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Characterization of antibiotic resistance and pathotypes of avian pathogenic *Escherichia coli* isolates in Korea

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Characterization of antibiotic resistance and pathotypes of avian pathogenic *Escherichia coli* isolates in Korea

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Abstract

Characterization of antibiotic resistance and pathotypes of avian pathogenic *Escherichia coli* isolates in Korea

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Antimicrobial therapy has been an important tool in reducing the enormous losses caused by avian pathogenic *Escherichia coli* (APEC) infections in the poultry industry. However, it has been less effective due to antibiotic resistance in APEC which has also been increased dramatically. 101 APEC Korean isolates from 1985 to 2005 were tested for fundamental information of antibiotic resistance in Korea. Antibiograms and relevant genotypes of these isolates were assessed via

disc diffusion test, polymerase chain reactions (PCR), restriction enzyme analysis (REA), and sequencing. Significant increases of resistances to several antibiotics were observed during these periods. Resistance to streptomycin and tetracycline (both at 84.2%) was highest, followed by enrofloxacin (71.3%), ampicillin (67.3%), trimethoprim/sulfamethoxazole (37.6%), gentamicin (26.7%). Relevant resistance genes (*tetA*, *tetB*, *aadA*, *strA-strB* and *TEM*) and mutations in certain region (*gyrA* and *parC*) were increased during same period. As shown in this test results, the tendency of antibiotic resistance has been increased and another protection tool will be needed urgently.

One hundred-one APEC Korean isolates were examined for the characteristics that affect pathogenicity. The serotypes and virulence genes of isolates were determined and they were classified into molecular pathotypes (MPs) on the basis of virulence gene content. Only twenty-eight isolates (27.8%) were serotyped and the frequencies of virulence genes were various. The frequency of *iroN* (100%) was highest, followed by *ompT* (94.1%), *fimC* (90.1%), *hlyF* (87.1%), *iss* (78.2%), *iucD* (73.3%), *tsh* (61.4%), *fyuA* (44.6%), *irp2* (43.6%), and *vat* (10.9%). All isolates were classified into 27 MPs on the basis of virulence gene content. The virulence gene profiles of MPs showed a cumulative pattern. Therefore, the molecular pathotyping can be used for

identifying higher pathogenic APEC isolates.

Eleven APEC isolates were selected to investigate correlation between MPs and pathogenicity. Seven-day-old chickens were inoculated subcutaneously with 10-fold serial dilutions of each isolates (10^9 to 10^6 CFU/0.2 ml) to improve reproducibility of virulence assay and obtain statistical significance. Necropsy, gross pathological examinations, and re-isolation in infected chickens were conducted. Based on LD_{50} of infected chickens, 11 APEC isolates were classified into lethality classes (LC) 1 to 3 as follows: LC1 ($LD_{50} \leq 5 \times 10^6$ CFU), LC2 (5×10^6 to 10^8 CFU), and LC3 ($\geq 5 \times 10^8$ CFU). The ratio LC1, LC2, and LC3 were 18.2% (2/11), 27.3% (3/11), and 54.5% (6/11), respectively. Chickens inoculated with LC1 isolates showed clinical signs of illness as early as 24 hours, and some of chickens were found dead 24 hours after infection. All samples collected from chickens inoculated APEC isolates showed positive re-isolation result. The MPs of higher pathogenic isolates (LC1; lower 50% lethal dose), E64 (MP26; *iroN-fimC-ompT-hlyF-iucD-iss-fyuA-irp2-vat*) and E89 (MP25; *iroN-fimC-ompT-hlyF-iucD-iss-fyuA-irp2-tsh*) were more abundant possession of virulence genes than others, which suggests the correlation between MPs and pathogenicity of APEC isolates.

The present study supplied fundamental information of antibiotic

resistance in Korean APEC isolates and showed molecular pathotyping might be a powerful tool for identifying higher pathogenic APEC isolates. The efficacy of higher pathogenic isolates (LC 1) as a vaccine candidate may be needed in further study.

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Key words: avian pathogenic *E. coli*, antibiotic resistance, virulence gene, molecular pathotypes, lethality class

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List of Abbreviations

ACRONYM	FULL NAME
APEC	Avian pathogenic *Escherichia coli*
PCR	Polymerase chain reactions
REA	Restriction enzyme analysis
SNPs	Single nucleotide polymorphisms
MPs	Molecular pathotypes
LD_{50}	50% lethal dose
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
EAggEC	Eenteroaggregative *E. coli*
EIEC	Eenteroinvasive *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
SXT	Trimethoprim/sulfamethoxazole
PAGE	Polyacrylamide gel electrophoresis
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration

tsh	Temperature-sensitive hemagglutinin
iucD	Iron uptake chelate gene D
fyuA	Ferric yersiniabactin uptake
irp2	Iron-repressible protein 2
iss	Increased serum survival gene
lt	Heat-labile toxin
st	Heat-stable toxin
vat	Vacuolating autotransporter toxin
LC	Lethality classes
DPI	Day-post-inoculation

I. General introduction

Escherichia coli (*E. coli*) is generally considered as a harmless bacterium in the normal intestinal microflora of humans and many animals. However, some strains were able to express pathogenic factors and to provoke intestinal or extra-intestinal diseases (Ambrozic *et al.*, 1998). Approximately 10-15% of *E. coli* were believed to be pathogenic, but definitive criteria for pathogenicity that encompass all pathogenic *E. coli* had not been identified (Horne *et al.*, 2000). Some researchers classified *E. coli* into the following heterogeneous groups based on its infection and pathogenic mechanisms: enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), and enterotoxigenic *E. coli* (ETEC) (Gyles, 1994a; Nataro and Kaper, 1998).

The other groups, which cause extra-intestinal infections, were belong to extra-intestinal pathogenic *Escherichia coli* (ExPEC). ExPEC strains might cause various infections in humans and animals. ExPEC strains were implicated in a large range of extra-intestinal infections in humans, such as neonatal meningitis, septicemia, urinary tract infections, and pneumonia, as well as in animals such as urinary tract infections in cats

and dogs, septicemia in calves and pigs, and systemic colibacillosis in birds (Gan *et al.*, 2013; Johnson and Russo, 2002; Mokady *et al.*, 2005; Ron, 2006). In ExPEC strains, *E. coli* that causes diseases in poultry was collectively known as avian pathogenic *E. coli* (APEC) (Kariuki *et al.*, 2002). Thus, APEC mostly caused extra-intestinal infection in poultry including colisepticemia (Dho-Moulin and Fairbrother, 1999; Kawanishi *et al.*, 2013; Vandemaele *et al.*, 2005). APEC strains showed similarities with human ExPEC strains. Even though no specific set of pathogenic factors could be associated with APEC strains, most of the virulence genes that they possess was similar to those identified in human ExPEC strains (Delicato *et al.*, 2003; Germon *et al.*, 2005; Johnson *et al.*, 2012; Lafont *et al.*, 1987; Mellata *et al.*, 2003; Ngeleka *et al.*, 2002; Rodriguez-Siek *et al.*, 2005a; Ron, 2006; Schouler *et al.*, 2012; Stordeur *et al.*, 2002).

APEC was mostly associated with infection of extra-intestinal tissues in chickens, turkeys, ducks and other avian species (Barbieri *et al.*, 2013; Dho-Moulin and Fairbrother, 1999). The hatcheries and the breeder farms could act as sources of bacterial contamination while the breeder farm was the main source of disease such as yolk-sac infection. Fecal contaminations of egg shell surface induced yolk sac infection of day old chicks. These vertical contaminations often resulted in embryonic

mortality or death of the young birds after hatching. Horizontal contaminations usually occurred through contact with other birds, or through feces, contaminated water and feed. Birds were frequently contaminated by inhalation of fecal contaminated particles in dust. Most of the diseases associated with them was secondary to environmental and host predisposing factors which are Newcastle disease virus, infectious bronchitis virus, *Mycoplasma gallisepticum*, live respiratory vaccine virus, ammonia gas, and stress from farm management (Barbieri *et al.*, 2013; Dho-Moulin and Fairbrother, 1999; Rosario *et al.*, 2004).

The disease, generally found in domesticated and wild birds, started as a respiratory tract infection, leading to a systemic infection of internal organs with sepsis finally setting in (Bonacorsi and Bingen, 2005; Ewers *et al.*, 2007; Ozawa and Asai, 2013). APEC caused several severe disease and syndromes in poultry farms such as colisepticemia, peritonitis, air sac disease, pericarditis, perihepatitis, salpingitis, omphalitis, yolk sac infection, cellulitis, and swollen head syndrome (Fernández *et al.*, 1986; Gan *et al.*, 2013; Gomis *et al.*, 1997; Kawanishi *et al.*, 2013; Morley and Thomson, 1984; Nagi and Mathey, 1972; Pourbakhsh *et al.*, 1997a).

Many isolates detected among APEC strains had already been implicated in various human diseases including new born meningitis and

urinary tract infections suggesting a possible transfer of avian strains to humans or vice versa and thus a potential zoonotic risk of avian strains (Ewers *et al.*, 2007; Johnson *et al.*, 2003; Porcheron *et al.*, 2012; Rodriguez-Siek *et al.*, 2005a).

APEC infection could be prevented by controlling environmental contamination in order to avoid predisposing respiratory infections. Infection of the respiratory tract of birds could be reduced by maintaining mycoplasma-free birds and by controlling the environmental factors (ventilation, dust, and ammonia gas) (Barbieri *et al.*, 2013; Dho-Moulin and Fairbrother, 1999). Control of APEC was important to public health and poultry industry. Antimicrobial therapy had been an important tool in reducing both the incidence and mortality associated with avian colibacillosis (Freed *et al.*, 1993; Watts *et al.*, 1993). However, the prevalence of antibiotic resistance and public concern to antibiotics were increased (Allan *et al.*, 1993; Amara *et al.*, 1995; Cloud *et al.*, 1985; Gan *et al.*, 2013; Ozawa and Asai, 2013; Zhao *et al.*, 2005). Misuse of antimicrobials was likely to be the major cause of the increasing prevalence of antibiotic-resistance, and mobile DNA elements, including plasmids, transposons, and integrons are the major tools to transfer antimicrobial resistance between bacterial strains (Kawanishi *et al.*, 2013; Leibert *et al.*, 1999; Speer *et al.*, 1992). Therefore, in chapter 1, it

was attempted to characterize one of mobile DNA elements (integrons), related factors, and relationships with several antibiotics in APEC isolated between 1985 and 2005. Additionally, the frequencies of certain resistance genes and antibiograms were chronologically compared.

Some researchers suggested that many APEC isolates are non-pathogenic strains and that these strains may act as opportunistic agents in causing disease (Barbieri *et al.*, 2013; Da Silveira *et al.*, 2003; Da Silveira *et al.*, 2002). Therefore, researchers had been trying to develop specific typing method to identify virulent isolates. A variety of methods had been used to study avian pathogenic *E. coli*, including somatic antigen serologic typing (Blanco *et al.*, 1998; Cloud *et al.*, 1985; Hemsley *et al.*, 1967; Schoulder *et al.*, 2012; Whittam and Wilson, 1988), phylogenetic groups (Barbieri *et al.*, 2013; Bingen *et al.*, 1998; Herzer *et al.*, 1990; Johnson *et al.*, 2008b; Zhao *et al.*, 2009), and molecular characterization (Ewers *et al.*, 2009; Johnson *et al.*, 2008a; Kemmett *et al.*, 2013; Ling *et al.*, 2013; Schouler *et al.*, 2012). Recently, potential virulence genes had been reported more often in clinical isolates than fecal isolates of *E. coli* from avian sources (Altekruse *et al.*, 2002a; Maurer *et al.*, 1998; Pfaff-McDonough *et al.*, 2000; Reingold *et al.*, 1999). The frequency of various virulence genes in APEC strains also had been reported, but knowledge of the frequencies of combined virulence genes and patterns of virulence

gene accumulation in APEC strains was limited, which limits the understanding of the evolution of APEC in pathogenicity (Dziva and Stevens, 2008). To address this shortcoming, serotyping, phylogenetic grouping, and molecular pathotyping were performed in chapter 2. It was determined the serotypes, phylogenetic groups, and molecular pathotypes (MPs) of 101 APEC isolates collected in Korea from 1985 to 2005.

Additionally, in chapter 3, 11 isolates of APEC were selected on the basis of MPs in chapter 2 and tested the LD_{50} of these isolates in chickens. The objective of this study was sought to investigate correlation MPs with pathogenicity.

In Korea, some researchers had studied APEC. But, most of their studies had been related to biochemical and cultural characteristics of APEC, such as colicin production, hemolysin catalase, indole, methyl-red test, congo red binding ability, HA activity, R-plasmid (Kim and Namgoong, 1988; Kim and Tak, 1984; Kim and Tak, 1983; Seo *et al.*, 1990; Woo *et al.*, 1991; Woo *et al.*, 1990), antibiotic resistance of APEC (Cho *et al.*, 2006; Kim *et al.*, 2007b; Kim, 2000; Kim and Namgoong, 1988; Kim and Namgoong, 1987; Lee *et al.*, 2005; Seo *et al.*, 1990), serotyping (Cho and Shin, 1985; Kim and Namgoong, 1987; Kim and Tak, 1983; Oh *et al.*, 2011; Seo *et al.*, 1990), bacterial isolation from livestock

meats (Lee *et al.*, 2005), and molecular characterization (Kwon *et al.*, 2002; Oh *et al.*, 2011). Therefore, this study will provide fundamental information of antibiotic resistance in Korean APEC isolates and potential selection markers for pathogenic APEC for diagnosis and vaccine candidate.

II. Main text

Chapter 1. Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic *Escherichia coli* isolates

1.1. Abstract

APEC can cause colibacillosis in all ages of chickens and other avian species. Antimicrobial therapy was an important tool in reducing both the incidence and mortality associated with avian colibacillosis. However, the increasing incidence of antibiotic resistance to APEC was a worldwide problem not only for economic losses in the poultry farms but also for the public health concern. It might be induced by misuse of antimicrobials and virulence gene transfer by mobile DNA elements, including plasmids, transposons, and integrons. Therefore, it was important to know antibiograms and relevant genotypes of Korean APEC isolates. In this study, antibiograms and relevant genotypes of 101 APEC Korean isolates from 1985 to 2005 were assessed via disc diffusion test, polymerase chain reactions (PCR), restriction enzyme analysis (REA), and sequencing.

Multi drug resistance (MDR) APEC isolates were detected in 76.2% during observation period. Korean APEC isolates were highly resistant to streptomycin and tetracycline (both at 84.2%) was highest, followed by

enrofloxacin (71.3%), ampicillin (67.3%), trimethoprim/sulfamethoxazole (37.6%), gentamicin (26.7%). Among them, most of tetracycline, streptomycin, enrofloxacin and ampicillin resistances were associated with *tetA* and/or *tetB*, *aadA* and/or *strA*−*strB*, mutations in *gyrA* and/or *parC*, and *TEM*, respectively. Class 1 integrons were detected in 40 isolates (39.6%), and a variety of gene cassettes conferring streptomycin (*aadA*), gentamicin (*aadB*) and trimethoprim (*dfr*) resistances were identified: *aadA1a* (27.5%), *dfrV*−*orfD* (2.5%), *aadB*−*aadA1a* (2.5%), *dfr I*−*aadA1a* (47.5%), *dfr XVII*−*aadA5* (12.5%), and *dfr XII*−*orfF*−*aadA2* (7.5%).

In addition, several types of common promoters (P_{ant}) of the gene cassettes (hybrid P1, weak P1, or weak P1+P2) and single nucleotide polymorphisms (SNPs) in *aadA1a* were identified. The results of a chronological analysis demonstrated significant and continuous increases in resistances to several antibiotics (tetracycline, streptomycin, enrofloxacin, ampicillin, and trimethoprim/sulfamethoxazole), and frequencies of the relevant resistance genes (*tetA*, *strA*−*strB*, and *TEM*), mutations in *gyrA* and *parC*, and multi−drug resistant APEC isolates during the period 2000−2005. This study supplied fundamental informations of antibiotic resistance in Korean APEC isolates and showed that the tendency of antibiotic resistance was increased. Therefore,

another control tools will be needed.

1.2. Introduction

Escherichia coli (*E. coli*) was generally considered as a harmless bacterium in the normal intestinal microflora of humans and many animals. Approximately 10–15% of *E. coli* was believed to be pathogenic (Barbieri et al., 2013; Horne *et al.*, 2000). *E. coli* that causes disease in chickens was collectively known as avian pathogenic *E. coli* (Kariuki *et al.*, 2002; Vandemaele *et al.*, 2005). The APEC induced colibacillosis in chickens including polyserositis, septicemic shock, and cellulitis (Dho-Moulin and Fairbrother, 1999; Vandemaele *et al.*, 2005), and APEC infections were major cause of enormous economic losses in poultry industry (Altekruse *et al.*, 2002b; Blanco *et al.*, 1997a; Dho-Moulin and Fairbrother, 1999; Gyles, 1994b).

Antimicrobial therapy was an important tool in reducing the enormous losses caused by *E. coli* infection (colibacillosis) in the poultry industry (Freed *et al.*, 1993). However, resistance to existing antimicrobials was widespread after introduction of antibiotic therapies, and increasing incidence of antibiotic resistances among APEC had been recognized as an increasing problem in the veterinary and medical fields (Blanco *et al.*, 1997a; Gan *et al.*, 2013).

