Differences in Antibiotic Resistance of MDR Enterobacteriaceae between Broiler and Laying Farms in Which Two mcr-1 Positive Plasmids Revealed their Risk of Resistance

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Research Article

Keywords: Difference, ARGs, MGEs, multidrug-resistant Enterobacteriaceae, broiler and laying farms

Posted Date: January 24th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1274477/v1>

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1 **Differences in antibiotic resistance of MDR *Enterobacteriaceae* between**
2 **broiler and laying farms in which two *mcr-1* positive plasmids revealed their risk**
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Abstract

Background: Existing studies on poultry (broilers and layers) suggest that strong screening of antibiotics leads to the wide spread of antibiotic resistance genes (ARGs). However, due to the different breeding cycle, antibiotic use and breeding environment characteristics of layers and broilers, they have different antibiotic resistance risks. Until now, there are few studies on the difference in risk of antibiotic resistance between broilers and layers, which is still a gap in our knowledge.

Results: In the current study, high-throughput sequencing-based metagenomic revealed that *firmicutes* and *bacteroidetes* were the dominant bacteria in broiler and layer farms. *Aph(3''')-III* and *ermF* were the ARGs with the highest absolute abundance in broiler farms, which was higher than that of laying farms. *TetW* was the highest absolute abundance of ARG in laying hens, but it was still lower than broiler chickens. Three hundred fecal samples were used to isolate multidrug-resistant *Enterobacteriaceae* (MDRE). It was found that the detection rates of *mcr-I*, *aph(3')-Ia*, *aadA2*, *bla_{OXA-1}*, *bla_{CTX-M}*, *fosB*, *qnrD*, *sulI*, *tetA* and *catA1* in broiler source MDRE were significantly higher than that in laying farms ($P < 0.05$). Further studies showed that a chimeric plasmid p20432-mcr which carried the novel integron *In1866* was isolated from broiler source MDRE. High frequency of conjugation (10^{-1} to 10^{-3}) and wide range of hosts made p20432-mcr likely play an important role for the high detection rate of *mcr-I*, *aph(3')-Ia* and *aadA2* in broiler farms.

Conclusions: We comprehensively compared the prevalence of pathogen MDRE and their ARGs in the two environments and the mechanism of transmission. It is found that the *mcr-I* positive plasmid p20432-mcr has a wide host range which may play an important part in the high detection rate of ARGs in broiler source MDRE. These findings will help to optimize disinfection and antibiotic resistant bacteria surveillance

38 programs in poultry farms, especially in broilers.

39 **Keywords:** Difference, ARGs, MGEs, multidrug-resistant *Enterobacteriaceae*,

40 broiler and laying farms

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Introduction

Antibiotic resistance has been characterized as a global public health concern, which catalyzed by the large amounts of antibiotics used in the production of food-producing animals [1,2]. Every year, hundreds of tons of antibiotics used to treat diseases and growth promoters in livestock and poultry [3, 4]. This has caused the accumulation of antibiotics in farmed environment, creating the constant selective pressure for antibiotics resistance to microorganisms [4], which make poultry become the reservoirs of resistant bacteria and resistant genes [5]. Then antibiotics resistance genes (ARGs) which can be transferred to human pathogens and enter the human food chain [6]. Therefore, transmission of resistant genes and their hosts in the farm environment needs to be monitored more frequently.

China is home to the largest layer chicken industry in the world, and is the second-largest producer of broiler chickens [7, 8]. These industries cause massive pollution of the environment by animal excretion [9]. The high numbers of resistant bacteria that are often isolated in feces turn farms into outposts for the spread of resistant bacteria [10]. ARGs mostly derived from the *Enterobacteriaceae* which have become increasingly common in human and animal infections [11]. Polymyxin is generally considered the last resort for the treatment of multidrug-resistant *Enterobacteriaceae* (MDREs) [12]. Although the Chinese government has banned colistin as a growth-promotor in livestock and poultry production [13]. With the wide spread of colistin resistance genes and increasing rates of MDRE [14], contamination of livestock and poultry products with these foodborne pathogens remains a major

risk to public health and may jeopardize the treatment of serious bacterial infections [14-16].

To date, most studies on antibiotic resistance in poultry production mainly focus on antibiotic residues and specific ARGs, and there are few studies on separate comparison between broilers and layers [16]. There is a knowledge gap in our understanding of the differences in bacterial community structure, antibiotic resistome, and mobile genetic elements (MGEs) between layers and broilers environment.

