저작자표시-비영리-변경금지 2.0 대한민국

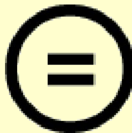
이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

수의학석사학위논문

Phenotypic and Genotypic Analysis of Antimicrobial Resistance Patterns of *Actinobacillus pleuropneumoniae* Korean Isolates

국내분리 *Actinobacillus pleuropneumoniae*의 항생제 내성 패턴에 대한 표현형과 유전적 특성 분석

2013년 2월

서울대학교 대학원
수의학과 수의미생물학 전공
유 안 나

Phenotypic and Genotypic Analysis of Antimicrobial Resistance Patterns of *Actinobacillus pleuropneumoniae* Korean Isolates

By

An Na Yoo

February, 2013

Department of Veterinary Medicine
(Major : Veterinary Microbiology)
The Graduate School
Seoul National University

Phenotypic and Genotypic Analysis of Antimicrobial Resistance Patterns of *Actinobacillus pleuropneumoniae* Korean Isolates

A Dissertation
Submitted to the Graduate School in Partial Fulfillment of the Requirements for Degree of Master

To the Faculty of College of Veterinary Medicine
Department of Veterinary Medicine
The Graduate School
Seoul National University

By

An Na Yoo

2013

Phenotypic and Genotypic Analysis of Antimicrobial Resistance Patterns of *Actinobacillus pleuropneumoniae* Korean Isolates

국내분리 *Actinobacillus pleuropneumoniae*의 항생제 내성 패턴에 대한 표현형과 유전적 특성 분석

지도교수 : 유 한 상

이 논문을 수의학석사 학위논문으로 제출함

2012 년 10 월

서울대학교 대학원

수의학과 수의미생물학 전공

유 안 나

유안나의 수의학석사 학위논문을 인준함

2012 년 12 월

위 원 장 박 봉 균 (인)

부위원장 유 한 상 (인)

위 원 박 세 창 (인)

Abstract

Phenotypic and Genotypic Analysis of Antimicrobial Resistance Patterns of *Actinobacillus pleuropneumoniae* Korean Isolates

An Na Yoo

(Supervisor: Han Sang Yoo, D.V.M., Ph.D)

Department of Veterinary Medicine
The Graduate School
Seoul National University

The antimicrobial susceptibility patterns of 102 *Actinobacillus pleuropneumoniae* isolated from pigs with pleuropneumonia in Korea from 2006 to 2010 were analyzed using the disk diffusion test and microdilution method. Amoxicillin/clavulanic acid, cephalothin, and ceftiofur were active against *A. pleuropneumoniae*, while most of the isolates were resistant to lincomycin (100 %), erythromycin (99.9 %), and kanamycin (93.2 %). The susceptibility to florfenicol decreased considerably by year from 2006 to

2010, as did that of a minor percentage of ampicillin and amoxicillin in minor percentage. Further studies were focused on the resistance to tetracycline and florfenicol based on the phenotypic analysis of antibiotic susceptibility patterns. Of 11 tetracycline resistance genes [*tet* (A), *tet* (B), *tet* (C), *tet* (D), *tet* (E), *tet* (G), *tet* (H), *tet* (K), *tet* (M), *tet* (L), and *tet* (O)], *tet* (B) was predominant (62 %), followed by *tet* (H) (12 %) and *tet* (O) (8 %). The florfenicol resistance (*flo* R) gene was detected in 35 strains (34%) and β-lactam antibiotic resistance ($bla_{\text{ROB-1}}$) gene was detected in 15 strains (15 %) of all isolates. To determine the factor related with the MIC value, the expression levels of the tetracycline repressor gene and resistance gene, as well as biofilm formation, were compared with the MIC values of tetracycline-resistant strains. There was, however, no significant degree of relation. Interspecies transferability of the florfenicol resistance (*flo* R) gene and tetracycline resistance [*tet* (B)] / β-lactams antibiotic resistance ($bla_{\text{ROB-1}}$) gene were observed with 5.7 X 10^{-3} and 3.5 X 10^{-2}, respectively. In analyzing digested DNA patterns using PFGE, they showed slight relatedness to serotypes but not to isolated location and year. This study acknowledges the antimicrobial resistance of *A. pleuropneumoniae* based on the current situation in Korea and may help to control the disease caused by *A. pleuropneumoniae*.

Keywords: *Actinobacillus pleuropneumoniae,* antimicrobial susceptibility,

resistance gene, gene transfer, PFGE

Student Number : 2011-21689

Contents

Abstract -- i

Contents -- iv

List of Figures -- vi

List of Tables --- viii

List of Abbreviations -- ix

1. Introduction --- 1

2. Materials and Methods -- 4

2.1. Bacterial strains and growth conditions ------------------------ 4

2.2. Antimicrobial susceptibility testing ----------------------------- 4

2.3. Polymerase Chain Reaction (PCR) for the detection of antibiotic resistance genes -- 5

2.4. Real-time PCR assay --- 6

2.5. Biofilm formation assay -- 10

2.6. Bacterial conjugation -- 10

2.7. Pulsed field gel electrophoresis (PFGE) ------------------------ 11

3. Results -- 13

3.1. Serotyping and antimicrobial resistance profile ---------------- 13

3.2. Relatedness of MIC values to resistance-gene expression and biofilm formation -- 20

3.3. Transfer of resistance gene --------------------------------------- 23

3.4. PFGE -- 25

4. Discussion -- 28

5. References -- 34

국문초록 -- 43

List of Figures

Figure 1 Relative concentration of *tet* R gene and average of expression levels of *tet* gene under the condition of tetracycline in the medium according to MIC values

Figure 2 Distribution of OD values of biofilm formation according to MIC values

Figure 3 Detection of transferability of antimicrobial resistance genes in transconjugants which came from *A. pleuropneumoniae* and *Escherichia coli*. A) Lane 1, *A. pleuropneumoniae* DCA 1630 *flo* R^R; lane 2, *Escherichia coli* 612 *flo* R^S; lane 3-4, transconjugants; M, Molecular weight DNA marker (100bp). B) Lane 1-2, *A. pleuropneumoniae* DCA 2018 *tet* $(B)^R$ / bla_{ROB-1}^R; lane 3, *Escherichia coli* 612 *tet* $(B)^S$ / bla_{ROB-1}^S; lane 4, transconjugants; M, Molecular weight DNA marker (100bp)

Figure 4 Dendrogram of 48 *A. pleuropneumoniae* with serotype, *tet* gene, isolated location and year. PFGE cluster analysis of *AscI* and *ApaI* restriction enzyme double digests of *A. pleuropneumoniae* clinical isolates in Korea

GB, Gyeongsangbukdo; GN, Gyeongsangnamdo; GG,

Gyeonggido; JB, Jeollabukdo; JN, Jeollanamdo; CN, Chungcheongnamdo; JJ, Jeju Special Self-Governing Province

List of Tables

Table 1 Oligonucleotides used in this study

Table 2 Distribution of serotypes according to year

Table 3 Results of antimicrobial susceptibility test and the resistance rates of 102 strains of *Actinobacillus pleuropneumoniae* against 16 antimicrobial agents

Table 4 Number of resistant antimicrobial agents and resistant strains by year

Table 5 Distribution of MIC values and their antimicrobial resistance gene

List of Abbreviations

BHI	Brain Heart Infusion
bla$_{\text{ROB-1}}$	Beta lactam antibiotic resistance gene
BLAST	Basic Local Alignment Search Tool
CAMH	Cation-Adjusted Mueller-Hinton
cDNA	Complementary DeoxyriboNucleic Acid
DNA	DeoxyriboNucleic Acid
dNTP	Deoxyribonucleotide
EDTA	EthyleneDiamineTetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
flo R	Florfenicol resistance gene
KAHPA	Korea Animal Health Products Association
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
mRNA	Messenger RiboNucleic Acid
NAD	β-Nicotinamide Adenine Dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical Density
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis

qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	RiboNucleic Acid
rRNA	Ribosomal RiboNucleic Acid
TCP	Tissue Culture Plate
tet	Tetracycline resistance gene
tet R	Tetracycline repressor gene
TSB	Tryptone Soy Broth
UV	UltraViolet ray
VFM	Veterinary Fastidious Medium

