Jpn. J. Infect. Dis., 56, 2003

Laboratory and Epidemiology Communications

Drug Resistance Genes Encoded in Integrons and in Extra-Integrons: Their Distribution and Lateral Transfer among Pathogenic *Enterobacteriaceae* including Enterohemorrhagic *Escherichia coli* and *Salmonella enterica* Serovars Typhimurium and Infantis

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Communicated by Kazue Tabita

(Accepted July 2, 2003)

Salmonella enterica serovars Typhimurium and Infantis have been major causes of *Salmonella* infections in Japan during the past decades, though *S*. Enteritidis suddenly emerged in 1989 and continues to prevail (1). While rare in *S*. Enteritidis, multidrug resistance (MDR) is frequent among *S*. Typhimurium and *S*. Infantis (1). The drug resistance genes are transferred among these *Salmonella* spp. along with the class 1 integrons (1) present in transposons and conjugative plasmids (1,2). Class 1 integrons are predominant within (2,3) and outside (4,5) the family *Enterobacteriaceae*. In Japan, enterohemorrhagic *Escherichia coli* (EHEC), particularly O157, O26, and O111 serotypes, has prevailed since 1996. Though well documented for O157 and O111 serotypes, integron-mediated antibiotic resistance among O26 serotype has remained relatively unknown (3). Here, we present a systematic investigation of drug resistance genes carried by integrons (2 - 5) or extra-integrons (2,4,6) in *S*. Typhimurium, *S*. Infantis, and EHEC with special reference to their transferability.

The strains used in this study were collected by authors. *Salmonella* strains were those used previously (1) and one additional *S*. Infantis strain (Inf32). EHEC strains were 29 serotype O26 strains (including two verotoxin non-producers), one O111, and three O157 strains, all collected in 1996 - 2003. The O111 and O157 EHEC strains were chosen randomly from a small number of MDR strains (resistant to more than three drugs) found in approximately 550 strains collected in 1996 - 2002.

The strains were tested for sensitivities to ampicillin (Am), cefotaxime, kanamycin (Km), gentamicin, streptomycin (Sm), tetracycline (Tc), trimethoprim (Tm), ciplofloxacin, fosfomycin (Fm), chloramphenicol (Cm), sulphamethoxazole (Su), and nalidixic acid (Na). We used antibiotic disks (Becton Dickinson Microbiology Systems, Cockeysville, Md., USA) on Mueller-Hinton agar (MH) plates and agar dilutions on MacConkey (MAC) and/or MH agar plates (1). Table 1 shows the antibiograms of 14 MDR *S*. Typhimurium strains (among 22 strains tested), all the tested 12 MDR *S*. Infantis strains, and all the tested 11 MDR *E. coli* strains. A susceptible *E. coli* ('02-S.031) and a susceptible *Salmonella* (Inf01) were included for comparison. By means of polymerase chain reaction (PCR) (1), we searched for class 1 integrons, for *ant* (*3″*)-*1a* and *qac*Δ*E1sul1* in close association with the 3'-conserved segment (3'-CS) of integron (2-5), and for drug resistance genes often located in integrons (2-5) or outside of integrons (2,4,6). The primer pairs used for PCR and their PCR products are shown in Fig. 1. Primer concentration was 0.2 μM and Taq polymerase concentration 0.25 units/50 μl for all the genes except for *tet* genes (*tetA*, *B*, *C*, *D*, and *E* [4]). For PCR of *tet*, the primer concentration was 0.4 μM, and Taq polymerase concentration 0.5 units/50 μl.

Table 1 summarizes the characteristics of bacterial strains examined in the present and previous (1) reports.

① When the int l primer pair was used, various sizes of PCR products were obtained. We conveniently classified them in terms of the size of PCR product. Among 22 drug-resistant *S*. Typhimurium strains, seven had 1.0-kb and 1.2-kb integrons (such strains are called A type) and five strains had a 2.0 kb-integron only (they are called B type). All the 12 MDR *S*. infantis strains had a 1.0 kb-integron only (they are called C type). One non-EHEC *E. coli* strain ('96-E.094) was C type.

② All the pathogenic species of *E. coli* and *Salmonella* harboring integrons possessed an *ant*(*3″*)-*1a* gene (0.75 or 2.0 kb in PCR product size) encoding an aminoglycoside-modifying enzyme.

③ *aadA2* encoding aminoglycoside-adenyltransferase was detected in one half of *S*. Typhimurium and in one EHEC ('00-E.051).

