consumption of pork as the likely contamination source.

## 4. Discussion

Diarrhoea caused by *Salmonella* is a severe foodborne disease in humans. After the first report of *mcr-1*-mediated colistin resistance in Enterobacteriaceae, *Salmonella* were also surveyed for the presence of *mcr-1*-harbouring plasmids. A study in Portugal found a prevalence rate of 1.5% for *mcr-1*-harbouring *Salmonella* 1,4,[5],12:i:- strains in 2011–2015 [23], and *mcr-1*-harbouring *Salmonella* isolates have been reported in sporadic cases [24], healthy carriers [11], and non-human sources, such as food and farm animals [13]; however, the epidemiology-based prevalence of *mcr-1*-mediated colistin resistance in *Salmonella* spp. infecting outpatients remained unclear. Here, our 11-year retrospective analysis of *Salmonella* from a successive diarrhoeal outpatient surveillance revealed the appearance and increasing prevalence of *mcr-1*-mediated colistin resistance in community-acquired *Salmonella* infections. The strains screened in this study were collected via a designed, continuous laboratory-based surveillance; this approach can provide comparable data groups to assess the emergence, positive rates, age-group distribution, and strain-serotype distribution of *mcr-1*-harbouring *Salmonella* strains. Our study highlights the emerging problem of *mcr-1*-carrying *Salmonella* infection and spread in the community.

We observed that the *mcr-1*-positive *Salmonella* strains were recovered from outpatients in Shanghai only after 2012, with the most explosive increase in detection occurring after 2015. This pattern warns of the threat posed by an expansion in the community of *Salmonella* with *mcr-1*-mediated colistin resistance. Although the total positive rate of *mcr-1*-harbouring *Salmonella* strains in this study was low, our conjugation experiments show that all the plasmids harboured by these *Salmonella* strains can transfer from/to *Salmonella*, demonstrating the ability of *Salmonella* to spread these plasmids as either the recipient or vector. A similar dramatic increase in *mcr-1* detection was also found in clinical *E. coli* isolates from China [25]. Together with our data, these findings may support a common expansion of *mcr-1*-harbouring strains in humans after 2012 and expose the potential threat presented by *Salmonella* strains carrying *mcr-1* plasmids via their role in transmitting the *mcr-1* resistance gene.

Our *Salmonella* surveillance covered all age groups of outpatients; however, 90% (33/37) of outpatients suffering from infection with *mcr-1*-harbouring *Salmonella* were children aged <5 years, revealing a much higher positive rate of *mcr-1*-harbouring *Salmonella* in the child group than in the adult group. Diarrhoea is the second leading cause of death in children under five years old and is responsible for killing 2195 children every day worldwide [26]. Furthermore, *Salmonella* is a prominent cause of bacterial diarrhoea in children aged <5 years. The higher positive rates of *mcr-1*-harbouring *Salmonella* in children highlights the threat posed by these pathogens. Notably, 26 of the 33 *mcr-1*-positive strains isolated in the present study simultaneously carried ESBL genes, which confer resistance to the common antibiotics used for the treatment of diarrhoea in children, such as cephalosporins [27]. Children are particularly vulnerable in MDR Enterobacteriaceae epidemics due to the lack of broad-spectrum antibiotics approved for use in this age group. Here, because of the co-resistance to third-generation cephalosporins and colistin in *Salmonella* strains isolated from children, the use of cephalosporin may result in treatment failure and enrich the *mcr-1*-positive strains in gut flora. These data indicate that the infection of children by bacteria with *mcr-1*-mediated colistin resistance has reached the forefront of the emerging antibiotic resistance problem.

The higher rate of *mcr-1*-harbouring *Salmonella* in the low-age group may be related to the diets, intra-familial transmissions, behaviours, and compromised immune systems of children. Previous work has reported some infection risk factors, such as immunosuppression associated with *mcr-1*-harbouring Enterobacteriaceae infection [25] and mother–child transmission as a potential means of new-born colonization by

**Fig. 7.** Dendrograms of *mcr-1*-positive *S.* Typhimurium isolates from different hosts. In addition to the 35 *mcr-1*-harbouring *S.* Typhimurium strains from humans found in this study, another 10 *mcr-1*-positive *S.* Typhimurium strains from swine obtained by our team and 55 *mcr-1*-negative *S.* Typhimurium genomic sequences retrieved from GenBank (comprised of 19 from poultry, 11 from swine, and 25 from humans) were also included in this analysis. Red: *mcr-1*-harbouring *S.* Typhimurium strains isolated from humans; Green: *S.* Typhimurium strains isolated from swine; Purple: *S.* Typhimurium strains isolated from poultry; Blue: *mcr-1*-negative *S.* Typhimurium strains isolated from humans. Right phylogenetic tree: the strains carrying *mcr-1* plasmids are marked with light-brown spots, the branches containing swine-source strains are marked with light green boxes as the background, and the branches containing poultry-source strains are marked with light purple boxes. Bottom-left phylogenetic tree: the branches containing strains from different sources are marked with the same colour format as the phylogenetic tree on the right, to more clearly present the separation of the branches and locations of the *mcr-1*-harbouring strains.

ESBL-producing strains [28]. Bed and toy sharing in the day-care setting can also be considered as a risk for community transmission of *mcr-1*-harbouring strains. Additionally, a survey conducted in a paediatric hospital in Hangzhou, a city near Shanghai, found that a high rate (9.8%) of non-diarrhoeal paediatric patients carried *mcr-1*-harbouring *E. coli* in their gut flora, and more than half of these strains also carried ESBL genes [29]. This indicates a relatively higher carriage of *mcr-1*-positive strains in children and may support the possibility of plasmid transfer between bacterial species in the intestine. To monitor the low-age-group infection preference of *mcr-1*-harbouring *Salmonella* and identify possible transmission factors, continuous surveillance and detailed epidemiological investigations are needed.

