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Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea

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Objectives: Antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea were characterized.

Methods: *E. coli* isolates were examined for susceptibility to antimicrobial agents. Integrase genes were amplified. Gene cassette regions for classes 1 and 2 integrons were amplified and sequenced. Conjugal transfer and Southern hybridization were performed to determine the genetic localization of class 1 integrons. The clonal relationship of *E. coli* isolates carrying an identical cassette array was analysed by PFGE.

Results: Commensal *E. coli* isolates from animals were highly resistant to commonly used antimicrobial agents such as tetracycline, sulfamethoxazole, streptomycin, ampicillin and carbenicillin. Integrons were most prevalent in commensal *E. coli* isolates from poultry (44%), followed by clinical isolates from humans (33%), commensal isolates from swine (23%) and humans (13%). *dfrA17-aadA5*, *dfrA12-orfF-aadA2* and *aadA1* were found most frequently in *E. coli* isolates from humans, poultry and swine, respectively. Class 1 integrons were mostly located in conjugative plasmids. *E. coli* isolates carrying an identical cassette array were phylogenetically unrelated.

Conclusions: The use of antibiotics is strongly associated with antimicrobial resistance. *E. coli* isolates from different sources may select a specific gene cassette by antibiotic selective pressure, which results in differences in class 1 integrons. The horizontal transfer of class 1 integrons through conjugative plasmids seems to be responsible for wide dissemination of a particular type of class 1 integron.

Keywords: antibiotics, gene cassettes, plasmids

Introduction

Escherichia coli isolates from clinical specimens may be resistant to multiple antimicrobial agents[1–3] and a substantial proportion of multiresistant *E. coli* isolates carry integrons.[4] Commensal *E. coli* isolates from humans and animals can cause extra-intestinal diseases, including urinary tract infection, pneumonia, meningitis and bacteraemia.[5] These bacterial strains are a potential reservoir for antimicrobial resistance genes and play an important role in the ecology of antimicrobial resistance of bacterial populations. In addition, enteric faecal flora from food-producing animals such as poultry and swine can transfer antimicrobial resistance to human pathogens via the food chain.

Since many gene cassettes of integrons contain antimicrobial resistance genes in Gram-negative bacteria, the horizontal transfer of integrons through plasmids and transposons has been

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found to play an important role in the dissemination of antimicrobial resistance genes and the development of multiresistance.[6] Three classes of integrons that encode antimicrobial resistance have been described. Class 1 integrons are most commonly found in clinical isolates of Gram-negative bacteria and more than 60 distinct gene cassettes have been identified.[7] In our previous studies, class 1 integrons were detected in 54% of urinary *E. coli* isolates, and the presence of integrons was strongly associated with multiresistance.[8]

Apart from being carried by clinical pathogens, few studies have reported the prevalence of integrons and gene cassettes in the enteric faecal flora of humans and animals.[9,10] The transfer of integrons between human and animal isolates is not fully understood, although it is known that resistance to antimicrobial agents is transferred from animals to humans through transferable plasmids.[11] The aim of the current study was to characterize antimicrobial susceptibility and integrons found in *E. coli* isolates from humans and animals. The study was also to determine if there was any possible transfer of antimicrobial resistance between *E. coli* isolates from humans and animals.

Materials and methods

Bacterial strains

A total of 664 *E. coli* isolates were obtained from four sources: clinical specimens from hospitalized patients at Kyungpook National University Hospital ($n = 201$), the stools of healthy humans (Kyungpook National University students, $n = 167$), large intestine swabs from poultry ($n = 163$) and swine ($n = 133$) grown in Kyungpook province during 2001–2003.

The isolates from poultry and swine were obtained from their respective slaughterhouses. The animals from which *E. coli* isolates were obtained consumed feed containing antibiotics. The patients and students from whom isolates were obtained consumed the animal products from these slaughterhouses.

Antimicrobial susceptibility testing

The MICs were determined by agar dilution in a Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA) according to the guidelines of the NCCLS.[12] The inoculated plates were incubated at 37°C for 20 h, and the MIC defined as the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Antimicrobial resistance was determined according to their MICs of ampicillin (≥ 32 mg/L), carbenicillin (≥ 64 mg/L), piperacillin (≥ 128 mg/L), cefazolin (≥ 32 mg/L), cefoxitin (≥ 32 mg/L), ceftazidime (≥ 32 mg/L), streptomycin (≥ 64 m/L), kanamycin (≥ 64 mg/L), gentamicin (≥ 16 mg/L), tobramycin (≥ 16 mg/L), sulfamethoxazole (≥ 512 mg/L), trimethoprim (≥ 16 mg/L), ciprofloxacin (≥ 4 mg/L), chloramphenicol (≥ 32 mg/L) and tetracycline (≥ 16 mg/L).

