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Characterization of antimicrobial resistance and related resistance genes in *Escherichia coli* strains isolated from chickens in China during 2007-2012

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In the present study, the prevalence of antimicrobial-resistant chicken *Escherichia coli* strains and the resistance genes in *E. coli* was investigated. For this purpose, 1002 chicken *E. coli* strains isolated from layer and broiler flocks in Shaanxi, Henan and Gansu provinces in China during 2007-2012 were examined. Antimicrobial susceptibility of these *E. coli* strains against 18 antimicrobials was determined by the Kirby-Bauer disk diffusion method. Eight out of the twenty antimicrobial resistance genes were detected by polymerase chain reaction (PCR). The sequences of the resistance genes in chicken *E. coli* strains were compared with the previously published sequences. Our results revealed that the antimicrobial resistance prevalence of *E. coli* strains in western China to ampicillin, doxycycline, tetracycline and nalidixic acid were consistently kept at 62-100%. The *E. coli* resistance to nalidixic acid and ciprofloxacin had an increasing trend, as high as 100% for nalidixic acid while the resistance prevalence to gentamicin had a decreasing trend. The detection rates of the genes for *tetA*, *tetB*, *blaTEM,* and *aac(3)-II* in chicken *E. coli* strains were positively correlated with their antimicrobial resistance (P <0.01) during 2007-2012. Among 1002 chicken *E. coli* strains tested, all *E. coli* strains were resistant to more than three kinds of antimicrobials. Our results revealed that 499 of the 1002 (49.8%) chicken *E. coli* strains were resistant to more than eight kinds of antimicrobials. Considering all the 1002 isolates, the detection prevalence of the genes for *tetA*, *tetB, blaTEM* in chicken *E. coli* strains were constantly over 88.9%. The detection prevalence of the genes for *floR*, *sul-I* and *cmlA* in chicken *E. coli* strains increased, while *aac(3)-II* declined from 75.0 to 28.6%.

Key words: *Escherichia coli*, antibiotic resistance, antibiotic resistance genes, polymerase chain reaction (PCR), chicken.

INTRODUCTION

Escherichia coli is one of the common pathogens in chicken production. For a long time, antibiotics have been widely used in the treatment and prevention of colibacillosis, even increasingly being used as animal growth promotion agents (Sarmah et al., 2006; Martinez, 2009). *E. coli* resistance rises with the selective pressure of the antibiotics, and then reduces the clinical efficacy of antibacterial drugs and increase the mortality of sick animals,

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Table 1. *E. coli* strains isolated from chickens in three provinces in China during 2007-2012.

Year	Number of total strains/number of strains from each source							
	Shaanxi Province			Henan Province		Gansu Province		Total
	Xi'an	Tongchuan	Yangling	Sanmenxia	Luoyang	Tianshui	Dingxi	
2007	14	20	16	18	22	24	20	134
2008	18	22	20	20	22	22	24	148
2009	12	20	16	16	18	17	16	115
2010	18	20	20	20	25	26	16	145
2011	22	42	24	25	20	26	24	183
2012	30	63	44	34	32	40	34	277
2007-2012	114	187	140	133	139	155	134	1002

thereby causing economic losses (Dho-Mouline and Fairbrother, 1998; Barnes et al., 2008; Pan et al., 2009). Avian pathogenic *E. coli* (APEC) cause aerosacculitis, polyserositis, septicemia and other mainly extraintestinal diseases in chickens, turkeys and other avain species (Dho-Moulin and Fairbrother, 1998). This disease results in significant morbidity and mortality, which gives rise to multimillion-dollar annual losses for all facets of the world's poultry industry. Plasmid-mediated antibiotic resistance genes are an important mechanism of resistance in *E. coli.* The resistance genes are not only vertical to offspring but also horizontally transmitted between different microbes, potentially affecting human health and causing economic loss in the breeding industry (Collignon and Angulo, 2006; James et al., 2007; Ben et al., 2010; Lu et al., 2010). In recent years, many reports have been published on the plasmid-mediated β-lactams, aminoglycosides, chloramphenicol, sulfonamides, quinolones and tetracyclines in *E. coli*, but few reports have been published regarding chicken sources of *E. coli* resistance and resistance genes. Therefore, study of *E. coli* resistance and resistance genes in chickens is of great significance to public health (Collignon and Angulo, 2006).

In previous studies, it was found that quinolones and the first-generation of cephalosporins resistant strains occurred in the 1990s and drug-resistant strains to the third-generation of cephalosporins were found in 2003 (Li et al., 2010). The detection prevalence of resistance gene (*blaCTX-M*) was up to 75% (Li et al., 2010). Furthermore, previous study also suggested that the majority of *E. coli* strains from swine in China were resistant to streptomycin, chloramphenicol, norfloxacin and doxycycline, showing that the detection prevalence of the genes for *cmlA* and *floR* were 65 and 57%, respectively (Wang et al., 2011). The chicken *E. coli* strains in South Australian were found to be resistant to tetracycline, ampicillin, cotrimoxazole, streptomycin and neomycin, having the detection prevalence of the genes for *tetA* (19.1%) and *blaTEM* (17.1%) (Obeng et al., 2012). Karah et al. (2010) studied the plasmid-mediated quinolone resistance gene in *E. coli* strains isolated from human in Norway and Sweden. Among isolates that were ESBL producers and were resistant to nalidixic acid and/or had reduced susceptibility to ciprofloxacin, the detection prevalences of the genes for *qnr* and *aac (6')-ib-cr* genes were 9.1 and 52.3%, respectively (Karah et al., 2010).

