




Complete Genetic Analysis of Plasmids Carrying *mcr-1* and Other Resistance Genes in Avian Pathogenic *Escherichia coli* Isolates from Diseased Chickens in Anhui Province in China

Dongdong Yin,^a Baoyan Cheng,^a Kankan Yang,^a Mei Xue,^a Yanfeng Lin,^b Zhonghong Li,^c Xiangjun Song,^a Ying Shao,^a Jian Tu,^a  Peng Li,^b Kezong Qi^a

^aAnhui Province Key Laboratory of Veterinary Pathobiology and Disease Control College of Animal Science and Technology, Anhui Agricultural University, Hefei, China

^bChinese PLA Center for Disease Control and Prevention, Beijing, China

^cCollege of Environmental and Chemical Engineering, Nanchang Hangkong University, Nanchang, China

Dongdong Yin, Baoyan Cheng, Kankan Yang, and Mei Xue contributed equally to this work. Author order was determined by their contributions.

ABSTRACT Antimicrobial resistance associated with colistin has emerged as a significant concern worldwide, threatening the use of one of the most important antimicrobials for treating human disease. This study aimed to investigate the prevalence of colistin-resistant avian-pathogenic *Escherichia coli* (APEC) and shed light on the possibility of transmission of *mcr-1* (mobilized colistin resistance)-positive APEC. A total of 72 APEC isolates from Anhui Province in China were collected between March 2017 and December 2018 and screened for the *mcr-1* gene. Antimicrobial susceptibility testing was performed using the broth dilution method. Pulsed-field gel electrophoresis, Southern blot analysis, and conjugation assay were performed to determine the location and conjugative ability of the *mcr-1* gene. Whole-genome sequencing and analysis were performed using Illumina MiSeq and Nanopore MinION platforms. Three APEC isolates (AH25, AH62, and AH65) were found to be positive for the *mcr-1* gene and showed multidrug resistance. The *mcr-1* genes were located on IncI2 plasmids, and conjugation assays revealed that these plasmids were transferrable. Notably, strains AH62 and AH65, both belonging to ST1788, were collected from different places but carried the same drug resistance genes and shared highly similar plasmids. This study highlights the potential for a possible epidemic of *mcr-1*-positive APEC and the urgent need for continuous active monitoring.

IMPORTANCE In this study, three plasmids carrying *mcr-1* were isolated and characterized from APEC isolates from Anhui Province in China. The *mcr-1* genes were located on IncI2 plasmids, and these plasmids were transferrable. These three IncI2 plasmids had high homology with the plasmids harbored by pathogenic bacteria isolated from other species. This finding showed that IncI2 plasmids poses a risk for the exchange of genetic material between different niches. Although colistin has been banned for use in food-producing animals in China, the coexistence of the broad-spectrum β -lactamase and *mcr-1* genes on a plasmid can also lead to the stable existence of *mcr-1* genes. The findings illustrated the need to improve the monitoring of drug resistance in poultry systems so as to curb the transmission or persistence of multidrug-resistant bacteria.

KEYWORDS APEC, colistin resistance, IncI2, *mcr-1*, plasmids

Colistin is considered the last defense against multidrug-resistant (MDR) Gram-negative bacteria, and the World Health Organization has classified it as one of the most critical antibiotics (1). Before 2016, colistin was generally used as a feed additive in farms to prevent diseases caused by members of the *Enterobacteriaceae*. This resulted in a significant increase in the rate of resistance to colistin among organisms isolated from livestock and poultry farms, threatening public health (2).

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Address correspondence to Peng Li, jjiekenlee@126.com, or Kezong Qi, qkz@ahau.edu.cn.

