Detection of resistance phenotype and genotype of avian *Escherichia coli* in Hebei Province

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ABSTRACT We investigated the resistance of avian *Escherichia coli* to commonly used clinical antibiotics in Hebei Province. It is of significance to reveal the extent and mechanism of drug resistance, as well as to prevent and control colibacillosis. We investigated the resistance of 132 *E. coli* isolates to 5 kinds of antibiotics (including β-lactams, aminoglycosides, tetracyclines, sulfonamides, and chloramphenicol) using the Kirby-Bauer drug susceptibility test, and resistance genes were detected by PCR. The results showed that the *E. coli* had a higher resistance rate to ampicillin, cephalotin, gentamicin, streptomycin, tetracycline, doxycycline, sulfamethoxazole trimethoprim, sulfamonomethoxine, and florfenicol, but it had higher sensitive rate to cefepime, imipenem, spectinomycin, and minocycline. Of the resistance genes, the *TEM*, *aac*(3)-IIa, *tet*A, *Sul1*, *Sul2*, and *forl* had higher positive rates. Therefore, drug resistance of avian *E. coli* in Hebei Province is very serious. The resistance mechanisms may include structure changes of the target enzyme mediated by multiresistance genes, and resulting in reduced affinity and increased efflux of antibiotics.

Key words: avian *Escherichia coli*, drug resistance, resistance gene, polymerase chain reaction

2013 Poultry Science 92:2326–2332
http://dx.doi.org/10.3382/ps.2013-03180

INTRODUCTION

Hebei Province, an area of about 190,000 km^2 with more than 100 million chickens, is a large poultry breeding province in China. With the expansion of intensive poultry breeding, the prevalence of infectious diseases has become increasingly serious. Colibacillosis is one of the most common infectious diseases. Chicken colibacillosis is a local or systemic disease caused by pathogenic *Escherichia coli*. The most common symptoms of colibacillosis are septicemia, airsacculitis, perihepatitis, pericarditis, vitelline peritonitis, and salpingitis (Diao and Zhang, 2000). Chickens of various breeds and at different ages can be infected by pathogenic *E. coli*. After suffering colibacillosis, chickens have increased susceptibility to *Salmonella*, Newcastle disease virus, infectious bursal disease virus, and other pathogenic microorganisms, which leads to severe economic losses to the poultry industry (Liu et al., 2010). Although improved feeding conditions and the use of vaccines can reduce the occurrence of colibacillosis, the main preventive measure for colibacillosis is the use of antibacterial drugs. The widespread application of antibacterial drugs, especially the abuse of antibiotics, has led to widespread resistance of *E. coli*. The β-lactams, aminoglycosides, tetracyclines, sulfonamides, and chloramphenicol antibiotics have advantages such as broad antimicrobial spectrum and low price. They are widely used for the prevention and control of livestock and poultry bacterial diseases. As a result of selection pressure on the antibiotics, bacteria have produced widespread resistance to the antibiotics.

The plasmids of resistance genes carried by animal source bacteria can be transferred to human through animal feeding or animal products processing (Piddock et al., 2000). In recent years, the incidence and mortality rates of colibacillosis continue to rise due to the existence and prevalence of a large number of drug-resistant strains. It not only restricts the healthy development of the poultry industry, but also increases the risk of food contamination and human infection. Therefore, the World Health Organization has issued a serious warning, "the nascent super bacteria resistant to all drugs will put human back to a wanton age of infectious diseases" (Hao et al., 2009). Thus, the study on drug resistance of *E. coli* has important significance in veterinary, food safety, and human health.

To reveal the resistance mechanisms of avian *E. coli* and to provide a basis for reasonable clinical medication, we monitored resistance of 132 *E. coli* isolates (from large-scale chicken farms in Hebei Province) to β-lactams, aminoglycosides, tetracyclines, sulfonamides, and chloramphenicol antibiotics using drug susceptibil-

©2013 Poultry Science Association Inc.
Received March 14, 2013.
Accepted May 27, 2013.
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ity, and the resistance genes [including *TEM*, *SHV*, *CTX-M*, *aph*(3′)-IIa, *aac*(3)-IIa, *aac*(6′)-Ib, *ant*(3″)-Ia, *tetA*, *tetC*, *tetM*, *Sul1*, *Sul2*, *Sul3*, *cat1*, *cmltA*, and *flor*] were detected by PCR.