The aim of this study was to investigate the occurrence of antimicrobial resistance of *E. coli* strains and the correlation between plasmid-mediated resistance genes and antimicrobial resistance of *E. coli* strains isolated from chickens in Shaanxi, Henan, and Gansu provinces in China during 2007-2012.

MATERIALS AND METHODS

Source of strains

One thousand and two *E. coli* strains were isolated from liver samples of sick and dead layer and broiler flocks (Hy-Line Variety Brown) from Xi'an, Tongchuan City, Yangling Demonstration Zone in Shaanxi province; Sanmenxia and Luoyang Cities in Henan province; Tianshui, Dingxi Cities in Gansu province in China during 2007-2012. Samples were collected with visible enlargement of the liver, pericarditis, bladder inflammation and peritonitis during necropsy. The isolation information and *E. coli* source and distribution in three provinces are shown in Table 1. *E. coli* standard strain ATCC25922 was kindly provided by preventive veterinary medicine laboratory of Northwest Agriculture and Forestry University, China.

Medium, susceptibility paper and reagents

MacConkey agar and nutrient agar were purchased from Beijing Aobo Star Biotechnology Co. Bacteria trace biochemical reaction tubes were purchased from Hangzhou Tianhe Microorganism Reagent Co. Eighteen kinds of antibiotics susceptibility papers, including ampicillin (AMP), amoxicillin (AMX), ceftazidime (CAZ), cefotaxime thiophene (CEF), new neomycin (NEO), streptomycin (STR), gentamicin (GEN), kanamycin (KAN), tobramycin (TOB), amikacin (AMK), florfenicol (FFC), tetracycline (TET), doxycycline (DOX), trimethoprim sulfamethoxazole (SXT), nalidixic acid (NAL), norfloxacin (NOR), ciprofloxacin (CIP) and ofloxacin (OFZ) were purchased from Hangzhou Tianhe Microorganism Reagent Co. PCR Master Mix (containing Taq DNA polymerase, dNTP, PCR buffer) and DNA Marker DL2000 were purchased from TaKaRa Biotechnology (Dalian) Co. Plastic recycling kit was purchased from TianGen bio-technology(Beijing) Co.

Table 2. Primers used for PCR amplification of resistance genes in chicken *E. coli*.

Detected gene	Description	Fragment sizes (bp)	Primer sequences	Accession number	Position
blaSHV F[a]	β-lactams	450	CGCGAGCGGCTCATACAGG	GU732836	350-367
blaSHV R[b]			TCGTCGGGCAGCGTTTCT		778-799
blaCTX-M F	β-lactams	301	ACACGTCAACGGCACAATG	AB545872	323-341
blaCTX-M R			GAGCCACGTCACCAACTGC		605-623
blaCMY-2 F	β-lactams	470	GGGAGCTTGCCACCTACAGC	AF373218	392-411
blaCMY-2 R			CCCGCCTACCGAGTAATGC		843-861
blaTEM F	β-lactams	293	CGGTATTATCCCGTGTTG	GU550123	374-391
blaTEM R			GTCGTTTGGTATGGCTTC		649-666
aac(3)-IV F	Aminoglycosides	357	GCCGTGGTTGGCTTGTAT	EU784153	3169-3186
aac(3)-IV R			CGTTCTCGAAATCAGCTCTTG		3505-3525
aac(3)-II F	Aminoglycosides	412	GGCGACTTCACCGTTTCT	FQ482074	344-361
aac(3)-II R			GGACCGATCACCCTACGAG		737-755
ant(3')-I F	Aminoglycosides	400	GACATTGATCTGGCTATCTTGCTG	JN108887	382-405
ant(3')-I R			CTACCTTGGTGATCTCGCCTTTC		759-781
aph(3)-II F	Aminoglycosides	325	TTGCTCGGAAGAGTATGAA	JN609224	193-211
aph(3)-II R			GCCACTTACTTTGCCATCT		499-517
sul-I F	Sulfonamides	925	TCGGACAGGGCGTCTAAG	EU598449	1801-1818
sul-I R			GGGTATCGGAGCGTTTGC		2708-2725
sul-II F	Sulfonamides	792	CTTGCGGTTTCTTTCAGC	JX869967	11-28
sul-II R			CATCATTTTCGGCATCGT		785-802
cmlA F	Chloramphenicols	467	GGGTGGCGGGCTATCTTT	HM175865	2057-2074
cmlA R			GCGACACCAATACCCACTAG		2504-2523
floR F	Chloramphenicols	601	GAACACGACGCCCGCTAT	AY775258	665-682
floR R			TTCCGCTTGGCCTATGAG		1248-1265
cat-I F	Chloramphenicols	307	GTCAGTTGCTCAATCTACCTAT	AB670687	138-159
cat-I R			ACCGTAAGACGCCACATC		427-444
qnrA F	Quinolones	633	ATTGATAAAGTTTTTCAGCAAGAGG	EU195836	10-34
qnrA R			TATTACTCCCAAGGGTTCCAGC		621-642
qnrB F	Quinolones	427	CTATGATCGTGAAAGCCAGAAAGG	EU093091	171-194
qnrB R			CCGAATATCTAAGTCACCCAACTCC		573-597
qnrS F	Quinolones	300	ATCGAAGGCTGCCACTTT	EF571010	40-57
qnrS R			TGATGCACCCGCTAGGTT		322-339
aac(6')-ib-cr F	Quinolones	679	TGACCTTGCGATGCTCTAT	HM175873	76-94
aac(6')-ib-cr R			GGCTTACTTGTCTGCGTTCTT		734-754
tetA F	Tetracyclines	344	TTGGCATTCTGCATTCACTCG	FJ794040	117-137
tetA R			CCACCCGTTCCACGTTGTT		442-460
tetB F	Tetracyclines	388	TTCACCGCATAGTCCCTT	FJ917423	237-254
tetB R			TGCAATAAATCCGAGCAG		607-624
tetC F	Tetracyclines	427	TCACTATGGCGTGCTGCTA	JQ966989	15-33
tetC R			GCTGTCCCTGATGGTCGT		875-892