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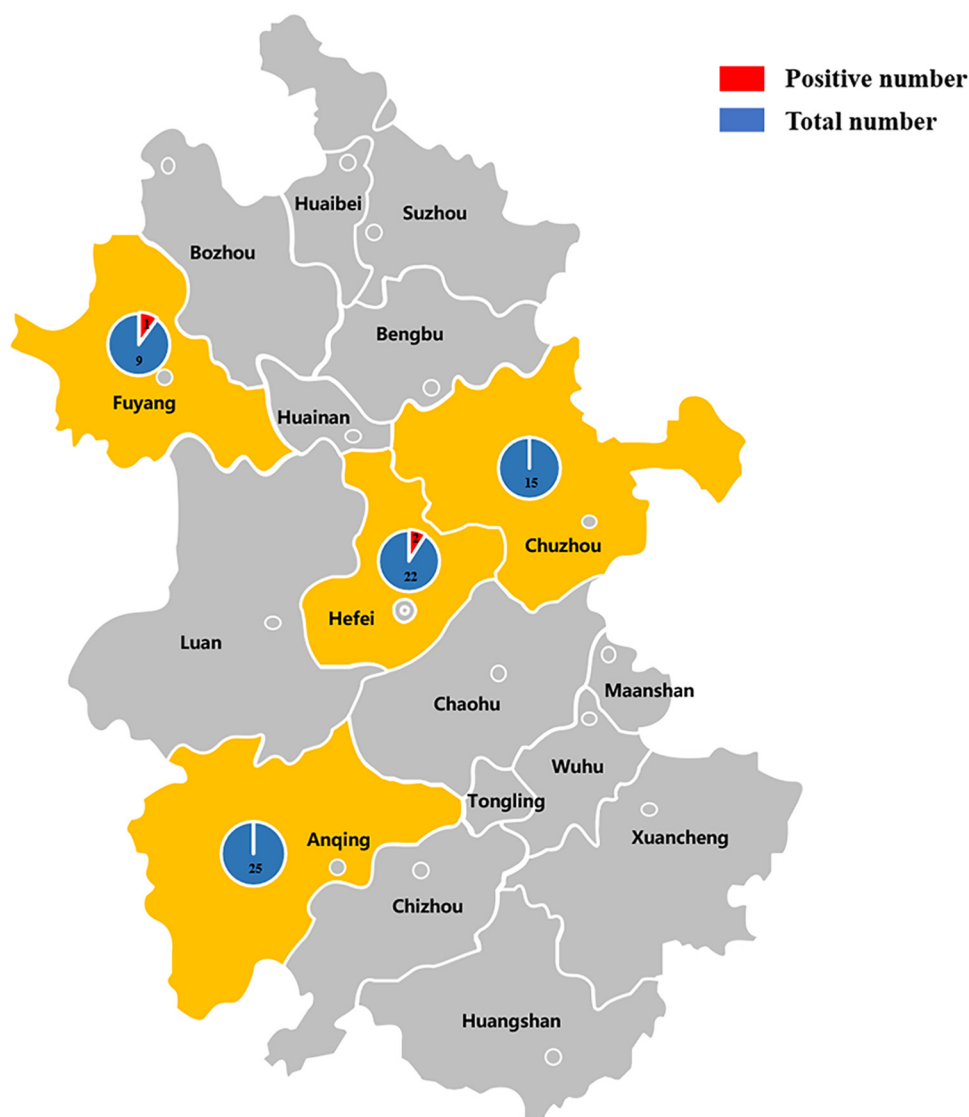


FIG 1 Anhui Province (in gray) and cities (in yellow) where APEC-infected chickens were collected in this study.

The mobilized colistin resistance gene (*mcr-1*) is a transferable phosphoethanolamine transferase-encoding gene that can modify the lipopolysaccharide of the outer membrane of bacteria, resulting in a weakened binding of colistin to the outer membrane and resistance to the drug (3, 4). The *mcr-1* gene was first identified in 2016 and has been found in more than 50 countries on six continents, indicating that *mcr-1* has become an epidemic worldwide (5–7).

Numerous retrospective studies have shown that chickens are a reservoir of resistance genes, and it is important to identify the origins of their MDR plasmids (8–10). The present study aimed to characterize three avian-pathogenic *Escherichia coli* (APEC) isolates by Illumina short-read and MinION long-read whole-genome sequencing (WGS) and identify the genetic features of plasmids containing the *mcr-1* genes. A total of 72 APEC isolates were collected from diseased chickens in Anhui Province in China between March 2017 and December 2018 and screened for the *mcr-1* gene.