MATERIALS AND METHODS

Reagents

Enterobacteriaceae bacterial biochemical tubes, drug-sensitive paper discs, MacConkey medium, and MHA medium were purchased from Hangzhou Tianhe Microorganism Reagent Co. Ltd. (Hangzhou, China). *Taq* DNA polymerase, PCR kits, DNA markers, dNTP, Gold-view dye, agarose, and other reagents were purchased from TaKaRa (Dalian, China). Control strain *E. coli* ATCC25922 was supplied by China Institute of Veterinary Drugs Control (Beijing, China).

Isolation and Identification of E. coli

Avian *E. coli* isolates were isolated from 8 large-scale chicken farms in Hebei Province. According to the clinical symptoms and pathological changes, the dead chickens were preliminarily diagnosed with colibacillosis. The livers and pericardial fluid were collected from dead chickens under sterile conditions and cross-inoculated in MacConkey medium. Pink single punctate colony was picked and transferred onto fresh MacConkey medium plates several times to ascertain culture purity. Each purified isolate was observed by microscope after Gram stain, and incubated into a micro-biochemical reaction test tube to do biochemical testing, including sugar fermentation experiment, indole test, methyl red test, Voges-Prolauer test, citrate utilization test, urea hydrolysis test, and H_2S production test.

Drug Susceptibility Test

The investigated antibiotics included β-lactams (ampicillin, cephalotin, cefuroxime, ceftriaxone, cefepime, imipenem, and aztreonam), aminoglycosides (streptomycin, gentamicin, kanamycin, neomycin, amikacin, and spectinomycin), tetracyclines (tetracycline, doxycycline, and minocycline), sulfonamides (sulfamethoxazole trimethoprim and sulfamonomethoxine), and chloramphenicol (thiamphenicol and florfenicol).

Drug susceptibility testing was performed by the Kirby-Bauer disc diffusion method. Bacteria liquid (10^7 cfu/mL, 0.2 mL each plate) was uniformly spread onto the MHA medium by a sterile cotton swab, and drug-sensitive paper discs were placed with at least 30-mm intervals on the medium. After incubation for 18 to 24 h at 37°C, the inhibition zone diameters were examined. Drug resistance was identified using the standard proposed by the Clinical and Laboratory Standards Institute (CLSI, 2011).

PCR Amplification of Resistance Genes

Primers, shown in Table 1, were designed as described previously (Wang et al., 2007; Zhang et al., 2007; Zhou et al., 2007; Xia, 2008; Yang, 2008; Ye, 2009) and synthesized by Beijing Sunbiotech Co. Ltd. (Beijing, China). Bacterial chromosomal DNA was extracted by the boiling method (Sambrook and Russell, 2008). The PCR was performed in a MyCycler thermal cycler (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

The PCR reaction mixture contained 0.8 m*M*/L of dNTP, 0.2 μ*M*/L of each primer, 1.25 units of *Taq* DNA polymerase, 5.0 μL of 10 × *Taq* buffer, 2.5 μL of template DNA, and was made up to a final volume of 50.0 μL with double-distilled H_2O. The PCR temperature profile was as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 55 s, and extension at 72°C for 60 s; and final extension at 72°C for 5 min. The PCR products were preserved at 4°C for use.

