

MICROBIOLOGY AND FOOD SAFETY

Changes in antibiotic resistance of *Escherichia coli* during the broiler feeding cycle

Tianfei Han,^{*,†,1} Qingqing Zhang,[†] Na Liu,[†] Juan Wang,[†] Yuehua Li,[†] Xiumei Huang,[†] Junhui Liu,[†] Junwei Wang,[†] Zhina Qu,^{†,1} and Kezong Qi^{*}

^{*}Anhui Province Key Laboratory of Veterinary Pathobiology and Disease Control, College of Animal Science and Technology, Anhui Agricultural University, Hefei, Anhui, China; and [†]Livestock and Poultry Products Quality & Safety Risk Assessment Laboratory (Qingdao) of Ministry of Agriculture and Rural Affairs, China Animal Health and Epidemiology Center, Qingdao, Shandong, China

ABSTRACT The purpose of this study was to investigate the drug-resistant phenotypes and genes of *Escherichia coli* in animal, environmental, and human samples before and after antibiotic use at a large-scale broiler farm to understand the respective effects on *E. coli* resistance during the broiler feeding cycle. The antibiotic use per broiler house was 143.04 to 183.50 mg/kg, and included tilmicosin, florfenicol, apramycin, and neomycin. All strains isolated on the first day the broilers arrived (**T1**; day 1) were antibiotic-resistant bacteria. *E. coli* strains isolated from animal samples were resistant to ampicillin, tetracycline, and sulfamethoxazole (100%), and those isolated from environmental samples were resistant to 5 different drugs (74.07%, 20 of 27). *E. coli* strains isolated

on the last day before the broilers left (**T2**; day 47) had a higher resistance rate to florfenicol (100%, 36 of 36) than at T1 ($P < 0.05$). Multidrug resistance increased from T1 (84.21%, 32 of 38) to T2 (97.22%, 35 of 36). Most strains were resistant to 5 classes of antibiotics, and 2 strains were resistant to 6 classes of antibiotics. Among 13 identified drug resistance genes, 11 and 13 were detected at T1 and T2, respectively. *NDM-1* was detected in 4 environmental samples and 1 animal sample. In conclusion, the use of antibiotics during breeding increases *E. coli* resistance to antibacterial drugs. Drug-resistant bacteria in animals and the environment proliferate during the feeding cycle, leading to the widespread distribution of drug resistance genes and an increase in the overall resistance of bacteria.

Key words: *Escherichia coli*, animal-environment-human, drug resistance, drug resistance gene

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INTRODUCTION

Bacterial antibiotic resistance has posed significant challenges in the animal breeding industry. The misuse and abuse of veterinary antibiotics have contributed to the development of bacterial resistance (Garcia-Migura et al., 2014). *Escherichia coli*, one of the most common bacteria in farms, is present in animal intestines, excrement, soil, water, and air (Adachi et al., 2013; Sun et al., 2017; Yang et al., 2018; Falgenhauer et al., 2019; Qiu et al., 2019; Zhang et al., 2019). *E. coli* can transmit drug resistance genes between drug-resistant and non-drug-resistant bacteria, contributing to the spread of

drug resistance genes. In recent years, studies have shown that bacteria have developed resistance to commonly used clinical antibiotics. From 2006 to 2016, the resistance rate to ampicillin, tetracycline, and sulfadiazine of *E. coli* strains isolated from samples in a veterinary laboratory of Minnesota, United States, was >50% (Hayer et al., 2020). Similarly, from 2008 to 2015, >80% of *E. coli* strains isolated from chickens and pigs in China showed resistance to ampicillin, tetracycline, and sulfadiazine (Zhang et al., 2017). β -lactams (Klimiene et al., 2018; Ye et al., 2018; Alegría et al., 2020; Wang et al., 2020), tetracycline (Hu et al., 2013; Seifi et al., 2016; Bourély et al., 2019), and plasmid-mediated resistance genes such as *PMQR* (Hricová et al., 2017; Seo et al., 2019a; Seo et al., 2019a,b) are prevalent among *E. coli* isolated from poultry.