Bacterial antibiotic resistance was frequently caused by the acquisition of new genes rather than by mutation (Hall and Collis, 1995). Mobile

DNA elements, including plasmids, transposons, and integrons, facilitated the proliferation of resistance genes in bacteria and played an important role in the dissemination of antimicrobial resistance genes as a horizontal transfer (Kang *et al.*, 2005; Kawanish *et al.*, 2013; Leibert *et al.*, 1999; Speer *et al.*, 1992). Plasmids carrying one or more antibiotic resistance genes were isolated in many countries and the common association of antibiotic resistance with plasmids was now widely accepted (Hall and Collis, 1998). The integrons were themselves mobile elements, namely transposons or defective transposon derivatives, and sometimes they were found within other transposons. Transposition enabled integrons to move onto plasmids and hence acrossed species boundaries. As consequence, the resistance genes found in gene cassettes and thus associated with integrons were widely distributed amongst the *Enterobacteriaceae* and were also found in *Peudomonas aeruginosa* (Hall and Collis, 1998).

Class 1 integrons had been identified as the most prevalent class among five classes of integron in clinical isolates. Class 1 integrons were most commonly found in clinical isolates of Gram-negative bacteria. Class 1 integron had been known to be associated with multi-drug resistance in pathogenic bacteria (Hall and Stokes, 1993; Kang *et al.*, 2005; Recchia and Hall, 1995). The class 1 integron acquired resistance

genes in the form of gene cassettes via site-specific recombination. Resistance of gram-negative organisms to antibiotics such as β-lactams, aminoglycosides, trimethoprim and chloramphenicol was caused by many different acquired genes, and a substantial proportion of these part of small mobile elements was known as gene cassettes (Hall and Collis, 1998). Integrons generally contained an integrase gene (*intI*) and a cassette integration site (*attI*), into which antibiotic resistance gene cassettes had been integrated. A gene cassette contained an antibiotic resistance gene and a 59-bp element, a short inverted repeat element with a core recombination site (Bass *et al.*, 1999).

Among the same gene cassettes, some extent of nucleotide changes tended to be present, and some of these were shared in common by other bacteria. Some of those nucleotide changes could be classified as single nucleotide polymorphisms (SNPs), and SNPs had been proven useful in the fine differentiation of bacterial isolates, as well as in studies of the molecular evolution of class 1 integrons (Kim *et al.*, 2007a; Sokurenko *et al.*, 1999). Since gene cassettes lacked their own promoters, their expression levels were affected by their proximity to the common promoter, P_{ant} (P1), the strength of the promoter P1, and the presence of P2 (Collis and Hall, 1995). P1 could be classified into three groups according to their promoter activity; the strong, hybrid, and weak

promoters. The insertion of three guanosines into 119 nucleotides downstream from P1 resulted in the creation of a new weak promoter, designated P2 (Collis and Hall, 1995; Fluit *et al.*, 1999; Lévesque *et al.*, 1994). Thus far, integron studies had focused primarily on the contents of gene cassettes, and had largely overlooked their common promoter structures (Kim *et al.*, 2007a).

Tetracyclines, streptomycin, enrofloxacin, and ampicillin were commonly utilized in the treatment of diseases and to promote growth in livestock animals. Although resistances to various antibiotics including these compounds had clearly increased, the relevant resistance genes in Korea remained to be elucidated thoroughly. Therefore, I had attempted to characterize the class 1 integrons in APEC isolated between 1985 and 2005 from the perspective of their gene cassettes as well as their promoters, and had delineated the relationships between tetracycline, streptomycin, enrofloxacin, ampicillin, trimethoprim/sulfamethoxazole (SXT), and gentamicin resistances to relevant genes (*tetA*, *tetB*, *strA*–*strB*, *gyrA*, *parC*, and *TEM*). Additionally, the frequencies of certain resistance genes (*tetA*, *strA*–*strB*, and *TEM*) and antibiograms were chronologically compared. Resistance surveillance tests were usually aimed at assessing resistance phenotypes. These resistance phenotypes might arise from many different genetic determinants and each

determinant might present specific epidemiological features. The aim of the current study was to provide fundamental information for characterizing Korean isolates of APEC and for further study in these isolates.

1.3. Materials and Methods

1.3.1. Bacteria

One hundred-one APEC isolates between 1985 and 2005 were isolated from Korean chickens suffering from colibacillosis. All of the APEC isolates were identified using VITEK® Gram-Negative Identification (GNI) Cards (bioMerieux Vitek, Hazelwood, MO). Once identified, all isolates were preserved at −70℃ in LB broth containing 20% glycerol (v/v), until further studies.

1.3.2. Antimicrobial susceptibility testing

All isolates were tested for their susceptibility to 7 antimicrobial agents (tetracycline, streptomycin, enrofloxacin, ampicillin, trimethoprim/sulfamethoxazole, gentamicin, and chloramphenicol) via disk diffusion assay following CLSI (formerly NCCLS) guidelines (National Committee for Clinical Laboratory Standards, 2003).

1.3.3. Detection of class 1 integrons by PCR

Total APEC DNA was extracted with G-spin genomic DNA extraction kit (for Gram-negative; iNtRON Biotechnology Co., Seoul, Korea) in accordance with the manufacturer's instructions. All isolates were

evaluated for the presence of class 1 integrons using the 5'CS and 3'CS primer set (Table 1.1) (Lévesque *et al.*, 1995). PCR was conducted by cycling conditions as follows: 95°C, 5 min; 35 cycles at 95°C, 40 sec; 53°C, 40 sec; and 72°C, 2 min; and a final extension step at 72°C, 5 min. Amplicons were analyzed through electrophoresis on 1.0% agarose gels. Size determination was achieved using a 1 kb ladder (iNtRON Biotechnology Co., Seoul, Korea) as molecular weight marker (Kwon *et al.*, 2002).

1.3.4. Characterization of P_{ant} by restriction enzyme analysis (REA) and Polyacrylamide gel electrophoresis (PAGE)

For the rapid and simple characterization of the class 1 integron promoter structures, PCR primer sets for REA and sequencing, and for PAGE were designed as described previously (Table 1.1) (Kim *et al.*, 2007). The amplicons generated by the IntProF/ IntProR primer set were subsequently treated with *Hinc*II and *Alu*I (for P1) as described previously (Kim *et al.*, 2007), and *Bsrg*I (for P2) in separate tubes, then they were subjected to electrophoresis in 2% agarose gel. PAGE was also conducted with the amplicon for four hours using 12% (19:1) polyacrylamide gel at 90V, and the gel was stained with ethidium bromide. The expected fragments and promoter types were summarized in Figure 1.1.

Table 1.1. Primer sets for class 1 integrons and antibiotic resistance genes of avian pathogenic *E. coli* isolates

Gene	Primer sequence (5'–3')	Amplicon size (bp)	Reference
Class 1 integron	F: GGCATCCAAGCAGCAAG R: AAGCAGACTTGACCTGA	1000–2000	Lévesque *et al.*, 1995
Class 1 integron for REA[a]	F: ATGCCTCGACTTCGCTGCT R: ACTTTGTTTTAGGGCGACTGC	327	Kim *et al.*, 2007a
Class 1 integron for PAGE[b]	F: TGGTAACGGCGCAGTGGC R: TTGCTGCTTGGATGCCCG A	82	Kim *et al.*, 2007a
tetA	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210	Ng *et al.*, 1999
tetB	F: TTGGTTAGGGGCAAGTTTTG R: GTAATGGGCCAATAACACCG	659	Ng *et al.*, 1999
strA–strB	F: TCTATCTGCGATTGGACCCTC R: ATTGCTCATCATTTGATCGGC	520	Sunde and Norström, 2005.
tem	F: TACTCACCAGTCACAGAAAAGC R: TGCTTAATCAGTGAGGCACC	548	Bradford, 2001 De Gheldre *et al.*, 2003
gyrA	F: TACACCGGTCAACATTGAGG R: TCRATACCRCGACGACCGTT	166–355	Everett *et al.*, 1996
parC	F: TGTATGCGATGTCTGAACTG R: CTCAATAGCAGCTCGGAATA	137–401	Everett *et al.*, 1996

[a] Restriction enzyme analysis.

[b] Polyacrylamide gel electrophoresis.

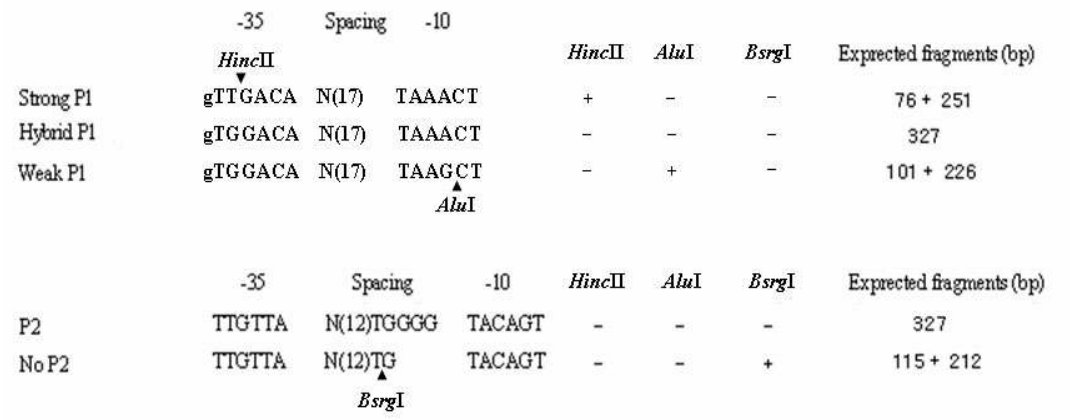

Figure 1.1. Strategy for the differentiation of common promoters (P1 and P2) of class 1 integron gene cassettes via restriction enzyme analysis (REA). Arrowheads indicate enzymatic cleavage sites.

1.3.5. Detection of antibiotic resistance genes by PCR

Tetracycline and streptomycin-resistance genes were PCR amplified using primers targeting the tetracycline efflux genes, *tetA* and *tetB*, and the streptomycin-phosphorylation genes, *strA-strB*. The primer sets were designed as described in previous studies (Table 1.1) (Ng *et al.*, 1999; Sunde and Norström, 2005). The PCR solution was composed of 10 × buffer (2 μl), dNTPs (2.5 mM, 2 μl), forward and reverse primer (10 pmol/μl, 1.0 μl each), Taq DNA polymerase (5 U/μl; iNtRON Biotechnology Co., 0.2 μl), distilled water 12.8 μl and template DNA (50 ng/μl, 1 μl). In negative control reaction, the DNA template was replaced by sterile deionized water. Cycling conditions of *tetA* and *tetB* were 94℃, 5 min; 40 cycles at 94℃, 20 sec; 57℃, 20 sec; 72℃, 30 sec, and final elongation at 72℃, 5 min. Cycling conditions of *strA-strB* were 94℃, 5 min; 35 cycles at 94℃, 30 sec; 58℃, 20 sec; 72℃, 40 sec, and final elongation at 72℃, 5 min. Amplicons were analyzed by electrophoresis on 1.0% agarose gels, and 100bp ladder, 1Kb ladder (iNtRON Biotechnology Co., Seoul, ROK) was used as the molecular size marker. Of ampicillin resistance genes, *TEM* was targeted for PCR amplification. The primer set was designed for amplification of *TEM* mutants (Table 1.1) (Bradford, 2001; De Gheldre *et al.*, 2003). Resistance to

enrofloxacin was assessed by determining mutations in *gyr A* with a primer set for PCR and sequencing and in *parC* with a primer set for PCR and sequencing (Table 1.1).

1.3.6. Sequencing and sequence analysis

The PCR amplicons were purified using a PCR quick Spin Kit (iNtRON Biotechnology Co., Seoul, Korea) in accordance with the manufacturer's instructions. The DNA sequences obtained were compared to the information in the GenBank database of the BLAST network of the National Center for Biotechnology Information (Altschul *et al.*, 1990).

1.3.7. Statistical analysis

The increases and decreases of antibiotic resistances and the frequency of resistance genes were compared between the periods Studied by Chi-square and Fisher's exact tests (with 95% confidence interval), using SPSS for Windows, version 12.0. All experiments in this study were confirmed by more than two times of repetitions.

1.4. Results

1.4.1. Antimicrobial susceptibility

The highest rate of resistance detected was against streptomycin and tetracycline (both at 84.2% prevalence) followed by enrofloxacin (71.3%), ampicillin (67.3%), SXT (37.6%), gentamicin (26.7%), and chloramphenicol (15.8%) (Table 1.2). Significant increase or decrease of resistances to tetracycline, streptomycin, enrofloxacin, ampicillin, SXT, and chloramphenicol were observed during these periods ($P<0.05$) (Table 1.2). Remarkably, the MDR APEC evidencing resistance against at least three different classes of antibiotics had increased from 60.0% in the period from 1985–1989 and 57.1% in 1990–1999 to 94.1% in 2000–2005 period ($P=0.003$ and $P=0.000$ for the comparisons of 2000 to 2005 with 1985 to 1989 and 1990 to 1999, respectively; $P < 0.05$ for both comparisons averaged) (Table 1.3).

Table 1.2. Antimicrobial susceptibility of APEC isolates collected in Korean chickens

Antimicrobial drugs	Frequency of resistance			
	1985−1989	1990−1999	2000−2005	Average
Streptomycin (S)	60.0% (9/15)	80.0% (28/35)	94.1% (48/51) [a]	84.2% (85/101)
Tetracycline (Te)	86.7% (13/15)	68.6% (24/35)	94.1% (48/51) [b]	84.2% (85/101)
Enrofloxacin (ENR)	26.7% (4/15)	60.0% (21/35)	92.2% (47/51) [a,b]	71.3% (72/101)
Ampicillin (AM)	46.7% (7/15)	51.4% (18/35)	84.3% (43/51) [a,b]	67.3% (68/101)
Trimethoprim/Sulfamethoxazol (SXT)	0.0% (0/15)	31.4% (11/35) [a]	52.9% (27/51) [a,b]	37.6% (38/101)
Gentamicin (G)	13.3% (2/15)	22.9% (8/35)	33.3% (17/51)	26.7% (27/101)
Chloramphenicol (C)	40.0% (6/15)	14.3% (5/35)	9.8% (5/51) [a]	15.8% (16/101)

[a] significant increase or decrease ($P<0.05$) compared to the 1985−1989 period.

[b] significant increase ($P<0.05$) compared to the 1990−1999 period.

Table 1.3. Number of multi-drug resistance (MDR) avian pathogenic *E. coli* (APEC) isolates collected in Korean chickens

Period	1985-1989	1990-1999	2000-2005	Total
No. of tested APEC	15	35	51	101
No. of MDR APEC	9	20	48	77
Frequency of MDR APEC	60.0%	57.1%	94.1% [a,b]	76.2%

[a] significant increase ($P<0.05$) compared to the 1985-1989 period.

[b] significant increase ($P<0.05$) compared to the 1990-1999 period.

1.4.2. Molecular characterization of antibiotic resistance

Class 1 integrons were detected in 39.6% of APEC (40/101) and the amplicon sizes were variable at 1,000, 1,478, 1,662, 1,680, and 2,000bps (Figure 1.2, Table 1.4). Nucleotide sequencing showed *aadA1a* in the 1,000bp, *dfrV-orfD* in the 1,478bp, *dfrI-aadA1a* or *aadB-aadA1a* in the 1,662bp, *dfrXVII-aadA5* in the 1,680bp, and *dfrXII-orfF-aadA2* in the 2,000bp amplicons (Table 1.4). *aadA1a* and *aadA5*, *aadB*, and *dfr* were associated with streptomycin, gentamicin, and trimethoprim resistance, respectively. The nucleotide sequences of *aadA1a*, *dfrV-orfD*, *dfrI-aadA1a*, *aadB-aadA1a*, *dfrXVII-aadA5*, and *dfrXII-orfF-aadA2* were similar to those of GenBank sequences with accession numbers AB188263, AM231806, AJ884723, AY139602, AY748452, and AB154407, respectively. Nucleotide changes suspected of being single nucleotide polymorphisms (SNPs) were then evaluated with regard to their frequency via BLAST searches (www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences of *aadA1a* in the 1,000bp and 1,662bp amplicons differed slightly at codons 201 and 250: AAG (K) vs. AGA (R) and GTC (V) vs. GTT (V), respectively. Both corresponding codons at 201 and 250 were common, shared by significant numbers of *aadA1* registered in the GenBank database. Among the APEC isolates harboring *dfrXVII-aadA5*, one isolate evidenced a single nucleotide mutation at codon 7 of

dfrXVII, which resulted in an amino acid change from S (TCT) to P (CCT), and another isolate evidenced a silent mutation at codon 149 of *aadA5*, TCC (S) to TCA (S). However, both codons, CCT in *dfrXVII* and TCA in *aadA5*, were rare, but TCT in *dfrXVII* and TCC in *aadA5* were common in the GenBank database.

The array of gene cassettes, *dfrI–aadA1a*, the prevalence of which increased steeply during the 2000–2005 period (P=0.003 and P=0.007 for the comparisons of 2000 to 2005 with 1985 to 1989 and 1990 to 1999, respectively; P<0.05 for comparisons averaged), was the most prevalent (47.5%), and *aadA1a* was the second most frequently detected (27.5%) (Table 1.4). The oldest APEC isolated in 1985 harbored *aadA1a*. One isolate harboring *dfrXVII–aadA5* was found, unexpectedly, to be susceptible to both streptomycin and SXT.