A total of 5.0-μL PCR products were mixed into 1.0 μL of 6 × loading buffer. Then the mixture was subjected to electrophoresis at 80 V on a 1.5% (wt/

Table 1. Primers of resistance genes of avian *Escherichia coli*

Resistance gene		GenBank accession number	Size (bp)
β-Lactams	*TEM*	AY956315	719
	CTX-M	AY293071	365
	SHV	EF650037	502
Aminoglycosides	*aac(3)-IIa*	AY138987	384
	aac(6′)-Ib	AF282595	486
	ant(3″)-Ia	AF453999	284
	aph(3′)-IIa	AY286001	677
Tetracyclines	*tetA*	X75761	915
	tetC	Y19114	480
	tetM	DQ060148	580
Sulfonamides	*Sul1*	GQ293501	238
	Sul2	AY360321	793
	Sul3	AY494779	443
Chloramphenicol	*Cat1*	AY617066	550
	flor	AY517519	650
	cmltA	NC006856	900

Table 2. Resistance rate and sensitive rate of avian *Escherichia coli* to antibiotics

Antibiotic		Resistant	Intermediate	Sensitive
β-Lactams	Ampicillin	87.9 (116/132)^{A}	7.6 (10/132)	4.5 (6/132)^{E}
	Cephalotin	79.5 (105/132)^{AB}	16.7 (22/132)	3.8 (5/132)^{E}
	Cefuroxime	71.2 (94/132)^{BC}	12.9 (17/132)	15.9 (22/132)^{D}
	Ceftriaxone	54.5 (72/132)^{CD}	12.1 (16/132)	33.3 (44/132)^{C}
	Cefepime	16.7 (22/132)^{E}	4.5 (6/132)	78.8 (104/132)^{B}
	Imipenem	0 (0/132)^{F}	0 (0/132)	100 (132/132)^{A}
	Aztreonam	45.5 (60/132)^{D}	12.1 (16/132)	42.4 (56/132)^{C}
Aminoglycosides	Gentamicin	70.5 (93/132)^{A,a}	16.7 (22/132)	12.9 (17/132)^{C,d}
	Amikacin	28.8 (38/132)^{BC,b}	41.7 (55/132)	29.5 (39/132)^{B,c}
	Streptomycin	62.9 (83/132)^{A,a}	16.7 (22/132)	20.5 (27/132)^{BC,bc}
	Kanamycin	33.3 (44/132)^{B,b}	45.5 (60/132)	21.2 (28/132)^{BC,bc}
	Neomycin	25.0 (33/132)^{BC,bc}	12.1 (16/132)	62.9 (83/132)^{A,b}
	Spectinomycin	16.7 (22/132)^{C,c}	8.3 (11/132)	75.0 (99/132)^{A,a}
Tetracyclines	Tetracycline	74.2 (98/132)^{A}	8.3 (11/132)	17.4 (23/132)^{B}
	Doxycycline	58.3 (77/132)^{B}	12.1 (16/132)	29.5 (39/132)^{B}
	Minocycline	7.6 (10/132)^{C}	9.1 (12/132)	83.3 (110/132)^{A}
Sulfonamides	Sulfamethoxazole trimethoprim	66.7 (88/132)	12.9 (17/132)	20.5 (27/132)^{a}
	Sulfamonomethoxine	70.5 (93/132)	19.7 (26/132)	9.8 (13/132)^{b}
Chloramphenicol	Thiamphenicol	46.2 (61/132)	15.9 (21/132)	37.9 (50/132)^{A}
	Florfenicol	54.5 (72/132)	25.0 (33/132)	20.5 (27/132)^{B}

$^{A–E}$Different uppercase letters indicate an extremely significant difference ($P < 0.01$).

$^{a–f}$Different lowercase letters indicate a significant difference ($P < 0.05$).

vol) agarose gel for 50 min using a JY1000C universal electrophoresis power supply (Beijing Liuyi Instrument Factory, Beijing, China). After electrophoresis, the gels were observed by using a WD-9413C UV gel imaging system purchased from the Beijing Liuyi Instrument Factory. The PCR products were sequenced by Beijing Sunbiotech Co. Ltd. (Beijing, China). In this study, DL-2000DNA marker was used as a molecular weight standard and the dye was Gold-view.