[a] Forward; [b]Reverse.

Antimicrobial susceptibility test

The *E. coli* strains were tested for susceptibility to 18 antimicrobial drugs by Kirby-Bauer disk diffusion method on ordinary agar plates. The standard procedure of the clinical and laboratory standards institute guidelines were strictly followed throughout the testing procedure and the determination of results (CLSI, 2008a, b). The criteria for a drug to be classified as resistant or sensitive were judged as described previously (CLSI, 2008a, b).

Primers for amplification of resistance genes in chicken *E. coli* strains

The twenty sets of primer pairs (Table 2) used for polymerase chain reaction (PCR) amplification of β-lactams, aminoglycosides,

Table 3. Antimicrobial resistance of *E. coli* isolated from chickens of Shaanxi province during 2007-2012.

Antimicrobial	Percentage of resistance % (number of resistant strains)						
	2007 (n=50)	2008 (n=60)	2009 (n=48)	2010 (n=58)	2011 (n=88)	2012 (n=137)	2007-2012 (n=441)
AMP	100.0(50)	100.0(60)	100.0(48)	100.0(58)	100.0(88)	100.0(137)	100.0(441)
AMX	26.0(13)	28.3(17)	12.5(6)	29.3(17)	0.0	6.6(9)	14.1(62)
CAZ	50.0(25)	71.7(43)	87.5(42)	89.7(52)	77.3(68)	80.3(110)	77.1(340)
CEF	26.0(13)	0.0	12.5(6)	10.3(6)	9.1(8)	13.1(18)	11.6(51)
NEO	50.0(25)	28.3(17)	25.0(12)	20.7(12)	0.0	0.0	15.0(66)
STR	88.0(44)	71.7(43)	75.0(36)	39.7(23)	38.6(34)	33.6(46)	51.2(226)
GEN	76.0(38)	56.7(34)	50.0(24)	50.0(29)	46.6(41)	53.3(73)	54.2(239)
KAN	20.0(10)	15.0(9)	12.5(6)	17.2(10)	35.2(31)	37.2(51)	26.5(117)
TOB	12.0(6)	28.3(17)	12.5(6)	10.3(6)	15.9(14)	19.7(27)	17.2(76)
AMK	12.0(6)	28.3(17)	0.0	10.3(6)	9.1(8)	0.0	8.4(37)
FFC	26.0(13)	28.3(17)	25.0(12)	29.3(17)	38.6(34)	27.0(37)	29.5(130)
TET	70.0(35)	83.3(50)	100.0(48)	100.0(58)	100.0(88)	100.0(137)	94.3(416)
DOX	100.0(50)	100.0(60)	100.0(48)	89.7(52)	92.0(81)	93.4(128)	95.0(419)
SXT	26.0(13)	28.3(17)	12.5(6)	39.7(23)	30.7(27)	27.0(37)	27.9(123)
NAL	62.0(31)	85.0(51)	87.5(42)	89.7(52)	100.0(88)	100.0(137)	90.9(401)
NOR	76.0(38)	85.0(51)	50.0(24)	60.3(35)	77.3(68)	73.0(100)	71.7(316)
CIP	50.0(25)	60.0(36)	62.5(30)	69.0(40)	92.0(81)	93.4(128)	77.1(340)
OFZ	26.0(13)	43.3(26)	50.0(24)	31.0(18)	46.6(41)	59.9(82)	46.3(204)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

chloramphenicol, sulfonamides, quinolones and tetracycline resistant genes, respectively were designed with Primer5.0 software based on the sequences deposited in GenBank.

All *E. coli* strains and reference strains were grown on Luria-Bertani (LB) agar plates at 37°C overnight. *E. coli* colonies were suspended in 500 µL of deionized water and boiled for 10 min, followed by chilling on ice for 5 min and centrifugation at 10,000 xg for 5 min, the supernatant was used as the DNA templates for PCR amplification. The PCR mixture contained 10 µL of 2X PCR Master mix (including 2X Taq DNA polymerase, 2X PCR Buffer and 2X dNTP mixture) (TaKaRa), 1 µL of primer pairs, 4 µL of DNA template, and deionized water to a final volume of 25 µL. PCR was completed by an initial heat activation of 5 min at 95°C and then 30 cycles of 30 s at 94°C, 30 s at annealing temperatures and 45 s at 72°C; and an extension of 10 min at 72°C. PCR products were analyzed by 1% agarose gel electrophoresis and visualized after staining with ethidium bromide on a UV transilluminator.