In this study, high-throughput sequencing-based metagenomic was used to analysis the abundance of ARGs and their potential bacterial hosts in the microbial communities. We conducted collect 300 fresh fecal samples from farm environmental of 3 broiler farms (BFs) and 3 laying farms (LFs) in Zhejiang, China, to characterize the difference of antimicrobial resistance in MDR *Enterobacteriaceae* (MDRE) between broiler and laying husbandry and analyze the multidrug resistance profiles of broilers and layers. We identified the detection of antibiotic resistance genes in MDRE that are prohibited or have different antibiotic requirements in broilers and layers. In particular, polymyxins are critically important for the treatment of MDRE infections in humans. Polymyxin resistance gene *mcr-1* was detected in both BFs and LFs. However, we analyzed two plasmids which carried *mcr-1*, and the chimeric plasmid p20432-mcr from BFs was a multidrug-resistant plasmid contains both IncHI2 type and IncN type replicons which make it have a wide range of hosts. Overall, this study provides a theoretical basis for the differential management of broiler and layer breeding systems in China and beyond.

Methods and Material

2.1 Study area and sample collection

Six farms corresponding to layers and broilers were selected for comparative study, all farms were located in Jiaxing, Zhejiang Province. None of the six farms were large intensive operations but small farms with chicken houses, which is very common in the local area. Each farm has four to six individual chicken houses and each house has about 80 chickens. Twenty fecal samples were randomly collected at each chicken house by swab. 150 fecal samples in BFs and 150 fecal samples in LFs were collected respectively in September 2021. In addition, soil (n=3), water (n=3), and faces (n=3) samples were collected from broiler farms and mixed into a composite sample. The same sampling scheme is also used in layer farms. A total of two composite samples were used for metagenomic analysis. All swab samples were transported to laboratory in normal saline within 8 hours for isolation of *Enterobacteriaceae*.

2.2 Metagenomic analysis

Metagenomic sequencing was performed using Illumina Hiseq 4000 with the sequencing strategy of Index 250 PE (paired-end sequencing). Raw data is filtered using fastp [17] in order to correct base and quality-filtered. Low-quality reads were filtered to ensure that (1) the reads were aligned to proper adaptors or primers, (2) the reads contained < 10% unknown bases, (3) the reads contained > 50% high-quality bases [18]. Bowtie2 [19] was used to remove reads from the host genome or contamination with high similarity. On average, 9.5 GB clean reads were generated

for each sample. ARG-OAP PROTO [20], an analysis pipeline, integrates two common resistance gene databases, including ARDB [21] and CARD [22], identifies assembly sequences based on hidden Markov model (HMM) was used to characterize the profile and quantify the relative abundance of ARGs.

2.3 Antimicrobial Susceptibility Test

The antimicrobial susceptibility of isolated strains were tested with the disk diffusion method which is recommended by Clinical & Laboratory Standards Institute (CLSI) [23] on Muleller-Hinton(MH) agar and evaluated based on the size of inhibition zones and classified as resistant, intermediate, or susceptible (the breakpoints were shown in Supplementary Table1). The strains exhibiting resistance to three or more classes of antimicrobials were considered to be multi-drug resistant organisms.

2.4 PCR amplification and sequencing of 16S rRNA.

The isolated strains were identified by VITEK-2 Compact and 16S rRNA gene identification using the universal 16s primers 27F and 1492R [24]. The system amplified by 5 μ L PCR was added into 1.2% agarose gel for electrophoresis, and the voltage was set at 120 V for 30 min. PCR amplification consisted of denaturation at 96 °C for 5 min followed by denaturation at 95 °C for 30s, annealing at 50 °C for 90s, and polymerization at 72 °C for 60s for a total of 30 cycles, and a final extension at 72 °C for 5 min. Western blot detection system was used to observe whether agarose gel had corresponding bands at 1500 bp after electrophoresis. PCR product of successful amplification was used paired-end sequencing (2x150bp) by Sangon

Biotech (Shanghai, China).

2.5 PCR amplification and sequencing of Antibiotic Resistance Genes

The isolated strains were screened for nine major classes of antibiotic resistance genes which are usually used for human clinical treatment or poultry by PCR using the primers listed in Supplementary Table2. PCR amplification consisted of denaturation at 95 °C for 5 min followed by denaturation at 94 °C for 30 s, annealing at their respective annealing temperature for 30 s, and polymerization at 72 °C for 40 s for a total of 30 cycles, and a final extension at 72 °C for 10 min. All the PCR products were subjected to Sanger sequencing.

