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Antimicrobial Resistance and Resistance Gene Determinants of Fecal *Escherichia coli* Isolated from Chicken

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Abstract: Antimicrobial resistance has emerged as a major problem and national antimicrobial resistance management program has been set up for antimicrobial resistance surveillance in human and veterinary medicine from 2009 in Korea. The aim of the present study was to assess the actual frequency of antimicrobial resistance in fecal *Escherichia coli* (*E. coli*) isolated from chicken at the phenotype level and to determine the genetic background for the two major resistance phenotypes (Streptomycin and tetracycline). One hundred and nine *E. coli* isolates were higher resistant to ampicillin (68.8%), cephalothin (63.3%), streptomycin (60.6%), ciprofloxacin (65.1%), enrofloxacin (79.8%), norfloxacin (66.1%), tripmethoprim/sulfamethoxazole (50.5%), erythromycin (89.0%) and tetracycline (96.3%). All of them showed resistance to two or more of the 18 antibiotics tested and one isolates was resistant to 13 antibiotics. In 51 (46.8%) isolates of 105 tetracycline resistant isolates only the resistant gene *tetA* was amplified while both the *tetA* and *tetB* gene were found in 22 (20.2%) isolates. The only *tetB* gene was found in 11 (10.1%) isolates and *tetA* and *tetC* gene was found in two isolates. About 30 (27.5%) isolates among 66 streptomycin resistant isolates were positive with *strA*, *strB* and *aadA* gene. The 25 (22.9%) and 4 (3.7%) isolates were positive with *strA* plus *strB* and *strB* plus *aadA* gene, respectively. The researchers could show that significant differences can be observed between isolates not only at the phenotype level but also at the genotype level.

Key words: Veterinary medicine, genotype, national antimicrobial resistance, phenotypes, isolates, Korea

INTRODUCTION

The use of antimicrobial agents in animal feeds for growth promotion for improved feed efficiency and for control and prevention of disease has been widespread since, the early 1950s (Guest, 1976; Hays, 1978; Kiser, 1976). Antibiotics once effective at controlling many infections are now ineffective due to the bacterium's acquired resistance to these compounds. Resistance in microbial pathogens like *E. coli* to two or more classes of antibiotics is now common place in both veterinary (Gonzalez and Blanco, 1989; Harnett and Gyles, 1984; Irwin *et al*., 1989) and human medicine.

Antimicrobial resistance has emerged as a major problem and many programs have been set up for its surveillance in human and veterinary medicine. These programs are aimed mainly at human pathogens, agents of zoonoses and indicator bacteria of the normal intestinal flora from animals (Lanz *et al*., 2003). Resistance surveillance programs are usually aimed at assessing resistance phenotypes. These resistance phenotypes may arise from many different genetic determinants features. Therefore, the assessment of the resistance situation at the genetic level would be an important asset in the understanding and control of antimicrobial resistance in general. In Korea, only sporadic studies have been devoted to antimicrobial resistance in animal pathogens during the past 20 years (Kim *et al*., 1995, 1997, 2001; Lee *et al*., 2004). But national antimicrobial resistance management program has been set up for antimicrobial resistance surveillance in human and veterinary medicine from 2009.

The aim of the present study was to assess in a first step the actual frequency of antimicrobial resistance in fecal *E. coli* isolated from chicken at the phenotype level. In a second step, the resistance determinants for the two major resistance phenotypes (streptomycin and tetracycline) observed in these isolates were identified. Because they seem to play a major role in the dissemination of resistance in gram-negative bacteria (Carattoli, 2001; Fluit and Schmitz, 1999; Jones *et al*., 1997; Rowe-Magnus and Mazel, 1999).

MATERIALS AND METHODS

Sampling of feces: One hundred and nine fresh fecal sample from healthy chickens at the 8 different farms (10 sample/farm) were collected from March to November

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2009. Fecal sample were place in sterile plastic specimen tubes on ice and transported to the laboratory for *E. coli* isolation within 2 days.

Isolation and identification: For isolation of *E. coli*, feces were plated on to EBM agar (Difco, USA) and incubated at 37°C for 24 h. Blue-black colonies with dark centers and greenish metallic sheen were randomly picked out. All of the isolates were identified by biochemical test (Gram stain, oxidase, TSI, indole, citrate, methyl red and urea agar) and kept at 4°C in MacConkey agar (Difco, USA) until use. Only two colonies per colonial morphology were selected for the purpose of determining susceptibility. If two colonies had the same resistant phenotype, only one colony was selected for further analysis.

Susceptibility testing: Antibiotic susceptibility was determined by disk diffusion method on Mueller-Hinton II agar (Difco, USA) with 18 antibiotics (Ampicillin, amoxicillin/clavulanic acid, cephalothin, cefazolin, cefoxitin, cefotaxime, cefepime, imipenem, streptomycin, gentamicin, amikacin, ciprofloxacin, enrofloxacin, norfloxacin, trimethoprim/sulfamethoxazole, erythromycin, chloramphenicol and tetracycline) according to the NCCLS standard method. *E. coli* ATCC 25922 were included as susceptibility control strains.

