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<Original Article>

Comparison of antibiotic resistance profiles for *Escherichia coli* isolated from wild boar and domestic pig fecal samples

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Abstract : Increasing presence of wild boar around cities and suburban areas is a growing concern with respect to agronomy, environmental ecology, and public safety. In this study, antibiotic resistance profiles of *Escherichia* (*E.*) *coli* isolated from wild boar and domestic pig fecal samples were compared. Eighty *E. coli* samples were isolated from wild boars. Resistance of the bacteria to 14 common antimicrobial agents used in human and veterinary medicine was evaluated. Ninety-five *E. coli* isolates from domestic pig farms were used for comparison. Common and distinct antibiotic resistance patterns were observed when comparing wild boar and domestic pig isolates, indicating that wild boars may significantly influence environmental microbiology.

Keywords: antibiotic resistance, domestic pigs, *Escherichia coli*, feces, wild boar

Introduction

The parallel growth of wild boar population and urban area caused boar migration to peri-urban area, which have resulted in the frequent appearance of wild boars around cities and suburban areas (Table 1) [18]. Subsequently, the transmission of microbes from wild boar fecal material to farms or domestic animals and the public via a number of routes is becoming more likely [13]. Because of the potential of contamination, the antimicrobial sensitivity profile of the wild boar is considered important. Even though many investigations about the antibiotic resistance of intestinal microflora in wild animals have been conducted and reported worldwide [17, 20], there are no reports about antibiotic resistance in wild boar fecal bacteria in Korea. Therefore, a surveillance study of antibiotic resistance in the wild boar was performed. Domestic pigs were selected as a representative group of livestock, and antibiotic resistance of this domestic group was investigated in order to assess the effect of indiscriminate domestic farm-use of antibiotics on antibiotic resistance in wild boars [9]. *Escherichia* (*E.*) *coli* was selected as a bacterium to survey antibiotic sensitivity for two reasons. First, *E. coli* is a common component of the gut flora in the animals used for meat production and can therefore serve as an indicator of enteric organisms acquiring resistance to various antimicrobials [16]. Second, changes in the antibiotic resistance of *E. coli* may serve as an early warning of the development of resistance by related pathogenic bacteria [4, 5]. In this study, resistance of wild boar and domestic pig fecal bacteria to various antimicrobial agents was investigated, and the two antibiotic sensitivity profiles of fecal bacteria were compared.

Table 1. Appearance and captured numbers of wild boars around cities in Korea (data provided by the Ministry of Environment, the Republic of Korea)

Year	Number of wild boar	
	Appearance	Captured
2010	79	27
2011	380	194
2012	641	195
Total	1,100	416

Materials and Methods

Sixty-seven fecal samples were collected from free-ranging wild boars hunted in the Gyeonggi province of Korea between March 2010 and March 2012. In order to avoid contamination, the fecal samples were taken directly from the rectum after death. According to the enterobacteria preservation protocol, the samples were transported immediately to the laboratory in ice-cooled containers and stored in a trypticase soy broth-glycerol freezing medium (Becton, Dickinson and Company, USA) at −70°C [24]. Sixty-four fecal samples

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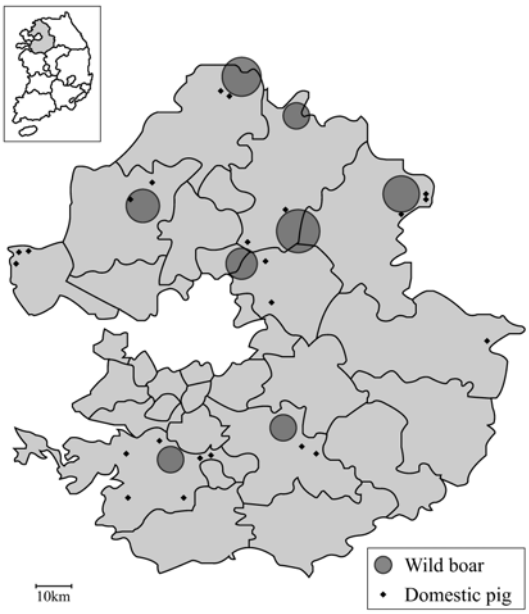

