J. Vet. Sci. (2005), **6**(3), 201–205

JOURNAL OF
Veterinary
Science

Changes in patterns of antimicrobial susceptibility and class 1 integron carriage among *Escherichia coli* isolates

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The worldwide use of antimicrobials in different fields has created enormous pressure for the selection of resistance among opportunistic bacterial pathogen. One hundred four *E. coli* isolates were collected and identified from swine with diarrhea in Korea during the period of 2002. The isolates showed highly resistant to streptomycin (99.0%), tetracycline (97.1%), neomycin (91.3%) and carbenicillin (84.6%) in antimicrobial susceptibility test. Moreover, all of the isolates showed multiple antimicrobial resistant to more than 3, and 85% of them were resistant to more than 7 of total 14 antimicrobial agents. In comparison with isolates in 1998, resistance to antimicrobials was more frequent among the isolates in 2002. Presence of class 1 integrons was investigated through amplification of the gene with PCR, and could be classified 8 groups by pattern of 4 different amplicons. Class 1 integrons were observed in 67 strains (64.2%) of *E. coli* from swine in Korea. One and 1.6 kbp of amplicons were revealed to contain *aadA1* and *aadB-aadA1* gene cassettes respectively. Two kbp of amplicon had three different gene cassettes, *dhfrXII-orfF-aadA2*, and 3.0 kbp of amplicon includes *aadB-cmlA1* gene cassettes.

Key words: class 1 integron, *E. coli*, multiple antimicrobial resistance, swine

Introduction

Coilbacillosis caused by *Escherichia coli* occur primarily in young animals and typically involve septicaemia and/or mild to severe diarrhea. Diarrhea syndrome attribute to *E. coli* infection in neonatal has become one of the most trouble diseases of livestock in Korea. The economic losses occurred by this has been increasing as no appropriate control [7,13,18,19,26]. Antimicrobial agents are often used without any laboratory assessment in the prevention and treatment of the infection, resulting in the emergence of antibiotic resistant strains. Excessive use of antibiotics in the treatment of animal diseases and large scale administration in the form of feed additives in pig industry could be responsible for free desemination of multiple drug resistance among *E. coli* isolates. Nowadays, it is difficult to control the disease using antimicrobial agents owing to emergence of new or multiple antimicrobial resistance [5,11,31].

The multiple antimicrobial resistance may arise from many different genetic determinants and each of them may present specific epidemiological features. Therefore, the assessment of the resistance situation at the genetic level would be important to understand and control antimicrobial resistance in general [21]. Integrons are known to be a new mechanism for spreading genes of resistance among Gram negative bacteria and act as natural expression vectors supplying a common promoter to a mobile gene cassette containing various antibiotic-resistance genes. The essential components of the integron are found within the 5'-conserved segment of the element and include an integrase gene *intI*, which encodes a site-specific recombinase, an adjacent site *attI*, which is recognized by the integrase and acts as a receptor for gene cassettes, and a common promoter region P_{ant}, from which integrated gene cassettes are expressed [12,20,27,28]. The horizontal transfer of integrons is considered as the most efficient means for dissemination of resistance genes and emergence of multi-resistant strains [6,14,29].

In this respect, the continuous monitoring for drug resistance of *E. coli* isolated from animals would contribute to assess future trends in the antimicrobial resistance pattern. The purpose of this study is to assess, in the first step, the actual frequency of antimicrobial resistance in pathogenic *E. coli* isolated from swine in Korea at the phenotype level. In the second, frequency of the presence of integron class 1 as resistant determinants in genotype level were identified.

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Materials and Methods

Bacterial isolates

The present study included 104 bacterial isolates obtained from pigs with diarrhea in Korea during the period of 2002. The bacteria were isolated by directly streaking with a cotton swab onto blood agar and MacConkey agar. The isolates were cultured at 37°C overnight and then identified on the basis of Gram-staining, convetional biochemical tests including oxidase and catalse test, and Vitek system (BioMérieux-Vitek, USA). Once identified, the isolates were preserved at −70°C in TSB broth containing 20% glycerol.

