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Antimicrobial Resistance, Virulence Genes and PFGE-profiling of *Escherichia coli* Isolates from South Korean Cattle Farms

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To estimate the prevalence of *Escherichia coli* with potential pathogenicity in cattle farm in South Korea, a total of 290 *E. coli* isolates were isolated from cattle farms over a period of 2 years in South Korea. These were examined for phenotypic and genotypic characteristics including antimicrobial susceptibility, serotype, and gene profiles of virulence and antimicrobial resistance. The most dominant virulence gene was *f17* (26.2%), followed by *stx2* (15.9%), *ehxA* (11.0%), *stx1* (8.3%), *eae* (5.2%), and *sta* (4.1%). Some shiga-toxin producing *E. coli* isolates possessed *eae* (15.9%). All isolates except for one showed resistance to one or more antimicrobials, with 152 isolates exhibiting multidrug-resistance. The most prevalent resistance phenotype detected was streptomycin (63.1%), followed by tetracycline (54.5%), neomycin (40.3%), cephalothin (32.8%), amoxicillin (30.0%), ampicillin (29.7%), and sulphamethoxazole/trimethoprim (16.6%). The associated resistance determinants detected were *strA-strB* (39.0%), *tet*(E) (80.0%), *tet*(A) (27.6%), *aac*(*3*)-*IV* (33.1%), *aphA1* (21.4%), *bla*$_{TEM}$ (23.8%), and *sul2* (22.1%). When investigated by O serotyping and PFGE molecular subtyping, the high degree of diversity was exhibited in *E. coli* isolates. These results suggest that *E. coli* isolates from South Korean cattle farms are significantly diverse in terms of virulence and antimicrobial resistance. In conclusion, the gastroinstestinal flora of cattle could be a significant reservoir of diverse virulence and antimicrobial resistance determinants, which is potentially hazardous to public health.

Keywords: *Escherichia coli*, prevalence, virulence, antimicrobial resistance, serotyping, PFGE

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Introduction

Although *Escherichia coli* is usually a non-pathogenic member of the gastroinstestinal flora of the host, some strains may cause diseases that represent a hazard to the public health and the food-producing animal industry. Bacterial pathogenicity is determined by the presence of virulence factors which are mostly encoded by genes located in chromosomes and/or plasmids. Therefore, it is very important to distinguish the pathogenic *E. coli* from gastroinstestinal flora by detection of the virulence factors, such as toxins, fimbriae, and non-fimbrial adhesion molecules.

Antimicrobial agents have been used as preventive measures against bacterial infections in the food-producing animal industry, with beneficial effects in decreasing morbidity and mortality (Berge *et al*., 2009). However, the intensive and indiscriminate usage of antimicrobials may induce the emergence and dissemination of antimicrobial resistance, not only in pathogenic bacteria but also in commensals. Also, until 2011, copious amounts of various antimicrobial agents were used as feed supplements in the livestock industry in South Korea. Furthermore, until 2013, antimicrobial agents could be used without a veterinarian's prescription. These factors may have accelerated the emergence and dissemination of antimicrobial resistance. Antimicrobial resistance genes have been considered as one of virulence factors (Davies and Davies, 2010). Moreover, commensal *E. coli* isolates, resistant to several antimicrobials, may constitute an important reservoir of antimicrobial resistance determinants, which may be transferred via transmissible plasmids intra and/or inter species (Wright, 2007). Thus *E. coli* strains isolated from healthy animals need to be assessed for the prevalence of resistance in animal populations, and these results can be used in several monitoring programs (Franklin *et al*., 2001).

Pathogenic *E. coli* strains isolated from animals can be transmitted from animals to humans through the food chain and give rise to severe disease in humans. Therefore it is important that the pathogenic *E. coli* strains are distinguished from gastroinstestinal flora in food-animals by identification of virulence factors. Serotyping has been widely used for differentiation of *E. coli* pathogenicity, and it is of value due to the common association of some serotypes with calf diarrhea (Acres, 1985). However, the pathogenicity of *E. coli* strains cannot be determined only by serotyping because there are many factors to affect the virulence of *E. coli*. According to the Center for Disease Control and Prevention (CDC), pathogenic *E. coli* isolates are classified as enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli*

(DAEC) (CDC, 2012). ETEC isolates can cause diarrhea in newborn calves by the expression of fimbriae (F5, F41, and F17) and enterotoxin (STa and LT) (Nagy and Fekete, 1999). STEC isolates, producing Stx1, Stx2, or both, known also as verotoxigenic *E. coli* (VTEC), are the significant causatives of diarrhea in calves (Nagy and Fekete, 1999). In humans, STEC can cause hemorrhagic colitis (HC) and the hemolytic uremic syndrome (HUS) (Nataro and Kaper, 1998). EPEC and STEC isolates, carrying *eae* genes, produce the adhesion protein intimin to initiate attachment to the enterocyte and effacement of the microvillus border (Jerse *et al*., 1990).