Fig. 1. Geographic regions in which wild boar and domestic pig fecal samples were collected in Gyeonggi province, Korea. The regions where wild boars were hunted are shown in grey circles with different diameter, while the site in which domestic pig farms were located are indicated as black diamond.

of domestic growing or finisher pigs were collected by picking up droppings in pigsties between April 2010 and October 2012 from 24 different pig farms located in Gyeonggi province. The samples were treated in the same manner as those from wild boars. All the farms were raising a cross breed between Durok males and Landrace × Yorkshire females. Geographic regions where wild boars were hunted (5 regions) and domestic pig farms (7 regions) were located are indicated in Fig. 1, and the area of Gyeonggi province is 10,185.6 km^2.

Approximately 4 g feces was taken from the preserved samples, placed in tubes with 10 mL brain heart infusion (BHI) broth (Becton, Dickinson and Company), and incubated at 35°C for 24 h. A sample (10 μL) of the turbid broth was removed, streaked onto MacConkey agar (Becton, Dickinson and Company), and incubated at 37°C for 18~20 h. The plates were examined for the development of red colonies that could precipitate bile and had dark red centers. To investigate the number of antibiotic-sensitivity profile types, 10 colonies were selected from each fecal sample [22] and streaked onto eosin methylene blue (EMB) agar (Becton, Dickinson and Company). The colonies were assumed to be *E. coli* if they showed a metallic sheen after incubation on EMB agar at 37°C for 24 h. The *E. coli* colonies were selected, inoculated into BHI, and incubated for 4~5 h until the culture became turbid. Bacteria from the broth were confirmed as *E. coli* based on Gram-staining and conventional biochemical tests such as the oxidase and catalase assays, using the BBL Crystal system (Becton, Dickinson and Company, Germany) and the Vitek system (BioMérieux, USA). Only bacterial isolates confirmed as *E. coli* were selected for antimicrobial agent sensitivity testing. Confirmed isolates were inoculated into fresh BHI and incubated until the turbidity measured 0.5 McFarland units (3~4 h).

The modified Kirby-Bauer disc diffusion method developed by the Clinical and Laboratory Standards Institute (CLSI) was used to determine the susceptibility of the *E. coli* isolates to 14 antimicrobial agents that were selected based on the suggested standard antimicrobial agents for Enterobactericeae [7] and the frequency of use of drugs in domestic pig farms. Because *E. coli* are naturally resistant to penicillin and spiramycin, these antibiotics were excluded from this study [21]. According to the initial screening and phenotypic confirmatory test for extended-spectrum beta-lactamases (ESBLs) provided by the CLSI, azetreonam for screening tests, and cefotaxime and ceftazidime, with or without clavulanic acid, for phenotypic confirmatory tests were also included [7]. These two tests were performed for *E. coli* isolates showing resistance to cefotaxime. The antimicrobial compounds used in this study were tetracycline (30 μg/disc), gentamicin (10 μg/disc), amoxicillin/clavulanic acid (20/10 μg/disc), norfloxacin (10 μg/disc), ampicillin (10 μg/disc), streptomycin (10 μg/disc), cefazolin (30 μg/disc), amikacin (30 μg/disc), trimethoprim (5 μg/disc), chloramphenicol (30 μg/disc), tobramycin (10 μg/disc), cefotaxime (30 μg/disc), cefoxitin (30 μg/disc), azetreonam (30 μg/disc), ceftazidime (30 μg/disc), and cefotaxime/clavulanic acid (30/10 μg/disc), ceftazidime/clavulanic acid (30/10 μg/disc). The antimicrobial discs (Becton Dickinson, USA) were obtained from Becton, Dickinson and Company.

Samples of the *E. coli* isolates grown in BHI to a turbidity of 0.5 McFarland Standard were swabbed onto Mueller-Hinton agar (Becton, Dickinson and Company). Fourteen prepared antimicrobial discs were placed onto the inoculated plates, and the plates were incubated at 35°C for 18 to 20 h. The diameters of the inhibition zones surrounding the antimicrobial discs were used to determine susceptibility or resistance based on the criteria suggested by the CLSI [7].

