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PAPER

Detection and coexistence of six categories of resistance genes in *Escherichia coli* strains from chickens in Anhui Province, China

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Abstract

The aim of this study was to characterise the prevalence of class 1 integrons and gene cassettes, tetracycline-resistance genes, phenicol-resistance genes, 16S rRNA methylase genes, extended-spectrum β -lactamase genes and plasmid-mediated fluoroquinolone resistance determinants in 184 *Escherichia coli* isolates from chickens in Anhui Province, China. Susceptibility to 15 antimicrobials was determined using broth micro-dilution. Polymerase chain reaction and DNA sequencing were used to characterise the molecular basis of the antibiotic resistance. High rates of antimicrobial resistance were observed; 131 out of the 184 (72.3%) isolates were resistant to at least six antimicrobial agents. The prevalences of class 1 integrons, tetracycline-resistance genes, phenicol-resistance genes, 16S rRNA methylase genes, extended-spectrum β -lactamase genes and plasmid-mediated fluoroquinolone resistance determinants were 49.5, 17.4, 15.8, 0.5, 57.6 and 46.2%, respectively. In 82 isolates, 48 different kinds of coexistence of the different genes were identified. Statistical (χ^2) analysis showed that the resistance to amoxicillin, doxycycline, florfenicol, ofloxacin and gentamicin had significant differences ($P < 0.01$ or $0.01 < P < 0.05$) among the strains that carried and did not carry the resistance genes, which showed a certain correlation between antimicrobial resistance and the presence of resistance genes.

Introduction

Recently, the frequency and spectrum of

antimicrobial-resistance infections have increased in the poultry industry. The emergence of *Escherichia coli* isolates with multiple antibiotic-resistance phenotypes has been previously reported and is regarded as a serious problem (Maynard *et al.*, 2003). Transfer of resistance determinants by mobile genetic elements, including plasmids, transposons, and gene cassettes in integrons (Carattoli, 2001; Hall and Collins, 1995) are important factors that contribute to the increase in antibiotic resistance in multiple-resistant *E. coli*. In a previous study (Saénz *et al.*, 2004), 17 multiple antimicrobial-resistance nonpathogenic *E. coli* strains of human, animal, and food origins showed a wide variety of antibiotic resistance genes, many of them carried by class 1 and class 2 integrons. The main purpose of the present study was to investigate the prevalence and characteristics of class 1 integrons, tetracycline-resistant genes, phenicol-resistant genes, 16S rRNA methylase genes, extended-spectrum β -lactamase (ESBL) genes and plasmid-mediated fluoroquinolone resistance (PMQR) genes and their coexistence in 184 *E. coli* isolates collected from chicken farms.

Materials and methods

Bacterial isolates

In this study, *E. coli* isolates ($n=184$) were collected from chicken cloacae at four different farms located in Anhui Province, China, from March to May 2012. The data and location of each farm are as follows: No. 1 chicken farm ($n=46$, located in Hefei city), No. 2 chicken farm ($n=44$, located in Changfeng county), No. 3 chicken farm ($n=44$, located in Feixi county), and No. 4 chicken farm ($n=50$, located in Feidong county). Sterile cotton swabs were used to collect fecal samples from chicken cloacae. The swabs were immediately transferred to sterile collection containers containing Luria-Bertani (LB) broth and were cultured at 37°C overnight. The cultures were inoculated in *E. coli* Chromogenic Medium and were grown at 37°C for 18-24 h. Then picked up a single colony which was routinely grown in LB or LB agar at 37°C for 18-24 h.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of 15 antimicrobials [amoxicillin (AMX), ceftriaxone (CRO), ceftiofur (CTF), amikacin (AMI), gentamicin (GEN), apramycin (APR), doxycycline (DC), oxytetracycline (OTC), florfenicol (FFC), enrofloxacin

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Key words: *Escherichia coli*; Antimicrobial resistance; Chicken; Coexistence.

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Contributions: LL and YW conceived and designed the experiments. YW and SF performed the experiments. XY and YF analysed the data. JN and MH contributed to reagents/materials/analysis tools. LL and YW contributed equally to the work and wrote the paper. YW, SF, XY and YF collected *E. coli* isolates from chickens at four farms.