2.6 Conjugal Transfer Experiments

To access the transferability of *mcr-I* positive plasmids, conjugal transfer experiments were performed with rifampin resistant *E.coli* strain EC600 used as a recipient and each of the *mcr-I* positive strain RB20432 and DB20D28 Isolates as a donor. The donor can grow in Brain Heart Infusion (BHI) agar plates with 2 µg/ml polymyxin and the recipient *E.coli* strain EC 600 can grow in BHI agar plates with 2.5 mg/mL rifampicin. After incubation at 37 °C for 24 h, colonies growing on selective plates were further confirmed by antimicrobial sensitivity experiments and VITEK-2 Compact.

2.7 Sequencing and Analysis of plasmids

The two conjugatable plasmids of the transconjugants RB20432-EC600 and DB20D28-EC600 were sequenced by the Illumina HiSeq platforms. The two plasmids DNA was extracted from the transconjugants RB20432-EC600 and

DB20D28-EC600 using the SanPrep Column Plasmid Mini-Preps Kit. After extraction, plasmid sequencing was performed by with a pair-end library with an average insert size of 300 bp on a Hiseq sequencer (Illumina). Quality control and removing low-quality data were performed with Trim Momatic 0.36 [25]. Gapcloser [26] was used to fill gaps. Finally, Cytoscape [27] was used to spline the sequence to obtain the final cyclized plasmids. The plasmid sequences were submitted to Rast [28], Genemarks, Glimmer, and Prodigal library for preliminary gene prediction then submitted to ISFinder、Tn Number Registry and Integrall [29] for MGEs.

2.8 Statistical analysis

The data of bacterial and ARGs were processed with Microsoft Excel 2016. Column was generated using Origin 2021. Heat map for resistance profile of MDRE was visualized via Heatmapper [30] (website URL: <http://www.heatmapper.ca/>). Independent sample t-tests were used by SPSS21.0 in analysis of antimicrobial susceptibility and detection of resistance gene of MDRE isolates, and $P < 0.05$ was considered statistically significant. Running a gene alignment program in Perl language with Ubuntu 18.04 LTS1 and Inkscape 0.48.1 were used to draw gene organization diagrams.

3.Results

3.1 Characterization of bacterial and ARG communities between LFs and BFs

The top ten relative abundance of the microbial communities at the phylum levels were shown in Fig. 1. *Firmicutes*, *Bacteroidetes* were the top two in all bacteria phyla, with 55.16% and 32.58% in BF's sample, 44.72% and 41.88% in LF's sample. It

can be inferred that *firmicutes*, *bacteroidetes* are the dominant bacteria in broiler and laying breeding environment which were potential host of ARGs. *Actinobacteria*, *proteobacteria*, *synergistetes* were the secondary dominant phyla.

The top ten antibiotic resistance genes in broiler and laying farms in absolute abundance were aminoglycosides, bacitracin, β -lactam, chloramphenicol, MLSBs (macrolide-lincosamide-streptomycin B), multidrug resistance genes, sulfonamides, tetracyclines, vancomycin and unclassified antibiotic resistance genes (Fig. 2). In general, ARGs of all ten classifications were detected in broilers, while MDR_transporter and *cpxR* were not detected in laying hens. A large number of aminoglycoside and tetracycline resistance genes were enriched in broilers and layers, and their absolute abundance in broilers was higher than that in layers. In broiler samples, *aph(3'')-III* had the highest absolute abundance, followed by *ermF*, 16S rRNA methylase, *tetQ*, etc. The absolute abundance of *tetW* in laying sample was the highest, followed by *aph(3'')-III*, *tetQ*, *aadE*, etc. The absolute abundance of vancomycin resistance gene *vanG* and *vanS* in laying samples was higher than that in broiler.

3.2 Detection of multidrug resistant *Enterobacteriaceae* in LFs and BFs

A total of 526 MDR *Enterobacteriaceae* were recovered from 300 fresh fecal samples, 272 in BFs and 254 in LFs. According to the total general detection of MDRE layers and broilers, *E.coli* whose percent reached 67.8% (357/526) were the dominant bacteria, most strains, the rest were *Salmonella* (8.6%, 45/526), *Shigella* (8.2%, 43/526), *Proteus* (7.8%, 41/526), *Citrobacter* (7.6%, 40/526) (Fig. 3).