Conjugal transfer of plasmids carrying integrons

E. coli RG488 Rif[r] and RG176 Nal[r] were used as the recipients for the conjugation experiment.[13] Prior to each experiment for conjugation, a single colony from a MacConkey agar plate (Difco Laboratories) was inoculated in trypticase soy broth (TSB, Difco Laboratories) and grown at 37°C for 20 h. The donor and recipient strains in a logarithmic phase were grown in TSB, mixed and incubated at 37°C for 20 h. The transconjugants were selected on Mueller–Hinton agar supplemented with trimethoprim (50 mg/L) or streptomycin (50 mg/L), and rifampicin (50 mg/L) or nalidixic acid (50 mg/L).

Plasmid DNA isolation and Southern hybridization

The plasmid DNA was isolated using the alkaline extraction method.[14] The extracted DNA was separated by electrophoresis in 0.7% agarose gel. After agarose gel electrophoresis of the plasmids, the denatured DNA was transferred onto a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) using the capillary method. For the hybridization assays, a DIG DNA labelling and detection kit (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. The purified integrase gene from the PCR products was used as the probe DNA and labelled with digoxigenin-11-dUTP according to the random labelling method.

PCR amplification of integrase genes

The conserved regions of integrase genes were amplified using the degenerate primer hep35 (5′-TGCGGGTYAARGATBTKGATTT-3′) and hep36 (5′-CARCACATGCGTRTARAT-3′), and the PCR products were further restricted using either *Rsa*I or *Hin*fI (Boehringer Mannheim) to determine the class of integrons as described previously.[15] The template was prepared by suspending a loopful of each isolate, which had been growing on a trypticase soy agar (Difco Laboratories) plate, in 200 μL of sterile water, followed by boiling for 10 min and centrifuging for 5 min.

Amplification and sequencing of gene cassette regions

The gene cassette regions for the class 1 integrons were amplified using hep58 (5′-TCATGGCTTGTTATGACTGT-3′) and hep59 (5′-GTAGGGCTTATTATGCACGC-3′) as described previously.[15,16] The gene cassette regions for the class 2 integrons were amplified using hep74 (5′-CGGGATCCCGGACGGCATGCACGATTTGTA-3′) and hep51 (5′-GATGCCATCGCAAGTACGAG-3′). To determine identical arrays of gene cassettes, same-sized amplicons were digested with *Rsa*I and *Hin*fI (Boehringer Mannheim). The PCR products of the gene cassette regions that showed different restriction patterns were ligated with a pGEM T-easy vector (Promega, Madison, WI, USA) and transformed to *E. coli* DH5α cells. Sequencing reactions were performed with a double-stranded plasmid preparation using dideoxy chain termination with T7 and Sp6 primers.

PFGE

The genomic DNA was digested with *Xba*I (Boehringer Mannheim) for 18 h and separated on a 1.0% agarose gel using a contour-clamped homogeneous-field apparatus (CHEF DRIII systems; Bio-Rad Laboratories, Hercules, CA, USA) in a $0.5 \times$ TBE buffer.[17] The conditions for electrophoresis were 6 V/cm for 20 h with an increasing pulse time of 5–40 s. A λ DNA ladder consisting of 48.5 kb concatemers (Bio-Rad Laboratories) was used as the size standard. The PFGE patterns were interpreted using the criteria established by Tenover *et al.*[18]

