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Apramycin and Gentamicin Resistances in Indicator and Clinical *Escherichia coli* Isolates from Farm Animals in Korea

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Abstract

A total of 1921 *Escherichia coli* isolated from healthy animals (501 from cattle, 832 from pigs, and 588 from chickens) and 237 isolates from diseased pigs were tested to determine the prevalence of apramycin and gentamicin resistance in Korea during 2004–2007. Apramycin/gentamicin resistances observed in healthy cattle, pigs, and chicken were 0.2%/0.6%, 11.2%/13.6%, and 0.5%/18.2%, respectively. Gentamicin/apramycin resistance was much higher in *E. coli* isolated from diseased pigs (71/237, 30.0%) than in those from healthy pigs (93/832, 11.2%). The aminoglycoside resistance gene content of all apramycin-gentamicin-resistant *E. coli* isolates ($n = 164$) was determined by polymerase chain reaction. Of seven different types of aminoglycoside resistance genes tested, five kinds were detected in the 164 isolates: *aac(3)-IV*, *aac(3)-II*, *aac(3)-III*, *ant(2″)-I*, and *armA*. All apramycin-resistant *E. coli* contained the *aac(3)-IV* gene. About half of the resistant isolates carried only the *aac(3)-IV* gene and the other half carried other genes in addition to *aac(3)-IV*. The results of the present study suggest that humans are at risk of gentamicin resistance from apramycin use in animals.

Introduction

Apramycin, an aminoglycoside antibiotic, has been used in animal husbandry around the world since late 1970s. Although it has not been used in human medicine, apramycin resistance has also been detected in human clinical isolates from patients who are hospitalized (Johnson *et al.*, 1995). Cross resistance between apramycin and other aminoglycosides used to treat serious infections in humans, such as gentamicin and tobramycin, has been well established (Johnson *et al.*, 1994; Yates *et al.*, 2004; Jensen *et al.*, 2006). Horizontal transfer of the apramycin resistance determinants, *aac(3)-IV* gene, is responsible for the dissemination of apramycin resistance in animals or between animals and humans (Yates *et al.*, 2004). It was reported that *Escherichia coli* from pigs may have been an important reservoir for transfer of gentamicin resistance genes or bacteria to humans (Johnson *et al.*, 2005). Jensen *et al.* (2006) also reported that occurrence of apramycin/gentamicin cross resistance in pigs was significantly correlated with the apramycin use. Spread of gentamicin resistance in humans is of great concern while considering the importance of this antibiotic in human medicine (Zarrilli *et al.*, 2005).

Apramycin has been used as feed additives in Korea since 1983, but little attention has been paid to this antibiotic. To date, no study on the prevalence of apramycin resistance has been conducted in Korea, although there have been reports on antimicrobial resistance in food animals (Lim *et al.*, 2007, 2009). In a previous study from Korea (Lim *et al.*, 2007), about 10% and 2% of gentamicin resistance was observed in *E. coli* isolates from healthy pigs and cattle, respectively, despite the limited use of gentamicin in food-producing animals in this country. Therefore, the objectives of this study were (1) to investigate apramycin and gentamicin resistance in *E. coli* isolates from healthy farm animals and diseased pigs; (2) to investigate the genetic content of the apramycin and gentamicin resistance among resistant *E. coli* isolates from pigs.

Materials and Methods

Sample collection

To isolate indicator *E. coli*, fresh feces were aseptically obtained from randomly selected individual healthy animals on farms (cattle) and slaughterhouses (pigs and chicken) throughout Korea during 2004–2007. A total of 2075 samples were collected, which included 601 adult cattle feces from 87

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cattle farms, 874 finishing pig feces from 79 pig farms, and 600 chicken feces from 60 chicken farms, respectively. For clinical *E. coli* isolates, 456 fecal or intestine samples were collected from diarrheic pigs submitted for necropsy examination to the diagnostic laboratory of National Veterinary Research and Quarantine Service in Korea during 2004–2007.

