

Characteristics of Antimicrobial Resistance in *Escherichia Coli* Isolated from Fecal and Semen of Pigs

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Abstract: This study was analyzed and explained about antimicrobial resistance distribution and those mechanisms for 81 *Escherichia E. coli* isolates from diseased porcine feces and boar semen. In the study of 41 *E. coli* isolates from boar semen they showed high resistance to some antibiotics such as sulfamethoxazole (100%), tetracyclin (100%), streptomycin (92.7%), trimethoprim (80.5%). However, they showed high susceptibilities to others antibiotics such as colistin (97.6%), amikacin (100%), ceftiofur (100%). On the other hand, 40 *E. coli* isolates from feces of diseased pigs resisted strongly to various antibiotics such as sulfamethoxazole (100%), chloramphenicol (100%), tetracyclin (97.5%), ampicillin (97.5%), spectinomycin (95%), streptomycin (95%), nalidixic acid (95%), kanamycin (92.5%), kanamycin (92.5%), trimethoprim (92.5%), apramycin (90%), florfenicol (82.5%), enrofloxacin (82.5%) and gentamicin (80%) but they showed high susceptibilities to some antibiotics such as ceftiofur (90%), amikacin (100%), colistin (100%). *E. coli* isolates from diseased porcine feces showed higher antimicrobial resistance with most of antibiotics than *E. coli* isolated from boar semen. In this study, 6 types of aminoglycoside resistance genes, between 8 types aminoglycoside resistance genes are detected from boar semen isolates and diseased porcine feces isolates (n = 42/81, 51.9%). Those types of genes are detected in diseased porcine feces isolates (95%) 9.5 times higher than boar semen isolates (9.8%). In this study, even if *E. coli* isolates from boar semen showed lower antimicrobial resistance than *E. coli* isolates from feces of diseased pigs. *E. coli* isolates from boar semen showed high antimicrobial resistance with most of antibiotics. In conclusion, the detection rates for antibiotics resistance genes has been increased internationally for several decades. Therefore, many livestock industries and associates are needed to start monitoring antimicrobial resistance changes consistently for proper selection of antibiotics and managing them efficiently.

Key words: Aminoglycoside resistance genes, antibiotics, boar semen, *Escherichia E. coli*, porcine feces, internationally

INTRODUCTION

In the pig industry in Korea, Artificial Insemination (AI) has been generalized to the degree that its penetration rate has reached 90%, making a great contribution to the development of the pig industry (Kim *et al.*, 2010; Maes *et al.*, 2008). In performing artificial insemination, the quality of diluted semen affects reproduction rate and litter size. Especially, an increased bacterial contamination results in abnormal sperm morphology, reduction of sperm motility and lifetime, ending up with a reduced reproduction rate as well as genital diseases, miscarriage, stillbirth, etc.

Therefore, in the process of artificial insemination, it is very crucial to keep the bacterial contamination low (Kim *et al.*, 2010; Martin *et al.*, 2010).

The commercial diluted semen of pig is the isotonic solution which increases sperm viability in case of having neutral pH (6.8-7.3) and contains energy source, various electrolytes and proper antibiotics (Althouse, 1997; Althouse *et al.*, 2000). The antibiotics in diluted semen is a fairly critical ingredient in terms of managing contaminated semen because the bacterial contamination is unavoidable at the time of semen collection (Althouse and Lu, 2005). Initially, the antibiotics in a diluted semen were limited to a mixture of

penicillin and streptomycine. But today the antibiotics have become diversified, including gentamycin, neomycin sulfate, ampicillin, polymyxin, enrofloxacin, lincomycin and spectinomycin (Althouse and Lu, 2005; Althouse *et al.*, 2000; Dziuk and Henshaw, 1958; Polge, 1956; Sone *et al.*, 1982). Among them, gentamicin and ceftiofur are mostly being used in Korea. These antibiotics work to restrict the development of bacteria or sterilize. However, due to misuses and abuses of the antibiotics, antibiotic-resistant bacteria emerged, so use of antibiotics has been limited.

Gentamicin which is most popular in Korea for diluted semen is an aminoglycoside antibiotic and has cross-tolerance with apramycin. And *E. coli* emitted from pigs serve as a major carrier for transmitting gentamicin resistance gene or transferring resistance from animals to humans (Jensen *et al.*, 2006). There were some reports that non-pathogenic and resident bacteria were observed in a healthy child the increased antibiotic resistance of non-pathogenic and resident bacteria escalates the possibility of antibiotic resistance transfer to pathogenic bacteria. It is known that contamination source of diluted semen would rather be some polluted bacteria from outside than inside of pig genitalia and the possible external contamination sources include decal material of pig, pig's skin, instrument used to manufacture liquid sperm and its containers and some pollutants originated from manufacturers (Althouse and Lu, 2005; Althouse *et al.*, 2000; Kim *et al.*, 2010; Sone *et al.*, 1982; Tamuli *et al.*, 1984). The polluted bacteria which is detected most often is known as enteric bacteria which includes *Escherichia E. coli*, *Pseudomonas*, *Staphylococcus*, *Proteus sp.* (Martin *et al.*, 2010).