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(ERO), ofloxacin (OFX), lomefloxacin (LOM), cefquinome (CQN), sarafloxacin (SAR), sulfamonomethoxine (SUL)] were determined using the broth micro-dilution method, according to the guidelines issued by the Clinical and Laboratory Standards Institute. *E. coli* ATCC 25922 was used as the reference strain.

Genotypic resistance characterisation

Class 1 integrons and gene cassettes, tetracycline-resistance genes (*tetA* and *tetM*), phenicol-resistance genes (*floR* and *fexA*), 16S rRNA methylase genes (*armA* and *rmtB*), ESBL genes (*bla_{CTX-M}*, *bla_{TEM}* and *bla_{SHV}*) and PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')*-*Ib-cr* and *qepA*) were detected by PCR using the primers listed in Table 1. All the PCR amplicons were confirmed by dideoxy DNA sequencing. The DNA sequences obtained were compared with those in GenBank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/>).

Results

Antimicrobial susceptibility of *Escherichia coli* isolates

High rates of resistance to OTC (97.8%), SUL (97.3%), DC (90.2%), AMX (82.6%), LOM (77.7%), CRO (70.1%), OFX (67.4%), ERO (55.9%), and FFC (52.7%) were observed among the 184 *E. coli* isolates. Low rates of resistance to AMI (7.6%) and SAR (2.2%) were observed. The resistance rates of *E. coli* isolates from four chicken farms to 15 antimicrobials can be seen in Figure 1. One hundred and thirty-one (72.3%) of the isolates were resistant to at least six antimicrobial agents, while 25 (13.6%) were resistant to at least 10 of these drugs (Figure 2).

Detection of the six categories of resistance determinants

The PCR results showed that 91 (49.5%) strains harbored class 1 integrons. Seventy of the 91 *int1*-positive isolates carried gene cassettes, which were *dfrA1-tnpAIS26-aadA1* (45/70), *dfrA2-aadA12* (16/70) and *dfrA1-aadA1* (9/70); 21 isolates did not carry gene cassettes.

Tetracycline-resistance genes were detected in 32 of 184 (17.4%) isolates (*tetA*, n=27; *tetM*, n=5). Twenty-nine (15.8%) isolates possessed phenicol-resistance genes (*floR*, n=29); no *fexA* genes were detected. Only one isolate carried a 16S rRNA methylase gene in the form of *rmtB*; no isolates were positive for *armA*.

ESBL genes were detected in 106 of the 184 (57.6%) isolates (*bla_{CTX-M}*, n=40; *bla_{TEM-1}*,

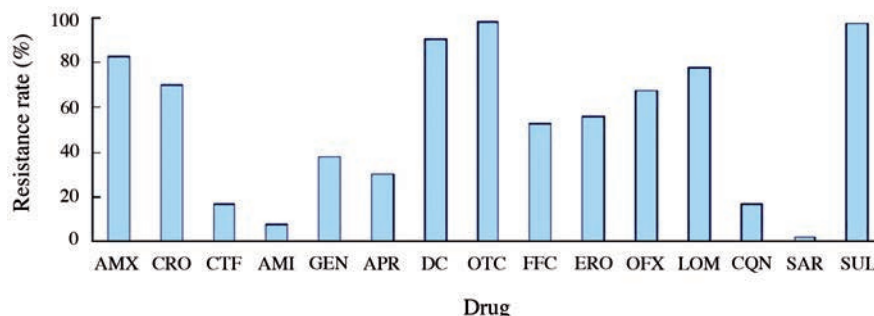


Figure 1. Resistance rates of *Escherichia coli* isolates from four chicken farms to fifteen antimicrobials. AMX, amoxicillin; CRO, ceftriaxone; CTF, ceftiofur; AMI, amikacin; GEN, gentamicin; APR, apramycin; DC, doxycycline; OTC, oxytetracycline; FFC, florfenicol; ERO, enrofloxacin; OFX, ofloxacin; LOM, lomefloxacin; CQN, cefquinome; SAR, sarafloxacin; SUL, sulfamonomethoxine.