Although several researches on antimicrobial resistance or virulence factors in pathogenic bacteria have been carried out in South Korea (Kang *et al*., 2005; Lim *et al*., 2007), there has been no report showing the relationship between antimicrobial resistance and virulence factors in bacteria isolated on cattle farms. Based on current knowledge, the genotypic and phenotypic prevalence of virulence factors and of antimicrobial resistance were investigated in enteric *E. coli* isolated from cattle farms in South Korea. Also, their genetic relationships were compared, using the macrorestriction profiling (PFGE). This information may give a new horizon to develop a new preventive measure against food-animal originated *E. coli* infection.

Materials and Methods

Bacterial isolates

A total 290 *E. coli* strains were isolated from 830 fecal samples collected from healthy beef cattle on eight cattle farms from six different cities (Asan, Anyang, Buyeo, Chilgok, Namyangju, and Gwangju) in South Korea between 2011 and 2012. The samples were obtained from cattle rectum and pats on the shed. The samples were placed at 4–8°C and delivered to the laboratory within 24 h. The fecal samples were plated onto eosin methylene blue (EMB) agar, and MacConkey agar for selection, and were incubated at 37°C for 18 h. From each sample, three to five colonies that were suspected to be *E. coli* were sub-cultured onto a blood agar plate (BAP). Isolates were confirmed as *E. coli* by a standard biochemical test (Indole, Methyl Red, Voges-Proskauer, and citrate utilization tests) and by the Vitek2 system (bioMérieux, France). The confirmed *E. coli* isolates were stored in tryptic soy broth (TSB), with 20% glycerol, at -70°C to await further analysis. The reference *E. coli* strains for virulence factor were O9:K35 ($K99^+$ and $F41^+$), O141:K85ab ($987P^+$ and STa^+) and O15:H11 (LT^+) which were kindly provided by the Animal and Plant Quarantine Agency, Anyang, Republic of Korea. In addition, the *E. coli* strains EC192 ($Stx1^+$, $Stx2^+$, $Intimin^+$, and $EhxA^+$) isolated in this study were used for reference.

Antimicrobial susceptibility test

The resistance against 15 antimicrobials was tested by the disk diffusion test. The following antimicrobial compounds were used: enrofloxacin (ENR), 5 μg (Bayer, Germany); ampicillin (AMP) 10 μg; amoxicillin (AML), 10 μg; streptomycin (S), 10 μg; gentamicin (CN), 10 μg; neomycin (N), 30 μg; tetracycline (TE), 30 μg; nalidixic acid (NA), 30 μg; ciprofloxacin (CIP), 5 μg; cephalothin (KF), 30 μg; ceftiofur (EFT), 30 μg; ceftazidime (CAZ), 30 μg; chloramphenicol (C), 30 μg; florfenicol (FFC), 30 μg; sulfamethoxazole/trimethoprim (SXT), 25 μg, which contains 1.25 μg of trimethoprim and 23.75 μg of sulfamethoxazole (Oxoid, England). All antimicrobial resistance tests were performed on Mueller-Hinton (MH) agar, and data were classified as susceptible or resistant, based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. *E. coli* ATCC 29522 was included as the quality control strain.

Detection of virulence and antimicrobial resistance genes

Bacterial genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, USA), and following the manufacturer's instructions. The concentration of DNA was measured using a Nanodrop N-1000 spectrophotometer (Thermoscientific, USA).

PCR assay for virulence genes: All isolates were analyzed for nine different virulence genes. The multiplex polymerase chain reactions (m-PCR) were used to detect toxins (Stx1, Stx2, STa, and LT), adhesions (F5, F17, F41, and Intimin) and enterohemolysin, and were carried out as described in earlier studies (Schmidt *et al*., 1995; Franck *et al*., 1998; Van Bost *et al*., 2001; López-Saucedo *et al*., 2003), with some modifications. The primers and PCR conditions used in this study are shown in Table 1.

PCR assay for antimicrobial resistance genes: Genes encoded for aminoglycoside resistance (*aadA*, *aadB*, *aphA1*, *aphA2*, *strA-strB*, and *aac(3)-IV*), β-lactam resistance (*ampC*, bla_{TEM}, bla_{OXA}, and bla_{SHV}), tetracycline resistance (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G)), phenicol resistance (*cat*, *cmlA*, and *floR*) and sulfonamide resistance (*sul1*, *sul2*, and *sul3*) were screened as described by previous study (Karczmarczyk *et al*., 2011a) with some modifications (Table 1). EC137, EC192, and EC277 isolated in this study were used as the positive controls for detection of *aadA*, *aphA1*, *strA-strB*, *aac(3)-IV ampC*, bla_{TEM}, *tet*(A), *tet*(B), *tet*(C), *tet*(E), *cat*, *cmlA*, *floR*, *sul1*, *sul2*, and *sul3*.