E. coli, among some polluting semen is gram negative bacteria and normal bacterial flora dwelling in intestines of animals which means such bacteria are widely distributed in nature. Not only is it to humans, a major pathogen causing the urinary tract infections such as orchitis and prostatitis but also it is most frequently detected as contamination bacteria in porcine semen (Althouse, 1997; Diemer *et al.*, 1996; Martin *et al.*, 2010). *E. coli* has been reported to reduce sperm motility and cause agglutination reaction (Diemer *et al.*, 1996). *E. coli* has mannose-binding structure so as to adhere to any surface of sperm. The adhesion causes sperm to agglutinate and damage the ultrastructure of sperm plasma cell membrane. In addition, *E. coli* takes spermatocidal action unless in an acidic milieu. These reactions of *E. coli* further affect litter size and bring about economical losses (Althouse *et al.*, 2000; Diemer *et al.*, 1996; Martin *et al.*, 2010; Ryu *et al.*, 2008).

E. coli has been used as an index to monitor antibiotic resistance and as studied on the interactions between *E. coli* and sperm, manufacturing diluted semen requires to take a strict control over bacteria and antibiotics. Today, researches on antibiotic resistance of pathogenic bacteria from fecal material are vital however, antibiotic resistance and mechanism of bacteria from semen have seldom been explored. This study based on this situation for the purpose of securing safe management, use and selection of antibiotics for diluents being used for artificial insemination, aimed to investigate over the antibiotic resistance characteristics of bacteria emitted from fecal material of pigs with diarrhea and from diluents in circulation.

MATERIALS AND METHODS

Bacterials and antibiotic susceptibility test (MIC): The total 81 *E. coli* isolates from diseased porcine feces and boar semen were used for this study. To determine antibiotic susceptibility over the 81 stains being tested, Microdilution Broth Method (MBD) was conducted in compliance with the method by Clinical Laboratory Standard Institute (CLSI). Using 96 well round bottom plates (Costar®, Corning, USA), antibiotics were twofold diluted into the final concentration of 128–2048 µg/mL, having 11 different phases with 50 uL each. The bacterial suspension was mixed with saline solution and adjusted to a turbidity of 0.5 Mcfarland (1X10⁸ CFU/mL). And then it was diluted at 1:100 with Mueller-hinton broth and divided into 50 uL per each well within 15 min, ending up being adjusted to 100 uL at last. Having both negative control well and positive control well on every place, it was done with aerobic culture in the environment of 37°C for 24 h. And then the degree of development was checked by eyes to determine MIC as the antibiotic concentration of the well in which a culture had been completely inhibited. *E. coli* ATCC 25922 was used for a positive control strain. In order to differentiate multi-drug resistant bacteria, 20 different kinds of antibiotics were categorized into 11 subclasses according to the standard of Clinical Laboratory Standard Institute subclass (CLSI), by which a comparative analysis was conducted on multi-drug resistance of *E. coli* separated from porcine semen and pigs with diarrhea.

Detection of resistance gene to aminoglycosides antibiotics: For DNA extraction from bacteria, 81 bacterial strains from porcine semen and pigs with diarrhea and *E. coli* ATCC 25922 were smeared on MacConkey agar (Difco, USA) which was followed by aerobic culture at 37°C for 18-24 h. And then a single colony was transferred

Table 1: Primers used for detecting aminoglycoside resistance genes

Target genes	Product size (bp)	Genes	Primer sequence (5'-3')	Annealing time (°C)	References
<i>aac(3)-I</i>	429	<i>aac(3)-I F</i>	GGGCATCATTCGCACATGTAGGC	65	27
		<i>aac(3)-I R</i>	CATCACTTCTCCCGTATGCC		
<i>aac(3)-II</i>	369	<i>aac(3)-II F</i>	TGAAacGCTGACGGAGCCTC	55	59
		<i>aac(3)-II R</i>	GTCGAacAGGTAGCACTGAC		
<i>aac(3)-III</i>	436	<i>aac(3)-III F</i>	GTGCATCGCAGCGCAAacCCC	65	27
		<i>aac(3)-III R</i>	CAAGCCACTGCACCGCAAacCG		
<i>aac(3)-IV</i>	628	<i>aac(3)-IV F</i>	GTGTGCTGCTGGTCCACAGC	62	59
		<i>aac(3)-IV R</i>	AGTTGACCCAGGGCTGTCCG		
<i>ant(2'')-I</i>	329	<i>ant(2'')-I F</i>	GGGCGCGTCATGGAGGAGTT	58	59
		<i>ant(2'')-I R</i>	TATCGCGACCTGAAAGCGGC		
<i>ArmA</i>	777	<i>Met-F</i>	GTGTGCTGCTGGTCCACAGC	55	18
		<i>Met-R</i>	AGTTGACCCAGGGCTGTCCG		
<i>aac(6)-Ib</i>	285	<i>aac(6)-Ib F</i>	CCGACACTTGCTGACGTACAG	55	44
		<i>aac(6)-Ib R</i>	TGGATCTTGGTGACCTCGG		
<i>AadB</i>	208	<i>aadB-F</i>	GAGGAGTTGGAATATGGATT	55	69
		<i>aadB-R</i>	CTTCATCGGCATAGTAAAA		

to Tryptic soy agar on the same condition. A single colony was mixed with distilled water of 500 uL and then boiled for 10 min which then was taken to centrifugal separation process at 1200×g for 10 min. Finally, Genomic DNA was extracted from the supernatant liquid.