Isolation and identification of indicator and pathogenic E. coli

Feces were directly plated on Chromogenic *E. coli*/coliform agar (Oxoid Ltd., Basingstoke, Hants, England) at 37°C for overnight. Colonies yielding typical results for *E. coli* on the agar (purple) were streaked onto eosin methylene blue (Becton Dickinson, Sparks, MD) and incubated at 37°C for 18–20 h. The eosin methylene blue agar plates were examined and selected for metallic sheen colonies, which were streaked again on MacConkey agar (Becton Dickinson). After overnight incubation at 37°C, one or two typical pink colonies were selected and further tested by indole, methyl red, Voges-Proskauer, and Simmons citrate tests for confirmation of *E. coli*. API 20 E test strips (bioMerieux Vitek, Hazelwood, MO) were also used to confirm the identification of suspected isolates *as E. coli.*

Pathogenicity of *E. coli* isolates from diseased pigs was confirmed by detecting fimbrial (K88, K99, 987P, and F41) and toxin (heat-labile toxin, heat-stable toxin, and *E. coli* heat-stable enterotoxin 1) genes using polymerase chain reaction, as a previously described method with slight modification of primers for K88 and K99 (Vu-Khac *et al.*, 2004). Modified Primers for K88 and K99 were as follows: K88/F, GGTGATTTC AATGGTTCGGTC; K88/R, AGTCCATTCCATTTATAGGC; K99/F, TGCGACTACCAATGCTTCTG; K99/R, TATCCACC ATTAGACGGAGC. Presence of more than one virulence factor in *E. coli* isolate was regarded as a pathogenic one.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations for *E. coli* isolates were determined by an agar dilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). Antimicrobials were obtained from Sigma Chemical Co. (St. Louis, MO). Breakpoints for gentamicin and tobramycin ($\geq 8\,\mu g/mL$) and apramycin ($\geq 32\,\mu g/mL$) were used as described by CLSI (2008) and the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP, 2004–2007), respectively. *E. coli* ATCC 25922 was used as a quality control strain.

Aminoglycoside resistance profile

The aminoglycoside resistance gene content of all apramycin-gentamicin-resistant *E. coli* was determined by polymerase chain reaction based on previously described methods (Sandvang and Aarestrup, 2000; Jakobsen *et al.*, 2008) using primer sets for seven different aminoglycoside resistance genes including *aac(3)-I*, *aac(3)-II*, *aac(3)-III*, *aac(3)-IV*, *ant(2″)-I*, *armA*, and *aac(6)-Ib.*

Statistical analyses

The significance of differences in resistance between groups was determined using *t*-test. A value of $p \leq 0.05$ was considered statistically significant.

Results

Antimicrobial resistance in indicator E. coli *isolates from food animals*

A total of 1921 *E. coli* isolates from healthy cattle ($n = 501$), pigs ($n = 832$), and chicken ($n = 588$) were tested for apramycin and gentamicin resistance. There was a remarkable difference in the prevalence of apramycin and gentamicin resistance in *E. coli* isolates among animal species. A particularly high rate of apramycin resistance was observed in pigs (11.2%) compared with those in cattle (0.2%) and poultry (0.5%). Gentamicin resistance was also markedly higher in pigs (13.6%) than in cattle (0.6%), although the highest resistance to this antibiotic was observed in poultry (18.2%) (Table 1).

Cross resistance of gentamicin and apramycin in indicator and clinical E. coli *isolates from pigs*

Occurrence of apramycin/gentamicin cross resistance in *E. coli* isolates from healthy ($n = 832$) and diseased pigs ($n = 237$) is shown in Table 2. In this study, much higher rate of gen-

Table 1. Gentamicin and Apramycin Resistance in Indicator *Escherichia coli* from Food-Producing Animals in Korea During 2004–2007

	Resistance, % (no. of resistant isolates/no. of tested isolates)					
	Cattle		*Pigs*		*Chicken*	
Year	*GM*	*APR*	*GM*	*APR*	*GM*	*APR*
2004 ($n = 525$)	0.8 (1/130)	0 (0/130)	15.0 (34/227)	12.3 (28/227)	23.8 (40/168)	0.6 (1/168)
2005 ($n = 419$)	0.8 (1/130)	0 (0/130)	21.0 (37/176)	17.0 (30/176)	19.5 (22/113)	1.8 (2/113)
2006 ($n = 448$)	0.8 (1/120)	0 (0/120)	11.1 (21/190)	9.5 (18/190)	12.3 (17/138)	0 (0/138)
2007 (n = 529)	0 (0/121)	0.8 (1/121)	8.8 (21/239)	7.1 (17/239)	16.6 (28/169)	0 (0/169)
Total ($n = 1921$)	0.6 (3/501)	0.2 (1/501)	13.6 (113/832)	11.2 (93/832)	18.2 (107/588)	0.5 (3/588)

APR, apramycin; GM, gentamicin.

