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Antibiotic resistance gene reservoir in live poultry markets

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SUMMARY

Objectives: The heavy use of antibiotics in farm animals contributes to the enrichment and spread of antibiotic resistance genes (ARGs) in "one-health" settings. Numerous ARGs have been identified in livestock-associated environments but not in Chinese live poultry markets (LPMs).
Methods: We collected 753 poultry fecal samples from LPMs of 18 provinces and municipalities in China and sequenced the metagenomes of 130 samples. Bioinformatic tools were used to construct the gene catalog and analyze the ARG content. PCR amplification and Sanger sequencing were used to survey the distribution of *mcr-1* gene in all 753 fecal samples.
Results: We found that a low number of genes but a high percentage of gene functions were shared among the poultry, human and pig gut gene catalogs. The poultry gut possessed 539 ARGs which were classified into 235 types. Both the ARG number and abundance were significantly higher in poultry than that in either pigs or humans. Fourteen ARG types were found present in all 130 samples, and tetracycline resistance (TcR) genes were the most abundant ARGs in both animals and humans. Moreover, 59.63% LPM samples harbored the colistin resistance gene *mcr-1*, and other *mcr* gene variants were also found.
Conclusions: We demonstrated that the Chinese LPMs is a repository for ARGs, posing a high risk for ARG dissemination from food animals to humans under such a trade system, which has not been addressed before.

Introduction

The emergence of antibiotic-resistant bacteria poses a serious risk to both public health and food safety worldwide.[1] It is estimated that about 10 million people will die each year due to antibiotic-resistant bacteria infections, costing approximately 2-3.5% of the gross domestic product (GDP) by 2050.[2]

Studies demonstrated that antibiotic-resistant bacteria can spread or circulate among humans, environments, and animals, thus leading to both medical and ecological concerns.[3,4] In recent years, the antibiotic resistance problem has been amplified by the continuous occurrence of multidrug resistance bacteria bearing new antibiotic resistance genes (ARGs), such as New

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Delhi Metallo-β-lactamase (NDM-1)[5] and the very recently identified mobilized colistin resistance gene (*mcr-1*).[6] Since the first identification of *mcr-1* in China by Liu et al.,[6] it has been detected in animals and humans in different countries and regions worldwide.[7,8]

Although antibiotic resistance is not a new phenomenon,[9] the use of antimicrobials undoubtedly speeds up the enrichment and spread of antibiotic-resistant bacteria and ARGs.[10,11] This situation may be even worse with respect to the heavy use of antibiotics for disease treatment and growth promotion in food animals.[12] The global use of antibiotics in food animal production was 131,109 tons in 2013, likely more than the sum of all human antibiotic consumption, and it may increase to 200,235 tons by 2030.[13] In China, antibiotics have been widely used for keeping animals healthy and maintaining productivity since the early 1990s,[14] and about half of the 210,000 tons of antibiotics produced in China end up in animal feed.[15]

Therefore, the microbes in the gut microbiota of food animals likely constitute a large reservoir for ARGs, which can be reflected by a large amount of ARGs found in livestock farms. Zhu et al. reported that manure samples from three large-scale swine farms in China harbored 149 unique genes, and the abundance of 63 ARGs was enriched 192-fold (median) up to 28,000-fold compared to controls.[16] Li et al. also revealed that 260 ARG types are widely distributed and co-exist in 50 samples from 10 environmental niches. The ARGs were most abundant in samples of pig or chicken feces and wastewater from livestock farms.[17] In addition to farm-associated environments, a typical environment in China is the live poultry market (LPM), where live birds are brought together for trading. It is anticipated that in such environments, live poultry-associated bacteria and viruses are at a relatively high density that is ready to disseminate. Copious evidence indicates that humans exposed to LPMs are at high risk for influenza virus infection during avian influenza outbreaks.[18,19] Given these facts, we hypothesized that LPMs are also a potential site for the dissemination of animal-origin ARGs to humans in China. However, the ARG profile in Chinese LPMs has not been characterized.

In this study, we focused on building the first poultry gut gene catalog and revealing the diversity and abundance of ARGs in the gut microbiomes of live poultry in Chinese LPMs by large-scale metagenomic sequencing of 130 fecal samples. We also surveyed the distribution of the previously discovered colistin resistance gene *mcr-1* in 753 samples from 18 provinces and municipalities in China using PCR amplification and Sanger sequencing techniques.

Results and discussion

Construction and assessment of poultry gut catalog

We collected 753 fecal samples from 22 cities in 18 provinces and municipalities in China (Supplementary appendix 1: Fig. S1). We sequenced the gut microbiomes of 130 representative samples, to generate a gene catalog containing 8,485,510 poultry gut microbial genes. The total size of the sequencing data is approximately 1.7 Tbp with an average of 13.3 Gbp per sample (min: 6.5 Gbp, max: 23.3 Gbp) (Supplementary appendix 2: Table S1). For comparison, we downloaded the 9,879,896 non-redundant (NR) genes from the human gut microbiome,[21] 7,685,872 NR genes from the pig gut microbiome.[22] A rarefaction analysis including all samples revealed a curve approaching saturation (Fig. 1(A)). Taxonomic annotation showed that more than 90.71% could be assigned to the bacteria super kingdom (Fig. 1(B)), which were classified into 12 phylum, 22 classes, 37 orders, 86 families, 173 genus, and 372 species. At the phylum level, the chicken gut microbiome is predominantly composed of three phyla: Firmicutes (55.57%), Bacteroidetes (24.10%), Proteobacteria (15.19%), with the first three

