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Avian-source *mcr-1*-positive *Escherichia coli* is phylogenetically diverse and shares virulence characteristics with *E. coli* causing human extra-intestinal infections

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ABSTRACT

Colisepticemia caused by bloodstream infection of the extraintestinal pathogenic *Escherichia coli* (ExPEC) has become a serious public health problem. The recent emergence of the colistin-resistant *Enterobacteriaceae*, especially *mcr-1*-positive *E. coli* (MCRPEC) exerts great concern around the world. The molecular epidemiology and zoonosis risk of avian-origin MCRPEC are reported to be substantially lower. Here, we presented a system-wide analysis of emerging trends and zoonotic risk of MCRPEC recovered from avian colibacillosis in China. Our results showed the majority of avian-source MCRPEC isolates were classified as ExPECs. We also found that not only MCRPEC in phylogroups B2 and D, but also several *E. coli* populations in groups B1 and F possessed high virulence in the two models of avian colibacillosis and three rodent models for ExPEC-associated human infections. The high-virulent MCRPEC clones belong to ST131, as well as ST-types (such as ST48, ST117, ST162, ST501, ST648, and ST2085). Our data suggested the zoonotic risk of MCRPEC appeared to be a close association with ColV/ColBM type virulence plasmids. A comprehensive genomic analysis showed the overlapped of ColV/ColBM plasmids contents between MCRPEC isolates from humans and poultry. Identification of ColV/ColBM plasmids among human MCRPEC isolates revealed the potential transmission of avian-source *mcr-1*-positive ExPECs to humans. Moreover, the presence of ColV/ColBM plasmid-encoded virulence determinants, could be used as a predictive label for pathogenic MCRPEC. These findings highlighted avian-origin MCRPEC isolates could be recognized as a foodborne pathogen.

1. Introduction

Poultry is a worldwide source of human food consumption. Poultry could also carry various food-borne pathogenic or drug-resistant microbes (Mitchell et al., 2015). The etiologic agent of colibacillosis is ExPEC, which causes respiratory and systemic disease in poultry (Zhu Ge et al., 2014). Unlike diarrhea-causing *E. coli*, ExPECs are normally the gut colonizing commensals and become pathogenic when colonize in extraintestinal tissues in humans and animals (Johnson et al., 2017; Manges et al., 2019; Rodriguez-Siek et al., 2005b). ExPEC infection also cause urinary tract infection (UTI), sepsis, and newborn meningitis et al. Based on the hosts and syndromes, the representing pathotypes of ExPEC are designated as avian pathogenic *E. coli* (APEC), uropathogenic *E. coli*, sepsis-associated *E. coli* (SEPEC), and neonatal meningitis *E. coli* (NMEC). ExPEC-associated diseases are a major threat to the poultry industry due to the productivity loss and medical costs (Manges and Johnson, 2012).

It is noteworthy that ExPEC increases the probability of poultry products contamination (Manges and Johnson, 2012; Mitchell et al., 2015). Avian-source ExPEC can be transmitted to humans by the consumption of uncooked/undercooked contaminated poultry products or direct human-animal contact (Manges and Johnson, 2012). In-depth understanding of the zoonotic potential of avian-source *E. coli* is of great importance for public health purposes. For the serious human

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infections caused by multidrug-resistant (MDR) bacteria, such as clinically carbapenem-resistant *Enterobacteriaceae* (CRE), colistin acts as the last treatment resort (Shen et al., 2018a). However, the emergence of the colistin-resistant *Enterobacteriaceae*, such as MCRPEC deteriorates the situation in the face "postantibiotic era" (Shen et al., 2018b).

The emergence of the plasmid-mediated *mcr-1* gene occurs first in animal production. The animal products and farming environment are suspected to be important routes to spread the *mcr-1* gene to human beings (Liu et al., 2016; Shen et al., 2018b). Compared with the global attention to the transferable colistin resistance mechanisms among bacteria populations, the systematic virulence test of MCRPEC isolates has not been investigated. In this study, we ascertained the clinical associations and zoonotic risk for avian-origin MCRPEC recovered from avian colibacillosis in Eastern China, 2015 to 2017.

2. Materials and methods

2.1. Sample information of avian-origin MCRPEC isolates

The chickens and ducks with avian colibacillosis were collected from poultry farms in Jiangsu, Anhui and Zhejiang provinces, 2015 to 2017 (Zhuge et al., 2019a). A total of 1360 *E. coli* isolates were recovered from the 1718 samples, which were collected from 46 poultry farms and 11 duck farms. MCRPEC isolates (n = 172) were preliminary screened by PCR amplification using special primer pairs for *mcr-1* gene (Rebelo et al., 2018). They had been previously identified *in vitro* for antimicrobial genotypic and phenotypic traits (Zhuge et al., 2019a). Among those MCRPEC, 129 strains were chicken-source *E. coli*, and the remaining were duck-source isolates (n = 43) (Zhuge et al., 2019a). Detailed statistics of MCRPEC strains were shown in Table S1. Phylogenetic groups and multilocus sequence typing (MLST) analysis of *E. coli* isolates were determined by the established PCR assay of seven housekeeping genes (Clermont et al., 2013).

2.2. Virulence factor screening

The popular occurrence of virulence factors specific for ExPEC was identified by established multiplex PCR-based assay with the slight modification (Luo et al., 2012; Rodriguez-Siek et al., 2005b). In total, 45 targeted virulence genes were detected by nine PCR reactions (Luo et al., 2012). The virulence factor score to evaluate ExPEC pathogenicity was calculated as the sum of positively detected virulence markers in each isolates. Avian-origin MCRPEC isolate was defined when ≧2 of the following 5 ExPEC-defining genes were presented: *papA* and/or *papC* (P fimbriae), *sfa/focDE* (S/F1C fimbriae), *afa/draBC* (Dr-binding adhesins), *kpsM* II (II capsule), and *iutA* (aerobactin system) (Johnson et al., 2017, 2012b).

2.3. Pathogenicity assessment of mcr-1-positive clinical isolates

Ethics statement: All animal experimental protocols were approved according to the guidelines of Experimental Animal Management Measures of Jiangsu Province and were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province, China.