Several studies have focused on *E. coli* resistance; however, there is little information on *E. coli* resistance during breeding (Montoro-Dasi et al., 2020). In this study, we investigated a large-scale broiler farm in Shandong Province, China. The objectives of this study were to 1) assess

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¹Corresponding authors: qkz@ahau.edu.cn (TH); 641117207@qq.com (ZQ)

the effect of *E. coli* resistance from different aspects (animal, environment, human, and antibiotic use) and 2) to evaluate *E. coli* resistance in depth to provide a reference for controlling bacterial resistance and promote healthy animal breeding.

MATERIALS AND METHODS

Sample Collection and *E. coli* Isolation and Identification

Animal samples (poultry cloacal swabs), environmental samples (ground swabs, cage swabs, drinking water, air, and feed), and hand swabs of breeders were collected from 6 houses at a large-scale broiler farm in Shandong Province, China, on July 10, 2019 (T1; the first day after the broilers arrived) and on August 26, 2019 (T2; the last day before the broilers left). The swab samples were stored in Cary-Blair Transport Medium (Beijing Lu Qiao Technology, Beijing, China), and the air samples were collected directly in MacConkey culture medium (Beijing Lu Qiao Technology). All samples were stored at 4°C during transportation.

An aliquot of the water samples (100 mL) was centrifuged, and the resulting precipitate was cultured for 12 h in tryptone soybean broth (Beijing Lu Qiao Technology) and subsequently in MacConkey culture medium. Feed sample (5 g) was mixed with 45 mL of tryptone soybean broth, cultured in 50 mL centrifuge tubes and placed in an incubator at 37°C and 120 rpm for 4 h, and subsequently in MacConkey culture medium. Swab samples were placed directly in MacConkey culture medium. All the samples in MacConkey medium were cultured for 18 to 24 h at 37°C and purified 3 times. The suspected colonies were analyzed using indicator tests (Qingdao Hai Bo Biotechnology, Qingdao, China) of *E. coli* (e.g., trisaccharide iron test, Kovacs' indigo matrix test, methyl red test, V-P test, and Simon's hydrochloric acid test). All strains identified as *E. coli* were stored in serum at -40°C.

Medication Survey

A questionnaire was administered to the breeders and managers of the farms to investigate disinfection protocols, drug use, and epidemic prevention of broilers during the breeding period.

Antimicrobial Susceptibility Testing

Representative *E. coli* strains isolated from T1 and T2 samples were screened for their resistance to antibiotics using the broth dilution method (Clinical and Laboratory Standards Institute, 2017). The antibiotics were ampicillin, amoxicillin-clavulanate, gentamicin, spectinomycin, tetracycline, florfenicol, sulfafurazole, sulfamethoxazole, ceftiofur, ceftazidime, enrofloxacin, ofloxacin, meropenem, and colistin. *E. coli* strain ATCC 25922 was used for quality control purposes. The strains with simultaneous resistance to 3 classes or more antibiotics were classified as multidrug resistant.

Thirteen antibiotic resistance genes in T1 and T2 samples were analyzed by PCR using the primers listed in Table 1. The antibiotic resistance genes were *CMY*, *OXA*, *CTX-M*, *TEM*, and *NDM-1* (β -lactams); *parC*, *gyrA*, *qnrS*, and *aac(6')-Ib-cr* (quinolones); and *tetA*, *tetB*, *tetM*, and *tetX* (tetracycline). PCR products were sequenced by Shanghai Bioengineering Co. (Shanghai, China), and the resulting sequences were compared against the GenBank database.

Statistical Analysis

SPSS 22 (IBM, Qingdao, China) was used for statistical analysis. A 2-sample *t* test was used to investigate the differences in antibiotic resistance between T1 and T2 samples. Statistical significance was set $P < 0.05$.

RESULTS

Isolation of *E. coli* Isolates and Medication Survey

The farm adopted a cage feeding mode, with 1 full-time keeper in charge of each henhouse. However, there was a situation in which a husband and wife managed 2 henhouses together. After a batch of chickens were raised, the henhouses were kept free for 15 D (known as the vacancy period), during which the henhouses were disinfected twice. Henhouses were disinfected for the last time 2 to 3 D before entering a new batch of chickens. The chickens were caged the following day for prevention and treatment. During the feeding cycle, the main antibiotics used were tilmicosin, florfenicol, apramycin, and neomycin, which belong to the macrolide, amphenicol, and aminoglycoside antibiotic classes.