1. Introduction

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumoniae, a respiratory disease in pigs of all ages that may result in sudden deaths detected in the post-mortem inspection at slaughter, or in severe symptoms characterized by fibrinous pleuritis and hemorrhagic and fibrinous pneumonia with necrotic lung lesions (Bosse *et al.*, 2002; Chiers *et al.*, 2010). Some animals exposed to the organism may remain in a state of chronic infection as asymptomatic carriers transmitting the disease to the healthy pigs (Rycroft and Garside, 2000). Symptoms depend on the serotype of the *A. pleuropneumoniae*, the immune condition of the host, and the amount of bacteria colonizing in the lung (Bosse *et al.*, 2002). *A. pleuropneumoniae* can be divided into 15 serotypes on the basis of the antigenic properties of capsular polysaccharides and the cell wall lipopolysaccharides; each serotype has different virulence factors, causing the exhibition of various clinical signs (Chiers *et al.*, 2010; Blackall *et al.*, 2002). Infection with *A. pleuropneumoniae* is a potential cause of great economic losses worldwide due to high mortality, reduction of feed conversion rate, and retarded growth rate among affected livestock. Thus, it is essential that an appropriate therapy be applied as accurately and quickly as possible (Rosendal and Mitchell, 1983).

Despite several researches and many studies seeking vaccines to control

the disease, vaccines offering complete protection against *A. pleuropneumoniae* have not been developed until recently (Ramjeet *et al.*, 2008). Current vaccines for *A. pleuropneumoniae* have consisted of heat-killed or formalin-treated whole cells (Goethe *et al.*, 2000). These can reduce morbidity and mortality, but do not provide cross-immune response for various serotypes or do not prevent the disease at the initial infection stage and the development to the carrier state (Ramjeet *et al.*, 2008). To compensate for the weaknesses of the vaccine, antimicrobial therapy is thus needed to control the disease at the initial stage of infection and to prevent its spread.

Unfortunately, the indiscriminate and abusive use of antibiotic agents has resulted in an increase in antimicrobial resistance in a large number of animal and human bacterial pathogens. In Korea, copious amounts of various antibiotic agents have been used as food supplements up to 2011, as veterinary medicine prescriptions, and for self-treatment by farmers to prevent the outbreak of diseases in the swine industry. According to the recorded data provided by the Korea Animal Health Products Association (KAHPA), the use of antibiotics as food supplements from 2009 to 2011 (840,191 kg) was almost equivalent to their use as medicine in therapy performed by veterinary practitioners (851,249 kg). The concern about this situation has led to a reduction in the use of antibiotics, primarily through

the banning of antibiotics as a component in feed since July 2011; since then, the reports of outbreaks of porcine pleuropneumonia have increased.

Based on the current state of *A. pleuropneumoniae* infection, it is clear that further knowledge of the antimicrobial susceptibility of infecting strains from clinical isolates in recent years is needed. There are many differences within resistance patterns according to serotypes, across different countries, and over time (Gutiérrez-Martín *et al.*, 2006; Kucerova *et al.*, 2011; Morioka *et al.*, 2008; Asawa *et al.*, 1995). In this study, the distribution of antibiotic resistance of *A. pleuropneumoniae* isolated from pigs with pleuropneumonia in Korea was evaluated. Moreover, to satisfy our curiosity regarding which factor determines the MIC value, the expression levels of tetracycline repressor gene and resistance gene, as well as biofilm formation, were investigated. It is well-known that the biofilm matrix contributes to increased resistance to antibiotics and host defenses (Hall-Stoodley *et al.*, 2004). Finally, the intra/interspecies transferability of resistance was investigated, and the digested DNA patterns were compared based on serotypes, resistance gene, origins and time span.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

All *A. pleuropneumoniae* used in this study were kindly provided by Choong-Ang Vaccine Laboratory Co. Ltd., in Daejeon, Korea. These 102 clinical isolates of *A. pleuropneumoniae* were aseptically collected over a period of five years (from 2006 to 2010) from specimens of pigs showing symptoms of pulmonary diseases in Korea. *A. pleuropneumoniae* was isolated and identified based on general bacterial isolation and identification methods, and cultured on chocolate agar (Hanil Comed Co. Korea) at 37 ℃ for 24 hours with 5 % CO_2. Once identification was complete, bacterial serotyping was performed as described previously (Rafiee and Blackall, 2000; NCCLS, 2008).

2.2. Antimicrobial susceptibility testing

Antimicrobial resistances of *A. pleuropneumoniae* were investigated by disk diffusion test and microdilution test conducted following NCCLS guidance for *in vitro* antimicrobial susceptibility tests. *A. pleuropneumoniae*

ATCC 27090 was used for quality control (QC) of the tests. In accordance with NCCLS regulations (NCCLS, 2008), Mueller–Hinton (MH) chocolate agar (BD, USA) was used for the disk diffusion test and Veterinary Fastidious Medium (VFM) (Cation-Adjusted Mueller-Hinton (CAMH) broth with 2 % lysed horse blood, 2 % supplement C and 5 % yeast extract) was used for the microdilution test. Sixteen kinds of antibiotics were used in disk diffusion tests: ampicillin (10 μg/ml), penicillin (10 μg/ml), amoxicillin (10 μg/ml), amoxicillin/clavulanic acid (30 μg/ml), gentamicin (10 μg/ml), neomycin (30 μg/ml), kanamycin (30 μg/ml), lincomycin (2 μg/ml), tetracycline (30 μg/ml), nalidixic acid (30 μg/ml), enrofloxacin (5 μg/ml), cephalothin (30 μg/ml), ceftiofur (30 μg/ml), florfenicol (30 μg/ml), erythromycin (15 μg/ml), and sulfamethoxazole/trimethoprim (25 μg/ml). Tetracycline, florfenicol, and β-lactam antibiotics (penicillin, ampicillin, cefoxitin, and cefotaxim) were used for the microdilution test.

2.3. Polymerase Chain Reaction (PCR) for the detection of antibiotic resistance genes

A loop of bacterial culture was resuspended in 50 μl of distilled water and boiled at 100 ℃ for 10 min and then stored at 4 ℃ for five min. The suspension was spun down for 15 sec, and supernatant was used as template

DNA. Eleven different oligonucleotide primer sets were used for detection of the tetracycline-resistant gene and other sets was used to find the florfenicol and β-lactam antibiotics resistant gene (Table 1). Each PCR mix of 20 μl contained 2 μl of *i*-Taq 10X PCR buffer, 0.015 mM of each dNTPs, 50 pmole of each primer, 1 μl of template DNA and 2.5 units of *i*-Taq DNA polymerase (iNtRON Biotechnology, Korea). After initial denaturation at 94 ℃ for five min, PCR cycling consisted of 35 cycles of denaturation at 94 ℃ for 30 sec, annealing at 54 - 55 ℃ for 30 sec and extension at 72 ℃ for 30 sec. Final extension was performed at 72 ℃ for 10 min. The PCR products were separated by electrophoresis in 1.5 % agarose gel and visualized under a UV transilluminator after staining with ethidium bromide. Amplified PCR products of expected sizes were subjected to direct sequencing by an automatic sequence and dye-termination sequencing system (Macrogen Co., Korea). A BLAST search for homogeneous sequences was conducted in the GenBank database at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Real-time PCR assay

To investigate the amount of tetracycline-repressor (*tet* R) gene and

tetracycline resistance [*tet* (B), *tet* (H), *tet* (O)] gene mRNA under the condition of tetracycline in the medium, real-time PCR was performed. Prior to real-time PCR, the band of products was confirmed from the conventional PCR method. To test the tetracycline resistance gene mRNA, the RNA was extracted from bacteria-inoculated TSB including 0.01 % β-nicotinamide adenine dinucleotide (NAD, Sigma, USA) and 4 μg/ml of tetracycline using RNeasy Mini Kit (Qiagen). The RNA extracts were converted into first-strand cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The synthesized cDNA was stored at -20 ℃ until further use.