RESULTS

Antimicrobial susceptibility testing. Three (~4%) colistin-resistant isolates (AH25, AH62, and AH65) were identified from a total of 72 APEC strains from Anhui Province during 2017–2018 (Fig. 1). AH25 and AH62 were collected from two different farms in

TABLE 1 Features of *mcr-1*-positive APEC strains

Strain	Yr	Serotype	MLST	Plasmid	Size (bp)	Plasmid type ^a	MDR gene(s)	Accession no.
AH25	2017	O125ab:H28	ST156	pAH25-1	64,941	Incl2	<i>bla</i> _{CTX-M-55} , <i>mcr-1</i>	CP055257
				pAH25-2	116,030	p0111	<i>strA</i> , <i>strB</i> , <i>fosA</i> , <i>rmtB</i> , <i>erm(B)</i> , <i>sul1</i> , <i>sul2</i> , <i>aadA2</i> , <i>aadA5</i> , <i>floR</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>qepA tet(A)</i> , <i>dfrA12</i> , <i>catA1</i> , <i>aph(3')-Ic</i> , <i>aph(4)-Ia</i> , <i>aac(3)-Iva</i> , <i>bla</i> _{TEM-1B}	CP055258
AH62	2018	O15:H40	ST1788	pAH62-1	60,960	Incl2	<i>mcr-1</i>	CP055260
				pAH62-2	84,033	Incl1-I	<i>strA</i> , <i>strB</i> , <i>aac(3)-IId</i> , <i>sul1</i> , <i>sul2</i> , <i>mph(A)</i> , <i>aadA5</i> , <i>dfrA17</i> , <i>tet(A)</i> , <i>floR</i> , <i>bla</i> _{TEM-1A}	CP055261
				pAH62-3	136,694	IncFIB, IncFIC		CP055262
AH65	2018	O15:H40	ST1788	pAH65-1	60,960	Incl2	<i>mcr-1</i>	CP058303
				pAH65-2	84,033	Incl1-I	<i>strA</i> , <i>strB</i> , <i>aac(3)-IId</i> , <i>sul1</i> , <i>sul2</i> , <i>mph(A)</i> , <i>aadA5</i> , <i>dfrA17</i> , <i>tet(A)</i> , <i>floR</i> , <i>bla</i> _{TEM-1A}	CP058304
				pAH65-3	139,513	IncFIB, IncFIC		CP058305

^aPlasmid type was determined with PlasmidFinder.