Table 1. Antimicrobial resistance of *E. coli* isolates from humans and animals

	No. (%) of isolates resistant to antimicrobial agents			
Antibiotics	clinical specimens from humans	stool from healthy humans	large intestine of poultry	large intestine of swine
Ampicillin	183 (91.0)	73 (43.7)	104 (63.8)	100 (75.2)
Carbenicillin	183 (91.0)	6 (3.6)	103 (63.2)	99 (74.4)
Piperacillin	98 (48.8)	28 (16.8)	69 (42.3)	35 (26.3)
Cefazolin	66 (32.8)	1 (0.6)	1 (0.6)	0 (0)
Cefoxitin	29 (14.4)	0 (0)	1 (0.6)	0 (0)
Ceftazidime	20 (10.0)	0 (0)	0 (0)	0 (0)
Streptomycin	179 (89.1)	61 (36.5)	141 (86.5)	114 (85.7)
Kanamycin	71 (35.3)	6 (3.6)	37 (22.7)	70 (52.6)
Gentamicin	67 (33.3)	3 (1.8)	37 (22.7)	48 (36.1)
Tobramycin	50 (24.9)	3 (1.8)	26 (16.0)	43 (32.3)
Sulfamethoxazole	186 (92.5)	44 (26.3)	150 (92.0)	111 (83.5)
Trimethoprim	126 (62.7)	25 (15.0)	114 (69.9)	52 (59.1)
Ciprofloxacin	66 (32.8)	2 (1.2)	83 (50.1)	10 (7.5)
Chloramphenicol	62 (30.8)	17 (10.2)	86 (52.8)	66 (49.6)
Tetracycline	137 (68.2)	66 (39.5)	131 (80.4)	132 (99.2)
Total	201	167	163	133

Results

Antimicrobial susceptibility of E. coli isolates from humans and animals

Of the 368 *E. coli* isolates of human origin, the overall rates of resistance to antimicrobial agents were higher in clinical *E. coli* isolates than in commensal *E. coli* isolates (Table 1). Fifty-five (32.9%) commensal *E. coli* isolates were all susceptible to antimicrobial agents tested, whereas three (1.5%) clinical isolates were all susceptible. The second or third generation of cephalosporins such as cefoxitin, cefuroxime, cefotaxime, ceftizoxime, ceftriaxone, cefoperazone and ceftazidime have been used to treat Gram-negative bacterial infections in humans in Korea. Only one commensal *E. coli* isolate was resistant to cephalosporins tested, whereas 66 (32.8%) clinical *E. coli* isolates were resistant to cefazolin.

Commensal *E. coli* isolates from animals were also characterized for antimicrobial susceptibility. The overall rates of resistance to antimicrobial agents were higher in commensal *E. coli* isolates from animals than in commensal *E. coli* isolates from humans. Among 296 commensal *E. coli* isolates from animals, >80% of isolates were resistant to tetracycline, sulfamethoxazole and streptomycin (Table 1). Resistance to quinolones was more prevalent in the isolates from poultry than that of the isolates from swine. Only one isolate from swine was susceptible to all antimicrobial agents tested among the commensal *E. coli* isolates from animals.

Integron carriage and characterization of gene cassettes

Of the 664 *E. coli* isolates tested, 189 (28.5%) carried an integrase gene (Table 2). Integrase genes were detected in 44.2% (72/163), 32.8% (66/201), 22.6% (30/133) and 12.6% (21/167) of commensal isolates from poultry, clinical isolates from humans, commensal isolates from swine and humans, respectively. Among the 189 integrase-positive isolates, 175 (92.6%)

carried *intI1*, nine (4.8%) carried *intI2* and five (2.6%) carried both *intI1* and *intI2*. No class 3 integrons were detected.

To identify gene cassettes in integrons, each gene cassette region in classes 1 and 2 integrons was amplified. Nine and two different gene cassette arrays were found in classes 1 and 2 integrons, respectively (Table 3). Ten gene cassettes that encoded resistance to trimethoprim (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17*), aminoglycosides (*aadA1*, *aadA2* and *aadA5*), β-lactams (*blaP1*) and truncated alcohol dehydrogenase (*adhEΔ*) were detected in nine different class 1 integrons. All gene cassettes detected have been previously described in Enterobacteriaceae from clinical specimens from humans in Korea.[8] *dfrA17-aadA5*, *dfrA12-orfF-aadA2* and *aadA1* were the most prevalent in *E. coli* isolates from humans, commensal *E. coli* isolates from poultry and swine, respectively. Two different types of class 2 integrons, *dfrA1-sat-aadA1* and *sat-sat1-aadA1*, were found in *intI2*-positive isolates, but *sat-sat1-aadA1* was only detected in commensal isolates from swine.

Table 2. Frequency of integrons among *E. coli* isolates from humans and animals

		No. of isolates carrying integrons		
Source	Total	class 1	class 2	class 1 and 2
Humans				
clinical specimens	201	62 (30.8%)	2 (1.0%)	2 (1.0%)
stool from healthy humans	167	19 (11.4%)	2 (1.2%)	0
Animals				
large intestine of poultry	163	69 (42.3%)	1 (0.6%)	2 (1.2%)
large intestine of swine	133	25 (18.8%)	4 (3.0%)	1 (0.8%)

