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Multiple Drug Resistance of *Vibrio cholerae* O1 and O139 Isolated from Various Regions of the World: Changes in the Past 10 Years

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Summary. **In vitro susceptibilities to 35 antimicrobial agents were investigated in 319 strains of *Vibrio cholerae* O1 and 213 strains of *V. cholerae* O139 isolated from various regions of the world between 1991 and 1999. Drug resistance was found with 12 of the 35 antimicrobial agents and was divided into chromosome-encoded multiple drug resistance (VC MAR, found in plasmid-less strains) and that encoded by drug resistance (R) plasmids. VC MAR was subtyped into eight major groups. In Bangladesh, the VC MAR pattern changed from that including tetracycline resistance (VC MAR2) since the 1990s to a tetracycline-susceptible, nalidixic acid-low resistant pattern (VC MAR3a), and then to a nalidixic acid-highly resistant pattern (VC MAR3b). The VC MAR3b type also showed low resistance to a newer quinolone, norfloxacin. R plasmids were isolated from a *V. cholerae* O139 strain from Thailand and *V. cholerae* O1 strains from Rwanda, Tanzania, and Peru. In the case of *V. cholerae* O139, this was the second isolate after the previous R plasmid from Indian strains. *V. cholerae* O1 isolated in 1997 from cholera patients in Japan who had never been abroad showed a VC MAR6 pattern, which was the same as that of *V. cholerae* O1 occurring in travelers to Bali, Indonesia in 1995. This study showed that the multiple drug resistance of *V. cholerae* O1 and O139 differed among regions and eras due to changes in VC MAR and the presence of R plasmids, and that the isolates were still susceptible to some antimicrobial agents such as azithromycin and tetracycline (e.g., in Bangladesh).**

Key words—multiple drug resistance, *Vibrio cholerae* O1, *Vibrio cholerae* O139, VC MAR pattern, R plasmid.

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INTRODUCTION

Cholera is currently in the 7th pandemic that started in Indonesia in 1961[1]. The pathogen is *Vibrio cholerae* O1 biotype El Tor and the reservoir of infection is such locations as India and Bangladesh. The pathogenicity of the El Tor biotype is considered to be weaker than the *V. cholerae* O1 biotype classical, which was the pathogen of up to the 6th cholera pandemic[1,2]. However, *V. cholerae* O1 El Tor still maintains a strong epidemic potential, and its epidemiological pathogenicity should not be neglected. In 1991, *V. cholerae* O1 El Tor appeared in South and Central America, starting in Peru, where no large cholera endemics have been recorded in this century, and ca. 3.9 millions cases with ca. 4 thousand deaths were recorded from Latin America[3,4]. In 1992, a new type *V. cholerae* with a new serotype O139, which was resistant to previous cholera vaccines, appeared in India and was rapidly transmitted to neighboring countries such as Bangladesh and Thailand which expanded the epidemic area[5-10]. *V. cholerae* O139 has been shown to be genetically related to *V. cholerae* O1 El Tor, and now recognized as an agent of another wave of epidemics within the 7th cholera pandemic[11]. In 1994, *V. cholerae* O1 El Tor attacked Rwandan refugees in Africa, resulting in about 58,057 cholera cases and 4,181 deaths in July, 1994[12].

For the treatment of cholera, oral and intravenous rehydration therapy has been successfully employed. Chemotherapy is also effective for reducing stool output and the duration of diarrhea, and has widely been used as a supplement therapy in epidemic regions[13]. With an extensive use of antimicrobial

agents, the appearance of drug-resistant strains of *V. cholerae* has increasingly been reported.

Kuwahara et al. have isolated drug resistance (R) plasmid in *V. cholerae* O1 isolated from Philippines in 1967[14]. Ramamurthy et al.[9] and Albert et al.[5] have shown that most *V. cholerae* O139 strains are resistant to sulfamethoxazole, trimethoprim, furazolidone, streptomycin, or vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine). Moreover, Albert et al. showed in 1993 that about 70% of *V. cholerae* O1 strains in Bangladesh were resistant to tetracycline[5].

Our previous studies on *V. cholerae* O1 and O139 isolated in India, Bangladesh, and Thailand have shown that the multiple drug resistance included two distinct types: one (designated VC MAR1 and 2)[15] being found in plasmid-less strains (and thus considered to be chromosome-encoded) and another due to conjugative R plasmid (e.g., pNON1 found in *V. cholerae* O139)[16]. However, the distribution of VC MAR types and R plasmids among *V. cholerae* O1 and O139 strains isolated in the world and in eras remains unclear.

In this study, we investigated in vitro susceptibilities to antimicrobial agents of *V. cholerae* O1 and O139 isolated from various regions of the world, and have summarized the changes in drug resistance due to VC MAR (found in plasmid-less strains) and conjugative R plasmid over the past 10 years.