The virulence status of *mcr-1*-positive clinical isolates and other control APEC strains to cause colibacillosis were determined by duck and chick colisepticemia models, according to the previous report (Zhuge et al., 2019b). For mouse sepsis model, 8-week-old imprinting control region (ICR) mice (10/group) were injected intraperitoneally with ~ 1.0×10^7 CFU/mouse with each MCRPEC strain (Zhuge et al., 2013). The ability of MCRPEC isolates to induce septicemia and enter the central nervous system was assessed with an established rat model of *E. coli* meningitis with slight modification (Tivendale et al., 2010; Zhu Ge et al., 2014). The ability of *mcr-1*-positive isolates to cause UTI was assessed in mice, as described previously (Allsopp et al., 2012; Thai et al., 2010).

2.4. Genome sequencing of MCRPEC isolates

For Illumina sequencing, > 2 μg genomic DNA of each strain was used to construct sequencing library. Paired-end libraries with the insert size of ~400bp were constructed according to the manufacturer's instructions (Biooscientific, AIR™ Paired-End DNA Sequencing Kit). Subsequently draft genomic sequence for each strain was obtained by sequencing 150bp at each end using Illumina Hiseq X Platform. For Pacific Biosciences sequencing, 20k insert whole genome shotgun library for each strain was generated and sequenced on a Pacific Biosciences RSII instrument using standard methods.

Virulence genes were identified by VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) plus extra genes, shown in Table S7 (% ID threshold is larger than 90% and minimum length coverage is larger than 60% of target genes) (Kallonen et al., 2017).

2.5. Statistical methods

Factorial analysis of correspondence (FAC), which uses a covariance matrix relying on χ^2 distances, is a method to study amounts of data (Johnson et al., 2006a).

2.6. Accession number(s)

Shotgun sequences of 7 MCRPEC isolates had been deposited in the NCBI database (BioProject accession no. PRJNA488670). And 3 complete genomes of CT29 (accession no. CP032073), E166 (accession no. CP032066), and CT30 (accession no. CP032078).

For information and detailed descriptions on Materials and Methods, see Text S1 in the supplemental material

3. Results

3.1. Identification of ExPEC virulence genotypes among MCRPEC isolates

Our previous study showed that avian-source MCRPEC isolates (n = 172) were widely distributed in phylogroups A, B1, B2, D, and F (Zhuge et al., 2019a). MCRPEC isolates were assigned to 52 unique STs, suggesting the substantial horizontal dissemination of *mcr-1* gene through *E coli* populations (Zhuge et al., 2019a). Moreover, the chicken-source MCRPEC isolates (n = 129) were assigned to 49 STs, apart from ST93, ST227, and ST62. The chicken-source MCRPEC isolates belong to 27 unique STs. ST profile showed the duck-source MCRPEC strains shared the most common STs with chicken-source MCRPEC, suggesting there was no phylogenetic diverse of duck-source MCRPEC population relative to the chicken. In this study, the analysis of virulence gene content among 172 MCRPEC isolates revealed the widespread prevalence of ExPEC virulence-related genes in several specific STs or phylogroups. Table S1 and Fig. 1A showed the detailed distribution of ExPEC virulence genes among MCRPEC isolates. Extended virulence genotyping of these isolates identified 35 of the 43 ExPEC virulence factors in at least one strain.

The ExPEC virulence-related genes were significantly prevalent among phylogroup B2, D, and F MCRPEC isolates. The virulence factor (VF) scores among five phylogroups were shown in Fig. 1B. MCRPEC isolates (n = 81) presented VF score ≥ 15, including 10 (16.4%, 10/61) strains in group A, 27 strains in B1 (67.5%, 27/40), 19 strains in B2 (100%, 19/19), 10 strains in D (38.5%, 10/26), and 16 strains in F (61.5%, 16/26). These 81 MCRPEC isolates were divided into 20 ST types, among which 2 STs (ST23 and ST48) were in group A, 8 STs (such as ST602, ST101, and ST5694) in group B1, 3 STs (ST95, ST127, and ST131) in group B2, 2 STs (ST501 and ST2732) to group D, 4 STs (ST117, ST648, ST2171, and ST2085) in group F. Although MCRPEC isolates (VF score ≥ 15) were from different poultry sources (60 chicken-source and 21 duck-source) (Table S1), the virulence genotypes showed that MCRPEC isolates in the same ST types shared most similar