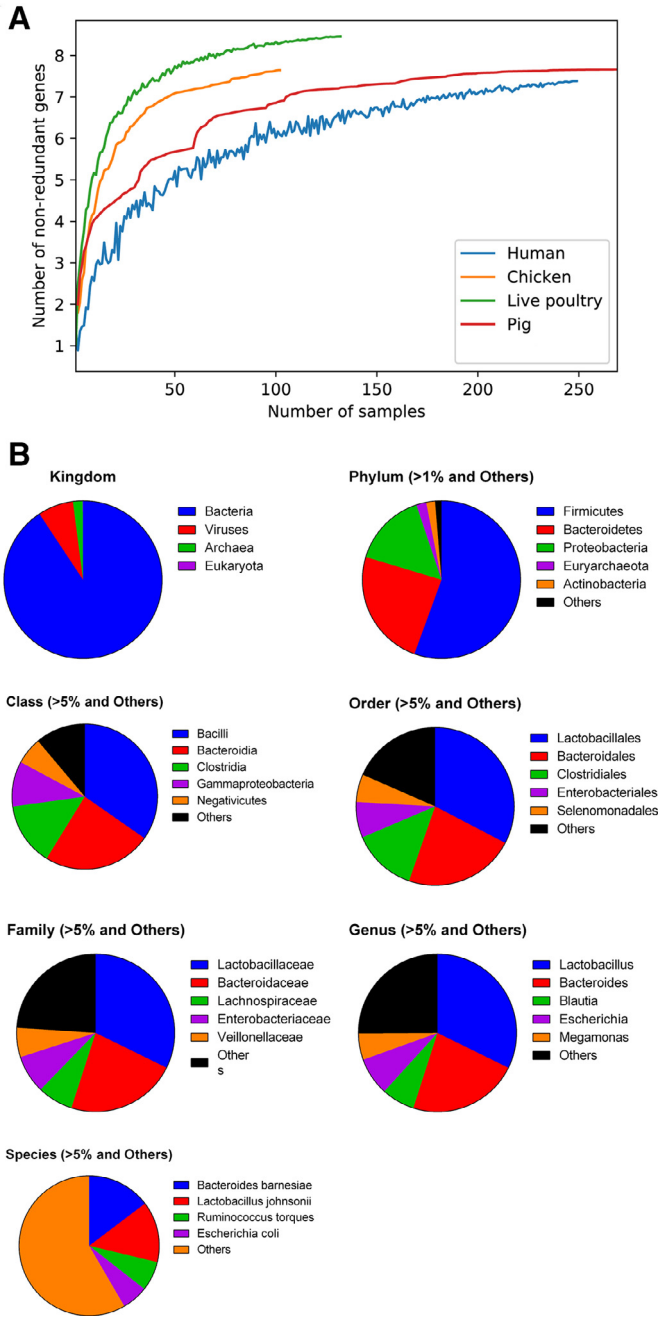

Fig. 1. Rarefaction curve and taxonomic annotation of the poultry gut microbiome catalog. (A) Rarefaction curve based on the gene numbers of the poultry (green), pig (red), and human (blue) samples. The rarefaction curves drown from the chicken (yellow) and from the whole set of 130 poultry samples confirm the complementarities of the three gene sets for providing good coverage of the gene catalog of poultry gut microbiome. Poultry: $n = 130$; Chicken: $n = 102$; Human: $n = 249$; Pig: $n = 287$. The y-axis means the number of non-redundant genes, and the order of magnitude is millions. (B) Taxonomic annotation of the poultry gut microbiome catalog.

contributing more than 94.85% of the total bacteria (Fig. 1(B)). At the genus level, most of these belonged to *Lactobacillus* (32.29%), followed by *Bacteroides* (22.67%), *Blautia* (6.79%), *Escherichia* (7.73%) and *Megamonas* (5.43%) (Fig. 1(B)). We also mapped the poultry gene catalog to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[20] and identified 8614 KEGG orthologous groups (KOs) in chicken gut microbiomes. We identified a common set of 2104 KOs shared by 100% of the 102 chicken samples, suggesting there exist a core of functions in the gut microbiome in chickens.