All PCRs were performed on Veriti thermocycler (Applied Biosystems, USA), and each run included a negative control and an appropriate positive control. The reactions were run in duplicate to confirm results. All PCR products were analyzed by electrophoresis on a 2.0% agarose gel for 1 h at 100 V, and photographed under UV light after staining with ethium bromide. Amplified PCR products of expected sizes were subjected to direct sequencing by an automatic sequencer and dye termination sequencing system (Macrogen Co., Korea). A BLAST search for homologous sequence was performed in the GenBank database at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

Serotyping

Out of a total 290 *E. coli* isolates, 53 isolates were selected for serotyping. These isolates showed multidrug-resistance and harbored more than one virulence gene. The O serogroup of *E. coli* isolates was determined using 181 O-antisera (O1-O187) by a standard slide agglutination test (Ørskov

Table 1. Primers and m-PCR conditions for virulence genes and antimicrobial resistance genes used in this study

Target gene	Nucleotide sequence (5′→3′)	PCR condition[a]			Amplicon (bp)	Reference
		Denaturing	Annealing	Extension		
f5[a]	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	94°C for 30 sec	50°C for 45 sec	70°C for 90 sec	314	Franck *et al.* (1998)
f41[a]	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT				380	Franck *et al.* (1998)
stx1[a]	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT				555	Franck *et al.* (1998)
stx2[a]	GTGCCTGTTACTGGGTTTTTCTTC AGGGGTCGATATCTCTGTCC				118	Franck *et al.* (1998)
sta[a]	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA				190	Franck *et al.* (1998)
eae[a]	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA				425	Franck *et al.* (1998)
f17[a]	GCAGAAAATTCAATTTATCCTTGG CTGATAAGCGATGGTGTAATTAAC	94°C for 60 sec	57°C for 60 sec	72°C for 60 sec	537	Van Bost *et al.* (2001)
lt[a]	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT				450	López-Saucedo *et al.* (2003)
ehxA[a]	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA				1551	Schmidt *et al.* (1995)
aadB[b]	GAGGAGTTGGACTATGGATT CTTCATCGGCATAGTAAAA	94°C for 60 sec	53°C for 60 sec	72°C for 60 sec	208	Travis *et al.* (2006)
aphA2[b]	GATTGAACAAGATGGATTGC CCATGATGGATACTTTCTCG				347	Travis *et al.* (2006)
aphA1[b]	ATGGGCTCGCGATAATGTC CTCACCGAGGCAGTTCCAT				600	Maynard *et al.* (2003)
aadA[b]	GTGGATGGCGGCCTGAAGCC AATGCCCAGTCGGCAGCG				525	Madsen *et al.* (2000)
strA-strB[b]	ATGGTGGACCCTAAAACTCT CGTCTAGGATCGAGACAAAG		58°C for 60 sec		893	Tamang *et al.* (2007)
aac(3)-IV[b]	TGCTGGTCCACAGCTCCTTC CGGATGCAGGAAGATCAA				653	Boerlin *et al.* (2005)
ampC[b]	CCCCGCTTATAGAGCAACAA TCAATGGTCGACTTCACACC	94°C for 60 sec	53°C for 60 sec	72°C for 60 sec	634	Féria *et al.* (2002)
bla_{OXA}[b]	TATCTACAGCAGCGCCAGTG CGCATCAAATGCCATAAGTG				199	Féria *et al.* (2002)
bla_{TEM}[b]	TACGATACGGGAGGGCTTAC TTCCTGTTTTTGCTCACCCA				716	Belaaouaj *et al.* (1994)
bla_{SHV}[b]	TCAGCGAAAAACACCTTG TCCCGCAGATAAATCACCA				475	M'Zali *et al.* (1996)
tet(A)[b]	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	94°C for 60 sec	58°C for 60 sec	72°C for 60 sec	210	Ng *et al.* (2001)
tet(B)[b]	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG				659	Ng *et al.* (2001)
tet(C)[b]	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC				418	Ng *et al.* (2001)
tet(D)[b]	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC				787	Ng *et al.* (2001)
tet(E)[b]	AAACCACATCCTCCATACGC AAATAGGCCACAACCGTCAG		58°C for 60 sec		278	Ng *et al.* (2001)
tet(G)[b]	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC				468	Ng *et al.* (2001)
cat[b]	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	94°C for 60 sec	55°C for 60 sec	72°C for 60 sec	547	Van *et al.* (2008)
cmlA[b]	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG				698	Keyes *et al.* (2000)
floR[b]	TATCTCCCTGTCGTTCCAG AGAACTCGCCGATCAATG				399	Keyes *et al.* (2000)
sul1[b]	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	94°C for 60 sec	57°C for 60 sec	72°C for 60 sec	433	Kerren *et al.* (2002)
sul2[b]	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC				721	Lanz *et al.* (2003)
sul3[b]	CAACGGAAGTGGGCGTTGTGGA GCTGCACCAATTCGCTGAACG				244	Kozak *et al.* (2009)

[a] All PCRs were carried out for 25 cycles
[b] All PCRs were carried out for 30 cycles

and Ørskov, 1984). The O-antisera were provided by the Animal and Plant Quarantine Agency, Anyang, South Korea.