Quantitative real-time PCR was conducted using a RotorGene Q Detection System. The conditions consisted of holding at 95 ℃ for five min, 35 cycles of step 1 at 95 ℃ for 10 sec and step 2 at 60 ℃ for 45 sec, and melting at 72 - 95 ℃. The 16S rRNA of *A. pleuropneumoniae* was used as a housekeeping gene. The oligonucleotide sets for *tet* R, *tet* (B), *tet* (H), *tet* (O), and 16S rRNA of *A. pleuropneumoniae* are described below (Table 1). Following real-time PCR, the result was analyzed by Rotor-Gene Q series software (Qiagen).

Table 1. Oligonucleotides used in this study

Gene	Name	5’ → 3’ sequence	Reference
tet (A)	tetA-L	GGCGGTCTTCTTCATCATGC	(Lanz *et al.*, 2003)
	tetA-R	CGGCAGGCAGAGCAAGTAGA	
tet (B)	tetB-L	CATTAATAGGCGCATCGCTG	(Lanz *et al.*, 2003)
	tetB-R	TGAAGGTCATCGATAGCAGG	
tet (C)	tetC-L	GCCGGAAGCGAGAAGAATCA	(Lanz *et al.*, 2003)
	tetC-R	GCTGTAGGCATAGGCTTGGT	
tet (D)	tetD-L	ACACTGCTGGACGCGATG	(Matter *et al.*, 2007)
	tetD-R	TCCGCACTCTGCTGTGTC	
tet (E)	tetE-L	GTGATGATGGCACTGGTCAT	(Guardabassi *et al.*, 2000)
	tetE-R	CTCTGCTGTACATCGCTCTT	
tet (G)	tetG-L	TGATCGTGGGTCTTGACG	(Matter *et al.*, 2007)
	tetG-R	TGCGAATGGTCTGCGTAG	
tet (H)	tetH-L	TTATACTGCTGATCACCG	(Matter *et al.*, 2007)
	tetH-R	CATCCCAATAAGCGACGC	
tet (K)	tetK-F	TTATGGTGGTTGTAGCTAGAAA	(Koo and Woo, 2011)
	tetK-R	AAAGGGTTAGAAACTCTTGAAA	
tet (L)	tetL-F	GAACGTCTCATTACCTGATATTGC	(Koo and Woo, 2011)
	tetL-R	CAAACCCTGCTACTGTTCCAA	
tet (M)	tetM-F	GTRAYGAACTTTACCGAATC	(Koo and Woo, 2011)
	tetM-R	ATCGYAGAAGCGGRTCAC	
tet (O)	tetO-F	AACTTAGGCATTCTGGCTCAC	(Koo and Woo, 2011)
	tetO-R	TCCCACTGTTCCATATCGTCA	

flo R	floF	GCGATATTCATTACTTTGGC	(Faldynova *et al.*,
	floR	TAGGATGAAGGTGAGGAATG	2003)
bla_{ROB-1}	rob-1F	TGTTGCAATCGCTGCC	(San Millan *et al.*,
	rob-1R	TTATCGTACACTTTCCA	2007)
tet R	tet R-F	AACCCGTAAACTCGCCCAGAAGC	This study
	tet R-R	TAAGGCGTCGAGCAAAGCCCG	
tet (B)	*tet* B_RT_F	CGCGTGAAGTGGTTCGGTTGG	This study
	tet B_RT_R	AGAAGGGGCAATGCGGTGAGA	
tet (H)	*tet* H_RT_F	GGCGCGGCACTCGACTATCT	This study
	tet H_RT_R	TACGGCACCTGTTGCGCCTG	
tet (O)	*tet* O_RT_F	TGCGAGCAGGGGCTGTATGGA	This study
	tet O_RT_R	GCAGCCGAAAGTCTGCGGGG	
16S	16S RNA_F	CGCTGGCGGCAGGCTTAACA	This study
rRNA	16S RNA_R	GTCCGCCACTCGTCGGCAAA	
PBP3-S	PBP3S-S	GATACTACGTCCTTTAAATTAAG	(Hasegawa *et al.*,
	PBP3S-R	GCAGTAAATGCCACATACTTA	2003)
PBP3-	PBP3BLN-S	TTCAAGTAACCGTGGTGTGAC	(Hasegawa *et al.*,
BLN	PBP3BLN-R	GCAGTAAATGCCACATATTTC	2003)

2.5. Biofilm formation assay

To investigate the biofilm formation of *A. pleuropneumoniae*, the tissue culture plate (TCP) method was used (Grasteau *et al.*, 2011). The 96 flat-bottomed tissue culture wells were filled with two-hundred microliters of cultured bacteria, diluted overnight in a 1 : 100 solution of Brain Heart Infusion (BHI) broth with 0.01 % NAD. After incubation at 37 ℃ for 20 hours, each well was washed three times with distilled water and was inverted onto towels to eliminate non-adherent bacteria. The cells attached to the wells were fixed with 200 μl of 99 % methanol (Duksan, Korea) for 15 min and stained using 2 % crystal violet solution for two min at room temperature, and then washed. After drying, 200 μl of 33 % glacial acetic acid were added to resolve the adherent biofilm on each well. To measure the optical density (OD), a microplate ELISA reader (E max, USA) at 570 nm was used.

2.6. Bacterial conjugation

To investigate the transferability of tetracycline, florfenicol, and β-lactam antibiotic resistance genes between intra/interspecies, *A. pleuropneumoniae* DCA 1630 *flo* R^R, DCA 2018 *tet* $(B)^R$ / $bla_{ROB-1}{}^R$ and *Escherichia coli* 612

flo R^S, DCA 2018 *tet* $(B)^S$ / $bla_{ROB-1}{}^S$ were prepared. Two broth cultures for conjugation were mixed as previously described (Kang *et al.*, 2005). After mating at 37 ℃ for 20 h in trypticase soy broth (TSB, Difco Laboratories, USA) with 0.01 % NAD, the cultures were spread onto MacConkey agar with florfenicol (4 μg/ml) or tetracycline (4 μg/ml) / ampicillin (4 μg/ml). The transconjugants were picked after 20 h and were used for detection of the resistant gene. Transfer frequency was calculated as the number of transconjugants per recipient.

2.7. Pulsed field gel electrophoresis (PFGE)

To make a plug including bacterial DNA, organisms were grown overnight on chocolate agar at 37 ℃ and suspended in three mL of cell suspension buffer (20 mM tris-HCl (pH 8), 20 mM NaCl, 50 mM EDTA and distilled water) to a turbidity of four McFarland. Each 2 mL bacterial suspension was spun down at 12,000 rpm for one min, and the concentrated cells which were mixed with 200 μl plug agarose were poured into the molds. After being washed with wash buffer (20 mM tris-HCl (pH 8), 50 mM EDTA and distilled water) and then going through the process of proteinase K solution overnight at 55 ℃, each genomic DNA was digested with *AscI* (New England Bio Labs Inc. USA) and *ApaI* (Takara Bio Inc. Japan) for 18 h, and they were then separated on 1 % SeaKem Gold agarose

(Lonza, Rockland, ME USA) using CHEF MAPPER™ (Bio Rad Laboratories, USA). Electrophoresis conditions were 6 V/cm for 20 h with an increasing pulse time of eight to 40 sec. Lambda ladder PFG marker (New England Bio Labs Inc. USA) was used as a standard line, and we analyzed the patterns of digested DNA using Gel Compar Ⅱ (Kang *et al.*, 2005).