MATERIALS AND METHODS

Bacterial strains

All *V. cholerae* strains used in this study were of clinical origin. A total of 295 O1 El Tor strains examined in this study included 13 strains from India isolated from 1995 to 1997, 71 strains from Bangladesh isolated from 1992 to 1999, 4 strains from Thailand isolated in 1998 and 1999, 41 strains from Japan isolated from 1991 to 1997, 54 strains from Peru isolated from 1991 to 1995, 30 strains from Bolivia isolated in 1994, 5 strains from Rwanda isolated in 1994, 15 strains from Tanzania isolated in 1991 and 1992, 8 strains from Nigeria isolated in 1991, 9 strains from Djibouti isolated in 1993, and 10 strains from USA isolated in 1992.

The remaining 35 strains of *V. cholerae* O1 were isolated from Japanese travelers who visited Indonesia (Bali) during February and March; among them, 26 were isolated at the Narita Airport Quarantine when the travelers returned back to Japan, and 8 were isolated in Japanese domestic areas 1 to 6 days after arrival at Narita.

V. cholerae O1 atypical biotype strains (24 strains) from Bangladesh isolated from 1991 to 1994 were also included in this study. *V. cholerae* O1 biotype El Tor is Voges-Proskauer (VP) reaction-positive, polymyxin B-resistant, chicken erythrocytes agglutination-positive, sheep erythrocytes lysis-positive, phage IV-resistant, and phage V-sensitive[17]. In contrast, *V. cholerae* O1 biotype classical showed different results on these tests[17]. The atypical strains employed in this study were negative for chicken erythrocytes agglutination and thus initially diagnosed as a classic biotype, but showed intermediate results between biotypes El Tor and classic in other tests.

The 213 O139 strains examined included 151 strains from India isolated from 1992 to 1997, 49 strains from Bangladesh isolated from 1992 to 1999, and 13 strains from Thailand isolated from 1992 to 1998.

The above strains were kindly provided by Yoshifumi Takeda (Kyoto University, Kyoto), Peter Echeverria (Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand), M. John Albert (International Center for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh), Carlos Carrillo Parodi (National Institute of Health, Lima, Peru), Haruo Watanabe (National Institute of Infectious Diseases, Tokyo), Masako Uchimura (Public Health Laboratory of Chiba Prefecture, Chiba), and Yoshinori Hayashi and Michiya Ootaka (Narita Airport Quarantine Station, Chiba). All isolates were stored frozen at −80℃.

V. cholerae O1 strain 569B (classic biotype)[18] and *V. cholerae* O139 strains MAC757 (kindly provided by Yoshifumi Takeda) were used as a standard strain. *E. coli* strains HB101 (a streptomycin-resistant hybrid between *E. coli* K12 and *E. coli* B lacking restriction ability), CSH2-1 (a nalidixic acid-resistant derivative of *E. coli* K12 strain CSH2), and W677-1 (a rifampicin-resistant derivative of *E. coli* K12) were used as a host of plasmids.

E. coli strain ML4905, which harbored an incompatibility group C (IncC) R plasmid R40a, IncJ R plasmid R391, IncP R plasmid RP4, IncT R plasmid Rtsl, or IncX R plasmid R6K was kindly provided by Shizuko Iyobe (Gunma University, Gunma).

Media and bacterial growth

For bacterial growth, we used L broth (Difco Laboratories, Detroit, Mich.) as a liquid medium which was inoculated and incubated at 37℃ for 12-18 h with agitation. Nutrient agar (Eiken Chemical, Tokyo), TCBS agar (Eiken Chemical), MacConkey agar (Eiken Chemical), and Mueller-Hinton agar (Difco Laboratories) were used as the solid media.