Adult gastroenteritis is usually treated with Norfloxacin, Ciprofloxacin, and Levofloxacin. In this study, most *mcr-1*-harbouring strains were resistant to fluoroquinolone, and four *mcr-1*-harbouring plasmids carried *qnrS*2 genes, with three of these four also simultaneously carrying ESBL genes. Fluoroquinolone-resistant *Salmonella* were regarded by the WHO in 2017 as priority pathogens for which new antibiotics were urgently needed [30]; therefore, the spread of *mcr-1*-ESBL-*qnrS*2 MDR *Salmonella* may lead to clinical treatment failure. Importantly, although colistin is rarely used in the treatment of salmonellosis, the administration of antibiotics that are used commonly in clinics may unintentionally promote the co-selection and preservation of colistin-resistant *Salmonella* strains in humans due to the presence of *mcr-1*-harbouring plasmids in *Salmonella* strains that are also resistant to other antibiotics.

Here, based on the results of short- and long-read sequencing, we assembled the complete sequences of all 37 *mcr-1*-harbouring plasmids. The use of complete plasmid sequences ensured accurate analyses of the co-existence of different resistance genes and accurate similarity comparisons between the MDR plasmids. Whole genome sequencing and comparisons among the *mcr-1*-harbouring plasmids and their hosts verified that a variety of *mcr-1*-carrying *Salmonella* clones have spread in the community in recent years. The same or highly similar plasmids were found in host strains belonging to different genomic subclades, showing the broad and active transfer abilities of these plasmids into different host strains. Bacterial genome sequencing can be applied to identifying genetic clusters as well as to detecting outbreaks and tracking sources [31]. Here, some clusters of cases were identified that had extremely similar chromosomal and plasmid sequences, the same antimicrobial resistance patterns, and even similar onset dates; together, these findings strongly suggest that *mcr-1*-harbouring *S.* Typhimurium triggered outbreaks and that the rapid expansion of colistin-resistant strains in the community presents a potential public health threat. The early identification of *mcr-1*-harbouring *S.* Typhimurium through active surveillance may help prevent their expansion.

Our genomic phylogeny analysis provided evidence of a clonal relationship among the *mcr-1*-harbouring strains from the outpatients in this study and those from swine. Agricultural animals, particularly pigs, have been singled out as the most likely reservoir for the amplification and spread of Enterobacteriaceae that are resistant to colistin and other antibiotics [32–34]. The same major *mcr-1*-carrying plasmid types as those in the present study were also detected in isolates from a pig farm located in Shanghai [35]. Therefore, our study provides strong genome epidemiology-based evidence that the consumption of pork is the likely contamination source of *mcr-1*-harbouring *S.* Typhimurium.

Since the first description of *mcr-1*, seven other *mcr*-like genes (*mcr-2* to *mcr-8*) have been identified [36–42]. Despite the phylogenetic diversity of these *mcr*-like genes, it seems likely that functional unification occurs among them [7,43–46]. Over the course of our study, several *mcr*-like genes have been reported in *Salmonella*, such as *mcr-3*[46], *mcr-4*[38], and *mcr-5*[39]. Only the *mcr-1* gene was screened in the *Salmonella* isolates in this study, but adding screening for other *mcr*-like genes in future work will provide more data for a better overview of the spread of *mcr*-mediated resistance.

In summary, our study revealed the increasing prevalence of *mcr-1*-harbouring *Salmonella* in outpatients with community-acquired diarrhoea and indicated that these strains were also found in potential outbreaks. The skewed distributions of the *mcr-1*-harbouring strains across outpatient age groups (more prevalent in patients aged <5 years) and *Salmonella* serotypes (more prevalent in *S.* Typhimurium) may provide insight into the distinct manner of their transmission; hence, further studies on the epidemiology and microbiology of these strains are needed. Importantly, MDR *Salmonella* strains that carry a *mcr-1*-ESBL-*qnrS*2 plasmid pose threats to both food safety and health management via two factors: 1) the active horizontal transfer of MRD genes in Enterobacteriaceae, and 2) their preservation through co-selection with other antibiotics, even if colistin is not used in treatment. China has taken a first step towards limiting the development of antimicrobial resistance by banning the use of colistin as a feed additive [47], but sustained surveillance needs be conducted to monitor the epidemic trends of *Salmonella* with *mcr-1*-mediated colistin resistance in animal products, food, the community, and hospitals and to estimate the effectiveness of control measures in the face of antibiotic selective pressure.

## Author contributions

X Lu contributed to all the experiments, data analysis and interpretation, and manuscript writing; M Zeng contributed to the data collection, analysis, and interpretation, study design, and article writing; J Xu contributed to the gene detection, genome sequencing, and plasmid conjugation experiments, data analysis and interpretation, and article writing; H Zhou contributed to the data analysis and interpretation; B Gu, H Jin, and W Xiao contributed to the data collection, analysis, and interpretation; Z Li, W Zhang, and Y Hu contributed the genome sequence analysis and interpretation; X Wang contributed to the gene detection experiments and resistance analysis; B Zhu contributed to the data interpretation and manuscript revision; X Xu contributed to the data collection, analysis, and interpretation and the study design and supervision; B Kan contributed to the study conception, design, and supervision and manuscript revision.

## Declaration of interests

We declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.006.

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