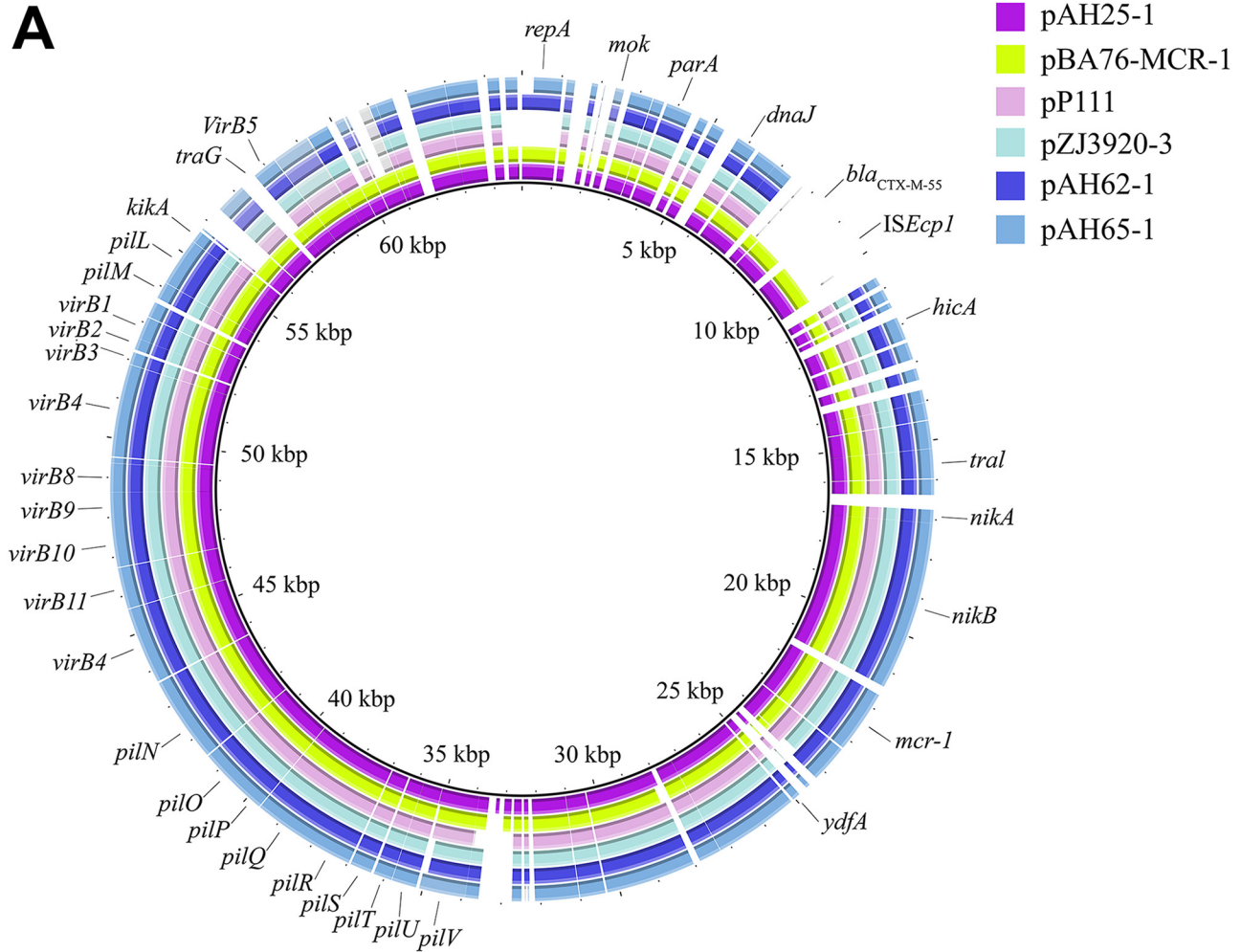
Hefei, while AH65 was collected from a farm in Fuyang. Susceptibility testing indicated that AH25 coexpressed cefotaxime and colistin resistance and exhibited an MDR phenotype, which included resistance to kanamycin, fosfomycin, florfenicol, tetracycline, and polymyxin (see Table S1 in the supplemental material). Both AH62 and AH65 were susceptible to fosfomycin and cefotaxime. The MIC of colistin for the donor strains and their transconjugants was 8 µg/ml (Table S1).

Microbiological and genomic features of *mcr-1*-positive APEC strains. All three isolates were subtyped by multilocus sequence typing (MLST), and the corresponding serotypes were identified *in silico* following analysis of the WGS data. AH25 belonged to sequence type 156 (ST156) (serotype O125ab:H28), and AH62 and AH65 belonged to ST1788 (O15:H40) (Table 1). S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) showed that AH25 contained two different plasmids and that both AH62 and AH65 contained three plasmids (Fig. S1). Southern blot analysis revealed that the *mcr-1* genes were located on an ~64-kb plasmid (named pAH25-1), an ~60-kb plasmid (named pAH62-1), and an ~60-kb plasmid (named pAH65-1) (Fig. S1).

WGS analysis. Multiple resistance genes were identified in the three isolates. AH25 carried the *bla*_{CTX-M-55} and *bla*_{TEM-1B} genes, while AH62 and AH65 produced extended-spectrum beta-lactamase (ESBL) with the *bla*_{TEM-1A} gene (Table 1). In addition to the aforementioned MDR genes, the strains were also found to harbor multiple resistance elements, including but not limited to *aadA5*, *floR*, *fosA*, *strA*, *strB*, *sul1*, *sul2*, and *mph(A)*, which were consistent with the antimicrobial-resistant phenotypes in the study (Table 1).