④ *qacE*Δ*1sul1* is responsible for insecticide and sulfonamide resistances. All the Su-resistant *S*. Typhimurium except Tym04 or Infantis had the gene, while all the Su-resistant EHEC strains except '96-E.094 were negative for the gene.

⑤ *TetA* and *TetB* are known to be responsible for Tc^r. Among Tc-resistant *E. coli* strains, a half of the strains had *tetA* or *tetB*. The remaining half was negative for *tetA* or *tetB*. All the MDR *S*. Infantis had *tetA*. Most Tc-resistant *S*. Typhimurium had *tetB*. *S*. Typhimurium strains of the B integron type had *tetB* and those of A integron type had *cmlAtetR* (Cm^r and a regulatory gene for Tc^r). Tym22 of the A integron type (1) having *cmlAtetR* and *tetA* (2) is an exception.

⑥ All the Km-resistant strains had *aphA1-LAB* gene.

⑦ All the Am-resistant *E. coli* harbored TEM (bla_{TEM})

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Table 1. List of multidrug-resistant enterohemorrhagic *Escherichia coli* and *Salmonella enterica* serovars Typhimurium and Infantis showing their antibiograms and relevant genes for drug resistances[1)]

Strain	Source and year of isolation	Serotype	Antibiogram[2)]									Presence (+) or absence (–) of								
			Am	Km	Sm	Tc	Fm	Cm	Tm	Su	Na	*intI*[3)] (type)	*ant(3″)-Ia*[4)] (size in kb)	*qacE* Δ*1sul1*	*aadA2*	*tet*	*cmlAtetR*	PSE-1	TEM	*aphA1-LAB*
Enterohemorrhagic *Escherichia coli*[5)]																				
’96-E.094	human 1996	O26 : HNM		Km	Sm	Tc		Cm		Su		+ (C)	+ (0.75)	+	–	*tetB*	–	–	–	+
’96-E.152	cattle 1996	O26 : H11	Am		Sm					Su		–	–	–	–	–	–	–	+	–
’98-E.001	human 1998	O26 : HNM	Am	Km	Sm	Tc	Fm			Su		–	–	–	–	–	–	–	+	+
’98-E.002	human 1998	O26 : HNM	Am	Km	Sm	Tc	Fm			Su		–	–	–	–	–	–	–	+	+
’99-E.003	human 1999	O26 : H11	Am	Km	Sm	Tc				Su		–	–	–	–	*tetA*	–	–	+	+
’02-E.001	human 2002	O26 : HNM			Sm	Tc				Su		–	–	–	–	*tetB*	–	–	–	–
’02-E.092	human 2002	O26 : HND			Sm	Tc				Su		–	–	–	–	*tetB*	–	–	–	–
’02-S.031	human 2002	O26 : H11										–	–	–						
’99-E.041	human 1999	O111 : HNM	Am	Km	Sm	Tc				Su	Na	–	–	–	–	*tetA*	–	–	+	+
’99-E.024	human 1999	O157 : H7	Am		Sm	Tc				Su		–	–	–	–	–	–	–	+	–
’99-E.025	human 1999	O157 : H7	Am		Sm	Tc				Su		–	–	–	–	–	–	–	+	–
’00-E.051	human 2000	O157 : H7			Sm	Tc		Cm		Su		–	–	–	+	–	–	–	–	–
Salmonella enterica serovars Typhimurium and Infantis																				
Tym03	human 1992	Typhimurium	Am		Sm	Tc		Cm		Su	Na	+ (A)	+ (0.75)	+	+	–	+	+	–	–
Tym04	human 1992	Typhimurium	Am	Km	Sm	Tc				Su		–	–	–	–	*tetB*	–	+	+	
Tym05	human 1992	Typhimurium	Am		Sm	Tc		Cm		Su		+ (A)	+ (0.75)	+	+	–	+	+	–	–
Tym06	human 1992	Typhimurium	Am		Sm	Tc		Cm		Su		+ (A)	+ (0.75)	+	+	–	+	+	–	–
Tym13	human 1993	Typhimurium	Am		sm	Tc		Cm		Su		+ (B)	+ (2.0)	+	–	*tetB*	–	–	–	–
Tym14	human 1993	Typhimurium	Am	Km	sm	Tc		Cm		Su		+ (B)	+ (2.0)	+	–	*tetB*	–	–	–	+
Tym15	human 1993	Typhimurium	Am	Km	sm	Tc		Cm		Su		+ (B)	+ (2.0)	+	–	*tetB*	–	–	–	+
Tym16	human 1993	Typhimurium	Am	Km	sm	Tc		Cm		Su		+ (B)	+ (2.0)	+	–	*tetB*	–	–	–	+
Tym18	human 1994	Typhimurium	Am		Sm	Tc		Cm		Su		+ (A)	+ (0.75)	+	+	–	+	+	–	–
Tym20	human 1996	Typhimurium	Am	Km	sm	Tc		Cm		Su		+ (B)	+ (2.0)	+	–	*tetB*	–	–	–	+
Tym21	human 1996	Typhimurium	Am		Sm	Tc		Cm		Su		+ (A)	+ (0.75)	+	+	–	+	+	–	–
Tym22	human 1997	Typhimurium	Am		Sm	Tc		Cm	Tm	Su		+ (A)	+ (0.75)	+	+	*tetA*	+	+	–	–
Tym25	human 1997	Typhimurium	Am		Sm	Tc		Cm		Su		+ (A)	+ (0.75)	+	+	–	+	+	–	–
Tym29	human 2002	Typhimurium		Km		Tc						–	–	–	–	*tetB*	–	–	–	+
Inf 01	human 1992	Infantis										–	–	–		–				
Inf 05	chicken 1994	Infantis		Km	sm	Tc				Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 07	chicken 1994	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 13	human 1998	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*	–	–	–	+
Inf 14	human 1998	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 18	human 1999	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 21	human 2001	Infantis			sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*	–	–	–	–
Inf 22	chicken 2001	Infantis		Km	sm	Tc				Su		+ (C)	+ (0.75)	+	–	*tetA*	–	–	–	+
Inf 27	human 2001	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 28	human 2001	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 29	human 1995	Infantis		Km	sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				+
Inf 31	chicken 2002	Infantis			sm	Tc			Tm	Su		+ (C)	+ (0.75)	+	–	*tetA*				–
Inf 32	human 2003	Infantis		Km	sm	Tc			Tm	Su		+ (C)								