RESULTS

Resistance to 5 Kinds of Antibiotics in Avian E. coli Isolates

A total of 132 isolates were identified as *E. coli* by Gram staining and biochemical tests. The resistance to β-lactam, aminoglycoside, tetracycline, sulfonamide, and chloramphenicol antibiotics was respectively detected by using a drug susceptibility test. In β-lactam antibiotics, the avian *E. coli* isolates had obvious resistance to penicillins and 1- to 2-generation cephalosporins, and the resistance rate to ampicillin was 87.9%. The resistance rate and sensitive rate to 3-generation cephalosporins and monocyclic lactams were close. The *E. coli* was extremely sensitive to 4-generation cephalosporins and carbapenems, and the sensitive rate to cefepime and imipenem had reached 78.8 and 100%, respectively. In aminoglycosides antibiotics, the resistance rate to gentamicin was highest (70.5%), followed by streptomycin and kanamycin; the sensitive rate to spectinomycin was highest (75.0%), followed by neomycin. In tetracycline antibiotics, the sensitive rate to minocycline was very high and reached 83.3%. The resistance rate to sulfonamides and chloramphenicol antibiotics was about 70 and 50%, respectively. Resistance rate and sensitive rate of avian *E. coli* to antibiotics are shown in Table 2. The above results indicated that all avian *E. coli* strains were multidrug resistant. They had resistance to at least 4 kinds of drugs, and the most up to 15 kinds of drugs.

Distribution of Resistance Genes in Avian E. coli Isolates

The resistance genes were found in all 132 avian *E. coli* isolates and the total positive rate was 100%, as evidenced by the results of PCR. Three β-lactam resistance genes, *TEM* (Figure 1), *CTX-M*, and *SHV*, were detected; the positive rate was 65.9% (87/132), 32.6% (50/132), and 4.5% (6/132), respectively. Of the 132 isolates, 49 (accounting for 37.1%) had more than 2 commensal β-lactam resistance genes. Four aminoglycoside resistance genes, *aac*(3)-IIa (Figure 2), *ant*(3″)-Ia, *aac*(6′)-Ib, and *aph*(3′)-IIa, were detected, and the positive rate was 50.1% (67/132), 37.1% (49/132), 24.2% (32/132), and 6.8% (9/132), respectively. Of the isolates, 35.6% (47/132) had more than 2 commensal aminoglycoside resistance genes. The positive rate of tetracycline resistance genes *tetA* (Figure 3) and *tetM* was 52.3% (69/132) and 15.9% (21/132), respectively. The *tetC* gene was not found in the 132 isolates. The positive rate of sulfonamide resistance genes *Sul1* (Figure 4), *Sul2* (Figure 5), and *Sul3* was 68.2% (90/132), 73.5% (97/132), and 47.0% (63/132), respectively. More than 2 sulfonamide resistance genes were commensal in 74.2% (98/132) of the isolates. The positive rates of chloramphenicol resistance genes *flor* (Figure 6), *cat1* and *cmltA* were 49.2% (65/132), 31.8% (42/132), and 23.5% (31/132), respectively. More than 2 chloramphenicol resistance genes were commensal in 33.3% (44/132) of the isolates. Detection of multiple resistance genes in avian *E. coli* isolates is shown in Table 3.

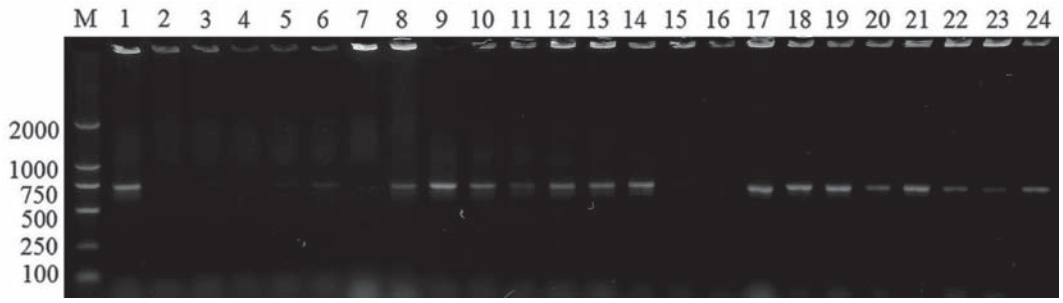

Figure 1. Gel electrophoresis analysis of PCR product of the *TEM* gene; the *TEM* gene can mediate avian *Escherichia coli* to resist β-lactam antibiotics. Lane M: DNA marker; lanes 1–24: 1–24 strains; lanes 1, 6, 8–14, and 17–24 have the specific bands (719 bp).