3. Results

3.1. Serotyping and antimicrobial resistance profile

Among the total of 102 *A. pleuropneumoniae* isolates, 58 strains (57 %) belonged to serotype 5 predominantly, followed by serotype 1 (15 %), 2 (15 %), 4 (4 %), 7 (4 %) and 8 (1 %). Four strains were defined as unknown (Table 2). All of the tested *A. pleuropneumoniae* isolates were perfectly susceptible to amoxicillin/clavulanic acid, cephalothin, and ceftiofur, while the isolates were resistant to lincomycin (100 %), erythromycin (100 %), and kanamycin (93.1 %) (Table 3). Only half of all isolates were susceptible to gentamicin, and few strains showed efficiency to neomycin and kanamycin. The susceptibility to florfenicol decreased considerably by year from 2006 to 2010, as did that of a minor percentage of ampicillin and amoxicillin in minor percentage. The susceptibility to florfenicol, which in 2006 had been over 94 %, was significantly decreased to just over 50 % in 2010. In the case of ampicillin and amoxicillin, they were active against over 88 % of *A. pleuropneumoniae* isolates in 2007, while only 65 % showed the susceptibility in 2010. Analysis of the number of resistant strains and the number of resistant antibiotic agents showed that both of

them had increased up to 2010, and more than half of the *A. pleuropneumoniae* isolates showed resistance to more than three antibiotics. Moreover, multi-drug-resistant strains had been increasing year by year, and a strain resistant to 11 antimicrobial drugs out of 16 antibiotic agents was discovered in the cultures of 2010 (Table 4). This suggests greater difficulty in choosing effective antibiotics to control the disease.

As a result of PCR, 79 strains (77 %) out of all the isolates harbored *tet* (B), *tet* (H), or *tet* (O) among 11 tetracycline resistance genes. *tet* (B) was predominant (n = 63), followed by *tet* (H) (n = 12) and *tet* (O) (n = 8). Only four strains had two kinds of tetracycline resistance genes. Three of them had *tet* (B) and *tet* (O) genes, and the other had *tet* (H) and *tet* (O) genes. Strains defined as susceptible against tetracycline (n = 19) by disk diffusion test tested negative for all tetracycline resistance genes, while all the strains with intermediate susceptibility (n = 64) to tetracycline appeared to have the resistance genes. The florfenicol resistance (*flo* R) gene was detected in 35 strains (34%) in accordance with the disk diffusion test and β-lactam antibiotic resistance gene ($bla_{\text{ROB-1}}$) was detected in 15 strains (15 %) of all isolates. Interestingly, we could not detect either $bla_{\text{ROB-1}}$ or $bla_{\text{TEM-1}}$ in some strains with ampicillin resistant phenotype.

The distribution of MIC values for the *A. pleuropneumoniae* isolates having tetracycline resistance genes, florfenicol resistance genes or β-lactam

antibiotic resistance gene are shown in table 5. All tetracycline-resistant strains displayed MIC values over the level of breakpoints (8 μg/ml). Out of 79 strains, the MIC values of 46 strains were 8 μg/ml, those of 26 strains were 16 μg/ml, and those of all others were over 32 μg/ml. In the case of florfenicol-resistant strains, the MIC of one strain was shown under the breakpoint (8 μg/ml), but the other strains were spread between eight and 32 μg/ml. The MIC of ampicillin resistant strains were between 8 to 512 μg/ml and all strains in this experiment showed susceptibility to second and third generation cephalosporins (cefoxitin and cefotaxim, respectively) as well as to the amoxicillin-clavulanic acid.

Table 2. Distribution of serotypes according to year

Serotype	No. of strains					Total
	2006	2007	2008	2009	2010	
APP 1		1	1	2	12	15
APP 2	2	3	3	3	4	15
APP 4		2			2	4
APP 5	4	11	15	15	13	58
APP 7	1	1			2	4
APP 8					1	1

Table 3. Results of antimicrobial susceptibility test and the resistance rates of 102 strains of *Actinobacillus pleuropneumoniae* against 16 antimicrobial agents

Antimicrobial agents	Susceptible strains (*n*)	Intermediate strains (*n*)	Resistant strains (*n*)	Resistance rate (%)
Ampicillin (10 µg/ml)	80	7	15	21.57
Penicillin (10 µg/ml)	21	47	34	79.41
Amoxicillin (10 µg/ml)	77	6	19	24.51
Amoxicillin/clavulanic acid (30 µg/ml)	102	0	0	0
Gentamicin (10 µg/ml)	58	25	19	43.14
Neomycin (30 µg/ml)	27	36	39	73.53
Kanamycin (30 µg/ml)	7	63	32	93.13
Lincomycin (2 µg/ml)	0	0	102	100
Tetracyline (30 µg/ml)	20	63	19	80.39
Nalidixic acid (30 µg/ml)	48	5	49	52.96
Enrofloxacin (5 µg/ml)	97	5	0	4.9
Cephalothin (30 µg/ml)	102	0	0	0
Ceftiofur (30 µg/ml)	102	0	0	0
Florfenicol (30 µg/ml)	67	3	32	34.31
Erythromycin (15 µg/ml)	0	9	93	100
Sulfamethoxazole/ trimethoprim (25 µg/ml)	91	4	7	10.78

Table 4. Number of resistant antimicrobial agents and resistant strains by year

No. of resistant antimicrobial Agents	No. of resistant strains					Total (%)
	2006	2007	2008	2009	2010	
2	3	3	2	3	2	13 (12.8)
3	6	8	4	7	6	31 (30.4)
4	2	2	6	2	6	18 (17.6)
5	3		4	3	3	13 (12.7)
6	2	2	3	2	2	11 (10.8)
7		2		1	1	4 (3.9)
8	3		1		3	7 (6.9)
9				2	1	3 (2.9)
10					1	1 (1)
11					1	1 (1)
Total	19	17	20	20	26	102 (100)

Table 5. Distribution of MIC values and their antimicrobial resistance genes

Antibiotics		Resistance genes	MIC (mg/L)				
			< 4	8	16	32	64 <
Tetracycline		*tet* (B) (n = 60)		41	18	1	
		tet (H) (n = 11)		3	8		
		tet (O) (n = 4)				3	1
		tet (B), (O) (n = 3)		2		1	
		tet (H), (O) (n = 1)				1	
Florfenicol		*flo* R (n = 35)	1	9	11	13	1
β-lactam antibiotic	Penicillin	$bla_{\text{ROB-1}}$ (n = 15)		1	2	6	13
	Ampicillin			2	4	7	9
	Cefotaxime		22				
	Cefoxitin		22				

3.2. Relatedness of MIC values to resistance-gene expression and biofilm formation

To investigate a factor which might be related to MIC values of tetracycline, qRT-PCR for the *tet* R gene of tetracycline-resistant strains was performed with *A. pleuropneumoniae* specific primers for the 16S rRNA gene. The relative concentration of *tet* R gene with its MIC values is shown in fig 1. In the same way, the expression levels of tetracycline resistance genes were evaluated under the condition of tetracycline and compared the average of expression levels of *tet* (B), (H), and (O) genes to MIC values. The amount of *tet* R was not in accordance with the activity of resistance, but the expression levels of the tetracycline resistance genes of *tet* (B) and (O) were slightly related to MIC values; this, however, was not so in the case of the *tet* (H) gene.

Biofilm formation (OD 590nm > 0.1) was observed in 53 strains (89.8 %) out of 59 strains which were resistant to tetracycline or florfenicol. Twenty strains (33.9 %) developed a moderate biofilm matrix showing over 0.2 OD value, and seven strains (11.9 %) formed a severe biofilm showing over 0.3 OD value. There is no significant correlation between biofilm formation and MIC values (Fig 2).

