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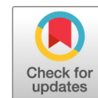

The *mcr-9* Gene of *Salmonella* and *Escherichia coli* Is Not Associated with Colistin Resistance in the United States

Gregory H. Tyson,[a] Cong Li,[a] Chih-Hao Hsu,[a] Sherry Ayers,[a] Stacey Borenstein,[a] Sampa Mukherjee,[a] Thu-Thuy Tran,[a] Patrick F. McDermott,[a] Shaohua Zhao[a]

[a]U.S. Food and Drug Administration, Center for Veterinary Medicine, Office of Research, Laurel, Maryland, USA

ABSTRACT Reports of transmissible colistin resistance show the importance of comprehensive colistin resistance surveillance. Recently, a new allele of the mobile colistin resistance (*mcr*) gene family designated *mcr-9*, which shows variation in genetic context and colistin susceptibility, was reported. We tested over 100 *Salmonella enterica* and *Escherichia coli* isolates with *mcr-9* from the National Antimicrobial Resistance Monitoring System (NARMS) in the United States for their susceptibility to colistin and found that every isolate was susceptible, with an MIC of ≤1 μg/ml. Long-read sequencing of 12 isolates revealed *mcr-9* on IncHI plasmids that were either independent or integrated into the chromosome. Overall, these results demonstrate that caution is necessary when determining the clinical relevance of new resistance genes.

KEYWORDS colistin, genomics, PacBio, conjugation, *mcr*

Colistin is an antimicrobial drug of last resort used to treat serious Gram-negative bacterial infections. It is not used as a first-line antimicrobial agent due to its toxicity, but it may be used to treat resistant infections (1). Colistin works by targeting the negatively charged membrane of Gram-negative bacteria, resulting in membrane disruption and cell death (2). Colistin has also been used as a growth promoter in food animals in Europe and Asia (3), until recent governmental actions restricted or banned these uses (4, 5). Colistin has not been used in food animals in the United States.

Colistin resistance has historically been rare, with known mechanisms being restricted to certain mutations, such as those in *pmrA* and *pmrB* (6). In 2015, the mobile colistin resistance gene *mcr-1*, conferring transmissible colistin resistance, was discovered (7). This gene has been found worldwide in many bacterial species, including *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica*, among others (8–10). In subsequent years, an additional eight *mcr* variants have been described and assigned new numerical designations (11, 12). Despite these reports, *mcr* gene prevalence in the United States has remained low, with most findings in human patients attributed to infections acquired as part of international travel (13, 14). Prior to this study, the National Antimicrobial Resistance Monitoring System (NARMS), a resistance surveillance program in the United States, had not identified any retail meat isolates with any *mcr* variant.

In 2019, the latest variant, *mcr-9*, was reported (15). This allele had only 64.5% amino acid identity with *mcr-3*, the most closely related allele. In addition, it was most commonly found in *Salmonella*, whereas other *mcr* genes were more common in *E. coli* and *Klebsiella*. The first isolate identified with *mcr-9* was susceptible to colistin, with an MIC of 0.25 to 0.5 μg/ml, although *mcr-9* did confer colistin MICs of >2.5 μg/ml when cloned and overexpressed in the laboratory (15). Further work found that *mcr-9* expression was inducible in the presence of colistin when located upstream of the two-component system *qseBC* (16). A subsequent study with 30 *mcr-9*-positive isolates

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Address correspondence to Gregory H. Tyson, Gregory.Tyson@fda.hhs.gov.

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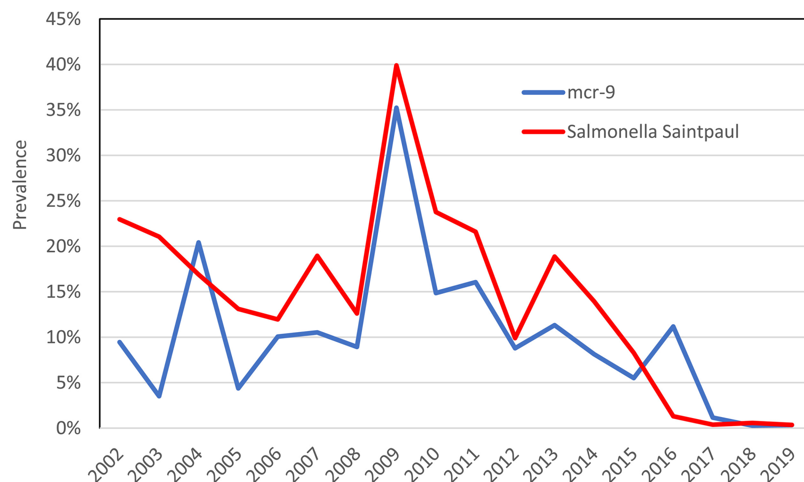