Antimicrobial susceptibility testing

Fourteen antibiotics were purchased from Becton, Dickinson and Company (USA) and assayed in this study (Table 1). All bacterial samples were tested with susceptibility discs containing each antibiotic according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [8]. Briefly, preliminary tests were conducted to show that all bacterial strains were able to grow in Mueller-Hinton (MH; Difco, USA) medium. Working cultures were obtained in liquid MH medium after 24 h incubation at 37°C. Discs containing each antibiotic were then loaded on MH agar medium being spotted with each bacterial strain. The plates were then incubated for 24 h at 37°C and interpreted by measuring inhibition diameters according to the criteria recommended by CLSI. Three references strains (*E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Actinobacillus pleuropneumoniae*) were used for quality control [24,30].

Detection of class 1 integrons by PCR

All strains were tested more than once for the presence of class 1 intergron using primers 5'CS, 5'-GGCATCCAAGC AGCAAG-3' and 3'CS, 5'-AAGCAGACTTGACCTGA-3' [21]. Total DNA of *E. coli* was extracted using Genomic DNA Extraction kit (Promega, USA) following manufacture's protocol for Gram-negative bacteria. The PCR solution was composed of 10 × buffer 2 μl, dNTPs (2.5 mM) 0.4 μl, 5'CS/3'CS (10 pmol/ μl) 0.5 μl each, Taq DNA polymerase (5 U/ μl, Promega, USA) 0.2 μl, distilled water 15.4 μl and template DNA (50 ng/ μl) 1 μl. Amplification consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min 30 s and a final extension at 72°C for 5 min. Amplicons were analyzed through electrophoresis on 1.0% agarose gels, and 1 kb ladder (Takara, Japan) was used as a molecular size marker.

Sequencing of amplicons

PCR amplicons to be sequenced were purified from 1% agarose gels with QIAquick Gel Extraction kit (Qiagen, Germany), according to the manufacturer's instruction. Purified amplicons were sequenced using an automated DNA sequence (ABI PRISM 377 × L; Perkin Elmer, USA) and compared to the GenBank database of the National Center for Biotechnology Information BLAST network [3].

Results

Antimicrobial susceptibility

Results of the antimicrobial susceptibility test are summaried in Table 1. The isolates showed highly susceptible to ceftiofur (87.5%) and ampicillin (72.1%), but resistant to streptomycin (99.0%), tetracycline (97.1%), neomycin (91.3%) and carbenicillin (84.6%) in antimicrobial susceptibility test. Moreover, all of the isolates showed multiple antimicrobial resistant to more than 3, and 85% of them were resistant to more than 7 of total 14 antimicrobial agents (data not shown).

Table 1. Antimicrobial susceptibility of *Escherichia coil* isolated from swine with diarrhea

Antimicrobial drugs	Potency/disc	Number of resistant isolate (n=104)
Amikacin (AM)	10 μg	84 (80.8%)
Ampicillin (AN)	30 μg	29 (27.9%)
Chloramphenicol (C)	30 μg	58 (55.8%)
Carbenicillin (CB)	100 μg	88 (84.6%)
Ceftiofur (XNL)	30 μg	13 (12.5%)
Colistin (CL)	10 μg	50 (48.1%)
Enrofloxacin (ENR)	5 μg	59 (56.7%)
Gentamycin (GM)	10 μg	86 (82.7%)
Neomycin (N)	30 μg	95 (91.3%)
Nalidixic acid (NA)	30 μg	74 (71.2%)
Norfloxacin (NOR)	10 μg	55 (52.9%)
Streptomycin (S)	10 μg	103 (99.0%)
Sulfamethoxazole/Trimethoprim (SXT)	23.5 μg/1.25 μg	80 (76.9%)
Tetracycline (Te)	30 μg	101 (97.1%)