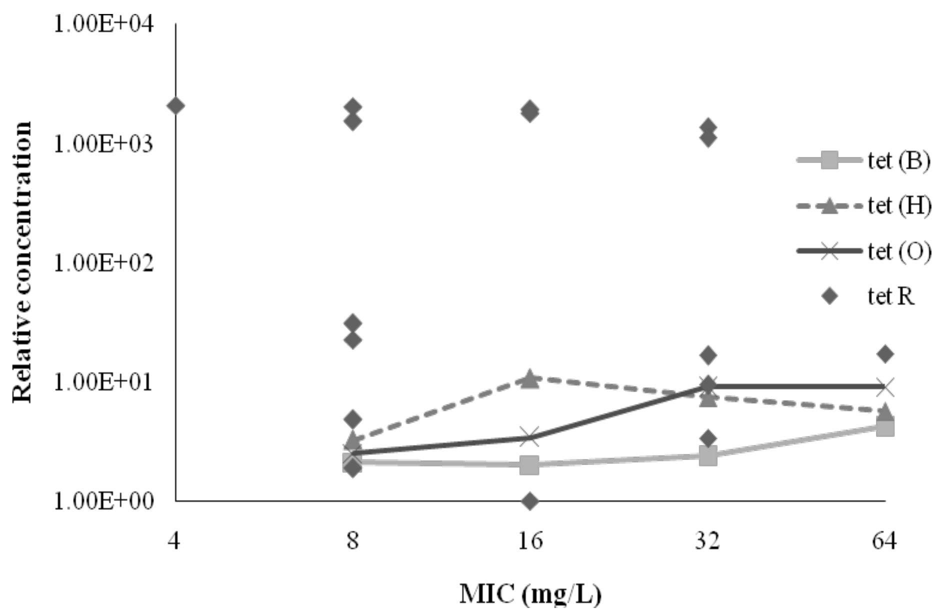


Fig 1. Relative concentration of *tet* R gene and average of expression levels of *tet* gene under the condition of tetracycline in the medium according to MIC values

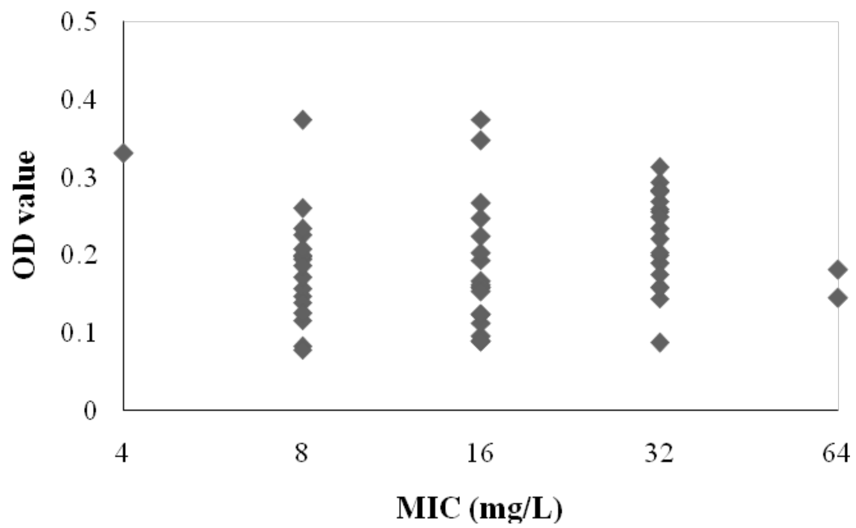


Fig 2. Distribution of OD values of biofilm formation according to MIC values

3.3. Transfer of resistance gene

Florfenicol resistance and tetracycline / β-lactam antibiotics resistance were transformed from *A. pleuropneumoniae* to *E. coli* with 5.7×10^{-3} and 3.5×10^{-2}, respectively. Gene transfer was confirmed by detecting the *flo* R and *tet* (B) / $bla_{ROB\text{-}1}$ genes in transconjugants (Fig 3). However, transfer of resistance genes within species was not observed.

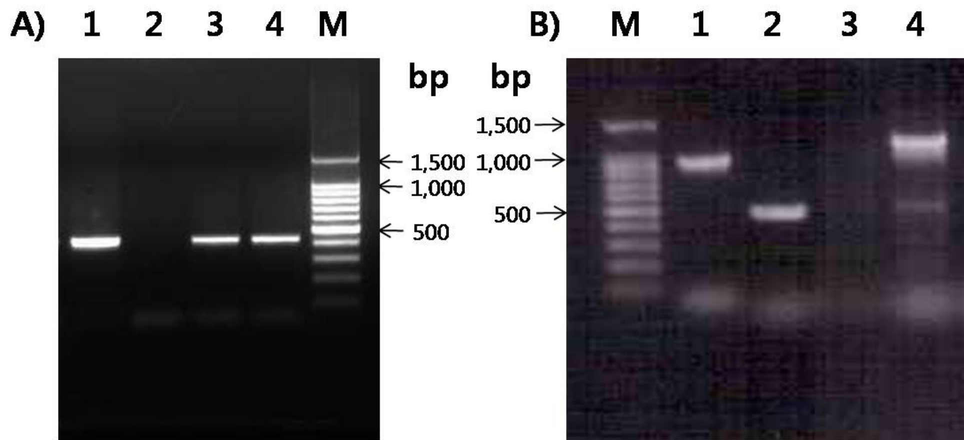


Fig 3. Detection of transferability of antimicrobial resistance genes in transconjugants which came from *A. pleuropneumoniae* and *Escherichia coli*. A) Lane 1, *A. pleuropneumoniae* DCA 1630 *flo* R^R; lane 2, *Escherichia coli* 612 *flo* R^S; lane 3-4, transconjugants; M, Molecular weight DNA marker (100bp). B) Lane 1-2, *A. pleuropneumoniae* DCA 2018 *tet* $(B)^R$ / $bla_{ROB-1}{}^R$; lane 3, *Escherichia coli* 612 *tet* $(B)^S$ / $bla_{ROB-1}{}^S$; lane 4, transconjugants; M, Molecular weight DNA marker (100bp)

3.4. Pulsed field gel electrophoresis (PFGE)

To compare the digested chromosome patterns of tetracycline-resistant strains, the relative position of each DNA fragment was determined from double restriction digests. Cleaving with *AscI* and *ApaI* caused six to 16 segments to appear on the gel, and we analyzed the bands based on their serotypes, *tet* gene, isolated location and year (Fig 4). A high degree of similarity was observed among serotypes regardless of differences in isolated origin and year. As time goes by, new combinations of serotype and *tet* gene appeared.