FIG 1 *mcr-9* prevalence among ground turkey isolates over time.

from various Gram-negative organisms found that all were susceptible to colistin and lacked the *qseBC* regulatory operon (17). Additional studies reported conflicting results as to the phenotype associated with *mcr-9* depending upon the isolate collection and species, indicating uncertainty regarding when this gene confers elevated colistin MICs (18, 19). The genomic structure of *mcr-9*-associated plasmids or chromosomes have not been substantially characterized, although there has been some association with IncHI plasmids (17, 20). To help determine whether *mcr-9* conferred decreased susceptibility to colistin and may have clinical relevance, we characterized 100 isolates with *mcr-9* recovered from the NARMS retail meat program by whole-genome sequencing (WGS) and colistin susceptibility testing.

RESULTS

Prevalence of *mcr-9*. After *mcr-9* was first identified, we sought to determine whether this gene was in any bacterial isolates collected as part of routine NARMS retail meat surveillance. Unlike previous *mcr* genes, most isolates identified in the NCBI Isolates Browser (1,052/1,862) were from *Salmonella enterica*. Upon additional analysis, we found that many of these *S. enterica* isolates (301 [28.6%]) were from NARMS retail meat sampling.

We further analyzed the assembled genomes and confirmed that each was indeed positive for *mcr-9* by BLAST analysis. An additional six *E. coli* isolates from NARMS retail meat sampling also had *mcr-9*. Each isolate had *mcr-9* with 100% identity and length to the reference sequence. NARMS performs sampling of retail beef, pork, turkey, and chicken products as part of its surveillance. However, 95.3% (287/301) of isolates with *mcr-9* were from retail turkey products. Despite the recent identification of *mcr-9*, we found that its occurrence was not recent. In fact, it was found in all years of NARMS retail meat testing, from 2002 to 2019, with its prevalence among turkey isolates peaking at 35.2% in 2009 (Fig. 1). Further analysis showed the 301 *mcr-9* isolates comprised 12 different serotypes, with *Salmonella enterica* serovar Saintpaul representing 75.1% of all isolates (Table 1). Therefore, the *mcr-9* prevalence in ground turkey tracks closely with that of *Salmonella* Saintpaul (Fig. 1). All Saintpaul isolates were in the same single nucleotide polymorphism (SNP) cluster, PDS000004383.101, in the NCBI Isolates Browser, indicating that they were all within 50 SNPs of one another. For *E. coli*, the six isolates with *mcr-9* were all from 2018 and 2019 retail sampling, with one isolate from ground turkey, four from retail chicken, and one from ground beef.

Additional analysis determined whether any isolates had the *qseBC* two-component system, which is thought to induce *mcr-9* expression (16). We found these genes in 10 isolates, including 4 *E. coli* and 6 *Salmonella* isolates (see Table S1 in the supplemental material).

TABLE 1 *Salmonella enterica* serotypes among NARMS retail isolates with *mcr-9*

	No. of isolates in:			
Serotype	Ground turkey	Retail chicken	Pork chop	Ground beef
I 4,[5],12:i:-	1		2	
Agona	1			
Albany	6			
Heidelberg	30			1
Johannesburg		1		
Kentucky	2	1		
Litchfield		1		
Mbandaka		2		
Montevideo	1			
Saintpaul	221		2	3
Schwarzengrund	23			
Senftenberg	2	1		
Total	287	6	4	4

Susceptibility testing. Broth microdilution testing was performed to determine the relationship between MIC and the presence of the *mcr* gene. We included a variety of different isolates, representing those isolated in each year from 2002 to 2019, and included those from all retail meat sources in testing (Table S1). A total of 105 isolates were tested, including 99 *S. enterica* and 6 *E. coli* isolates, and all were susceptible to colistin, with MICs of $\leq$1 μg/ml (Fig. 2). These results were true for all isolates tested, including all 10 isolates with *qseBC* (Table S1), which in some other studies had elevated colistin MICs. Furthermore, all isolates had MICs of $\leq$2 μg/ml for polymyxin B (Table S1), which had not previously been tested in other studies with *mcr-9*.