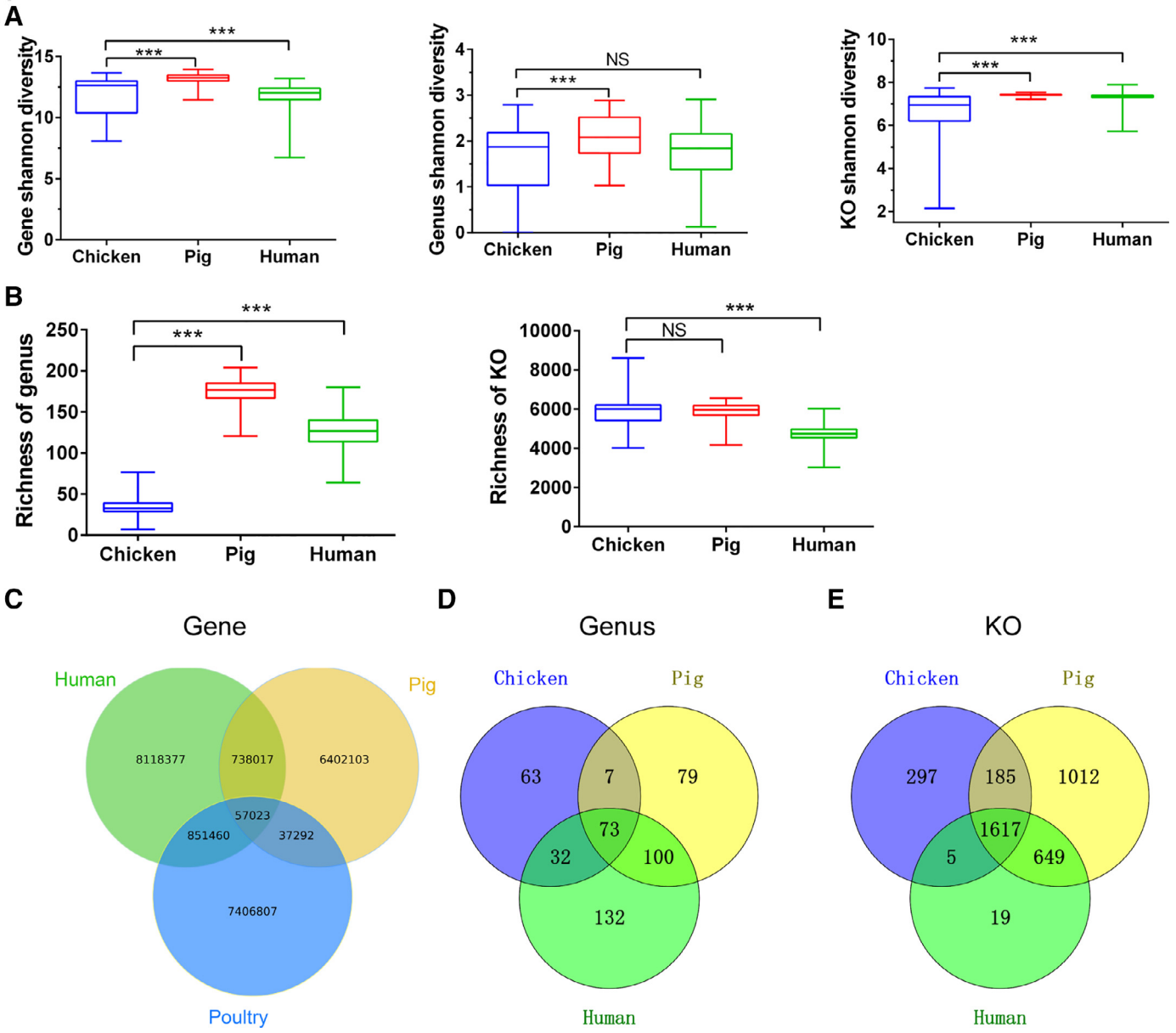

Fig. 2. Comparison of the gut microbiome gene catalog of humans, pigs, and poultry. (A)-(B) Alpha diversity (Shannon index) (A) and richness (B) at the NR gene counts, KO and genus levels. Boxes denote the interquartile (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the line inside denotes the median. Whiskers denote the lowest and highest values within 1.5 times and the IQR from the first and third quartiles, respectively. The asterisks on the top indicate $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, (Mann–Whitney *U* test). (C) Venn diagram of shared NR genes between the human (green), poultry (blue) and pig (yellow). Only 0.67% (57,023 genes) of the genes from the poultry catalog are present in the human and pig catalogues, and the poultry catalog shared 10.71% of the genes with the human catalog. (D) Venn diagram of genus shared by the human (green), pig (yellow), and chicken (blue). (E) Venn diagram of KO functions present in 100% of the individuals in each data set shared by the human (green), pig (yellow), and chicken (blue). Based on a 100% inter-individual sharing within each group, 1617 KO identifiers are shared by the chickens, pigs, and humans.

Comparison with the human and pig gut microbiomes

Only a low number of genes (57,023 genes) were shared among the poultry, human, and pig gut gene catalogs (Fig. 2(C)), accounting for 0.67%, 0.58%, and 0.74% of the gene number in the respective catalogs, underscoring the marked differences among the gut microbiomes of these three groups. In the poultry gut gene catalog, the pairwise overlap at the gene level was also modest, 10.71% (908,483 genes) of the genes are present in the human gut catalog, 1.11% (94,315 genes) of the genes are included in the pig gut catalog. The chicken gut metagenome exhibited a lower alpha diversity (Fig. 2(A)) than the pig at the gene, genus and KO levels, and higher alpha diversity than human microbiomes at the gene level. Genus richness in chicken was lower than that observed in pigs and humans (Fig. 2(B)), while KO richness was higher than that in humans (Fig. 2(B)).

We also identified bacterial genus that occurred in each group samples. Comparing the core genera from the chickens, humans, and pigs, we found 73 genus that are shared between the three species (Fig. 2(D)), and 105 and 80 genus identified in the chicken gut catalog were also found in the human and pig gut catalog, respectively, but we also noted that the abundance of these genus are different between hosts. Among the 20 most abundant genus in each species, only three genus, *Bacteroides, Blautia* and *Escherichia*, were common among the chicken, pig and human (Supplementary appendix 1: Fig. S2), suggesting a different gut microbiota