A total of 468 samples were collected: 234 samples from T1 and 234 samples from T2 (Table 2). The overall *E. coli* isolation rate was higher in T2 (58.12%, 136 of 243) than in T1 (30.77%, 72 of 234). Except for hand swabs, the *E. coli* isolation rate was higher in T2 than in T1. The separation rate of cloacal swabs increased from 28.33% (17 of 60) to 90.00% (54 of 60), which was the highest increase among the sample types. One *E. coli* strain was isolated from a water sample at T1, and at T2, 2 strains were isolated from a water sample and 5 strains were isolated from feed.

Antimicrobial Resistance

The antibiotic resistance results are shown in Figure 1. All 38 strains of *E. coli* in T1 were resistant to 12 drugs, but not to meropenem and colistin. *E. coli* strains were resistant to ampicillin, tetracycline, and sulfafurazole (100%, 7 of 7) in animal samples; ampicillin, tetracycline, florfenicol, sulfafurazole, and sulfamethoxazole (74.07%, 20 of 27) in environmental samples; and ampicillin (100%, 4 of 4), ceftiofur, spectinomycin, and tetracycline (50%) in human samples. Among the environmental samples, the resistance rate to tetracycline and florfenicol was 100% (27 of 27),

Table 1. Antibiotic resistance genes.

Gene name	Primer	sequences (5'-3')	Primer size (bp)	References
<i>CMY</i>	F	CAATGTGTGAGAAGCAGTC	1,432	Hanson et al., 2002
	R	CGCATGGGATTTTCCTTGCTG		
<i>OXA</i>	F	TTCAAGCCAAAGGCACGATAG T	814	Briñas et al., 2002
	R	TCCGAGTTGACTGCCGGGTTG		
<i>CTX-M</i>	F	AGTGAAAGCGAACC GAATC	365	Tian et al., 2011
	R	CTGTCACCAATGCTTTACC		
<i>TEM</i>	F	CAGAAACGCTGGTGAAAGTA	719	
	R	ACTCCCCGTCGTGTAGATAA		
<i>NDM-1</i>	F	GGTTTGGCGATCTGGTTTTTC	621	Wang et al., 2012
	R	CGGAATGGCTCATCACGATC		
<i>parC</i>	F	TGGGCTTAAAACCCACCACT	319	Shimada et al., 2010
	R	CGGGTTTCTGTGTAACGCAT		
<i>gyrA</i>	F	CGTCGTGTTCTTTATGGTGC	230	
	R	ATAACGTTGTGCAGCAGGTC		
qnrS	F	ACCTTCACCGCTTGCACATT	571	Jiang et al., 2008
	R	CCAGTGCTTCGAGAATCAGT		
<i>aac (6)-Ib-cr</i>	F	TGACCTTGCGATGCTCTATG	616	
	R	TTAGGCATCACTGCGTGTTC		
<i>tetA</i>	F	GGCCTCAATTTCTGACG	372	Guillaume et al., 2000
	R	AAGCAGGATGTAGCCTGTGC		
<i>tetB</i>	F	GAGACGCAATCGAATTCGG	228	
	R	TTTAGTGGCTATTCTTCTCGCC		
<i>tetM</i>	F	ACAGAAAGCTTATTATATAAC	171	Aminov et al., 2001
	R	TGGCGTGTCTATGATGTTTAC		
<i>tetX</i>	F	CGCGGATCCATGACAATGCGAATA	1,167	Wen et al., 2020
		GATACAG		
	R	CCGCATATGTTATACATTTAACA ATTGCT		

followed by ampicillin 96.30% (26 of 27). The resistance rate to 6 drugs was >74.07% (20 of 27). *E. coli* strains from human samples had 100% resistance to 7 drugs (3 of 3) and 100% nonresistance to meropenem and ceftazidime.