Antimicrobial agents

The antimicrobial agents were a gift from their manufacturers. They included: ampicillin (ABPC) for penicillins; faropenem (FRPM) for carbapenems; cefoperazone (CPZ) and cefixime (CFIX) for broad-spectrum cephems; tetracycline (TC), doxycycline (DOXY), and minocycline (MINO) for tetracyclines; chloramphenicol (CM); streptomycin (SM), kanamycin (KM), and gentamicin (GM) for aminoglycosides; spiramycin (SPM), oleandomycin (OL), midecamycin (MDM), josamycin (JM), rokitamycin (RKM), kitasamycin (LM), roxithromycin (RXM), clarithromycin (CAM), erythromycin (EM), azithromycin (AZM)[19] for macrolides; lincomycin (LCM); clindamycin (CLDM); nalidixic acid (NA), norfloxacin (NFLX), ofloxacin (OFLX), tosufloxacin (TFLX), ciprofloxacin (CPLX), sparfloxacin (SPFX), and sitafloxacin (STFX)[20] for quinolones. TC, DOXY, MINO, NA, NFLX, CPFX, EM, CAM, and AZM were from Wyeth Lederle Japan (Tokyo), Pfizer Pharmaceuticals (Tokyo), Wyeth Lederle Japan, Daiichi Pharmaceutical Co. (Tokyo), Daiichi Pharmaceutical, Bayer Yakuhin (Osaka), Shionogi & Co. (Osaka), Taisho Pharmaceutical Co. (Tokyo), and Pfizer Pharmaceuticals, respectively. Furazolidone, 3-(5-nitrofurfurylideneamino)-2-oxazolidinone (FZ), was kindly provided by G. Balakrish Nair (National Institute of Cholera and Enteric Diseases, Calcutta, India). Sulfamethoxazole (SMX) and trimethoprim (TMP) (Shionogi) were used either alone or in combination at a ratio of 5 : 1 (as in the combination drug) or 20 : 1 (the expected ratio in the human body). O/129 (2, 4-diamino-6,7-diisopropylpteridine phosphate)[15] and polymyxin B (PL-B) were purchased from Sigma Chemical (St. Louis, MO). Colistin (CL) was acquired from Kayaku Co., Tokyo.

Susceptibility testing

Susceptibility testing of bacterial strains was done by the agar dilution method with Mueller-Hinton agar according to standard procedures[21]. The final concentrations of antimicrobial agents were from 0.004 to 128 mg/ml. The test bacteria were grown for 18 h at 37℃ with agitation in L broth, and diluted to approximately 10^6 CFU/ml. Aliquots of the bacterial suspension (approximately 10^4 CFU of bacteria per spot) were inoculated on the surface of antimicrobial agent-containing agar plates. Incubation was for 20 h at 37℃. The MIC was determined as previously described[21]. *E. coli* NIHJ JC-2 was used as a reference strain for quality control[21]. When the susceptibility to sulfamethoxazole or trimethoprim was tested, Mueller-Hinton agar supplemented with 7.5% (vol/vol) defibrinated horse blood (frozen and thawed) was also used, in addition to Mueller-Hinton agar alone[22].

Plasmid analysis

Plasmids of *V. cholerae* O1 and O139 strains were analyzed as previously described[16,23]. Briefly, bacterial cells grown in L broth (5 ml) were suspended in 100 μl of 40 mM Tris-acetate (pH 7.9) containing 2 mM EDTA (pH 7.9) in a 1.5-ml microcentrifuge tube. This was followed by an addition of 200 μl of lysis solution (3% SDS, 50 mM Tris, and 0.128 N NaOH) at room temperature. After being mixed by brief agitation, the solution was heated to 55℃ for 20 min. The solution was then mixed with 600 μl of phenol-chloroform (1 : 1, vol/vol) by brief shaking. It was then centrifuged at room temperature, and the resultant upper aqueous phase (~160 μl) was retained. Plasmid DNA thus prepared (10 μl) was electrophoresed in 0.3% or 0.7% agarose with reference plasmid DNAs of a known molecular size (including the NR1 plasmid 94.5 kb in size[24] and 236-kb *Shigella flexneri* plasmid kindly provided by Chihiro Sasakawa, Institute of Medical Science, University of Tokyo, Tokyo).

Bacterial mating

Bacterial mating was conducted for 3 h at 37℃ in L broth or for 18 h at 37℃ on membrane filters placed on agar media, as previously described[25]. Transconjugants were selected on agar plates for both donor and recipient resistance markers.

Incompatibility group testing

E. coli strains that carried a drug resistance plasmid from *V. cholerae* O1 and O139 and a standard R plasmid of the known incompatibility group were constructed by bacterial mating or transformation. Stability of the two plasmids within the same *E. coli* cells was then tested, as previously described[26,27].

RESULTS

Drug resistance and resistance patterns of clinical isolates of *V. cholerae* O1 and O139

Drug resistance was detected with 12 of 35 antimicrobial agents examined in the *V. cholerae* O1 and O139 strains isolated from Bangladesh, India, Thailand,

Japan, Indonesia, Rwanda, Tanzania, and Peru. None of the Djibouti and Nigerian strains of *V. cholerae* O1 examined were drug-resistant, nor were there drug resistant strains from Bolivia and USA.

Drug resistance patterns and resistance levels of *V. cholerae* O1 and O139 are summarized in Table 1. In Asia, the majority of drug resistance was not due to plasmids. Those chromosome-encoded multiple drug resistance patterns (designated VC MAR) were subtyped into eight major groups, VC-MAR1 to VC-MAR6 (Table 2).