3.3 Differences in antibiotic-resistant phenotypes of MDRE in BF_s and LF_s

MDRE isolated from 6 farms showed different resistance rates to 25 antibiotics (Fig. 4). Overall, oxacillin had the highest resistance rate among the 25 antibiotics, 96% in BF_s and 92% in LF_s. In addition, MDRE in BF_s and LF_s showed high resistance rates of more than 80% to five antibiotics which were amoxicillin, oxacillin, sulfamethoxazole, trimethoprim-sulfamethoxazole, and tetracycline. Comparison between the two farms showed that resistance rates of polymyxin B, meropenem, oxacillin, amikacin, streptomycin, gentamicin, ciprofloxacin, sulfamethoxazole and chloramphenicol were significantly higher in BF_s than in LF_s ($P < 0.05$). Among them, polymyxin B, which has been banned for use in poultry production in recent years, has a high resistance rate in BF_s, indicating that its resistance gene has been widely spread in the broiler environment.

3.4 Antibiotics resistance of *Enterobacteriaceae* in broilers was more serious than that in layers.

In order to reflect the multidrug resistance profile of MDRE in the culture environment more directly, they were divided into three resistance levels of mild antibiotics resistance (4R~10R), moderate antibiotics (11R~17R) and severe drug resistance (18R~24R) according to the number of antibiotics which MDRE is resistance to (Fig. 5). The proportion of moderate and severe resistance MDRE in BF_s was higher than LF_s, indicating that the MDR profile of *Enterobacteriaceae* in BF_s was more serious. As the heatmaps show that the MDRE in LF_s most resistant to amoxicillin, trimethoprim-sulfamethoxazole, oxacillin, tetracycline, trimethoprim,

sulfamethoxazole, erythrocine and chloramphenicol (Fig. 6A). While the MDRE in BFs most resistant to amoxicillin, oxacillin, trimethoprim, sulfamethoxazole, erythrocine, tetracycline and cefazolin (Fig. 6B). It should be noticed that 24R MDRE in BFs and LFs were only sensitive to polymyxins, but 17R MDRE in LFs contain resistant strains of polymyxin which should be monitored more frequently. In addition, tetracycline, macrolides, sulfonamides in folic acid metabolic pathway inhibitors and amoxicillin in β -lactam were resistant, so the use of these antibiotics alone should be avoided as far as possible in poultry.

3.4 Differences of ARGs detection between broiler and layer farms

To investigate the prevalence of ARGs in the poultry environment, resistance genes of nine categories of antibiotics were detected by PCR analysis. The detection rates of ARGs, *bla_{TEM}* (β -lactamases), *tetA* (tetracycline), *sulI* (sulfonamides), *floR* (chloramphenicol), *fosA3* (fosfomycin), *mph(A)* (macrolides), *mcr-1* (polymyxins), *qnrS* (quinolones) and *aac(6')-Ib-cr* (aminoglycoside), were significant higher than other ARGs in the same category ($P < 0.05$). The detection rates of each of the genes is detailed in the Supplementary Data1 and figure S1. In addition, we focused on antibiotic resistance genes that are banned in poultry production and used differently in layers and broilers for subsequent analysis. Some of these antibiotics, such as carbapenems and polymyxin, are only available for clinical use but not for use in animals. To avoid antibiotic residues in eggs, some antibiotics are banned during laying period, such as tetracycline, quinolones (danofloxacin, pefloxacin, norfloxacin, ciprofloxacin, enrofloxacin, ofloxacin), macrolides (kitasamycin), β -lactam

(amoxicillin, ampicillin), sulfonamides (sulfachlorpyridazine sodium), polymyxin (polymyxin B).

A total of three kind of tetracycline resistance genes were detected, the detection rate of *tetA* all above 60% in LFs and BFs. The detection rate of *TetA* in BFs was significant higher than in LFs ($P < 0.05$). Although *TetD* was detected in both LFs and BFs, there was no significant ($P > 0.05$). difference (Fig. 7A). MDRE containing quinolone resistance genes *qnrA*, *qnrB*, *qnrD*, *qnrS* were detected in LFs and BFs. There is a significant difference in the detection rates of *qnrD* between BFs and LFs ($P < 0.05$), and the detection rates of *qnrA*, *qnrB* and *qnrD* were all higher in BFs. While the detection rate of *qnrS* was higher in LFs than in BFs (Fig. 7B). Among the macrolide resistance genes, the detection rate of *mphA* was the highest in broiler and layer farms, both exceeding 60% (Fig. 7C). The detection rates from high to low were *mph(A)*, *mph(E)*, *mph(B)*, *mph(D)* *ermA* and other macrolide resistance genes were not detected. The detection rate of *mph(B)* in BFs was significantly higher ($P < 0.05$). Only three β -lactam resistance genes were identified: *bla_{TEM}*, *bla_{OXA-1}* and *bla_{CTX-M}*. Except that *bla_{TEM}* had high detection rates in both broiler and layer farms, the detection rates of *bla_{TEM}* and *bla_{OXA-1}* in broiler farms were significantly higher than those in LFs ($P < 0.05$) (Fig. 7D). The sulfonamides resistance genes only *sulI* and *folpI* were amplified in MDRE. The total detection rate of *sulI* was the higher than *folpI* in BFs and LFs (Fig. 7E). And the detection rate of *sulI* in BFs was significantly higher than that in LFs ($P < 0.01$). The detection rate of polymyxin resistance gene in broiler and layer farms was not high, and *mcr-1* was detected in both farms and was