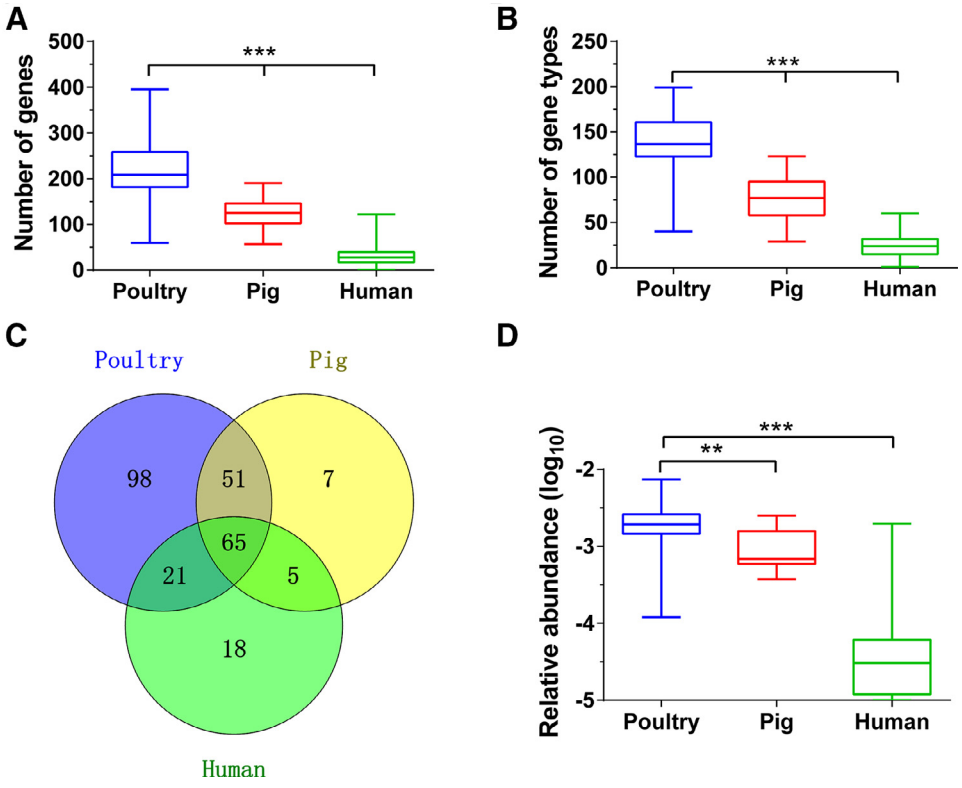

Fig. 3. Comparison of the number of ARGs, ARG types and ARG relative abundance in the gut microbiomes of poultry, pigs, and humans. (A) ARG number comparison. (B) ARG type number comparison. Explanation of the box plotting can be found in Fig. 2 legend. The asterisks on the top indicate $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, (Mann–Whitney *U* test). Poultry, $n = 130$; Human, $n = 1267$; and Pig, $n = 287$. (C) Venn diagram of shared ARG types among poultry (blue), humans (green), and pigs (yellow). (D) Comparison of ARG relative abundance in poultry, pigs, and humans.

composition among three species in the major genus. At the functional level, a large number of KO functions was shared (1617 KO identifiers, 42.73% of the total number, 3784) at the functional level between chicken, pig, and humans, showing a functional core in these three groups (Fig. 2(E)). Of note, pigs shared more KO identifiers with humans than chickens. These data highlight the consistency of the microbiomes at the functional level in food animals and humans, suggesting a significant set of chicken-specific KEGG functions.

High diversity of ARGs in live poultry gut microbiomes

We then searched ARGs from food animals and human datasets by BLAST against the core set of the Comprehensive Antibiotic Resistance Database (CARD) using an 80% identity cutoff.[23–25] We found the gut microbiomes of poultry, humans, and pigs harbored 539, 249, and 224 unique ARGs, respectively. Both the number of ARGs and ARG types in live poultry were significantly higher than that in humans and pigs, suggesting that ARGs are highly enriched in live poultry (Fig. 3(A), (B) and Supplementary appendix 1: Fig. S3A, B). Interestingly, the number of ARGs and ARG types in pigs from China is significantly higher than in pigs from France or Denmark (Supplementary appendix 1: Fig. S4A, B).

The 539 ARGs in live poultry were classified into 235 different ARG types, while only 109 and 128 ARG types were found in humans and pigs, respectively. Among the different poultry species, no significant differences were observed between avian species (Supplementary appendix 1: Fig. S5A, B), which may be caused by the small number of samples. Among the 130 live poultry samples, one chicken sample from Jiangxi (JX), JX5-C, contained the highest number of ARG types (199 types), while a chicken sample from Anhui (AH), AH7-C, possessed the lowest number of ARG types (40 types). We then compared the distribution of ARGs and ARG types between the sampling sites. We found that both the number of ARGs and ARG types were the highest in JX samples, and the lowest in Shanxi (SX) samples (Supplementary appendix 1: Fig. S6A, B). In view of different regions, both the numbers of ARGs and ARG types in southern China were significantly higher than in northern China, suggesting that ARGs are enriched in LPMs in southern China (Supplementary appendix 1: Fig. S6C, D).

Shared and unique ARG types among live poultry, pigs, and humans

We then investigated the shared and unique ARG types among the three groups. We found that a total of 65 ARG types were shared among live poultry, humans, and pigs, accounting for 27.66%, 59.63%, and 50.78% of the total ARG types identified in each group, respectively (Fig. 3(C)). Interestingly, live poultry and pigs shared more ARG types (116 types) live poultry and humans (86 types) or between pigs and humans (70 types) (Fig. 3(C)). This result may be indicative of the different usages of antibiotics in food animals and humans causing different antibiotic resistance mechanisms. However, there were 98 unique ARG types in live poultry (For example, CTX-M-53, CTX-M-55, and CTX-M-65), but only 18 in humans and seven in pigs, suggesting a greater diversity and enrichment of ARGs in live poultry. Among these shared ARG types, the resistance genes for tetracycline, lincosamide, macrolide, and streptogramin were dominant in poultry samples, those for tetracycline, lincosamide, and macrolide were abundant in pig samples, and tetracycline, beta-lactam, and fluoroquinolone resistance genes were more abundant in human fecal samples (Supplementary appendix 2: Table S2). Notably, 14 ARG types were found in all 130 live poultry samples, and 17 types were common in 287 pig samples (Supplementary appendix 2: Table S3). None were found in

the human samples, suggesting that microbes in the guts of food animals face similar antibiotic selection pressures, likely due to commonly used antibiotics during husbandry.