Table 2. Comparison of Gentamicin and Apramycin Resistance in Indicator *Escherichia coli* and Pathogenic *Escherichia coli* Isolated from Pigs

	Resistance, % (no. of resistant isolates/no. of tested isolates)					
	Indicator E. coli			*Pathogenic* E. coli		
Year	*GM*	*GM + APR*	*APR*	*GM*	*GM + APR*	*APR*
2004 ($n = 246$)	2.6 (6/227)	12.3 (28/227)	0 (0/227)	0 (0/19)	21.1 (4/19)	0 (0/19)
2005 ($n = 247$)	4.0 (7/176)	17.0 (30/176)	0 (0/176)	1.4 (1/71)	38.0 (27/71)	0 (0/71)
2006 ($n = 264$)	1.6 (3/190)	9.5 (18/190)	0 (0/190)	0 (0/74)	36.5 (27/74)	0 (0/74)
2007 ($n = 312$)	1.7 (4/239)	7.1 (17/239)	0 (0/239)	0 (0/73)	17.8 (13/73)	0 (0/73)
Total ($n = 1069$)	2.4 (20/832)	11.2 (93/832)	0 (0/832)	0.4 (1/237)	30.0 (71/237)	0 (0/237)

tamicin/apramycin resistance was observed in clinical *E. coli* than in indicator one: 30.0% (71/237) and 11.2% (93/832) of clinical and indicator *E. coli* isolates showed resistance to both antimicrobials, respectively. About 2.4% (20/832) and 0.4% (1/237) of indicator and clinical *E. coli* isolates showed resistance to gentamicin but not to apramycin, respectively. None of the *E. coli* presented sole resistance only to apramycin.

Detection of aminoglycoside-resistant genes in apramycin-resistant E. coli *isolates from pigs*

Of seven different types of aminoglycoside resistance genes tested, five kinds were detected in apramycin-resistant *E. coli* ($n = 164$): *aac(3)-IV*, *aac(3)-II*, *aac(3)-III*, *ant(2″)-I*, and *armA* (Table 3). All the apramycin-resistant *E. coli* contained at least one of those five resistance genes: *aac(3)-IV* gene was detected in all *E. coli* resistant to apramycin. The other genes *aac(3)-II*, *aac(3)-III*, *ant(2″)-I*, and *armA* were detected in 11 (11.8%), 42 (45.2%), 1 (1.1%), and 0 (0%) of indicator *E. coli* resistant to apramycin, respectively. Meanwhile, among apramycin-resistant *E. coli* isolated from diseased pigs, 13 (18.3%), 22 (31.0%), 5 (7.0%), and 2 (2.8%) isolates contained the *aac(3)-II*, *aac(3)-III*, *ant(2″)-I*, and *armA*, respectively. About half of all apramycin-resistant isolates carried only *aac(3)-IV* gene, and the other half carried other genes in addition to *aac(3)-IV*. Also, three different genes were found in 11 (6.7%) isolates. Except *aac(3)-IV*, the most frequently observed aminoglycoside resistance gene was *aac(3)-III*, followed by *aac(3)-II*. *E. coli* isolates containing *armA* showed much higher level of resistance to gentamicin and tobramycin (>256 μg/mL), compared with those containing genes other than *armA* (≥8–256 μg/mL).

Discussion

In this study, apramycin resistance in *E. coli* isolated from pigs was markedly higher than those from cattle and poultry ($p < 0.001$). The difference in resistance rates among those animal species might be related to the amounts of apramycin those animals are exposed to. Although apramycin has been used for prevention of digestive disease in food animals since 1983 in Korea, the most amount of this antibiotic has been consumed in pig husbandry: about 3800–5600 kg of apramycin was annually consumed in Korea during the period of this study, almost 90% of the amounts were used in pigs, and the rest was in cattle farms (NARMP, 2004–2008). A number of studies have also reported a positive correlation between the amounts of aminoglycoside consumption and the prevalence of resistance to this group of antimicrobials (Jensen *et al.*, 2006; Iosifidis *et al.*, 2008).