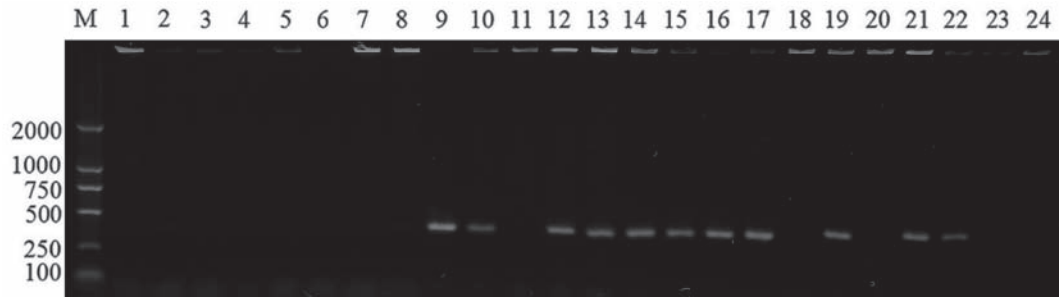

Figure 2. Gel electrophoresis analysis of PCR product of the *aac*(3)-IIa gene; the *aac*(3)-IIa gene can mediate avian *Escherichia coli* to resist aminoglycoside antibiotics. Lane M: DNA marker; lanes 1–24: 1–24 strains; lanes 9, 10, 12–17, 19, 21, and 22 have the specific bands (384 bp).

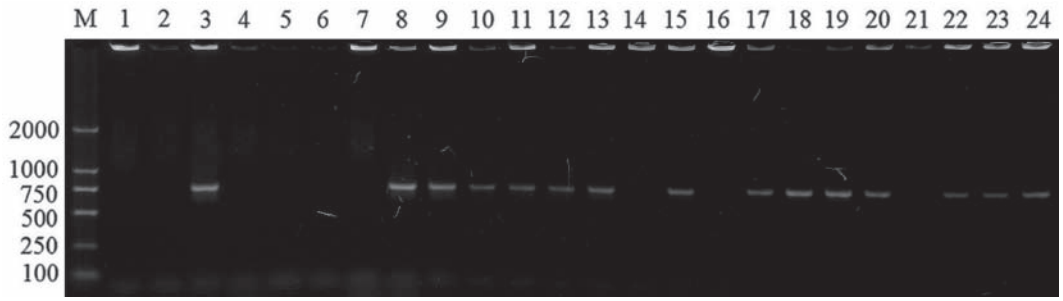

Figure 3. Gel electrophoresis analysis of PCR product of the *tetA* gene; the *tetA* gene can mediate avian *Escherichia coli* to resist tetracycline antibiotics. Lane M: DNA marker; lanes 1–24: 1–24 strains; lanes 3, 8–13, 15, 17–20, and 22–24 have the specific bands (915 bp).