Pulsed-field gel electrophoresis (PFGE) profiling

Overall, 53 *E. coli* isolates determined serogroups were analyzed by PFGE, according to a standard protocol of the Center for Disease Control and Prevention (CDC), with some modifications. *E. coli* isolates were incubated on tryptic soy agar (TSA) at 37°C for 18 h. Bacteria were suspended in a cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0), and adjusted to OD_{600} of 1.3–1.4 using a spectrophotometer. The cell suspension (400 µl) was mixed with 20 µl of proteinase K and 400 µl of melted 1% SeaKem Gold Agarose (Lonza, USA). The mixture was dispensed into appropriate wells of a disposable plug mold (Bio-Rad Laboratories, USA). After solidification, the plugs were transferred to 15 ml conical tubes containing 5 ml of cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) and 0.5 mg/ml proteinase K. The plugs were lysed in a 55°C hybridization incubator for 2 h. After lysis, the plugs were washed twice with distilled water, and four times with TE buffer, for 15 min per wash at 55°C hybridization. After washing, the plugs were digested with 50 U of *Xba*I (TaKaRa, Japan) at 37°C for 4 h. The digested plugs were loaded into appropriate wells in a 1% SKG gel. The gel was electrophoresed using a CHEF-MAPPER (Bio-Rad Laboratories) with pulse times of 2–30 sec at 14°C for 18 h in 0.5× tris-borate EDTA (TBE) buffer at 6 V/cm. Then the gels were stained with ethium bromide, and photographed using the Gel Doc XR system (Bio-Rad Laboratories). Gel images were analyzed using GelCompar II software (Applied Maths, Belgium). PFGE dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA) analysis based on Dice coefficients.

Table 2. **The distributions of antimicrobials resistances in phenotypes and genotypes of *E. coli* isolated from South Korean cattle farms**

Antimicrobial class	Antimicrobials (No. of isolates)	Resistance gene (No. of isolates)
Aminoglycosides	Gentamicin (42) Neomycin (117) Streptomycin (183)	*aac(3)-IV* (96) *aadA* (57) *aadB* (0) *aphA1* (62) *aphA2* (0) *strA-strB* (113)
β-Lactams	Ampicillin (86) Amoxicillin (87) Cephalothin (95) Ceftiofur (1) Ceftazidime (3)	*ampC* (287) bla_{TEM} (69) bla_{OXA} (0) bla_{SHV} (0)
Phenicols	Chloramphenicol (52) Florfenicol (21)	*cat* (22) *cmlA* (12) *floR* (22)
Sulfonamide	Sulphamethoxazole/ trimethoprim (48)	*sul1* (64) *sul2* (97) *sul3* (11)
Tetracycline	Tetracycline (158)	*tet*(A) (80) *tet*(B) (78) *tet*(C) (65) *tet*(D) (0) *tet*(E) (232) *tet*(G) (0)
Fluoroquinolones	Ciprofloxacin (29) Enrofloxacin (32)	ND
Others	Nalidixic acid (55)	ND

[a] ND, not determined

Results

Antimicrobial susceptibility

A total of 205 isolates were resistant to one or more antimicrobials. The prevalence of antimicrobial resistances was as follows: streptomycin (63.1%), tetracycline (54.5%), neomycin (40.3%), cephalothin (32.8%), amoxicillin (30.0%), ampicillin (29.7%), nalidixic acid (19.0%), chloramphenicol (17.9%), sulphamethoxazole/trimethoprim (16.6%), gentamicin (14.5%), enrofloxacin (11.0%), ciprofloxacin (10.0%), florfenicol (7.2%), ceftiofur (1.0%), and ceftazidime (1.0%) (Table 2). Two isolates showed resistance against 13 antimicrobials. Among all the isolates, 116 (40.0%) showed resistance against more than three different classes of antimicrobials. The most frequent multidrug-resistance patterns were [ampicillin-amoxicillin-neomycin-streptomycin-tetracycline] and [ampicillin-amoxicillin-cephalothin-gentamicin-neomycin-streptomycin-tetracycline], both detected in eight of the total 116 multidrug-resistant isolates (6.9%) (Table 3).