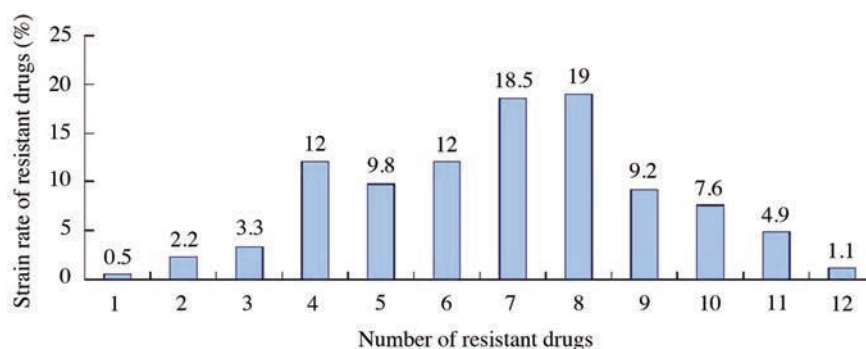


Figure 2. Resistance profiles of 184 *Escherichia coli* isolates from four chicken farms to fifteen antimicrobials. One to twelve in the X axis represents resistance to one antimicrobial to twelve antimicrobials. Y axis represents the rates of the isolate resistance to one antimicrobial to fifteen antimicrobials.

Table 1. Polymerase chain reaction primers and annealing temperatures.

Gene	Sequence (5'-3')	Annealing temperature, °C	Reference
<i>bla_{TEM}</i>	F:ATGAGTATTCAACATTTCCGR:CCAATGCTTAATCAGTGAGG	50	This study
<i>bla_{CTX-M}</i>	F:CGATGGGACGATGTCACCTGR:CGGCTTTCTGCCTTAGGTT	59	This study
<i>bla_{SHV}</i>	F:ATTATCTCCCTGTTAGCCACCCGR:CGCCTCATTGAGTTCGGTTT	55	This study
<i>qnrA</i>	F:AGAGGATTTCTACGCCAGGR:TGCCAGGCACAGATCTTGAC	54	Wang et al. (2012)
<i>qnrB</i>	F:GGAATCGAAATTCGCCACTGR:TTTGCCGTTCCGCAGTCGAA	58	This study
<i>qnrC</i>	F:GGGTTGTACATTTATTGAATCR:TCCACTTTACGAGGTTCT	50	Dai et al. (2008)
<i>qnrD</i>	F:CGAGATCAATTTACGGGAATAR:AACAAGCTGAAGCGCCTG	50	Xia et al. (2010)
<i>qnrS</i>	F:CACTTTGATGTCGAGATR:CAACAATACCCAGTGCTT	52	Yuan et al. (2009)
<i>aac(6')-Ib-cr</i>	F:CGATGCTCTATGGGTGGCTAAR:GGTCCGTTTGGATCTTGGTGA	58	This study
<i>qepA</i>	F:CCGATGACGAAGCACAGGGR:TCTACGGGCTCAAGCAGTTGG	50	This study
<i>int1</i>	F:GCCTTGCTGTTCTTCTACR:GATGCCTGCTTGTCTAC	55	del Castillo et al. (2013)
<i>Int-class1</i>	F:GGCATCCAAGCAGCAAGCR:AAGCAGACTTGACCTGAT	58	del Castillo et al. (2013)
<i>armA</i>	F:CCGAAATGACAGTTCCTATCR:GAAATGAGTGCCTTGGAGG	56	Yan et al. (2004)
<i>rmtB</i>	F:ATGAACATCAACGATGCCCTCR:CCTTCTGATTGGCTTATCCA	56	Yan et al. (2004)
<i>floR</i>	F:CACGTTGAGCCTCTATATR:ATGCAGAAGTAGAACGCG	55	Sáenz et al. (2004)
<i>fexA</i>	F:GTACTTTGAGGTGCAATTACGGCTGAR:CGCATCTGAGTAGGACATAGCGC	57	Kehrenberg and Schwarz (2005)
<i>tetA</i>	F:GTAATTCTGAGCACTGTCGR:CTGCCTGGACAACATTGCTT	62	Sáenz et al. (2004)
<i>tetM</i>	F:GAACCTGAACAAGAGGAAR:ATGGAAGCCAGAAAGGA	60	Giovanetti et al. (2003)

n=49; and *bla*_{TEM-206}, n=17); no isolates were positive for the *bla*_{SHV} gene. Eighty-five out of the 184 (46.2%) isolates possessed PMQR determinants. Twenty-two (11.9%) isolates carried the *qnr* determinant and 63 (34.2%) carried *aac*(6')-Ib-cr. Among the *qnr* determinants, only the *qnrS*-type gene was detected; no isolates were positive for *qnrA*, *qnrB*, *qnrC*, *qnrD* or *qepA* genes.