Sequence analysis of PCR products

Resistance gene sequences were aligned and compared with related sequences in GenBank by DNAStar program. Longitudinal data on resistance and resistance genes in *E. coli* in the same farms at Shaanxi province, China was analyzed. The six farms of Tongchuan which raised about one million of layer at Shaanxi province in China were selected for study and the changes of resistance and resistance gene in *E. coli* strains were examined during 2007-2012.

Statistical analysis

Student's *t*-test was used to measure the correlations between resistance and resistance gene and to compare resistance prevalence between years. In all tests, $p<0.05$ was considered statistically significant.

RESULTS

Susceptibility test of *E. coli* strains

The resistance information of chicken *E. coli* strains in three provinces in China to 18 common clinically used antibiotics is shown in Tables 3, 4 and 5. As shown in Table 3, the resistance prevalence of chicken *E. coli* strains to ampicillin in Shaanxi province was 100% during 2007-2012. Resistance prevalence to tetracycline and doxycycline was 70-100%. The increase in chicken *E. coli* strains resistance to nalidixic acid and ciprofloxacin was seen. The resistance rate to nalidixic acid has increased to 100% but a downward trend for neomycin and streptomycin was found. In 2007-2010, the resistance prevalence of chicken *E. coli* strains to ceftazidime increasingly was observed, but there was a slight decline in 2011-2012. A downward trend in resistance to gentamicin in

Table 4. Antimicrobial resistance of *E. coli* strains isolated from chickens of Henan province during 2007-2012.

Antmicrobial	Percentage of resistance (%) (no. of resistant isolates)						
	2007 (n=40)	2008 (n=42)	2009 (n=34)	2010 (n=45)	2011 (n=45)	2012 (n=66)	2007-2012 (n=272)
AMP	100.0(40)	100.0(42)	100.0(34)	100.0(45)	100.0(45)	100.0(66)	100.0(272)
AMX	17.5(7)	40.5(17)	41.2(14)	33.3(15)	13.3(6)	15.2(10)	25.4(69)
CAZ	32.5(13)	59.5(25)	67.6(23)	44.4(20)	51.1(23)	39.4(26)	47.8(130)
CEF	0.0	11.9(5)	0.0	6.7(3)	13.3(6)	0.0	5.1(14)
NEO	32.5(13)	23.8(10)	20.6(7)	20.0(9)	13.3(6)	0.0	16.5(45)
STR	50.0(20)	19.0(8)	41.2(14)	60.0(27)	51.1(23)	60.6(40)	48.5(132)
GEN	50.0(20)	42.9(18)	41.2(14)	40.0(18)	37.8(17)	30.3(20)	39.3(107)
KAN	7.5(3)	14.3(6)	17.6(6)	20.0(9)	15.6(7)	27.3(18)	18.0(49)
TOB	0.0	0.0	0.0	8.9(4)	13.3(6)	30.3(20)	11.0(30)
AMK	17.5(7)	0.0	0.0	0.0	11.1(5)	0.0	4.4(12)
FFC	17.5(7)	19.0(8)	20.6(7)	20.0(9)	37.8(17)	39.4(26)	27.2(74)
TET	67.5(27)	81.0(34)	100.0(34)	100.0(45)	75.6(34)	80.3(53)	83.5(227)
DOX	100.0(40)	100.0(42)	100.0(34)	100.0(45)	100.0(45)	100.0(66)	100.0(272)
SXT	0.0	19.0(8)	20.6(7)	40.0(18)	13.3(6)	19.7(13)	19.1(52)
NAL	67.5(27)	81.0(34)	100.0(34)	100.0(45)	100.0(45)	100.0(66)	92.3(251)
NOR	50.0(20)	19.0(8)	41.2(14)	80.0(36)	62.2(28)	80.3(53)	58.5(159)
CIP	32.5(13)	40.5(17)	58.8(20)	80.0(36)	86.7(39)	89.4(59)	67.6(184)
OFZ	32.5(13)	40.5(17)	20.6(7)	40.0(18)	24.4(11)	39.4(26)	33.8(92)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

Table 5. Antimicrobial resistance of *E. coli* strains isolated from chickens in Gansu province during 2007-2012.

Antimicrobials	Percentage of resistance (%) (no. of resistant isolates)						
	2007 (n=44)	2008 (n=46)	2009 (n=33)	2010 (n=42)	2011 (n=50)	2012 (n=74)	2007-2012 (n=289)
AMP	100.0(44)	100.0(46)	100.0(33)	100.0(42)	100.0(50)	100.0(74)	100.0(289)
AMX	20.5(9)	26.1(12)	0.0	28.6(12)	0.0	0.0	11.4(33)
CAZ	59.1(26)	76.1(35)	81.8(27)	100.0(42)	72.0(36)	66.2(49)	74.4(215)
CEF	20.5(9)	26.1(12)	0.0	21.4(9)	0.0	21.6(16)	15.9(46)
NEO	40.9(18)	0.0	18.2(6)	28.6(12)	0.0	0.0	12.5(36)
STR	59.1(26)	50.0(23)	33.3(11)	42.9(18)	42.0(21)	55.4(41)	48.4(140)
GEN	79.5(35)	76.1(35)	81.8(27)	71.4(30)	58.0(29)	55.4(41)	68.2(197)
KAN	40.9(18)	50.0(23)	33.3(11)	45.2(19)	66.0(33)	82.4(61)	57.1(165)
TOB	20.5(9)	26.1(12)	33.3(11)	42.9(18)	42.0(21)	55.4(41)	38.8(112)
AMK	0.0	10.9(5)	0.0	0.0	10.0(5)	9.5(7)	5.9(17)
FFC	0.0	8.7(4)	33.3(11)	28.6(12)	42.0(21)	21.6(16)	22.1(64)
TET	68.2(30)	89.1(41)	97.0(32)	100.0(42)	100.0(50)	100.0(74)	93.1(269)
DOX	100.0(44)	100.0(46)	100.0(33)	100.0(42)	100.0(50)	100.0(74)	100.0(289)
SXT	0.0	26.1(12)	0.0	14.3(6)	28.0(14)	21.6(16)	16.6(48)
NAL	86.4(38)	93.5(43)	100.0(33)	92.9(39)	100.0(50)	100.0(74)	95.8(277)
NOR	59.1(26)	76.1(35)	66.7(22)	57.1(24)	72.0(36)	66.2(49)	66.4(192)
CIP	40.9(18)	76.1(35)	81.8(27)	85.7(36)	82.0(41)	82.4(61)	75.4(218)
OFZ	40.9(18)	50.0(23)	33.3(11)	42.9(18)	42.0(21)	44.6(33)	42.9(124)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