The detection for aminoglycosides resistance gene was conducted in compliance with the method of Jakobsen *et al.* (2008) and primer and PCR conditions used in this study are shown in Table 1. And it was done with 39 bacterial strains from porcine semen and 40 strains from pigs with diarrhea that had shown resistance to aminoglycosides antibiotics-amikacin, apramycin, gentamicin, kanamycin, neomycin and streptomycin. PCR was conducted, using commercial AccuPower® HFPCR PreMix (Bioneer, Korea) with PCR mixture (30 mM Kcl, 2.5 mM MgCl₂, 10 mM Tris/HCl (pH 9.0) 1 unit Taq DNA polymerase, 4×deoxy Nucleotide Triphos Phate (dNTP) 250 mM (Bioneer, Daejeon, Korea) that was adjusted to the final volume of 20 uL by distilled water, following the addition of primer forward and reverse of 1 uL diluted by 10 pmole/uL of genomic DNA of 2 uL.

PCR-amplified products, for their band to be verified were taken, using 1.5% agarose gel mixed with 0.5 µg/mL ethidium bromide to electrophoresis under 140 V for 40 min and then to an examination under UV transilluminator (BIO-RAD Laboratories, Inc., USA). Gene sequencing for the PCR products was conducted by using BLAST tool that was available online, National center for Biotechnology information web site (www.ncbi.nlm.nih.gov/BLAST). Buffer and temperature to treat with restriction enzyme in detecting *aac(6)-Ib-cr* were matched with the instruction of the manufacturer (BtsCI, New England Biolabs, Ipswich, MA), ending up with PCR product of 10 uL being added with restriction enzyme BtsCI of 15-20 U. To check on DNA cleavage, Electrophoresis under 140 V for 60 min by using 1.5% agarose gel and examination of UV transilluminator (BIO-RAD Laboratories, Inc., USA) were conducted.

Because the deformed *aac(6)-Ib-cr* was not digested, only a band (482 bp) was observed while 2 bands (272 bp, 210 bp) were observed for wild-type *aac(6)-Ib*.

RESULTS

Antibiotic resistance patterns: The results from investigating over 41 strains of bacterial contaminants isolated from porcine semen and 40 stains of pathogenic bacteria separated from pigs with diarrhea were shown in Table 2. *E. coli* from porcine semen was found to have very high-level resistance to sulfamethoxazole (100%), tetracyclin (100%), streptomycin (92.7%) and trimethoprim (80.5) while having intermediate-level resistance to florfenicol (75.6%), chloramphenicol (65.9%), spectinomycin (56.1%), ampicillin (56.1%) and apramycin (51.2%). However, colistin was confirmed to have resistance only in one strain and amikacin and ceftiofur have 100% susceptibility. The susceptibility of gentamicin, being mostly used in Korea was found as 92.7%. In the meantime, bacteria isolated from pigs with diarrhea had higher resistance to most antibiotics than bacteria from porcine semen especially, sulfamethoxazole and chloramphenicol showed 100% resistance. In addition, bacteria isolated from pigs with diarrhea showed high-level resistance to such antibiotics as tetracyclin (97.5%), ampicillin (97.5%), spectinomycin (95%), streptomycin (95%), nalidixic acid (95%), kanamycin (92.5%), trimethoprim (92.5%), apramycin (90%), florfenicol (82.5%), enrofloxacin (82.5%) and gentamicin (80%) while having intermediate-level resistance to ciprofloxacin (75%), cephalothin (65%) and ceftioxin (42.5%). Only in 4 strains, antibiotic resistance was confirmed to ceftiofur, amikacin and ceftiofur had 100% susceptibility which result was the same as in bacteria from semen in terms of having low-level resistance.