Tetracycline resistance was not associated with the class 1 integron, and *tetA* and *tetB* were detected via PCR. *tetA* was detected in 40.0% and 42.9% of APEC during the 1985–1989 and 1990–1999 periods, respectively, but its frequency during 2000–2005 increased steeply compared with both periods, to 78.4% (P=0.009 and P=0.001 for the comparisons of 2000 to 2005 with 1985 to 1989 and 1990 to 1999, respectively; P<0.05 for both comparisons averaged) (Table 1.5). *tetB* was present in an average of 18.8% of APEC, and its frequency remained

relatively stable during the periods of observation (Table 1.5). Isolates positive for both *tetA* and *tetB* (5.9%) and negative for both but still tetracycline-resistant (5.0%) were observed (Table 1.2 and 1.5). Therefore, the majority of tetracycline resistance in APEC could be attributed to *tetA* and/or *tetB* (94.1%, 80/85).

In an effort to investigate streptomycin resistance genes other than *aadA*, PCR directed to *strA-strB* was conducted. The *strA-strB* was detected in 33.3% and 48.6% of isolates during 1985-1989 and 1990-1999, respectively, but its frequency increased steeply to 84.3% during 2000-2005 (P=0.000 and P=0.001 for the comparisons of 2000 to 2005 with 1985 to 1989 and 1990 to 1999, respectively; P<0.05 for both comparisons averaged) (Table 1.5). Of the APEC isolates, 29.7%, 34.7%, and 8.9% of isolates harbored both *aadA* and *strA-strB*, *strA-strB* alone, and *aadA* alone, respectively, and 10.9% of isolates were negative for both but still streptomycin resistant (Table 1.2 and 1.5). Therefore, 76.5% (65/85) of the streptomycin resistance in APEC isolates could be attributed to *strA-strB*.

Most of ampicillin resistance was associated with *TEM*. *TEM* was detected in 33.3% and 31.4% of APEC isolates during periods 1985-1989 and 1990-1999, respectively, but its frequency during 2000-2005 increased, to 60.8%. The frequency of *TEM* during 2000-2005 was

significantly higher than that in 1990–1999 (P=0.009) (Table 1.5).

Most of enrofloxacin resistant APEC isolates possessed mutations at residues 83 and 87 in quinolone–resistance–determining region (QRDR) of *gyrA* (88.9%, 64/72) and at residue 80 in its analogue of *parC* (72.2%, 52/72). Leucine and isoleucine substituted for serines at residue 83 in *gyrA* and residue 80 in *parC*, respectively, but various amino acids (asparagine, alanine, glycine, histidine, and tyrosine) replaced the aspartic acid at residue 87 in *gyrA* (Table 1.6). The frequency of the double mutations of *gyrA* and the single mutation of *parC* were significantly increased from the period 1990–1999 (28.6 and 38.1%, respectively) to the period 2000–2005 (87.2 and 91.5%, respectively) (P=0.000 and P<0.05, respectively) (Table 1.6).

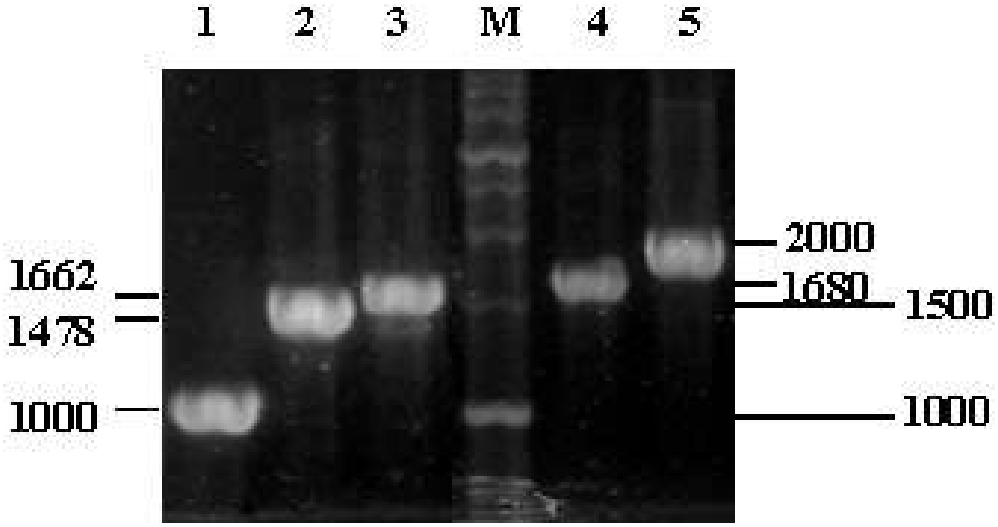

Figure 1.2. Amplified class 1 integron gene cassettes of APEC isolates collected in Korean chickens. Lanes: M, 1−kb molecular weight marker (iNtRON Biotechnology, Seoul, Republic of Korea); 1, 1,000 bp; 2, 1,478 bp; 3, 1,662 bp; 4, 1,680 bp; 5, 2,000 bp.

Table 1.4. Molecular characterization of Class 1 integrons in APEC isolates

Amplicons (bp)	Gene cassettes	Relevant resistance[a]	Promoters		No. of APEC isolates			Frequency[c]
			P1	P2	1985–1989	1990–1999	2000–2005	
1,000	*aadA1a*	S	Weak	+	2	5	4	27.5 (11/40)
1,478	*dfrV–orfD*	S–SXT	Weak	–	0	1	0	2.5 (1/40)
1,662	*dfrI–aadA1a*	S–SXT	Weak	–	0	2	17[b]	47.5 (19/40)
	aadB–aadA1a	G–S	Hybrid	–	1	0	0	2.5 (1/40)
1,680	*dfrXVII–aadA5*	S–SXT	Hybrid	–	1	3	0	10.0 (4/40)
		–			0	0	1	2.5 (1/40)
2,000	*dfrXII–orfF–aadA2*	S–SXT	Weak	–	0	0	2	5.0 (2/40)
			Hybrid	–	0	1	0	2.5 (1/40)
Frequency[c]					26.7 (4/15)	34.3 (12/35)	47.1 (24/51)	39.6 (40/101)

[a] S, Streptomycin; SXT, Sulfamethoxazole and Trimethoprim; G, Gentamicin; –, susceptible.

[b] Significantly high frequency ($P<0.05$) compared to the periods, 1985–1989 and 1990–1999.

[c] Percentage (No. of isolates showed gene cassettes/No. of isolates tested).

Table 1.5. Relationships between antibiotics resistances and genotypes

Antibiotics	Genotypes	Frequency of APEC isolates per year			
		1985−1989	1990−1999	2000−2005	Average
Tetracycline	*tetA*$^{+}$/*tetB*$^{+}$	0.0% (0/15)*	5.7% (2/35)	7.8% (4/51)	5.9% (6/101)
	tetA^{+}	40.0% (6/15)	37.1% (13/35)	70.6% (36/51)	54.5% (55/101)
	tetB^{+}	26.7% (4/15)	22.9% (8/35)	13.7% (7/51)	18.8% (19/101)
	tetA^{-}/*tetB*$^{-}$	33.3% (5/15)	34.3% (12/35)	7.8% (4/51)	20.8% (21/101)
Streptomycin	*aadA*$^{+}$/*strA*−*strB*$^{+}$	6.7% (1/15)	22.9% (8/35)	41.2% (21/51)	29.7% (30/101)
	aadA^{+}	20.0% (3/15)	8.6% (3/35)	5.9% (3/51)	8.9% (9/101)
	strA−*strB*$^{+}$	26.7% (4/15)	25.7% (9/35)	43.1% (22/51)	34.7% (35/101)
	aadA^{-}/*strA*−*strB*$^{-}$	46.7% (7/15)	42.9% (15/35)	9.8% (5/51)	26.7% (27/101)
Ampicillin	*TEM*	33.3% (5/15)	31.4% (11/35)	60.8% (31/51)	46.5% (47/101)

* Percentage (No. of isolates showed genotype/No. of isolates tested).

Table 1.6. Molecular characterization of enrofloxacin-resistant APEC isolates

gyrA		*parC*	1980-1989	1990-1999	2000-2005	Total
Ser[a]-83	Asp[b]-87	Ser-80				
wt[c]	wt	wt	3	4	1	8
wt	mt	wt	0	1	0	1
mt[d]	wt	wt	0	8	3	11
mt	wt	mt	0	2	2	4
mt	mt	mt	0	0	1	1
mt	mt	mt	1	1	8	10
mt	mt	mt	0	0	1	1
mt	mt	mt	0	4	26	30
mt	mt	mt	0	1	5	6
Total			4 (25.0%*, 25.0%**)	21 (28.6%*, 38.1%**)***	47 (87.2%*, 91.5%**) ***	72 (66.7%*, 72.2%**)

[a] Serine. [b] Aspartic acid. [c] wild type. [d] mutant type.

* The frequency of double mutations in *gyrA.*

** The frequency of mutation in *parC.*

*** Significant difference (P=0.000, P<0.05).

1.4.3. Molecular characterization of common promoters of gene cassettes

The amplicons harboring weak P1 promoters were not digested by *HincII*, but were digested by *AluI*, and amplicons harboring the hybrid P1 promoters were not digested by either of the enzymes. Strong P1 promoters, digested only by *HincII*, were not detected among the APEC isolates assessed in this study. A 3-nucleotide insertion in P2 resulted in a gel shift, and the amplicons either were (P2 negative) or not (P2 positive) digested by *BsrgI*. Therefore, 7, 22, and 11 isolates of APEC harbored hybrid, weak P1, and weak P1 plus P2 promoters, respectively (Table 1.4, Figure 1.1, Figure 1.3, Figure 1.4). All amplicons utilized in this promoter study were sequenced, and all of the nucleotide variations were verified.

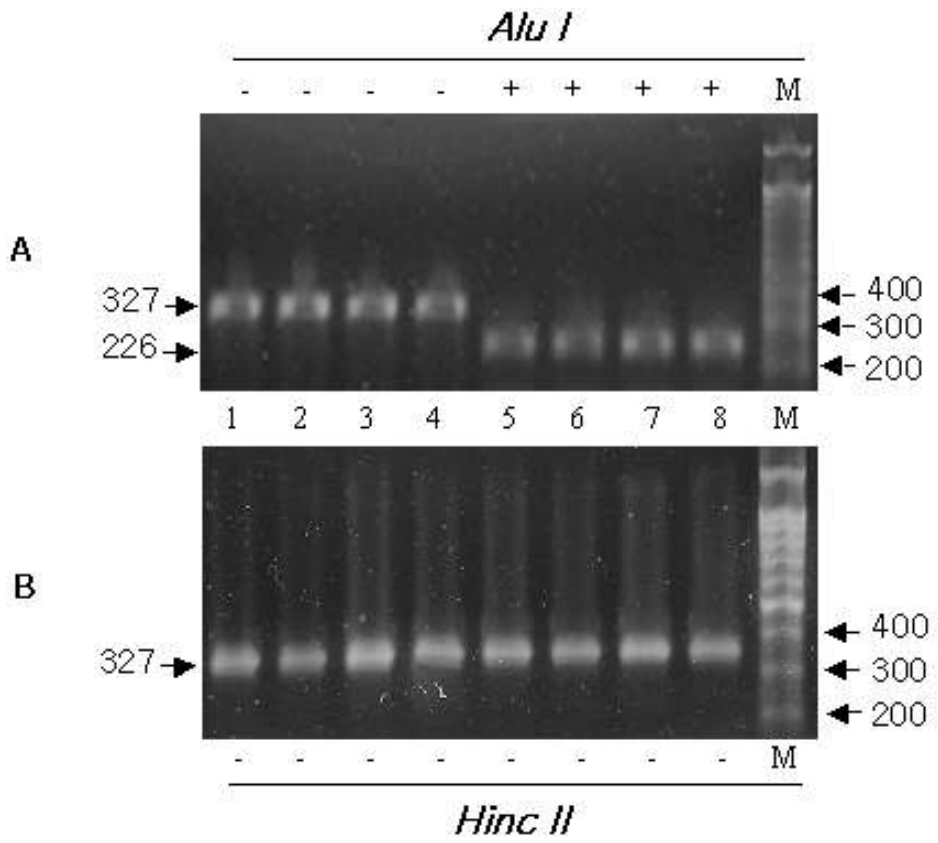

Figure 1.3. Molecular typing of P1 by restriction enzyme analysis (REA). Panel A (REA with *Alu*I): lanes 1–4, no cut by *Alu*I; lanes 5–8, cut by *Alu*I; M, 100bp molecular weight marker (iNtRON Biotechnology, Seoul, Korea); Panel B (REA with *Hinc*II): lanes 1–8, no cut by *Hinc* II.

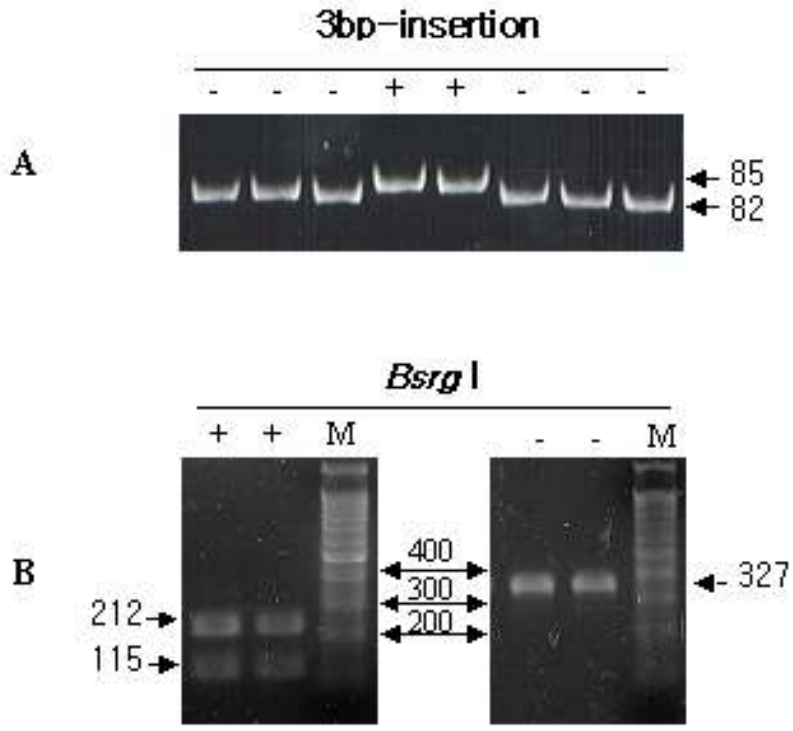

Figure 1.4. Molecular typing of P2. Each lane represents an individual isolate. Panel A: molecular typing via polyacrylamide gel electrophoresis (PAGE). A 3-bp (GGG) insertion (85bp) resulted in the creation of a new promoter, P2, which could be distinguished from 82bp by an upward shift of the amplicon. Panel B: molecular typing via REA (*Bsrg*I). The 3-bp insertion destroys the enzyme site of *Bsrg*I, and the enzyme does not recognize the P2.

1.5. Discussion

Antimicrobial therapy was an important tool in reducing the enormous losses by APEC. However, resistance to existing antimicrobials was widespread and the spread of antibiotic resistances among bacteria also had been recognized as an increasing problem in the veterinary and medical fields (Blanco *et al.*, 1997a).

The APEC antibiograms generated in the present study were similar to those of recent intestinal *E. coli* isolates obtained from poultry in Korea (Kang *et al.*, 2005). Tetracycline and streptomycin had been utilized for several decades, and their resistances increased to more than 80% (Bass *et al.*, 1999; Kang *et al.*, 2005; Zhao *et al.*, 2005). Streptomycin had remained in regular use in Korea, and tetracycline had been one of the most frequently employed antibiotics, primarily as a feed additive in the poultry industry until government banned antibiotics in animal feed in 2011. The average resistances of the APEC isolates during the observed periods were found to be approximately 80%, but resistance during the 2000 to 2005 period was in excess of 94%. MDR APEC had increased in same period. Therefore, preventive and therapeutic effects on APEC should no longer be expected from these antibiotics.

The relevant antibiograms varied from nation to nation, and also differ in accordance with the origins of bacteria, as the result of differing

exposures and selections by different antibiotics. Consistent with previous reports from other studies, rates of resistance against enrofloxacin and ampicillin were highest, but resistance to chloramphenicol during the period 2000 to 2005 was lowest (Bass *et al.*, 1999; Kang *et al.*, 2005; Yang *et al.*, 2004; Zhao *et al.*, 2005). Enrofloxacin was one of the most frequently employed prophylactic antibiotics, but chloramphenicol was seldom used in the Korean poultry industry during 2000 to 2005.

Only some of the detected resistance against streptomycin, gentamicin, and trimethoprim-sulfamethoxazole could be attributed to the class 1 integrons. The *dfrXII-orfF-aadA2* array of gene cassettes was most frequently detected in avian intestinal *E. coli* isolates during the 2000 to 2003 period(Kang *et al.*, 2005), but *dfrI-aadA1a* was most frequently detected in APEC during 2000 to 2005 in the present study. Therefore, the structures and prevalence of gene cassettes differed substantially among *E. coli* isolates. However, the structure and frequency of the gene cassettes of APEC from different countries were similar to each other (Nógrády *et al.*, 2006; Yang *et al.*, 2004). The high frequency of *aadA* and *dfr* could be attributed to selection pressures exerted by streptomycin and trimethoprim, and this observation might be attributed to the frequent prophylactic use of streptomycin and trimethoprim in

poultry farms.

