Variable Gene Cassette Patterns of Class 1 Integron-associated Drug-resistant *Escherichia coli* in Taiwan

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This study characterized class 1 integrons in *Escherichia coli* in Taiwan. The stability and changes in gene cassettes inserted into integrons were also evaluated. The study included 436 clinical strains of *E. coli* isolated in 2002. Class 1 integrons were characterized by polymerase chain reaction and direct sequencing. Genetic localization of class 1 integrons was determined by conjugal transfer and Southern hybridization. The results indicated that 64% of *E. coli* isolates carried class 1 integrons. Molecular analysis revealed that the class 1 integrons harbored 13 different antimicrobial resistance gene cassettes and two unknown gene cassettes; the predominant cassettes were *aadA* and *dfrA*. Novel gene cassettes first recovered from *E. coli* were *aacA4* and *linF*. Cassette arrays *orfD-aacA4-catB8* and *aadA1-linF* were also observed. Gene cassette *dfrA12-orfF-aadA2* was stable. The class 1 integron and *dfrA17-aadA5* gene cassette were located on the same transferable plasmids and were capable of transmission. Therefore, the increased drug resistance of clinical isolates may be explained by antibiotic selective pressure and widespread presence of integrons. Under antibiotic selective pressure, gene cassette-mediated resistance may not be easily lost. The potential role of integrons in the uptake and dissemination of resistance genes by plasmid between species of bacteria may decrease the therapeutic effectiveness of antibiotics.

Key Words: *aadA1-linF*, conjugative plasmid, *dfrA17-aadA5*, *Escherichia coli*, *orfD-aacA4-catB8*
(*Kaohsiung J Med Sci* 2007;23:273–80)

The rapid spread of antimicrobial-resistant bacteria is an alarming and increasing problem which often complicates treatment of infections. Such complications often result from rapid dissemination of antibiotic-resistant genes carried by plasmids, transposons, and integrons [1,2]. Integrons are potentially mobile genetic elements frequently located on transposons and have been identified at loci in which site-specific incorporation and excision of gene cassettes frequently occur [3]. Most of the integrons characterized to date contain gene cassettes coded for resistance to antimicrobial agents. Since integrons can act as natural expression vectors for any gene cassettes inserted, recent studies have examined the role of integrons in the carriage and dissemination of antimicrobial resistance genes [4].

As an increasing number of bacterial isolates demonstrate resistance to a wide spectrum of antimicrobial agents [5] in both clinical and community settings, the dissemination of antimicrobial-resistant genes between bacteria is of great concern. Several studies have documented the widespread prevalence of integrons in clinical isolates [6–9]. However, the stability of these gene cassettes after insertion into integrons requires further investigation to clarify the development of resistance.

Received: August 1, 2006 Accepted: December 6, 2006
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In Taiwan, *Escherichia coli* has shown a high overall rate of resistance to commonly used "first-line" antibiotics including ampicillin, cephalothin, gentamicin, and trimethoprim/sulfamethoxazole [10]. Because of this selective pressure, it would be of interest to characterize the antimicrobial-resistant gene cassettes located on the class 1 integrons of *E. coli* isolated in 2002. Furthermore, in comparison with our previous study [11], the stability and changes in gene cassettes over a 10-year period in Taiwan were also evaluated.

Materials and Methods

Clinical isolates and antimicrobial susceptibility profiles

Four hundred and thirty-six *E. coli* strains were obtained from 436 inpatients at Kaohsiung Medical University Hospital in southern Taiwan in 2002. The *E. coli* strains were isolated from clinical specimens taken by various hospital departments, including urine ($n=233$), pus ($n=85$), blood ($n=45$), sputum ($n=25$), bile ($n=8$), genital tract samplings ($n=10$), and other samples ($n=30$). Once identified, the isolates were preserved at –70°C in Tryptic Soy Broth (Difco Laboratories, Detroit, MI, USA) containing glycerol (15%, v/v).