The multidrug resistance of *E. coli* isolated from T1 and T2 samples is shown in Figure 2. The overall multiple drug resistance rate in T1 samples was 84.21% (32 of 38). There were no multidrug-resistant bacteria in either feed or water (environmental samples) of T1. Multidrug-resistant bacteria were collected from animal and human samples (most had resistance to 4 or 5 drugs). In T2 samples, multidrug resistance increased to 97.22% (35 of 36), whereas multi-drug resistance to 5 classes of antibiotics increased in both water and feed. The other environmental samples showed an increase in the number of resistant bacteria, among which 2 strains with resistance to 6 classes of antibiotics were identified in cage samples.

We obtained a wider distribution of drug-resistant bacteria and a higher number of drug-resistant bacteria in T2 samples than in T1 samples.

Prevalence of Antibiotic Resistance Genes

Several antibiotic resistance genes were identified among the *E. coli* isolates (Figure 3). Among the 13 drug resistance genes, 11 were detected to be of different degrees in T1, except *NDM-1* and *tetX*. All 13 drug resistance genes were detected in T2. Furthermore, *NDM-1* was detected in 1 animal and 4 environmental samples, and *tetX* was detected in 1 environmental sample. Among the β -lactam resistance genes in T1 and T2, *TEM* was the most predominant followed by *CTX-M*, *CMY*, and *OXA*. *NDM-1* was not detected in T1 but was detected in T2 animal and environmental samples. In T1, *gyrA* and *parC* (quinolone resistance genes)

Table 2. *Escherichia coli* isolated from samples collected at T1 and T2.

House collection sample type	T1			T2		
	Sample size	Positive detection	Isolation rate (%)	Sample size	Positive detection	Isolation rate (%)
Animal						
Cloacal ¹	60	17	28.33	60	54	90.00
Environment						
Ground	60	22	36.67	60	29	48.33
Cage	60	25	41.67	60	32	53.33
Air ¹	30	3	10.00	30	11	36.67
Water	6	1	16.67	6	2	33.33
Feed ¹	6	0	0.00	6	5	83.33
Human						
Hand	12	4	33.33	12	3	25.00
Total	234	72	30.77	234	136	58.12

Abbreviations: T1, first day the broilers' arrival; T2, last day before the broilers left.

¹*P* < 0.05.