DNA extraction and PCR: About 1 mL of the preenriched sample was transferred to a microcentrifuge tube with a capacity of 1.5 mL. The cell suspension was centrifuged for 10 min at 14,000×g. The supernatant was discarded. The pellet was resuspended in 300 uL of D.W. by vortexing and incubated for 10 min at 100°C. An aliquot of 2 uL of the supernatant was used at the template DNA. All the PCR experiments were performed in a PCR mix consisting of 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 uM of each dNTP and 0.25 uM of each primer (Table 1). All amplification consisted of an initial denaturation of 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at specific temperature (Table 1) and elongation at 72°C for 60 sec and a final elongation at 72°C for 7 min.

Table 1: Primers and PCR conditions

Genes	Primers[a]	Code	Annealing temperature (°C)	Fragment size (bp)
tetA	TetA-L	GGCGGTCTTCTTCATCATGC	64	502
	TetA-R	CGGCAGGCAGAGCAAGTAGA		
tetB	TetB-L	CATTAATAGGCGCATCGCTG	64	930
	TetB-R	TGAAGGTCATCGATAGCAGG		
tetC	TetC-L	GCTGTAGGCATAGGCTTGGT	65	888
	TetC-R	GCCGGAAGCGAGAAGAATCA		
aadA	4F	GTGGATGGCGGCCTGAAGCC	68	525
	4R	AATGCCCAGTCGGCAGCG		
strA	2F	CCTGGTGATAACGGCAATTC	55	546
	2R	CCAATCGCAGATAGAAGGC		
strB	3F	ATCGTCAAGGGATTGAAACC	56	509
	3R	GGATCGTAGAACATATTGGC		

[a]Lanz *et al.* (2003)

RESULTS AND DISCUSSION

The resistance of 109 *E. coli* isolates to 18 antibiotics was determined (Table 2). *E. coli* isolates were higher resistant to ampicillin (68.8%), cephalothin (63.3%), streptomycin (60.6%), ciprofloxacin (65.1%), enrofloxacin (79.8%), norfloxacin (66.1%), tripmethoprim/ sulfamethoxazole (50.5%), erythromycin (89.0%) and tetracycline (96.3%) and some isolates also showed intermediate resistance to amoxicillin/clavulanic acid (31.2%), cephalothin (27.5%), cefozolin (18.3%), streptomycin (37.6%) and ciprofloxacin (13.8%). All isolates were susceptible to cefepime and imipenem.

The frequencies of the resistance genotypes for tetracycline and streptomycin are shown in Table 3 and 4. In 51 (46.8%) isolates of 105 tetracycline resistant isolates, only the resistant gene *tetA* was amplified while both the *tetA* and *tetB* gene were found in 22 (20.2%) isolates. The only *tetB* gene was found in 11 (10.1%) isolates and *tetA* plus *tetC* was found in 2 isolates. In eighteen tetracycline resistant isolates and one tetracycline intermediate resistant isolates, no *tetA*, *tetB*, or *tetC* genes were detected but *tetA* gene was detected in 2 of 3 tetracycline sensitive isolates.

About 30 (27.5%) isolates among 66 streptomycin resistant isolates were positive with *strA*, *strB* and *aadA* gene. The 25 (22.9%) and 4 (3.7%) isolates were positive with *strA* plus *strB* and *strB* plus *aadA* gene,

Table 2: Antimicrobial resistance frequency of 109 fecal *E. coli* isolates from chicken

	No. of resistant isolates[a] (%)																	
Types	AM	AmC	CF	CZ	FOX	CTX	FEP	IMP	S	GM	AN	CIP	ENO	NOR	SXT	E	C	TE
Resistant	75.0	1.0	69.0	2.0	2.0	1.0	0	0	66.0	25.0	1.0	71.0	87.0	72.0	55.0	97.0	13.0	105.0
	(68.8)	(0.9)	(63.3)	(1.8)	(1.8)	(0.9)	(0)	(0)	(60.6)	(22.9)	(0.9)	(65.1)	(79.8)	(66.1)	(50.5)	(89.0)	(11.9)	(96.3)
Intermediate	16.0	34.0	30.0	20.0	1.0	4.0	0	0	41.0	0.0	1.0	15.0	7.0	5.0	8.0	12.0	5.0	1.0
	(14.7)	(31.2)	(27.5)	(18.3)	(0.9)	(3.7)	(0)	(0)	(37.6)	(0.0)	(0.9)	(13.8)	(6.4)	(4.6)	(7.3)	(11.0)	(4.6)	(0.9)

[a]AM: Ampicillin; AmC: Amoxicillin/Clavulanic acid; CF: Cephalothin; CZ: Cefozolin; FOX: Cefoxitin; CTX: Cefotaxime; FEP: Cefepime; MP: Imipenem; S: Streptomycin; GM: Gentamicin; AN: Amikacin; CIP: Ciprofloxacin; ENO: Enrofloxacin; NOR: Norfloxacin; SXT: Trimethoprim/Sulfamethoxazole; E: Erythromycin; C: Chloramphenicol; TE: Tetracycline

Table 3: Frequency tetracycline resistance determinants among 109 *E. coli* isolates from chicken