Coexistence of different resistance genes

Coexistence of different resistance genes was identified in 82 *E. coli* isolates. There were 48 different kinds of coexistence of resistance genes (Table 2). Twenty-six isolates carried three different genes, five isolates carried four genes and three carried five genes. Only one isolate harbored six genes, which were *dfrA2-aadA12*, *tetA*, *tetM*, *bla*_{TEM-1}, *qnrS* and *aac*(6')-Ib-cr. The combination of *dfrA1-tnpAIS26-aadA1* and *aac*(6')-Ib-cr was observed in eight *E. coli* isolates.

Relationship between resistance genes and antibiotic resistance of the 184 *Escherichia coli* isolates

A χ^2 test was used to determine the relationship between resistance genes and antimicrobial resistance of the 184 *E. coli* isolates.

Compared the resistance to AMX, DC, FFC, OFX and GEN, the results of strains carrying resistance genes were significantly different ($P<0.01$ or $0.01<P<0.05$) from the strains not carrying resistance genes, while resistance to the remaining 10 antimicrobials showed no difference ($P>0.05$) (Table 3).

Discussion

No information is available about the occurrence and distribution of the different resistance determinants, such as integrons and gene cassettes, tetracycline-resistance genes, phenicol-resistance genes, 16S rRNA methylase genes, ESBL and PMQR genes in *E. coli* from chickens in Anhui Province, China, therefore we screened 184 *E. coli* isolates for the presence of these resistance determinants. 71.2% of the isolates were resistance to at least six antimicrobial agents, while 13.6% were resistance to at least 10 of these drugs, indicating a high prevalence of multiple antibiotic-resistance *E. coli* in chickens in Anhui Province. In Henan Province, the proportion of multiple antibiotic resistance isolates in chickens was 76.5% (Yuan *et al.*, 2010). In Shanghai, 56.9% *E. coli* isolates were resist-

ance to 10 to 15 antimicrobial agents (Ma *et al.*, 2009). Additionally, 95.1% *E. coli* isolates from different regions showed multiple antibiotic resistance (Lin *et al.*, 2009). The differences in multiple antibiotic resistance could be caused by different antimicrobial usage in these areas.

In this study, 184 *E. coli* isolates were assayed for the presence of class 1 integrons. 49.5% of isolates carried class 1 integrons, harbouring gene cassettes such as *dfrA1-tnpA* *IS26-aadA1*, *dfrA2-aadA12* and *dfrA1-aadA1*, which encode resistance to sulfonamides and aminoglycosides. There are differences in the prevalence of the class 1 integrons in China and abroad. 52% of *E. coli* isolated from poultry food possessed class 1 integrons (Soufi *et al.*, 2011) in Tunisia. In Spain, a study suggested that the frequency of class 1 integrons was 51% (Marchant *et al.*, 2013). In China, one study showed that 60.4% of *E. coli* isolates from chickens had class 1 integrons (Zhang *et al.*, 2009). In addition, 89.9% *E. coli* collected from food-producing animals were positive for class 1 (Lin *et al.*, 2011). In this study, the frequency of class 1 integrons is similar to the data obtained abroad, but lower than the domestic data.

In addition, we detected tetracycline-resistance genes, phenicol-resistance genes and

Table 2. Coexistence of different resistance genes.