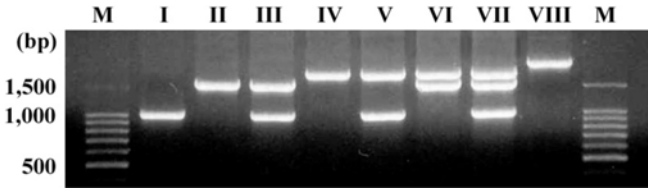

Fig. 1. Agarose gel (1.0%) electrophoresis pattern of the amplicons generated with 5'CS-3'CS primers. Lane M is 100 bp ladder as a molecular size standard. Lanes 1 to 8 represented gene groups, I to VIII, respectively.

Relationships of class 1 integrons, resistance genes, and resistance patterns

Presence of class 1 integrons was investigated through amplification of the gene with PCR (Fig. 1). Class 1 integrons were present in 67 strains (64.4%) of *E. coli* (104 strains) from swine in Korea. Using 5'CS and 3'CS, 1.0, 1.6, 2.0 and 3.0 kbp of amplicons were amplified. Presences of class 1 integrons were classified 8 groups by pattern of amplicons (Table 2). Out of 67 isolates, which have amplicons, 53.7% (36 strains) contained 1 kbp integron in the variable region. One kbp of amplicon was revealed to contain *aadA1* gene cassette encoding aminoglycoside 3'-(9)-O-adenyltransferase related to streptomycin and spectinomycin resistance. Comparing with 1.0 kbp amplicon, 1.6 kbp had additionally *aadB* gene cassette which encodes aminoglycoside 2"-adenyltransferase related to gentamycin and kanamycin resistance. Two kbp amplicon contained three different gene cassettes, *dhfrXII-orfF-aadA2*. Both of *dhfrXII* encoding dihydrofolate dehydrogenase and *orfF* encoding dihydrofolate reductase are related to trimethoprim resistance. Three kbp of amplicon holds *aadB-CmlA1* gene cassettes. *CmlA* cassette encodes chloramphenicol transporter (exporter) known as chloramphenicol resistance gene.

Dissucussion

Antimicrobial susceptibility

In comparison with isolates in 1998 [26], resistance to antimicrobials was more frequent among the isolate in 2002. Especially number of isolates resistant amikacin and colistin were dramatically increased from 4.9 to 80.8%, from 3.9 to 48.1% respectively. However, number of isolate resistant ampicillin was decreased from 76.5 to 27.9%. This phenomenon is due to change in the use of antimicrobial agents. The isolate showing resistance to ceftiofur was newly emerged in 2002. Ceftiofur is a newer broad-spectrum cephalosporin antimicrobial agent originally developed for the treatment of bovine respiratory disease in 1991. It is open used as first-line agents for invasive gram-negative infections in swine [9,30,36,37]. Nevertheless, nearly all enteric bacilli including a large number of *E. coli*, produce β-lactamases that can compromise successful β-lactam chemotherapy of *Enterobacteriaceae* infections [8,31]. The new emergency of strains resistant to ceftiofur may be explained by newly development and usage of this antimicrobial agent in Korea. Most of all isolates show resistant to tetracycline. Tetracycline resistance is frequently found in zoonotic, pathogenic and intestinal bacteria. Most consequence of the selection pressure is resulted from the extended use of tetracycline, which is used for all different food animal species [34]. The tetracycline resistance is not concerned with the integron and associated cassette. The *tet*(A) and *tet*(B) genes is occurred predominantly in the intestinal environment of food animals and/or the presence of specific conjugative plasmids [17,20,33,34].