Table 2: MIC of 81 *E. coli* from boar semen and feces of diarrheic pigs

Antibiotics	Sources	No. of isolates with indicated MIC (µg/mL) of <i>E. coli</i>										MIC ₅₀		MIC ₉₀		Resistance (%)	
		0.03	0.06	0.13	0.25	0.50	1	2	4	8	16	1	2	1	2	1	2
Ampicillin	A	-	-	-	1.00	-	1	10	3	2	1	256.00	>512.00	>512.00	>512.00	56.1	97.5
	D	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Amikicin	A	-	-	-	-	10.0	12	7	11	1	-	1.00	1.00	4.00	4.00	0.0	0.0
	D	-	-	-	-	5.0	19	8	7	-	1	-	-	-	-	-	-
Apramycin	A	-	-	-	-	-	-	-	1	7	12	32.00	>512.00	64.00	>512.00	51.2	90.0
	D	-	-	-	-	-	-	-	1	-	3	-	-	-	-	-	-
Cefoxitin	A	-	-	1.00	-	-	-	2	25	10	1	4.00	8.00	8.00	128.00	4.9	42.5
	D	-	-	-	-	-	1	2	10	9	1	-	-	-	-	-	-
Ceftiofur	A	-	-	-	2.00	10.0	14	11	4	-	-	1.00	2.00	2.00	4.00	0.0	10.0
	D	1.00	-	-	2.00	8.0	7	8	10	2	1	-	-	-	-	-	-
Cephalothin	A	-	-	-	-	-	-	1	5	22	8	8.00	32.00	32.00	>256.00	12.2	65.0
	D	-	-	-	-	-	1	-	1	5	7	-	-	-	-	-	-
Ciprofloxacin	A	27.00	-	-	2.00	3.0	2	1	3	1	1	<0.06	8.00	4.00	32.00	14.6	75.0
	D	2	-	-	1.00	-	1	6	4	6	7	-	-	-	-	-	-
Chloramphenicol	A	-	-	-	-	-	-	-	1	10	3	128.00	256.00	256.00	>256.00	65.9	100.0
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colistin	A	-	-	40.00	-	-	-	-	-	-	-	<0.25	<0.25	<0.25	<0.25	2.4	0.0
	D	-	-	36.00	-	1.0	-	-	1	2	-	-	-	-	-	-	-
Enrofloxacin	A	27.00	-	-	-	2.0	6	1	-	1	2	<0.06	8.00	8.00	>64.00	14.6	82.5
	D	3.00	-	-	1.00	-	3	2	5	8	6	-	-	-	-	-	-
Florfenicol	A	-	-	-	-	-	-	-	3	3	4	128.00	256.00	>256.00	>256.00	75.6	82.5
	D	-	-	-	-	-	-	-	-	3	4	-	-	-	-	-	-
Gentamicin	A	-	-	-	4.00	9.0	11	8	5	1	1	1.00	16.00	4.00	64.00	7.3	80.0
	D	-	-	-	-	2.0	-	-	1	5	15	-	-	-	-	-	-
Kanamycin	A	-	-	-	-	-	4	11	12	3	2	4.00	>256.00	>256.00	>256.00	19.5	92.5
	D	-	-	-	-	-	-	1	-	-	2	-	-	-	-	-	-
Nalidixic acid	A	-	-	-	-	-	11	12	4	-	2	2.00	>256.00	>256.00	>256.00	29.3	95.0
	D	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-
Neomycin	A	-	-	10.00	-	5.0	6	5	2	3	2	1.00	64.00	64.00	256.00	24.4	92.5
	D	-	-	-	-	2.0	1	-	-	-	1	-	-	-	-	-	-
Spectinomycin	A	-	-	-	-	-	-	-	-	-	13	64.00	1024.00	1024.00	>1024.00	56.1	95
	D	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Streptomycin	A	-	-	-	-	-	-	-	2	1	-	256.00	256.00	>256.00	>256.00	92.7	95.0
	D	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-
Sulfamethoxazole	A	-	-	-	-	-	-	-	-	-	-	>1024.00	>2048.00	>1024.00	>2048.00	100.0	100
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetracyclin	A	-	-	-	-	-	-	-	-	-	-	256.00	128.00	256.00	256.00	100.0	97.5
	D	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Trimethoprim	A	-	-	5.00	-	2.0	-	1	1	1	13	16.00	32.00	64.00	64.00	80.5	92.5
	D	-	-	1.00	-	1.0	1	-	-	1	3	-	-	-	-	-	-

Antibiotics	Sources	No. of isolates with indicated MIC (µg/mL) of <i>E. coli</i>									MIC ₅₀		MIC ₉₀		Resistance (%)	
		32	64	128	256	512	1024	2048	4096	1	2	1	2	1	2	
Ampicillin	A	-	-	-	5	11	7	-	-	-	256.00	>512.00	>512.00	>512.00	56.1	97.5
	D	-	-	3	3	10	23	-	-	-	-	-	-	-	-	-
aacAmikicin	A	-	-	-	-	-	-	-	-	-	1.00	1.00	4.00	4.00	0.0	0.0
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Apramycin	A	13	6	-	-	-	2	-	-	-	32.00	>512.00	64.00	>512.00	51.2	90.0
	D	-	-	-	1	4	31	-	-	-	-	-	-	-	-	-
Cefoxitin	A	2	-	-	-	-	-	-	-	-	4.00	8.00	8.00	128.00	4.9	42.5
	D	3	9	3	1	1	-	-	-	-	-	-	-	-	-	-
Ceftiofur	A	-	-	-	-	-	-	-	-	-	1.00	2.00	2.00	4.00	0.0	10.0
	D	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Cephalothin	A	2	-	1	-	2	-	1	-	-	8.00	32.00	32.00	>256.00	12.2	65.0
	D	6	2	-	-	18	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin	A	1	-	-	-	-	-	-	-	-	<0.06	8.00	4.00	32.00	14.6	75.0
	D	11	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloramphenicol	A	1	3	5	16	2	-	-	-	-	128.00	256.00	256.00	>256.00	65.9	100.0
	D	1	3	13	14	9	-	-	-	-	-	-	-	-	-	-
Colistin	A	-	-	-	1	-	-	-	-	-	<0.25	<0.25	<0.25	<0.25	2.4	0.0
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enrofloxacin	A	2	-	-	-	-	-	-	-	-	<0.06	8.00	8.00	>64.00	14.6	82.5
	D	5	2	5	-	-	-	-	-	-	-	-	-	-	-	-
Florfenicol	A	5	4	6	6	10	-	-	-	-	128.00	256.00	>256.00	>256.00	75.6	82.5
	D	3	2	3	10	15	-	-	-	-	-	-	-	-	-	-