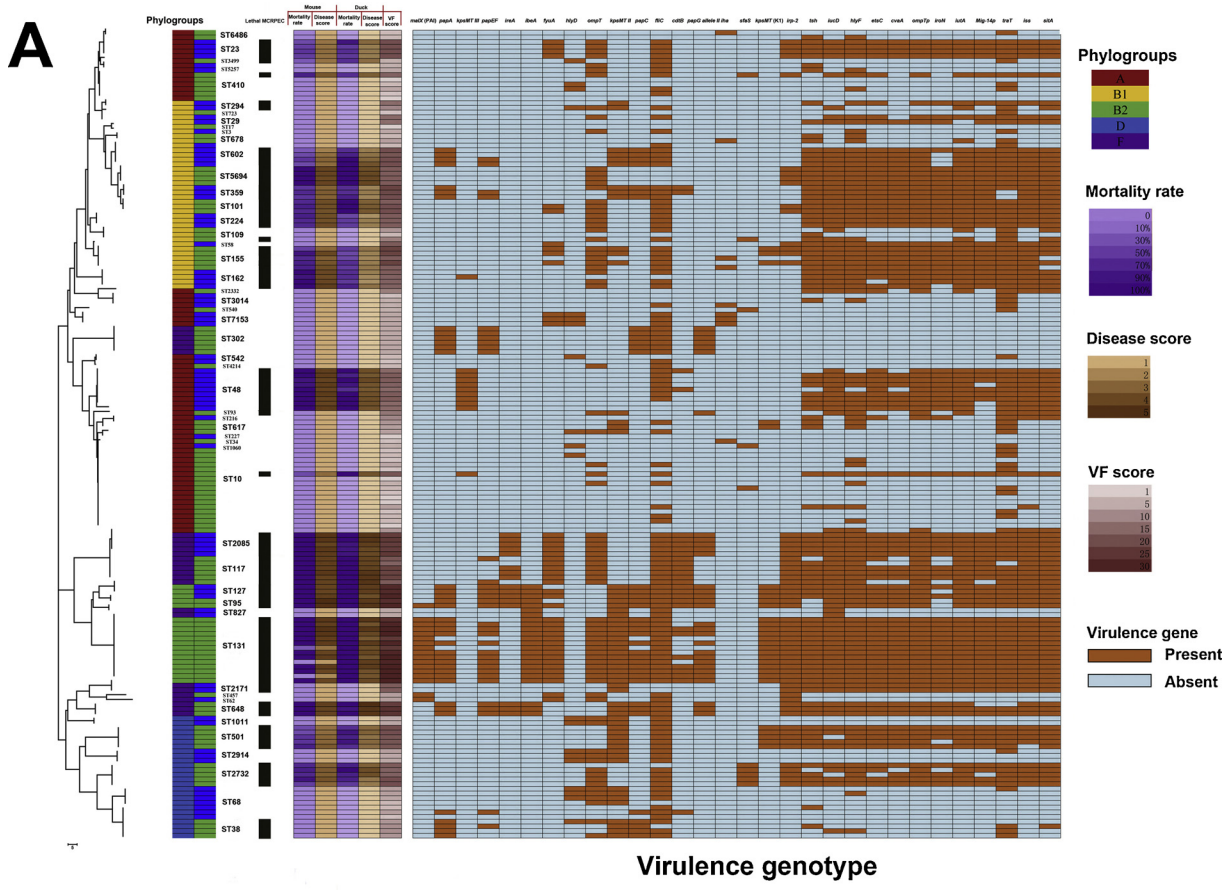

Fig. 1. The association of ExPEC virulence genotypes and ST types with extraintestinal infection among 172 MCRPEC isolates. (A) Distribution profiles of ExPEC virulence-related genes and the pathogenic potential of avian-source 172 MCRPEC isolates. On the left was the MLST-based phylogenetic tree. The first two columns adjacent the phylogenetic tree represented the phylogroups and ST types for each isolate. Immediately following the first panel were the classification of lethal MCRPEC strains. The next panel for 5 columns represented the pathogenic level of MCRPEC isolates assessed by duck and mouse infectious models, and the parameters included number of dead animals, disease score, and corresponding VF score for each MCRPEC strain. The rightmost panel for 35 columns indicated that the presence or absence of ExPEC virulence genes, respectively. VF score for each MCRPEC isolates was based on the distribution profiles of ExPEC virulence genes. (B) Number of MCRPEC isolates with different VF scores among five phylogroups(A, B1, B2, D, and F).

gene profiles, especially in these dominant STs (ST48, ST68, ST117, ST131, ST155, ST302, ST410, ST602, and ST2085).

Based on the criterion to identify an ExPEC pathotype, 57 (33.1%) MCRPEC isolates were classified as ExPECs, and the remaining strains were qualified as non-ExPEC strains (Fig. 1A and Table S1). Moreover, 49 classified ExPEC isolates presented VF score ≥ 15, except for 8 strains in 4 STs (ST38, ST93, ST109, and ST294), which didn't harbor ColV/ColBM plasmid virulence traits. Even without fimbriae, group II capsule and aerobactin determinants, it was noteworthy that 33 so-called non-ExPEC strains, identified by ExPEC-defining criteria, presented VF score ≥ 15 and might be endowed with the ExPEC pathogenic characteristics. These non-ExPEC strains were divided into 9 STs (such as ST23, ST48, ST117, and ST5694) and belonged to phylogroup A, B1, and D.

3.2. Association of MCRPEC infection with virulence genotypes and ST types

Here, the pathogenic potential and zoonotic risk of avian-source MCRPEC isolates were further assessed by duck/chick models of avian colibacillosis and three rodent models for ExPEC-associated human infections.

3.2.1. Ability to cause avian colibacillosis

To assess the infection severity caused by MCRPEC (~ 5.0×10^7 CFU/duck dose), we categorized *E. coli* isolates as "lethal" if they lead to mortality for at least one duck, and "nonlethal" if all ducks survive. Half of MCRPEC isolates (85, 49.4%) lead to duck death. Then, each group of 10 ducks was inoculated intratracheally with every lethal MCRPEC strain (~ 1.0×10^7 CFU/duck dose). The tested 85 isolates presented high pathogenicity, comapred to the negative control MG1655 in duck colibacillosis model (Fig. S1) ($P < 0.01$). These isolates resulted in a wide range of mortality rate, and each clinical isolate was assigned to an average disease score within 3-day post-infection (dpi) (Fig. S1). Pathogenic MCRPEC isolates (n = 85) belonged to 20 STs, and most of pathogenic MCRPEC isolates presented VF score ≥ 15. Moreover, the virulence level (disease score and mortality rate) of MCRPEC isolates in 8 STs (ST48, ST101, ST5694, ST95, ST131, ST117, ST2085, and ST648) was close to the highly virulent DE205B (the positive control) and exhibited near 100% mortality rate and with the disease score greater than 2.63 (Fig. 1A and S1). Based on the ExPEC identification criterion, 77.2% (44/57) typical ExPEC isolates presented pathogenicity in duck model. Moreover, 38 non-ExPEC strains in 11 STs (such as ST48, ST117, and ST602) led to the mortality in duck model (Fig. 1A and S1). Similar to the results of duck infection model, half of MCRPEC exhibited pathogenicity in chick infection model. As shown in Fig. S2, every strain presented virulence in duck model, also caused the colibacillosis in the chick model. These above results showed the high VF-score strains possessed the ability to cause avian colibacillosis either in chicken or duck.

ColV/ColBM plasmids have critical roles in the APEC/ExPEC pathogenesis (Johnson et al., 2006b, c). The virulence genes (*iucD*, *hlyF*, *etsC*, *ompTp*, *iroN*, *iutA*, *iss*, and *sitA*) were encoded by ColV/ColBM plasmids. It was worth noting that these virulence genes were significantly associated with pathogenic MCRPEC isolates. Therefore, it was not yet possible to define whether these avian-source strains as ExPECs on the basis of the current screening standard (presence of≧2 of the following 5 ExPEC-defining genes). We speculated that this "ExPEC-defining" criteria might not identify ExPEC strain accurately, only a group of human ExPEC wherein these factors were known to be commonly associated with this *E. coli* pathotype.