In the case of *V. cholerae* O1 strains from Bangladesh (Table 1), the major drug resistance pattern (found in 54.1%) in 1992 was VC MAR2, seven-drug resistance to tetracycline, chloramphenicol, furazolidone, streptomycin, sulfamethoxazole, trimethoprime, and O/129. The major resistance pattern changed to VC MAR1, in which tetracycline resistance was excluded from VC MAR2, in 1994. A eight-drug resistance pattern VC MAR4a, in which nalidixic acid-low resistance was added to VC MAR2, emerged as a minor pattern in the same year. In 1999, all the O1 strains examined showed nalidixic acid resistance; the major resistance pattern was VC MAR3a (VC MAR1 plus nalidixic acid-low resistance) and the minor pattern was VC MAR3b (VC MAR1 plus nalidixic acid-high resistance).

Atypical *V. cholerae* O1 strains, which was initially diagnosed as *V. cholerae* O1 biotype classical, appeared in Bangladesh during 1991–1994 and exhibited the VC MAR1 and VC MAR2 phenotypes in 83.3% (Table 1).

V. cholerae O139 strains appeared during 1992 and 1993 in Bangladesh and showed a major resistance pattern of VC MAR1 (found in 96.7%), which has become a minor pattern since 1995 (Table 1).

In India, major resistance patterns of *V. cholerae* O1 strains isolated in 1995 and 1997 were VC MAR3a or VC MAR3a-FZ (VC MAR3a derivative lacking furazolidone resistance). Those resistance patterns included nalidixic acid resistance similar to the case in Bangladesh (Table 1). Tetracycline resistance was not detected. In addition to the above multiple drug resistance patterns, one *V. cholerae* O1 strain from India isolated in 1993 showed a VC MAR3a-SM pattern plus ampicillin resistance (MIC, $\geq$256) and cefoperazone resistance (MIC, $\geq$256).

In 1992, *V. cholerae* O139 strains emerged in India and showed a VC MAR1 phenotype. Nalidixic acid-low or high resistance-including VC MAR types (VC MAR3a and VC MAR4b-FZ) existed as a minor pattern during 1992 and 1993 and in 1995 (Table 1). Some O139 strains isolated during 1992 and 1993 showed higher resistance to tetracycline and chloramphenicol compared with the resistance levels of VC MAR2 or VC MAR1; this higher resistance was due to the presence of R plasmid (pNON1).

In Thailand, recent isolates of *V. cholerae* O1 showed VC MAR2 (Table 1). VC MAR1 was found in *V. cholerae* O139 strains. One O139 strain isolated in 1994 showed a higher resistance to tetracycline and chloramphenicol compared with the resistance levels of VC MAR2 or VC MAR1; this higher resistance was due to R plasmid (pNON T1).

In Japan, VC MAR1 existed in *V. cholerae* O1 strains in 1991 (Table 1). In summer 1997, many cases of cholera in patients who had never been abroad were reported in Japan. The *V. cholerae* O1 strains from those Japanese cholera patients in 1997 showed resistance to drugs that were not applied to cholera treatment in Japan. The most frequent resistance pattern was three-drug resistance to furazolidone, streptomycin, and sulfamethoxazole (VC MAR6). This multiple drug resistance pattern was found in 58.8% of isolates, and some isolates were only resistant to one or two of the drugs. Isolates susceptible to all drugs accounted for 2.9%. Fig. 1 shows the nationwide distribution of the resistance patterns. VC MAR6 was more frequent in the Kanto area.

The multiple drug resistance pattern (VC MAR6) of *V. cholerae* O1 strains found in cholera patients in 1997 who had never been abroad was identical to the drug resistance pattern (VC MAR6) detected in the *V. cholerae* O1 strains isolated from Japanese travelers to Indonesia (Bali) in 1995 (Table 1).

In African cases, multiple drug resistance was detected in specimens from Rwanda and Tanzania (Table 1). Those *V. cholerae* O1 strains showed higher levels of resistance to tetracycline and chloramphenicol compared with the resistance levels of VC MAR2 or VC MAR1; this higher resistance was due to R plasmid (pELT R1 and pELT T1, respectively).

V. cholerae O1 strains from Peru had R plasmid (pELT P1) whose presence resulted in higher levels of resistance to tetracycline and chloramphenicol (Table 1).

Conjugal transfer and drug resistance patterns of R plasmids

V. cholerae O1 and O139 strains showing higher levels of resistance to tetracycline (MIC, $\geq$16) or chloramphenicol (MIC, $\geq$32) carried a self-transmissible R plasmid. Characteristics of the isolated R plasmids are summarized in Table 3.