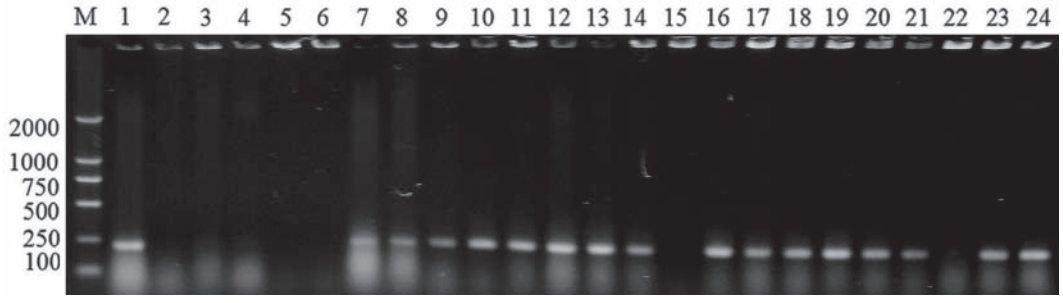

Figure 4. Gel electrophoresis analysis of PCR product of the *Sul1* gene; the *Sul1* gene can mediate avian *Escherichia coli* to resist sulfonamide antibiotics. Lane M: DNA marker; lanes 1–24: 1–24 strains; lanes 1, 7–14, 16–21, 23, and 24 have the specific bands (238 bp).

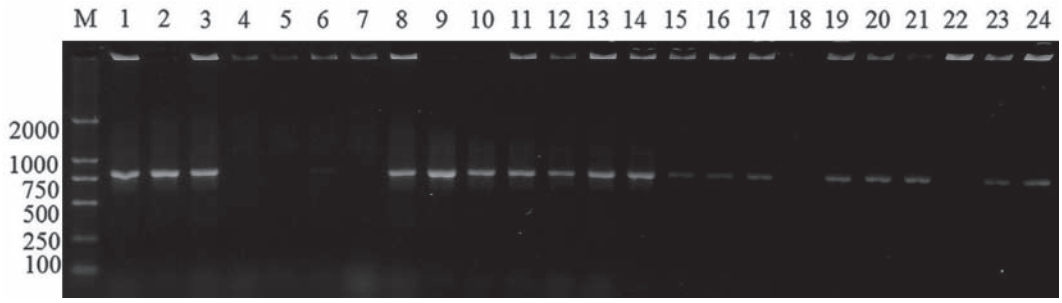

Figure 5. Gel electrophoresis analysis of PCR product of the *Sul2* gene; the *Sul2* gene can mediate avian *Escherichia coli* to resist sulfonamide antibiotics. Lane M: DNA marker; lanes 1–24: 1–24 strains; lanes 1–3, 8–17, 19–21, 23, and 24 have the specific bands (793 bp).

The amplified resistance genes had higher than 96% homology to the corresponding sequences published in GenBank, indicating the PCR products were the target genes.

DISCUSSION

Resistance Phenotype of Avian E. coli

In this study, the results of drug susceptibility test verified that the resistance of avian *E. coli* in Hebei Province is very serious. The longer antibiotics were used, the more drug-resistant strains were found. *Escherichia coli* was sensitive to cefepime, imipenem, spectinomycin, and minocycline due to the late clinical application. The resistance to *E. coli* is associated with the habits of use, and as a result the reported resistance of *E. coli* is different in different areas. In Hubei Province, the resistance rate of 21 *E. coli* isolates in chickens to tetracycline, amoxicillin, cotrimoxazole, and sulfamethoxazole was up to 100%, but florfenicol and kanamycin were at only 42.9 and 28.6%, respectively (Luo et al., 2011). In Shandong Province, the 76 *E. coli* isolates in chickens had the highest resistance to penicillin and ampicillin, followed by the compound sulfamethoxazole, ciprofloxacin, and tetracycline, but they were sensitive to cefoperazone, cefazolin, and cefatrizine (Yu, 2009). In Liaoning Province, the resistance rate of chicken *E. coli* to oxytetracycline, tylosin tartrate, sulfamonomethoxine, ampicillin sodium, and amoxicillin was up to above 90% (Hao et al., 2009).