significantly higher in BF_s than in LF_s ($P < 0.01$) (Fig. 7F). However, *mcr-2* was only detected in BF_s, and the detection rate was 0.32%.

3.5 The *mcr-1* positive plasmids p20432-*mcr* and p2021-*mcr* are conjugative.

The transconjugants isolated by mating RB20432 and DB20D28 strains with EC600 were resistant to rifampicin and polymyxin antibiotics, possibly indicating plasmids transfer. Experiments showed that polymyxin resistance could be successfully transferred from RB20432 and DB20DB to EC600 at a frequency of 10^{-1} to 10^{-3} (p20432-*mcr*) and 10^{-2} to 10^{-3} (p2021-*mcr*) cell per recipient cell by conjugation. As can be seen from the table (Table 1), the minimum inhibitory concentrations for polymyxin B for the transconjugant increasing 16-fold compared with the untransformed control. In addition, the minimum inhibitory concentration for ampicillin (32-fold) and amoxicillin (32-fold), tetracycline (32-fold), gentamicin (8-fold, only RB20432) and sulfamethoxazole (16-fold) were increasing to different degree, suggesting that resistance genes of these antibiotics also exist in p20432-*mcr* and p2021-*mcr* and be transferred through conjugation.

3.6 Characterization of multi-Drug plasmids p20432-*mcr* and p2021-*mcr*

Two *mcr-1* positive multidrug-resistant plasmids p20432-*mcr* and p2021-*mcr* were found in the polymyxin resistant MDRE of fecal samples of layers and broilers, respectively. As shown in Fig. 8, the total length of plasmid 20432-*mcr* was 228.7kb, contained 275 ORFs in total. And it has a complex region, which has two replicons (IncHI2 and IncN), and the backbone was interrupted by accessory modules. The content of GC was 47.3%, and the plasmid contained four accessory modules which

were MDR-1 region, MDR-2 region, *In1866*-related region and metal resistant region. The single replicon IncN plasmid R46 (GenBank: AY046276.1) was chosen as the reference plasmid which had similar *repA*, *intI1* and resistance genes.

The IncI2-type plasmid p2021-mcr was extracted from *E. Coli* strain DB20D28, with a total length of 60.2kb, the content was 42.3%, and a total of 78 ORFs in total (Fig. 9A). Its structure was relatively simple which the whole plasmid consisted of a backbone region and a single *mcr-1-pap2*. *mcr-1* was the only ARG in p2021-mcr. The conjugation transfer region accounted for a large proportion of the plasmid, including flagellum formation gene *pilP* and conjugation transfer regulation gene *virB9*. Among them, the Shufflon DNA-specific recombinase secreted by *rci* gene in this structure is the most important member of the recombinant system of IncI plasmid, which obtained exogenous DNA through specific recombination. While there were no MGEs such as IS, Tn and integron in p2021-mcr, and *mcr-1* is the only ARG. IncI2 plasmids have a high overall structural similarity, while most of difference is caused by gene-specific recombination upstream and downstream of *rci* [31]. Linear comparison between p2021-mcr and reference plasmid pAH62-1 (Genbank: CP055260.1) (Fig. 9B) showed that *mcr-1-pap2* structure also existed in the upstream of *rci* and the main difference was that partial sequence inversion occurred in the downstream of this gene due to recombination (Fig. 9C).