3.2.2. Ability to cause mouse sepsis

In the mouse sepsis model, the pathogenicity of 85 virulent ExPEC isolates was determined, and the illness severity score and mortality of these isolates were compared with the control DE205B and MG1655. Most of the selected ExPEC isolates caused mouse mortality post-inoculation, whereas two ST131 isolates (E048 and AE64) were non-lethal (Fig. 1A and S3). These lethal MCRPEC isolates resulted in a wide range of mortality rate from 30% to 100%, and 48 *E. coli* in 9 ST-types (ST48, ST95, ST117, ST127, ST131, ST162, ST648, ST2085, and ST5694) behaved like DE205B, killing more than 90% of mice, and presented higher disease scores close to that of DE205B (Fig. 1A and S3). Similar to the result of avian colibacillosis model, 38 non-ExPEC strains with VF score ≥ 15 in 11 STs (such as ST48, ST117, and ST602), presented pathogenicity in the mouse sepsis model. Our results showed that the vast majority of pathogenic MCRPEC isolates still possessed high virulence to cause bloodstream infections in mouse sepsis model.

3.2.3. Ability to cause meningitis

ExPEC strains with *kpsMT* (K1 capsule) and invasion gene *ibeA* gene were classified as NMECs (Mitchell et al., 2015). Thus, in a neonatal rat model, 13 K1 or *ibeA*-positive pathogenic isolates in 6 ST types (ST95, ST127, ST131, ST155, ST501, and ST648) and 11 high-virulent ExPECs in 6 STs (ST48, ST101, ST117, ST162, ST2085, and ST5694) were selected to assess the ability to cause meningitis compared to the NMEC strain RS218 and negative control MG1655. Our result showed that all of the tested pathogenic isolates could induce bacteremia in the blood of neonatal rats at 24hpi (Fig. 2A) ($P < 0.01$). The number of bacteria in the blood of neonatal rats infected with the MCRPEC isolates were varied, and many isolates in 8 ST types (ST48, ST95, ST117, ST131, ST162, ST648, ST2085, and ST5694) exhibited higher bacteria proliferation level similar to that of RS218-inoculated neonatal rats (Fig. 2A). The MCRPEC isolates in ST48, ST95, ST117, ST131, ST162, ST648, ST2085, and ST5694, which were capable of inducing high bacteremia in blood, also caused higher colonization in the brain of neonatal rats (Fig. 2A). Even without K1 or *ibeA*, these strains in ST types (ST48, ST117, ST162, ST2085, and ST5694) could still induce high-level of bacteremia in blood and colonization in brain of neonatal rats.

3.2.4. Ability to urinary tract infection

In the mouse UTI model, 24 MCRPEC isolates in 12 STs (such as ST48, ST648, and ST5694), which presented high virulence in duck and mouse model, were further assessed the capability to cause UTI. The number of bacteria in the bladder and kidney of mice at 48hpi after inoculated with MCRPEC strains was shown in Fig. 2B. Our results showed that all the ST131 isolates could grow in mouse bladder and presented a significantly greater degree of infection than that observed in the negative control of MG1655 ($P < 0.01$) (Fig. 2B). The proliferation/colonization level of ST131 isolates in mouse bladder and kidney equaled or exceeded the infection of positive control of CFT073 ($P > 0.05$). Moreover, the tested MCRPEC isolates in other 9 ST-types could also cause UTI. The bacterial counts showed that many non-ST131 isolates (such as tested ST48, ST95, and ST648 strains) induce UTI, which was close to that of ST131 isolates in mouse bladder and kidney (Fig. 2B).

3.2.5. Factorial analysis of correspondence (FAC)

FAC was performed to further explore the relationship among ST types, phylogroups, and virulence genotypes of MCRPEC lethal in duck colibacillosis or mouse sepsis models. For duck mortality status, the F1/F2 plane accounted for 28.72% of all statistical variance, accompanied by factors F1 and F2 accounting for 17.34% and 11.38%, respectively (Fig. 2C). This group mainly contained ColV plasmid-related virulence genes, which were closely related to mortality status and occurred significantly in high virulent MCRPEC. Phylogroups (B2, F, and B1) and almost ST-types (such as ST131, ST48, ST117, and ST648) for high-virulent MCRPEC were also closely related to mortality status (Fig. 2C). For mortality status in mouse sepsis model, a similar statistical result showed that ColV plasmid-related virulence factors could play critical roles in ExPEC bloodstream infection (Fig. 2D).