2007-2011 was seen, but there was a slight increase in 2012.

Table 4 shows that the resistance prevalence of chicken *E. coli* strains isolated from Henan province to ampicillin and doxycycline were 100% during 2007-2012 while tetracycline resistance prevalence was 60-100%. The resistance of chicken *E. coli* strains to tobramycin, florfenicol, nalidixic acid and cefaloxime showed an upward trend, and resistance rate of nalidixic acid has been up to 100% while resistance to neomycin and gentamicin showed a declining trend.

As seen in Table 5, the resistance prevalence of chicken *E. coli* strains isolated in Gansu province to ampicillin and doxycycline has remained at 100% from 2007-2012 while the resistance prevalence of tetracycline and nalidixic acid were 60-100%. The resistance prevalence to tobramycin showed an upward trend while gentamicin resistance prevalence showed a downward trend. Resistance rates to ceftazidime and ciprofloxacin increased in 2007-2010, but there was a slight decline in 2011-2012.

Overall, resistance prevalence of chicken *E. coli* strains to ampicillin in Shaanxi, Henan and Gansu provinces in China has been maintained at 100%. Doxycycline and tetracycline resistance prevalence were more than 80% and 60%, respectively. An upward trend to nalidixic acid and ciprofloxacin was seen while gentamicin resistance prevalence showed a downward trend. Resistance to kanamycin, tobramycin and trimethoprim sulfamethoxazole showed significant differences ($P<0.05$) and the rest of antibiotic resistance showed no significant difference ($P>0.05$).

Isolates showed multi-drug resistance (resistant to more than three kinds of antibiotics), and more than 3 were up to 100% of the drug-resistant strains, in which 8 resistant strains had the highest count for 19.2% (192/1002); isolates resistant to more than 8 antibiotics were up to 49.8% (499/1002), of which 14 chicken *E. coli* isolates resistant to 18 antibiotics, accounted for 1.4% (14/1002).

PCR detection of resistant genes in *E. coli* isolates

Among 1002 chicken *E. coli* strains, 8 of 20 resistance genes were detected by PCR. The electrophoretic patterns of these 8 resistance genes were indicated in Figure 1. The detection rates of the genes for *tetA*, *tetB*, *blaTEM*, *aac(3)-II*, *sul-I*, *cmlA*, *floR* and *qnrB* in three provinces in China during 2007-2012 were shown in Table 6. In 2007-2012, the *tetB* and *tetA* genes were detected with the highest prevalence in 86.6%-100% in chicken *E. coli* strains isolated from Shaanxi, Henan and Gansu provinces in China while the detection rates of the genes for *floR*, *sul-I*, *cmlA* and *blaTEM* increased gradually. It is interesting to note that a downward trend of detection prevalence of *aac (3)-II* gene and low detection rate of *qnrB* gene (0.4%) were seen (Table 6).

A continuous monitoring of *E. coli* resistance and resistance genes in the Tongchuan chicken farm

In 2007-2012, a continuous monitoring of *E. coli* resistance and resistance genes in the Tongchuan chicken farms was performed. The results are shown in Tables 7 and 8. Table 7 shows that *E. coli* strains had resistance to kanamycin since 2008 while that *E. coli* strains were resistant to florfenicol and ciprofloxacinb since 2009. *E. coli* strains were resistant to ceftazidime and norfloxacin since 2010 but were sensitive to gentamicin and neomycin. The number of antibiotics of *E. coli* resistance increased from 7 to 13 between 2007 and 2012. Table 8 indicates that, in 2007-2012, detection prevalence of the genes for *blaTEM*, *tetA,* and *tetB* in *E. coli* strains in this farm was more than 80%. The detection prevalence of the genes for *sul-I* and *cmlA* increased while the detection prevalence of the gene for *aac(3)-II* showed a declining trend.

The correlation between resistance and resistance genes of the *E. coli* isolates

Resistance of 1002 chicken *E. coli* strains to different antibiotics and the related resistance genes are shown in Table 9. The ampicillin, streptomycin, trimethoprim sulfamethoxazole, florfenicol, ciprofloxacin and doxycycline resistance genes in 1002 chicken *E. coli* strains are shown in Table 9. Among the 1002 *E. coli* strains detected, detection prevalence of the genes for *tetA*, *tetB*, *blaTEM*, *aac(3)-II*, *sul-I*, *cmlA*, *floR*, and *qnrB* were 97.4, 99.6, 88.9, 85.3, 41.7, 40.3, 26.5 and 0.5%, respectively.