The results of WGS revealed that the three *mcr-1*-harboring plasmids (pAH25-1, pAH62-1, and pAH65-1) belonged to the Incl2 type (Table 1). A BLAST search revealed that the three *mcr-1*-harboring plasmids were highly similar to pBA76-MCR-1 (accession no. [KX013540](#)) (100% coverage and 99.56% identity), pZJ3920-3 (accession no. [CP020548](#)) (100% coverage and 99.97% identity), and pP111 (accession no. [KY120365](#)) (100% coverage and 99.99% identity). The genetic environments of the *mcr-1* gene in the three plasmids and the data available in GenBank were almost the same (Fig. 2A; Fig. S2). The WGS data on the genetic context of the three plasmids in this study revealed a typical plasmid backbone responsible for plasmid replication, maintenance, and transfer (Fig. 2B). Upstream structures were identical to those seen in pAH25-1, pAH62-1, and pAH65-1. In the three plasmids, the *mcr-1*-*pap* cassette was inserted in the conserved cassette position, that is, downstream of the *nikB* locus, and pAH25-1 also harbored an *ISEcp1*-driven *bla*_{CTX-M-55} gene inserted independently of *mcr-1* at the plasmid scaffold (Fig. 2B).

A



B

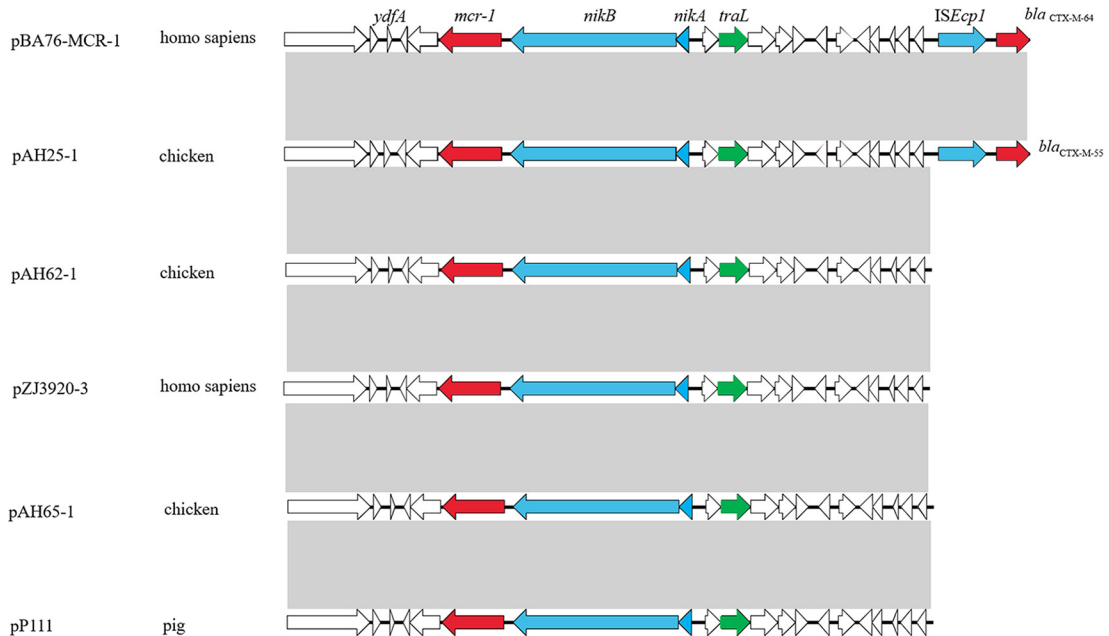


FIG 2 Sequence alignment analysis of Incl2 *mcr-1*-harboring plasmids. (A) Sequence comparison of *mcr-1*-harboring plasmids. (B) Comparative schematic representation of the flanking regions of the *mcr-1* genes in Incl2 plasmids. Areas shaded in gray indicate homologies in the (Continued on next page)

DISCUSSION

APEC strains are a versatile group of bacteria revealing a complex phylogeny and considerable genomic plasticity. APEC can cause various disease syndromes, such as pericarditis, perihepatitis, peritonitis, and yolk sac infections in poultry (11). Certain subpopulations of APEC have been suggested to be zoonotic agents (12, 13), which are of great concern because they also have been associated with multidrug-resistant clonal lineages.