Single nucleotide polymorphisms (SNPs) could provide selective advantages to bacteria over the course of a single infection, epidemic spread, or the long-term evolution of pathogenicity (Sokurenko *et al.*, 1999). To be accepted as a SNP, a single nucleotide mutation should be fairly common among compared genetic populations, and should also be associated with the biological functions of the protein. One APEC isolate harboring *aadB-aadA1a* evidenced a single nucleotide mutation, which resulted in the R201K amino acid change in *aadA1a*. This mutation was detected in other *aadA1a* in the GenBank (AY046276, AY309066, AY602405, D1166553, DQ663487, Y18050), but the biological effects of the mutation remain to be elucidated thoroughly. In addition, the nucleotide sequence difference at codon 250 of *aadA1*, GTC (V) *vs*. GTT (V), results in no amino acid alteration, but GTC and GTT were selected preferentially by the single cassette (*aadA1a*) and two cassettes (*aadB-aadA1a* or *dfrI-aadA1a*) class 1 integrons, respectively. Our BLAST search revealed that GTC was also detectable in the oligocassette class 1 integrons in the GenBank database (AF550679, DQ522235, and DQ522239). The *aadA1* cassette had spread throughout a variety of bacterial species over several decades (Yu *et al.*, 2003), and associated nucleotide changes were likely to occur. The distribution of different

genotypes of *aadA1* in different arrays of gene cassettes implied frequent exchanges of gene cassettes under conditions of antibiotic selection pressure and other environmental, metabolic, or physiological stressors. Therefore, the provisional SNPs observed in *aadA1a* allowed for a more delicate analysis of the evolution pathways of class 1 integrons, as well as the molecular differentiation of clinical isolates harboring the same gene cassette arrays.

The massive and long-term use of antibiotics for therapy and animal growth promotion in the livestock industry had resulted in drug resistances (Lévesque *et al.*, 1995). In Korea the resistance to tetracycline was the highest and has been increased significantly during the period from 2000 to 2005 over than in 1990 to 1999. In contrast to *tetA*, which confers resistance against tetracycline, oxytetracycline, and chlortetracycline, *tetB* provided additional resistance against doxycycline (Chopra *et al.*, 1982). The relative prevalences of *tetA* and *tetB* differed in accordance with the origins of *E. coli* isolates, but *tetA* was more prevalent than *tetB* in Korea, as had also been reported in studies conducted in other countries (Bryan *et al.*, 2004; Lanz *et al.*, 2003). The steep increase in the proportion of *E. coli* isolates encoding *tetA* during the period from 2000 to 2005 could be attributed to the continued utilization of chlorotetracycline and oxytetracycline as a feed additive, as

well as for prophylaxis. Relatively low frequency of *tetB* during the observation periods could be explained by a strong negative association between *tetA* and *tetB*, which was probably due to plasmid incompatibility (Boerlin *et al.*, 2005; Jones *et al.*, 1992; Maynard *et al.*, 2003).

The frequency of APEC isolates positive for *strA*−*strB* only or for both *aadA* and *strA*−*strB* had been increased markedly during the 2000 to 2005. The increase in *strA*−*strB* and the acquisition of *strA*−*strB* in *aadA* harboring APEC isolates reflected the selection of APEC isolates harboring *strA*−*strB*. In contrast to *aadA*, the *strA* and *strB* genes had been suggested to confer high−level resistance to streptomycin, and *E. coli* strains positive for both evidenced the highest observed minimum inhibitory concentrations (MICs) (Sunde and Norström, 2005). The majority of streptomycin products in Korea were used on poultry, and antibiotic selection might be related to the increase in the frequency of *strA*−*strB*.

According to the results of the promoter study with a chloramphenicol acetyltransferase (CAT) assay, the relative strengths of strong P1, weak P1 plus P2 and hybrid P1 to weak P1 promoters increased approximately 32−fold, 16−fold, and 3.5−fold, respectively (Leibert *et al.*, 1999). Among the APEC isolates evaluated in the present study, strong P1 was

absent, but P1 and P2 positive isolates were detected at a percentage of 27.5% (11/40). The frequency of *strA*–*strB* in the weak P1 plus P2 positive APEC was 27.3% (3/11) but the frequencies of weak P1– and hybrid P1–positive APEC isolates were 96.6% (28/29). All of the weak P1 plus P2–positive APEC variants harbored the single cassette, *aadA1a*, but others possessed more than two cassettes with streptomycin resistance genes, positioned distantly from the promoter. The expression level of a gene cassette was affected by its proximity to the common promoter (Collis and Hall, 1995). Therefore, it appeared that the low level of expression of streptomycin resistance among APEC isolates resulted in the recruitment of the additional resistance genes, *strA*–*strB*, under conditions of streptomycin pressure. The antibiograms of the APEC isolates that harbored class 1 integrons were coincident with their gene cassettes, with the exception of one isolate. This isolate harbored hybrid P1 and was sensitive to streptomycin and SXT.

In some cases, the discordance of *aadA* with the antibiogram had been reported, and the influence of nonintegrated and nonexpressed gene cassettes had been suggested (Lanz *et al.*, 2003). However, I detected class 1 integron, and was unable to detect any nonsense mutations, deletion or insertion mutations resulting in stop codons within the coding regions of *dfrXVII* and aadA5. Therefore, different mechanisms, other

than the non-integration of the gene cassette into the class 1 integron might be involved in the silence of gene cassettes.

Resistance to quinolones in APEC was primarily related to mutations in *gyrA* (Yang *et al.*, 2004; Zhao *et al.*, 2005), and double mutation of *gyrA* and additional mutations in *parC* produced higher levels of resistance (Everett *et al.*, 1996; Yang *et al.*, 2004; Zhao *et al.*, 2005). The substitution, S83L, was most frequently observed among substitutions in quinolone resistance-determining region (QRDR) of *gyrA* (87.5%) and isolates with the substitutions S83L, D87N, and S80I, were most frequent (41.7%) among quinolone-resistant APEC isolates, results similar to those of other reports (Yang *et al.*, 2004; Zhao *et al.*, 2005). The frequencies of double mutations in *gyrA* and the single mutation in *parC* increased in steep during the period from 2000 to 2005. Thus quinolone resistance became more serious problem with regard to the quantity as well as the quality. Most of quinolone-resistant APEC isolates possessed at least one mutation in *gyrA* and/or *parC* but eight enrofloxacin-resistant isolates (11.1%) in the present study showed no known mutations in *gyrA* and *parC*. Mutations of other genes such as *gyrB* and *parE* were also associated with quinolone resistance, but usually they were coincident with mutations of *gyrA* and/or *parC* (Everett *et al.*, 1996; Yang *et al.*, 2004; Zhao *et al.*, 2005). In mutants of

Salmonella enterica serovar Typhimurium the expression level of the AcrAB efflux pump was strongly correlated with resistances to ciprofloxacin and a wide variety of compounds such as fusidic acid, chloramphenicol, tetracycline, norfloxacin, and penicillin (Giraud *et al.*, 2000; Nikaido *et al.*, 1998). Seven out of the eight isolates showed intermediate resistance to chloramphenicol and six isolates were resistant or intermediate resistant to tetracycline without *tetA* and *tetB*. Therefore, further study on association of AcrAB efflux pump to enrofloxacin resistance in the APEC isolates is required.

The frequency of *TEM*-positive APEC increased markedly during from 2000 to 2005. The frequency of *TEM* among ampicillin resistant APEC isolates was 71.4% (5/7), 61.1% (11/18), and 72.1% (31/43) during 1985-1989, 1990-1999, and 2000-2005, respectively, and *TEM* turn out to be the major beta-lactamase among APEC isolates in Korea.

In conclusion, the frequency of resistances to several antibiotics (tetracycline, streptomycin, enrofloxacin, ampicillin, and SXT), relevant resistance genes (*tetA*, *strA-strB*, and *TEM*) and mutations in *gyrA* and *parC*, and MDR APEC isolates had increased during the observation periods of this study. Studies of SNPs and promoter structure might prove beneficial for our knowledge of class 1 integron transmission, as well as for the evolution of and relationship between antibiotic

resistances and genotypes. As shown in this study, antibiotics treatment should no longer be desirable to Korean chicken industry and there is an urgent need to investigate another protection tool.

Chapter 2. Pathotyping of avian pathogenic *Escherichia coli* isolates in Korea

2.1. Abstract

Many APEC isolates were non-pathogenic strains as act opportunistic agents in causing disease. Researchers had been trying to develop many typing method to find virulent isolates. To understand the background of APEC pathogenicity, I determined serotypes, characterized the virulence genes and classified as molecular pathotypes of 101 APEC isolates isolated from diseased chickens from 1985 to 2005. Twenty-eight isolates were serotyped to be O1 (2.0%), O18 (3.0%), O20 (1.0%), O78 (19.8%), and O115 (2.0%). Tested virulence genes included type 1 fimbriae (*fimC*), iron uptake-related genes (*iroN*, *irp2*, *iucD*, and *fyuA*), toxins (*lt, st*, *stx1, stx2*, and *vat*), and other genes (*tsh*, *hlyF*, *ompT*, and *iss*). The frequency of *iroN* (100%) was highest, followed by *ompT* (94.1%), *fimC* (90.1%), *hlyF* (87.1%), *iss* (78.2%), *iucD* (73.3%), *tsh* (61.4%), *fyuA* (44.6%), *irp2* (43.6%), and *vat* (10.9%). All toxin genes except *vat* were negative. All the isolates were classified into 27 molecular pathotypes (MPs). MP25, MP19, and MP10 possessing *iroN-fimC-ompT-hlyF-iucD-tsh-iss-irp2-fyuA* (22.8%), *iroN-fimC-ompT-hlyF-iucD-tsh-iss* (21.8%) and *iroN-fimC-ompT-hlyF-iss*

(11.9%), respectively, were predominant. The redundancy of iron uptake-related genes was apparent. These results supplied fundamental information of virulence genes in Korean APEC isolates and showed molecular pathotyping may be a diagnostic tool for higher pathogenic APEC. Further studies on the biological meaning of redundant iron uptake-related genes and improvement of virulence assay may be valuable.

2.2. Introduction

The extra-intestinal infection of avian pathogenic *E. coli* induced colibacillosis in chickens, which is characterized by polyserositis, septicemic shock, and cellulitis (Dho-Moulin and Fairbrother, 1999; Ozawa and Asai, 2013). In APEC infection, preventive or therapeutic effects of antibiotics should not be expected longer (Amara *et al.*, 1995; Cloud *et al.*, 1985; Gan *et al.*, 2013). Vaccine was an important tool in reducing the losses caused by APEC infection (Dho-Moulin and Fairbrother, 1999). To develop vaccine, homologous strain induced APEC infection was important to protect breeder or progeny. Therefore, selection of higher pathogenic APEC was important in APEC vaccine. Many researchers had used serotyping, phylogenetic typing, and detecting of virulence genes as selection tools for higher pathogenic APEC (Blanco *et al.*, 1997b; Johnson *et al.*, 2008a; Kemmett *et al.*, 2013; Zhao *et al.*, 2009).

More than 50,000 different *E. coli* serotypes existed and more than 180 serogroups had been identified in *E. coli* (Ozawa *et al.*, 2010; Ozawa and Asai, 2013; Rosario *et al.*, 2004). Distribution and prevalent serotypes of *E. coli* showed a great diversity depending on sampling time and regional differences. Although many pathogenic isolates did not

belong to identified pathogenic serogroup, which were classified as untypable, O1, 2, 4, 5, 6, 7, 8, 9, 10, 15, 16, 18, 20, 21, 35, 49, 75, 78, 79, 85, 88, 109, 111, 115, 124, 128, 139, and 173 were known to be related to pathogenic *E. coli* in poultry (Blanco *et al.*, 1997b; Bonacorsi and Bingen, 2005; Ewers *et al.*, 2007). But, among them, the most commonly encountered serogroups of APEC were O1, O2, O35, and O78 (Barns *et al.*, 2003; Orskov and Orskov, 1992; Ozawa and Asai, 2013), although their orders of prevalence varied with countries and farms (Cortes *et al.*, 2010; Wang *et al.*, 2010).

The problems associated with the serotyping of *E. coli* were cross-reaction, multiple serogroups, or autoagglutinated, increasing untypable groups (Allan *et al.*, 1993; Kemmett *et al.*, 2013; Rodriguez-Siek *et al.*, 2005a). Some researchers reported that the ratio of untypable strains in their tests ranged between 0 to 84% (Blanco *et al.*, 1998; Cloud *et al.*, 1985; Hemsley *et al.*, 1967). Therefore, serotyping could not be recommended as the sole diagnostic tool for the identification of APEC, especially in light of the fact that the designation of a serogroup did not reflect the pathogenicity of the strains (Ewers *et al.*, 2005; Kemmett *et al.*, 2013). Some people suggested the application of PCR as a further analytic tool supplementing serotyping (Ewers *et al.*, 2004; Schouler *et al.*, 2012).

E. coli is composed of four main phylogenetic groups: A, B1, B2, and D. Although it was not clear which is for human and for chicken, among them, B2 and D, and A were frequent in human pathogenic *E. coli* and in APEC, respectively (Barbieri et al., 2013; Bingen *et al.*, 1998; Herzer *et al.*, 1990; Johnson *et al.*, 2008b; Zhao *et al.*, 2009).

Many genes were significantly more likely to be found in the disease-associated *E. coli* strains (Rodriguez-Siek *et al.*, 2005b). Recently, various virulence genes were identified in APEC and their distribution and frequency among APEC isolates had been reported (Ewers *et al.*, 2009; Ewers *et al.*, 2004; Janben *et al.*, 2001; Johnson *et al.*, 2008a; Schouler *et al.*, 2012). These virulence genes might play roles in various aspects of extra-intestinal pathogenesis of APEC, and their functions could be categorized as adhesions (F1-, P-, AC/I-, and F17 fimbriae, curli fibers, and afimbrial adhesions), iron acquisition systems (aerobactin and yersiniabactin), hemolysins (hemolysin E and a temperature-sensitive hemagglutinin), antibactericidal factors (outer membrane protein A, protein for increased serum survival lipopolysaccharide, K1-capsule, and colicin production), and toxins (heat stable toxin, cyto-/verotoxin, flagella toxin, and vacuolating autotransporter toxin) (Dho-Moulin and Fairbrother, 1999; Dozois *et al.*, 1992; Dziva and Stevens, 2008; Johnson, 1991; Kemmett *et al.*, 2013;

Schouler *et al.*, 2012).

Fimbriae (pili) were proteinaceous structures on the outer membrane of *E. coli* that mediate adherence of the bacteria to host epithelial cells, allowing them to overcome resident defence mechanisms of the respiratory tract and more successfully colonize (Pourbakhsh *et al.*, 1997c). Two main groups of fimbriae, type 1 (F1) and P, had been associated with APEC. Type 1 fimbriae, encoded by *fim* (*pil*) or related gene clusters, were commonly found on both commensal and pathogenic *E. coli* isolates (Pourbakhsh *et al.*, 1997b; Schouler *et al.*, 2012). Type 1 fimbriae were detected mainly in the trachea, in the lungs, and in the air-sacs (Pourbakhsh *et al.*, 1997c). Thus, type 1 fimbriae might mediate *E. coli* adherence to host epithelial cells of the respiratory tract and colonization. Genetic regions encoding type 1 fimbriae consisted of *fimA*, *fimB*, *fimC*, *fimD*, *fimE*, *fimF*, *fimG*, and *fimH*. Among these genes, *fimC* was important in fimbrial assembly and in anchoring assembled fimbriae (Jones *et al.*, 1993; Pourbakhsh *et al.*, 1997c). *fimC* (type 1 fimbriae) was found more frequently in *E. coli* isolated from diseased birds compared to isolates from the feces of healthy birds (McPeake *et al.*, 2005).

Iron was needed by all living cells. *E. coli* used iron for oxygen transport and storage, DNA synthesis, electron transport, and

metabolism of peroxides (Johnson, 1991). Most of the avian pathogenic strains of *E. coli* showed the ability to grow under iron-starvation conditions because of the existence of iron uptake systems that compete with host transferrin for the available iron (Lafont *et al.*, 1987; Williams, 1979). Iron acquisition systems had been recognized to be associated with bacterial pathogenicity especially in bacteria causing septicemia (Janben *et al.*, 2001; Lafont *et al.*, 1987). Most APEC strains (73-98%) possessed and expressed the aerobactin iron-acquisition system (Dozois *et al.*, 1992; Schouler *et al.*, 2012).

