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Drug susceptibility and its genetic basis in epidemic *Vibrio cholerae* O1 in Vietnam

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SUMMARY

The drug susceptibility and genes responsible for the drug resistance of *Vibrio cholerae* O1 isolated in Vietnam in 1995, 2000 and 2002 were studied. The strains isolated in 1995 were resistant to streptomycin and harboured the class I integron which contained the *aadA1* gene responsible for streptomycin resistance. The strains isolated in 2000 were devoid of a class I integron but were multiple-drug resistant and harboured SXT constin, with several drug-resistant genes. The genes responsible for streptomycin resistance were *strA* and *strB*. The strains isolated in 2002 were sensitive to all drugs examined, and the organisms were devoid of both class I integron and SXT constin. Cholera outbreaks in the three periods examined (1995, 2000 and 2002) were apparently due to different categories of *V. cholerae* O1.

INTRODUCTION

Vibrio cholerae O1, a causative agent of cholera, has in the past been shown to be susceptible to various antibiotics, as expected. Although outbreaks of cholera due to drug-resistant *V. cholerae* O1 were occasionally reported in some areas, the resistant strains soon disappeared, especially after preventive medication was stopped [1, 2]. In those cases, the organisms were highly resistant to tetracycline and the resistance was mediated by plasmids. However, worldwide in the late 1990s, *V. cholerae* O1 simultaneously started to become resistant to a variety of antibiotics [3–6]. Most of these strains had specific genetic elements such as class I integron or SXT constin (a conjugative, self-transmissible, integrating element) that could be associated with the spread of genetic determinants of resistance to antimicrobial agents. Integrons are gene-capturing systems that usually contain antibiotic-resistance genes. SXT constin is a transmissible genetic element that contains some regions (hot-spots) for insertion of additional DNA such as drug-resistance genes [7]. Dalsgaard et al. characterized *V. cholerae* O1 isolated in Vietnam from 1979 to 1996 and found that strains isolated after 1990 were resistant to streptomycin and harboured class I integrons containing a gene cassette encoding resistance to streptomycin [8]. Nguyen et al. reported that *V. cholerae* O1 isolates in Vietnam in 1995 were sensitive to, and those in 2000 were resistant to, tetracycline and chloramphenicol [9]. It thus seems that the characteristics of *V. cholerae* O1 in the recent epidemic are variable. The purpose of this study was to compare the genetic composition of the organisms

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Table 1. *Primers used in this study*

Primer	Sequence (5′ to 3′)	Locus (direction)	Ref.
inDS-F	CGG AAT GGC CGA GCA GAT C	*intI1* (–)	[5]
inDS-B	CAA GGT TCT GGA CCA GTT GCG	*intI1* (+)	[5]
in-F	GGC ATC CAA GCA GCA AGC	5′-CS	[5]
in-B	AAG CAG ACT TGA CCT GAT	3′-CS	[5]
aadA-B	ATT GCC CAG TCG GCA GCG	*aadA* (–)	[5]
TetA-F	GTA ATT CTG AGC ACT GTC GC	*tetA* (+)	[11]
TetA-R	CTG CCT GGA CAA CAT TGC TT	*tetA* (–)	[11]
INT1	GCT GGA TAG GTT AAG GGC GG	int_{SXT} (–)	[10]
INT2	CTC TAT GGG CAC TGT CCA CAT TG	int_{SXT} (+)	[10]
strB-F	GGC ACC CAT AAG CGT ACG CC	*strB* (–)	[This study]
strB-R	TGC CGA GCA CGG CGA CTA CC	*strB* (+)	[This study]
STRA-F	TTG ATG TGG TGT CCC GCA ATG C	*strA* (+)	[10]
STRA-B	CCA ATC GCA GAT AGA AGG CAA	*strA* (–)	[10]
FLOR-F	TTA TCT CCC TGT CGT TCC AGC G	*floR* (+)	[10]
FLOR-B	TCG TCG AAC TCT GCC AAA TG	*floR* (–)	[10]
SUL2-F	AGG GGG CAG ATG TGA ATC GAC	*sulII* (+)	[10]
SUL2-B	TGT GCG GAT GAA GTC AGC TCC	*sulII* (–)	[10]

Locus, target genes: (direction)/(+), oligonucleotides corresponding to the coding strand (forward primer); (direction)/(–), oligonucleotides corresponding to the non-coding strand (backward primer).

responsible for the drug resistance of epidemic *V. cholerae* O1 in 1995, 2000 and 2002 in Vietnam.