E. coli isolates having the same antibiotic resistance pattern for each fecal sample were counted as one. In total, eighty *E. coli* isolates from wild boars and 95 *E. coli* isolates from domestic pigs were included for the comparative and statistical analysis to compare antimicrobial profiles between the two groups (Tables 2 and 3). The Fisher's exact test with a 95% confidence interval (95% CI) was performed using SPSS 19.0 (SPSS, USA) to clarify the difference between the group of domestic pigs that were directly exposed to antibiotics and wild boars that had not been exposed to antibiotics directly. At $p < 0.05$, the associations were considered statistically significant and odds ratios (ORs) and 95% confidence intervals were calculated.

Results

For each wild boar fecal sample, 10 colonies were selected

Table 2. Antibiotic resistance of *Escherichia* (*E.*) *coli* isolated from wild boars and domestic pigs

Class	Antimicrobials	Diffusion zone breakpoint (mm)	Number (%) of resistant isolates	
			Wild boar (n = 80)	Domestic pig (n = 95)
Penicillins	Ampicillin*	13	5 (6.3)	70 (73.7)
	Amoxicillin/clavulanic acid	13	2 (2.5)	25 (26.3)
Tetracyclines	Tetracycline*	11	4 (5.0)	69 (72.6)
Cephalosporin	Cefazolin†	19	12 (15.0)	30 (31.6)
	Cefotaxime†	22	3 (3.8)	11 (11.6)
	Cefoxitin	14	0 (0)	2 (2.1)
Aminoglycoside	Streptomycin*	11	0 (0)	72 (75.8)
	Gentamicin	12	0 (0)	12 (12.6)
	Amikacin	14	0 (0)	22 (23.2)
	Tobramycin	12	0 (0)	4 (4.2)
Sulfonamides	Trimethoprim	10	0 (0)	52 (54.7)
Phenicols	Chloramphenicol	12	0 (0)	58 (61.1)
Fluoroquinolones	Ciprofloxacin	15	0 (0)	20 (21.1)
	Norfloxacin	12	0 (0)	17 (17.9)

*Over 70% of domestic *E. coli* isolates showed resistance to three antimicrobial agents (ampicillin, tetracycline, streptomycin). †High levels of resistance to cefazolin and cefotaxime were detected in wild boar isolates. Otherwise, the resistance to these antibiotics was relatively low in domestic pig isolates when compared to that of other antibiotics.

Table 3. Antibiotic resistance patterns of *Escherichia coli* isolated from wild boars and domestic pigs

Resistance patterns	Number (%) of resistant isolates	Resistance patterns	Number (%) of resistant isolates
	wild boar (n = 80)		domestic pig (n = 95)
–	62 (77.5)	S-AM-T-C-TM	19 (20.0)
CZ	6 (7.5)	AM-T-C-TM-CZ-AmC	13 (13.7)
CTX	3 (3.8)	S-T-AN	10 (10.5)
AmC-AM-CZ	2 (2.5)	S-AM-T-TM	9 (9.5)
AM-CZ	2 (2.5)	S-CZ-AN	9 (9.5)
T	2 (2.5)	AM-T-C-CIP-NOR-CTX	8 (8.4)
T-CZ	2 (2.5)	S-AM-C	6 (6.3)
AM	1 (1.3)	S-AM-C-AmC	5 (5.3)
		S-AM-T-C-AmC-CIP-NOR-GM	4 (4.2)
		AM-T-C-TM-C-NOR-GM	3 (3.2)
		S-TM-CZ-CIP-GM	3 (3.2)
		S-TM-CZ-GM-CTX-NN	3 (3.2)
		AM-T-C-AmC-AN-CIP-NOR-FOX	1 (1.1)
		AM-T-C-TM-CZ-AmC-AN	1 (1.1)
		S-AM-T-C-TM-CZ-AmC-AN-CIP-NOR-GM-NN-FOX	1 (1.1)

S; streptomycin, AM; ampicillin, T; tetracycline, C; chloramphenicol, TM; trimethoprim, CZ; cefazolin, AmC; amoxicillin/clavulanic acid, AN; amikacin, CIP; ciprofloxacin, NOR; orfloxacin, GM; gentamicin, CTX; cefotaxime, NN; tobramycin, FOX; cefoxitin.

from the MacConkey agar plates, yielding 670 total colonies, of which 643 were identified as *E. coli*. Among the 640 colonies isolated from domestic pig fecal samples, 621 were identified as *E. coli*. Antibiotic sensitivity profiles of confirmed *E. coli* isolates from each fecal sample were determined by the CLSI disc diffusion method. The average number of different antibiotic sensitivity profiles for wild boar and domestic pig fecal samples was 1.2 ± 0.4 and 1.5 ± 0.7, respectively.