Hydroxamate siderophore aerobactin (an iron-sequestering system) encoded by *iuc* gene cluster allowed the bacteria to survive in condition of iron deprivation within host (Ngeleka *et al.*, 2002). Iron uptake chelate gene D (*iucD*) was involved in the biosynthesis of aerobactin and pathogenicity of APEC (De Lorenzo *et al.*, 1986; Ngeleka *et al.*, 1996). Ferric yersiniabactin uptake A (*fyuA*) and iron-repressible protein 1 and 2 (*irp2*), which were involved in iron acquisition in *Yersinia*, were detected in human *E. coli* and APEC strains (Gophna *et al.*, 2001; Janben *et al.*, 2001; Schubert *et al.*, 1998). The outer membrane siderophore receptor gene *iroN*, which was first reported in *Salmonella enterica*, was related to the pathogenicity of APEC (Baumler *et al.*, 1998; Dozois *et al.*, 2003; Hantke *et al.*, 2003).

Temperature-sensitive hemagglutinin (*tsh*) was first demonstrated in *E. coli* by Provence and Curtiss (1994). This gene was more frequently found in pathogenic than commensal isolates (Dozois *et al.*, 2000; Dozois *et al.*, 1992; Maurer *et al.*, 1998; Stordeur *et al.*, 2002) and primarily responsible for infections that cause agglutination of bird erythrocytes, leading to airsacculitis and colisepticemia (Dozois *et al.*, 2000). Temperature-sensitive hemagglutinin was also considered to be involved in the mechanisms of adherence to the avian respiratory tract at earlier stages of infection (Dozois *et al.*, 2000; Janben *et al.*, 2001; Schouler *et al.*, 2012). *tsh* genes were probably the most important and widely distributed known virulence markers of APEC isolated from clinical specimens (Ngeleka *et al.*, 2002). A new class of hemolysin, *hlyF*, was identified in *E. coli* strains from broilers and reported to be related to the pathogenicity of APEC (Johnson *et al.*, 2006; Morales *et al.*, 2004).

The *iss* (increased serum survival) gene encoded a protein that plays an important role in serum resistance and provides protection against the actions of complement. Previous work showed that complement resistance may play an important role in APEC pathogenicity (Vidotto *et al.*, 1990). Thus, the presence of this gene in pathogenic avian strains had been shown to be highly significant for their pathogenicity (Binns *et al.*, 1979; Pfaff-McDonough *et al.*, 2000). The gene of *iss* was found

more frequently in *E. coli* isolated from diseased birds compared to isolates from the faeces of healthy birds (McPeake *et al.*, 2005). The *iss* gene was strongly associated with APEC of various serogroups but not with fecal *E. coli* isolates from apparently healthy birds (Pfaff-McDonough *et al.*, 2000; Rodriguez-Siek *et al.*, 2005b). Isolates from speticemic chickens showed strong association with serum resistance (Wooley *et al.*, 1992). *ompT*, which cleaves an antimicrobial peptide protamine and plasminogen, was reported in human *E. coli* from urinary tract infections and APEC (Johnson *et al.*, 2006; Lundrigan and Webb, 1992; Schouler *et al.*, 2012; Stumpe *et al.*, 1998).

The presence of different toxins had also been reported in APEC as with different prevalences (Blanco *et al.*, 1997b; Reingold *et al.*, 1999). Heat-labile and heat-stable toxins (*lt* and *st*, respectively) and shiga toxins (*stx1* and *stx2*) were reported in pathogenic *E. coli* and vacuolating autotransporter toxin (*vat*) was reported in APEC (Salvadori *et al.*, 2001; Schouler *et al.*, 2012).

Several factors had been associated with the pathogenicity of *E. coli* for avian hosts, but no specific pathogenic factor that had been discovered contributed entirely to the pathogenicity of APEC (Horne *et al.*, 2000). In addition, while a number of factors had been associated with pathogenicity in epidemiological studies, confirmation of their role

had generally been limited to reproduce (Tivendale *et al.*, 2004; Vidotto *et al.*, 1990). Thus, many researchers had been tried to find specific virulence genes and combination of virulence genes. To date, the frequency of various virulence genes in APEC strains had been reported. However, knowledge for the frequencies of combined virulence genes and patterns of virulence gene accumulation in APEC strains was largely unknown, which limits the understanding of the evolution of APEC in pathogenicity (Dziva and Stevens, 2008).

To address this shortcoming, I determined the serotypes and phylogenetic groups of 101 APEC isolates collected in Korea from 1985 to 2005, and investigated the frequencies of virulence genes, *fimC*, *tsh*, *hlyF*, *iroN*, *iucD*, *fyuA*, *irp2*, *iss*, *ompT*, *vat*, *lt*, *st*, *stx1*, and *stx2*. Also, I determined combination of virulence genes and divided by molecular pathotypes (MPs) based on the different combination of virulence genes.

2.3. Materials and Methods

2.3.1 Bacteria preparation

One hundred and one APEC isolates were collected in Korean chickens suffering from colibacillosis from 1985 to 2005. All of the APEC isolates were identified using VITEK® Gram−Negative Identification (GNI) Cards (bioMerieux Vitek, Hazelwood, MO) as described in chapter 1.

2.3.2. Serotyping

Each isolates was streaked onto MacConkey agar plate (Difco), grown overnight at 37°C (BP−701, Biofree, Korea), and checked visually for purity. One colony of each isolates was transferred by sterile loop to a microtube containing 3 ml of Luria−Bertani (LB) broth (Duchefa Biochemie) and grown for 12 hours at 37°C with shaking at 250 r.p.m. (NB−205L, N−biotec, INC.). Liquid culture was spread onto Tryptic Soy Agar plate (Difco) and incubated overnight at 37°C (BP−701, Biofree). The following day, cells from this agar culture were harvested by adding 2 ml of autoclaved 0.85% NaCl, transferred to a microtube and centrifuged (gyrospin, Gyrozen) at 13,000 rpm for 3 min. Supernatant was removed and cell pellet was diluted to an OD_{600} of 1.8 (BioPhotometer, Eppendorf). Bacterial suspension was heated to 100°C

for 1 hour and incubated for 10 min at room temperature. Antigen solution was prepared by mixing 250 ㎕ of antigenic suspension, 5 ㎕ of formaldehyde (Duksan pure chemical Co., Ltd., Korea), and 5 ㎕ of 0.01% crystal violet (Showa chemicals Inc., Japan). Antibody solution was prepared by mixing 125 ㎕ of Somatic (O) monovalent sera (Denka Seiken Co., LTD.) of O1, O6, O8, O15, O18, O20, O78, and O115 and 875 ㎕ of 1% Sodium azide (NaN3) (Junsei chemical Co., Ltd., Japan). Serotyping was performed by mixing 50 ㎕ of antigen solution and 50 ㎕ of antibody solution onto a 96-well microplate. Plate was vortexed and incubated overnight at 37°C (BP-701, Biofree). Then, all of results were interpreted. Antigen solution without bacterial suspension and antibody solution without monovalent serum were used as negative controls.

2.3.3. Phylogenetic typing

Phylogenetic grouping of *E. coli* was performed by a rapid and simple method as described previously (Clermont *et al.*, 2000) with modification of some primers as shown in table 2.1. The nucleotide sequences of the previously-described primers were compared with the genome sequences of *E. coli* in the GenBank by BLAST search (http://blast.ncbi.nlm.nih.gov). As shown in table 2.1, the YjaAF, YjaAR, and TspE4C2R primers were modified to bind to the variable nucleotides

[*e.g.* YjaAF (the fifth nucleotide from the 3'-end)/YjaAR (the fourth nucleotide from the 5'-end), IHE3034 strain (CP001969); TspE4C2R, IAI1 strain (NC_011741)]. The both *chuA* and TspE4C2 negative and positive *E. coli* isolates were grouped into group A and B2, respectively, and the *chuA*-negative and TspE4C2-positive, and the *chuA*-positive and *yjaA*-negative *E. coli* isolates were grouped into B1 and D, respectively.

Table 2.1. Primer sets for genotyping of avian pathogenic *E. coli* isolates

Gene	Primer sequence (5'–3')	Amplicon size (bp)	Reference
fimC	F: GGAAATAACATTCTGCTTGC R: TTTGTTGCATCAAGAATACG	288	This study
fyuA	F: CAACATCGTCACCCAGCAG R: CGCAGTAGGCACGATGTTGTA	949	This study Schubert *et al.*, 1998
hlyF	F: GGCGATTTAGGCATTCCGATACTC R: ACGGGGTCGCTAGTTAAGGAG	599	Johnson *et al.*, 2006
irp2	F: AAGGATTCGCTGTTACCGGAC R: TCGTCGGGCAGCGTTTCTTCT	280	Schubert *et al.*, 1998
iroN	F: AAGTCAAAGCAGGGGTTGCCCG R: GACGCCGACATTAAGACGCAG	667	Johnson *et al.*, 2006
iss	F: AGCAACCCGAACCACTTGATG R: TAATAAGCATTGCCAGAGCGG	329	This study
iucD	F: GTGAGTTGTACCACCGTTTT R: CCATTCCAGAGTGAAGTCAT	278	This study
lt	ATGAGTACTTCGATAGAGG ATG GTATTCCACCTA ACGC	279	This study
ompT	F: ATCTAGCCGAAGAAGGAGGC R: CCCGGGTCATAGTGTTCATC	559	Johnson *et al.*, 2006

Table 2.1. Continued

Gene	Primer sequence (5'–3')	Amplicon size (bp)	Reference
st	TCTGTATTGTCTTTTTCACCTTTC TTAATAGCACCCGGTACAAGC	165	This study
stx1A	F: CAGTTAATGTGGTGGCGAAG R: CTGCTAATAGTTCTGCGCATC	895	This study
stx2A	F: CTTCGGTATCCTATTCCCGG R: GGATGCATCTCTGGTCATTG	482	This study
tsh	F: GGGAAATGACCTGAATGCTGG R: CCGCTCATCAGTCAGTACCAC	420	Maurer *et al.*, 1998
vat	F: TCCTGGGACATAATGGTCAG R: GTGTCAGAACGGAATTGT	981	Ewers *et al.*, 2004
chuA	F: GACGAACCAACGGTCAGGAT R: TGCCGCCAGTACCAAAGACA	279	Clermont *et al.*, 2000
yjaA	MF: TGAAGTGTCAGGAGAYGCTG MR: ATGRAGAATGCGTTCCTCAAC	211	Modified in this study Modified in this study
TspE4C2	F: GAGTAATGTCGGGGCATTCA MR: CGCGYCAACAAAGTATTRCG	152	Clermont *et al.*, 2000; Modified in this study

2.3.4. DNA extraction and PCR

Each isolate was streaked onto MacConkey agar plate (Difco), grown overnight at 37°C (BP-701, Biofree), and checked visually for purity. One loopful of each isolates was transferred by sterile loop to a microtube containing 50 ㎕ of LB broth (Duchefa Biochemie). DNA was extracted with the G-spin for Bacteria kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The PCR solution was composed of 10 x buffer (2 μl), dNTPs (2.5 mM, 0.4 μl), forward and reverse primers (10 pmol/μl, 0.5 μl each), Taq DNA polymerase (5 U/μl; MACROGEN, Seoul, Korea, 0.2 μl), and distilled water (15.4 μl). This solution was added to 0.2 ml PCR-tube (Eppendorf) and template DNA (50 ng/μl, 1 μl) was added also to this tube. In negative control reaction, the DNA template was replaced by sterile deionized water. Cycling conditions were as follows: 94°C, 3 min; 35 cycles at 94°C, 30 s; 55°C, 30 s; and 72°C, 1 min; and a final extension step at 72°C, 5 min. Amplicons were analyzed through electrophoresis on 1.0% agarose gels. Gel was run at 100V/cm (Mupid-21, Cosmo Bio Co., Ltd.) for 15 minutes and visualized using a UV transilluminator (Wealtec). Size determination was achieved using a 1 kb ladder (iNtRON Biotechnology Co., Seoul, Korea) as molecular size marker.

2.3.5. Molecular pathotyping

Iron uptake system or aerobactin synthesis, adhesion-related, resistance to serum, and toxin producing genes were PCR amplified using primers targeting the gene encoding ferric yersiniabactin uptake related system (*fyuA*), the iron repressible gene associated with yersiniabactin synthesis (*irp2*), the gene encoding temperature-sensitive hemagglutinin (*tsh*), the gene encoding the new class of hemolysin (*hlyF*), the gene encoding the aerobactin iron uptake system (*iucD*), the gene encoding type 1 fimbriae (*fimC*), the gene encoding outer membrane siderophore receptor (*iroN*), the gene encoding increased serum survival gene (*iss*), the gene encoding shiga toxins (*stx1* and *stx2*), the gene encoding vacuolating autotransporter toxin(*vat*), the gene encoding outer membrane proteases(*ompT*), the gene encoding heat-labile toxin (*it*), and the gene encoding heat-stable toxin (*st*). The primer sets were designed for amplification of *fyuA*, *irp2*, *tsh*, *hlyF*, *iucD*, *fimC*, *iroN*, *iss*, *ompT*, *stx1* and *stx2*, *vat*, *lt*, *chuA*, and *st* (Table 2.1). MPs were determined according to the combinations of virulence genes.

2.3.6. Statistical analyses

The frequencies of virulence genes were compared between the

periods(1985–1989, 1990–1999, and 2000–2005) with Chi–square and Fisher's exact tests (with 95% confidence interval) using SPSS for Windows (version 12.0; SPSS, USA). The *P*–value less than 0.05 was considered as significant.

2.4. Results

2.4.1. Serotyping

One hundred and one APEC isolates were serotyped with anti-O1, O6, O8, O15, O18, O20, O78, and O115 antisera. Among 101 APEC isolates, 73 (72.2%) isolates were untypable (Table 2.2). Serotype O78 was the most frequent serotype (19.8%, 20/101), followed by O18 (3.0%, 3/101), O1 (2.0%, 2/101), O115 (2.0%, 2/101), and O20 (1.0%, 1/101). Isolates of serotype O78 were E24, E28, E30, E31, E36, E43, E49, E61, E75, E102-108, E114, E115, E123, and E125. Isolates of serotype O18 were E22, E35, and E127. Isolates of serotype O1 were E129 and E144. Isolates of serotype O115 were E26 and E146. Isolate of serotype O20 was E79.

Table 2.2. Serotyping results of avian pathogenic *E. coli* isolates

Serotype	Frequency	Isolates
O1	2.0%	E129, E144
O18	3.0%	E22, E35, E127
O20	1.0%	E79
O78	19.8%	E24, E28, E30, E31, E36, E43, E49, E61, E75, E102-108, E114, E115, E123, E125
O115	2.0%	E26, E146
UT*	72.2%	E1, E5, E9-21, E23, E25, E27, E29, E32, E33, E37-39, E41, E42, E48, E51-53, E55, E59, E62, E64-66, E68-70, E84, E86, E88-91, E95, E101, E109-113, E116-120, E124, E126, E130-133, E135-143, E145

*untypable.

2.4.2. Phylogenetic typing

Eighty-six Korean APEC isolates were divided into different phylogenetic groups. Group A was the largest phylogenic type (39.5%, 34/86), and groups B1 (23.3%, 20/86) and B2 (22.1%, 19/86) were similar in size, whereas group D (15.1%, 13/86) was notably smaller (Table 2.3).

2.4.3. Molecular pathotyping

The frequencies and combinations of virulence genes among 101 APEC isolates were determined (Figure 2.1, Table 2.3, and Table 2.4). *iroN* was carried by 100% of APEC isolates and 90.1%, 94.1%, 87.1%, 78.2%, 73.3%, 61.4%, 44.6%, 43.6%, and 10.9% of APEC isolates carried *fimC*, *ompT*, *hlyF*, *iss*, *iucD*, *tsh*, *fyuA*, *irp2*, and *vat*, respectively (Table 2.4). The toxin genes *lt*, *st*, *stx1*, and *stx2* were not detected in any isolates. Chronological increases of *iss* and *fyuA*/*irp2* frequencies between 1985-1989 and 2000-2005, between 1985-1989 and 1990-1999/2000-2005, and between 1985-1989 and 2000-2005 were significant ($p < 0.05$, Table 2.4).

The 101 APEC isolates were divided into 27 MPs based on different combinations of virulence genes. Three major MPs, MP25 (22.8%, *iroN-fimC-ompT-hlyF-iucD-iss-fyuA-irp2-tsh),* MP19 (21.8%, *iroN-*

fimC–*ompT*–*hlyF*–*iucD*–*iss*–*tsh*), and MP10 (11.9%, *iroN*–*fimC*–*ompT*–*hlyF*–*iss*), covered 56.5% of APEC isolates. The frequencies of *iroN*, *fimC*, *ompT*, *hlyF*, *iucD*, *iss*, *fyuA*, *irp 2*, *tsh*, and *vat* among the MPs were 100%, 70.4%, 63.0%, 59.3%, 59.3%, 59.3%, 51.9%, 48.1%, 33.3%, and 14.8%, respectively. Comparing the frequencies of virulence genes in the examined APEC isolates and in the MPs, the frequencies of *fimC*, *ompT*, *hlyF*, *iss*, *iucD*, and *tsh* were lower in MPs than in the APEC isolates. In contrast, the frequencies of *fyuA*, *irp2*, and *vat* in MPs were higher than those in the APEC isolates (Table 2.4). The virulence gene profiles of MPs showed a cumulative pattern, and the hypothetical steps of virulence gene acquisition of MPs were diagrammed according to the frequencies of virulence genes in MPs (Figure 2.2). I hypothesized that the probability of virulence gene transmission is similar each other, and genes with higher frequency would be introduced earlier into the APEC isolates than other genes with lower frequencies. MP1 microorganisms, which had acquired *hlyF*, evolved to MP8 and MP12 by acquisitions of *iss* and *iucD*–*iss*, respectively, and MP12 further evolved to MP21 by acquisition of *fyuA*–*irp2*. MP1 organisms, which had not acquired *hlyF* but acquired *iucD*, later evolved to MP16 and MP17 through acquisition of *tsh* and *iss*–*fyuA*–*irp2*, respectively. APEC isolates that had not gained *fimC* and *ompT* evolved to MP18 and MP9 by acquisition of *hlyF*–

iucD–*iss*–*fyuA*–*irp2* and *iucD*–*fyuA*–*irp2*, respectively. APEC isolates possessing *iroN* and *fimC* evolved to MP2, MP3, and MP7 by acquisition of *ompT*, *hlyF*, and *iucD*–*iss*, respectively. MP3 evolved to MP6 by acquisition of *iss*. MP2 evolved to MP5, MP15, MP11, and MP4 by acquisition of *tsh*, *iss*–*fyuA*–*irp2*, *fyuA*–*irp2*, and *hlyF*, respectively. MP15 further transformed into MP20 by gaining of *vat*. MP4 acquired *iss* evolved to MP10. MP4 that acquired *iucD* evolved to MP13, MP23, and MP14 by acquisition of *tsh*, *fyuA*–*irp2*–*tsh*, and *iss*, respectively. MP23 further evolved to MP24 by acquisition of *vat*. MP14 further evolved to MP19, MP22, MP25, and MP26 by acquisitions of *tsh*, *fyuA*–*tsh*, *irp2*–*tsh*, and *fyuA*–*irp2*–*vat*, respectively. MP25 further developed into MP27 *via* the acquisition of *vat*.