MATERIALS AND METHODS

Bacterial strains

Vibrio cholerae O1 strains used in this study were isolated from cholera patients in Vietnam in 1995, 2000 and 2002.

The strains were stored in a butt of soft agar at room temperature, and they were subcultured when necessary. The phenotypes of the strains were examined at isolation, and re-examined in the present study. From this strain collection, 36 strains (12 strains each year) that showed the same phenotypes as in the initial examination at isolation were randomly selected and used in this study.

Drug susceptibilities

Seven therapeutic drugs: ampicillin (Meiji Seika Co., Tokyo, Japan), tetracycline (Nacalai Tesque Co., Kyoto, Japan), chloramphenicol (Wako Pure Chemical Ind., Osaka, Japan), erythromycin (Dainihon Pharmaceutical Co., Osaka, Japan), ofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan), nalidixic acid (Wako), sulphamethoxazole–trimethoprim (sulphamethoxazole: Wako; trimethoprim: Sigma-Aldrich Japan K.K., Tokyo, Japan), and streptomycin (Wako) were used to determine the minimum inhibitory concentrations (MICs) against the organisms. Additionally, susceptibility to polymyxin B (Pfizer Japan Inc., Tokyo, Japan) was examined to distinguish between El Tor biotype and classical biotype. The MICs were examined by the plate dilution technique. A series of heart infusion agar plates containing two-fold dilutions of the drugs from 100 to 0·0125 μg/ml were prepared. The compound drug sulphamethoxazole–trimethoprim was separately examined. The ratio of sulphamethoxazole to trimethoprim in the mixture was 19 to 1, and the concentration of the drug was expressed as the total amount of sulphamethoxazole–trimethoprim. A dilution series from 640 to 0·078 μg/ml of the drug combination in heart infusion agar plates (608 to 0·74 μg/ml of sulphamethoxazole and 32 to 0·0039 μg/ml of trimethoprim) was prepared. A 10-fold dilution of overnight broth culture was inoculated onto each plate using a Microplanter (model MITP no. 00257, Sakuma Co. Ltd, Chiba, Japan), and incubated at 37 °C for 24 h. The susceptibility was expressed as the MIC (μg/ml) of each drug.

Detection of specific genes

The presence of class I integron, SXT constin and drug-resistance genes was examined by PCR. Class I integron was determined by detection of the class I

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Table 2. *Drug susceptibility and the associated genes*