Coexistence of different genes	Strains, n	Coexistence of different genes	Strains, n
<i>dfrA2-aadA12+tetA+tetM+bla</i> _{TEM-1} <i>+qnrS+aac</i> (6')-Ib-cr	1	<i>floR+bla</i> _{TEM-1} <i>+qnrS</i>	1
<i>dfrA1-tnpAIS26-aadA1+tetA+bla</i> _{CTX-M} <i>+qnrS</i>	1	<i>dfrA1-aadA1+tetM+bla</i> _{TEM-1}	1
<i>dfrA1-tnpAIS26-aadA1+tetA+bla</i> _{TEM-1} <i>+qnrS+aac</i> (6')-Ib-cr	1	<i>dfrA1-aadA1+floR+bla</i> _{CTX-M}	1
<i>dfrA1-aadA1+tetM+floR+bla</i> _{TEM-1} <i>+aac</i> (6')-Ib-cr	1	<i>bla</i> _{CTX-M} <i>+qnrS+aac</i> (6')-Ib-cr	1
<i>dfrA1-tnpAIS26-aadA1+tetA+bla</i> _{CTX-M} <i>+bla</i> _{TEM-1}	1	<i>bla</i> _{TEM-1} <i>+qnrS+aac</i> (6')-Ib-cr	1
<i>dfrA1-aadA1+tetA+bla</i> _{CTX-M} <i>+bla</i> _{TEM-1}	1	<i>floR+tetA</i>	1
<i>dfrA2-aadA12+bla</i> _{TEM-1} <i>+qnrS</i>	1	<i>dfrA1-tnpAIS26-aadA1+aac</i> (6')-Ib-cr	8
<i>bla</i> _{CTX-M} <i>+bla</i> _{TEM-1} <i>+qnrS+aac</i> (6')-Ib-cr	1	<i>dfrA1-aadA1+floR</i>	4
<i>dfrA1-tnpAIS26-aadA1+qnrS+aac</i> (6')-Ib-cr+ <i>bla</i> _{CTX-M}	1	<i>bla</i> _{CTX-M} <i>+aac</i> (6')-Ib-cr	4
<i>dfrA1-aadA1+floR+bla</i> _{TEM-1} <i>+aac</i> (6')-Ib-cr	1	<i>dfrA1-tnpAIS26-aadA1+tetA</i>	4
<i>dfrA1-aadA1+floR+aac</i> (6')-Ib-cr	3	<i>bla</i> _{CTX-M} <i>+bla</i> _{TEM-1}	4
<i>dfrA1-tnpAIS26-aadA1+bla</i> _{CTX-M} <i>+bla</i> _{TEM-1}	2	<i>dfrA1-tnpAIS26-aadA1+bla</i> _{TEM-1}	2
<i>dfrA1-tnpAIS26-aadA1+tetA+aac</i> (6')-Ib-cr	4	<i>floR+aac</i> (6')-Ib-cr	2
<i>tetA+bla</i> _{TEM-1} <i>+qnrS</i>	2	<i>bla</i> _{TEM-1} <i>+aac</i> (6')-Ib-cr	4
<i>bla</i> _{TEM-1} <i>+qnrS+aac</i> (6')-Ib-cr	2	<i>dfrA2-aadA12+bla</i> _{TEM-1}	1
<i>dfrA2-aadA12+bla</i> _{TEM-1} <i>+bla</i> _{CTX-M}	1	<i>dfrA1-tnpAIS26-aadA1+bla</i> _{OXA}	1
<i>dfrA1-tnpAIS26-aadA1+qnrS</i>	1	<i>tetA+aac</i> (6')-Ib-cr	1
<i>dfrA1-tnpAIS26-aadA1+floR+qnrS</i>	1	<i>dfrA1-aadA1+aac</i> (6')-Ib-cr	1
<i>tetM+aac</i> (6')-Ib-cr	1	<i>tetA+bla</i> _{TEM-1}	3
<i>dfrA1-aadA1+bla</i> _{TEM-1} <i>+aac</i> (6')-Ib-cr	1	<i>dfrA1-tnpAIS26-aadA1+floR</i>	1
<i>dfrA2-aadA12+bla</i> _{TEM-1} <i>+aac</i> (6')-Ib-cr	1	<i>floR+bla</i> _{TEM-1}	1
<i>dfrA1-tnpAIS26-aadA1+floR+aac</i> (6')-Ib-cr	1	<i>floR+bla</i> _{CTX-M}	1
<i>dfrA1-tnpAIS26-aadA1+tetA+bla</i> _{TEM-1}	1	<i>bla</i> _{CTX-M} <i>+aac</i> (6')-Ib-cr	1
<i>dfrA2-aadA12+bla</i> _{TEM-1} <i>+tetA</i>	1	<i>tetA+bla</i> _{CTX-M}	1

Table 3. Comparison of resistance between the strains carrying and not carrying resistance genes.