The detection rates of the genes for *tetA*, *tetB*, *blaTEM* and *aac(3)-II* were positively correlated with the doxycycline, ampicillin and streptomycin-resistant *coli* strains, respectively ($P < 0.01$). Only 41.7% of the 223 trimethoprim sulfamethoxazole-resistant chicken *E. coli* strains carried the *sul-I* gene, showing no significant difference with the strains resistance to trimethoprim sulfamethoxazole ($P > 0.05$). In the 268 florfenicol-resistant chicken *E. coli* strains, the detection prevalence of *cmlA* and *floR* were 40.3 and 26.5%, respectively, showing no significant difference with the strains resistance to florfenicol ($P > 0.05$). Only 0.4% of the 742 ciprofloxacin-resistant strains carried the *qnrB* gene, showing no correlation with strains resistance to ciprofloxacin.

Sequence analysis of resistance genes in chicken *E. coli* strains

Among 1002 chicken *E. coli* strains tested, eight kinds of resistance genes in *E. coli* strains were detected by PCR (Figure 1). The sequences of these resistance genes have been sequenced and deposited with GenBank accession numbers JQ362472 (cmlA), JQ362473 (floR),

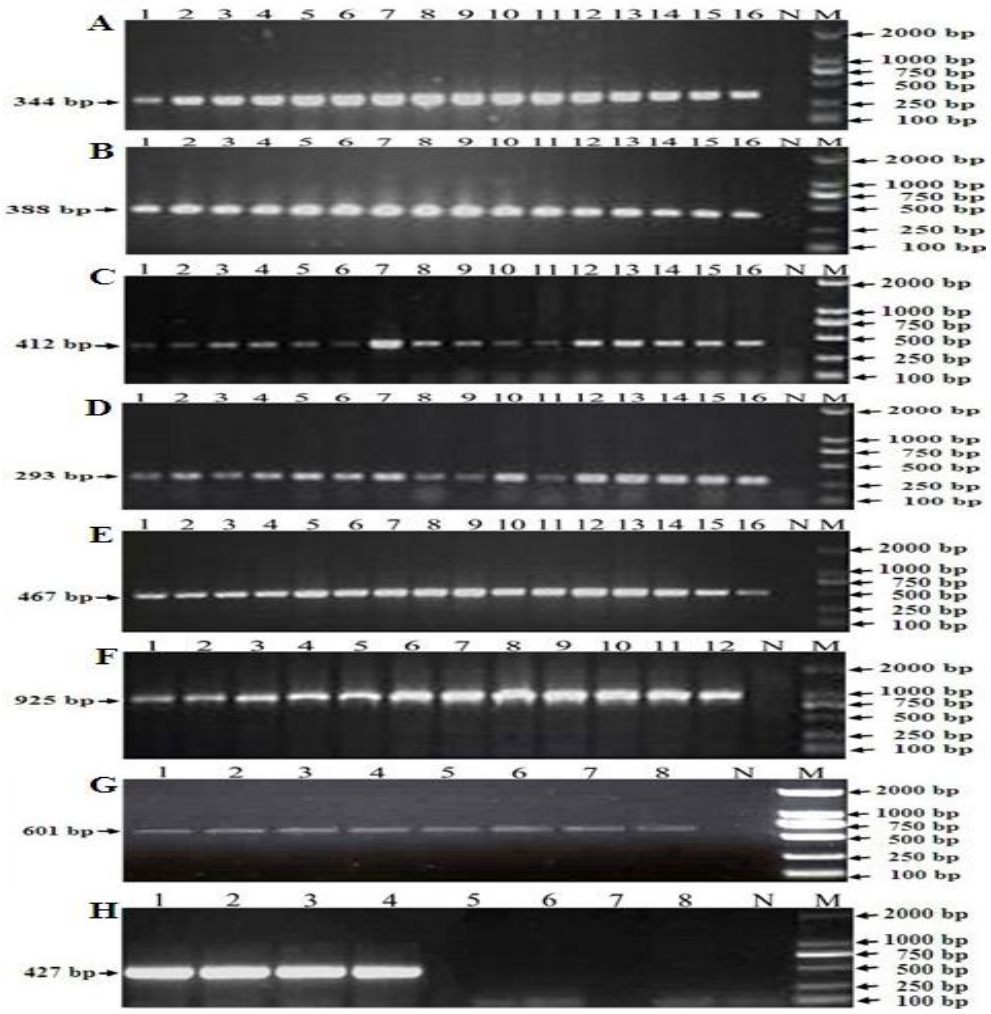

Figure 1. PCR amplification of 8 resistance genes in chicken *E. coli* strains. Panel A: lanes 1-16, *E. coli* strains carried *tetA*; Panel B: lanes 1-16, *E. coli* strains carrying *tetB*; Panel C: lanes 1-16, *E. coli* strains carrying *aac(3)-II*; Panel D: lanes 1-16, *E. coli* strains carrying *blaTEM*; Panel E: lanes 1-16, *E. coli* strains carrying *cmlA*; Panel F: lanes 1-12, *E. coli* strains carrying *sul-I*; Panel G: lanes 1-8: *E. coli* strains carrying *floR*; Panel H: lanes 1-8, *E. coli* strains carrying *qnrB*. Lanes M: DL 2000 marker and lanes N: a negative control.