Strains	TC	EM	ABPC	CP	NA	ST	SM	SXT constin	Class I integron	*tetA*	SMr gene
95-1	0·4	6·25	3·13	0·8	0·4	0·313	50	−	+	−	*aadA1*
-2	0·4	12·5	3·13	0·8	0·2	2·5	50	−	+	−	*aadA1*
-3	0·4	12·5	1·6	0·8	0·1	1·25	25	−	+	−	*aadA1*
-4	0·4	12·5	3·13	0·8	0·2	0·313	50	−	+	−	*aadA1*
-5	0·4	12·5	1·6	0·8	0·2	0·625	6·25	−	−	−	—
-6	0·4	12·5	3·13	0·8	0·2	0·313	50	−	+	−	*aadA1*
-7	0·2	6·25	3·13	0·8	0·2	0·313	6·25	−	−	−	—
-8	0·4	12·5	3·13	1·6	0·2	0·313	50	−	+	−	*aadA1*
-9	0·4	6·25	3·13	0·8	0·2	0·313	50	−	+	−	*aadA1*
-10	0·4	12·5	3·13	0·8	0·2	0·313	100	−	+	−	*aadA1*
-11	0·2	3·13	3·13	0·8	0·2	0·313	50	−	+	−	*aadA1*
-12	0·4	6·25	1·6	0·8	0·2	0·313	50	−	+	−	*aadA1*
00-1	3·13	12·5	3·13	6·25	0·2	>640	100	+	−	+	*strA*, *B*
-2	3·13	12·5	3·13	6·25	0·2	>640	50	+	−	+	*strA*, *B*
-3	0·4	12·5	3·13	0·8	0·2	0·313	12·5	+	−	−	−
-4	3·13	12·5	3·13	6·25	0·8	>640	100	+	−	+	*strA*, *B*
-5	3·13	12·5	3·13	12·5	0·8	>640	100	+	−	+	*strA*, *B*
-6	3·13	12·5	3·13	6·25	0·2	>640	50	+	−	+	*strA*, *B*
-7	6·25	12·5	3·13	6·25	0·2	>640	100	+	−	+	*strA*, *B*
-8	3·13	12·5	3·13	6·25	0·8	>640	50	+	−	+	*strA*, *B*
-9	3·13	12·5	3·13	6·25	0·2	>640	100	+	−	+	*strA*, *B*
-10	3·13	12·5	3·13	6·25	0·2	>640	50	+	−	+	*strA*, *B*
-11	3·13	12·5	3·13	6·25	0·2	>640	100	+	−	+	*strA*, *B*
-12	3·13	12·5	3·13	6·25	0·2	>640	100	+	−	+	*strA*, *B*
02-1	0·4	12·5	3·13	0·8	0·4	1·25	6·25	−	−	−	−
-2	0·4	12·5	3·13	0·8	0·4	1·25	6·25	−	−	−	−
-3	0·4	6·25	3·13	0·8	0·4	2·5	6·25	−	−	−	−
-4	0·4	6·25	3·13	0·8	0·4	0·625	6·25	−	−	−	−
-5	0·4	12·5	3·13	0·8	0·4	1·25	6·25	−	−	−	−
-6	0·2	12·5	3·13	0·8	0·4	2·5	6·25	−	−	−	−
-7	0·4	6·25	3·13	0·8	0·4	0·625	6·25	−	−	−	−
-8	0·4	6·25	3·13	0·8	0·4	0·625	6·25	−	−	−	−
-9	0·4	6·25	3·13	0·8	0·4	2·5	12·5	−	−	−	−
-10	0·4	12·5	3·13	0·8	0·4	2·5	6·25	−	−	−	−
-11	0·2	12·5	3·13	0·8	0·4	2·5	6·25	−	−	−	−
-12	0·4	12·5	3·13	0·8	0·4	2·5	6·25	−	−	−	−

Drug susceptibility was expressed as MIC (μg/ml). Strains 95, 00, 02 indicate isolation in the year 1995, 2000, 2002 respectively. TC, tetracycline; EM, erythromycin; ABPC, ampicillin; CP, chloramphenicol; NA, nalidixic acid; ST, sulphamethoxazole–trimethoprim (19:1 compound); SM, streptomycin.

integrase gene (*intI1*) using the primers inDS-F and inDS-B [5]. The presence of SXT constin was examined by detection of the SXT integrase gene (int_{SXT}) using the primers INT1 and INT2 [10]. Streptomycin-resistance genes were detected using the primers in-F and aadA-B [5] for *aadA1*, STRA-F and STRA-B [10] for *strA*, and strB-F and strB-R (designed in this study) for *strB*. The chloramphenicol-resistance gene *floR* was detected using FLOR-F and FLOR-B [10]. The sulphamethoxazole-resistance gene *sulII* was detected using SUL2-F and SUL2-B [10]. The tetracycline-resistance gene *tetA* was detected using the primers TetA-F and TetA-R [11]. DNA was extracted by the method of Yokoyama [12]. All primers used in this study are listed in Table 1.

Analysis of drug-resistance gene clusters

The genetic organization of the gene cluster in SXT constin was determined essentially by primer walking referring to the sequence of SXT^{MO10} [7]. Amplified DNA was purified before sequencing using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany) and the

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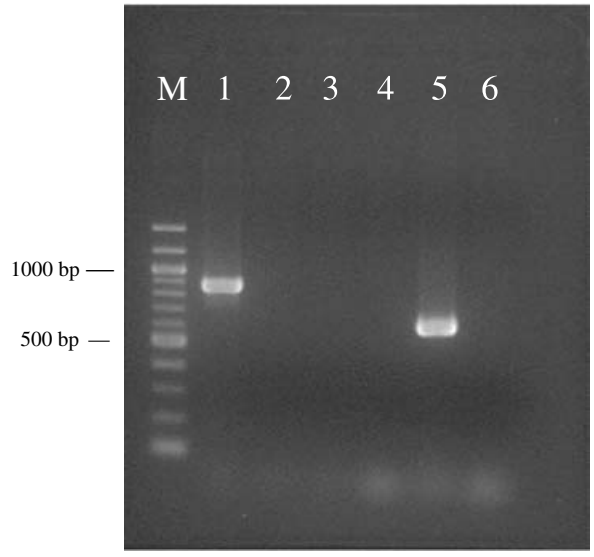