Resistance Genotype of Avian E. coli

The *E. coli* resistance may be due to innate resistance gene, gene mutation, or gene transfer. The present studies suggest that resistance mechanisms of *E. coli* are mainly 4 kinds, including target site change and producing new target site of antibacterial drugs, producing the enzyme to modify or destroy the antibiotic, educing the permeability of cell membrane to decrease antibiotic intake, and increase active efflux of the antibiotic (Yu, 2009). The resistance mechanism of *E. coli* to β-lactam antibiotics is mainly producing extended-spectrum β-lactamases that destroy the β-lactam ring and lead to antibiotic inactivation (Fluit et al., 2001). The encoding extended-spectrum β-lactamase genes are mainly *TEM*, *SHV*, and *CTX-M* (Ye et al., 2010). The resistance mechanism of *E. coli* to aminoglycoside antibiotics is mainly producing aminoglycoside-modifying enzymes that modify the active molecules and lead to activity loss of drugs, and it is mediated by resistance genes *aph*(3′)-IIa, *aac*(3)-I, *aac*(6′)-Ib, and *ant*(3′′)-Ia (Llano-Sotelo et al., 2002). The resistance mechanism of *E. coli* to tetracycline antibiotics is complex and mainly mediated by the active efflux genes (*tetA*, *tetB*, and *tetC*) and the ribosomal protection genes (*tetM*, *tetW*, *tetO*, *tetK*, and *tetL*; Benacer et al., 2010). The resistance mechanism of *E. coli* to sulfonamide antibiotics is mainly producing dihydrofolate synthetase and decreasing affinity to sulfa drugs, and it is mediated by resistance genes *Sul1*, *Sul2*, and *Sul3* (Zhou, 2007). The resistance mechanism of *E. coli* to chloramphenicol

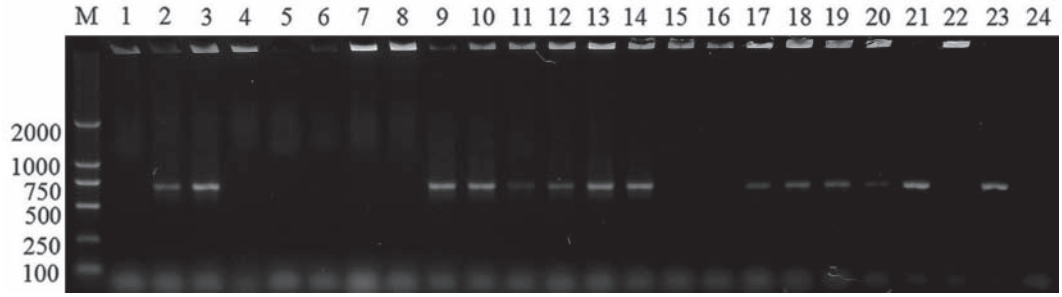

Figure 6. Gel electrophoresis analysis of PCR product of the *flor* gene; the *flor* gene can mediate avian *Escherichia coli* to resist chloramphenicol antibiotics. Lane M: DNA marker; lanes 1–24: 1–24 strains; lanes 2, 3, 9–14, 17–21, and 23 have the specific bands (650 bp).

Table 3. Detection of multiple resistance genes in avian *Escherichia coli* isolates