Drugs	Resistance, %		χ^2	P
	Carrying resistance genes	Not carrying resistance genes		
AMX	87.3	70.0	7.58	<0.01
CRO	73.1	62.0	2.14	>0.05
CTF	18.7	12.0	1.17	>0.05
AMI	9.0	4.0	0.68	>0.05
CQN	14.9	22.0	1.13	>0.05
GEN	44.0	24.0	6.15	0.01< and <0.05
APR	29.1	24.0	0.47	>0.05
DC	95.5	78.0	11.28	<0.01
OTC	98.5	96.0	0.22	>0.05
FFC	61.2	32.0	12.47	<0.01
ERO	60.4	46.0	3.07	>0.05
SAR	3.0	0	0.45	>0.05
OFX	73.9	48.0	11.02	<0.01
LOM	78.4	74.0	0.40	>0.05
SUL	96.3	90.0	1.73	>0.05

AMX, amoxicillin; CRO, ceftriaxone; CTF, ceftiofur; AMI, amikacin; CQN, cefquinome; GEN, gentamicin; APR, apramycin; DC, doxycycline; OTC, oxytetracycline; FFC, florfenicol; ERO, enrofloxacin; SAR, sarafloxacin; OFX, ofloxacin; LOM, lomefloxacin; SUL, sulfamonomethoxine. P>0.05, no differences; 0.01<P<0.05, difference; P<0.01, significant difference.

16S rRNA methylase genes. The results showed that in Anhui Province, the frequencies of *tetA*, *tetM*, and *floR* were 14.7, 2.7 and 15.8%, respectively. Only one isolate harbored a 16S rRNA methylase gene (*rmtB*). No isolates carried *fexA* and *armA*. In other studies, the frequencies of *tetA* and *tetM* were 87.9 and 15.5%, respectively (Zhang *et al.*, 2010), the prevalence of *floR* was 45.1% (Du *et al.*, 2007) and the frequency of *rmtB* was 30.3% (Zhou *et al.*, 2010). Thus the frequencies of these three genes in the current study were lower than those reported previously.

The *bla*_{TEM-1} gene was the most common β -lactamase gene among *E. coli* isolates in Anhui Province. This was in agreement with previous findings (Xia *et al.*, 2010). With respect to PMQR genes, *qnrS* and *aac(6')-Ib-cr* were predominant, while other PMQR genes were not detected in this study.

Notably, 82 isolates carried more than two types of genes, resulting in 48 different kinds of coexistence of class 1 integrons and gene cassettes, tetracycline-resistance genes, phenicol-resistance genes, ESBL and PMQR genes. Thus the coexistence of resistance genes was very common and varied in Anhui Province. The most frequent coexistence was *dfrA1-tmpAIS26-aadA1* and *aac(6')-Ib-cr*, which was found in eight *E. coli* isolates, while the others were found in \leq four isolates. Six determinants, *dfrA2-aadA12*, *tetA*, *tetM*, *bla*_{TEM-1}, *qnrS* and *aac(6')-Ib-cr*, were detected in an individual *E. coli* isolate. To the best of our knowledge, this is the first report of these six genes coexisting in an *E. coli* strain in China. In addition, statistical analysis indicated a cor-

relation between antimicrobial resistance and the presence of resistance genes. Except for CQN, the frequency of resistance to the other 14 antimicrobial agents in those isolates that carried resistance genes was higher than those without resistance genes. This was especially the case for resistance to AMX, DC, FFC, OFX and GEN (P<0.01 or 0.01<P<0.05), which suggested that the prevalence of ESBL genes, tetracycline-resistance genes, phenicol-resistance genes, PMQR genes and *aadA* was related, to a certain extent, to the observed resistance to AMX, DC, FFC, OFX and GEN.

Conclusions

In conclusion, this is the first study describing the prevalence and characteristics of six categories of resistance determinants in *E. coli* isolates from chickens in Anhui Province, China. The most abundant genes were *ESBL* genes (57.6%), class 1 integrons (49.5%) and *PMQR* genes (46.2%). In 82 isolates, 48 different types of coexistence of the different genes were identified. Six genes, *dfrA2-aadA12*, *tetA*, *tetM*, *bla*_{TEM-1}, *qnrS* and *aac(6')-Ib-cr*, were detected in an individual *E. coli* isolate for the first time. Resistance to AMX, DC, FFC, OFX and GEN was significantly different (P<0.01 or 0.01<P<0.05) among the strains that carried and did not carry resistance genes, which indicated that resistance genes contributed to the corresponding antimicrobial resistance.

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