Genomic structure of *mcr-9*-associated plasmids and chromosomes. To further characterize the genetic context of the *mcr-9* genes, we performed long-read sequencing using Pacific Biosciences technology. We sought to sequence diverse isolates to understand the various plasmids that carried this gene. Surprisingly, 6 of the 12 isolates had *mcr-9* on the chromosome, with the remaining isolates having it on IncHI plasmids (Table S2).

The IncHI plasmids ranged from 269 to 340 kb, and all contained genes encoding mercury, tellurium, and copper resistance (Fig. S1 to S3). The presence of additional resistance genes varied among the plasmids; notably, the *E. coli* plasmid in isolate N18EC0432 possessed bla_{TEM-1}, bla_{SHV-12}, *sul1*, *sul2*, *ereA*, and *aac(6')-IIc* (Fig. S2). These genes encode resistance to beta-lactams, sulfonamides, macrolides, and aminoglycosides. Of importance is bla_{SHV-12}, an extended-spectrum-beta-lactamase gene whose presence could negatively impact treatment of associated infections. While most sequences did not have significant homology to known plasmids, the plasmid from

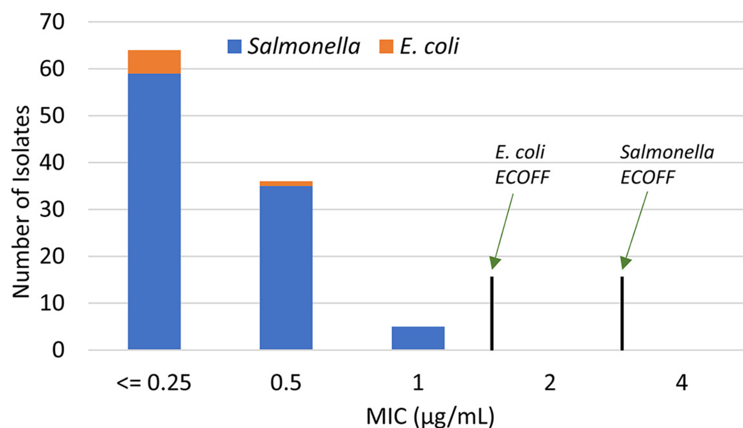

FIG 2 Colistin MICs of isolates with *mcr-9*.

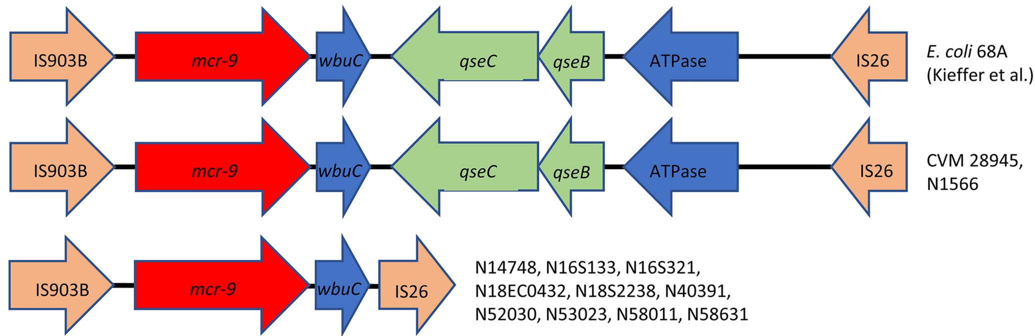

FIG 3 Genetic context of *mcr-9* genes. The *mcr-9* genes and their surrounding environments are shown. GenBank accession numbers for these are as follows: GCA_900500325 (*E. coli* 68A), CP048303 (CVM 28945), CP048299 (N1566), CP048926 (N14748), CP049986 (N16S133), CP049313 (N16S321), CP048293 (N18EC0432), CP049312 (N18S2238), CP049983 (N40391), CP049981 (N52030), CP049310 (N53023), CP049309 (N58011), and CP049307 (N58631).

isolate N53023 was highly related to an unknown plasmid in *Enterobacter hormaechei* (GenBank accession no. CP027144), which has *mcr-9* and has public data in GenBank listing the colistin MIC as ≤0.25 μg/ml (Fig. S3). All chromosomal copies of *mcr-9* appear to have been mobilized by integration of either entire or partial IncHI plasmids.