Table 2.3. Molecular pathotyping and phylogenetic grouping of avian *E. coli* isolates

Molecular Pathotype (MP)	Virulence genes	Frequency	Isolates (phylogenetic group)
MP1	*iroN−ompT*	1.0%	E68(A)
MP2	*iroN−fimC−ompT*	3.0%	E19(D), E31 (A), E127 (B1)
MP3	*iroN−fimC−hlyF*	1.0%	E18(A)
MP4	*iroN−fimC−ompT−hlyF*	3.0%	E10(A), E51(A), E103 (A)
MP5	*iron−fimC−ompT−tsh*	1.0%	E21(B1)
MP6	*iroN−fimC−hlyF−iss*	2.0%	E136(B1), E145(A)
MP7	*iroN−fimC−iucD−iss*	1.0%	E43 (B2)
MP8	*iroN−ompT−hlyF−iss*	1.0%	E66(D)
MP9	*iroN−iucD −fyuA−irp2*	1.0%	E20(A)
MP10	*iroN−fimC−ompT−hlyF−iss*	11.9%	E11(B1), E24 (A), E55(D), E84(A), E90(D), E95(B2), E101, E114, E116(B1), E117(A), E118, E125
MP11	*iroN−fimC−ompT −fyuA−irp2*	1.0%	E36 (A)

Table 2.3. Continued

Molecular Pathotype (MP)	Virulence genes	Frequency	Isolates (phylogenetic group)
MP12	*iroN−ompT−hlyF−iucD−iss*	1.0%	E26 (A)
MP13	*iroN−fimC−ompT−hlyF−iucD−tsh*	5.9%	E5(B1), E16(B1), E17(B1), E25(B1), E29(B1), E141
MP14	*iroN−fimC−ompT−hlyF−iucD−iss*	2.0%	E132, E142
MP15	*iroN−fimC−ompT−iss−fyuA−irp2*	1.0%	E144
MP16	*iroN−ompT−iucD −fyuA−irp2−tsh*	1.0%	E28 (D)
MP17	*iroN−ompT−iucD−iss−fyuA−irp2*	1.0%	E41(A)
MP18	*iroN−hlyF−iucD−iss−fyuA−irp2*	1.0%	E133(A)
MP19	*iroN−fimC−ompT−hlyF−iucD −iss−tsh*	21.8%	E1(A), E12(B1), E13(B1), E14(B1), E15 (A), E48(B2), E52(B2), E53(B1), E69 (B1), E70(B2), E79(B1), E86(A), E109(D), E110(B2), E111(B2), E112(A), E115(A), E119(B2), E120(B2), E124 (B2), E131, E138(A)

Table 2.3. Continued

Molecular Pathotype (MP)	Virulence genes	Frequency	Isolates (phylogenetic group)
MP20	*iroN−fimC−ompT−iss−fyuA−irp2−vat*	1.0%	E129
MP21	*iroN−ompT−hlyF−iucD−iss−fyuA−irp2*	3.0%	E32(A), E33(A), E37(D)
MP22	*iroN−fimC−ompT−hlyF−iucD−iss−fyuA−tsh*	1.0%	E146
MP23	*iroN−fimC−ompT−hlyF−iucD−fyuA−irp2−tsh*	2.0%	E39(B1), E104 (B1)
MP24	*iroN−fimC−ompT−hlyF−iucD−fyuA−irp2−tsh−vat*	2.0%	E22 (B2), E35 (D)
MP25	*iroN−fimC−ompT−hlyF−iucD−iss−fyuA−irp2−tsh*	22.8%	E27(A), E30(A), E42(A), E49 (B2), E61 (B2), E62(B2), E75 (B1), E89(D), E91(A), E102(B1), E105(A) E106(A), E107, E108(A), E113(A), E123(A), E126(D), E130(B2), E135(A), E137, E139(A), E140, E143
MP26	*iroN−fimC−ompT−hlyF−iucD−iss−fyuA−irp2−vat*	3.0%	E23(D), E38(D), E64(B2)
MP27	*iroN−fimC−ompT−hlyF−iucD−iss−fyuA−irp2−tsh−vat*	4.0%	E9(B2), E59(B2), E65(B2), E88(D)

Table 2.4. Prevalence of virulence genes in avian pathogenic *E. coli* isolates

Virulence gene	Frequency of virulence genes				Frequency among genotypes (n=27)
	1985-1989 (n=15)	1990-1999 (n=35)	2000-2005 (n=51)	Total (n=101)	
iroN	100%	100%	100%	100%	100%
fimC	93.3%	77.1%	98.0%	90.1%	70.4%
ompT	86.7%	97.1%	94.1%	94.1%	63.0%
hlyF	80.0%	82.9%	92.2%	87.1%	59.3%
iss	46.7%[a,b]	71.4%[a]	92.2%[b]	78.2%	59.3%
iucD	66.7%	80.0%	70.6%	73.3%	59.3%
tsh	66.7%	54.3%	64.7%	61.4%	33.3%
fyuA	13.3%[a,b]	57.1%[a]	45.1%[b]	44.6%	51.9%
irp2	13.3%[a]	57.1%[a]	43.1%	43.6%	48.1%
vat	6.7%	20%	5.9%	10.9%	14.8%

Table 2.4. Continued

Virulence gene	Frequency of virulence genes				Frequency among genotypes (n=27)
	1985–1989 (n=15)	1990–1999 (n=35)	2000–2005 (n=51)	Total (n=101)	
lt[*]	0.0%	0.0%	0.0%	0.0%	0%
st[**]	0.0%	0.0%	0.0%	0.0%	0%
stx1[***]	0.0%	0.0%	0.0%	0.0%	0%
stx2	0.0%	0.0%	0.0%	0.0%	0%

[a] Significant difference between 1985–1989 and 1990–1999.

[b] Significant difference between 1985–1989 and 2000–2005.

[*] Heat–labile toxin; [**] Heat–stable toxin; [***] Shiga toxin.

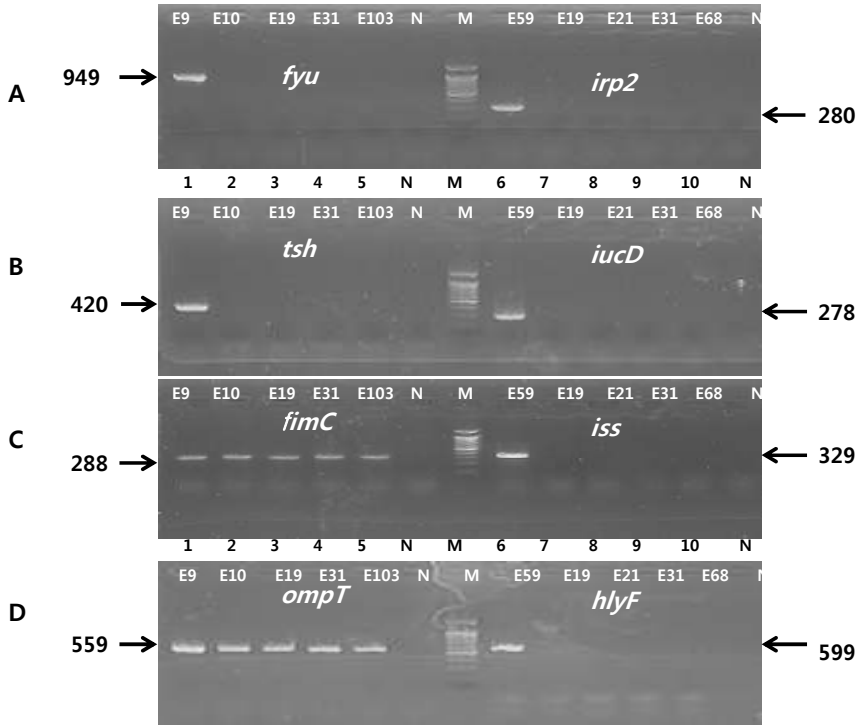

Fig 2.1. Amplified virulence genes of APEC isolates collected in Korean chickens. Lanes: M, 1-kb molecular weight marker (iNtRON Biotechnology, Seoul, Republic of Korea); N, negative controls. Panel A, *fyuA* (949 bp) and *irp2* (280 bp); Panel B, *tsh* (420 bp) and *iucD* (278 bp); Panel C, *fimC* (288 bp) and *iss* (329 bp);

Panel D, *ompT* (559 bp) and *hlyF* (599).

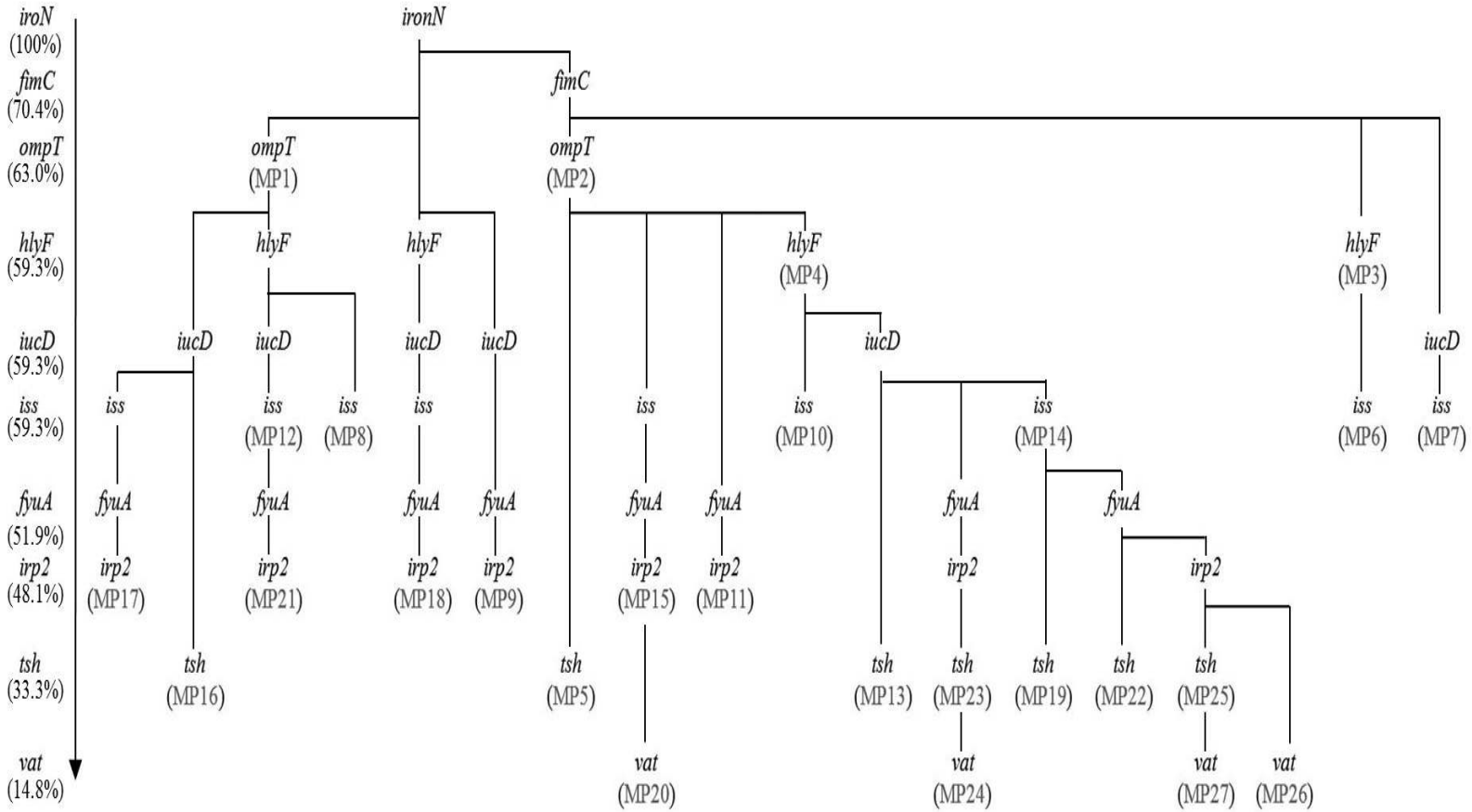

Fig 2.2. Accumulation of virulence genes and evolution of molecular pathotypes (MPs) of APEC.

2.5. Discussion

More than 50,000 different *E. coli* serotypes existed and more than 180 serogroups had been identified in *E. coli* (Ozawa and Asai, 2013; Rosario *et al.*, 2004). However, disease-causing *E. coli* typically consisted of a relatively few serogroups (Yang *et al.*, 2004). Many researchers had serotyped many kinds of samples of poultry and a variety of serotypes had been identified. These results were some different from each other depending on the types of samples and, times and countries of study conducted. Although many pathogenic isolates did not belong to these identified pathogenic serogroup (untypable), O1, O2, and O78 were the most commonly encountered serogroups among APEC (Altekruse *et al.*, 2002a; Mokady *et al.*, 2005; Ozawa and Asai, 2013). The frequencies of O78 among APEC isolates varied according to countries and hosts. In one study conducted in Germany, O78 was the second most frequent serotype (14.7%) following O2 (28.7%) (Ewers *et al.*, 2004). These two serotypes were the most frequent (45.6% and 20%) in Ireland (McPeake *et al.*, 2005), and in Turkey (Altekruse *et al.*, 2002a). Although tested serotypes only with a limited battery of antisera, O78 was also the most frequent serotype (19.8%) in Korea. This result was different from previous data reported around 1970s to 1980s in

Korea. Kim and Namgoong (1987) reported that O119 (3.7%), O25 (3.1%), and O78 (2.5%) were most predominant serogroups in Korea. The other report showed that O128 (22.7%), O1 (7.2%), and O124 (5.8%) were predominant serogrups in Korea (Kim and Tak, 1983). The discrepancy between present results and previous report in Korea could be explained by time differences between present study and others. Moreover, several reports from several countries showed similar results to present finding (Altekrus *et al.*, 2002a; Ewers *et al.*, 2004; McPeake *et al.*, 2005).

Previous studies had reported frequencies of A, B1, B2, and D groups in APEC strains as 34.5%−71.0%, 4.1%−21.3%, 7.9%−44.5%, and 12.0%−29.9%, respectively, and, on average, group A was the most frequent group in APEC strains (Barbieri *et al.*, 2013; Dissanayake *et al.*, 2008; Ewers *et al.*, 2009; Ghanbarpour *et al.*, 2011; Johnson *et al.*, 2008b; Zhao *et al.*, 2009). Present results showed similar result to previous reports. The B2 group was closely related to human extra−intestinal pathogenic *E. coli* (ExPEC) and was frequent in human uropathogenic and neonatal meningitis *E. coli* strains (Bingen *et al.*, 1998; Johnson *et al.*, 2008b; Zhao *et al.*, 2009). Therefore, further studies on the correlation of B2 group in APEC and human ExPEC strains may be valuable in aspects of zoonosis. Recently, using genome sequences

derived from 17 strains of *E. coli* and comparing the nucleotide sequences of phylogenetic grouping primer sets (Clermont *et al.*, 2000), it was revealed some mutations in yjaA.1 (the fifth C to T from the 3'-end), yjaA.2 (the fourth G to A from the 5'-end), and TspE4.C2 (the fifth C to T from the 5' end and the third A to G from the 3'-end). Those mutations might occasionally cause weak signals or false negatives of *yjaA* and TspE4.C2, resulting in increases of the frequency of the A and D groups. Therefore, the modified forward and reverse primers of *yjaA* and reverse primer of TspE4.C2 (Table 2.1) might be useful for minimizing errors in the phylogenetic grouping.