Table 3. Gene cassette arrays among _E. coli_ isolates from humans and animals

	No. of isolates carrying gene cassette				
Cassette array	clinical specimens from humans	stool from healthy humans	large intestine of poultry	large intestine of swine	Total
Class 1 integrons					
aadA1	–	2	7	15	24
aadA2	–	–	2	–	2
dfrA5	5	1	–	–	6
dfrA7	–	2	–	–	2
adhEΔ	5	–	–	–	5
blaP1-aadA1	2	–	–	–	2
dfrA1-aadA2	2	6	12	7	27
dfrA12-orfF-aadA2	22	1	49	3	75
dfrA17-aadA5	30	7	1	3	41
Class 2 integrons					
dfrA1-sat-aadA1	4	2	3	1	10
sat-sat1-aadA1	–	–	–	4	4

Genetic localization of class 1 integrons

To determine the genetic localization of class 1 integrons, 42 _E. coli_ isolates, comprising 16 isolates carrying _dfrA1-aadA2_, 14 isolates carrying _dfrA12-orfF-aadA2_ and 12 isolates carrying _dfrA17-aadA5_, were selected on the basis of isolation date and origin. Conjugation experiment, plasmid analysis and Southern hybridization with _intI1_ probe were performed. Class 1 integrons were conjugally transferred to recipients in 32 (76.2%) of the 42 isolates. The transfer rates of class 1 integrons carrying _dfrA1-aadA2_, _dfrA12-orfF-aadA2_ and _dfrA17-aadA5_ were 68.8% (11/16), 85.7% (12/14) and 75.0% (9/12), respectively. To determine the genetic localization of non-transferable class 1 integrons, Southern hybridization with an _intI1_ probe was performed, which revealed that all class 1 integrons were located in plasmids, except for two class 1 integrons carrying _dfrA1-aadA2_ and _dfrA12-orfF-aadA2_ that were located in a chromosome. The plasmid profiles of original isolates carrying identical gene cassettes were very different from each other, but _intI_ was hybridized at plasmids of similar sizes (73–85 kb) (data not shown).

Figure 1. Representative PFGE patterns of _E. coli_ isolates. Chromosomal DNA was digested with _Xba_ I. Lanes 1–5, _E. coli_ isolates carrying _dfrA1-aadA2_; lanes 6–10, _E. coli_ isolates carrying _dfrA12-orfF-aadA2_; and lanes 11–13, _E. coli_ isolates carrying _dfrA17-aadA5_.

Epidemiological typing of E. coli isolates carrying identical cassette arrays

To determine whether the high prevalence of a particular type of class 1 integron in _E. coli_ isolates from different sources was caused by the spread of a specific clone, 42 _E. coli_ isolates, which had been selected previously, were analysed by PFGE profiles. _E. coli_ isolates carrying the identical type of class 1 integrons showed distinct patterns (Figure 1). This finding suggested that the spread of a specific clone did not contribute to a wide dissemination of a particular type of class 1 integron.

Discussion

The present study demonstrated that commensal _E. coli_ isolates from animals were highly resistant to antimicrobial agents commonly used as feed additives or therapeutics. Resistance to antimicrobial agents among commensal _E. coli_ isolates from
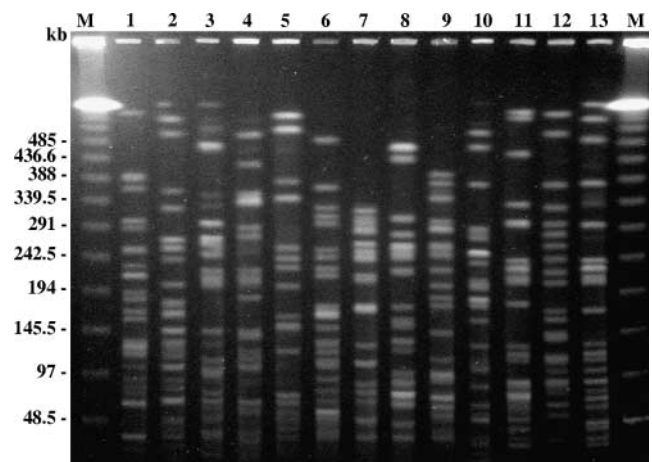
animals was higher than that of the commensal _E. coli_ isolates from humans. Approximately 1600 tons of antimicrobial agents are consumed every year as feed additives, in veterinary hospitals and pharmaceuticals for animals in Korea.[19] Half of all antimicrobial agents are used for feed additives. Tetracycline (48.3%) was the most commonly consumed during 2001–2003 in Korea, followed by sulphonamides (14.0%), penicillins (8.0%), aminoglycosides (3.4%) and others (26.3%).[19] This may reflect the high prevalence of resistance to tetracycline (88.9%), sulfamethoxazole (88.2%), streptomycin (86.1%), ampicillin (68.9%) and carbenicillin (68.2%) in commensal _E. coli_ isolates from animals. The rate of resistance to carbenicillin was high in clinical _E. coli_ isolates from humans and commensal _E. coli_ isolates from animals, although carbenicillin was rarely used for humans and animals. Amoxicillin and penicillin G were the most commonly used for animals. Resistance to ciprofloxacin was more prevalent in the isolates from poultry than in the isolates from swine. Big differences in resistance to ciprofloxacin