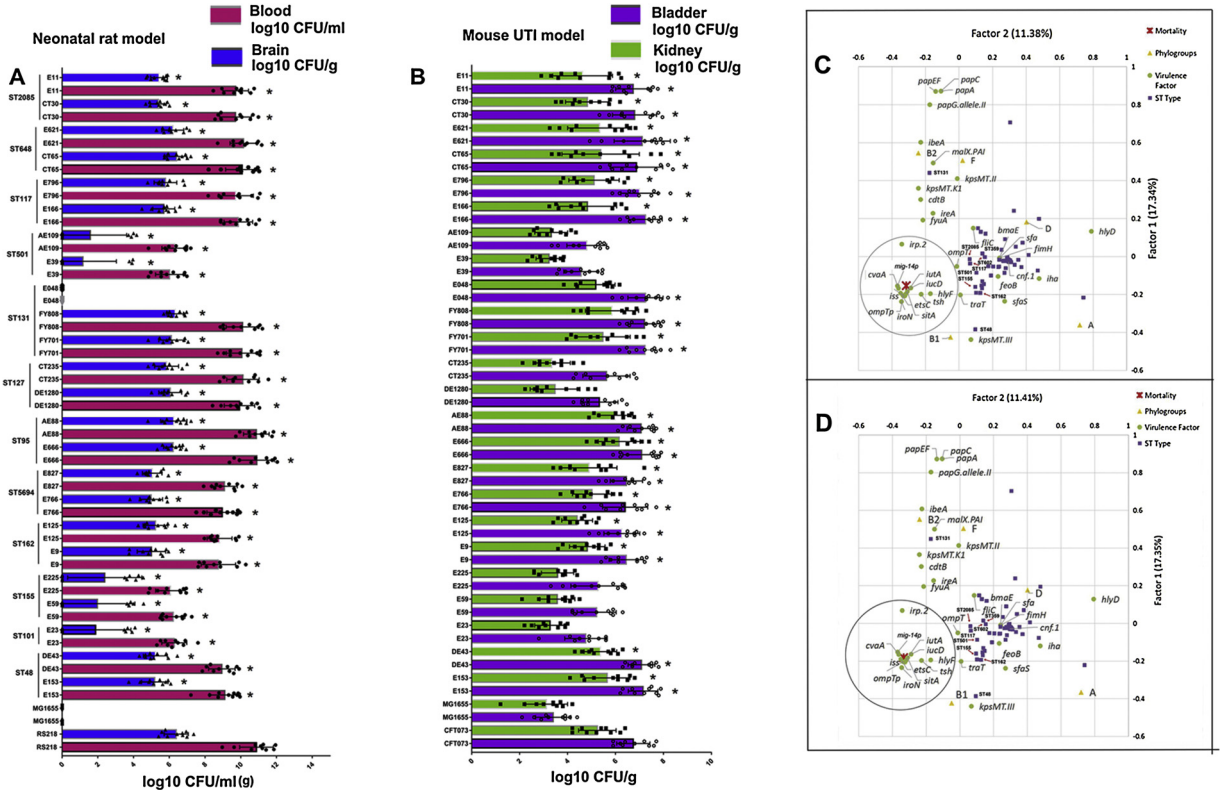

Fig. 2. The pathogenic and zoonotic potential of avian-source MCRPEC isolates assessed by meningitis and UTI models. (A) Virulence level of MCRPEC isolates to cause rat meningitis. Number of CFU in infected rat blood (red) and brain (blue) expressed as log10 CFU. The significant differences compared with the negative control MG1655 were identified using one-way ANOVA statistical analysis (*, $P < 0.01$), and the mean values ± SEs were shown in each histogram.. (B) Ability of MCRPEC isolates to cause mouse UTI. The significant differences compared with the negative control MG1655 were identified using one-way ANOVA statistical analysis (*, $P < 0.01$). (C) Factorial analysis of correspondence (FAC) to identify the relationship among MCRPEC ST types, phylogroups, and virulence genotypes with the lethality status in duck colibacillosis. The duck mortality status acted as the positive value of factors F1 and F2. The loading score for special variables positioned on the F1/F2 plane could be pointed out by variable's *X* and *Y* coordinates. (D) FAC between *E. coli* traits and mortality status in mouse sepsis model, among 172 MCRPEC isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Characterization of large plasmids in MCRPEC isolates

We performed the whole-genomes sequencing of 10 selected MCRPEC (based on the ST types, phylogroups, antimicrobial susceptibility, and virulence) to reveal the plasmid content or profile in *mcr-1*-positive ExPEC. The *mcr-1*-carrying plasmid and contigs were extracted from the sequenced genomes of MCRPEC isolates (Table S2 to S5). Diverse plasmid replicons were identified in *mcr-1*-containing plasmids or contigs (Fig. S4) (Table S2 to S5). The plasmid replicon was not detected in *mcr-1*-containing contig of strain E15, which might be separated with its replicon-carrying contig. The mobile genetic determinants at upstream and downstream of the *mcr-1* gene were identified among the *mcr-1*-carrying plasmids or contigs. The genetic context in the immediate proximity of *mcr-1* of 10 isolates was shown in Fig. S4. Our results showed that the *ISApl1* insertion sequence, first reported in *mcr-1*-positive plasmid pHNSHP45 from swine-origin *E. coli* (Liu et al., 2016), was located immediately upstream of the IncHI2-related *mcr-1*-carrying contigs from strains (E66 and E796), while the other 6 IncI2-related *mcr-1*-carrying contigs from isolates harbor *nikB*, encoding relaxase domain protein, other than *ISApl1* were adjunct to *mcr-1* gene (Fig. S4).

The non-pathogenic MDR strain CT29 harbored 3 large plasmids (Fig. 3A and Table S3). Plasmid pCT29-1 didn't carry any antimicrobial resistance gene or the known plasmid backbones. This accessory region shared identity with the *E. coli* S56 plasmid B (NZ_CP010244.1). The drug-resistant plasmid pCT29-2 carrying the resistance genes bla_{TEM-1B}, *oqxA*, *oqxB*, and *qnrS*, was similar to plasmid pH1038-142 (NC_025141.1) from one human *E. coli* isolate. As shown in Fig. 3A, IncX4-type pCT29-MCR1 was a derivative of pICBEC72Hmcr (NZ_CP015977.1) from a human invasive ST101 *E. coli* (Fernandes et al., 2016). Moreover, strain CT29 contained a small *β*-lactamase-containing plasmid pCT29-4, which carried bla_{OXA-10} and flanking antimicrobial resistance genes.

The high-virulent MDR MCRPEC strain E166 harbored 3 large plasmids (Fig. 3B). The IncY-type pE166-1 carried multiple non-lactamase resistance genes. The other IncFIB virulence plasmid pE166-2 harbored intact ColB and ColM operons (cba/cbi; cma/cmi, respectively) shared remarkable similarity with other ColV/ColBM plasmids (Fig. 3B) (Johnson et al., 2006b, c; Zhu Ge et al., 2014). The *mcr-1*-carrying plasmid pE166-MCR1, belonging to IncI2 replicon, contained a conjugal transfer region, *mcr-1* inserting elements, and *β*-lactamase $bla_{CTX-M-64}$ resistance gene (Fig. 3B). pE166-MCR1 shared high identity with plasmid pMTY17668-MCR1.5 (62,375bp, NZ_AP018110.1) in an invasive *E. coli* from a Japanese infant(Ishii et al., 2018).