3.7 Analysis of Multi-Drug Resistant Region in p20432-mcr

The MDR-1 region of p20432-mcr is composed of MGEs and ARGs in order $\triangle IS2$ -*merR*-*terC*- $\triangle IS2$ -*aph(3')*-*Ia-IS186B*-*mcr-1*-*PAP2-1-sul*- $\triangle Tn2$. *mcr-1* was

followed by a PAP2 family of proteins, and *mcr-1-pap2* has been reported in many
 plasmids, most of which are related to IS*Ap11*. But in p20432-*mcr*, IS*Ap11* has been
 replaced by IS*186B*. IS*186B-mcr-1-PAP2* has no similar sequence in Genebank, but
 IS*186B* has the ability to transpose *mcr-1* into chromosomes [32], which promote the
 vertical transmission of polymyxin resistance genes (Fig 10A). The multi-drug
 resistance region MDR-2 of the following mobile element in sequence IS*4321*,
 IS*6100*, IS*26*, Tn*Asl*. IS*26-mph(A)-mrx-mphR(A)-IS6100* is a classic antibiotic
 resistance locus which contains a macrolide resistance gene *mph(A)* and a regulator
mphR(A). A new type 1 integron was found and the number In*1866* was assigned by
 Integrall database. The ARGs in gene cassette of In*1866* contained *dfrA12*
 (Trimethoprim), *aadA2* (aminoglycoside), *cmlA1af* (chloramphenicol), *aadA1*
 (aminoglycoside) and the 3 prime-CS region contained *sul3* (sulfanilamide) (Fig.
 10B). It is worth noting that the chloramphenicol resistance gene *cmlA1af* is a new
 variant of *cmlA1* which is first discovered in the gene cassette. The main difference is
 that the final two amino acids of the product encoded by *cmlA1af* changed from
 arginine - valine to serine - methionine.

Discussion

China is the country with the highest use of antibiotics in husbandry each year
 and there was a long period that antibiotics(e.g. C, TET, AMP,TMP) were used to treat
 bacterial infections or as antimicrobial growth promoters [33, 34]. As a result,
 selection for resistant bacteria and has promoted gene mutation, recombination, and
 horizontal gene transfer events in poultry farming environments which can lead to the

enrichment of multidrug resistant bacteria. China is the largest producer and consumer of eggs in the world, and second only to the United States in chicken production [35]. The enormous and still increasing demand for poultry products in China has meant that there is plenty of room to expand production of layers and broilers [36]. Identification of the phenotype and genotype of MDRE isolated from BFs and LFs indicated that different treatment methods and culture-cycle could cause the development of antimicrobial resistance in culture environment, but lead to the spread of different ARGs. Few studies have examined differences among broilers and layers, which have only focused on antimicrobial residues or individual resistance genes. Our understanding of the differences in antibiotic resistance profiles between broilers and layers and how they spread is still limited. Therefore, it is necessary to make effective tactics for monitor antibiotic resistance in BFs and LFs, and the monitoring focus should be different [37].

In this study, a high prevalence of MDR *Enterobacteriaceae* and high resistance-gene diversity has been demonstrated in poultry environment. 3 BFs and 3 LFs were selected in Jiaying, Zhejiang Province. 526 MDRE were isolated, of which *Escherichia* was the most MDR bacteria, followed by *Proteus*, *Shigella*, *Salmonella* and *Citrobacter*. There was no significant ($P>0.05$) difference in MDRE composition between laying hens and broiler farms, indicating that they are important reservoirs of ARGs in poultry farming environment. By identifying the antibiotic resistance phenotype of MDRE, the results showed that tetracycline, sulfonamides, amoxicillin and macrolides antibiotics all had high resistance rates in both types of farms. Among

them, the resistance rates of oxacillin, amoxicillin, trimethoprim, tetracycline and sulfamethoxazole used in this study have reached more than 90%, while the resistance rates of polymyxin B and meropenem are lower than 10%. Tetracycline-erythromycin-trimethoprim-sulfamethoxazole-sulfamethoxazole-trimethoprim-benzacillin-amoxicillin are common antibiotic resistance spectrum of MDRE in this study. The comparison of MDRE in BF and LF shows that the risk of MDRE in BF is higher than those from LF, which is mainly reflected in the following two aspects: (1) multidrug resistance rates were higher in BF samples. (2) in terms of meropenem, amoxicillin, streptomycin, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole, enrofloxacin, ofloxacin and polymyxin B, the resistance rates of MDRE isolated from BF were higher. This result may be caused by: (1) short breeding period and weak resistance of broilers; (2) compared with the cage mode of layers, free-range broilers had a wider range of activities and were more susceptible to MDR infection; (3) some antibiotics are forbidden in laying hens breeding or laying period, but not as strictly in broilers. There were also significant differences in the detection rates of some ARGs between broiler and layer farms. The detection rate of *aadA2*, *catA1*, *fosB*, *mph(B)*, *qnrB*, *bla_{oxa-1}*, *bla_{CTX-M}* and *tetB* in BF was significantly higher than that of LF ($P < 0.05$). The difference of ARGs was consistent with that of antibiotic resistance phenotype, indicating that the difference was largely caused by the difference of transmission of ARGs.