Antimicrobial susceptibility was analyzed by disk diffusion methods in Mueller-Hinton agar (Difco Laboratories). Results were interpreted according to CLSI criteria [12]. *E. coli* strain ATCC 25922 was used as a control strain.

Incidence of class 1 integron

The template DNA for polymerase chain reaction (PCR) were prepared as described by Bass et al [13]. Integrons were detected by PCR with primers *intI1* (F: 5′-CCTCCCGCACGATGATC-3′, R: 5′-TCCACGCATCGTCAGGC-3′), which hybridize to conserved regions of integron-encoded integrase genes *intI1* [14]. The PCR reactions were performed at 94°C for 1 minute and then for 30 cycles at 94°C for 30 seconds, 58°C for 20 seconds and 72°C for 30 seconds. Plasmid pUB2401, which harbors transposon Tn*21* containing the In2 class 1 integron, was used as control [1].

DNA sequencing

To determine the possibility of inserting of antimicrobial-resistant gene cassettes, primers specific for the 5′-conserved (5′-GGCATCCAAGCAGCAAG-3′) and 3′-conserved segment (5′-AAGCAGACTTGACCTGA-3′) were used to amplify the entire integron cassette insertion region. The PCR products were purified using the Wizard PCR kit (Promega, Madison, WI, USA) and used as templates for direct nucleotide sequencing. The Prism Ready Reaction DyeDeoxy Termination Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions. The samples were run on a 377 DNA sequencer (Applied Biosystems), and the sequences were analyzed by Sequence Navigator software (Applied Biosystems).

Conjugation, plasmid DNA isolation, and Southern hybridization

Five clinical isolates carrying class 1 integron and *dfrA17-aadA5* cassette were randomly selected for use as a donor; *E. coli* K12 20R 764 *Rif*^{r} was used as the recipient for the conjugation experiment [15]. In accordance with the method described by Kado and Liu [16], plasmid DNA was extracted, electrophoresed on 1% agarose gels, then blotted onto nylon membrane. The membranes were hybridized with digoxigenin-labeled probe and then auto-radiographed in accordance with the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Two DNA probes were amplified by PCR. Primers 5′-AGTGTCAAAGAACGGAATTTCAAGCTCA-3′ and 5′-GGATAGCGCCAAGGCACTAC-3′, including part of the *dfrA17* gene and the *aadA5* gene were used for *dfrA17-aadA5* cassette detection. A 727 bp fragment was amplified. The amplification reaction consisted of 35 cycles of 1 minute of denaturation at 94°C, 30 seconds of annealing at 58°C, and 90 seconds of extension at 72°C. The other probe used was integrase gene *intI1*.

Results

The percentage of isolates containing class 1 specific integron was 64% (280/436). The integron cassette region could not be amplified by PCR in 54 of the class 1 integron-containing isolates. No cassette inserts were detected in two isolates (Figure 1, lane 9); however, the other 224 strains of *E. coli* carried gene cassettes conferring antimicrobial resistance. Of the 224 isolates, 215 yielded one amplicon, and nine yielded two amplicons of different sizes (Table 1). The amplicon lengths present within each integron ranged in size from 700 bp

to 3 kb (Figure 1). The integrons were classified into nine groups according to the length and the numbers of amplicons yielded by a single isolate (Table 1). Groups 1, 2, 6 and 7 were further divided into subgroups based on the kinds of gene cassette types identified. As Table 1 illustrates, 15 different gene cassettes were found, including genes encoding resistance to aminoglycosides (*aadA1*, *aadA2*, *aadA5*, *aadB*, *aacA4*), chloramphenicol (*cmlA, catB8*), trimethoprim (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12, dfrA17*), lincosamide (*linF*) and unknown genes (*orfD, orfF*). The most common type of cassette, *aadA*, carried by class 1 integrons were those conferring resistance to streptomycin and spectinomycin, which represented 92% (207/224) of all cassettes. In the second most prevalent cassette, *dfrA*, resistance to trimethoprim was observed in 76% (170/224). In the present study, novel gene cassettes *aacA4* and *linF* were recovered, and cassette arrays *orfD-aacA4-catB8* and *aadA1-linF* were found.