All the *mcr-1*-positive transconjugants in this study belonged to IncI2, consistent with the results of previous studies showing that the *mcr-1*-carrying IncI2 plasmid had the advantages of suitability and was more beneficial for the isolation of the host *E. coli* than either IncHI2 or IncX4 plasmid (14). Since the first report of a plasmid-borne *mcr-1* gene, located on the IncI2 plasmid pHNSHP45, the IncI2 plasmid type has been widely reported as the main vector for *mcr-1* gene transmission worldwide (1). At the same time, the IncI2 plasmid type was also an important carrier of the *bla*_{CTX-M} gene (15). Studies have found that IncI2 plasmids carried the *bla*_{CTX-M-55} gene in *E. coli* in Chinese pets and food animals. Liu et al. found that IncI2 plasmids harbored the *bla*_{CTX-M-64} gene, which was widely distributed among members of the *Enterobacteriaceae* in different animals and different regions in China (16). Ho et al. reported that IncI2 plasmids in *E. coli* from pigs and chickens in China carried the *bla*_{CTX-M-64} and *bla*_{CTX-M-132} genes (17). All these findings indicated that the IncI2 plasmid type in different *Enterobacteriaceae* species spread *mcr-1* in food and animals around the world and then to clinical isolates producing carbapenemase.

The existing research results suggest that farms may be the source of the spread of *mcr-1*. This is because the isolation rate of *mcr-1*-positive strains in farms is relatively high, while the isolation rate in hospital-infected patients is relatively low (1). Moreover, farms are closely related to human life, and foodborne bacteria can infect humans through the food chain (18, 19). The *mcr-1*-positive strains are predominantly *Enterobacteriaceae*. On the one hand, bacteria can spread by contaminating animal-derived food; on the other hand, animal excrement returned to the field after being composted may also contaminate crops such as vegetables and wheat (20), thereby threatening public safety.

Fortunately, the use of colistin in animal feed was banned by the Ministry of Agriculture and Rural Affairs of the People's Republic of China (MARA, PRC) (announcement no. 2428; <http://www.moa.gov.cn/>) in November 2016. The implementation of this policy reduced the selection pressure of drugs on the intestinal flora. In the absence of selective pressure of colistin, the detection rate of the *mcr-1* gene might gradually decrease, but plasmids carrying the *mcr-1* gene also often carry the *bla*_{CTX-M} gene (21). Under the action of amoxicillin, cephalosporin, and other drugs, plasmids carrying both the *bla*_{CTX-M} and *mcr-1* genes or only the *bla*_{CTX-M} gene still persist, which might be the reason why *mcr-1* remained detectable but with a decreased detection rate.

In this study, pAH25-1 was closely aligned with IncI2 plasmid pBA76-MCR-1 from *E. coli* in humans in the Arabian Peninsula (22). pAH62-1 was closely aligned with IncI2 plasmid pZJ3920-3 from *E. coli* in humans in Hangzhou, China, and pAH65-1 was closely aligned with IncI2 plasmid pP111 from *Salmonella enterica* serovar Typhimurium in pigs in Taiwan (23). This phenomenon emphasized that IncI2 plasmid posed a risk for the exchange of genetic material between different niches. Additionally, these *mcr-1*-harboring plasmids lacked the IS*ApI1* element, a key element that mediated the translocation of *mcr-1* into various plasmid backbones and chromosomes (24, 25). It was speculated that the spread of *mcr-1* genes might be dependent on the diffusion of promiscuous plasmids, rather than on the clonal expansion of *mcr-1*-bearing bacteria.

The AH-25 strain belonged to ST156, which is distributed in humans and animals, and was related to human *E. coli* urinary tract and blood infection isolates (5, 26, 27). It is worth noting that isolates AH-62 and AH-65 both belonged to ST1788, indicating the presence of a clonal kinship. At the same time, the two isolates contained three plas-

FIG 2 Legend (Continued)

corresponding genetic environment on each plasmid. The open reading frames are shown as boxes or arrows. Antibiotic resistance-encoding genes are indicated with red boxes/arrows. Blue boxes/arrows denote transposon- and integron-associated genes. White boxes/arrows indicate hypothetical proteins or mobile element proteins.

mids of the same type and virulence, but the isolation sites were different, suggesting a trend of potential clonal outbreaks.