[1)] –: negative, +: positive, ND : not determined, blank: not tested.

[2)] Sensitivity to antibiotics was tested by means of disk diffusion (on MH)- and agar dilution (on MAC or MH)-methods. Am: ampicillin (30 μg/ml for dilution method), Km: kanamycin (25 μg/ml), sm (≦50 μg of MICs) and Sm (>50 μg/ml of MICs): streptomycin (12.5, 25, 50 μg/ml), Tc: tetracycline (25 μg/ml), Fm: fosfomycin (25 μg/ml), Cm: chloramphenicol (25 μg/ml), Tm: trimethoprim (25 μg/ml), Su: sulfamethoxazole (125 μg/ml), Na: nalidixic acid (25 μg/ml).

[3)] Alphabetical symbols in parentheses indicate the integron types produced by PCR.

[4)] Numerals in parentheses are the amplicon sizes (kb) produced by PCR.

[5)] EHEC, ’98-E.001 and ’98-E.002 (case A), and ’99-E.024 and ’99-E.025 (case B) were obtained from infections in a single family, respectively. The patterns of chromosomal DNA digests with *Xba*I were identical (case A) or different (case B) on PFGE (Gene Path Typing Sytem, Program No. 5: Bio-Rad). VT (verotoxin) was tested by using PCR employing EVT- (for VT1) and EVS- (for VT2) primers (Takara Shuzo) by following manufacturer’s protocol. Three O157 serotypes were positive for both VT1 and VT2. O26 and O111 serotypes produced VT1 except ’96-E.094, a non-producer of VT.

Table 2. Conjugal transfer and transformation by plasmid DNA of antibiotic resistance determinants of enterohemorrhagic *Escherichia coli*