Table 2: Continue

Antibiotics	Sources	No. of isolates with indicated MIC (µg/mL) of <i>E. coli</i>								MIC ₅₀		MIC ₉₀		Resistance (%)	
		32	64	128	256	512	1024	2048	4096	1	2	1	2	1	2
Gentamicin	A	1	1	-	-	-	-	-	-	1.00	16.00	4.00	64.00	7.30	80.0
	D	11	3	3	-	-	-	-	-	-	-	-	-	-	-
Kanamycin	A	1	-	1	1	6	-	-	-	4.00	>256.00	>256.00	>256.00	19.50	92.5
	D	-	-	2	-	35	-	-	-	-	-	-	-	-	-
Nalidixic acid	A	-	1	2	2	7	-	-	-	4.00	>256.00	>256.00	>256.00	29.30	95.0
	D	-	-	1	-	37	-	-	-	-	-	-	-	-	-
Neomycin	A	3	5	-	-	-	-	-	-	1.00	64.00	64.00	256.00	24.40	92.5
	D	5	11	13	3	4	-	-	-	-	-	-	-	-	-
Spectinomycin	A	5	3	3	7	4	6	-	-	64.00	1024.00	1024.00	>1024.00	56.95	95.0
	D	1	1	1	7	8	5	16	-	-	-	-	-	-	-
Streptomycin	A	1	2	6	13	16	-	-	-	256.00	256.00	>256.00	>256.00	92.70	95.0
	D	2	2	4	11	19	-	-	-	-	-	-	-	-	-
Sulfamethoxazole	A	-	-	-	-	-	-	4	37	>1024.00	>2048.00	>1024.00	>2048.00	100.00	100.0
	D	-	-	-	-	-	-	5	35	-	-	-	-	-	-
Tetracyclin	A	-	1	14	26	-	-	-	-	256.00	128.00	256.00	256.00	100.00	97.5
	D	-	2	19	16	2	-	-	-	-	-	-	-	-	-
Trimethoprim	A	13	5	-	-	-	-	-	-	16.00	32.00	64.00	64.00	80.50	92.5
	D	28	5	-	-	-	-	-	-	-	-	-	-	-	-

A, boar semen; D, diarrheic feces

Table 3: Multi-drug resistance percentage of *E. coli* from boar semen and feces of diarrheic pigs

Antibiotics	No. of resistance isolates (%)		
	Boar semen (n = 41)	Feces of diarrheic pigs (n = 40)	Total (n = 81)
No. resistance detected	0 (0.0)	0 (0.0)	0 (0.0)
Resistance 1 CLSI subclass	0 (0.0)	0 (0.0)	0 (0.0)
Resistance 2 CLSI subclasses	0 (0.0)	0 (0.0)	0 (0.0)
Resistance 3 CLSI subclasses	3 (7.3)	0 (0.0)	0 (0.0)
Resistance 4 CLSI subclasses	9 (22.0)	0 (0.0)	0 (0.0)
Resistance 5 CLSI subclasses	10 (24.4)	0 (0.0)	10 (12.3)
Resistance 6 CLSI subclasses	10 (24.4)	1 (2.5)	11 (13.6)
Resistance 7 CLSI subclasses	2 (4.9)	4 (10.0)	6 (7.4)
Resistance 8 CLSI subclasses	4 (9.8)	12 (30.0)	16 (19.8)
Resistance 9 CLSI subclasses	2 (4.9)	8 (20.0)	10 (12.3)
Resistance 10 CLSI subclasses	0 (0.0)	13 (32.5)	13 (16.0)
Resistance 11 CLSI subclasses	0 (0.0)	2 (5.0)	2 (2.5)

Multi-drug resistance patterns: The results from investigating over multi-drug resistance patterns of bacteria isolated from porcine semen and from pigs with diarrhea were shown in Table 3. There were no strains from porcine semen that belonged to lower than resistance 2 CLSI subclasses. 9 strains, 10 strains and 10 strains were respectively belonging to resistance 4 CLSI subclasses, resistance 5 CLSI subclasses and resistance 6 CLSI subclasses which means 70.7% of bacteria from semen belonged to resistance 4-6 CLSI subclasses. On the other hand, bacteria from pigs with diarrhea were found to belong to higher subclass than bacteria from semen which means to have higher-level resistance. Among strains of bacteria from pigs with diarrhea, no strain was found lower than resistance 5 CLSI subclasses; 82.5% of those belonged to resistance 8-10 CLSI subclasses which means to have 4-level higher resistance than bacteria from semen. In particular, among those, a single strain was found to belong to resistance 12 CLSI subclasses which means resistance to all kinds of antibiotics.