The frequencies of *iroN*, *fimC*, *ompT*, *hlyF*, *iucD*, *iss*, *fyuA*, *irp2*, *tsh*, and *vat* were 85.4%-89.0%, 90.4%-92.7%, 60.0%-81.6%, 0%-81.7%, 78.0%-100%, 38.5%-100%, 58.2%-71.3%, 68.0%-100%, 39.5%-93.9%, and 33.4%-64.3%, respectively (Delicato *et al.*, 2003; Ewers *et al.*, 2004; Ewers *et al.*, 2005; Johnson *et al.*, 2006; Ozawa *et al.*, 2008; Zhao *et al.*, 2009). In the present study, the frequencies of *fimC*, *hlyF*, *iucD*, *iss*, and *tsh* were similar to previous reports, the frequencies of *iroN* and *ompT* were higher, and the frequencies of *irp2*, *fyuA*, and *vat* were lower. The similar frequencies of *hlyF*, *iucD* and *iss*, and *fyuA* and *irp2* reflected possible co-transmission of those genes but the co-transmitted gene contents were different from previous reports (Johnson

et al., 2006; Schubert *et al.*, 1998).

The frequencies of virulence genes varied according to the countries and hosts of APEC strains, but the redundant possession of iron uptake-related genes (*iroN*, *chuA*, *iucD*, *fyuA*, and *irp2*) was common in APEC strains (Ling *et al.*, 2013). In these genes, *iroN* gene was the most frequent, followed by *iucD*, *fyuA*, and *irp2* in the present study. To date, the roles of redundant iron-uptake-related genes had been unclear, and they were expected to function in different niche conditions. Considering the essential role of iron-uptake in APEC pathogenicity, various redundant iron uptake-related proteins might be useful for evasion of humoral immunity.

Toxin genes except *vat* gene (10.9%), *lt*, *st*, *stx1*, and *stx2*, were not detected in this study. It was similar to some of previous reports that only few strains isolated from chickens can produce toxins (Reynard *et al.*, 1976; Schouler *et al*, 2012).

The functions of virulence genes tested in the present study were well-documented, and the accumulation of virulence genes might be potential risk factors of APEC isolates. Therefore, monitoring of MPs with multiple virulence genes in poultry farms and products, and comparative studies on the distribution of MPs in different hosts might be helpful to diminish economic loss in the poultry industry and lessen

potential zoonotic risks of APEC in the community (Johnson *et al.*, 2008b; Kemmett *et al.*, 2013; Moulin-Schouleur *et al.*, 2006).

In conclusion, most of APEC isolates were untypable. Therefore, serotype cannot be sole diagnostic tools for APEC and need another tool. In the frequencies of virulence gene, iron uptake-related genes (*fyuA* and *irp2*) were increased chronologically. Therefore, further studies on the biological meaning of redundant iron uptake-related genes and the presence of new high mortality-related genes may be valuable. APEC isolates were divided into 27 MPs on the basis of gene combination. It will be provided hypothetical steps of gene combination and fundamental information according to APEC pathogenicity. The molecular pathotyping can be target of diagnosis and selection tool of candidates for APEC vaccine in the chicken farms.

Chapter 3. Virulence assay of avian pathogenic *Escherichia coli* isolates in Korea

3.1. Abstract

Chemotherapy has been first choice to reduce the enormous economic losses caused by APEC infections in the poultry industry. However, it is a double-edged sword, as antibiotic resistance in APEC has also been increased dramatically. To overcome these limitations, vaccines against APEC infections may be one of important strategies against APEC infections. To develop ideal vaccine strains possessing multiple virulence genes are preferable but they should be tested for the pathogenicity in chickens. For this reason, I selected 11 APEC isolates based on the MPs, phylogenetic groups, and serotypes, and then tested pathogenicity in chickens by a virulence assay. Seven-day-old chickens were inoculated subcutaneously with 10-fold serial dilutions of each APEC isolates (10^9 to 10^6 CFU/0.2 ml) to measure the 50% lethal dose (LD_{50}). Then infected chickens were monitored and recorded daily for clinical signs and mortality for 7 days. Necropsy, gross pathological examinations, and re-isolation of bacteria were conducted with infected chickens. Based on the LD_{50} of APEC isolates, 11 APEC isolates were classified into lethality

classes (LC) 1 to 3 as follows: LC1 ($LD_{50} \leq 5 \times 10^6$ CFU), LC2 (5×10^6 to 10^8 CFU), and LC3 ($\geq 5 \times 10^8$ CFU).

The ratio LC1, LC2, and LC3 were 18.2% (2/11), 27.3% (3/11), and 54.5% (6/11), respectively. The LC1 isolates caused clinical signs as early as 24 hours and acute death within 24 hours at all titers of challenges. The LC2 isolates caused death which started first 7-day-post-inoculation at higher titer challenge. The LC3 isolates caused death in only a few chickens at the highest titer of challenge and no clinical signs in all of the surviving chickens. The MPs of high pathogenic isolates (LC1), E64 (MP26; *iroN-fimC-ompT-hlyF-iucD-iss-fyuA-irp2-vat*) and E89 (MP25; *iroN-fimC-ompT-hlyF-iucD-iss-fyuA-irp2-tsh*), possessed more abundant virulence genes than others, which suggests the correlation between MPs and pathogenicity of APEC isolates. Taken together, these data suggests that molecular pathotyping might be a powerful tool for identifying higher pathogenic APEC isolates and selecting effective vaccine candidates against APEC infections. Therefore, further study on the efficacy of APEC vaccine which is made of the highly pathogenic APEC isolates may be valuable.

3.2. Introduction

Chemotherapy has been the first choice to reduce the incidence and mortality associated with avian colibacillosis (Freed *et al.*, 1993; Watts *et al.*, 1993). However, multiple resistances to available antibiotics are frequent among APEC isolates and confronting threat to the poultry industry (Allan *et al.*, 1993; Amara *et al.*, 1995; Cloud *et al.*, 1985; Gan *et al.*, 2013; Zhao *et al.*, 2005). For this reason, vaccine is one of promising alternatives of chemotherapy to reduce the economic losses and selection of optimal vaccine strains among APEC isolates is most important for vaccine development (Dho-Moulin and Fairbrother, 1999).

In contrast to commensal *E. coli* multiple virulence genes are common in the disease-associated *E. coli* strains (Kemmett *et al.*, 2013; Rodriguez-Siek *et al.*, 2005b). In the previous study (Chapter 2), 101 APEC isolates were classified into 27 MPs and the virulence gene contents were variable. To date, host- or pathotype-specific virulence genes are not confirmed, therefore pathogenicity of APEC isolates should be determined by virulence assay (McPeake *et al.*, 2005; Ewers *et al.*, 2007).

Various methods for virulence assay have been applied to reproduce clinical signs and pathological lesions by APEC. The virulence assays

were performed by using embryo mortality (Wooley *et al.*, 1992) and pathological lesions of chickens (Barbieri *et al.*, 2013; Dias da Silveira *et al.*, 2002; Fantinatti *et al.*, 1994), which were challenged by APEC via intra-tracheal route with or without preliminary challenge of triggering agents such as virus, mycoplasma, or ammonia (Bree *et al.*, 1989; Goren, 1978; Tivendale *et al.*, 2004; Vidotto *et al.*, 1990). However, the pathogenicity of APEC was clearly classified into lethality classes (LC1 to LC3) by calculation of LD_{50} of APEC isolates after subcutaneous inoculation into chickens (Dozois *et al.*, 2000; Giovanardi *et al.*, 2005).

In this study, eleven APEC isolates were selected on the basis MPs, serotypes, and phylogenetic groups and their lethality classes were determined to understand the relationship between virulence genes and pathogenicity and also select highly pathogenic vaccine candidates.

3.3. Materials and Methods

3.3.1. Preparation of bacteria

The eleven isolates of APEC (Table 3.1) were selected from the 101 APEC isolates on the basis of MPs, phylogenetic groups, and serotypes which had been determined in the previous chapter. These included E9 (MP27/B2), E22 (MP24/O18/B2), E29 (MP13/B1), E30 (MP25/O78/A), E43 (MP7/O78/B2), E64 (MP26/B2), E89 (MP25/D), E104 (MP23/O78/B1), E115 (MP19/O78/A), E129 (MP20/O1), and E138 (MP19/A). Each isolate was streaked onto MacConkey agar plate (Difco) that was incubated overnight at 37°C (BP-701, Biofree). One colony of each isolate was transferred by sterile loop into a 250 ml glass flask containing 20 ml LB broth (Duchefa Biochemie) and incubated overnight at 37°C with shaking at 250 r.p.m. (NB-205L, N-biotec, INC.). Each flask was grown for 6 hours in same condition after 70 ml of LB broth was added into each flask. This broth culture was centrifuged at 2,500 r.p.m. for 20 min (Allegra® X-15RC, Beckman Coulter Inc.), washed twice in LB broth and diluted to an OD_{600} of 5.0 (BioPhotometer, Eppendorf) with LB broth after supernant was discarded. Each broth was diluted with LB broth up to 10^{-3} fold to prepare a series of 10-fold dilution. Each diluted broth of each isolates was held on ice before use.

Table 3.1. Molecular pathotype, virulence gene accumulation, phylogenetic group, and serotype of 11 APEC isolates

Isolates	MP[a]	Virulence genes	Phylogenetic group	Serotype
E64	MP26	*iroN–fimC–ompT–hlyF–iucD–iss–fyuA–irp2–vat*	B2	UT[b]
E89	MP25	*iroN–fimC–ompT–hlyF–iucD–iss–fyuA–irp2–tsh*	D	UT
E22	MP24	*iroN–fimC–ompT–hlyF–iucD–fyuA–irp2–tsh–vat*	B2	O18
E104	MP23	*iroN–fimC–ompT–hlyF–iucD–fyuA–irp2–tsh*	B1	O78
E138	MP19	*iroN–fimC–ompT–hlyF–iucD –iss–tsh*	A	UT
E9	MP27	*iroN–fimC–ompT–hlyF–iucD–iss–fyuA–irp2–tsh–vat*	B2	UT
E29	MP13	*iroN–fimC–ompT–hlyF–iucD–tsh*	B1	UT
E30	MP25	*iroN–fimC–ompT–hlyF–iucD–iss–fyuA–irp2–tsh*	A	O78
E43	MP7	*iroN–fimC–iucD–iss*	B2	O78
E115	MP19	*iroN–fimC–ompT–hlyF–iucD –iss–tsh*	A	O78
E129	MP20	*iroN–fimC–ompT–iss–fyuA–irp2–vat*	ND[c]	O1

[a] MP: molecular pathotype.

[b] UT: untypable.

[C] ND: not determined.

3.3.2. Chicken and growth condition

Unvaccinated brown layer male chicks from healthy breeders without a history of colibacillosis (Yangji hatchery Co., Anseong, Korea) were obtained at 1 day old from commercial hatchery. All of chicks were housed on the floor in isolated rooms, allowed to eat and drink *ad libitum*, and underwent 7 days of acclimatization before experiments. Rearing temperatures were similar to those used commercially.

3.3.3. Virulence assay

At day seven, these chickens were randomly selected, marked and randomly allocated into 12 groups of 20 chickens and housed in four rooms with 3 groups of chickens in each isolated rooms.

For the LD_{50} test, a series of 10-fold dilution of each isolates (10^9 to 10^6 CFU/0.2 ml) or LB broth (for control group) were administered by delivering 0.2 ml by the subcutaneous route in the back of the neck. The colony forming unit of each inoculums was then titered by plating dilutions onto MacConkey plates. The plates were incubated overnight at 37°C and colonies counted next day.

The chickens were monitored and recorded daily for signs of illness and for deaths for 7 days after challenge. Necropsy, gross pathological examinations and bacteriological cultures were done immediately on

chickens that were found dead before 14 days old (7 days post-inoculation). After the observation period, all of remaining birds were euthanized by cervical dislocation, necropsied, and observed for lesions (such as pericarditis and perihepatitis) consistent with colibacillosis. Gross pathologic examinations of heart and liver were performed. For re-isolation, each organ was swabbed with a sterile cotton swab and streaked onto MacConkey agar plates and incubated overnight at 37°C.

3.3.4. Lethality classes

The LD_{50} was calculated by the method of Reed and Muench Lethality classes (LC) as described previously and APEC isolates were classified into lethality classes as below (Dozois *et al.*, 2000). LC was defined as follows: LC1, LD_{50} less than or equal to 5 X 10^6 CFU; LC2, LD_{50} is from 5 X 10^6 to less than 5 X 10^8 CFU; LC3, LD_{50} greater than or equal to 5 X 10^8 CFU.

3.4. Results

3.4.1. Virulence assays in chickens

The LD_{50} of the 11 isolates were variable and they were classified into LC1 (18.2%), LC2 (27.3%), and LC3 (54.5%) (Table 3.2). E64 (MP26/B2) and E89 (MP25/D) showed less than 5 X 10^6 CFU LD_{50} value, 3.3 X 10^6 CFU and 4.7 X 10^6 CFU, respectively, and were classified into LC1. E22 (MP24/O18/B2), E104 (MP23/O78/B1), and E138 (MP19/A) were classified into LC2 because the LD_{50} values were in the range from 5 X 10^7 CFU to less than 5 X 10^8 CFU. On the other hand, E9 (MP2/B2), E29 (MP13/B1), E30 (MP25/O78/A), E43 (MP7/O78/B2), E115 (MP19/O78/A), and E129 (MP20/O1) were classified into LC3 because the LD_{50} values were always higher than 5 X 10^9 CFU.

Table 3.2. Clinical signs, mortality, LD_{50}, and lethality classes of 11 APEC isolates

Isolate	Inoculation dosage (cfu/0.2 ml)	Detected clinical signs (day post inoculation)	mortality[a]	APEC re-isolation	LD_{50}(CFU/ml)[b]	LC[c]
E64	$3.3X10^6$	2	100%	Yes	$< 3.3X10^6$	LC1
	$3.3X10^7$		100%	Yes		
	$3.3X10^8$		100%	Yes		
	$3.3X10^9$		100%	Yes		
E89	$4.72X10^6$	2	100%	Yes	$< 4.7X10^6$	LC1
	$4.72X10^7$		100%	Yes		
	$4.72X10^8$		100%	Yes		
	$4.72X10^9$		100%	Yes		
E22	$7.7X10^6$	2	0%	Yes	$5.5X10^7$	LC2
	$7.7X10^7$		60%	Yes		
	$7.7X10^8$		60%	Yes		
	$3.8X10^9$		100%	Yes		
E104	$3.3X10^6$	2	0%	Yes	$1.8X10^8$	LC2
	$3.3X10^7$		40%	Yes		
	$3.3X10^8$		60%	Yes		
	$3.3X10^9$		80%	Yes		
E138	$2.67X10^6$	2	20%	Yes	$2.5X10^7$	LC2
	$2.67X10^7$		60%	Yes		
	$2.67X10^8$		100%	Yes		
	$2.67X10^9$		80%	Yes		
E9	$9.0X10^6$	NC[d]	0%	Yes	$> 4.5X10^9$	LC3
	$9.0X10^7$		0%	Yes		
	$9.0X10^8$		0%	Yes		
	$4.5X10^9$		0%	Yes		

Table 3.2. Continued

Isolate	Inoculation dosage (cfu/0.2 ml)	Detected clinical signs (day post inoculation)	mortality[a]	APEC re-isolation	LD_{50}(CFU/ml)[b]	LC[c]
E29	$7.7X10^6$	7	0%	Yes	$2.9X10^9$	LC3
	$7.7X10^7$		0%	Yes		
	$7.7X10^8$		0%	Yes		
	$3.8X10^9$		60%	Yes		
E30	$9.5X10^6$	NC	0%	Yes	$> 5.5X10^9$	LC3
	$9.5X10^7$		0%	Yes		
	$9.5X10^8$		0%	Yes		
	$5.5X10^9$		0%	Yes		
E43	$9.4X10^6$	NC	0%	Yes	$> 5.1X10^9$	LC3
	$9.4X10^7$		0%	Yes		
	$9.4X10^8$		0%	Yes		
	$5.1X10^9$		20%	Yes		
E115	$5.2X10^6$	NC	0%	Yes	$> 2.6X10^9$	LC3
	$5.2X10^7$		0%	Yes		
	$5.2X10^8$		0%	Yes		
	$2.6X10^9$		20%	Yes		
E129	$3.3X10^6$	6	0%	Yes	$> 3.3X10^9$	LC3
	$3.3X10^7$		0%	Yes		
	$3.3X10^8$		20%	Yes		
	$3.3X10^9$		40%	Yes		

[a] Total mortality of 7 day post inoculation. [b] LD_{50}; 50% lethal dose.

[c] LC: lethality classes; LC 1, $LD_{50} \leq 5 \times 10^6$ cfu; LC2, 5×10^6 to 10^8 cfu; LC3, $\geq 5 \times 10^8$.

[d] NC: no clinical signs.

3.4.2. Clinical signs and post-mortem observation

Chickens inoculated with the LC1 isolates were depressed with rough feathering appearance, droopy eyelids, anorexia, disinclination to move, and sternal recumbancy as early as 1-day-post-inoculation (dpi). The effects of the infection were rapid and pericardial lesions were observed in some of the birds that died around 1 dpi. In this test group, most chickens had died with typical lesions of colibacillosis before 2 dpi although lesions were mild (Figure 3.1). Chickens dying later showed more severe lesions in the heart and the liver (Figure 3.2). In spite of lesions in the heart and the liver, air sacculitis was not observed in any chickens. *E. coli* was reisolated from all chickens in the LC 1 group.