Gentamicin resistance rate seems to be directly proportional to the rate of apramycin resistance in both cattle and pigs, although the former is slightly higher than the latter in both cases. In poultry, however, a striking difference was observed in resistance rates between these two antimicrobials: 18.2% and 0.5% of resistance to gentamicin and apramycin, respectively ($p < 0.0005$). The total amount of gentamicin used per year in animals in Korea was about 2500–4000 kg during the period of this study, and about 21%, 42%, and 37% were reportedly used in cattle, pigs, and chicken, respectively (NARMP, 2004–2008). Gentamicin resistances observed in indicator *E. coli* isolated from cattle, pigs, and chicken were 0.6% (3/501), 13.6% (113/832), and 18.2% (107/588), respectively. Although few data are available for comparison, apramycin resistance observed in this study is much higher than that of Denmark (0%–3.0%) (DANMAP, 2004–2007) and Japan (0%–3.3%) (JVARM, 2000–2003), particularly in pigs. Gentamicin resistance in pigs is also much higher than that reported from Denmark (0%–3.0%) (DANMAP, 2004–2007), Canada (0%–2.2%) (CIPARS, 2004–2007), Japan (1.9%–4.0%) (JVARM 2004–2007), and Sweden (2%) (SVARM, 2005). Unlike pigs, cattle showed a very low level of gentamicin resistance, which is similar to that reported from other countries mentioned above, despite the proportion (21%) of gentamicin used in cattle farms. Our finding of the relatively lower correlation between antimicrobial usage and resistant bacterial populations in cattle may be due, in large part, to differences in animal husbandry practices and regimen of antimicrobial administration between animal species. Unlike pig farms, feed additives are generally not used in cattle farms. Also, antimicrobials for treatment might be individually given to diseased cattle mostly by veterinarians.

To investigate whether isolates were cross-resistant to gentamicin and apramycin, we compared occurrence of resistance to gentamicin only, apramycin only, or gentamicin and apramycin simultaneously in pathogenic and indicator *E. coli* isolates from pigs. Neither pathogenic nor indicator *E. coli* showed sole resistance only to apramycin in this study, whereas some

Table 3. Distribution of Aminoglycoside Resistance Genes in Apramycin-Resistant *Escherichia coli* Isolates from Pigs

	No. of isolates (%)			*Gentamicin (μg/mL)*		*Tobramycin (μg/mL)*	
Resistant gene pattern	*Indicator* E. coli (n = *93*)	*Pathogenic* E. coli (n = *71*)	*Total* (n = *164*)	*Range*	MIC_{50}	*Range*	MIC_{50}
aac(3)-IV	43 (46.2)	36 (50.7)	79 (48.2)	8 to 256	16	8 to >256	64
aac(3)-IV+aac(3)-II	7 (7.5)	9 (12.7)	16 (9.8)	8 to >256	64	16 to >256	128
aac(3)-IV+aac(3)-III	38 (40.9)	17 (23.9)	55 (33.5)	8 to 256	16	8 to >256	64
aac(3)-IV+ant(2″)	1 (1.1)	1 (1.4)	2 (1.2)	16 to >256	>256	16 to >256	>256
aac(3)-IV+armA	0 (0)	1 (1.4)	1 (0.6)	>256	>256	>256	>256
aac(3)-IV+aac(3)-II + aac(3)-III	4 (4.3)	3 (4.2)	7 (4.3)	16 to >256	64	32 to 128	64
aac(3)-IV+aac(3)-II + ant(2″)	0 (0)	1 (1.4)	1 (0.6)	64	64	64	64
aac(3)-IV+aac(3)-III + ant(2″)	0 (0)	2 (2.8)	2 (1.2)	16 to 32	32	128	128
aac(3)-IV+ant(2″) + armA	0 (0)	1 (1.4)	1 (0.6)	>256	>256	>256	>256

MIC, minimum inhibitory concentration.

of the pathogenic (0.4%) and indicator *E. coli* (2.4%) presented resistance only to gentamicin. We found that all apramycin-resistant *E. coli* were also resistant to gentamicin, but not all gentamicin-resistant *E. coli* showed apramycin resistance simultaneously. Our finding is similar to the findings of previous studies from other countries (Sandvang and Aarestrup, 2000; Jensen *et al.*, 2006). Cross resistance was known to be common within aminoglycosides group. Jensen *et al.* (2006) reported that the apramycin use at farm level is most likely driving the increasing occurrence of apramycin and gentamicin cross resistance in pigs at the national level. In a recent study, apramycin-resistant *E. coli* were detected from two farms in which gentamicin was used but apramycin was not (Zhang *et al.*, 2009).