The high-virulent MDR MCRPEC strain CT30 also harbored 3 large plasmids (Fig. 3C). The IncHI2A-type pCT30-1 was a drug-resistant plasmid, carrying antimicrobial resistance genes shared identity with the plasmid (NZ_CP015833.1) from human *E. coli* O157:H7 strain 180-PT54 (Cowley et al., 2016). The hybrid IncFIB/IncFII-types pCT30-2 was also a ColBM-type plasmid (Fig. 3C). IncI2-type pCT30-MCR1 was a derivative of pMTY17668-MCR1.5 (62,375bp, AP018110.1) identified in ST7278 *E. coli* from the stool of a Japanese female infant (Ishii et al., 2018).

Many replicon types were detected in E15 and E825 contigs, which also harbored the plasmid-carrying antimicrobial resistance genes (Fig. 3D and Table S6). However, ColV or ColBM plasmid-carrying

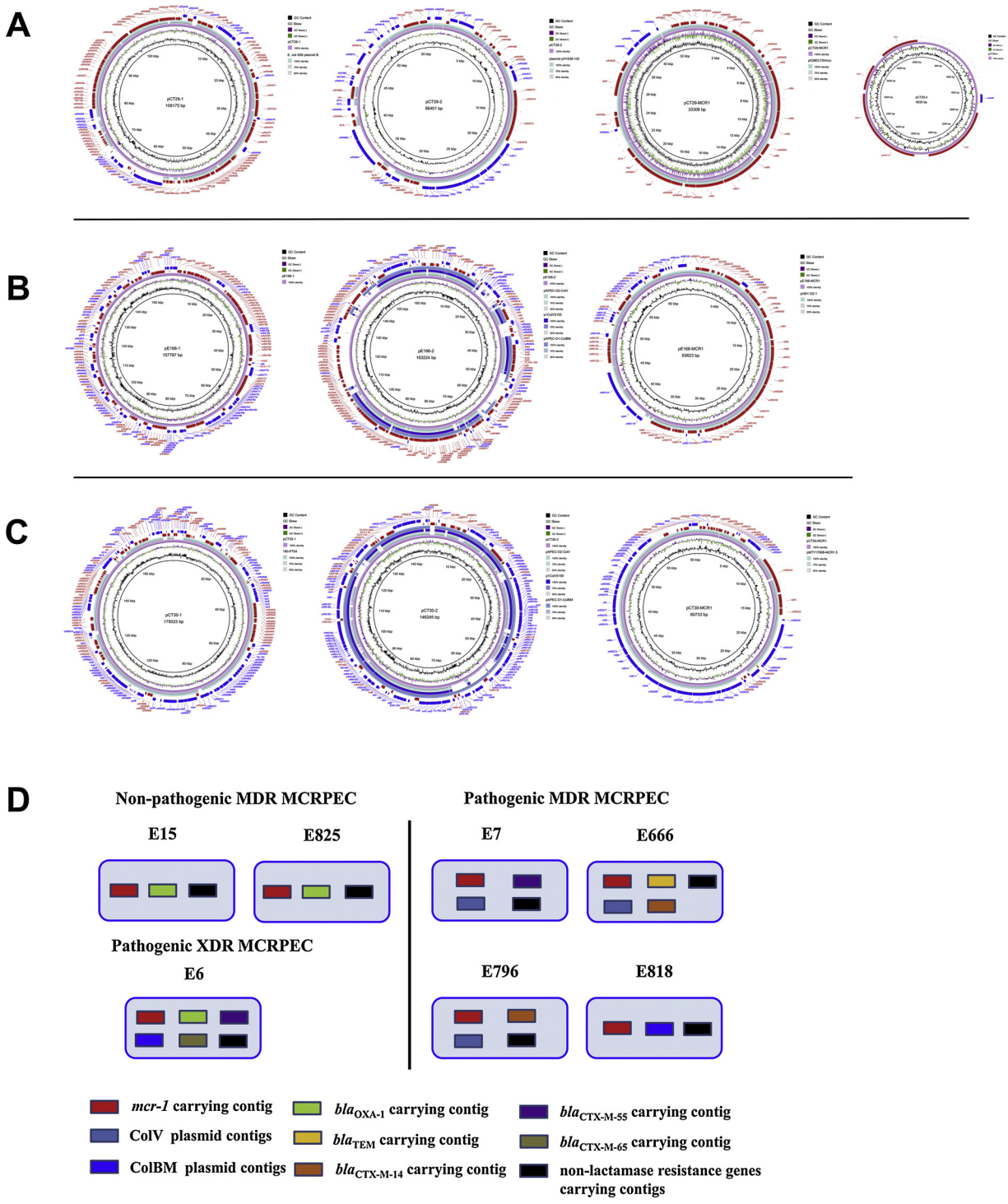

Fig. 3. The plasmid profiles of MCRPEC isolates. (A) Gene map of the complete plasmid sequences carried by non-pathogenic MDR strain CT29. This strain harbored 3 large plasmids (pCT29-1, pCT29-2, and pCT29-MCR1) and a small β-lactamase-containing plasmid pCT29-4. The circular genome comparison maps were produced by BLAST Ring Image Generator. From inside to outside, circular representation of gene alignments showed GC content (%), GC skew, common gene map with reference large plasmids, and plasmid ORFs prediction. (B) Gene map of the complete plasmid sequences carried by high-virulent MDR MCRPEC strain E166. This strain harbored 3 large plasmids pE166-1, pE166-2, and pE166-MCR1. From inside to outside, circular representation of gene alignments showed GC content (%), GC skew, common gene map with reference large plasmids, and plasmid ORFs prediction. (C) Gene map of the complete plasmid sequences carried by the high-virulent MDR MCRPEC strain CT30. This strain harbored 3 large plasmids pCT30-1, pCT30-2, and pCT30-MCR1. (D) Schematic depiction for the potent cocktails of plasmid-carrying virulence and drug-resistant contigs among MCEPEC draft genomes by BLSTN. The functional plasmid contigs of 7 MCRPEC isolates were be summarized, and 9 categories of plasmid contents with special colour keys were shown at the bottom.

virulence genes were not detected in the genomes of E15 and E825. The ColV plasmid-carrying virulence genes were identified in genomes of virulent MDR MCRPEC strains E7, E666, and E796. (Fig. 3D and Table S6). Moreover, many contigs carrying resistance genes were identified in these strains (Table S6). Multiple resistance genes, including $bla_{CTX\text{-}M\text{-}14}$ and $bla_{TEM\text{-}1B}$, were detected in several contigs of high-virulent MDR ST95 strain E666. Non-lactamase resistance genes and ColBM plasmid-carrying virulence genes were detected in high-virulent MDR E818 genome, but not β-lactamase gene. The ST131 *E. coli* is recognized as an international multidrug-resistant clone (Mathers et al., 2015). ColBM plasmid-related contigs and multiple plasmid-carrying resistance genes were identified in the genome of extensively drug-resistant (XDR) strain E6 (Fig. 3D).