Relationships of class 1 integrons, resistance genes, and resistance patterns

The *aadA* (*aadA1* and *aadA2*) gene cassette (97.0%) was the most frequently found resistance gene in the variable region of integrons. The similar predominant pattern has been reported in *E. coli* strains from natural habitat and clinical isolates, *Vibrio cholerae* O139, and *Salmonella enterica* serotype Gallinarum [4,11,20,21,28]. The predominance of the *aadA* suggests that this gene may either the first cassette to be acquired by an integron and/or may be more stably integrated into the integron than other gene cassette

Table 2. Relationships between amplicon size, resistantce genes, and resistance patterns found within *E. coli* isolates

Groups	Pattern of amplicon	Resistance gene	Type of resistance	Number of isolate
I	1.0 kbp	*aadA1*	S-Te	26 (25.0%)
II	1.6 kbp	*aadB-aadA1*	GM-S-SXT-Te	12 (11.5%)
III	1.0, 1.6 kbp	*aadA1, aadB-aadA1*	AM-CB-ENR-GM-NA-NOR-S-SXT-Te	3 (2.9%)
IV	2.0 kbp	*dhfrXII-orfF-aadA2*	S-SXT-Te	14 (13.5%)
V	1.0, 2.0 kbp	*aadA1, dhfrXII-orfF-aadA2*	N-NA-NOR-S-SXT-Te	5 (4.8%)
VI	1.6, 2.0 kbp	*aadB-aadA1, dhfrXII-orfF-aadA2*	C-GM-N-NA-S-SXT-Te	3 (2.9%)
VII	1.0, 1.6, 2.0 kbp	*aadA1, aadB-aadA1, dhfrXII-orfF-aadA2*	AM-CB-CL-ENR-GM-N-NA-NOR-S-SXT-Te	2 (1.9%)
VIII	3.0 kbp	*aadB-CmlA1*	C-GM-N-NA-S-SXT-Te	2 (1.9%)
	No Amplicon		S-Te	37 (35.6%)

*Abbreviation of antimicrobial agents is same as Table 1.

[28]. In addition, both the selection and dispersion of *aadA* genes in integrons could be related to the extensive use of streptomycin in the control of animal diseases. The clinical isolated *E. coli* resistant to gentamycin have been increased from 18.3% in 1992 [7] to 82.7% in 2002. The acquisition of the *aadB* gene cassette by integron could be responsible for the observed increase of resistance phenotype during last decade. The *dhfrXII-orfF-aadA2* genes for the majority of resistant dihydrofolate reductase occur as gene cassettes that are site specificially inserted into the recombinationally active site of class 1 and class2 integrons. The high incidence of trimethoprim resistance gene cassettes inserted into class 1 integrons [2,16]. The *dhfrXII* gene cassette with *aadA2*, *dhfrXII-orfF-aadA2* also, was reported in urinary tract pathogenic *E. coli* and *Shigella* strains [15]. *AadA2* gene encoding aminoglycoside 3'-adenyltransferase, as well as *aadA1*, is one of the six genetic subtypes of *aadA,* which show streptomycin and spectinomycin resistance [20,32]. The *CmlA* gene confers nonenzymatic resistance to chloramphenicol and functions as a drug efflux pump [5,35]. Chloramphenicol is a broad-spectrum antibiotic that was used extensively in veterinary medicine until Food and Drug Administration ban its use in food animals in the 1980s [10]. Thus, our result of 55.8% rate of resistance to chloramphenicol is an unexpected, but it is very similar to the report of 53% rate in beta-hemolytic *E. coli* associated with diarrhea in neonatal swine [5]. They and some other European researchers indicated that antimicrobial resistance can persist, as a concequence of coselection with other antimicrobials [1,25].

Integrons seem to play a major role in the epidemiology of resistance to these antimicrobial agents in clinical isolates from animals. However, we observed some isolates showing resistant to several antimicrobial agents in susceptible test, which probably have other resistant gene cassettes. These resistances may not be acquired by integron, but the other vehicles to transport it into the isolates, such as resistant plasmid, bacteriophages, or transposons [6,23].

Acknowledgments

This work was supported by Brain Korea 21 Project, Korea.

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