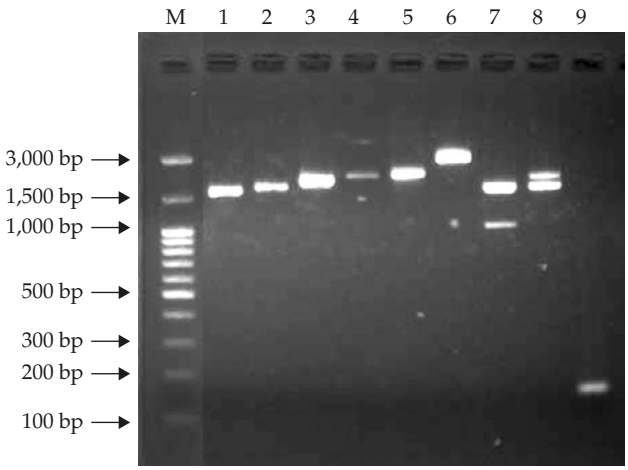

Figure 1. *Polymerase chain reaction (PCR) amplification of the integron-variable regions with 5′-CS and 3′-CS primers. The amplicons were separated by electrophoresis through an agarose 2% gel. Lane M, size marker; lanes 1–9, amplicons from clinical E. coli isolates. Lane 9, no cassette inserts into integron when 150 bp PCR product was found.*

The *dfrA12-orfF-aadA2* cassette array conferring resistance to streptomycin/spectinomycin and to trimethoprim was frequently found in class 1 integrons containing clinical isolates of *E. coli* (33%, 74/224) (Table 1). The cassette array *dfrA17-aadA5* was found in 81 (36%) class 1-containing isolates (Table 1). Southern hybridization with probes specific for the *intI1* and *dfrA17-aadA5* genes revealed that class 1 integron containing *dfrA17-aadA5* genes were located on the same plasmids and conjugatively transferable, ranging in size from 126 kb to 168 kb (Figure 2, lanes 2, 4, 6). Additionally, nonconjugative plasmid from isolates

Table 1. Amplicons and corresponding gene cassettes inserted in class 1 integrons and antibiotic resistance phenotype of gene cassettes of *Escherichia coli* isolates in 2002

Integron group	Approximate length of amplicons (kb)	Identity and order of inserted gene cassettes	Resistance phenotype	Number of isolates in 2002
1a	1	*aadA1*	STR	26
1b	1	*aadA2*	STR	5
2a	1.6	*dfrA1-aadA1*	TMP-STR	7
2b	1.6	*dfrA17-aadA5*	TMP-STR	72
3	1.9	*dfrA12-orfF-aadA2*	TMP-STR	69
4	3	*aadB-aadA1-cmlA*	GEN-STR-CHL	18
5	1.6 and	*dfrA17-aadA5*	TMP-STR	5
	1.9	*dfrA12-orfF-aadA2*	TMP-STR	
6a	0.7	*aadB*	GEN	2
6b	0.7	*dfrA5*	TMP	6
6c	0.7	*dfrA7*	TMP	7
7a	2	*aadA1-linF*	STR-LIM	1
7b	2	*orfD-aacA4-catB8*	GEN-CHL	2
8	1 and	*aadA1*	STR	4
	1.6	*dfr17-aadA5*	TMP-STR	
9	0.15	Empty integron	–	2
		Unidentified	–	54
Total				280

STR = streptomycin; TMP = trimethoprim; GEN = gentamicin; CHL = chloramphenicol; LIM = lincosamide.