The sequenced MCRPEC isolates shared identity with a variety of known resistant genes (β-lactamase, *mcr*-1, etc.). Meanwhile, the plasmid profiles of these sequenced MCRPEC isolates possessed the diversity of genetic contents with each other (Fig. 3 and Table S2 to S6). This result indicated that the drug-resistant plasmids exhibited remarkably flexible and heterogeneous transformation into avian-origin ExPEC and might just limit the incompatibility replicons. In contrast, the transfer of IncFIB ColV/ColBM plasmids still acted as the major horizontal dissemination route among avian-origin MCRPEC.

3.4. ColV/ColBM plasmids in MCRPEC isolates from humans and poultry

Given the significant correlation of avian-origin MCRPEC between zoonotic risk, we analyzed the virulence gene contents and ST genotypes of human MCRPEC isolates. In the NCBI nucleotide database, we found 616 whole genomes of *mcr-1*-positive isolates, including 412 human MCRPEC strains, 139 avian isolates, and 66 isolates from the environment, etc. A midpoint-rooted maximum-likelihood phylogenetic tree of these MCRPEC was constructed using core-genome SNPs. There was no clear phylogenetic difference among human, avian-origin, and environmental isolates, which were distributed throughout the five phylogroups (A, B1, B2, D, and F) (Fig. 4A). Our previous study showed that overlapped distribution of MLST genotypes between human and avian-origin MCRPEC isolates share the popular ST types (such as ST48, ST101, ST117, and ST410) (Zhuge et al., 2019a). The heatmap showed the distribution of ExPEC-related virulence genes in whole-sequenced MCRPEC. Apart from ColV/ColBM-carrying virulence genes, MCRPEC isolates from phylogroups B2, D, and F possessed the greater number of ExPEC-related virulence factors, while the isolates of phylogroup A and B1 displayed low distribution of these genes (Fig. 4B).

ColV/ColBM plasmids were recognized as a characteristic of animal-source ExPEC, but were rarely detected in human prototypic ExPEC strains (Rodriguez-Siek et al., 2005a, b). Therefore, ColV plasmids might be used as a definitive trait to reveal the zoonotic transmission of MCEPEC from poultry or livestock to humans. Here, we analyzed the presence of ColV plasmid accessory traits with a high-resolution phylogenetic tree to identify the portion of avian-source MCRPEC among human infections (Fig. 4A). The ColV/ColBM-carrying virulence genes were presented in 138 whole genomes of MCRPEC isolates (avian-source: 34.5%; human clinical source:18.9%) (Fig. 4A). Even though 18.9% instead of 34.5% was less in human MCRPEC isolates, our result showed not nearly as much less than the referenced reports citing the presence of ColV plasmids, as rarely detected. One major difference herein was that the tested *E. coli* isolates were all *mcr-1* containing resistant strains. We speculated ColV/ColBM plasmids could not only promote virulence, but also possibly lead to co-transfer of the *mcr-1* genes on the second plasmid, followed by the co-transfer of virulence and resistance gene-containing plasmids in *E. coli*. The above results suggested that poultry might be as an important reservoir for human MCRPEC clinical isolates. However, more evidence are needed to confirm the transfer route of MCRPEC from poultry or food sources to human. Based on the ExPEC pathotype identification standard, only 22 (3.5%) MCRPEC from NCBI were classified as ExPECs (Table S7). However, the ColV plasmids were mainly present in the remaining MCRPEC strains.