Fig. 1. Lanes 1 and 4, a strain isolated in 1995; lanes 2 and 5, a strain isolated in 2000; lanes 3 and 6, a strain isolated in 2002. Primers inDS-F and inDS-B were used for the first 3 lanes, and primers INT1 and INT2 were used for the next 3 lanes. The strain isolated in 1995 (lane 1) produced an 800-bp DNA fragment of the class I integrase gene, and the strain isolated in 2000 (lane 5) produced a 592-bp DNA fragment of the SXT integrase gene.

nucleotide sequence was determined using a cycle sequencer with an AmplitaqFS Dye Terminator kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, PerkinElmer, Foster City, CA, USA).

RESULTS

All *V. cholerae* strains examined in this study were resistant to polymyxin B, with an MIC higher than 50 μg/ml, which was compatible with the El Tor biotype. The MICs of the other drugs are shown in Table 2.

Isolates in 1995. The isolates were sensitive to all of the seven therapeutic antibiotics examined except for streptomycin. Ten out of the 12 isolates harboured a class I integron (Fig. 1) with the streptomycin-resistance gene *aadA1* as a gene cassette. The two isolates without a class I integron were sensitive to streptomycin. The MICs of streptomycin in the strains with a class I integron were higher than 25 μg/ml, whereas the MIC was 6·25 μg/ml in the strains without a class I integron (Table 2).

Isolates in 2000. Eleven out of 12 isolates were moderately resistant to tetracycline and chloramphenicol, and highly resistant to sulphamethoxazole–trimethoprim. The MIC of tetracycline was 3·13 μg/ml for 10 strains, 6·25 μg/ml for one strain (00-7), and 0·4 μg/ml for one susceptible strain (00-3). The MIC of chloramphenicol was 6·25 μg/ml for 10 strains, 12·5 μg/ml for one strain (00-5), and 0·8 μg/ml for one strain (00-3). The MIC of sulphamethoxazole–trimethoprim was higher than 640 μg/ml for 11 strains but was 0·313 μg/ml for one strain (00-3). The level of streptomycin resistance of these isolates was the same as that of the isolates in 1995. The strains isolated in 2000 did not have a class I integron or *aadA1* gene, but harboured SXT constin (Fig. 1). The genetic organization of the drug-resistance gene cluster within SXT constin was elucidated (Fig. 2), and it was found that SXT constin contained a gene cluster of approximately 10 kbp in which *floR*, *tetA*, *strA*, *strB* and *sulII* were located. The sequence of the gene cluster was determined, and it appears in the DNA Databank of Japan (DDBJ) with the accession number AB114188. The SXT constin of a strain (00-3) that was susceptible to all seven drugs was examined and found not to contain drug-resistance genes.

Isolates in 2002. All 12 isolates were susceptible to all seven drugs. These organisms did not harbour a class I integron or SXT constin (Fig. 1).

DISCUSSION

Cholera, in this seventh pandemic, appeared in Vietnam in 1964 [13], and repeated outbreaks have occurred since then. We studied *Vibrio cholerae* O1 strains isolated in 1995, 2000 and 2002, and found remarkable changes in drug susceptibility and in the corresponding genes of the organisms. These extensive changes of the epidemic strains are considered as being likely to have some epidemiological importance.