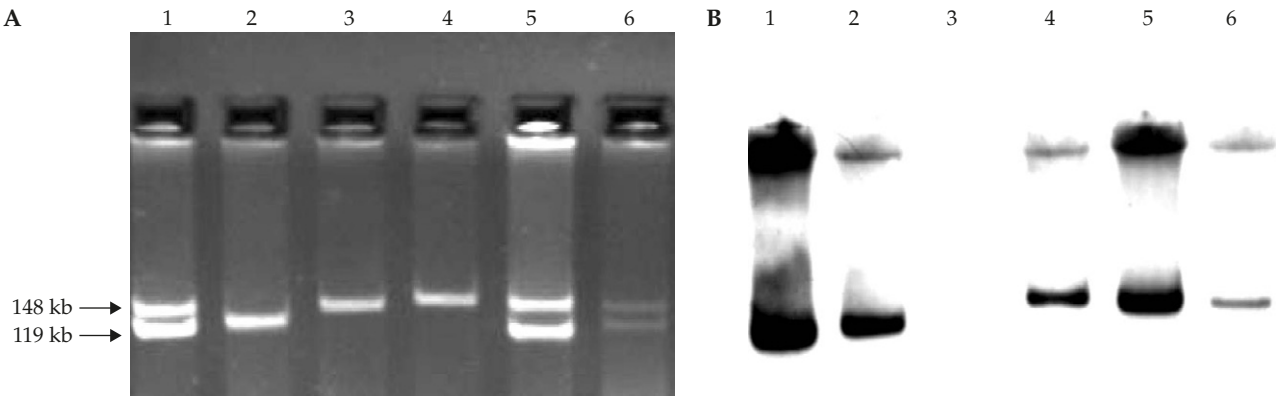

Figure 2. *(A) Agarose gel electrophoresis and (B) Southern hybridization of plasmid DNA of clinical E. coli isolates. Southern hybridization was performed with two probes specific for the intI1 and dfrA17-aadA5 gene, respectively. Lane 1, 119 kb conjugative plasmid pEC1072 containing dfrA17-aadA5 was used as positive control; lanes 2–6, E. coli clinical isolates. The two probes were hybridized to the same conjugative plasmids in lanes 2, 4, 6, and to the nonconjugative plasmid in lane 5.*

Table 2. Association between antibiotic resistance and integrons in *Escherichia coli* isolates

Antibiotic	Integron (+) (n = 280) % (no. of resistant isolates)	Integron (–) (n = 156) % (no. of resistant isolates)	p*
Trimethoprim	90 (251)	30 (48)	< 0.01
Streptomycin	94 (263)	70 (109)	< 0.01
Chloramphenicol	74 (206)	35 (55)	< 0.01
Ampicillin	95 (265)	72 (113)	< 0.01
Amikacin	5 (14)	0.7 (1)	NS
Aztreonam	12 (35)	0.7 (1)	< 0.01
Ceftazidime	11 (32)	0 (0)	< 0.01
Gentamicin	17 (48)	0.6 (1)	< 0.01
Moxalactam	10 (29)	0 (0)	< 0.01
Cefalothin	13 (38)	4 (5)	< 0.01
Trimethoprim/sulfamethoxazole	86 (241)	18 (28)	< 0.01
Piperacillin	80 (223)	44 (68)	< 0.01
Cefoperazone	18 (50)	4 (6)	< 0.01
Cefazolin	24 (67)	10 (15)	< 0.01
Amoxicillin/clavulanic acid	28 (77)	10 (16)	< 0.01
Ceftriaxone	13 (39)	1 (2)	< 0.01
Imipenem	0.3 (1)	0 (0)	NS
Ticarcillin/clavulanic acid	20 (56)	6 (9)	< 0.01
Ofloxacin	27 (75)	3 (5)	< 0.01
Piperacillin/tazobactam	3 (8)	0 (0)	NS
Cefepime	2 (7)	0.7 (1)	NS
All sensitive†	0 (0)	10 (15)	< 0.01

*$p < 0.01$, χ^2 test or Fisher's exact test; †isolates susceptible to antibiotics tested. NS = no statistical significance.

(Figure 2, lane 5) was also hybridized with the *dfr17-aadA5* and *intI1* specific probes.