Statistical analysis of differences between the domestic and wild boar groups revealed that resistance to 11 antimicrobial agents (streptomycin, ampicillin, tetracycline, chlorampheni-

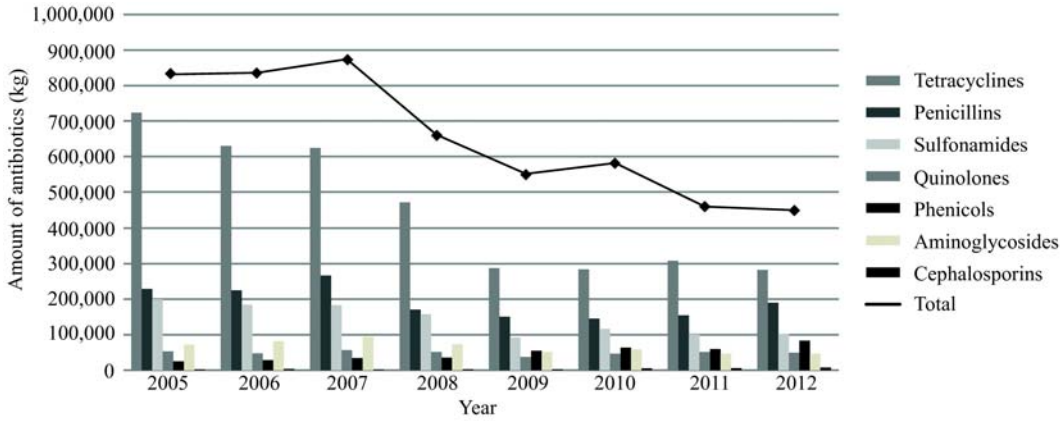

Fig. 2. The amount of antibiotics used in the Korean livestock industry from 2005 to 2012. The total amount (in kg) of each series of antibiotics used in swine production is indicated by the heights of the bars. 'Total' means the total amount of antimicrobial agents used in swine production (data provided by the Korea Animal Health Product Association).

col, trimethoprim, cefazolin, amoxicillin/clavulanic acid, amikacin, ciprofloxacin, norfloxacin and gentamicin) was statistically significant ($p < 0.05$). ORs (and 95% confidence intervals) for ampicillin, tetracycline, amoxicillin/clavulanic acid, and cefazolin were 44.4 (16.0-122.7), 50.4 (16.8-151.8), 13.9 (3.2-60.9), and 2.6 (1.2-5.5), and the p values of cefotaxime, tobramycin, and cefoxitin were 0.091, 0.126, and 0.501, respectively.

The most frequently observed antibiotic resistance in the wild boar samples was to cefazolin (15.0%), followed by ampicillin (6.3%), cefotaxime (3.8%), tetracycline (5%), and amoxicillin/clavulanic acid (2.5%). On the other hand, nearly 70% of domestic pig-derived isolates showed resistance to streptomycin, ampicillin, and tetracycline. Resistances to chloramphenicol (61.1%), trimethoprim (54.7%), cefazolin (31.6%), amoxicillin/clavulanic acid (26.3%), amikacin (23.1%), norfloxacin (17.9%), gentamicin (12.6%), cefotaxime (11.6%), tobramycin (4.2%), and cefoxitin (2.1%) were also observed in the domestic isolates (Table 2). In addition, all of the wild boar and domestic pig-derived *E. coli* isolates resistant to cefotaxime were identified as non-ESBL producers.