The highest abundance of ARGs in live poultry

Next, we calculated the relative abundance of each ARG and compared the total abundance of ARGs in live poultry, humans, and pigs. Similar to the results of our ARG number analysis, the abundance of ARGs was also the highest in live poultry, followed by pigs and then humans (Fig. 3(D)). Among the different avian species, chickens harbored a relatively higher abundance of ARGs than others (Supplementary appendix 1: Fig. S7). The JX samples possessed the highest abundance of ARGs, while the AH samples showed the lowest. Live poultry, humans, and pigs also differed in their dominant ARG types. Among the top 20 most abundant ARG types in each group, only three (*lnuC, sat-4*, and *tet40*) were shared, and six, 16 and seven were unique to live poultry, humans, and pigs, respectively. However, poultry and pigs shared more group-dominant ARG types (13 types) than poultry and humans (four types), and pigs and humans (three types) (Supplementary appendix 1: Fig. S8 and Supplementary appendix 2: Table S4). However, the dominant ARG types varied from market to market (Supplementary appendix 2: Table S5): the most abundant type was *tet32*, in Guangdong (GD), JX, Henan (HeN), Inner Mongolia (NM), Qinghai (QH), Sichuan (SC), Jiangsu (JS), and Zhejiang (ZJ); *ErmB* in Fujian (FJ), Hunan (HuN), and Tibet (TB); *tetQ* in Shangdong (SD), Ningxia (NX), SX, Guangxi (GX), Yunnan (YN), and AH; and *ErmF* in Jilin (JL). This may be attributed to the regional differences in antibiotic usage.

Taken together, we found that the poultry gut microbiome contained the highest diversity and abundance of ARGs, which is in agreement with previous findings that ARGs are enriched in adult chicken feces compared to pigs and humans.[26] It is estimated that the consumption of antibiotics is higher in pigs than chickens in China.[27] However, compared to pork production, a wider range of intensity of antibiotics is used for chicken production worldwide,[28] which may be one of the reasons responsible for the high ARG loads in chickens.

Tetracycline resistance (TcR) genes are the most abundant ARGs in food animals and humans

We mapped the ARG types to their corresponding antibiotics and summed the relative abundances of types belonging to the same antibiotic class. In general, the resistance profiles were more similar between live poultry and pigs, with resistance genes for tetracycline, lincosamide, macrolide, and streptogramin, accounting for more than 60% of the total abundance of ARGs (Fig. 4(A)), while in human, resistance genes for tetracycline, beta-lactam, macrolide, and fluoroquinolone were more abundant, accounting for 51% of the total abundance. In agreement with previous reports,[11,29] the TcR genes were the most abundant ARGs in live poultry, pigs, and humans, accounting for approximately 22.24%, 23.11%, and 21.34% of the total ARG abundance, respectively. Resistance genes for most antibiotic classes were found across the three groups, with the exception of resistance genes for fosfomycin only being found in live poultry and humans, and the resistance gene for linezolid (*cfrA*) found in live poultry ($n = 113$). Correlation analyses based on mean relative abundances indicated that the resistance profiles in 18 provinces were clustered into three groups (Fig. 4(B)). The first group gathered two provinces (QH and JX), the second contained five provinces (FJ, JL, TB, HuN, and JS), and the third contained 11 provinces (ZJ, YN, HeN, AH, GX, SC, NM, GD, SX, SD, and NX). The provinces were clustered together (Figs. 4(B) and S9), suggesting that the resistance profiles have similar trends may be due to illegal poultry inter-provincial transportation is frequent as suspected.

Table 1
Prevalence of colistin resistance gene *mcr-1* in China.

Sample	Positive rate (%)	Positive number	Total
Qinghai (QH)	53.33	16	30
Inner Mongolia (NM)	66.67	10	15
Jiangxi (JX)	90.91	30	33
Hunan (HuN)	62.50	20	32
Shanxi (SX)	55.00	22	40
Ningxia (NX)	85.00	34	40
Tibet (TB)	44.44	4	9
Henan (HeN)	80.00	32	40
Zhejiang (ZJ)	59.15	42	71
Jiangsu (JS)	63.75	51	80
Shandong (SD)	60.00	54	90
Anhui (AH)	18.42	7	38
Yunan (YN)	59.09	26	44
Guangxi (GX)	72.22	26	36
Fujian (FJ)	55.00	22	40
Guangdong (GD)	33.33	12	36
Jilin (JL)	35.00	14	40
Sichuan (SC)	69.23	27	39

We also investigated the distribution of CTX-M beta-lactam resistance genes, which are frequently found in the *Escherichia coli* isolates from food animals in China.[30] We found that the most dominant CTX-M type was CTX-M-65 ($n = 90$), followed by CTX-M-55 ($n = 62$), CTX-M-53 ($n = 48$), and CTX-M-123 ($n = 14$). In addition, CMY-102 was found in 27 poultry fecal samples. The high prevalence of CTX-M beta-lactam resistance genes suggests the significant role of Chinese LPMs as extend-spectrum beta-lactamase (ESBL) gene reservoirs, which poses an additional risk to human health. Moreover, we found two fosfomycin resistance genes, *fosA3* ($n = 79$) and *fosB3* ($n = 56$), in live poultry. The former has been found in pets[31] and food samples[32] in China, and the latter is a determinant for clinical *Enterococcus faecium* resistance to fosfomycin.[33]

Prevalence of the mcr-1 *gene in Chinese LPMs*

To explore the distribution of the *mcr-1* gene in fecal samples of live poultry, we first searched for this gene in our metagenomic sequencing data. Among the 130 samples, the *mcr-1* gene was found (100% nucleotide identity) in 71 samples, accounting for 54.62% of the total sequenced samples. The abundance of the *mcr-1* gene differed significantly from sample to sample, and interestingly, the highest abundance was found in a chicken sample from QH.