In the LC2 isolates, most chickens died before 7 dpi especially in higher dosage inoculation. Clinical signs in some chickens were similar to the LC 1 group. The effects of the infection were not rapid and most dead chickens showed typical lesions of colibacillosis. Some chickens sacrificed after 7 dpi showed less severe lesions in the heart and the liver than those in the LC1. The others did not show any kind of lesions. Swab samples from all the chickens in this group showed positive results for *E. coli* culture.

In the LC3 isolates, only few chickens died around 2 dpi. Most chickens in this group did not show any kind of clinical signs. All of dead chickens

showed mild lesions in the heart and the liver. Surviving chickens did not show any kind of clinical signs after 7 dpi but showed mild polyserositis and cellulitis. All swab samples showed positive re-isolation results. The uninoculated controls showed no clinical signs and mortality, negative for lesions, and no re-isolation of *E. coli*.

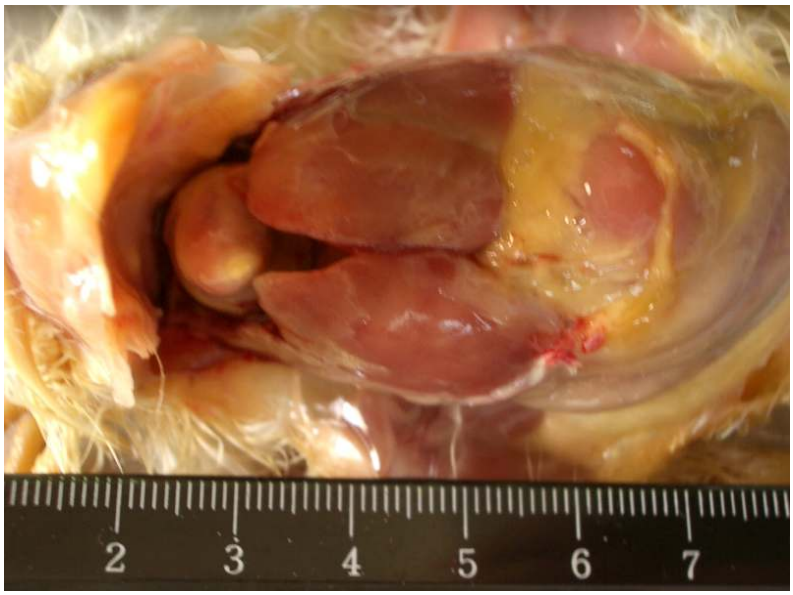

Figure 3.1. Gross pathological findings of a dead chicken subcutaneously infected with APEC isolate (E89) at 2 days after inoculation. The bird had mild fibrinous pericarditis and perihepatitis. Scale in mm.

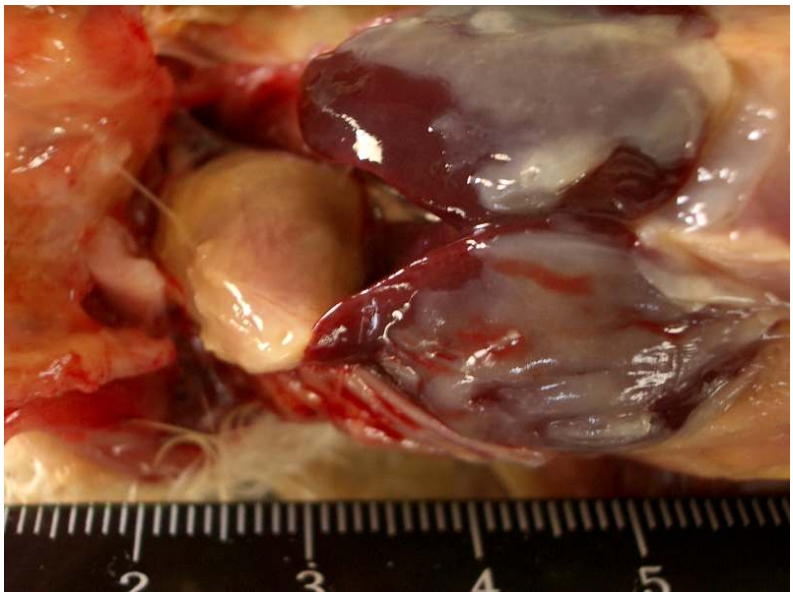

Figure 3.2. Gross pathological findings of a dead chicken subcutaneously infected with APEC isolate (E64) at 7 days after inoculation. The bird had severe fibrinous pericarditis and perihepatitis. Scale in mm.

3.5. Discussion

To date, the poultry industry has been expanded and large numbers of birds are kept at high stocking rates in automatic housing system. These birds are exposed to virus, mycoplasma infection, and environmental stresses (dust and ammonia), which predispose colibacillosis caused by APEC infection. Colibacillosis has induced enormous economic losses included mortality, morbidity, and therapeutic cost (Altekruse *et al.*, 2002b; Blanco *et al.*, 1997a; Dho-Moulin and Fairbrother, 1999; Gyles, 1994b).

Chemotherapy is an important tool in reducing both the incidence and mortality associated with avian colibacillosis (Freed *et al.*, 1993; Watts *et al.*, 1993). However, misuse of antibiotics has been the major cause of the increasing prevalence of antibiotic-resistance (Leibert *et al.*, 1999; Speer *et al.*, 1992) and multiple resistances to available antibiotics are already widespread (Allan *et al.*, 1993; Amara *et al.*, 1995; Cloud *et al.*, 1985; Gan *et al.*, 2013; Zhao *et al.*, 2005). This situation evoked increased interest to vaccine as alternative methods against colibacillosis.

The pathogenicity of APEC cannot be explained by the presently known virulence factors and additional determinants remain to be characterized (Brown and Curtiss, 1996; Kemmett *et al.*, 2013). Although

there is no host- or pathotype specific virulence gene pattern, certain gene combination with a greater linkage to a certain pathotype was proposed (Bingen-Bidois *et al.*, 2002; Ewers *et al.*, 2007).

The LC1, LC2, and LC3 are high pathogenic, pathogenic, and apathogenic, respectively. The frequencies of LC1, LC2, and LC3 of tested APEC isolates in the present study were similar to a previous study (Fantinatti *et al.*, 1994). Apathogenic isolates, E9, E29, E30, E43, E115, and E129 showed very high LD_{50} values, although surviving chickens in these isolates showed typical symptoms of colibacillosis. Most of these isolates except E9 and E30 possessed less abundant virulence genes. However, pathogenic isolates, E22, E104, and E138 possessed multiple virulence genes.

Methods related to virulence assay were various. Bree *et al.* (1989) and Goren *et al.* (1978) inoculated APEC directly into the trachea, following preliminary challenge with triggering agents such as virus, mycoplasma or ammonia. Peighambari *et al.* (2002) sprayed APEC into air-way of 14-day-old chicken. Some researchers used the method which inoculated APEC into air-sac of 8-day-old chickens. When highly and low pathogenic *E. coli* isolates were inoculated via the air sac route all isolates had colonized the respiratory tract and internal organs at 6 hours post-inoculation. However, only highly pathogenic isolates were

recovered from the pericardial fluid and blood (Dho-Moulin and Fairbrother, 1999). The reproduction of colibacillosis via air sac route is complicated and not easy because it is affected by age of host, housing condition, and the kind of predisposing factors (Dias da Silveira *et al.*, 2002; Giovanardi *et al.*, 2005; Ngeleka *et al.*, 2002; Ngeleka *et al.*, 1996).

Although the inoculation route is not natural, the subcutaneous inoculation of chickens without any predisposing factors is simple and easy to reproduce acute death and colibacillosis. In the present study only the mortality of chickens inoculated within 7 days was considered for classification of LC, but some of them showed polyserositis and cellulitis without mortality. Therefore, an extended observation period (e.g. 14 days) and grading of gross lesions of organs and tissues may improve resolution of virulence assays in the future (Peighambari *et al.*, 2002).

In conclusion, APEC isolates which possess more abundant virulence genes tend to more pathogenic in chickens and the highly pathogenic APEC isolates may be useful for vaccine development in the future.

General Conclusion

E. coli that causes disease in chickens was collectively known as avian pathogenic *E. coli* (APEC) (Barbieri *et al.*, 2013; Kariuki *et al.*, 2002). APEC induced enormous losses in poultry industry. Antimicrobial therapy was an important tool in reducing both the incidence and mortality associated with avian colibacillosis (Watts *et al.*, 1993). However, resistance to existing antimicrobials was widespread and of concern to poultry industry (Owawa and Asai, 2013; Zhao *et al.*, 2005).

In chapter 1, antibiograms and relevant genotypes of 101 Korean APEC isolates between 1985 and 2005 were assessed via disc diffusion test, PCR, restriction enzyme analysis (REA), and sequencing. Studies on single nucleotide polymorphisms (SNPs) and promoter structure might prove beneficial for our knowledge of class 1 integron transmission, as well as for the evolution of and relationship between antibiotic resistances and genotypes. The frequency of resistances to five antibiotics (tetracycline, streptomycin, enrofloxacin, ampicillin, and trimethoprim-sulfamethoxazole) had increased during the observation periods of this study. Also, MDR (multi-drug resistance) APEC evidencing resistance against at least three different classes of antibiotics had increased. Significant increase of MDR APEC was observed during the 2000 to 2005. The frequency of relevant resistance

genes (*tetA*, *strA*–*strB* and *TEM*) and mutations in *gyrA* and *parC* had increased during the observation periods of this study. Therefore, antibiotics' preventive and therapeutic effects on APEC should no longer be desirable. Additionally, restriction of the use of antibiotics in poultry production related to drug residues in meat and the costs associated with administration of drugs had led to increased interest in alternative methods of protecting flocks against APEC infections.

To make alternative methods, it should be needed to understand the pathogenicity of APEC. Therefore, 101 APEC isolates collected in Korea from 1985 to 2005 were classified by serotype, phylogenetic groups, virulence genes, and virulence gene combination (molecular pathotypes; MPs) in chapter 2. The purpose of this test was to take more information and to develop diagnostic tools and to select vaccine candidate for APEC.

Serotype O78 was the most frequent (19.8%), followed by O18 (3.0%), O1 (2.0%), O115 (2.0%), and O21 (1.0%). In phylogenetic groups, group A was the most frequent group (39.5%). Groups B1 (23.3%) and B2 (22.1%) were similar in frequency, while group D (15.1%) was notably less prevalent. According to virulence genes, the frequencies of *iroN*, *fimC*, *ompT*, *hlyF*, *iucD*, *iss*, *fyuA*, *irp2*, *tsh*, and *vat* among the MPs were 100%, 70.4%, 63.0%, 59.3%, 59.3%, 59.3%, 51.9%, 48.1%, 33.3%, and 14.8%, respectively. All APEC isolates were classified into 27 MPs on

the basis of their different combinations of virulence genes. Frequencies of MP25 (*iroN-fimC-ompT-hlyF-iucD-iss-fyuA-irp2-tsh*) and MP19 (*iroN-fimC-ompT-hlyF-iucD -iss-tsh*) were higher than the others with 30.8% and 21.8% of frequency, respectively.

According to the virulence assay the virulent APEC isolates tend to possess more abundant virulence genes. However, the fact that some less pathogenic APEC isolates have same virulence genes as more pathogenic isolates may reflect additional virulence genes and/or difference in gene expression of each virulence gene in vivo.

The virulence assay in the present study was simple and reproducible for colibacillosis, and it may be useful to evaluate efficacy of vaccine. Thus, the MDR of APEC isolates are confronting threat to poultry industry, and molecular pathotyping and virulence assay results in the present study may be useful to develop efficacious vaccine colibacillosis in the future.

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국 문 초 록

국내 조류 병원성 대장균 분리주의 항생제 저항성 및 병원성 인자에 관한 연구

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가금류 산업에서 조류 병원성 대장균 (avian pathogenic *Escherichia coli*; APEC)에 의한 피해가 매우 크며, 이를 최소화 하기 위해 항생제를 이용한 치료 방법을 활용해오고 있다. 하지만, 조류 병원성 대장균 내에서의 항생제 저항성 발현 빈도가 크게 증가되는 이유로 인해 항생제를 이용한 치료 효과는 낮아지고 있다. 1985년부터 2005년 동안 분리된 국내 병원성 대장균 101개 분리주를 대상으로 항생제 저항성과 관련된 기본적인 정보를 얻기 위한 실험을 실시하였다. 국내 조류 병원성 대장균 분리주들을 대상으로 디스크 확산법,

중합효소 연쇄반응(PCR), 제한효소 분석법(REA) 그리고 유전자배열 분석을 통해 항생제 감수성 검사와 항생제 저항성 관련 유전자에 대하여 조사하였다. 해당 기간 내에서 몇 가지 종류의 항생제에 대한 저항성이 상당한 비율로 증가된 것이 관찰되었다. 테트라싸이클린과 스트렙토마이신에 대한 저항성이 가장 높게 나타났으며 (각각 84.2%), 그 다음으로 엔로플록사신 (71.3%), 암피실린 (67.3%), 트리메소프림 및 설파메속사졸 합제 (37.6%), 그리고 겐타마이신 (26.7%) 순으로 나타났다. 항생제 저항성 관련 유전자들 (*tetA*, *tetB*, *aadA*, 그리고 *strA-strB*)의 발현비율과 특정 유전자 부위 (*gyrA*와 *parC*)에서의 돌연변이 발생 빈도도 동일한 기간 동안에 증가하는 것으로 나타났다. 이러한 실험 결과에 비추어 볼 때, 항생제 저항성의 발현 빈도는 계속 증가하는 것으로 보이며, 따라서 항생제 이외에 백신과 같은 또 다른 방어 수단이 필요하다.

백신을 개발하는데 있어서 병원성에 대한 이해와 병원성이 상대적으로 높은 분리주의 확보는 필수적인 사항이다. 이에 따라, 101개 국내 분리주를 대상으로 병원성에 영향을 미치는 특성들에 대해 실험을 실시하였다. 해당 분리주들에 대해서 혈청형을 검사하였고, 병원성 유전자들의 분포도를 조사하였으며, 병원성 유전자들의 누적 정도에 따라서 분자 병원형 (Molecular Pathotypes; MPs)을 구분을 하였다. 혈청형 검사 결과, 28개 분리주 (27.8%)에서만 혈청형이 확인되었고, 나머지 분리주들에서는 혈청형이 확인되지 않았다. 병원성 유전자들의 발현빈도는 다양하여, *iroN* (100%)의 분리율이 가장 높았으며, 그 다음으로 *ompT* (94.1%), *fimC* (90.1%), *hlyF* (87.1%), *iss* (78.2%),

iucD (73.3%), *tsh* (61.4%), *fyuA* (44.6%), *irp2* (43.6%), 그리고 *vat* (10.9%) 순이었다. 병원성 유전자들의 누적 정도에 따라 모든 분리주들은 27개 분자 병원형으로 분류하였다. 병원성 유전자들의 분포 양상을 보면 특정 분자 병원형에서 더욱 더 누적되는 경향을 보이고 있었다. 따라서 분자 병원성을 이용하여 병원성이 더 높은 조류 병원성 대장균의 구분이 가능할 것으로 판단되었다.

분자 병원형과 닭에서의 실제 병원성 사이의 관련성을 조사하기 위해 분자 병원형을 기준으로 하여 11개의 조류 병원성 대장균 분리주를 선발하였다. 병원성 실험의 재현성을 높이고 비교 가능한 결과치를 얻기 위해서 각 분리주들을 네 단계의 희석비율 (10^9–10^6 CFU/0.2 ml)로 준비하여 7일령 닭에 접종하는 방법으로 실험을 진행하였다. 실험에 사용된 모든 닭들을 대상으로 부검, 육안 병리검사 그리고 세균 재분리 등의 실험을 진행하였다. 50% 치사량 (50% lethal dose; LD_{50}) 수치를 기준으로 11개 분리주들을 대상으로 LC1 ($LD_{50} \leq 5 \times 10^6$ CFU), LC2 (5×10^6–10^8 CFU), 그리고 LC3 ($\geq 5 \times 10^8$ CFU)와 같이 세 단계의 치사율 등급 (lethality class)으로 각각 구분하였다. LC1에 속하는 분리주를 접종한 실험군에서는 접종 24시간 후부터 임상증상과 폐사가 발견되었다. 접종을 실시한 모든 실험군 내의 닭에서 채취한 샘플에서 조류 병원성 대장균이 재분리 되었다. 병원성이 상대적으로 높은 (LC1) 분리주들 (E64와 E89)이 속한 분자 병원형들은 상대적으로 병원성 유전자들이 더욱 더 많이 분포되어 있는 특징을 가지고 있다. 이러한 특징들을 볼 때, 분자 병원형과 조류 병원성 대장균의 병원성 사이에 관련성이 존재

하는 것으로 판단된다.

위와 같은 일련의 연구를 통해 국내 조류 병원성 대장균에서 항생제 저항성과 관련된 기본적인 정보를 얻을 수 있었고, 분자 병원형 분류 방법을 이용하여 병원성이 더 높은 분리주들을 구분할 수 있다는 점이 확인되었다. 본 실험에서 확인된 병원성이 더 높은 조류 병원성 대장균 분리주들을 활용한 백신의 효능 실험이 추가로 필요할 것이다.

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주요어: 조류 병원성 대장균, 항생제 저항성, 병원성 유전자, 분자 병원형, 치사율 등급

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