The strains isolated in 1995 harboured a class I integron, as Dalsgaard et al. reported previously [8]. Only one drug-resistant gene, *aadA1*, was detected within the class I integron, which was compatible with the antibiogram of the strains. Amita et al. [14] reported that a class I integron with an *aadA1* gene cassette was widely distributed among *V. cholerae* O1 strains isolated before 1992 (Pre-O139 period) in India; however, in Vietnam it was not detected in the strains isolated before 1992 [8]. Class I integron in *V. cholerae* O1 in Vietnam appeared after 1994 [8]. The strains isolated in 2000 were devoid of class I integron, but they contained SXT constin, with several drug-resistance genes. SXT constin, a mobile genetic element, was originally found in *V. cholerae* O139 [10], and it is speculated that the element has been transmitted to *V. cholerae* O1. Nevertheless,

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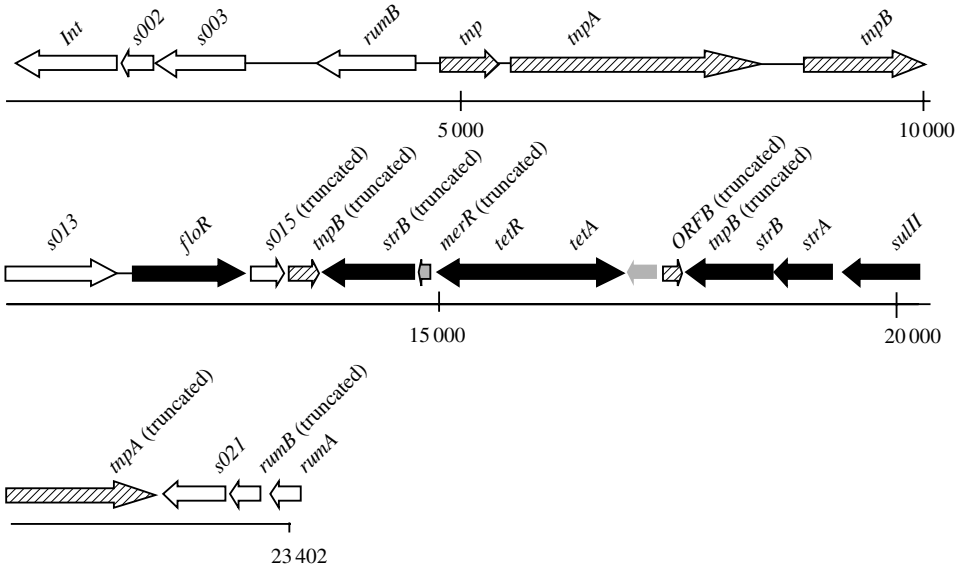

Fig. 2. Genetic organization and ORF map of the drug-resistance gene cluster within SXT constin (strain V21 corresponding to the strain 00-9 in Table 2). Putative ORFs are depicted by arrows showing the orientations. Integrase (*int*), antibiotic resistance (black), transposase (hatched), mercury resistance (grey). Antibiotic-resistance genes *floR* for chloramphenicol, *strB* for streptomycin, *tetR* and *tetA* for tetracycline, *strA* for streptomycin, and *sulII* for sulphamethoxazole.

V. cholerae O139 has not been isolated in Vietnam. It seems that Vietnamese strains isolated in 2000 are similar to Indian strains isolated after 1993 (Post-O139 period), since they harbour SXT constin and show resistance to sulphamethoxazole–trimethoprim, chloramphenicol, and streptomycin. The resistance genes for these antimicrobial agents are located in SXT constin [14]. However, strains from Vietnam in 2000 are tetracycline-resistant in constrast to Indian strains, which are susceptible to this antibiotic [9, 14]. The tetracycline resistance of *V. cholerae* O1 in Vietnam in 2000 was due to the *tetA* gene. We investigated the sequence of the integrated antibiotic-resistance gene cluster, and found the location of *tetA* within SXT constin. However, the trimethoprim-resistance gene was not found in the present study. The strains in 2002 had regained susceptibilities to all of the drugs examined, as if they were the strains isolated at the very beginning of this seventh cholera pandemic. Reflecting the susceptibility, the strains in 2002 were devoid of class I integron, SXT constin and each of the drug-resistance genes. Lao People's Democratic Republic (Lao PDR) is a neighbouring country sharing a long border with Vietnam. There were many outbreaks of cholera in Lao PDR in 2000, and the drug susceptibility [6] and genetic composition of the pathogens were almost identical to those in Vietnam in 2000 [15]. However, in Lao PDR, there was no cholera thereafter.

Cholera outbreaks in the three periods examined (1995, 2000 and 2002) were apparently due to different categories of *V. cholerae* O1. A question then arises 'How did the different categories appear and disappear?' In answer to this question, we propose some possibilities. First, all these categories are constantly present in the environmental flora in Vietnam and one category proliferates at a time. Secondly, these categories come from other countries, one by one, and produce outbreaks of one category of *V. cholerae* O1. Thirdly, one category of the organisms serially undergoes insertion and deletion of the drug-resistance genes, giving the appearance of belonging to a new category.