Donor[1]	Method[2]	Selection[3]	Antibiogram[4]						Frequency[5]	Plasmid[6]
			Am	Km	Sm	Tc	Fm	Su		
'96-E.152 (O26)			R		R			R		
	CT-I	Am	R		R			R	26/29	
	(7.8×10^{-6})		R		S			S	3/29	80 kb
	TF	Am	R		R			R	50/50*	90 kb
		Sm	R		R			R	50/50	
DH5α (Am^r-201)*			R		R			R		
	CT-II	Am	R		R			R	63/63	
	($4.4\sim6.3 \times 10^{-3}$)	Sm	R		R			R	59/60	
			S		R			R	1/60	
'98-E.001 (O26)			R	R	R	R	R	R		
	CT-I	Am	R	R	R	R	S	R	21/27	
	($1.8\sim2.6 \times 10^{-7}$)		R	S	S	S	S	S	6/27	
		Km	R	R	R	R	S	R	31/37	
			S	R	S	R	S	S	4/37	
			R	R	R	S	S	S	1/37	
			S	R	S	S	S	S	1/37	
		Tc	R	R	R	R	S	R	21/38	
			S	S	S	R	S	S	9/38	
			S	R	S	R	S	S	8/38	
	TF	Am	R	S	S	S	S	S	9/9	90 kb
'98-E.002 (O26)			R	R	R	R	R	R		
	CT-I	Am	R	R	R	R	S	R	16/18	
	($1.1\sim1.5 \times 10^{-7}$)		R	S	R	R	S	R	1/18	
			R	S	S	S	S	S	1/18	
		Km	R	R	R	R	S	R	20/25	
			S	R	S	R	S	S	5/25	
		Tc	R	R	R	R	S	R	17/25	
			S	S	S	R	S	S	5/25	
			S	R	S	R	S	S	2/25	
			R	S	R	R	S	R	1/25	
	TF	Am	R	S	S	S	S	S	10/10	
'99-E.003 (O26)			R	R	R	R		R		
	CT-I	Am	R	R	R	R		R	29/37	
	($2.8\sim4.0 \times 10^{-6}$)		R	S	S	S		S	5/37	
			R	S	R	R		R	3/37	
		Km	R	R	R	R		R	42/46	
			S	R	S	R		S	4/46	
		Tc	R	R	R	R		R	34/42	
			S	S	S	R		S	5/42	
			S	R	S	R		S	3/42	
	TF	Am	R	S	S	S		S	12/12	135 kb
		Km	R	R	R	R		R	4/4**	135 kb
		Sm	R	R	R	R		R	4/4***	135 kb
		Tc	R	R	R	R		R	5/5****	135 kb
DH5α (Km^r-801)**			R	R	R	R		R		
	CT-II	Am	R	R	R	R		R	35/35	
	($1.0\sim1.7 \times 10^{-5}$)	Km	R	R	R	R		R	35/35	
DH5α (Sm^r-801)***			R	R	R	R		R		
	CT-II	Am	R	R	R	R		R	35/35	
		Sm	R	R	R	R		R	35/35	
DH5α (Tc^r-801)****			R	R	R	R		R		
	CT-II	Am	R	R	R	R		R	35/35	
		Tc	R	R	R	R		R	35/35	
'99-E.041 (O111)			R	R	R	R		R		
	TF	Am	R	S	S	S		S	16/16	
'99-E.024 (O157)			R		R	R		R		
	TF	Am	R		S	S		S	37/38	90 kb
			R		R	R		R	1/38	90 kb
'99-E.025 (O157)			R		R	R		R		
	TF	Am	R		S	S		S	14/14	

[1] *, **, ***, ****: one of the DH5α transfectants with the same symbols at the right column, respectively.

[2] CT-I (conjugal transfer I): *E. coli* donor x a *Na^{r}Rf^r* derivative of *S.* Litchfield AOLac$^+$ (8) (*lac$^+$Na^{r}Rf^r*), TF (transformation): *E. coli* DH5α (*lac gylA*) cells were infected with plasmid DNA fractions from *E. coli* O26, O111, or O157 cells, CT-II (conjugal transfer II): DH5α transfectant x a *lac$^+$* revertant of *E. coli* WA921-3 (8) (*lac$^+$Na^{r}Rf^r*). Na: naldixic acid, Rf: rifampicin. Numerals in parentheses indicate the transfer frequency/h.

[3] Cells were grown on the MAC containing 0.5% sucrose (conjugal transfer I) or lactose (transformation and conjugal transfer II). To select the transconjugants and tranfectants, Am, Km, Sm, or, Tc was added to the plates, and to eliminate the donors in conjugation Na and/or Rf were also added. The drug concentrations each were stated in the text.

[4] Am: ampicillin, Km: kanamycin, Sm: streptomycin, Tc: tetracycline, Fm: fosfomycin, Su: sulfamethoxazole. Sensitivity to the drugs was determined by means of agar dilution method on the MAC containing lactose or sucrose except Su on the MH. The drug concentrations each were stated in the footnote of Table l.

[5] Number of isolates showing the antibiograms indicated/total isolates tested.

[6] Each one of the transductants or DH5α transfectants shown on the same lines was used. The approximate sizes of the plasmid relevant for drug resistances were determined by using PFGE (Gene Path Typing System, Program No. 5: Bio-Rad).