Detection of resistance gene to aminoglycosides antibiotics: In this study, any strains showing antibiotic resistance to more than 1 aminoglycoside antibiotics were investigated through PCR. As its result, 6 resistance genes [*aac(3)-IV*, *aac(3)-II*, *ant(2'')-I*, *aac(6)-Ib*, *aadB*, *aac(3)-III*] out of 8 resistance genes to aminoglycoside antibiotics were detected from bacteria from both semen and pigs with diarrhea (n = 42/81, 51.9%) but *aac(3)-I* and *armA* were not detected at all.

The 3 strains (7.4%) from semen and 32 strains (80%) from pigs with diarrhea were found to be resistant to gentamicin which is mainly used as the semen diluents along with other 3-5 antibiotics. More than a resistance gene were detected from all the 35 strains. All those 35 strains contained *aac(3)-IV* except for 3 others which contained *aac(3)-II* alone. In addition, among bacteria resistant to gentamicin, 3 strains from semen contained only 1 resistance gene. On the other hand, 26 strains, 4 strains and 2 strains from pigs with diarrhea have 1, 2, 3 resistance genes respectively which means majority bacteria contained only one resistance gene. Among 42

Table 4: Characterization of aminoglycosides including gentamicin resistant in 81 *E. coli*

Phenotype	Origin (%)		Genotype (No. of positive strain)
	Boar semen (n = 41)	Feces of diarrheic pigs (n = 40)	
AM GEN NEO SPX	0	1	aac(3)-II (n = 1)
AM GEN KAN SPX	1	0	aac(3)-II (n = 1)
GEN APR KAN NEO SPX	0	2	aac(3)-IV (n = 2)
AM APR GEN KAN NEO	0	1	aac(3)-IV (n = 1)
AM APR GEN KAN SPX	1	0	aac(3)-II (n = 1)
AM APR GEN KAN SPX	0	1	aac(3)-IV (n = 1)
AM APR GEN KAN SPX	0	1	aac(3)-IV aac (6)-Ib (n = 1)
AM APR GEN KAN NEO SPX	1	21	aac(3)-IV (n = 22)
AM APR GEN KAN NEO SPX	0	2	aac(3)-IV aac (3)-II (n = 2)
AM APR GEN KAN NEO SPX	0	1	aac(3)-IV aac (3)-III (n = 1)
AM APR GEN KAN NEO SPX	0	2	aac(3)-IV ant (2'')-I aadB (n = 2)
AM APR SPX	1	0	aac(3)-IV (n = 1)
AM APR NEO SPX	0	1	aac(3)-IV aac (6)-Ib (n = 1)
AM KAN NEO SPX	0	1	ant(2'')-I aadB (n = 1)
AM APR KAN NEO SPX	0	3	aac(3)-IV (n = 3)
AM APR KAN NEO SPX	0	1	aac(3)-IV aac (6)-Ib (n = 1)

AM, Amikacin; APR, Apramycin; GEM, Getamicin; KAN, Kanamycin; NEO, Neomycin; SPX, Streptomycin

strains where resistance genes were detected, aac (3)-IV was detected most frequently from 39 strains (48.1%) all the bacteria not only from semen but from pigs with diarrhea that contained aac (3)-IV had resistance to apramycin which shares a cross-tolerance with gentamicin. MIC toward apramycin was higher than 512 mg/L in all the strains which was high-level resistance, except for a strain (MIC 64mg/L). Genes such as aac(3)-II, aac(6)-Ib, ant(2'')-I, aadB and aac(3)-III, except for aac(3)-IV were respectively detected from 5 strains (6.2%), 3 strains (3.7%), 3 stains (3.7%), 3 stains (3.7%) and 1 stain (1.2%). Among bacteria from semen, aminoglycoside resistance gene was detected from 4 strains (9.8%), out of which only gene was detected. That is to say, aac(3)-IV (4.9%) and aac(3)-II (4.9%) were each detected from 2 strains and these 2 strains had antibiotic resistance to more than 3 kinds of aminoglycoside antibiotics. On the other hand, bacteria from pigs with diarrhea (95%) showed 9.5 times higher appearance ratio of resistance gene than bacteria from semen (9.8%). Bacteria from semen contained only two kinds of gene that is, aac(3)-IV, aac(3)-II whereas more diverse patterns as 5 different resistance genes were detected from bacteria from pigs with diarrhea. Among 38 strains (95%) from which resistance genes were detected, all except 2 strains contained aac(3)-IV. The results of phenotype and genotype detection in relation to antibiotic resistance to aminoglycoside antibiotics were shown in Table 4.