To better understand the genetic context of *mcr-9* in our isolates, we compared the sequences from our closed genomes to that from the colistin-resistant isolate *E. coli* 68A (16). Two of the isolates had genetic structures identical to that of *E. coli* 68A, including the regulatory genes *qseBC*, but still had colistin MICs of ≤0.5 μg/ml (Fig. 3). There was only one SNP difference in our sequences compared to that of *E. coli* 68A in the entire 8.4-kb sequence, which was in the coding region of IS903B and unlikely to affect the function of *mcr-9* or *qseBC*. The remaining 10 isolates with closed genomes had identical *mcr-9* genetic structures to one another, and all lacked the *qseBC* genes.

Conjugations. To further confirm the phenotype and transmissibility of plasmids associated with *mcr-9*, we performed conjugation assays on four different isolates. Conjugation was successful for one isolate, N53023, into the recipient strain DH5α, indicating that the IncHI plasmid is transmissible. Conjugations may have been more successful had they been conducted 26°C, as has been found optimal for IncHI plasmids (21). The transconjugant remained susceptible to colistin and polymyxin B (Table S1) but was resistant to other antimicrobials as predicted by the resistance genes encoded by the *mcr-9* IncHI plasmid. These results confirm the prior susceptibility testing results that *mcr-9* does not confer polymyxin resistance in our isolate collection. However, the initial isolate and transconjugant both had MICs of ≤0.25 μg/ml, so differences in MICs below the level of detection would not have been found based on our testing.

DISCUSSION

Microbial WGS has greatly expanded the data set of presumptive coding regions from which to infer function through gene relatedness surveys. The putative colistin resistance gene *mcr-9* was first identified by whole-genome sequencing by its 64.5% amino acid identity to *mcr-3*. We examined our database of over 4,000 NARMS genomes and found *mcr-9* in hundreds of isolates. This discovery was concerning since other *mcr* alleles had not previously been found in our isolate collection. However, our susceptibility testing and conjugation assays show that the presence of *mcr-9* was not associated with resistance to colistin or polymyxin B.

With the rapidly growing body of genomic data, it is especially important to study the genetic context and test large numbers of isolates to make firm assertions about novel resistance alleles. By testing over 100 *Salmonella* and *E. coli* isolates with *mcr-9* and closing the genomes of many of these, we have provided a basis to further understand differences in resistance phenotypes of bacteria with *mcr-9*.

Based on our results, it appears that *mcr-9* is not as concerning as other *mcr* alleles, most of which consistently display colistin resistance. In the absence of a colistin

resistance phenotype in our isolates, it is also unclear what function, if any, *mcr-9* may have. However, it is worth noting that not all isolates with *mcr* genes have been found to be colistin resistant (22). These results also demonstrate the continued value of antimicrobial susceptibility testing in certain contexts, where conclusions resulting from whole-genome sequencing may not be clear. Although *mcr-9* does not appear to be an emerging public health threat for now, changes in genetic context or the accumulation of mutations may affect its ability to impact colistin resistance. The closest reported allele to *mcr-9* is *mcr-3*, and some bacteria with this allele are also colistin susceptible (23). It is important to note that all copies of *mcr-9* in our collection had 100% identity and length to one another, so it is possible that changes in sequence could alter the effects of *mcr-9* on colistin susceptibility. In addition, each isolate had *mcr-9* on an IncHI plasmid or integrated into the chromosome from an IncHI plasmid, so a different genetic context could result in increased expression and a different resistance phenotype. These findings of association with IncHI plasmids are consistent with some previous findings (17, 20). Interestingly, the IncHI plasmids in our isolates were diverse both in overall genomic structure and in genomic context around the *mcr-9* genes (Supplemental Fig. S1 to S3).