Variety of antibiotics	Number of genes	Commensal gene	Positive rate (%)
β-Lactams	0	—	30.3 (40/132)
	1	*TEM*	31.1 (41/132)
		CTX-M	1.5 (2/132)
		SHV	0 (0/132)
	2	*TEM* + *CTX-M*	32.6 (43/132)
		TEM + *SHV*	0.8 (1/132)
		CTX-M + *SHV*	2.3 (3/132)
	3	*TEM* + *CTX-M* + *SHV*	1.5 (2/132)
Aminoglycosides	0	—	20.5 (27/132)
	1	*aph*(3′)-IIa	1.5 (2/132)
		aac(6′)-Ib	4.6 (6/132)
		ant(3″)-Ia	17.4 (23/132)
		aac(3)-IIa	20.5 (27/132)
	2	*aph*(3′)-IIa + *ant*(3″)-Ia	1.5 (2/132)
		aac(6′)-Ib + *ant*(3″)-Ia	2.3 (3/132)
		aac(6′)-Ib + *aac*(3)-IIa	12.9 (17/132)
		ant(3″)-Ia + *aac*(3)-IIa	14.4 (19/132)
		aph(3′)-IIa + *aac*(6′)-Ib	1.5 (2/132)
	3	*aph*(3′)-IIa + *aac*(6′)-Ib + *aac*(3)-IIa	1.5 (2/132)
		aac(6′)-Ib + *ant*(3″)-Ia + *aac*(3)-IIa	0.8 (1/132)
	4	*aph*(3′)-IIa + *aac*(6′)-Ib + *ant*(3″)-Ia + *aac*(3)-IIa	0.8 (1/132)
Tetracyclines	0	—	37.9 (50/132)
	1	*tetA*	46.2 (61/132)
		tetM	9.9 (13/132)
		tetC	0 (0/132)
	2	*tetA* + *tetM*	6.1 (8/132)
Sulfonamides	0	—	0 (0/132)
	1	*Sul1*	6.8 (9/132)
		Sul2	5.3 (7/132)
		Sul3	13.6 (18/132)
	2	*Sul1* + *Sul2*	40.2 (53/132)
		Sul1 + *Sul3*	6.1 (8/132)
		Sul2 + *Sul3*	12.9 (17/132)
	3	*Sul1* + *Sul2* + *Sul3*	15.2 (20/132)
Chloramphenicol	0	—	32.6 (43/132)
	1	*Cat1*	4.6 (6/132)
		cmltA	9.1 (12/132)
		flor	20.5 (27/132)
	2	*Cat1* + *cmltA*	4.6 (6/132)
		Cat1 + *flor*	18.9 (25/132)
		cmltA + *flor*	6.1 (8/132)
	3	*Cat1* + *cmltA* + *flor*	3.8 (5/132)

antibiotics is mediated by genes *cat*, *cmltA*, and *flor*. The *cat* gene mediates the producing chloramphenicol acetyltransferase and thus leads to chloramphenicol inactivation. In addition, the *cmltA* and *flor* genes can mediate the drug efflux (Du et al., 2004).

In our study, we found that most of the *E. coli* isolates carried multiple resistance genes. The results of PCR amplification of resistance genes in *E. coli* isolates indicated that the resistance mechanism of *E. coli* in Hebei Province is mainly mediated by multiple resistance genes that cause expression of modifying enzymes or changes of target enzyme structure, and thus decrease the antibacterial drug affinity and increase drug efflux.

Prevention and Control of Chicken Colibacillosis

Changes in climate, environment, and the method of raising are the main factors causing colibacillosis. Therefore, a good environment including health sanitation, good ventilation, and low stress is very important for chicken feeding. To reduce the production of drug resistance, we propose the following for the use of antibacterial drugs in the prevention and control of chicken colibacillosis: 1) understanding the local bacterial drug-resistant spectrum, and selecting effective drugs according to the results of drug susceptibility tests; 2) circulating application of different antimicrobial drugs with enough dosage according to the use cycle of antimicrobial drugs; 3) prompt transformation from empirical treatment to target treatment, namely suspending treatment or using the narrow-spectrum antibiotics when the situation of the diseased poultry has improved or the pathogenic bacteria is identified; 4) prophylactic administration in feeds should avoid long-term and high-dose application of the same antibiotic; and 5) strictly implementing the disinfection and isolation measures to prevent cross infection, and immunoprophylaxis protection should be done well in the process of feeding birds.

To reduce drug resistance, in addition to seeking new antibiotics based on traditional ideas, we should strengthen the research and development of alternative products of antibiotics and bacterial drug resistance inhibitors. From the current technology and conditions, we also should establish the detection system of bacterial drug resistance to achieve the accurate, systematic, and continuous detection of drug resistance in local bacteria, thus tracking and controlling the trend of drug resistance.

ACKNOWLEDGMENTS

This study was financially supported by the Ministry of Science and Technology, China (project no. 2011BAD34B02) and Hebei Key Technology R&D Program grant (project no. 10220414) from the Department of Science and Technology, Hebei Province, China.

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