Since the plasmid carrying *mcr-1* was first reported in China in 2015, colistin resistance has spread rapidly around the world by now, and the resulting development

of *Enterobacteriaceae* from extensive drug resistance to pan-drug resistance is inevitable and will ultimately become global [38, 39]. A study based on the prevalence of *mcr-1* in the human intestine showed that a decline in colistin-resistant bacteria isolated from both animals and humans decreased significantly after the implementation of the colistin prohibition policy in China in 2017 [40, 41]. However, this study found that there are still transmission risks in the breeding environment. Two *mcr-1* positive plasmids were found to have high in-vitro transfer rates between *Escherichia coli* which is key human pathogen in broilers and layers respectively. In particular, p20432-mcr, found in broiler MDRE, carries a novel integron *In1866* and multiple drug resistance genes, which is a worrying event. The plasmid p20432-mcr isolated from broiler source *E.coli* RB20432 belongs to a complex chimeric plasmid, which contains both IncHI2 type and IncN type replicons. The structure of multiple replicons is conducive to increasing the host range of this plasmid, which likely to make *mcr-1* spread more widely [42]. The cytoskeleton region contains the key genes of IncHI2 type plasmid, and some fragments of IncN type plasmid were also obtained with plasmid recombination. In addition to polymyxin resistance genes, there are antibiotic resistance genes of chloramphenicol, aminoglycoside and metal resistance genes on plasmids, which make the plasmid transfer under a variety of selective pressures.

Conclusion

We conducted a comprehensive analysis of MDRE from typical broiler and laying farms in Zhejiang Province, China, and the results showed that antibiotic

394 prohibition had a positive effect on reducing resistance genomes in the breeding
395 environment. The detection rate of some antibiotic resistance genes that were banned
396 in layers but not suppressed in broilers was significantly increased in broiler source
397 MDRE ($P < 0.05$). However, our study shows that although certain antibiotics such as
398 polymyxin have been banned in poultry farming, the polymyxin resistance gene *mcr-1*
399 persists in the environment for a long time due to the widespread spread of mobile
400 elements such as plasmids. The analysis results of the multidrug-resistant plasmid
401 P20432-MCR showed that the multi-replicon structure enabled it to transmit *mcr-1*
402 and other antibiotic resistant genes to different hosts, resulting in a higher detection
403 rate of *mcr-1*, *aph(3')-Ia* and *aadA2* in broiler farms. Therefore, it is necessary to
404 implement and improve cleaning and disinfection procedures in broiler farms, and to
405 detect resistant bacteria from broilers more frequently to prevent further transmission
406 to humans through the poultry production chain.

Abbreviations

BFs: Brolier Farms; LFs: Laying Farms; ARGs: Antibiotic Resistance Genes; MGEs: Mobile Genetic Elements; MDR: Multidrug-Resistance; MDRE: Multidrug-Resistant Enterobacteriaceae; IS: Insertion Sequence; Tn: transposon; AMX: Amoxicillin; CZ: Cefazolin; CAZ: Ceftazidime; FOX: Cefoxitin; IPM: Imipenem; MEM: Meropenem; AT: Aztreonam; OX: Oxacillin; AK: Amikacin; KAN: Kanamycin; S: Streptomycin; GM: Gentamicin; CIP: Ciprofloxacin; ENR: Enrofloxacin; OFX: Ofloxacin; TMP: Trimethoprim; SMZ: Sulfamethoxazole; SXT: Trimethoprim-Sulfamethoxazole; C: Chloramphenicol; FOS: Fosfomycin; E: Erythromycin; AZM: Azithromycin; PB: Polymyxin B; PB: Tetracycline; MI: Minocycline.

Acknowledgements

The authors are grateful to the anonymous reviewers for their insightful suggestions and comments.

Funding

This work was supported in part by the National Natural Science Foundation of China (no. 32172188)

Availability of data and materials

All metagenomic files have been uploaded to NCBI and are under review. Once approved by NCBI, we will upload the metagenomic data number immediately.

Authors' contributions

ZSZ analyzed the data and wrote the manuscript.; XDT, ZXE analyzed the data and prepared figures. WP collected samples and helped with software. HJZ and QDF conceived the study and revised the original manuscript. All authors edited the manuscript and approved the final draft.