We then investigated the distribution of the *mcr-1* gene in all of our 753 live poultry samples using PCR and Sanger sequencing. Interestingly, the *mcr-1* gene was found in 449 samples, accounting for 59.63% of the total, which was similar to the ratio in the metagenomics sequencing samples. This positive rate was significantly higher than that reported by Liu et al. (28%), where the *mcr-1* gene was firstly discovered.[6] The *mcr-1* gene was present in all 18 provinces, with high positive rates in JX (90.91%, $n = 33$), NX (85%, $n = 40$), and HeN (80%, $n = 40$) samples, but low positive rates in AH (18.42%, $n = 38$), GD (33.33%, $n = 36$), and JL (35%, $n = 40$) samples (Fig. 5(A) and Table 1). Geographically, the positive rate of the *mcr-1* gene was relative higher in southern China than that in northern China (Fig. 5(B)), while no significant difference was observed between east-west or coastal-inland provinces. Although a previous study indicates that the NDM-1 gene is prevalent in chickens,[34] we only found five samples possessing this gene (Table 2). The correlation analysis revealing *mcr-1* has a significantly positive correlation with many ARG types such as *acrE, APH(6)-Id, floR, mexD, sul2, APH(3'')-Ib, linA*, and *ErmT*,

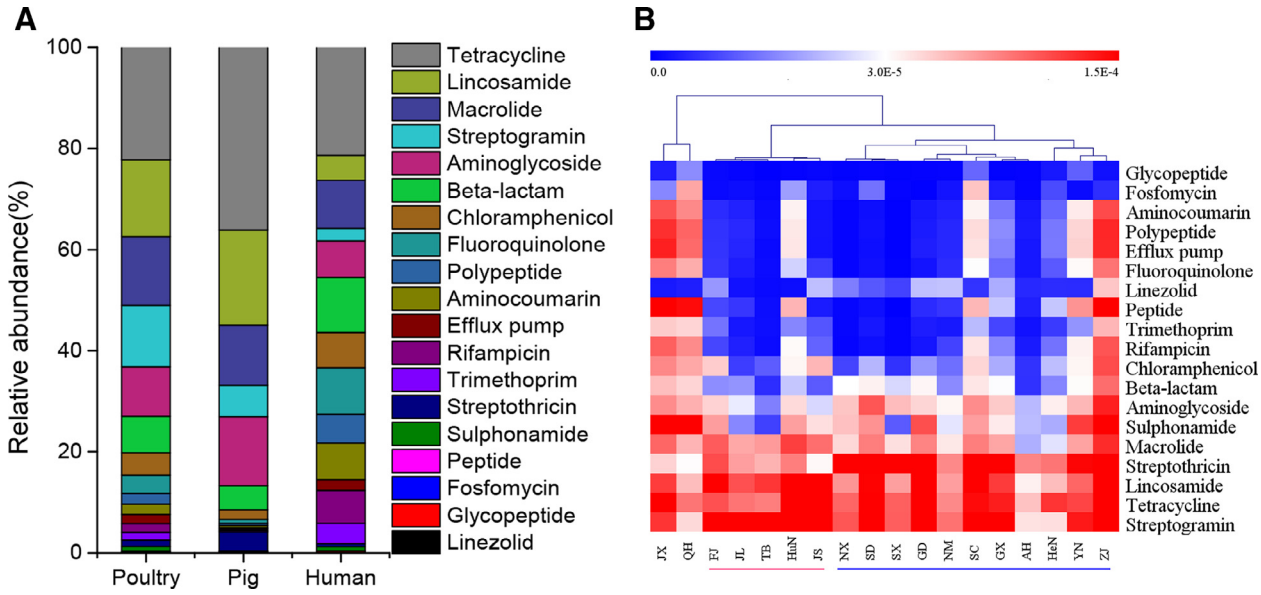

Fig. 4. Antibiotic resistance gene abundance and hierarchical clustering according to antibiotic classes. (A) The relative abundance of ARG types assigned to different antibiotic classes. The ARG types were mapped to their corresponding antibiotic classes according to the CARD classification. Genes resistant to more than one antibiotic were separately calculated and summed. Poultry: $n = 130$; Human: $n = 1267$; Pig: $n = 287$. (B) Heat map and hierarchical clustering of the relative abundance of each ARG type in different provinces. The gene types (rows) and samples (columns) were clustered with the MultiExperiment Viewer (MeV version 4.9) using the Spearman rank correlation and complete linkage. The antibiotic classes are listed on the right. The indicator on the top denotes the relative abundance and color range.