An R plasmid (pNON1) isolated from an Indian O139 strain belonged to IncC, and was able to trans-

Table 1. Multiple drug resistance patterns of *V. cholerae* O1 and O139 isolated from various regions of the world[a)]

Isolation and serotype	Year (Number of isolates)	Incidence of resistance strains (%)	MIC (μg/ml)								Resistance pattern
			TC	CM	FZ	SM	SMX	TMP	O/129	NA	
Bangladesh											
O1 El Tor	1992(n=24)	13/24 (54.1%)								0.125-0.25	VC MAR 2
		8/24 (33.3%)	0.25-0.5	1				0.125-0.25	2-4	0.125-0.25	VC MAR 6
		1/24 (4.2%)	0.5							0.125-0.25	VC MAR 1
	1994 (n=42)	18/42 (42.9%)	0.5-1							0.25	VC MAR 1
		17/42 (40.5%)								0.25	VC MAR 2
		4/42 (9.5%)	0.5	1				0.25-0.5	4-8	0.25	VC MAR 6
		2/42 (4.8%)									VC MAR 4a
		1/42 (2.4%)	0.5	1	0.25	16		0.5	4	0.25	SMX
	1999 (n=5)	4/5 (80.0%)	0.25-0.5								VC MAR 3a
		1/5 (20.0%)	0.25								VC MAR 3b
O1 atypical	1991-1994 (n=24)	18/24(75.0%)	0.5							0.125-0.25	VC MAR 1
		2/24 (8.3%)								0.125	VC MAR 2
		1/24 (4.2%)	0.25			64				0.125	(VC MAR 1)
		1/24 (4.2%)	0.5	1				0.25	4	0.25	VC MAR 6
		1/24 (4.2%)	0.5	1		16	2			0.125	FZ • TMP
		1/24 (4.2%)	0.25	0.5	2	16	16			0.25	TMP
O139	1992-1993 (n=30)	29/30(96.7%)	0.25-0.5							0.25	VC MAR 1
		1/30 (3.3%)	0.25	1		8		0.5	4	0.25	FZ • SMX
	1995 (n=14)	8/14 (57.1%)	0.25-0.5	1		8	2-4	0.13-0.25	2-4	0.25	FZ
		5/14 (35.7%)	0.25-0.5							0.25	VC MAR 1
		1/14 (7.1%)	0.5	1		8	4	0.25	4		FZ • NA
	1999 (n=5)	5/5 (100%)	0.25-0.5	1		16-32	2-4	0.125-0.25	2	0.125-0.25	FZ
India											
O 1 El Tor	1995 (n=8)	8/8 (100%)	0.25-0.5								VC MAR 3a
	1997 (n=5)	3/5 (60.0%)	0.25		0.25						VC MAR 3a-FZ
		2/5 (40.0%)	0.25								VC MAR 3a
O 139	1992-1993 (n=134)	120/134(90.0%)	0.25								VC MAR 1
		7/134 (5.2%)	0.25-0.5								VC MAR 3a
		6/134 (4.5%)	8-16	32			≥256	≥256	≥256	0.25-0.5	VC MAR[b)] +R (pNON1)
		1/134 (0.7%)	0.5	1				0.5	2	0.25	VC MAR 6