In conclusion, *E. coli* has a wide range of hosts, being isolated from healthy and diseased animals and humans. This study showed that APEC is an important drug resistance gene storehouse, and the prevalence of *mcr-1*-positive APEC posed a potential risk to public health. The surveillance of resistance in the poultry system should be reinforced to curb the transmission or persistence of MDR bacteria due to the wide spread of *mcr-1* around the world; in particular, *E. coli* easily harbored the plasmid that carried *mcr-1*.

MATERIALS AND METHODS

Bacterial collection and detection of resistance genes. A total of 72 APEC strains were collected from the livers or hearts of diseased chickens in the four urban areas of Anhui Province (Hefei, Anqing, Chuzhou, and Fuyang) from March 2017 to December 2018. The diseased chickens from these farms where the disease occurred were sent to the veterinary hospital of Anhui Agricultural University for testing. The strains were cultured in Luria-Bertani medium at 37°C. The existence of the *mcr-1* gene was determined by PCR amplification (1). MICs of colistin were determined separately using a broth dilution method. An MIC of $>2 \mu\text{g/ml}$ was considered resistant to colistin according to the European Committee on Antimicrobial Susceptibility Testing. In addition, all isolates were tested for their susceptibility to a panel of antimicrobial compounds by the broth dilution method, as described in previous studies (28, 29).

S1 nuclease-pulsed-field gel electrophoresis, Southern blot analysis, and conjugation assay. An S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) assay and Southern blot hybridization were performed to determine the location of the *mcr-1* gene. Genomic DNAs from strains AH25, AH62, and AH65 were digested with the S1 endonuclease (TaKaRa, Dalian, China). DNA fragments were separated by PFGE through a CHEF-DR III system (Bio-Rad, California, USA) with *Salmonella enterica* serotype Braenderup strain H9812 as a reference size standard. The plasmid DNA was transferred to a positively charged nylon membrane (Solabio, Beijing, China) and hybridized with the digoxigenin-labeled specific probe to *mcr-1*. The *mcr-1* gene conjugation experiments were performed by filter mating using AH25, AH62, and AH65 as the donor and a standard *E. coli* J53 strain (sodium azide resistant) as the recipient. Cultures of donor and recipient cells in the logarithmic phase were mixed at a ratio of 4:1 and incubated on a brain heart infusion (BHI) agar plate overnight at 37°C without shaking. The transconjugants were selected on BHI plates containing $4 \mu\text{g/ml}$ colistin and $200 \mu\text{g/ml}$ sodium azide. Antimicrobial susceptibility testing and PCR were subsequently performed to confirm the transconjugants.

WGS and bioinformatics analysis. The genomic DNA of the three isolates was extracted using a High Pure PCR template preparation kit (Roche, Basel, Switzerland). The genomic DNA was subjected to high-throughput sequencing using both Illumina MiSeq and Oxford Nanopore MinION platforms. The *de novo* hybrid assembly of Illumina short reads and MinION long reads was performed using Unicycler version 0.4.8 as the reported method (30). Genome annotation was done by using RAST (31). Acquired antibiotic resistance genes were identified using ResFinder. The sequence type was determined through the multilocus sequence typing (MLST) web server (32). Standard methods were used to annotate the serotype based on the WGS data by CGE platforms. Plasmid replicon types were identified using PlasmidFinder v2.0. Plasmid replicon types and the sequence types of IncF plasmids were identified using PlasmidFinder and pMLST (33). Sequence comparisons and map generation were performed using BLAST and visualized using Easyfig version 2.1 (34).

Data availability. The complete sequences of the chromosomes of strains AH25, AH62, and AH65 and plasmids pAH25-1, pAH25-2, pAH62-1, pAH62-2, pAH62-3, pAH65-1, pAH65-2, and pAH65-3 have been deposited in GenBank under the accession numbers CP055256, CP055259, CP058302, CP055257, CP055258, CP055260, CP055261, CP055262, CP058303, CP058304, and CP058305, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.6 MB.

FIG S2, TIF file, 2.6 MB.

TABLE S1, DOCX file, 0.03 MB.

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We declare no potential conflicts of interest.

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