4. Discussion

Pathogens in poultry are a serious threat to poultry farming and

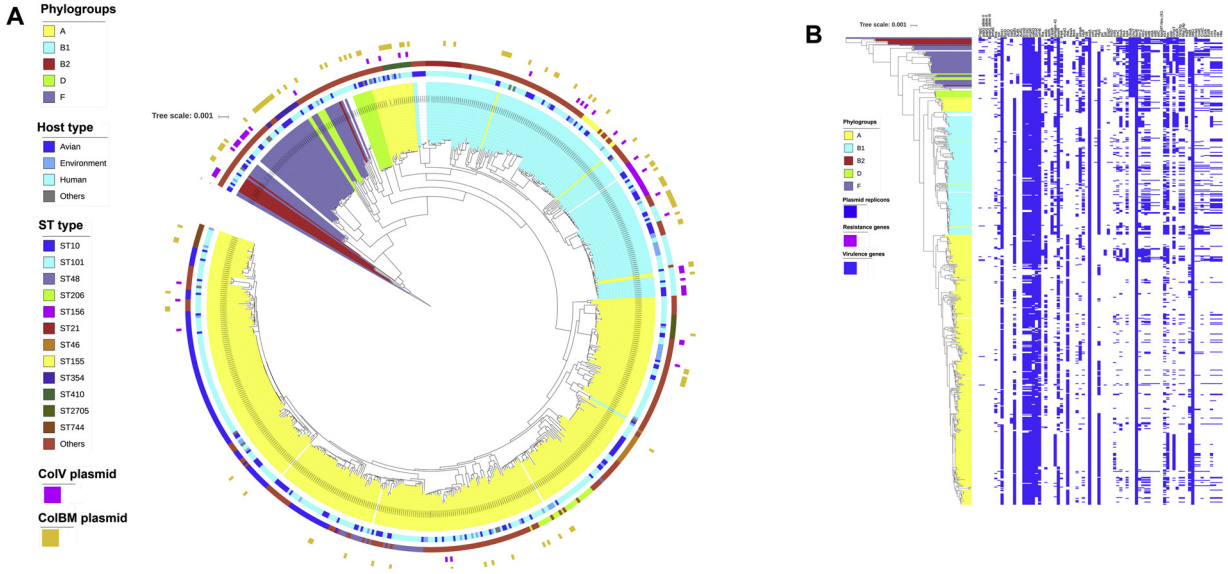

Fig. 4. Overlapped distribution of resistance gene contents among MCRPEC isolates from humans and poultry. (A) Genetic relationships among 623 MCRPEC isolates from humans, avian, environment, etc. These MCRPEC genomes were mainly obtained from NCBI online database, additionally our sequenced MCRPEC genomes. The maximum-likelihood phylogenic tree of all MCRPEC strains was generated using core-genome SNPs. Isolates from different hosts were depicted in specific colors. Phylogroups (A, B1, B2, D, and F) for almost MCRPEC isolates were presented by tree background shading. Outer ring of phylogenic tree showed host information, the ST types containing more than 5 strains, and the presence of ColV or ColBM plasmids. (B) Genomic analysis of MCRPEC isolates from NCBI online database. Distribution profiles of plasmid replicon types, antimicrobial resistance genes, and ExPEC virulence factors across core-genome phylogenic tree of MCRPEC isolates from NCBI online database. On the left was the MCRPEC phylogenetic tree, depicted with each phylogroup. The right panel revealed the distribution profile of ExPEC virulence-related genes. Details of these genes distribution in each category were shown in Table S7.

public health. The colibacillosis caused by ExPEC infection is the major bacterial disease of poultry (chickens, ducks, and turkeys) (Mitchell et al., 2015). In 2015, the occurrence of animal-origin MCRPEC was reported in China, and the *mcr-1*-carrying plasmids rapidly disseminate from animals to human (Liu et al., 2016). Since *E. coli* is the most common Gram-negative bacteria, the high prevalence of MCRPEC caused both a clinical and an epidemiological challenge. The isolates in groups B2 and D are recognized as the ExPECs that cause extraintestinal infections (Salipante et al., 2015; Zhu Ge et al., 2014). In this study, we identified that nearly half of MCRPEC isolates possessed the pathogenicity in five animal models. Moreover, we found that not only MCRPEC in phylogroups B2 and D, but also several *E. coli* populations in groups B1 and F presented high pathogenicity. Most of the virulent MCRPEC isolates belonged to 12 STs. MCRPEC isolates in 8 STs (ST48, ST95, ST101, ST117, ST131, ST648, ST2085, and ST5694) exhibited similarly high pathogenicity in duck and chick colibacillosis model. Apart from ST101, the tested MCRPEC strains in these ST-types also possessed high virulence in the mouse sepsis model. We found the MCRPEC isolates in ST127 and ST162 exhibited high pathogenicity to cause bloodstream infections. Moreover, the majority of tested MCRPEC strains with high virulence in avian colibacillosis and/or mouse sepsis model, could induce high-level of meningitis in neonatal rats model and cause urinary tract infection in mouse UTI model. Strict host specificity for these highly pathogenic MCRPEC strains was not identified at these five infection models.

Besides ST95 and ST131, the known pathogenic ExPEC clones (Johnson et al., 2012a), many MCRPEC isolates other than those from the “classic ExPEC groups” were also virulent in the animal models, which was largely associated with the presence of ColV/ColBM plasmids. Moreover, the virulent ExPEC ST95 and ST131 isolates from avian sources probably also harbored the ColV/ColBM plasmids. Our FAC analysis further revealed that the zoonotic risk of *mcr-1*-positive ExPEC appeared to be mainly associated with ColV plasmid-mediated virulence factors. The presence of ColV plasmids is considered as a predictive label for avian-source ExPECs (Johnson et al., 2006c; Shaik et al., 2017). The criteria to predefine ExPEC in a way that limits them to a certain group of human ExPEC, those often may not have a ColV plasmid. Poultry-source *E. coli* strains, associated with extraintestinal disease, may not possess some or all of these fimbriae/adhesin systems and *kps* capsule, which need not to be present in all ExPECs. Therefore, these criteria used to classify ExPECs may not be adequate for ExPEC screening or identification in poultry. Combined with our study, it is necessary to optimize the identification standard for avian-source ExPEC. These ExPEC-defining genes might mainly include the marks of ColV/ColBM plasmids, yersiniabactin system, P fimbriae, and group II capsule. In the future, we will establish the specific PCR assay to screen zoonotic ExPECs from poultry-source *E. coli*.

Our epidemiology study showed that the highly virulent MCRPEC clones belong to ST131, as well as ST48, ST117, ST162, ST501, ST648, and ST2085. Of particular concern, the characterization of large plasmids further demonstrated that the combinations of plasmids, mediating the coexistence of *mcr-1* along with ESBLs, exhibited remarkably heterogeneous combinations among avian-origin or human MCRPEC, even in same ST clones. Recently, Yossi Paitan summarizes the current situation and trend of *E. coli* antimicrobial resistance, and this review asserts a great concern that the occurrence of *mcr-1* in highly epidemical *E. coli* ST131 will be popular (Paitan, 2018). Our study confirmed the emergence of ST131 MCRPEC strains. However, the more serious concern would be the co-location of ColV/ColBM with resistance plasmids on poultry-source ExPECs. Comparative genomics analysis showed that ColV-carrying virulence factors were widely distributed in human MCRPEC isolates. Based on our results, we speculated that human MCRPEC isolates from the phylogoups A and B1, might have potential extraintestinal infection risk (Boswell et al., 2018).

Our data indicated the overlapped distribution of ST-types and plasmids contents in MCRPEC isolates from humans and poultry. The zoonotic MCRPEC clones, recognized as high-virulent ExPECs, belong to ST131, as well as ST-types (such as ST48, ST117, ST162, ST501, ST648, and ST2085). Identification of ColV/ColBM plasmids among human MCRPEC isolates revealed the potential transmission of avian-source MCEPEC to humans. The zoonotic risk of MCRPEC appeared to be a close association with ColV/ColBM type virulence plasmids. Given the significant zoonotic risk of avian-origin MCRPEC, poultry would be as an important source for human clinical MCRPEC isolates. Avian-origin *mcr-1*-positive ExPEC isolates, especially high-virulent clones, could be recognized as a foodborne pathogen.

Declaration of Competing Interest

All the authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108483.

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