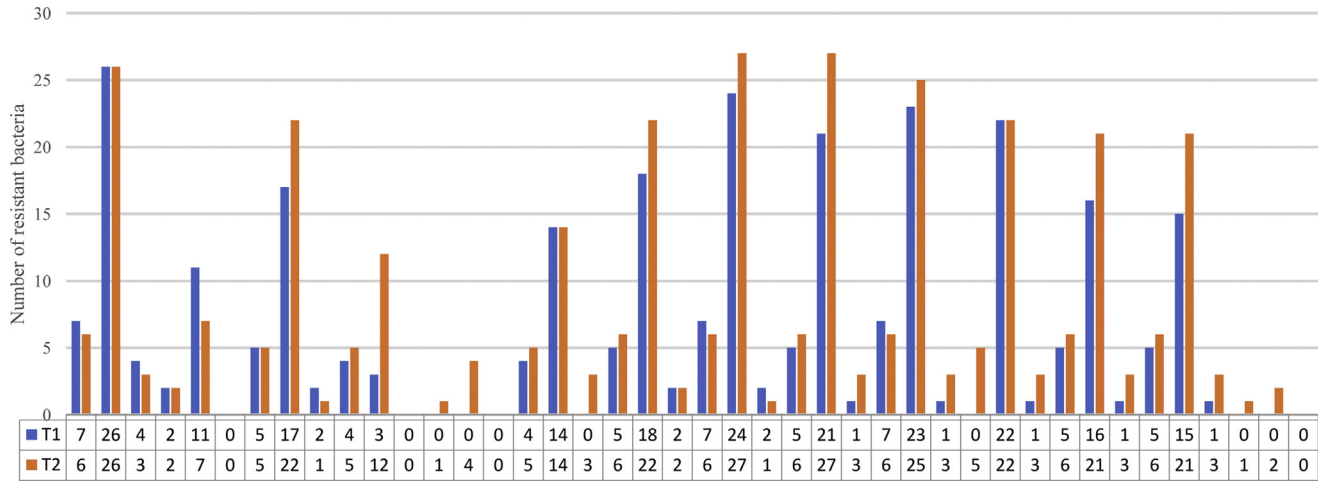


Figure 1. Antibiotic resistance of *Escherichia coli* isolated from samples collected at T1 and T2. **P* < 0.05. Abbreviations: A, animal; AMP, ampicillin; A/C, amoxicillin clavulanate; CAZ, cefazolin; CEF, cephalothin; CL, colistin; E, environment; ENR, enrofloxacin; FFC, florfenicol; GEM, gentamicin; H, human; MEM, meropenem; OFL, Ofloxacin; SPT, spectinomycin; SF, sulfafurazole; SXT, sulfamethoxazole; TE, tetracycline; T1, first day the broilers' arrival; T2, last day before the broilers left.

were second in prevalence to *aac (6)-Ib-cr* (100% detection rate). Among the tetracycline resistance genes, *tetB* was the most predominant in T1 and T2 (100%) followed by *tetA* (100%) in T1 human samples and T2 animal samples. The detection rates of *tetM* and *tetX* were relatively low; *tetM* was identified only in human samples.

DISCUSSION

Aminoglycosides and amido alcohols (e.g., florfenicol, apramycin, and neomycin) are used in the prevention and/or treatment of avian colibacillosis (Wang et al., 2003; Becker et al., 2013). In our study, gentamicin resistance was not observed in T1 human samples. However, gentamicin resistance was observed in T1 environmental samples (51.85%, 14 of 27) and T1 animal samples (57.14%, 4 of 7). However, all T2 human samples showed

drug resistance. Gentamicin resistance did not increase in environmental samples but increased to 83.33% (5 of 6) in animal samples. In T1 and T2 environment samples, streptomycin resistance was 66.67% (18 of 27) and 81.48% (22 of 27), respectively. Drug resistance increased from 71.43% (5 of 7) at T1 to 100% (6 of 6) at T2 in animal samples and from 50% (2 of 4) at T1 to 66.67% (2 of 3) at T2 in human samples. In general, resistance toward aminoglycosides was more evident at T2 than at T1 probably owing to the use of apramycin and neomycin that resulted in cross-resistance of *E. coli* during breeding. Streptomycin-resistant *E. coli* have different degrees of resistance to the other 6 aminoglycoside drugs (Lin et al., 2012; Reeves et al., 2013). The overall resistance toward florfenicol was 71.05% (27 of 38) at T1 and 100% (36 of 36) at T2 (*P* < 0.05). Our findings revealed the effect of antibiotic use on bacterial drug resistance during breeding process