Ethics approval and consent to participate

There is no ethics involved in this experiment.

Consent for publication

434 Not applicable.

435 **Competing interests**

436 The authors declare that they have no competing interests.

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Figures

Figure 1 Top ten relative abundance of taxonomic composition at the phylum level in broiler and laying farm samples.

Figure 2 The absolute abundance of top ten antibiotic resistance gene classification and their subtypes

584 Figure 3 Collection and isolation of MDR *Enterobacter*.

585 Figure 4 Resistance rates of MDRE isolated from broilers and layers to 25 antibiotics.

586 Figure 5 Proportion of resistant bacteria in different drug resistance situations in
587 broiler farms and laying farms.

588 Figure 6 (A) Heat map of multiple drug resistance of resistant strains in broiler farms.
 589 6 (B) Heat map of multiple drug resistance of resistant strains in laying farms. The
 590 right column shows the number of antibiotics which were resisted by MDRE.

Figure 7 The detection rate of antibiotic resistance genes which are banned in layers or both. (A) The detection rate of tetracyclines resistance genes. (B) The detection rate of quinolones resistance genes. (C) The detection rate of macrolides resistance genes. (D) The detection rate of β -lactam resistance genes. (E) The detection rate of sulfonamide antibiotics resistance genes. (F) The detection rate of polymyxins resistance genes.

B

C

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F

Figure 8 The complete sequence circular diagram of p20432-mcr and R478. The innermost ring represents (G-C)/ (G+C); the blue ring indicates GC content, and the outward depression indicates that the GC content is higher than the mean value. Black area in the outer circle represents the backbone area and gray area represents the accessory modules. The outermost circle shows the distribution of genes represented by colored arrows in the plasmid.

609

610 Figure 9 The complete sequence circular diagram of p2021-mcr. (A) The innermost
611 ring represents (G-C)/ (G+C); the blue ring indicates GC content, and the outward
612 depression indicates that the GC content is higher than the mean value. Black area in
613 the outer circle represents the backbone area and gray area represents the accessory
614 modules. The outermost circle shows the distribution of genes represented by colored
615 arrows in the plasmid. (B) Linear comparison of p2021-mcr and pAH62-1. Shadows
616 indicate nucleotide homology is greater than 95%. (C) The diversity of *mcr-1*
617 upstream environment of *rci* in Inc2 plasmid.

Figure 10 The organization and alignment of MDR-1 region of p20432-mcr. (A)

Different colors indicate different moving elements, and arrows indicate genes.

Shading indicates that the homology of the two parts of nucleic acids is greater than

95%, and the number in parentheses indicates the location of the fragment in the

plasmid. (B) The organization and alignment of MDR-2 region and In/866-related

region of p20432-mcr. Different colors indicate different moving elements, and

arrows indicate genes. Shading indicates that the homology of the two parts of nucleic

acids is greater than 95%, and the number in parentheses indicates the organization

and alignment of MDR-2 region and In/866-related region of p20432-mcr.

B

650 **Table**

651 Table1 The result of MIC by using VITEK-2

652

Category	Antimicrobial agent	MIC (mg/L)/antimicrobial susceptibility				
		EC600	RB20432	RB20432- <i>mcr-I</i> -EC600	DB20D28	DB20D28- <i>mcr-I</i> - EC600
Penicillin	Ampicillin	8/S	≥256/R	≥256/R	≥256/R	≥256/R
	Amoxicillin	≤4/S	≥256/R	≥256/R	≥256/R	≥256/R
β-lactam	Ceftazidime	≤4/S	≤4/S	≤4/S	≤4/S	≤4/S
	Imipenem	≤1/S	≥16/R	≤1/S	≤1/S	≤1/S
Colistin	Polymyxin B	≤0.5/ S	≥8/R	≥8/R	4/I	≥8/R
Tetracycline	Tetracycline	4/S	≥128/R	≥128/R	≥128/R	≥128/R
Aminoglycosides	kanamycin	8/S	≥128/R	32/I	≥64/R	≥64/R
	Azithromycin	8/S	≥128/R	≥64/R	≤4/S	≤4/S
	Gentamicin	≤1/S	≥16/R	8/I	≤1/S	≤1/S
Quinolones	Ciprofloxacin	≤0.25/S	≥4/R	≥4/R	≤0.25/S	≤0.25/S
Sulfonamides	Sulfamethoxazole	≤20/S	≥320/R	≥320/R	≥320/R	≥320/R

S, Susceptible; R, Resistant; I, Intermediate.

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