Prevalence of virulence genes

A total of 290 *E. coli* isolates were analyzed by m-PCR. As shown in Table 4, 147 (50.7%) isolates carried more than one virulence factor. The most prevalent virulence gene was *f17* (26.2%). No other virulence gene was detected in the *f17*-positive isolates. The frequencies of virulence genes detected by m-PCR were as follows: *stx2* (15.9%), *ehxA* (11.0%), *stx1* (8.3%), *eae* (5.2%), and *sta* (4.1%). None of the strains carried *f5*, *f41* or *lt*. Of 55 *stx*-positive isolates, 9 (16.4%) carried *stx1*, while the *stx2* gene was detected in 31 (56.4%) of the isolates. Fifteen (27.3%) isolates carried both *stx1* and *stx2*. Also, the *eae* and *ehxA* genes were detected in 10 (17.5%) and 15 (26.3%) isolates, respectively, of the *stx* gene positive isolates.

Table 3. **Multidrug-resistance patterns of *E. coli* isolates from South Korean cattle farms**

Resistance profile	No. of resistant antimicrobials	No. of strains (n=116)
AmpAmlNSTe	5	8 (6.9%)
AmpAmlKfCnNSTe	7	8 (6.9%)
KfNaSTe	4	6 (5.2%)
AmpAmlNSTeSxt	6	6 (5.2%)
AmpAmlKfCCipEnrNaCnNSTeSxt	12	6 (5.2%)
AmpAmlKfCCipEnrNaNSTeSxt	11	5 (4.3%)
KfNSTe	4	4 (3.4%)
KfCnNSTe	5	4 (3.4%)
KfSTe	3	3 (3.4%)

Amp, ampicillin; Aml, amoxicillin; S, streptomycin; N, neomycin; Cn, gentamycin; Kf, cephalothin; Enr, enrofloxacin; Cip, ciprofloxacin; Te, tetracycline; Na, nalidixic acid; Sxt, sulfamethoxazole/trimethoprim.

Table 4. The distribution of virulence genes of *E. coli* isolated from South Korean cattle farms

No. of isolates (%)	No. of genes[a]	Virulence gene								
		f5	*f41*	*f17*	*stx1*	*stx2*	*sta*	*lt*	*eae*	*ehxA*
1 (0.3)	4				+	+			+	+
6 (2.1)					+				+	+
2 (0.7)	3					+			+	+
5 (1.7)					+	+				+
9 (3.1)					+	+				
2 (0.7)					+		+			
2 (0.7)						+	+			
1 (0.3)						+			+	
6 (2.1)						+				+
5 (1.7)									+	+
4 (1.4)							+			+
76 (26.2)				+						
1 (0.3)					+					
20 (6.9)	1					+				
4 (1.4)							+			
3 (1.0)										+
143 (50.0)	0									
Total[b] (%) 290		0 (0)	0 (0)	76 (26.2)	24 (8.3)	46 (15.9)	12 (4.1)	0 (0)	15 (5.2)	32 (11.0)

[a] Number of the virulence genes
[b] Sum of each virulence genes

Prevalence of antimicrobial resistance genes

From twenty-two resistance genes investigated in the 290 *E. coli* isolates, sixteen were detected. Genes *aphA2*, *aadB*, bla_{OXA}, bla_{SHV}, *tet*(D), and *tet*(G) were not detected in any *E. coli* isolates. A total of 278 isolates (95.9%) carried more than one resistance gene. The prevalence of antimicrobial resistant genes is shown in Table 2. Regarding aminoglycoside resistance, the most prevalent gene was *strA-strB* (39.0%), followed by *aac(3)-IV* (33.1%), *aphA1* (21.4%), and *aadA* (19.7%). The predominant β-lactamase gene was bla_{TEM} (23.8%). Among the ampicillin/amoxicillin resistant isolates, the bla_{TEM} gene was identified in 72.1% (62/86). Additionally the *ampC* was detected in 287 isolates (99.0%). The determinants for phenicol resistance were identified as follows: *cat* gene (7.6%), *floR* gene (7.6%), and *cmlA* gene (4.1%). The *floR* gene encoding chloramphenicol/florfenicol efflux pump was detected in 90.5% (19/21) of the florfenicol resistant isolates. Sulfonamide resistance was attributable to *sul2* genes in 33.4% at the following frequencies: *sul1* (22.1%) and *sul3* (3.8%). The most prevalent tetracycline resistant gene was *tet*(E) (80.0%), followed by *tet*(A) (27.6%), *tet*(B) (26.9%), and *tet*(C) (22.4%). Among the tetracycline resistant isolates, 97.5% of isolates (154/158) carried *tet* genes. On the oher hand, 80.3% (106/132) of tetracycline susceptible isolates were positive for *tet* genes.