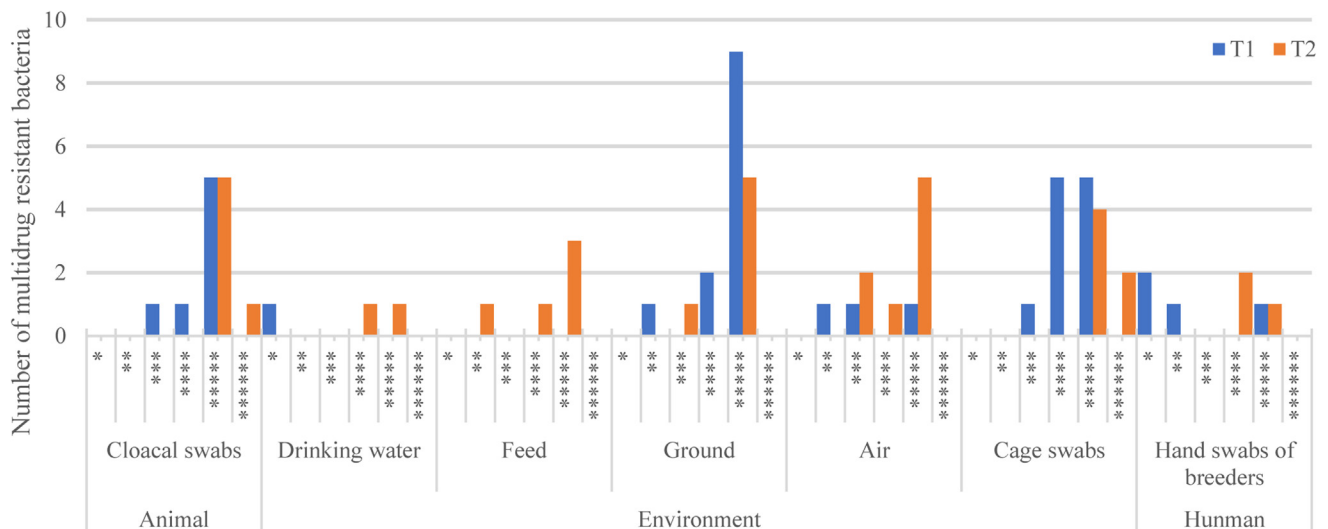


Figure 2. Distribution of multidrug resistant bacteria in samples from animals, environment, and humans. The number of * indicates the multiplicity of drug resistance.