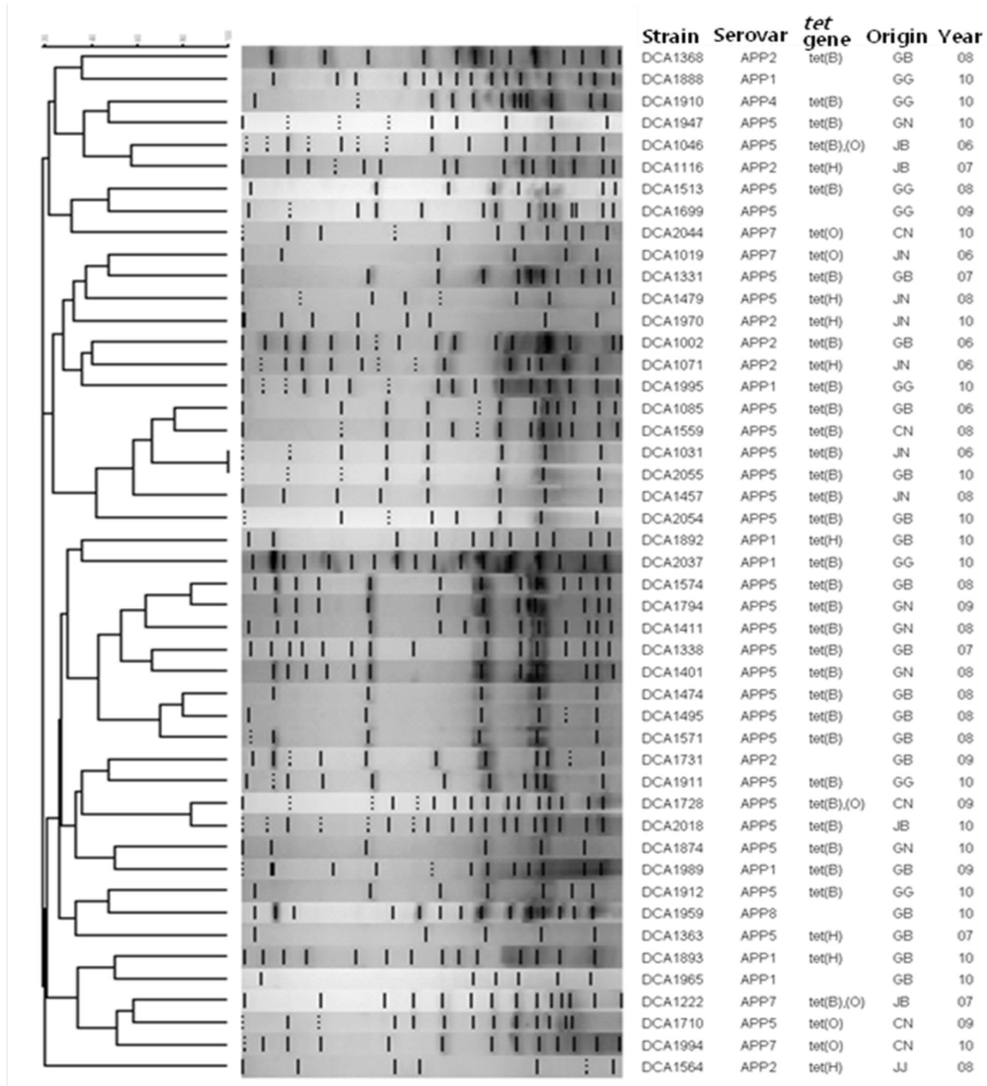


Fig 4. Dendrogram of 48 *A. pleuropneumoniae* with serotype, *tet* gene, isolated location and year. PFGE cluster analysis of *AscI* and *ApaI* restriction enzyme double digests of *A. pleuropneumoniae* clinical isolates in Korea. GB, Gyeongsangbukdo; GN, Gyeongsangnamdo; GG,

Gyeonggido; JB, Jeollabukdo; JN, Jeollanamdo; CN, Chungcheongnamdo; JJ, Jeju Special Self-Governing Province

4. Discussion

In this study, the antimicrobial susceptibility to 16 antibiotic agents of *A. pleuropneumoniae* isolates collected from pigs suspected in infection in Korea between 2006 and 2010 was investigated, and the antimicrobial susceptibility phenotype and genotype related to resistance were surveyed. Distribution of serotypes of *A. pleuropneumoniae* varies by region and time. Serotypes 9 and 2 were the most prevalent in the Czech Republic (Kucerova *et al.*, 2011), and serotypes 2 and 4 were dominant in Spain (Gutiérrez-Martín *et al.*, 2006). Changes in serotype appeared over the course of those years in Korea. While it had been reported that serotype 2 was predominant in Korea in 2000 (Kim *et al.*, 2001), as of 2010, serotype 5 was the most prevalent serotype, followed by serotype 1, 2, 4, 7 and 8. The appearance of serotype 1 from 2007 in this study was agreed with another study which showed the detection of serotype 1 in Korea (Shin *et al.*, 2010). Especially, the number of serotype 1 was significantly increased in 2010. This phenomenon might be due to the vaccination against only serotype 2 and 5 which were the most predominant serotype in Korea (Jung *et al.*, 1995; Lee *et al.*, 1999; Kim *et al.*, 1998) or the possibility of new introduction of serotype 1 from outside the country such as North America and China where the serotype 1 was predominant (Jacques, 2004; Lu *et al.*, 2002). However,

there is no correlation between the serovars of *A. pleuropneumoniae* and the resistance distributions to antimicrobial agents, as reported earlier (Kucerova *et al.*, 2011; Dom *et al.*, 1994; Kim *et al.*, 2001; Matter *et al.*, 2007).

Most β-lactam antibiotics were highly active against *A. pleuropneumoniae,* except penicillin. Penicillin had good activity with low MICs in Korea in 2000 (Kim *et al.*, 2001), but now showed a low degree of effectiveness, which suggested that penicillin has been used tremendously in pig industry and is no long a useful drug for the treatment of pleuropneumonia among swine. On the other hand, cephalosporin antibiotic agents such as cephalothin and ceftiofur were perfectly effective against *A. pleuropneumoniae* in this study, which was in agreement with previous reports (Blanco *et al.*, 2006; Kim *et al.*, 2001). Moreover, some ampicillin resistant strains without β-lactam antibiotic resistance gene were investigated according to previous report (Hasegawa *et al.*, 2003). To the best our knowledge, this is the first report of the presence of *A.pleuropneumoniae* strains carrying $bla_{\mathrm{ROB\text{-}1}}$ gene as well as β-lactamase-negative ampicillin-resistant (BLNAR) *A.pleuropneumoniae* in Korea, which was also reported in *Haemophilus influenza* recently (Kim *et al.*, 2007).

Until now, many studies had reported that most isolates were susceptible to florfenicol, and that a high rate of resistance was observed to tetracycline (Gutiérrez-Martín *et al.*, 2006; Kucerova *et al.*, 2011). That phenomenon also emerged in this study. The effectiveness of florfenicol was very high in 2006; interestingly, however, that has significantly decreased, and only 50 % of isolates were susceptible to florfenicol in 2010. From the results of this study, it is possible to conclude that resistance to florfenicol can be spread between species by conjugation of plasmids, including florfenicol resistance genes. Tetracycline showed a large portion of intermediate effectiveness and a small portion of susceptibility in this study. However, by PCR, the tetracycline resistance genes appeared in isolates determined as intermediate. The high ratio of tetracycline resistance could be related to the over use of this antimicrobial agent at sub-therapeutic doses in swine veterinary practice. According to KAHPA, tetracycline is one of the most frequently used antibiotics in Korea.

Tetracycline resistance is generally acquired by tetracycline resistance genes (*tet* genes) associated with plasmids or transposons between bacterial species (Kehrenberg *et al.*, 2001). The *tet* (B) gene coding for an efflux protein that reduces the intercellular tetracycline level was predominant among other *tet* genes in Korea, as in other countries (Morioka *et al.*, 2008; Blanco *et al.*, 2006; Wasteson *et al.*, 1996). Most strains containing

resistance genes showed resistant or intermediate phenotypes in disk diffusion tests, except for two strains. Also, in a comparison between *tet* genes and their MIC values, the strains having the *tet* (O) gene showed higher MIC values. Against our expectations, strains having two tetracycline-resistance determinants did not show higher MIC values.

Yu et al. developed real-time PCR assays for evaluation of genes encoding tetracycline resistance (Yu *et al.*, 2005). The *tet* R is an essential gene in the mechanism of resistance-coding a tetracycline repressor protein. Expression of the *tet* R gene is induced by tetracycline; in the absence of tetracycline, the tetracycline repressor protein turns off its own synthesis (Beck *et al.*, 1982). At first, it was expected that the amount of tetracycline repressor gene or tetracycline resistance gene might affect MIC values, but the amounts of *tet* R were not in accordance with resistance activity. Morsczeck also reported that *tet* R PCR is not reliable for the detection of all kinds of tetracycline-resistant *Escherichia coli* strains (Morsczeck *et al.*, 2004). Also, a slight relationship between MIC levels and the expression of the tetracycline resistance genes *tet* (B) and (O) was found, but none in the case of the *tet* (H) gene. From this result, the activity of resistance cannot be anticipated by real-time PCR, but it can help to confirm that real-time PCR is a quick method for the detection of microbes or antibiotic-resistant strains, as previously reported (Morsczeck *et al.*, 2004; Smith *et al.*, 2004; Dumas *et*

al., 2006).