	1995 (n=7)	4/7 (57.1%)	0.25-0.5	8	4	≥128	≥256	≥256	≥256	0.25	VC MAR 1
		1/7 (14.3%)	4	8	0.25	128	≥256	≥256	≥256	≥256	VC MAR 4b-FZ
		1/7 (14.3%)	0.5	8	4	128	≥256	≥256	≥256	8	VC MAR 3a
		1/7 (14.3%)	0.5	8	8	128	≥256	0.25	2	0.25	VC MAR 5
	1997 (n=10)	9/10 (90.0%)	0.25	4	4	128	≥256	0.25-0.5	2	0.25	VC MAR 5
		1/10 (10.0%)	0.25	0.25	4	8	8	0.5	2	0.25	FZ
Thailand											
O1 El Tor	1998 (n=2)	2/2	4	4-8	16	≥256	≥256	≥256	≥256	0.25	VC MAR 2
	1999 (n=2)	1/2	4	8	16	128	≥256	≥256	≥256	0.125	VC MAR 2
		1/2	0.25	1	16	128	≥256	0.25	4	0.25	VC MAR 6
O 139	1992-1993 (n=9)	9/9 (100%)	0.25	8	8-16	≥256	≥256	≥256	≥256	0.25	VC MAR[c]+R (pNON T1)
	1994 (n=2)	1/2	32	16	8	≥256	≥256	≥256	≥256	0.25	SMX[d]
		1/2	0.5	0.5	0.125	8	≥256	0.25	4	0.125	FZ
	1997 (n=1)	1/1	0.25	1	8	16	8	0.5	8	0.25	VC MAR 1
	1998 (n=1)	1/1	0.25	4	8	128	≥256	≥256	≥256	0.125	
Japan											
O1 El Tor	1991 (n=3)	1/3 (33.3%)	0.25	8	8	≥256	≥256	≥256	≥256	0.25	VC MAR 1
	1994 (n=2)	2/2	0.25	1	8	128-≥256	≥256	0.25-0.5	2-4	0.25	VC MAR 6
	1995 (n=2)	2/2	0.25	1	8	128-≥256	≥256	0.25-0.5	4	0.25	VC MAR 6
	1997 (n=34)	20/34(58.8%)	0.25	0.5	4-16	64-128	≥256	0.25-0.5	2-4	0.13-0.25	VC MAR 6
		6/34 (17.6%)	0.25	0.5	0.25	64-128	≥256	0.5	2	0.13	SM・SMX
		3/34 (8.8%)	0.25	0.5	0.25	32	≥256	0.25-0.5	2	0.13	(SM)・SMX
		2/34 (5.9%)	0.25	0.5	8	8	4	0.5	2	0.13	FZ
		2/34 (5.9%)	0.25	0.5	16	32	≥256	0.5	2-4	0.13	FZ・(SM)・SMX
Indonesia (Bali)											
O1 El Tor	1995 (n=35)	34/35 (97.1%)	0.25	1	8	128	≥256	0.25-0.5	2-4	0.13-0.25	VC MAR 6
Rwanda											
O1 El Tor	1994 (n=5)	5/5(100%)	16-32	32-64	0.25-0.5	≥256	≥256	≥256	≥256	8-16	R (pELT R1)
Tanzania											
O1 El Tor	1991-1992 (n=15)	15/15(100%)	16	32-64	8	≥256	≥256	≥256	≥256	0.25-0.5	VC MAR[c]+R (pELT T7)
Peru											
O1 El Tor	1991 (n=52)	1/52 (1.9%)	0.5	0.25	1	0.25-8	≥256	0.25	2	0.125	SMX
	1995 (n=2)	2/2	64	8	0.25	16	≥256	0.25-0.5	4	0.25	R (pELT P1)

a) Red color (and faint red color) represents VC MAR-related drug resistance, and yellow color represents R plasmid-related drug resistance.

b) Resistance to (at least) FZ and SM.

c) Resistance to (at least) FZ .

d) The strain was also resistant to ampicillin (MIC, 32 μg/ml).

Table 2. Major multiple drug resistance patterns of *V. cholerae* O1 and O139 found in Asian countries

VC MAR type or standard susceptible strain	Drug resistance pattern (MIC, μg/ml)							
	TC	CM	FZ	SM	SMX	TMP	O/129	NA
VC MAR 1		4-8	4-8	≥256	≥256	≥256	≥256	
VC MAR 2	4-8	4-16	4-16	128-≥256	≥256	≥256	≥256	
VC MAR 3a		4-16	2-16	64-≥256	≥256	≥256	≥256	4-16
VC MAR 3b		8	16	≥256	≥256	≥256	≥256	≥256
VC MAR 4a	4-8	8	8	128-≥256	≥256	≥256	≥256	8
(VC MAR 4b	4-8	8	8	128-≥256	≥256	≥256	≥256	≥256)[a]
VC MAR 4b-FZ	4	8		128	≥256	≥256	≥256	≥256
VC MAR 5		4-8	8	≥256	≥256			
VC MAR 6			4-16	128-≥256	≥256			
569B (O1)	0.13	0.5	0.13	8	128[b]	0.5	2	0.25
MAC757 (O139)	0.13	1	0.25	8	128[b]	0.5	2	0.25

a) VC MAR4b is a proposed pattern (not detected in this study).

b) Standard strains 569B and MAC757 of *V. cholerae* O1 and O139, respectively, were resistant to SMX. MICs (μg/ml) of SMX for many susceptible strains of *V. cholerae* O1 and O139 were 2-16.

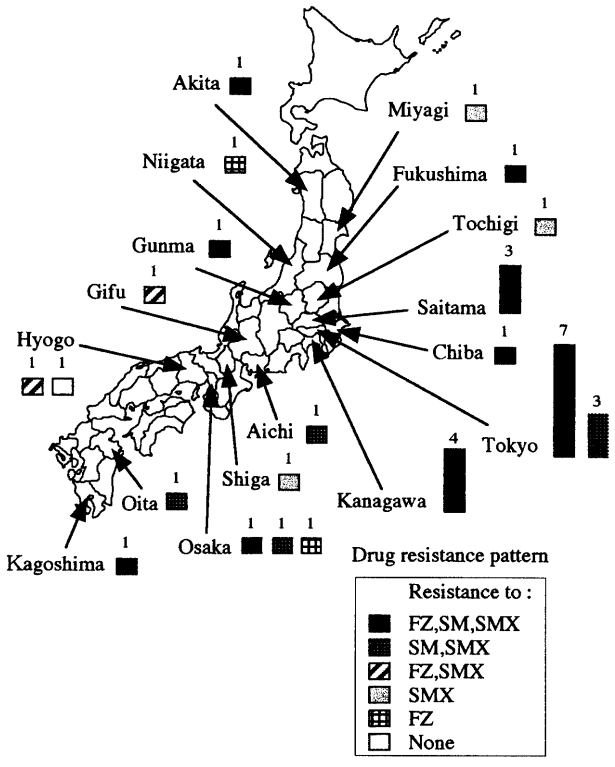

Fig. 1. Nationwide distribution of drug resistance patterns of *V. cholerae* O1 strains isolated in 1997 from cholera patients in Japan who had never been abroad. Numbers represent bacterial isolates.