While there were no antimicrobial agents that had an effect on all domestic *E. coli* isolates, all *E. coli* isolates from wild boar were susceptible to 9 of the 14 antimicrobial agents (amikacin, cefoxitin, chloramphenicol, ciprofloxacin, gentamicin, norfloxacin, streptomycin, tobramycin, trimethoprim). Among the 9 antimicrobial agents, 3 agents (chloramphenicol, streptomycin, and trimethoprim) had less of an inhibitory effect on domestic pig *E. coli* isolates than the other 6 agents. Especially, streptomycin inhibited only 24.2% of the domestic isolates. Resistance to 5 of the 14 antimicrobial agents (ampicillin, amoxicillin/clavulanic acid, cefazolin, cefotaxime, and tetracycline) was identified in both wild and domestic *E. coli* isolates. About 73% of the domestic *E. coli* isolates showed resistance to ampicillin or tetracycline (Table 2).

More *E. coli* isolates from wild boar showed resistance to a single antimicrobial agent (cephazolin, cefotaxime, tetracycline, and ampicillin) than to two or more antimicrobial agents. In contrast, only multidrug resistance was observed in domestic isolates. The most frequently observed antibiotic resistance patterns in wild boars and domestic pigs were cefazolin and streptomycin-ampicillin-tetracycline-chloramphenicol-trimethoprim, respectively (Table 3).

Discussion

A proportional relationship between antibiotic resistance and the amount of exposed antibiotics is well documented [3, 20]. While the prevalence of antibiotic resistance in domestic animals is generally high, that of wild animals is relatively low because wild animals are not intentionally exposed to large quantities of antimicrobial agents, and there is no known route for them to consume a large variety of antibiotics [20, 21]. This known correlation between the extent of exposure to antibiotics and antibiotic resistance was confirmed in the present study through the statistical analysis comparing the antimicrobial profiles of *E. coli* isolates derived from wild boars and domestic pigs. Even though the p-values of resistance to some antimicrobial agents such as cefotaxime, cefoxitin, and tobramycin were larger than 0.05, these exceptions are presumed to have been caused by the low use of these antibiotics in domestic pig farms, considering that the antibiotic resistance rate in domestic *E. coli* isolates is very low (Table 2).

The antibiotic resistance of pathogenic bacteria has become a social issue; therefore, many countries, including Korea, have been making efforts to regulate the use of antibiotics as additives to livestock food. As shown in Fig. 2, the total amount of antibiotics used in swine production and the quantity of tetracyclines, sulfonamides, and aminoglycosides used in livestock production have declined since 2005. On the other hand, the use of phenicols and cephalosporins has increased. Decreased resistance to tetracycline and increased resistance to phenicols and cephalosporins of domestic *E.*

coli isolates were observed compared to resistance patterns reported for 2003 and 2004 [12], which is consistent with the consumption changes of these antibiotics.

Tetracyclines and penicillins have been the most widely used antibiotics for 8 years in livestock production. In both wild boar and domestic pig-derived *E. coli* isolates, relatively high rates of resistance to ampicillin or tetracycline were observed. However, despite the small amount of use of aminoglycosides, domestic *E. coli* isolates showed the highest resistance to streptomycin (75.8%), which could be partially explained by the following two factors. High-level resistance to streptomycin is known to commonly occur by chromosomal mutations affecting ribosome affinity, and resistance to streptomycin via an altered ribosome-binding site occurs more readily than for the other aminoglycosides because streptomycin binds to a single site on the 30S subunit of ribosomes. Unlike streptomycin, the other aminoglycosides bind to multiple sites on both the 50S and 30S subunits [2, 8, 11, 14]. However, additional research, including genetic investigations of ribosome-coding genes, should be performed to establish the exact cause of high resistance rates to streptomycin. Furthermore, continuous efforts to reduce the quantity of penicillins, tetracyclines, and aminoglycosides being used in livestock production are needed to control antibiotic resistance to these agents.