between commensal *E. coli* isolates from poultry and swine may be caused by the use of different antibiotics. Quinolones such as enrofloxacin and norfloxacin have been frequently used for bacterial disease control in poultry farms, but rarely used in swine farms. These findings suggest that the use of antibiotics is highly associated with the prevalence of antimicrobial resistance.

Class 1 integrons are widely distributed in *E. coli* from humans and animals and directly contribute to the resistance to trimethoprim, aminoglycosides and sulphonamides. Differences in gene cassette arrays of class 1 integrons were found in *E. coli* isolates from different sources. *dfrA17-aadA5*, *dfrA12-orfF-aadA2* and *aadA1* were the most prevalent in *E. coli* isolates from humans, poultry and swine, respectively. In our previous study, *dfrA17-aadA5* has been the most prevalent in urinary *E. coli* isolates from humans since 1994.[8,20] *dfrA1-aadA2* first appeared in clinical *E. coli* isolates from the mid-1990s and the incidence of this type of class 1 integron was maintained at <10% after 1996.[8] This type of class 1 integron was prevalent among commensal *E. coli* isolates from humans in the current study. Class 1 integrons carrying a single gene cassette such as *aadA1* were the most prevalent in commensal *E. coli* isolates from swine, whereas most of the isolates carried multigene cassettes in clinical isolates from humans. Class 1 integrons carrying single gene cassettes were prevalent in clinical *E. coli* isolates from the 1980s, whereas class 1 integrons carrying multigene cassettes were prevalent in clinical isolates from the 1990s and 2000s.[8] Moreover, the incidence of each class 1 integron in commensal *E. coli* isolates from swine and poultry was similar to that of clinical *E. coli* isolates from humans in the 1980s and 1990s, respectively.[8] Accordingly, it would seem that *E. coli* isolates from different sources selected a specific gene cassette by antibiotic selective pressure. The prevalence of class 1 integrons carrying multigene cassettes was explained by the introduction of a new gene cassette encoding resistance to antimicrobial agents commonly used.

Wide dissemination of class 1 integrons carrying identical gene cassettes was observed in *E. coli* isolates from human and animal origins. We assessed whether wide dissemination of class 1 integrons carrying identical gene cassettes was due to clonal spread or horizontal transfer of plasmids carrying class 1 integrons. *E. coli* isolates carrying identical cassette arrays showed distinct PFGE patterns, which indicated little clonal relatedness between the *E. coli* isolates. Leverstein-van Hall *et al.*[21,22] proposed that the worldwide distribution of identical gene cassettes in multiresistant Enterobacteriaceae from clinical specimens and food-producing animals was due to the transfer of class 1 integrons between isolates from animals and humans via the food chain. Three prevalent class 1 integrons were mostly located in plasmids and conjugally transferable, which suggests the horizontal transfer of class 1 integrons through conjugative plasmids. However, the plasmids of the transconjugants carrying identical cassette arrays showed different restriction fragment length polymorphism patterns with *Hin*dIII, and the *intI* probe was hybridized with different-sized fragments of plasmids (data not shown). Accordingly, we could not directly demonstrate evidence for horizontal transfer of class 1 integrons between human and animal *E. coli* isolates.

In conclusion, the use of antibiotics is strongly associated with the prevalence of antimicrobial resistance in commensal *E. coli* isolates in food-producing animals. Class 1 integrons were found to be widely disseminated among *E. coli* isolates from humans and animals. *E. coli* isolates from different sources selected a specific gene cassette by antibiotic selective pressure. The prevalence of a particular type of class 1 integron in different sources may not be directly associated with clonal spread, but may be associated with horizontal transfer of conjugative plasmids. Therefore, the sequential analysis of class 1 integrons in Enterobacteriaceae from clinical specimens, faecal flora and environmental samples can provide information regarding the evolutionary changes of gene cassettes in class 1 integrons.

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