The two-component system *qseBC* was downstream of *mcr-9* in several of our isolates, including two with closed genomes (Fig. 3). It is unclear why the presence of *qseBC* did not discernibly change the observed phenotypes, considering that the genetic context around *mcr-9* was identical to that of *E. coli* 68A from Kieffer et al. (16). It is possible that the unusual O15:H6 serotype of *E. coli* 68A in some way contributed to the observed phenotype, as serotype-dependent colistin susceptibility has also been observed in *Salmonella* (24). Overexpression of *mcr* genes in *E. coli* TOP10 showed increased MICs from 0.03 μg/ml to 0.15 μg/ml for *mcr-9*, compared to 4 μg/ml for *mcr-1* (16). We did not perform induction experiments, but preinduction of *mcr-9* in the presence of colistin is not required to accurately measure MICs by broth microdilution (16). These results indicate that the effectiveness of *qseBC* in inducing *mcr-9* expression may be context dependent and differ among isolates with different strain backgrounds.

Interestingly, since *mcr-9* is frequently present on mobile genetic elements with other resistance genes, it means that the use of these other antimicrobials has the potential to coselect for the continued presence of *mcr-9*. In addition, various metal resistance genes, encoding resistance to silver, mercury, arsenic, copper, tellurium, and others, are present on these plasmids, providing another potential avenue for coselection for *mcr-9*.

NARMS will continue to detect colistin resistance genes as part of routine whole-genome sequencing and recently added colistin as part of routine antimicrobial susceptibility testing. Thus, ongoing work will determine whether colistin resistance emerges among bacteria collected as part of NARMS sampling and, if so, what the resulting mechanisms are.

MATERIALS AND METHODS

Data mining. Isolates with *mcr-9* were identified using the NCBI Pathogen Detection Isolates Browser, which annotates antimicrobial resistance genes using AMRFinderPlus (25). Initial results included investigation of all sequences with *mcr-9* among *Salmonella enterica* isolates present in the browser, among a total of more than 200,000 total *Salmonella enterica* genomes. Further analysis focused on NARMS isolates in BioProject PRJNA292661, which contains sequencing data from more than 4,000 isolates. Search results were collected on 13 December 2019.

Colistin susceptibility testing. Isolates with *mcr-9* were tested for susceptibility to colistin and polymyxin B using standard broth microdilution techniques, following CLSI standards (36). The panels GNX3F from Trek Diagnostics were used, which have colistin and polymyxin B concentrations of 0.25 μg/ml to 4 μg/ml. EUCAST epidemiological cutoffs (ECOFFs) and provisional ECOFFs were used to identify isolates with elevated MICs (26). Quality control strains *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used, along with a colistin-resistant WHO reference strain with *mcr-4* (*Salmonella* Kedougou WHO-S18-2) (27).

Whole-genome sequencing and analysis. Whole-genome sequencing was performed by short-read sequencing on the Illumina MiSeq using v3 chemistry, as previously described (28). Twelve isolates were selected for long-read sequencing, with DNA libraries prepared using a 10-kb template preparation protocol with SMRTbell template prep kit v1.0. Sequencing was performed using Pacific Biosciences technology on the Sequel platform with sequencing kit 3.0, as previously described (29). The long reads

were assembled to contigs by HGAP4, and contigs were circularized by Circlator (30, 31). Assembled genomes were then polished with Illumina reads by Pilon (32). Even and continuous coverage was assessed to determine that there were no misassemblies. Genes were annotated by Prokka in the depicted figures (33), and BLASTn was used to compare plasmid sequences with each other and with those in GenBank. PlasmidFinder was used to identify the plasmid types (34).

Conjugation. Conjugation assays were conducted as previously described (35). Briefly, recipient DH5α *E. coli* cells were resistant to nalidixic acid, with donor N53023 resistant to gentamicin, as encoded by *aac(3)-VIa* on the *mcr-9* IncHI plasmid. Mating occurred on blood agar plates at 37°C for 16 h. The conjugated cells were resuspended in 500 μl of LB broth, and 50 μl of the resulting mixture was plated onto doubly selective agars (nalidixic acid plus gentamicin). Individual isolates were confirmed not to grow on these doubly selective agars. Any resulting colonies were screened by whole-genome sequencing for recipient genomes and the presence of *mcr-9* plasmid sequence. Subsequent antimicrobial susceptibility testing was performed to identify any changes in colistin resistance phenotypes.

Accession number(s). Whole-genome sequencing data were submitted to NCBI with accession numbers for short-read sequences in Table S1 and long-read sequences in Table S2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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