Resistance of clinical isolates to almost every class of antimicrobial agent was noted. The resistance to ampicillin and extended-spectrum penicillin (e.g. piperacillin) was 87% and 67%, respectively. Additionally, resistance to penicillins combined with β-lactamase inhibitors was also observed, e.g. amoxicillin/clavulanic acid (21%) and ticarcillin/clavulanic acid (15%). However, resistance to third generation cephalosporins was observed in less than 10% of the isolates (Table 2). Integron-positive clinical isolates tended to have greater antibiotic resistance than integron-negative clinical isolates. Interestingly, integrons containing isolates

had a significantly high resistance to most of the antibiotics except amikacin, imipenem, cefepime, and piperacillin/tazobactam (Table 2).

Discussion

The increasing drug resistance of clinical isolates may be explained by antibiotic selective pressure and widespread presence of integrons. This study documented an increased prevalence of class 1 integrons from 52% [11] to 64% (this study) in *E. coli* isolates recovered in Taiwan over a 10-year period. Increased prevalence of the class 1 integron has also been reported elsewhere: 43% in Norway in 2000–2001 [17], 43% in Western and Central Europe in 1996–1997 [8], 50% in The Netherlands in 1994 [18], 59% in France in 1992 [7], 54% in Korea in 1980–2002 [19], and 86% in China in 2005 [20].

In the present study, most of the isolates with class 1 integrons contained at least one *aadA* cassette (*aadA1*, *aadA2* or *aadA5*), alone or in combination with other cassettes. However, the predominance of gene cassettes encoding resistance to streptomycin and spectinomycin (*aadA*) was unanticipated, as the clinical use of the drugs in Taiwan has been minimal. However, streptomycin-resistant bacteria can be isolated from animals, probably as a result of streptomycin and spectinomycin use in animal husbandry [21–25]. Furthermore, coliform bacteria isolated from the aquatic environment have also revealed a resistance to aminoglycosides [26]. It is therefore likely that humans became colonized with streptomycin-resistant bacteria via the food chain in a contaminated environment [27–29]. Another possibility is that integrons transferred from animal *E. coli* to human *E. coli* while transiently passing through the human intestine. A third possibility is that even when antibiotics cease to be used therapeutically, genes encoding resistance to these antibiotics were not easily lost [30].

aadA was the most common cassette carried by class 1 integron; however, cassette *aadA1* apparently decreased gradually during the 10-year period in Taiwan. Similar results have been reported in *E. coli* isolates in Korea [19]. Nevertheless, multigene cassette array *aadB-aadA1-cmlA* was found to increase from 2% in 1993 to 6% in 2002 in isolates in this study. The trimethoprim-resistant (*dfrA*) gene cassette was also frequently found in the *E. coli* strains under investigation. Five *dfrA* gene type cassettes were identified; these gene cassettes were not unexpected since trimethoprim-sulfamethoxazole is commonly used to treat bacterial infections in Taiwan. Such specific selective pressure may favor the acquisition and maintenance of a trimethoprim-resistant cassette by class 1 integrons containing *sul1* in the 3′ region.

Gene cassette *aacA4*, which codes for amikacin, netilmicin and tobramycin resistance was previously found in pan-resistant Gram-negative clinical isolates of *Pseudomonas aeruginosa* [31] and *Acinetobacter baumannii* [32]. Multigene cassette arrays *aacA4-catB8* found only in *A. baumannii* [32,33] were also found in *E. coli* in the present study. Although amikacin resistant *E. coli* was rarely isolated (3%, Table 2) from the clinical samples, class 1 integron carrying *orfD-aacA4-catB8* gene cassette was found in two isolates. An additional finding was a novel *aadA1-linF* cassette which differed from the *aadA2-linF* cassette previously identified in a Norway study [17]. These results indicate that resistant gene cassettes might disseminate through the integron between different bacteria species under antibiotic selective pressure. Furthermore, the same cassette may combine with other cassettes in different geographical areas. Most of the class 1 integrons identified in this study carried the *dfr12-orfF-aadA2* cassette array. This pattern was also reported in urinary *E. coli* isolates in a Korean study [19,22], and in *Shigella* strains isolated in Finland but originating from Asia [34]. This observation indicates that this combination of gene cassettes has achieved stable integration.