Table 3. Drug resistance plasmids isolated from *V. cholerae* O 1 and O 139

R plasmid	Original host (isolation)	Incompatibility group	Plasmid size (kb)	Drug resistance levels (MIC μg/ml) of: *V. cholerae* original strains (upper) and *E. coli* transconjugants[a] (in parentheses)									Frequency of conjugal transfer to *E. coli* (transconjugants/donor)
				TC	CM	SM	SMX	TMP	O/129	ABPC	KM	GM	
pNON 1[b]	*V. cholerae* O 139 (India,1992)	Inc C	200	8-16 (32)	32 (≥256)	No[c]	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	128->256 (128)	2.7×10^{-3} [d]
pNON T 1	*V. cholerae* O 139 (Thailand,1995)	Other than Inc C,J,P,T,X	>300	32 (≥256)	16 (128)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	No[c]	No[c]	No[c]	1.1×10^{-7} [e]
pELT T 7	*V. cholerae* O 1 (Tanzania,1992)	ND[f]	159	16 (64)	32-64 (128)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	No[c]	No[c]	4.4×10^{-8} [g]
pELT R 1	*V. cholerae* O 1 (Rwanda,1994)	Inc C	186	16-32 (64)	32-64 (128)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	≥256 (≥256)	No[c]	No[c]	2.7×10^{-5} [h]
pELT P 1	*V. cholerae* O 1 (Peru,1995)	Inc C	135	64 (≥256)	8 (128)	No[c]	≥256 (≥256)	No[c]	No[c]	No[c]	No[c]	No[c]	2.0×10^{-1} [i]

a) MICs (μg/ml) of TC, CM, SM, SMX, TMP, O/129, ABPC, KM, and GM for *E. coli* strains (CSH 2-1 and W 677-1) used as a recipient were 1-2, 4, 2, 8, 0.06-0.25, 16-32, 2-4, 2, and 0.25, respectively.

b) Reference 16.

c) No, No resistance.

d) Conjugal transfer was conducted in liquid media for 3 h (recipient *E. coli* strain, CSH 2-1).

e) Conjugal transfer was conducted on membrane filters for 18 h (recipient *E. coli* strain, CSH 2-1).

f) ND, Not determined.

g) Conjugal transfer was conducted on membrane filters for 18 h (recipient *E. coli* strain, W 677-1).

h) Conjugal transfer was conducted in liquid media for 3 h (recipient *E. coli* strain, W 677-1).

i) Conjugal transfer was conducted in liquid media for 3 h (recipient *E. coli* strain, W 677-1).

fer to *E. coli* strains at a relatively high frequency. In contrast, an R plasmid (pNON T1) isolated from a Thai O139 strain did not belong to IncC and had a very big molecular size, and its frequency to transfer to *E. coli* was low. Both pNON1 and pNON T1 did not confer furazolidone resistance.

The transfer frequency of pELT T7, isolated from a Tanzanian O1 strain, was very low. pELT T7 did not confer furazolidone resistance. pELT R1, isolated from a Rwandan O1 strain, belonged to IncC, was transferred to *E. coli* at a moderate level of frequency, and did not confer nalidixic acid resistance. pELT P1, isolated from a Peruvian O1 strain, belonged to IncC, and was transferred to *E. coli* at an extremely high frequency (Table 3).

In contrast, no plasmids were detected in other *V. cholerae* O1 and O139 strains with the VC MAR phenotypes.

Drugs active against the multiple drug resistant strains of *V. cholerae* O1 and O139

All the multiple drug resistant strains of *V. cholerae* O1 and O139, irrespective of the VC MAR phenotypes or the presence of R plasmids, were highly susceptible to minocycline, ciprofloxacin, and azithromycin (Table 4). The VC MAR3b and VC MAR4b-FZ types showed a moderate level of resistance to norfloxacin. No strains showed lower susceptibilities to erythromycin.