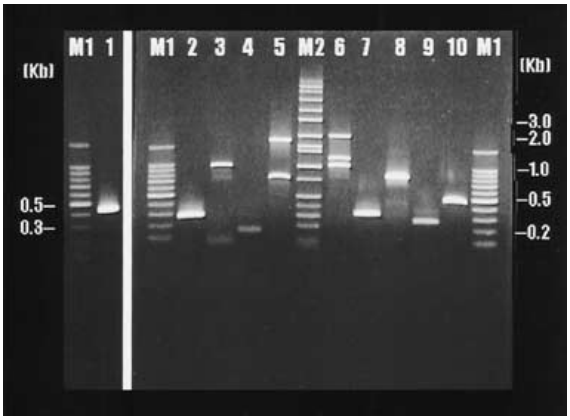

Fig. l. Different sizes of PCR products obtained with primer pairs targeting various genes in integrons and their surroundings and in extra-integrons in isolates of enterohemorrhagic *Escherichia coli* and *Salmonella enterica* serovars Typhimurium and Infantis in 1991 - 2003. The PCR products were separated using conventional agarose gel electrophoresis. M1 and M2 in the figure indicate 100 bp DNA ladder (Takara Shuzo) and wide-range DNA ladder (Takara Shuzo), respectively. Lane 1: 0.44-kb amplicons of *tetB* (4). Lane 2: 0.28-kb amplicons of *cmlAtetR* (7). Lane 3: 1.0-kb amplicons of *tetA* (4). Lane 4: 0.15-kb amplicons of PSE-1 (7). Lane 5: a mixture of 0.75- and 2.0-kb amplicons of *ant*(*3*")-*1a* (5). Lane 6: a mixture of PCR products of class 1 integron (1) amplicons, types A (1.0 and 1.2 kb) and B (2.0 kb). Lane 7: 0.31-kb amplicons of TEM (7). Lane 8: 0.80-kb amplicons of *qacE*Δ*lsull* (5). Lane 9: 0.25-kb amplicons of *aadA2* (6). Lane 10: 0.50-kb amplicons of *aphA1-LAB* (6).

genes. PSE-1 (bla_{PSE-1}) genes were found only among Am-resistant *Salmonella* of the A integron type. An exception was integron-negative Tym04 with both the *bla* genes. Am-resistant *S*. Typhimuriun of the B integron type having a 2.0 kb *ant* (*3*″)-*la* casette was devoid of both PSE and TEM-1 genes.

We examined 11 MDR strains of *E. coli* (Table 1) for transferability of drug resistance genes. Two protocols were used. Conjugal transfer I (CT-I): Each of the *E. coli* strains was conjugated with a rifampicin (Rf)-resistant derivative of *S*. Litchfield AOLac$^+$Nal^r-01 (*lac*$^+$*Na*^{r}) (1,8). The mating time was 4 h in liquid cultures at 37°C. The transconjugants were selected on sucrose (0.5%)-MAC plates containing Am (30 μg/ml), Km (25 μg/ml), Sm (25 and 50 μg/ml), or Tc (25 μg/ml). The donor strains were eliminated by Na (25 μg/ml) and/or Rf (25 μg/ml). Conjugal transfer II (CT-II): In order to know whether the resistance genes were present on transferable plasmids or not, we conducted transformation followed by conjugal transfer. For transformation, competent *E. coli* K12 DH5α (*lac gylA*) cells (Takara Shuzo, Co., Ltd., Kyoto) were transfected with plasmid DNA fractions prepared from the MDR strains. The transfectants were selected on the lactose (0.5%)-MAC plates containing Am, Km, Sm, or Tc. DH5α transfectants were crossed with a *lac*$^+$ revertant of *E. coli* K12 WA921-3 (*lacNa*^{r}*Rf*^{r}) (8). The MAC contained Rf in order to eliminate the DH5α transfectants used as donors. Table 2 shows CT-I and CT-II data. The results are summarized as follows:

(1) Among 11 MDR EHEC, conjugal transfer (CT-1) was successful for four strains, '96-E.152, '98-E.001, '98-E.002, and '99-E.003. Segregation of drug resistance genes, especially *Am*^{r} from other resistant genes, was observed. This suggests *Am*^{r} and the other genes are located on a plasmid and the choromosome, respectively (see also TF data in the table).

(2) CT-II was positive only for '96-E.152 and '99-E.003. In '96-E.152, there was a rare segregation of resistance genes, *Am*^{s}*Sm*^{r}*Su*^{r} from *Am*^{r}*Sm*^{r}*Su*^{r}, during CT-II. For '99-E.003 no such segregation was observed.

Thus, four of 11 EHEC were able to transfer drug resistance genes through conjugation, and at least two ('96-E.152 and '99-E.003) of them harbored all the drug resistance genes also on the same plasmids.

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