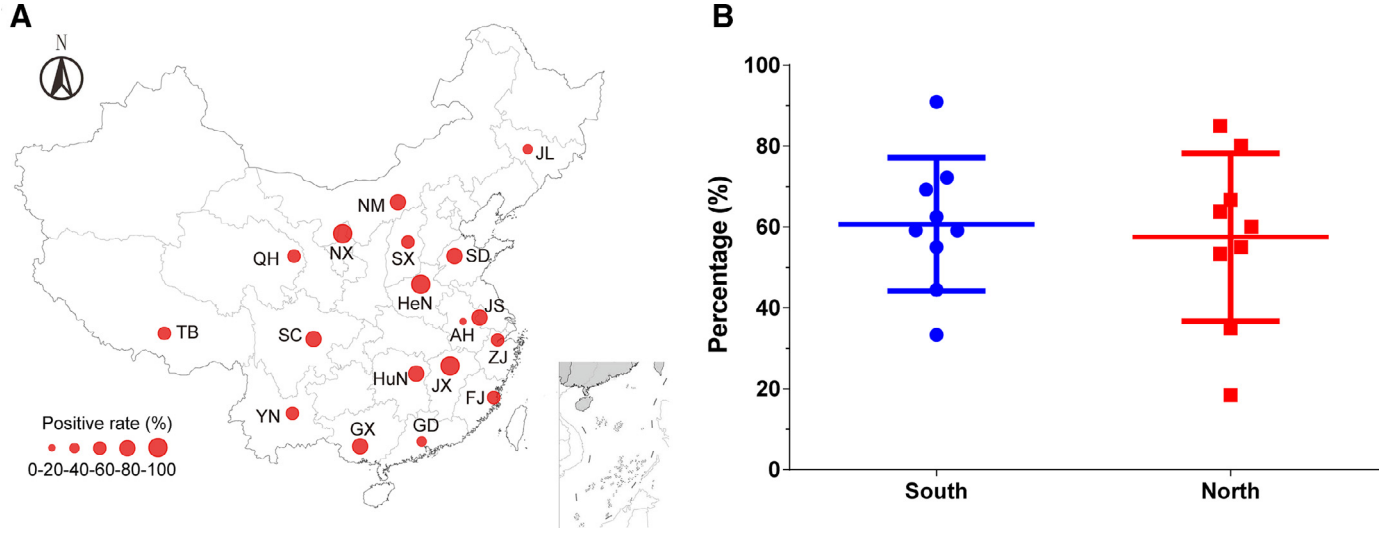

Fig. 5. Map of sampling sites and prevalence of *mcr-1* in China. (A) The different size of the red dots on the map indicate the positive detection rate of *mcr-1* in the market in that region, including 18 provinces and municipalities. "Positive rate" indicates the proportion of *mcr-1* in the feces sampled from the markets. The smallest red dot denotes a region (0–20%). The positive detection rate of samples is illustrated in Table 1. Abbreviations for the provinces and municipalities are as follows: SX, Shanxi; NX, Ningxia; NM: Inner Mongolia; JL, Jilin; HeN, Henan; SD, Shandong; JS, Jiangsu; QH, Qinghai; ZJ, Zhejiang; HuN, Hunan; JX, Jiangxi; AH, Anhui; FJ, Fujian; GD, Guangdong; GX, Guangxi; SC, Sichuan; TB, Tibet; and YN, Yunnan. The map in the square in the lower right corner indicates islands in the South China Sea. (B) Positive rates of *mcr-1* gene in Southern and Northern China. Man–Whitney test, $P = 0.8814$. Provinces with sampling sites were separated into two regions. North: NM, SX, JL, HeN, SD, AH, JS, QH, and NX; and South: HuN, JX, FJ, GD, GX, SC, TB, YN, and ZJ. The map in the square in the lower right corner indicates islands in the South China Sea.

but negatively correlated with *ErmF* and *tetQ* (Supplementary appendix 1: Fig. S10).

Additionally, we found that the *mcr-1* gene was present in all seven wild birds detected, while the positive rates were similar in chickens (60.46%, $n = 526$), ducks (59.43%, $n = 106$), pigeons (55.10%, $n = 49$), and geese (52.31%, $n = 65$) (Supplementary appendix 2: Table S5). Due to the limited number of wild bird samples we obtained, the prevalence of the *mcr-1* gene in wild birds is difficult to estimate. However, the presence of *mcr-1* in wild birds, which has also been reported in other studies,[34] poses a high risk for this gene to disseminate that may present a potential new dissemination pathway for *mcr-1*.

Interestingly, we found two *mcr-1* variants that were likely inactive. The first was found in a ZJ chicken sample. This gene variant harbored a C to A mutation at nucleotide position 853, leading to a Thr to Ala change at amino acid position 285, an essential site for *mcr-1* function.[35] The second gene variant was found in a HeN duck sample contained a mutation (C1266A) resulting in a premature stop codon (from UGC to UGA) at amino acid position 422, leading to a truncation of 120 amino acids containing the active site E468 and H478 residues.[36] To date, 13 slightly different variants (from *mcr-1.2* to *mcr-1.14)* and several new *mcr* alleles (from *mcr-2* to *mcr-8)* have been reported,[37–43] and all of them can be found in China except for *mcr-6*. However, all of these

Table 2
Prevalence of *mcr-3, mcr-4, mcr-5*, and NDM-1 in LPMs.

Sample name	*mcr-3*	*mcr-4*	*mcr-5*	*NDM-1*	*mcr-1*
ZJ-LA-J30-C	*				*
ZJ-LA-Y30-79-D	*				*
ZJ-LA-Y29-D	*	*			*
ZJ-LA-Y17-D	*		*		*
NX9-C			*		*
JX2-C				*	*
JX9-76-C				*	*
JX13-C				*	*
QH-G5-C				*	*
FJ15-D				*	*

reported genes are still active against colistin. In addition to *mcr-1, mcr-3* ($n=4$), *mcr-4* ($n=1$), and *mcr-5* ($n=2$) were also detected in our metagenomics samples, and all of these samples were positive for *mcr-1* (Table 2). Currently, the use of colistin in China is banned.[44] Whether there is a possibility that this will lead to the disappearance of colistin resistance deserves to be further investigated.

Conclusion

Here we constructed a gut microbiome catalog of poultry, consisted of 8,485,510 NR genes and compared it with the human and pig gut gene sets. This catalog represents the first gene set generated from poultry and provides a comprehensive reference resource for metagenomics-based research. Moreover, we characterized the ARG profiles in the gut microbiomes of poultry from Chinese LPMs and confirmed that the LPMs in China is a huge reservoir for ARGs. The *mcr-1* gene was more prevalent in LPMs than we ever expected. The LPM is a special environment in China, where city dwellers have the opportunity to contact live animals and the viruses and bacteria carried by them. Various avian influenza viruses spread to healthy humans in this environment.[18,45] Given the high diversity and abundance of ARGs harbored in LPMs, we propose that LPMs represent a high-risk environment for the dissemination of animal-origin ARGs to public health. We should stress that though many ARGs are in intrinsic to some gut bacteria, there is evidence that intrinsic ARGs can be horizontally transferred via homologous recombination.[54] In addition, there is a possibility that the ARG-containing bacteria can be transferred to humans by direct contact or by the clonal spread.