There was no significant correlation between biofilm formation and MIC values. Biofilm phenotype could be lost by introducing subcultures into tubes containing fresh broth after one or two passages, and phenotype transition from biofilm-positive to biofilm-negative would also occur irreversibly (Kaplan and Mulks, 2005). Because of these occurrences, it is difficult to observe exact biofilm formation of field isolates in the laboratory.

Distribution of the genotype of *A. pleuropneumoniae* was investigated by PFGE with *AscI* and *ApaI*, because the enzymes induce six to 12 fragments ranging from 11 kb to 1217 kb in *A. pleuropneumoniae* strains (Chevallier *et al.*, 1998). While a few serotypes of *A. pleuropneumoniae* have existed in Korea, there were many kinds of genotypes in the same serotype. PFGE patterns tended to have slight relatedness in serotypes, but specific differences cannot be found in the resistance gene, origin and time (Oswald *et al.*, 1999). Because the size of the tetracycline resistance gene is too small to be determined by PFGE patterns and the shipping of pigs in Korea is very extensive, crisscrossing the nation, so there are no differences according to specific locations.

It is important to acknowledge the antimicrobial resistance of *A. pleuropneumoniae* based on the current situation in Korea. The current results indicate that many antimicrobial resistances are already distributed

widely and will be wider-spread in the near future. It is needed to choose accurate antibiotics to control this disease and it is essential to find effective vaccines instead of continuing to abuse of antibiotic agents.

References

Asawa, T., Kobayashi, H., Mitani, K., Ito, N. & Morozumi, T. (1995). Serotypes and antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from piglets with pleuropneumonia. The Journal of Veterinary Medical Science 57(4): 757-759.

Beck, C., Mutzel, R., Barbe, J. & Müller, W. (1982). A multifunctional gene (*tet*R) controls Tn10-encoded tetracycline resistance. Journal of Bacteriology 150(2): 633-642.

Blackall, P., Klaasen, H., Van Den Bosch, H., Kuhnert, P. & Frey, J. (2002). Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Veterinary Microbiology 84(1-2): 47-52.

Blanco, M., Gutiérrez-Martin, C. B., Rodríguez-Ferri, E. F., Roberts, M. C. & Navas, J. (2006). Distribution of tetracycline resistance genes in *Actinobacillus pleuropneumoniae* isolates from Spain. Antimicrobial Agents and Chemotherapy 50(2): 702-708.

Bosse, J. T., Janson, H., Sheehan, B. J., Beddek, A. J., Rycroft, A. N., Simon Kroll, J. & Langford, P. R. (2002). *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes and Infection 4(2): 225-235.

Chevallier, B., Dugourd, D., Tarasiuk, K., Harel, J., Gottschalk, M., Kobisch, M. & Frey, J. (1998). Chromosome sizes and phylogenetic relationships between serotypes of *Actinobacillus pleuropneumoniae*. FEMS Microbiology Letters 160(2): 209-216.

Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R. & Haesebrouck, F. (2010). Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. Veterinary Research 41(5): 65-80.

Dom, P., Hommez, J., Castryck, F., Devriese, L. & Haesebrouck, F. (1994). Serotyping and quantitative determination of *in vitro* antibiotic susceptibility of *Actinobacillus pleuropneumoniae* strains isolated in Belgium (July 1991-August 1992). Veterinary Quarterly 16(1): 10-13.

Dumas, J. L., Delden, C., Perron, K. & Köhler, T. (2006). Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. FEMS Microbiology Letters 254(2): 217-225.

Faldynova, M., Pravcova, M., Sisak, F., Havlickova, H., Kolackova, I., Cizek, A., Karpiskova, R. & Rychlik, I. (2003). Evolution of antibiotic resistance in *Salmonella enterica serovar* Typhimurium strains isolated in the Czech Republic between 1984 and 2002.

Antimicrobial Agents and Chemotherapy 47(6): 2002-2005.

Goethe, R., Gonzáles, O. F., Lindner, T. & Gerlach, G. F. (2000). A novel strategy for protective *Actinobacillus pleuropneumoniae* subunit vaccines: detergent extraction of cultures induced by iron restriction. Vaccine 19(7): 966-975.

Grasteau, A., Tremblay, Y., Labrie, J. & Jacques, M. (2011). Novel genes associated with biofilm formation of *Actinobacillus pleuropneumoniae*. Veterinary Microbiology 153(1-2): 134-143.

Guardabassi, L., Dijkshoorn, L., Collard, J. M., Olsen, J. & Dalsgaard, A. (2000). Distribution and *in-vitro* transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. Journal of Medical Microbiology 49(10): 929-936.

Gutiérrez-Martín, C. B., Blanco, N. G., Blanco, M., Navas, J. & Rodríguez-Ferri, E. F. (2006). Changes in antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Spain during the last decade. Veterinary Microbiology 115(1): 218-222.

Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Microbiology 2(2): 95-108.

Hasegawa, K., Yamamoto, K., Chiba, N., Kobayashi, R., Nagai, K.,

Jacobs, M. R., Appelbaum, P. C., Sunakawa, K. & Ubukata, K. (2003). Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. Microbial Drug Resistance 9(1): 39-46.

Jacques, M. (2004). Surface polysaccharides and iron-uptake systems of *Actinobacillus pleuropneumoniae*. Canadian journal of veterinary research 68(2): 81.

Jung, B. Y., Cho, G.-j., Kim, B.-h. & CHo, K.-h. (1995). Biochemical characteristics and serotypes of *Actinobacillus pleuropneumoniae* isolated from pneumonic lungs of pigs. Korean Journal of Vetetrinary Research 36(1): 181-186.

Kang, H. Y., Jeong, Y. S., Oh, J. Y., Tae, S. H., Choi, C. H., Moon, D. C., Lee, W. K., Lee, Y. C., Seol, S. Y. & Cho, D. T. (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. Journal of Antimicrobial Chemotherapy 55(5): 639-644.

Kaplan, J. B. & Mulks, M. H. (2005). Biofilm formation is prevalent among field isolates of *Actinobacillus pleuropneumoniae*. Veterinary Microbiology 108(1): 89-94.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J. L., Chaslus-Dancla, E. & Schwarz, S. (2001). Antimicrobial resistance in *Pasteurella* and

Mannheimia: epidemiology and genetic basis. Veterinary Research 32(3-4): 323-339.

Kim, B., Min, K., Choi, C., Cho, W. S., Cheon, D. S., Kwon, D., Kim, J. & Chae, C. (2001). Antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Korea using new standardized procedures. The Journal of Veterinary Medical Science 63(3): 341-342.

Kim, I. S., Ki, C. S., Kim, S., Oh, W. S., Peck, K. R., Song, J. H., Lee, K. & Lee, N. Y. (2007). Diversity of ampicillin resistance genes and antimicrobial susceptibility patterns in *Haemophilus influenzae* strains isolated in Korea. Antimicrobial Agents and Chemotherapy 51(2): 453-460.

Kim, J.-a., Kim, S.-i., Woo, H.-c., Lim, Y.-k., Bae, J.-h., Kim, H.-s. & Lee, D.-s. (1998). Studies on biochemical characteristics and serotypes of *Actinobacillus pleuropneumoniae* isolated from pneumonic lungs of swine in Che-ju. Korean Journal of Vetetrinary Public Health 22(2): 147-155.