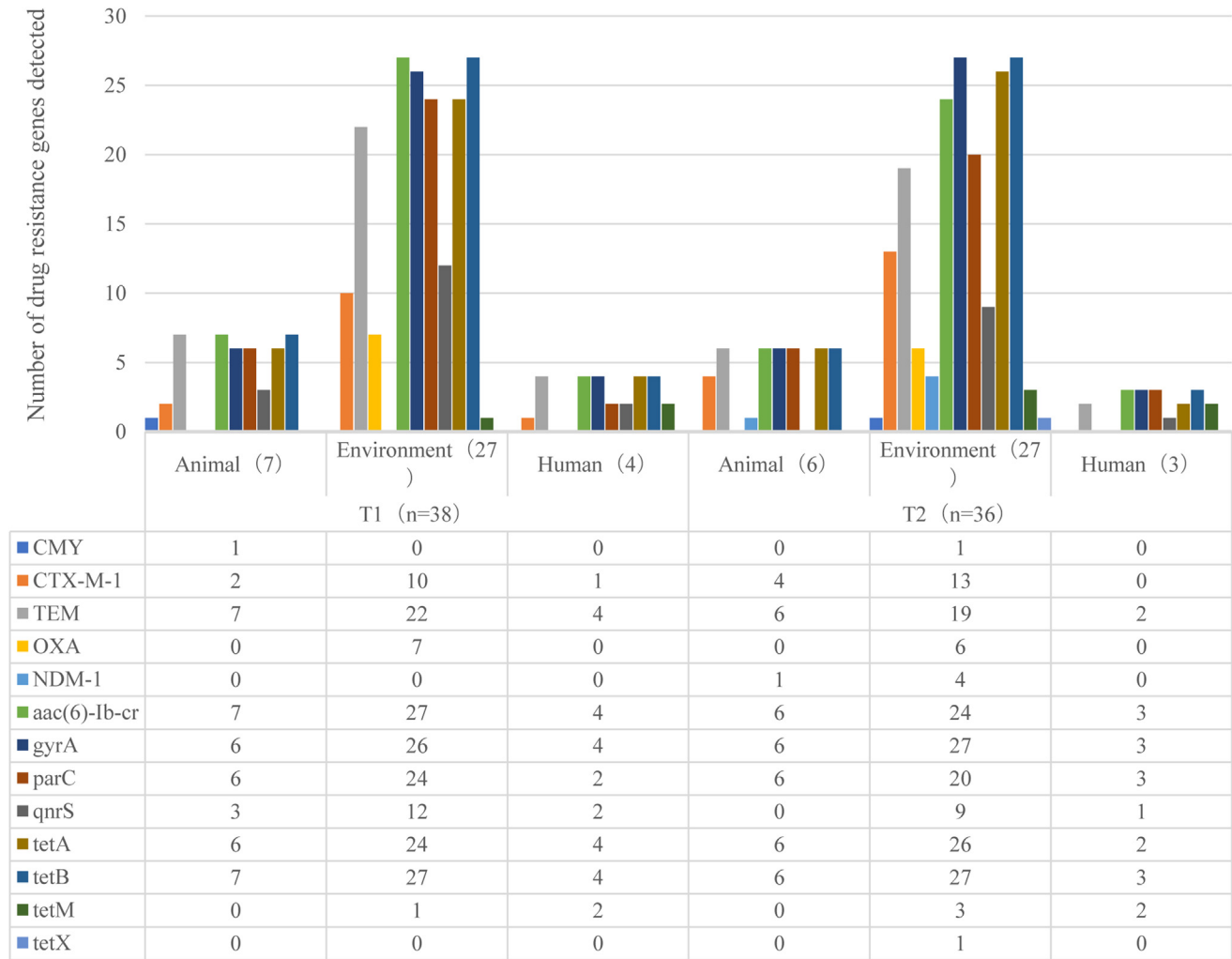


Figure 3. Detection results of antibiotic resistance genes in *Escherichia coli* isolated from samples collected at T1 and T2. Abbreviations: T1, first day the broilers' arrival; T2, last day before the broilers left.

(Mhondoro et al., 2019) and the resistance of *E. coli* to aminoglycosides and amide alcohols.

At T1, there was a large number of isolates on the ground and cages (environmental samples). Therefore, the disinfection measures were not sufficient to destroy *E. coli* in the environment, and these isolated strains were drug resistant (Figure 1). At T1, the detection rate of *aac* (β')-*Ib-cr* and *tetB* was 100% and remained high at T2. Therefore, *E. coli* had several drug resistance genes before the administration of antibacterial drugs. In addition, most of the drug resistance genes tested in this study can be mediated by plasmids (Park et al., 2019; Silvester et al., 2019; Suzuki et al., 2019; Cui et al., 2020; Koyama et al., 2020; Nair et al., 2020; Nishikawa et al., 2019), which is likely to be the source of bacterial drug resistance transmission during feeding. Newborn chicks had a certain carrier rate (Table 1), and these strains were drug resistant, which may be related to farm breeding conditions (Zhao et al., 2019). After a breeding cycle, the number of bacteria carried by the environment and chicken population increases, resulting in a higher detection rate of *E. coli* in water and air samples. The isolation rate of *E. coli* from animals increased

from 28.33 to 90.00% ($P < 0.01$); therefore, there was considerable *E. coli* proliferation in chickens.

The *E. coli* strain with meropenem resistance was isolated at T2. *NDM-1* was detected in all the positive strains. The number of bacteria resistant to 5 classes of antibiotics was the largest at T2, followed by the number of bacteria resistant to 4 classes of antibiotics, consistent with past studies. In addition, there were 3 colistin-resistant strains at T2, which were also resistant to 6 classes of antibiotics.

Among the β lactam resistance genes, the most prevalent was *TEM*, which was owing to the long-term use of this antibiotic (Sun et al., 2019). The main quinolone resistance genes were *gyrA*, *parC*, and *aac* (β')-*Ib-cr*. Mutations in *gyrA*, accompanied by mutations in *parC* (or *parE*), can result in quinolone resistance (Bagel et al., 1999). The *aac* (β')-*Ib-cr* gene may be helpful for strains to capture other resistant plasmids and improve their competitiveness (Zhang et al., 2016). Therefore, *aac* (β')-*Ib-cr* was widely present in multidrug-resistant strains. Among the tetracycline resistance genes, *tetA* and *tetB* were the most predominant ones. The detection rate was between 80 and 100%, as previously reported (Wu et al., 2017).

In conclusion, to improve the rational use of veterinary antibacterial drugs and achieve the antibacterial target as early as possible, it is important to implement strict disinfection measures to destroy residual bacteria in the environment. In addition, it is necessary to select breeding farms with a standardized use of antibacterial drugs. Drug-resistant bacteria affect the bacterial resistance of chicks. During daily feeding, it is essential to pay considerable attention to the disinfection of cages and ground in the enclosure, increase the disinfection measures of feed and water, and maintain adequate air circulation.

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