Table 5. The distribution of O serotypes of *E. coli* isolated from South Korean cattle farms

O serogroup	No. of isolates (%)	O serogroup	No. of isolates
O7	12 (22.6)	O140	1 (1.9)
O101	7 (13.2)	O157	1 (1.9)
O15	4 (7.5)	O165	1 (1.9)
O9	4 (7.5)	O168	1 (1.9)
O136	2 (3.8)	O174	1 (1.9)
O2	2 (3.8)	O182	1 (1.9)
O8	2 (3.8)	O26	1 (1.9)
O88	2 (3.8)	O3	1 (1.9)
O1	1 (1.9)	O45	1 (1.9)
O109	1 (1.9)	ND[a]	6 (11.3)
O14	1 (1.9)		

[a] ND, not determined

Serogroup determination

Overall, 47 of 53 isolates examined were serotyped, and belonged to 20 different O serogroups, while six were nontypeable. Only one isolate was identified as O157. The majority of isolates (62.3%) were classified in four serogroups, including O7 (22.6%), O101 (13.2%), O15 (7.5%), and O9 (7.5%) (Table 5).

Molecular subtyping of *E. coli* isolates using PFGE

PFGE of *Xba*I-digested chromosomal DNA of the 53 serotyped *E. coli* isolates showed 93 different PFGE subtype patterns, with 14–26 discernible bands, ranging from 30 to 600 kb in molecular size (Fig. 1). The similarity of PFGE profiles was 46.0%, with *Xba*I as analyzed by the Dice coefficient. However, these PFGE subtypes could be clustered into 26 groups of closely related PFGE subtypes, with more than 60% similarity by the Dice coefficient. 1 to 13 isolates were contained within each group. Although the PFGE profiling analyzed in this study showed a high degree of polymorphism, subgroup 14 showed high similarity (Dice coefficient similarity > 75%).

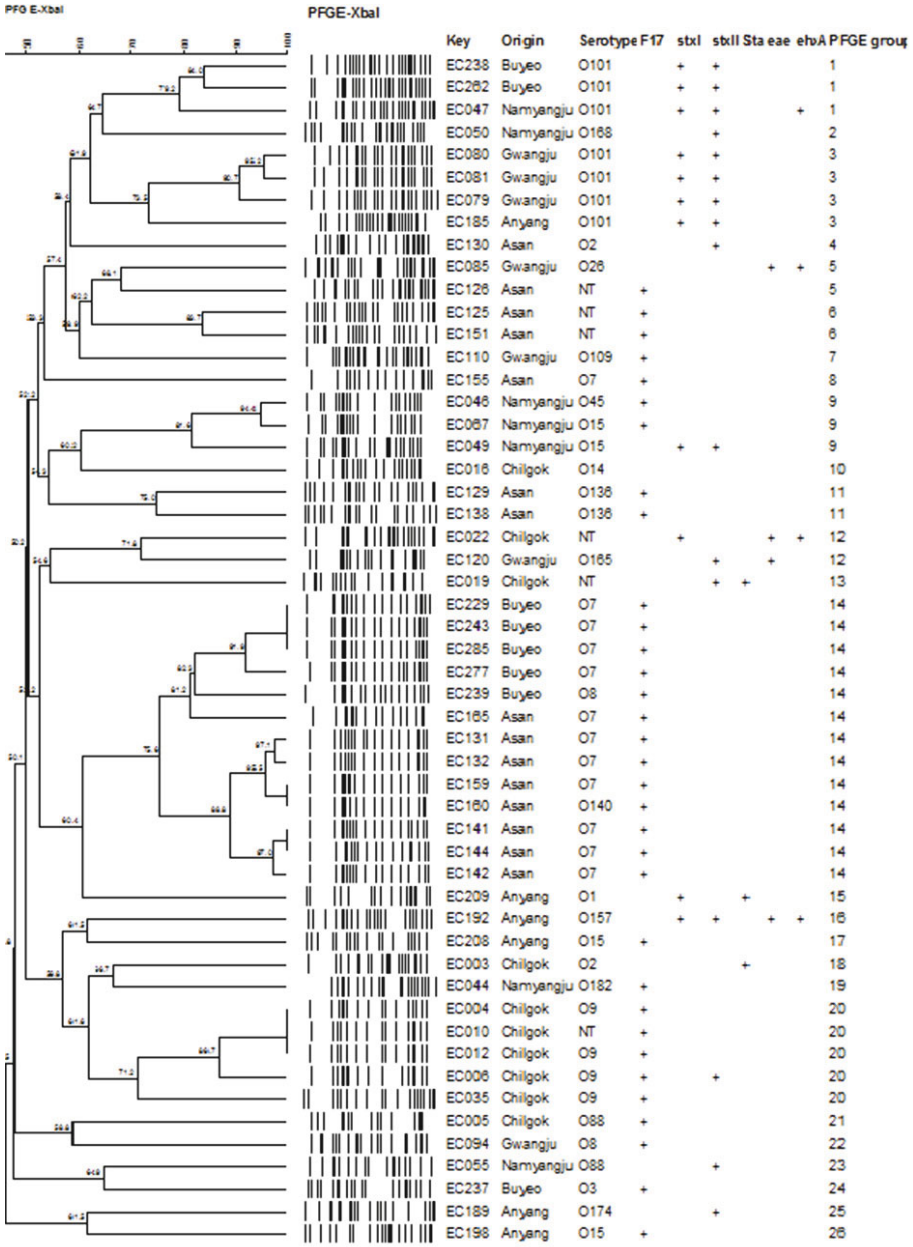

Fig. 1. ***Xba*I-PFGE dendrogram showing the prevalence of the 53 *E. coli* isolates from South Korean cattle farms.**

Discussion

In the present study, we investigated the prevalence of virulence factors and antimicrobial resistance to identify the distribution of the potentially pathogenic *E. coli* strains in cattle farms, South Korea.