Koo, H. J. & Woo, G. J. (2011). Distribution and transferability of tetracycline resistance determinants in *Escherichia coli* isolated from meat and meat products. International Journal of Food Microbiology 145(2-3): 407-413.

Kucerova, Z., Hradecka, H., Nechvatalova, K. & Nedbalcova, K. (2011). Antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolates from clinical outbreaks of porcine respiratory diseases. Veterinary Microbiology 150(1-2): 203-206.

Lanz, R., Kuhnert, P. & Boerlin, P. (2003). Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. Veterinary Microbiology 91(1): 73-84.

Lee, J. H., Jeong, J. Y., Jeon, Y. S. & Seok, H. B. (1999). Studies on serotyping and detection of *Actinobacillus pleuropneumoniae* by multiplex PCR techniques and immunodiffusion test. Korean Journal of Animal Science 41(3): 387-396.

Lu, Z., Zhao, P. & SHao, Y. (2002). Study on the inactivated trivalent vaccine against swine infectious pleuropneumoniae: selection of the seed strain, preparation and safety trials of the vaccine. Chinese Journal of Veterinary Science and Technology 32(3): 7-9.

Matter, D., Rossano, A., Limat, S., Vorlet-Fawer, L., Brodard, I. & Perreten, V. (2007). Antimicrobial resistance profile of *Actinobacillus pleuropneumoniae* and *Actinobacillus porcitonsillarum*. Veterinary Microbiology 122(1): 146-156.

Morioka, A., Asai, T., Nitta, H., Yamamoto, K., Ogikubo, Y., Takahashi,

T. & Suzuki, S. (2008). Recent trends in antimicrobial susceptibility and the presence of the tetracycline resistance gene in *Actinobacillus pleuropneumoniae* isolates in Japan. Journal of veterinary medical science 70(11): 1261-1264.

Morsczeck, C., Langendörfer, D. & Schierholz, J. M. (2004). A quantitative real-time PCR assay for the detection of *tet*R of Tn10 in *Escherichia coli* using SYBR Green and the Opticon. Journal of Biochemical and Biophysical Methods 59(3): 217-227.

NCCLS (2008). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. NCCLS documents M31-A3 28: 65-72.

Oswald, W., Konine, D. V., Rohde, J. & Gerlach, G. F. (1999). First chromosomal restriction map of *Actinobacillus pleuropneumoniae* and localization of putative virulence-associated genes. Journal of Bacteriology 181(14): 4161-4169.

Rafiee, M. & Blackall, P. (2000). Establishment, validation and use of the Kielstein-Rapp-Gabrielson serotyping scheme for *Haemophilus parasuis*. Australian Veterinary Journal 78(3): 172-174.

Ramjeet, M., Deslandes, V., Gouré, J. & Jacques, M. (2008). *Actinobacillus pleuropneumoniae* vaccines: from bacterins to new insights into vaccination strategies. Animal Health Research

Reviews 9(1): 25-45.

Rosendal, S. & Mitchell, W. (1983). Epidemiology of *Haemophilus pleuropneumoniae* infection in pigs: a survey of Ontario pork producers, 1981. Canadian Journal of Comparative Medicine 47(1): 1-5.

Rycroft, A. N. & Garside, L. H. (2000). *Actinobacillus* species and their role in animal disease. The Veterinary Journal 159(1): 18-36.

San Millan, A., Escudero, J. A., Catalan, A., Nieto, S., Farelo, F., Gibert, M., Moreno, M. A., Dominguez, L. & Gonzalez-Zorn, B. (2007). β-Lactam resistance in *Haemophilus parasuis* is mediated by plasmid pB1000 bearing bla_{ROB-1}. Antimicrobial Agents and Chemotherapy 51(6): 2260-2264.

Shin, D.-H., Byun, J.-W., Kim, H.-Y., Kim, D., Jang, W., Moon, O.-K., Lee, O.-S. & Jung, B. Y. (2010). Distribution of serotype and biofilm of *Actinobacillus pleuropneumoniae* isolated from pig. Korean Journal of Vetetrinary Public Health 35(1): 7-12.

Smith, M. S., Yang, R. K., Knapp, C. W., Niu, Y., Peak, N., Hanfelt, M. M., Galland, J. C. & Graham, D. W. (2004). Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. Applied and Environmental Microbiology 70(12): 7372-7377.

Wasteson, Y., Roe, D. E., Falk, K. & Roberts, M. C. (1996).

Characterization of tetracycline and erythromycin resistance in *Actinobacillus pleuropneumoniae*. Veterinary Microbiology 48(1-2): 41-50.

Yu, Z., Michel Jr, F. C., Hansen, G., Wittum, T. & Morrison, M. (2005). Development and application of real-time PCR assays for quantification of genes encoding tetracycline resistance. Applied and Environmental Microbiology 71(11): 6926-6933.

국문초록

국내분리 *Actinobacillus pleuropneumoniae*의 항생제 내성 패턴에 대한 표현형과 유전적 특성 분석

유 안 나

(지도교수: 유한상, D.V.M., Ph.D.)

서울대학교 대학원
수의학과 수의미생물학 전공

2006년부터 2010년까지 한국에서 흉막폐렴을 보이는 돼지에서 분리된 *Actinobacillus pleuropneumoniae* 102 개의 균주를 대상으로 디스크 확산법과 마이크로희석법을 이용하여 항생제 감수성 패턴을 분석하였다. Amoxicillin/clavulanic acid, cephalothin, 그리고 ceftiofur는 *A. pleuropneumoniae*에 모두 감수성이 있었지만 대부분의 균주는 lincomycin (100 %), erythromycin (99.9 %), kanamycin (93.2 %)에 내성을 보였다. 특히 florfenicol의 경우 2006년부터

2010년까지의 항생제 감수성이 눈에 띄게 감소하였고, ampicillin과 amoxicillin 또한 작은 부분 감소하였다. Tetracycline과 florfenicol에 내성을 보이는 균주에 대해서는 항생제 감수성 패턴에서 표현형적인 부분에 대하여 심도있는 분석을 하였다. 검사한 11개의 tetracycline 내성 유전자 (*tet* (A), *tet* (B), *tet* (C), *tet* (D), *tet* (E), *tet* (G), *tet* (H), *tet* (K), *tet* (M), *tet* (L), *tet* (O)) 중에서, *tet* (B) 가 가장 많았고 (62 %), *tet* (H) (12 %) 와 *tet* (O) (8 %) 가 그 뒤를 이었다. 전체 중 35개의 균주 (34 %) 는 florfenicol 내성 유전자를 가지고 있었으며, 이러한 결과는 디스크확산법의 결과와 일치하였다. MIC 수치를 결정하는 요인을 알아보기 위해 tetracycline 내성 균주를 대상으로 tetracycline 조절 유전자와 내성 유전자의 발현양, 그리고 바이오필름 형성 정도를 MIC 수치와 비교해 보았으나 큰 관련성은 찾아내지 못하였다. 종간 florfenicol 내성 유전자 (*flo* R) 와 tetracycline 내성 유전자 (*tet* (B)) / β-lactams 내성 유전자 (bla_{ROB-1}) 의 이동가능성이 각각 5.7×10^{-3} 와 3.5×10^{-2} 의 비율로 확인되었다. PFGE를 이용한 DNA 패턴을 분석한 결과 혈청형간 관계는 확인되었으나, 분리된 지역이나 시기와는 관계가 없는 것으로 나타났다. 이 연구 결과는 최근 한국의 *A.*

*pleuropneumoniae*의 항생제 내성에 대한 실태를 보여주고 있으며, *A. pleuropneumoniae*에 감염된 흉막폐렴을 제어하는 데 매우 유용할 것으로 사료된다.

핵심어 : 돼지 흉막폐렴 원인균, 항생제 감수성, 내성 유전자, 유전자 이동, PFGE

학번 : 2011−21689