As expected, *E. coli* isolated from clinical samples displayed more than double rate of resistance to apramycin/gentamicin compared with those from healthy pigs ($p < 0.005$). This could be related with the amounts of apramycin and gentamicin exposed to diseased pigs, as these antimicrobials are commonly used for prevention and treatment of enteric disease in pigs in Korea. A number of authors have also reported that pathogenic bacteria from diseased pigs were more likely to present antimicrobial resistance than those from healthy animals or from processed foods of animal origin (Jensen *et al.*, 2006; Garcia-Feliz *et al.*, 2008). The gap between resistance rates of indicator *E. coli* and clinical ones may also have been partly due to age difference between the sample groups. In this study, fecal samples for isolation of indicator *E. coli* were collected from healthy finishing pigs in slaughterhouses, whereas fecal samples for clinical isolates were originated from various age groups of diseased pigs. Previous studies have shown that resistance frequencies are usually higher in younger animals (Boerlin *et al.*, 2005).

We found that aminoglycoside-modifying enzyme (AME) 3-N-aminoglycoside acetyltransferase type IV [*aac(3)-IV*] was detected in all apramycin/gentamicin-resistant *E. coli* isolates. The *aac(3)-IV* gene confers apramycin resistance and also encodes cross resistance to other aminoglycosides such as gentamicin, tobramycin, and netilmicin (Chaslus-Dancla *et al.*, 1986). This gene was also found in *Salmonella* spp. and *E. coli* causing human infection (Pohl *et al.*, 1993; Zhang *et al.*, 2009).

Gentamicin-resistant *E. coli* isolated from pigs and cattle carried two or three different AME genes in the same strains (Sandvang and Aarestrup, 2000). Similarly, we found that about half of apramycin/gentamicin-resistant *E. coli* isolates carried other AMEs in addition to the *aac(3)-IV* gene. Among the four other genes, the most frequently observed was *aac(3)-III* (64/164, 39.0%), followed by *aac(3)-II* (24/164, 14.6%). This finding differs from the result of a study from Denmark (Sandvang and Aarestrup, 2000), in which *ant(2″)-I* and *aac(3)-II* were mainly present in gentamicin-resistant *E. coli* strains from cattle and pigs, respectively. Difference in usage pattern of antibiotics between the countries might be one of the reasons. Based on the variations in intensity and patterns of aminoglycoside usage, different combinations of AMEs have been selected in clinical strains in different countries and sometimes even in different hospitals within a given country. Both *aac(3)-II* and *aac(3)-III* enzymes produce resistance to gentamicin, tobramycin, sisomicin, netilmicin, and dibekacin (Vakulenko and Mobashery, 2003). In addition, *aac(3)-III* enzymes also produce resistance to kanamycin, neomycin, paromomycin, and lividomycin (Vakulenko and Mobashery, 2003). In Korea, neomycin accounts for about 50% of the amount of aminglycoside antimicrobials used in food-producing animals (NARMP, 2004–2008). Massive use of neomycin may have resulted in more selection of the *aac(3)-III* gene that encodes the resistance to neomycin in this study.

An interesting finding of this study is the detection of the *armA* gene in two pathogenic *E. coli* isolates, presenting high-level resistance to gentamicin and tobramycin. The two isolates were detected from diarrheic pigs in different farms in 2006. One isolate carried F6 (987P) and heat-labile toxin gene, whereas the other one carried F5 (K99). The isolates showed multiple resistances to antimicrobial agents such as amikacin, ampicillin, tetracycline, nalidixic acid, and trimethoprim/sulfamethoxazol. Unlike modifying enzymes that vary in their substrate ranges, the acquired methylases confer high-level resistance to most of the clinically important aminoglycosides (González-Zorn *et al.*, 2005). Similar to our finding, animal origin bacteria containing *armA* gene have also been previously reported (González-Zorn *et al.*, 2005). Various species of human Enterobacteriaceae isolates containing *armA* gene have also been reported from several European countries (González-Zorn *et al.*, 2005) and Korea (Kang *et al.*, 2008). Accordingly, the importance of coordinated surveillance of human and animal isolates has also been documented (González-Zorn *et al.*, 2005).

In the present study, we investigated the prevalence of resistance to apramycin in indicator *E. coli* isolates from food animals for the first time in Korea. Also, gentamicin/apramycin cross resistance and genotypes of these resistant isolates from healthy and diseased pigs were also determined for the first time in this country. The results of this study suggest that humans may be at risk of gentamicin resistance due to apramycin use in animals, indicating that more prudent use of apramycin in the pig production system is needed.

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Disclosure Statement

No competing financial interests exist.

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