Methods

Samples collection

A total of 753 samples were collected, from September 2016 to April 2017. All of the samples were animal feces from apparently healthy poultry (chickens, ducks, geese, pigeons, and wild birds). Sampling of poultry was conducted in 22 cities and counties across 18 provinces or municipalities in China. Sampling was conducted according to the animal welfare guidelines of the World Organization for Animal Health. All animal fecal samples were collected from LPMs, immediately transported to the laboratory with ice, and stored at −80 °C until use. The sampling sites we chose were according to Bi's report,[46] and these sites have been monitoring for avian influenza virus for a long time.

DNA extraction, library construction, and Illumina sequencing

Firstly, according to the user manual of PSP® Spin Stool DNA Plus Kit (collection, storage, stabilization), we carried out the sample collection and DNA extraction of 753 poultry feces. Total DNA was eluted in 50 μL of sterile water and stored at −20 °C. The concentration and purity of the DNAs were measured by a NanoDrop 2000 (Thermo Scientific). Secondly, all DNA samples were fragmented by Bioruptor sonicator and the libraries were prepared using NEXTflex Rapid DNA-Seq Kit (Bioo Scientific, Austin, Texas, USA). The quantity of each library was evaluated by Qubit. Ultimately, 130 metagenomics libraries, included 102 chickens, 19 ducks, six geese, and three pigeons were sequenced on Illumina HiSeq X Ten platform using the 150 bp paired-end module.

Pre-processing of data and metagenomics assembly

The raw reads were quality controlled to remove low-quality sequences (*e.g.*, included ≥10 bp N base or ≥ 15 bp adapter overlapped) and aligned to the chicken genome (alignment with SOAP2, parameters: identity ≥ 90%, -l 30, -v 10, -M 4, -m 200, -x 400), which is a filter step.[47] After quality control, high-quality short reads from each DNA sample were assembled by SOAPdenovo2 assembler (-D 1 -M 3 -L 124 -F -u).[48] Assembled contigs ≥ 500 bp were used for downstream analyses.

Gene prediction, construction of the NR gene set

We constructed the live poultry gut NR gene set according to the previous description.[49,50] The assembled contigs were used to predict open reading frames (ORFs) by MetaGeneMark[51] (prokaryotic GeneMark.hmm version 2.10), ignoring those < 100 nt. After redundancy was removed by CD-HIT,[52] a primitive gene catalogue was constructed (parameters: identity ≥ 95%, coverage ≥ 90%, -c 0.95, -G 0, -aS 0.9, -g 1, -d 0). We run MOCAT2 through do these things, including functional annotation, though many tools(such as SOAP, SOAPdenovo2, CD-HIT) have been embedded in MOCAT2. Functional annotations of gene set were performed using MOCAT2,[49] which was also used to generated abundance profiles. Functional annotations were made by translating the nucleotide sequences of gene set into amino acid sequences and search against KEGG[20] databases.

Taxonomic and functional annotation of gene catalog and abundance assessments

The composition of microbial communities from shotgun sequencing data was analyzed using metagenomic phylogenetic analysis (MetaPhlAn2).[53]

Comparison with human and pig gut gene set

The human and pig gut gene set were compared to the poultry gene catalog. If two or more genes had > 95% identify and > 90% overlap with the query, we considered the genes as the same one. For comparison at the functional level, shared KOs were identified and computed by unique KO ID. Rarefaction analysis was carried out to assess the gene richness.

ARG prediction

ORFs were aligned against the CARD[23] and it was annotated as an ARG if its best BLAST hit in the CARD database had a sequence identity > 80% and > 50% coverage of the original gene.[23–25] The databases were developed to classify the hits into different resistance gene types. In this paper, we downloaded the gut gene sets of human and pig, performed the same pipeline as poultry to annotate and analysis the ARGs, to ensure the comparability.

Determination of relative gene abundance

Gene abundance was determined as previously reported.[49] In short, to obtain the relative gene abundance for each gene, high quality reads from each sample were aligned against the gene catalogue by SOAP2 (identity >95%).[47]

PCR detection of the colistin resistance gene mcr-1 *in Chinese LPMs*

Detection of ARGs was conducted using PCR with total DNA according to the concentration of each sample, followed by confirmation via Sanger sequencing of PCR products. The total DNA from 753 samples was screened for *mcr-1* by using PCR as previously described.[6]

Statistical analysis

Averages were calculated using Excel 2016 (Microsoft Office, Microsoft, USA). Multiple comparisons and pairwise comparisons were performed by the Kruskal–Wallis test and Mann–Whitney *U* test, respectively, using Graph Pad Prism 6.0. Venn diagrams were generated using Venny 2.1.0. Stack column was generated using Origin Pro 9.0. Correlation analyses were performed in R using the "ggcorrplot" package. All statistical tests were considered significant at $P < 0.05$. A method based on geographical information systems, Region Map Generator 2.4.5, was used to map the sampling quantity and the positive rate of *mcr-1*.

Availability of data and materials

All sequences have been deposited in the NCBI SRA (National Center for Biotechnology Information Sequence Read Archive) under BioProject PRJNA408020.

Author contributions

G.F.G., B.Z., and X.J. designed the project. Y.W., J.C., and Y.B. collected samples. Y.W. and N.L. performed DNA extraction and organized the sequencing. Y.W., Y.H., J.C., F.L., S.L., Y.S., X.J., G.F.G., and B.Z. analyzed and interpreted the results. Y.W. and Y.H. created the figures and wrote the manuscript. Y.H., X.J., G.F.G., and B.Z. revised the paper. All authors discussed and interpreted the data and contributed to the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.03.012.

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