Most *E. coli* isolates displayed resistance to antimicrobials and a high number was also multi-resistant. Resistance to streptomycin, tetracycline, neomycin, cephalothin, amoxillin, and ampicillin was commonly detected in this study, while *E. coli* strains showed susceptibility against antimicrobial drugs, such as the fluoroquinolones and third-generation cephalosporins showed high susceptibility. According to the data provided by the Korea Animal Health Products Association (KAHPA), since the use of antimicrobials as feed supplements was banned by the government in July 2011, the quantity of antimicrobials used therapeutically is showing a growing trend. Therefore, it is necessary to choose the efficient antimicrobials for the reduction of them. Our results can be helpful for the antimicrobial selection.

ETEC strains are the most important agent causing diarrhea in cattle (Nagy and Fekete, 1999). Therefore, for the detection of ETEC, we screened the genes of fimbriae (F5, F41, and F17) and enterotoxins (LT and STa). As shown in the results, we found none of the *f5*, *f41*, and *lt* genes, and a relatively low percentage (4.14%) of *sta* genes. We identified that 75 (25.9%) of a total of 290 *E. coli* isolates carried the gene for F17 fimbriae, although these isolates were neg-

ative for other virulence genes. These results are in agreement with those of a previous study (Ghanbarpour and Oswald, 2009), which showed a greater prevalence of F17 fimbriae than of F5 and F41. The predominant distribution of F17 fimbriae may result from the environment and/or from genetic pressures on the *E. coli* isolates, such as extensive vaccination against the F5 and F41 fimbriae (Moon and Bunn, 1993). From this reason, the F17 fimbrial antigen has emerged, so it resulted in a high prevalence of *E. coli* being isolated from healthy cattle (Moon and Bunn, 1993).

Over the past three decades, studies on STEC infection in cattle have been carried out and have demonstrated that the prevalence of non-O157 STEC in cattle may vary according to the country (Hussein, 2007). The different prevalence rates of STEC might be due to patterns of shedding STEC, influenced by several factors, such as geographic differences, sampling and detection methods, age of host and seasonal variations (Menrath *et al.*, 2010). Although the results in this study is not enough to reflect true differences in prevalence rate, we could predict that STEC is widely distributed in South Korean cattle farms.

When analyzed by m-PCR, 46 STEC isolates (15.9%) harbored the *stx2* gene, 24 isolates carried (8.3%) *stx1* and 15 isolates (5.2%) had both genes. This result differed from a previous report (Bergamini *et al.*, 2007) that showed the dominance of the *stx1* gene in cattle. However, other studies have shown agreement with our study (Zschöck *et al.*, 2000). Based on epidemiologic data indicating the significance of the Stx2 toxin in the development of HUS (Bonnet *et al.*, 1998), the dominance of STEC strains carrying *stx2* gene in cattle might cause a serious risk to public health.

The intimin encoded by the chromosomal gene *eae* may be necessary for the development of the virulence of STEC, providing them with attaching and effacing activity (Jerse *et al.*, 1990). Several authors have described the significant association between the presence of the *eae* gene and the pathogenicity of STEC in causing severe diarrhea (HC) and disease (HUS) (Mainil *et al.* 1993). The presence of the *eae* gene was detected in 17.5% (10/55) of the STEC isolates, including one O157 bovine isolate in the present study. The percentage of *eae*-positive STEC in this study was higher than that found in healthy cattle (Blanco *et al.*, 1997) or in diarrheic calves (Nguyen *et al.*, 2011).

Enterohemolysin is widespread among STEC strains isolated from calves (Aidar-Ugrinovich *et al.*, 2007). It has been suggested that this virulence factor probably synergizes the effects of the Shiga toxin, and that it can be used as a diagnostic indicator because the presence of the *ehxA* gene is highly associated with the Shiga toxin (Beutin *et al.*, 1989). The prevalence of enterohemolysin among bovine STEC strains has been reported by several authors (Beutin *et al.*, 1989; Wieler *et al.*, 1992; Aidar-Ugrinovich *et al.*, 2007), with the prevalence of STEC ranging from 51.0% to 70.8%. About 62.5% of STEC isolates harbored the *ehxA* gene in our study, showing good agreement with these studies.