Interestingly, even though a lower percentage of domestic isolates were resistant to cefazolin and cefotaxime than to streptomycin, ampicillin, tetracycline, chloramphenicol, or trimethoprim, resistance to these cephalosporins in wild boar isolates was relatively high. From this result, two possible hypotheses can be proposed. One hypothesis is that *E. coli* easily acquires resistance to cephalosporins because even though the use of cephalosporins has been increasing, the amount of cephalosporins used relative to other antibiotics is still small. Cefoxitin should be excluded in this case because its use in domestic pig farms is thought to be very low, which is supported by the fact that the rate of resistance to cefoxitin in domestic isolates is low and the p-value corresponding to cefoxitin resistance was greater than 0.05.

Another possible hypothesis for the development of relatively high resistance to cefazolin and cefotaxime is that resistant bacteria and resistance genes reach the environment through sewage, for example, and are transmitted to wild boar. In fact, cephalosporins are used widely in veterinary medicine. For example, the third-generation cephalosporin ceftiofur is commonly used to prevent mastitis in dairy cattle, and high doses are administered shortly before their slaughter. Bacteria that are resistant to ceftiofur are frequently resistant to other important cephalosporins such as cefazolin [15]. Epidemiological surveys around wild boar habitats and investigations into cephalosporin resistance may be required to better understand these research findings.

Cephalosporins are widely used antibiotics in human medicine, especially for treating pneumonia and skin and soft tissue infections. Although there were no suspected ESBL-producing *E. coli* isolates in wild boars and domestic pigs, the development of ESBL-producing *E. coli* could occur in both species of pigs given that cephalosporin use has been increasing. While ESBL-producing *E. coli* is more likely to develop in domestic pigs than in wild boars because of much more chances to be located under direct exposure to cephalosporins, the possibility of the occurrence of ESBL-producing *E. coli* in wild boar cannot be ignored considering the relatively high prevalence of cephalosporin resistance in wild boar isolates. The occurrence of ESBL-producing *E. coli* in domestic pig represents an obvious risk for fecal-oral transmission to human especially through contaminated meat [23] and this problem could become persistent and widespread by the incidence of ESBL-producing *E. coli* isolates in wild animals including wild boar. That is because wild life has the potential to serve as reservoirs and transfer vector of antimicrobial resistance, which would severely threaten public health [1, 19]. There were no chloramphenicol-resistant *E. coli* isolates in the wild boar fecal samples. However, given the increase in the use of phenicols, as described in Fig. 2, and the high levels of resistance to chloramphenicol (61.1%) in domestic pig farms, resistance of chloramphenicol in wild boar is likely to occur and the presence of that in wild boar would threaten public health in the same way described above. Thus, regulation of cephalosporin and phenicol use in domestic animals farms is highly recommended.

All *E. coli* isolates from wild boars were susceptible to fluoroquinolones, but some isolates from domestic pigs were resistant. Considering that fluoroquinolones are among the most effective antimicrobials used in the treatment of human infections, and that development of resistance to one agent leads to cross-resistance to other fluoroquinolones [6], this result represents a substantial threat to public health. Many countries are reducing the use of these antimicrobials in livestock [10]. In Korea, the use of 4 types of fluoroquinolones (ciprofloxacin, norfloxacin, pefloxacin, and ofloxacin) in livestock has been prohibited by law since 2008. However, more strict restrictions on the use of fluoroquinolones in livestock are needed as soon as possible.

Previous research showed that restrictions on antimicrobial use in livestock decreased antibiotic resistance in livestock, which encourages efforts to reduce the amount of antibiotics used in livestock production. Both common and distinct aspects of the antibiotic resistance profiles were observed between domestic pig isolates and wild boar isolates in this study. Our results indicate that while there is a need for more detailed epidemiological studies to investigate the exposure to antimicrobial agents in the environment and analyze antibiotic resistance genes, wild boars could also be used as an indicator of antibiotic presence in the environment, and as a tool for research on the formation of antibiotic resistance, as demonstrated by the observed cephalosporin resistance. However, given that the frequency of contact between wild boar and human-dominated environments is increasing along with wild boar population growth and natural habitat distur-

bance, besides influencing the microflora of livestock, wild boar could substantially threaten public health and environmental ecology, especially via their droppings. Therefore, further efforts to control the wild boar population, as well as epidemiological investigations with periodical surveillance of the antibiotic resistance of the normal flora of wild boars and domestic pigs are necessary.

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