Table 6. Detection of 8 resistance genes in 1002 chicken *E. coli* strains during 2007-2012.

Resistance gene	Detection rates of resistance genes (%) (number of isolates carrying resistance genes)						
	2007 (n=134)	2008 (n=148)	2009 (n=115)	2010 (n=145)	2011 (n=183)	2012 (n=277)	2007-2012 (n=1002)
qnrB	0.0	0.7(1)	0.9(1)	0.7(1)	0.0	0.4(1)	0.4(4)
floR	0.0	6.1(9)	7.0(8)	6.9(10)	8.2(15)	10.5(29)	7.1(71)
sul-I	0.0	2.0(3)	5.2(6)	7.6(11)	13.1(24)	17.7(49)	9.3(93)
cmlA	3.7(5)	5.4(8)	9.6(11)	11.7(17)	13.7(25)	15.5(43)	10.9(109)
aac(3)-II	53.0(71)	50.0(74)	51.3(59)	47.6(69)	36.1(66)	31.8(88)	42.6(427)
blaTEM	64.2(86)	78.4(116)	85.2(98)	94.5(137)	97.8(179)	99.3(275)	88.9(891)
tetA	86.6(116)	89.2(132)	93.0(107)	96.6(140)	100.0(183)	100.0(277)	95.3(955)
tetB	91.0(122)	93.2(138)	96.5(111)	100.0(145)	100.0(183)	100.0(277)	97.4(976)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

Table 7. Antimicrobial resistance of chicken *E. coli* strains isolated from the Tongchuan farms at Shaanxi province during 2007-2012.

Year	Number of isolates	Sensitive drugs	Resistant drugs
2007	20	AMX, CAZ, CEF, KAN, TOB, FFC,AMK, SXT, CIP, OFZ, NOR	AMP, NEO, STR, GEN,TET, DOX, NAL
2008	22	AMX ,CAZ, CEF, AMK, TOB, FFC, SXT, NOR, OFZ, CIP	AMP, NEO, STR, GEN, KAN, TET, DOX, NAL
2009	20	AMX, CAZ, CEF, TOB, AMK, SXT , NOR, OFZ	AMP, NEO, STR, GEN, KAN, FFC, TET, DOX, NAL, CIP
2010	20	CEF, AMX, NEO, GEN, TOB, AMK, SXT , OFZ	AMP, CAZ, STR, KAN, FFC, TET, DOX, NAL, CIP, NOR
2011	42	AMX, CEF, NEO, GEN, TOB, AMK, OFZ	AMP, CAZ, STR, KAN, FFC, TET, DOX, SXT , NAL, NOR, CIP
2012	63	AMX, NEO, STR, GEN, AMK	AMP, CAZ, CEF, KAN, TOB, FFC, TET,DOX, SXT , NAL, NOR, CIP, OFZ

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

Table 8. Resistance genes in chicken *E. coli* strains isolated from the Tongchuan farms at Shaanxi province during 2007-2012.

Year	Number of isolates	Detection rates of resistance genes (%) (number of isolates carrying resistance genes /no. of total isolates)							
		qnrB	***floR***	***sul-I***	***cmlA***	***aac(3)-II***	***blaTEM***	***tetA***	***tetB***
2007	20	0	0	0	0	75.0(15)	85.0(17)	85.0(17)	90.0 (18)
2008	22	0	4.5(1)	0	0	72.7(16)	81.8(18)	86.4(19)	95.5 (21)
2009	20	0	0	5.0(1)	10.0(2)	65.0(13)	90.0(18)	90.0(18)	100.0(20)
2010	20	5.0(1)	5.0(1)	15.0(3)	15.0(3)	55.0(11)	95.0(19)	95.0(19)	100.0(20)
2011	42	0	14.3(6)	19.0(8)	21.4(9)	31.0(13)	100.0(42)	100.0(42)	100.0(42)
2012	63	0	12.7(8)	25.4(16)	25.4(16)	28.6(18)	100.0(63)	100.0(63)	100.0(63)

Table 9. Resistance and related resistance genes in 1002 chicken *E. coli* strains to different antibiotics.

Types of antimicrobials	Number of resistant isolates	Resistance genes	Number of isolates carrying resistance genes (detection prevalence)
Ampicillin	1002	blaTEM	891 (88.9%)
Streptomycin	498	aac(3)-II	425 (85.3%)
Trimethoprim sulfamethoxazole	223	sul-I	93 (41.7%)
Florfenicol	268	cmlA	108 (40.3%)
		floR	71 (26.5%)
Ciprofloxacin	742	qnrB	4 (0.5%)
Doxycycline	980	tetA	955 (97.4%)
		tetB	976 (99.6%)

JQ362474 (qnrB), JQ362475 (sul-I), Q362476 (blaTEM), JQ362477 (tetA), JQ362478 (tetB) and JQ362479 (aac(3)-II). The sequences homology of *cmlA*, *floR*, *qnrB*, *sul-I*, *blaTEM*, *tetA*, *tetB* and *aac (3)-II* sequences with the previously published sequences of *cmlA* (HM175865), *floR* (AY775258), *qnrB* (EU093091), *sul-I* (EU598449), *blaTEM* (GU550123), *tetA* (FJ794040), *tetB* (FJ917423) and *aac (3)-II* (FQ482074) are 99.30, 98.92, 97.14, 99.89,

100, 99.66, 98.84 and 97.29%, respectively.