Resistance determinants			No. of isolates (%)		
			Susceptible	Intermediate	Resistant
tetA	*tetB*	*tetC*	(n = 3)	(n = 1)	(n = 105)
-ve	-ve	-ve	1 (0.9)	1 (0.9)	19 (17.4)
+ve	-ve	-ve	2 (1.8)	0 (0.0)	51 (46.8)
-ve	+ve	-ve	0 (0.0)	0 (0.0)	11 (10.1)
+ve	+ve	-ve	0 (0.0)	0 (0.0)	22 (20.2)
+ve	-ve	+ve	0 (0.0)	0 (0.0)	2 (1.8)

Table 4: Frequency streptomycin resistance determinants among 109 *E. coli* isolates from chicken

Resistance determinants			No. of isolates (%)		
			Susceptible	Intermediate	Resistant
strA	*strB*	*aadA*	(n = 2)	(n = 41)	(n = 66)
-ve	-ve	-ve	1 (0.9)	0 (0.0)	0 (0.0)
+ve	-ve	-ve	0 (0.0)	0 (0.0)	1 (0.9)
-ve	+ve	-ve	1 (0.9)	3 (2.8)	5 (4.6)
-ve	-ve	+ve	0 (0.0)	1 (0.9)	1 (0.9)
+ve	+ve	-ve	0 (0.0)	21 (19.3)	25 (22.9)
-ve	+ve	+ve	0 (0.0)	10 (9.2)	4 (3.7)
+ve	+ve	+ve	0 (0.0)	15 (13.8)	30 (27.5)

respectively. Also, 21 (19.3%) isolates among 41 streptomycin intermediate resistant isolates were positive with strA plus strB while 15 (13.8%) isolates were positive with *strA*, *strB* and *aadA* gene. A similar streptomycin resistant determinants was observed between the intermediate and resistant isolates. Only one isolates of 2 streptomycin susceptible isolates were negative for all the streptomycin resistance determinants tested and one isolates were positive with *strB* gene.

E. coli strains isolated were higher resistant to ampicilline, cephalothin, streptomycin, ciprofloxacin, enrofloxacin, norfloxicin, trimethoprim/sulfamethoxazole, erythromycin and tetracycline and all of them exhibited multiple resistance to two or more antibiotics. High resistance to those antibiotics correspond to the continued use in veterinary medicine. Tetracycline, sulfas and streptomycin are currently recommended as a therapeutic agent and growth promotion in avian medicine in Korea. Ampicillin resistance may be a reflection of cross-resistance to cephalosporins currently available as a therapeutic for chicken. First generation cephalosporins are heavily used for the treatment in Korea. This use is clearly reflected in the higher resistance rate for cephalothin observed in chicken. Gentamicin resistance may be due to the inclusion of this antibiotic with the Marek's vaccine that is administrated to almost all chicken *in ovo* (Eidson and Kleven, 1976; Ricks *et al*., 1999). Recently, fluoroquinolones resistance of chicken isolates rapidly increased. Fluoroquinolones are registered for mass medication via feed in Korea. Nevertheless, these agents are heavily used for the bacterial treatment like colibacillosis and fowl typhoid by route of oral administration.

In most cases, tetracycline resistance genes are carried on bacterial plasmid in both gram-negative and gram-positive species (Chopra *et al*., 1981). Previous research (Langlois *et al*., 1986) has shown that tetracycline was the predominant drug in the resistance pattern in fecal coliform bacteria. The tetB tetracycline resistance determinant has been reported to be the most common tetracycline resistance determinant in members of the Enterobacteriaceae (Marshall *et al*., 1983). But the researchers found that the tetA was the most frequent tetracycline determinant in the sample whereas tetC was very infrequent. Specific associations of resistance determinants exist as for instance co-location on specific plasmid which may lead to different patterns of determinant co-selection dependent on the antimicrobial agents use pattern. The observed difference between the present study and those from other countries may consequently be explained by different uses of antimicrobial agents.

The *strA* and/or *strB* gene were with one single exception, always present in the isolates. In the single exception, an additional *aadA* gene could be responsible for the observed resistant phenotype. This finding is in agreement with the results of other researchers showing that both genes have to be present in order to obtain a functional streptomycin resistant (Chiou and Jones, 1995; Lanz *et al*., 2003). The *aadA* gene (Hollingshead and Vapnek, 1985), *strA* (Heinzel *et al*., 1988) and *strB* (Scholz *et al*., 1989) are the only genes previously reported to encode streptomycine resistance in *S. typhimurium*. In the present study, these genes were demonstrated in all resistant isolates characterized, confirming their importance for streptomycin resistance. As in the original reporting (Scholz *et al*., 1989), the *strA* and *strB* genes were located together. The expression of *strA* gene is significantly higher than the expression of *strB* gene (Scholz *et al*., 1989) but it has been suggested that both genes are needed to achieve streptomycin resistance (Chiou and Jones, 1995).

CONCLUSION

This research identified and compared for the first time resistance genotypes of fecal *E. coli* from chicken. The researchers could show that significant differences can be observed between isolates not only at the phenotype level but also at the genotype level.

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