DISCUSSION

Today, Artificial Insemination (AI) is in worldwide usage including in Korea, making enormous contribution to the development of pig industry in terms of the

improvement of litter size, minimization of diseases and improvement of breeding stock, etc. (Althouse and Lu, 2005; Kim *et al.*, 2010; Maes *et al.*, 2008). It is known that bacterial contamination which is unavoidable due to the reason of isolating semen by fusion method, adversely affects pig industry, causing to reduce sperm properties, sperm motility and litter size (Althouse and Lu, 2005; Kim *et al.*, 2010; Martin *et al.*, 2010; Tamuli *et al.*, 1984). It is known that bacterial contaminants of semen derived from external environments and their harmfulness depend on the concentration of polluting bacteria (Althouse and Lu, 2005). It has been reported that *E. coli* among bacteria with high appearance ratio, depending on its concentration has overall adverse effects to sperm as reducing sperm motility and increasing agglutination (Kohn *et al.*, 1980; Monga and Roberts, 1994; Tamang *et al.*, 2012). Antibiotics are added into diluted semen to minimize the bacterial contamination but misuses and abuses of antibiotics bring about the degradation of sperm properties and the creation of antibiotic resistant bacteria. However, it is challenging to select the proper type of antibiotics and the optimal concentration to fulfill the purpose (Althouse and Lu, 2005; Kim *et al.*, 2010; Rayamajhi *et al.*, 2008). Furthermore, out of worries about the increasing antibiotic resistant bacteria due to the misuses of antibiotics, some argue that there is a requirement to monitor bacterial contamination and to reevaluate on the effect of antibiotics periodically (Kim *et al.*, 2010; Tamuli *et al.*, 1984). This study was aimed to look into the distribution of antibiotic resistance and its mechanism which can also be used as an index for monitoring antibiotic resistance for *E. coli* that can adversely affect sperm properties in case of semen being contaminated (Althouse *et al.*, 2000; Diemer *et al.*, 1996; Martin *et al.*, 2010; Ryu *et al.*, 2008). The antibiotics used

in this study consisted of aminoglycosides antibiotics which contained gentamicin that is popularly used for diluted semen on the market today in Korea, cephalosporins antibiotics and quinolone antibiotics that are in the list of noteworthy antibiotics by WHO/OIE (Althouse and Lu, 2005; Althouse *et al.*, 2000; Kim *et al.*, 2010). Antibiotics to which *E. coli* from semen appeared resistant were sulfamethoxazole (100%), tetracyclin (100%), streptomycin (92.7%) and trimethoprim (80.5%) in the order of its level. Meanwhile, amikacin and ceftiofur showed 100% susceptibility. The streptomycin, that used to be added into diluted semen in the past, showed high-level resistance. If some AI centers in Korea use these antibiotics, it seems that its replacement should be seriously considered. Gentamicin that is mostly used in Korea at present showed 92.7% susceptibility. Kim *et al.* (2010) and Althouse *et al.* (2000) reported that *E. coli* from porcine semen had high-level resistance to gentamicin which showed some discrepancy from the result of this study. According to Lim *et al.* (2007), *E. coli* from normal pigs showed 10% resistance to gentamicin which confirmed a significant result of this study. From now on, some antibiotics that are expected to increase in demand for diluting semen include ceftiofur and apramycin. Amikacin and ceftiofur, the susceptibility of which was found to be 100% in this study toward *E. coli* isolated from semen in Korea, seem to be recommended as dilution antibiotics. And apramycin which showed intermediate-level resistance (51.2%) is considered to have to be monitored about its cross-tolerance with gentamicin. Ham *et al.* (1997), during the year 1980-1990, reported that antibiotic resistance of *E. coli* isolated from pigs with diarrhea to tetracyclin, streptomycin and penicillin was very high whereas gentamicin, kanamycin, nalidixic acid, ampicillin and cephalothin had susceptibility. In this study, it was found that *E. coli* from diseased pigs had higher resistance than those from semen that is revealing high-level resistance to 17 antibiotics other than amikacin, ceftiofur and colistin. Gentamicin, kanamycin, neomycin, cephalothin, ciprofloxacin, enrofloxacin and nalidixic acid to which *E. coli* from semen had relatively low resistance however, *E. coli* from pigs with diarrhea had high-level resistance to them, especially, to gentamicin. *E. coli* from pigs with diarrhea (80%) had 10 times higher resistance than *E. coli* from semen (7.3%). Only 4 strains were found to be resistant to ceftiofur and amikacin and colistin had 100% susceptibility, being confirmed as the effective antibiotics for semen polluting bacteria. Resistance of *E. coli* from semen to cefoxitin (42.5%) and ceftiofur (10%) was low but *E. coli* from pigs with diarrhea had intermediate-level resistance and low-level resistance