DISCUSSION

Antibiotics are widely used in the treatment and prevention of disease and can also promote the growth of animals. Under the pressure of antibiotic selectivity, drug-resistant bacteria appear. To date, there are many reports regarding *E. coli* resistance in many countries and regions (Harada et al., 2012; Holzel et al., 2012; Johns et al., 2012; Ryu et al., 2012a,b). The *E. coli* resistance has become a global problem (Alan et al., 2007; Szmolka and Nagy, 2013). To date, the comprehensive studies on chicken *E. coli* resistance genes are relatively few. Soufi et al. (2011) studied resistance of 166 chicken *E. coli* strains in Tunisia and found that resistant rates of different strains to ampicillin, streptomycin, nalidixic acid, sulfonamide and tetracycline are 66-95%. To date, *E. coli* resistance problem is very serious in China. Dai et al. (2008) found that the resistant rates of chicken *E. coli* strains in China between 2001 and 2006 to ampicillin and doxycycline is more than 70%. The resistance of chicken *E. coli* strains to ampicillin and doxycycline are very serious. Our results revealed that resistant rates of chicken *E. coli* strains isolated from three provinces in China between 2007-2012 to ampicillin and doxycycline was 100 and 80%, respectively while the resistant prevalence of amikacin was below 30%. In the present study, we also found that quinolone resistance among *E. coli* from chicken in China is rising, which is consistent with a previous study (Zhang et al., 2010).

The occurrence of antibiotic resistance of chicken *E. coli* strains isolated from three provinces in China during 2007-2012 is different, and this may be related to the use of different antibiotics in the farms in different provinces. In-feed or therapeutic antibiotics were used in these farms for all major classes of antibiotics except vancomycins. Ampicillin, tetracycline, doxycycline and nalidixic acid and ciprofloxacin were usually added into animal feed or drinking water in each sampling farm of these provinces, and the resistance of chicken *E. coli* strains isolated to the above antibiotics increased gradually. For example, a chicken farm in Gansu province in China used kanamycin to prevent and treat layer yolk peritonitis caused by *E. coli* for four years, the resistance rate of *E. coli* strains to kanamycin during 2009-2012 increased from 11.0 to 65.0%.

The resistance mechanism of *E. coli* is complicated. The resistance genes mediated by plasmid can make the resistance spread among different bacteria, which make bacteria obtain resistance genes more easily and thus produce multiple resistances (Li, 2005; Roberts, 2005; Zhang et al., 2009; Liu et al., 2012; Mosquito et al., 2012). This mechanism is that resistance genes can directly code enzymes which result in damage antibiotic effect (Skold, 2000; Yoo et al., 2003; Li et al., 2007; Ramirez and Tolmasky, 2010). Yu et al. (2009) found that the aminoglycoside resistance gene in human *E. coli* strain is main *aac (3)-II*. Previous studies on β-lactamase genes in the French *E. coli* strains indicated that the detection prevalence of the genes for *blaTEM and blaCTX-M* among 8 ceftiofur-resistant strains were 62.5 and 100%, respectively (Meunier et al., 2006). The results are similar to a previous study suggesting that *blaTEM* is the main β-lactamase resistance gene in the human *E. coli* (Yang et al., 2011). Tang et al. (2011) detected the *E. coli* drug-resistant gene in pigs in China during 2004-2007 and found that the β-lactamase resistance gene is mainly *blaTEM* and the detection rate is 87%. They also found that the resistance genes of aminoglycoside, tetracycline, and Sulfa are mainly *aphA*, *tet*B and *sul-II*, respectively. The detection prevalence for these resistance genes were 82.6, 49.8 and 55.4%, respectively (Tang et al., 2011). The detection rates for *E. coli* drug-resistant genes show differences which may be due to the strains from various countries and regions and the difference of serum type or antibiotic usage mode. During 2007-2012, we conducted the detection of resistance and resistance genes at chicken farms in Tongchuan City of Shaanxi province in China for 6 years, and found that the numbers of antibiotic resistance increased from 7 to 13. The detection prevalence of resistance genes for *sul-I* and *cmlA* increased gradually. The resistance genes of *tetB*, *tetA*, *blaTEM* and *aac(3)-II* in *E. coli* strains are positively correlated with the resistance of bacterial strain ($P < 0.01$).

In addition, only few quinolone resistance genes were detected from quinolone resistant strains in this study. Whether its resistance is associated with other types of resistance genes or other mechanisms of resistance remains to be further elucidated. Resistance genes were detected from several aminoglycosides and florfenicol-sensitive strains, indicating the resistance genes in a silent state under the pressure of antibiotic. These strains are likely to develop into drug-resistant strains. Therefore, the detection of resistance and associated resistance genes in animal source of pathogenic isolates will be of great significance to the rational use of antibiotics in clinical and public health.

In summary, our results revealed that 1002 chicken *E. coli* strains isolated in three provinces in China during 2007-2012 showed multiple drug resistance. Of all isolated strains, 499 of 1002 *E. coli* strains (69%) were resistant to more than eight kinds of antibiotics, of which resistance gene *tetB*, *tetA*, *blaTEM* and *aac(3)-II* showed a positive correlation ($P < 0.01$) with the *E. coli* strains resistance to antibiotics. The current results provide useful information on the drug prevention of chicken colibacillosis in China and resistance mechanisms of *E. coli.*

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