In the *strA-strB* determinants encoding enzymes required for streptomycin resistance (Chiou and Jones, 1995), the detection rates from our study were lower than in a previous report that described these determinants as being common in *E. coli* isolates resistant to aminoglycoside compounds (Karczmarczyk *et al.*, 2011b). Notably, among the *E. coli* isolates resistant to gentamicin, none of isolates harbored the *aadB* determinant conferring resistance to gentamicin, tobramycin and kanamycin. Therefore it might be suggested that resistance to gentamicin in this study resulted from the presence of the *aac(3)-IV* gene, mediating a broad spectrum aminoglycosides resistance that included gentamicin (Vinué *et al.*, 2010). It is interesting to note that resistance genes were largely found in isolates identified as susceptible by phenotype, and this could result from the existence of defective genes, and a reduced expression of these determinants (Karczmarczyk *et al.*, 2011b).

Only bla_{TEM} was detected as a β-lactamase gene in this study, which agreed with previous reports (Karczmarczyk *et al.*, 2011b; Wedley *et al.*, 2011). The prevalence of genes encoding amoxicillin- and ampicillin-resistance was lower than those of the phenotype to the antimicrobials in our study. This phenomenon may be attributed to the other β-lactamase genes not investigated in this study such as CTX-M or the over-expression of AmpC enzyme by mutations (Bergström and Normark, 1979; Tracz *et al.*, 2007).

Although the use of chloramphenicol in food-animals was banned in South Korea, the resistance to chloramphenicol was analyzed and was still identified with a relatively high percentage in our data. It could be resulted from the co-resistance to florfenicol by its similarity of molecular structure. The persistence of chloramphenicol has been reported by other authors (Kang *et al.*, 2005; Lim *et al.*, 2007). The persistence of chloramphenicol resistance could result from the presence of *floR* gene. This gene encodes a specific exporter for both chloramphenicol and florfenicol. Gene *cmlA* codify an exporter specific for chloramphenicol, while the *cat* gene codify for the enzymatic inactivation from chloramphenicol. The data on *sul2* and SXT-positive isolates of our study was consistent with the previous study (Enne *et al.*, 2001), which showed that *sul2* was the most prevalent mechanism for resistance to sulfonamides. Also most *sul2*-positive strains (94.8%) also showed positive association with *strA-strB* gene (Boerlin *et al.*, 2005). The *tet*(E) gene was the predominant tetracycline resistance determinant, detected in 80.0% of isolates, followed by *tet*(A) (27.6%), *tet*(B) (26.9%), and *tet*(C) (22.4%). These results showed a difference in relation to the previous study (Medina *et al.*, 2011), which showed the dominance of *tet*(A) and *tet*(B) determinants in tetracycline resistance. The difference might be due to the origin of the *tet*(E) gene from the environmental *Aeromonas* strains (Marshall *et al.*, 1986).

In the O serotyping of the 47 *E. coli* isolates, 21 serogroups were identified. These results showed similarity with a previous study showing 25 different O serogroups in healthy cattle (Kobayashi *et al.* 2001), even though there were differences in the diversity of the O serogroup distribution, which may be attributed to environmental differences, such as diet, antimicrobials used, sampling period and method, geographical difference, etc (Bettelheim *et al.*, 2005). A total of 31.9% of the isolates serotyped were identified as STEC strains, and 2 of 21 O serogroups (O157 and O174) belonged to the major bovine STEC O groups (Blanco *et al.*, 1993).

A genetic comparison of some isolates was carried out us-

ing PFGE in order to understand the correlation between virulence genes, antimicrobial resistance, O serotypes, and regional distribution. Our PFGE analysis showed high diversity. Only 4 PFGE patterns were observed more than once. However, isolates that showed the same PFGE pattern shared similarity in the prevalence of serogroups, virulence genes, antimicrobial susceptibility and antimicrobial resistant genes. But there was no isolate that showed co-identity in genotypes and phenotypes. As shown by subgroups 1 and 3, the isolates displaying serotype O101 had common virulence factors, Stx1 and Stx2. But the regional relationship was not found in these subgroups. On the other hand, *E. coli* isolates belonged to subgroup 14 was determined to share the same serogroup (O7) except two isolates and the same virulence factor (F17). In the view of regional respect, the isolates in subgroup 14 were originated from closed districts, Buyeo and Asan. However, despite this result, our analysis indicates the existence of diverse strains of *E. coli* in regards to virulence factors, antimicrobials resistance, and O serotypes in South Korean cattle farms.

Our results suggest that diverse determinants of virulence and antimicrobial resistance of *E. coli* are widespread in *E. coli* strains isolated from cattle farms in South Korea. Moreover, these determinants can disseminate into non-pathogenic *E. coli* isolates according pathogenicity to them. This suggests that the normal flora of cattle could be a significant reservoir of diverse virulence and antimicrobial resistance determinants, which is potentially threatening to public health. Therefore, surveillance of virulence and antimicrobial resistance in healthy cattle and their transfer mechanisms need to be pursued in further studies.

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