Drug-resistant pathogens usually appear after the use of therapeutic antibiotics, and the level of resistance increases step by step. However, in the past decade, drug-resistant *V. cholerae* O1 suddenly appeared regardless of antibiotic use, and in addition, they appeared simultaneously in a wide variety of districts for which there could not have been a single focus of infection. There must be many categories of *V. cholerae* in the environment, nevertheless the outbreaks due to *V. cholerae* O1 belonging to a certain category take place in a variety of districts. The reason why drug susceptibility of epidemic *V. cholerae* is uniform should be clarified. Some unknown environmental stimuli may enhance the proliferation of a certain category of *V. cholerae* in the environment

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and thereby induce outbreaks. Continuous surveillance for environmental *V. cholerae* O1 may lead to clues for answering these questions.

REFERENCES

1. Mhalu FS, Mmari PW, Ijumba J. Rapid emergence of El Tor *Vibrio cholerae* resistant to antimicrobial agents during the first six months of fourth cholera epidemic in Tanzania. Lancet 1979; **1**: 345–347.
2. Glass RI, Huq I, Alim ARMA, M Yunus. Emergence of multiply antibiotic-resistant *Vibrio cholerae* in Bangladesh. J Infect Dis 1980; **142**: 939–942.
3. Dalsgaard A, Forslund A, Serichantalergs O, Sandvang D. Distribution and content of class 1 integrons in different *Vibrio cholerae* O-serotype strains isolated in Thailand. Antimicrob Agents Chemother 2000; **34**: 642–650.
4. Dalsgaard A, Forslund A, Petersen A, et al. Class 1 integron-borne, multiple-antibiotic resistance encoded by a 150-kilobase conjugative plasmid in epidemic *Vibrio cholerae* O1 strains isolated in Guinea-Bissau. J Clin Microbiol 2000; **38**: 3774–3779.
5. Dalsgaard A, Forslund A, Sandvang D, Arntzen L, Keddy K. *Vibrio cholerae* O1 outbreak in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the *aadA2* gene located on class 1 integrons. J Antimicrob Chemother 2001; **48**: 827–838.
6. Phantouamath B, Sithivong N, Sisavath L, et al. Transition of drug susceptibilities of *Vibrio cholerae* O1 in Lao People's Democratic Republic. Southeast Asian J Trop Med Public Health 2001; **32**: 95–99.
7. Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R, Waldor MK. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob Agents Chemother 2001; **45**: 2991–3000.
8. Dalsgaard A, Forslund A, Tam NV, Vinh DX, Cam PD. Cholera in Vietnam: changes in genotypes and emergence of class I integrons containing aminoglycoside resistance gene cassettes in *Vibrio cholerae* O1 strains isolated from 1979 to 1996. J Clin Microbiol 1999; **37**: 734–741.
9. Nguyen BM, Higa N, Kakinohana S, Iwanaga M. Characterization of *Vibrio cholerae* O1 isolated in Vietnam. Jpn J Trop Med Hyg 2002; **30**: 103–107.
10. Hochhut B, Marrero J, Waldor MK. Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. J Bacteriol 2000; **182**: 2043–2047.
11. Schmidt AS, Bruun MS, Dalsgaard I, Larsen JL. Incidence, distribution and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl Environ Microbiol 2001; **67**: 5675–5682.
12. Yokoyama T. Study on *mec* gene in methicillin-resistant staphylococci. Kansenshogaku Zasshi 1993; **67**: 1203–1210.
13. Felsenfeld O. Areview of recent trends in cholera research and control. Bull Wld Hlth Org 1966; **34**: 161–195.
14. Amita SR, Chowdhury M, Thungapathra T, Ramamurthy G, Nair GB, Ghosh A. Class I integrons and SXT elements in El Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. Emerg Infect Dis 2003; **9**: 500–502.
15. Iwanaga M, Toma C, Miyazato T, Insisiengmay S, Nakasone N, Ehara M. Antibiotic resistance conferred by Class I integron and SXT constin in Vibrio cholerae O1 isolated in Lao People's Democratic Republic. Antimicrob Agents Chemother (in press).