Furthermore, *dfr17-aadA5* located on a 119 kb conjugative plasmid pEC1072 was isolated from an *E. coli* strain in 1993 in our previous study [35]. The quantitative presence of the same cassette arrays with the predominant class 1 integrons found on transferable plasmids in *E. coli* rapidly increased in 2002 in this work. The same *dfrA17-aadA5* cassettes were also detected in clinical isolates in Korea [19,22] and Australia [36] as well as in *E. coli* isolated from dogs, pigs, and other domestic livestock [28,29]. These data reveal that the *dfrA17-aadA5* cassette can disseminate by self-transferable plasmids in humans or animals and in different areas worldwide.

Of the class 1 integron-containing isolates recovered in 2002, the integron cassette region could not be amplified by PCR in 54 isolates. Possible causes are: (1) the number of inserted genes in the cassette exceeded the PCR extension capacity; (2) lack of the 3′ conserved

segment or insufficient homology to the 3′ conserved segment primer to produce a product [37].

Previous studies have not clarified whether imprudent or inappropriate use of first-line antimicrobials in Taiwan promotes cassette array formation. However, this study revealed a steady increasing prevalence of antibiotic resistant *E. coli* in Taiwan over a 10-year period. It is suggested that this increased resistance is partly attributable to the acquisition, dissemination and stable maintenance of a class 1 integron.

In conclusion, the extended survey period revealed a predominance of *dfrA* and *aadA* resistant gene cassettes conferring resistance to trimethoprim and aminoglycoside. However, genes conferring resistance to recently introduced antibiotics which were already part of the gene cassettes of pan-drug resistant bacteria were found in *E. coli*. Therefore, the potential role of integrons in the uptake and dissemination of resistant genes may be a continuing threat to the effectiveness of certain antibiotic therapeutic agents both in Taiwan and globally.

Acknowledgments

This work was supported by a grant from the National Science Council (91-2320-B-037-058).

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大腸桿菌第一型 Integron 相關抗藥性之基因片匣變異分析

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針對住院病人分離之大腸桿菌進行第一型 integron 之研究，並進一步分析其所攜帶基因片匣之穩定性及變異。實驗中挑選 436 株分離自 2002 年之大腸桿菌，以 PCR 及核甘酸定序探討第一型 integron 之特性並利用接合反應及南方雜交法分析第一型 integron 存在菌株位置。結果有 64% 之臨床菌株攜帶第一型 integron，分析顯示這些第一型 integron 攜帶 13 種不同抗藥基因片匣及兩種未知基因片匣，而主要之片匣為 *aadA* 及 *dfrA*。在大腸桿菌發現之新基因片匣及片匣組合分別為 *aacA4*、*linF* 及 *orfD-aacA4-catB8*、*aadA1-linF*。而 *dfrA12-orfF-aadA2* 是目前穩定存在的基因片匣，而第一型 integron 及 *dfrA17-aadA5* 片匣則發現位於菌株同一轉移性質體上，並且可藉質體轉移在菌株間傳遞。所以臨床抗藥菌株增加可能的解釋為抗生素篩選壓力及 integron 廣泛存在的結果。在抗生素篩選壓力下，攜帶抗藥基因之片匣可能不容易自菌株中遺失。因此認為 integron 攝入抗藥基因並藉質體於菌種間散佈抗藥性的角色將可能持續威脅臨床抗生素之使用及治療。

關鍵詞： *aadA1-linF*，接合性質體，*dfrA17-aadA5*，大腸桿菌，*orfD-aacA4-catB8*

(高雄醫誌 2007;23:273－80)

收文日期：95 年 8 月 1 日

接受刊載：96 年 12 月 6 日

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