to them respectively. Still, such level of *E. coli* from pigs with diarrhea was higher than those from semen-cefoxitin (4.9%) and ceftiofur (0%). In this study, amikacin showed 100% susceptibility to which *E. coli* both from semen and pigs with diarrhea had the lowest resistance. Based on this it can be inferred that amikacin had the lowest resistance because it was most stable for the inactivated enzyme of aminoglycoside. As the result of investigating over the distribution of multi-drug resistance, 70.7% strains of *E. coli* from semen belonged to resistance 4-6 CLSI subclasses whereas 82.5% strains of *E. coli* from pigs with diarrhea did to resistance 8-10 CLSI subclasses. It is generally known that resistance level of *E. coli* from pigs with diarrhea is higher than that of normal pigs (Slavic *et al.*, 2011; Yang *et al.*, 2004) reported that *E. coli* from pigs is an important carrier that transfers gentamicin-resistant gene to humans. According to the latest findings by Lim *et al.* (2007) in Korea, aac(3)-IV, aac(3)-II, aac(3)-III, ant(2'')-I and armA have been separated in order; apramycin-resistant strains contained more than 1 resistance gene. Furthermore, aac(3)-IV was separated from all the apramycin-resistant bacteria. In this study as the result of PCR with strains that appeared resistant to more than an aminoglycoside antibiotics, aac(3)-IV, aac(3)-II, ant(2'')-I, aac(6)-Ib, aadB and aac(3)-III among 8 kinds of aminoglycoside resistance genes were detected; however, aac(3)-I was neither detected, nor armA that encodes for the resistance to amikacin to which *E. coli* from both semen and pigs with diarrhea showed 100% susceptibility. In this study, as *E. coli* that have resistance to gentamicin which is mostly used to dilute semen, 3 strains (7.4%) from semen and 32 strains (80%) from pigs with diarrhea were confirmed and these strains showed resistance to 4-6 antibiotics including gentamicin. More than a resistance gene were detected from all the 35 strains of gentamicin resistance. From 32 out of 35 strains of gentamicin resistance was aac(3)-IV detected which was the highest detection ratio. According to some research literature, aac(3)-IV is stated to encode for the cross-tolerance of bacteria from animals to gentamicin, tobramycin, netilmicin and apramycin (Chaslus-Dancla and Lafont, 1985; Wray *et al.*, 1986). Shaw *et al.* (1991) and Klundert *et al.* (1984) reported that there was a significant correlation between the existence of resistance gene and the expression of resistance. In this study, as well, all the *E. coli* from both semen and pigs with diarrhea which contained aac(3)-IV showed resistance to gentamicin and apramycin; MIC to apramycin was higher than 512 mg/L, except for a strain (MIC 64 mg/L). Excluding aac(3)-IV, all other genes such as aac(3)-II, aac(6)-Ib, ant(2'')-I, aadB and aac(3)-III were respectively detected

from 5 strains (6.2%), 3 strains (3.7%), 3 strains (3.7%), 3 strains (3.7%) and 1 strain (1.2%). Among 4 strains (9.8%) of *E. coli* from semen, aac(3)-IV (4.9%) and aac(3)-II (4.9%) were detected from each group of 2 strains and these strains showed resistance to more than 3 kinds of aminoglycoside antibiotics. Meanwhile, *E. coli* from pigs with diarrhea (95%) showed 9.5 times higher appearance ratio of resistance gene than *E. coli* from semen (9.8%) and 5 resistance genes of diverse patterns were detected.

The resistant property of *E. coli* from semen was mostly lower than that of *E. coli* from pigs with diarrhea; however, *E. coli* from semen showed high-level resistance to most antibiotics. Moreover as detection frequency of resistance gene as well as antibiotic resistance increase not only in Korea but over the world, it is required to regularly monitor for antibiotic resistance for the purpose of making right choice of antibiotics for semen dilution, effective use and safe management. More than that this study has an importance in a sense of conducting a research on the distribution of antibiotic resistance gene for *E. coli* isolated from semen for the first time in comparison with *E. coli* separated from pigs with diarrhea.

CONCLUSION

As the results of investigating antibiotic resistance of 41 strains of *E. coli* from semen and 40 strains from pigs with diarrhea, *E. coli* from semen had high-level resistance to sulfamethoxazole (100%), tetracyclin (100%), streptomycin (92.7%) and trimethoprim (80.5); *E. coli* from pigs with diarrhea had higher resistance to most antibiotics than *E. coli* from semen-especially, 100% resistance to sulfamethoxazole and chloramphenicol.

Upon investigating on multi-drug resistance of *E. coli* from both semen and pigs with diarrhea in *E. coli* from semen was no strain that belonged to lower than resistance 2 CLSI subclasses; resistance 4-6 CLSI subclasses accounted for 70.7%. Meanwhile, *E. coli* from pigs with diarrhea belonged to higher subclasses than *E. coli* from semen, showing higher level resistance. In *E. coli* from pigs with diarrhea was no strain that belonged to lower than resistance 5 CLSI subclasses and resistance 8-10 CLSI subclasses accounted for 82.5%, having 4 phase higher resistance than *E. coli* from semen.

In this study, PCR was conducted for strains that had appeared to have resistance to more than an aminoglycoside antibiotic which resulted in the resistance gene detection in *E. coli* from both semen and pigs with diarrhea (n = 42/81, 51.9%) with 6 kinds [aac(3)-IV,

aac(3)-II, ant(2^{''})-I, aac(6)-Ib, aadB, aac(3)-III] out of 8 kinds of aminoglycoside resistant genes. However, aac(3)-I and armA were not detected at all.

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