DISCUSSION

Rehydration is an effective treatment for cholera[28,29]. Additionally, chemotherapy has also been recommended as a supplement therapy in epidemic regions to reduce the diarrhea stool volume or shorten the duration of diarrhea or the vibrios-shedding period[29]. Cheap antimicrobial agents such as tetracycline[30], doxycycline[31], furazolidone[30,32], trimethoprime-sulfamethoxazole (ST-compounding agent)[33,34], quinolones[34,35], and erythromycin[36] have been used for treating cholera in epidemic areas. The administration of antimicrobial agents for the purpose of preventing infection has also been recommended when the secondary infection is marked[13,37,38].

Through the pioneering studies by Kuwahara and Goto, transmissible multidrug resistant R plasmid showing resistance to chloramphenicol, tetracycline, streptomycin, and sulfamethoxazole was discovered in Philippine strains of *V. cholerae* O1 in 1967[14]. This was followed by the isolation by Prescott et al. in 1968 in Calcutta strains[39]. These R plasmids of *V. cholerae* O1 were self-transmissible, and belonged to IncC[27]. Multiple drug resistance was detected in 1-9% of isolates around 1970[40].

In 1992, a new serotype (O139) of the cholera agent appeared in India[9], and multiple drug resistance started attracting attention again. In *V. cholerae* O139, most isolates were resistant to the ST-compounding agent, furazolidone, and streptomycin[5,9]. We found a self-transmissible R plasmid in Indian O139 isolates in 1995[16]. In Bangladesh, 70% of *V. cholerae* O1 El Tor strains isolated during the same period were resistant to tetracycline[5].

In the Asian epidemics, the major multiple drug resistance of *V. cholerae* O1 and O139 strains was not due to plasmid, but was chromosomal[15]. The typical multiple drug resistant pattern was VC MAR1 showing resistance to chloramphenicol, furazolidone, streptomycin, sulfamethoxazole, trimethoprim, and O/129[15]. VC MAR2, in which tetracycline-resistance was added to VC MAR1, was found in Bangladesh *V. cholerae* O1 strains[15].

In this study, we further identified the following new VC MAR types; VC MAR3a in which nalidixic acid-low resistance was added to VC MAR1, VC MAR3b in which nalidixic acid-high resistance was added to VC MAR1, VC MAR4a in which nalidixic acid-low resistance was added to VC MAR2, VC MAR4b-FZ in which nalidixic acid-high resistance was added to VC MAR2 but furazolidone resistance was reduced, VC MAR5 in which resistance to trimethoprim and O/129 was reduced from VC MAR1, and VC MAR6 which was three-drug resistant to furazolidone, streptomycin, and sulfamethoxazole. It is conceivable that these VC MAR types are inter changeable, and the extensive use of antimicrobial agents selects one or more of the types, as shown in Fig. 2.

In some bacteria, a single specific mechanism called an efflux pump system plays a role in multiple drug resistance, in which resistance occurs when the drug entering the cell membrane(s) of the bacteria is pumped out[41,42,43]. Broad drug resistance patterns of *V. cholerae* O1 and O139 such as the chloramphenicol-, tetracycline-, and nalidixic acid-resistance found in this study may be explained by multiple drug resistance pumps. It has been shown that *V. cholerae* O1 strain 569B has genes which resemble the *E. coli* efflux pump genes[44]. However, strain 569B is susceptible to most of the drugs as shown in the text (Table 2), and thus the function of the 569B genes remains unclear. We are now investigating the possibility of the presence of "VC MAR pumps" in chromosomally-mediated, multiple drug-resistant strains of *V. cholerae* O1 and O139.

Table 4. Susceptibilities to tetracyclines, quinolones, and macrolides of multiple drug-resistant strains of *V. cholerae* O 1 and O 139

Resistance type	MIC (μg/ml)								
	Tetracyclines			Quinolones			Macrolides		
	TC	DOXY	MINO	NA	NFLX	CPFX	EM	CAM	AZM
VC MAR :									
VC MAR 1	0.25-0.5	0.25	0.13	0.25	0.007-0.02	0.03-0.06	4	4-8	0.25-0.5
VC MAR 2	8	0.5-1	0.13-0.25	0.125-0.25	0.007-0.02	0.06	4-8	4-8	0.5-1
VC MAR 3 a	0.25-0.5	0.13-0.25	0.06-0.13	4-16	0.06	0.03-0.06	4-8	2-8	0.25-1
VC MAR 3 b	0.25	0.25	0.13	$\geq$ 256	1	0.25	4	4	0.5
VC MAR 4 a	8	1	0.13	8	0.06	0.03	4	8	0.5
VC MAR 4 b-FZ	4	0.5	0.13	$\geq$ 256	1	0.03	4	4	0.5
VC MAR 5	0.25	0.25	0.13	0.25	0.12	0.06	4	4	0.5
VC MAR 6	0.5	0.25	0.13	0.13-0.25	0.02	0.06	4	4	0.5
R plasmid :									
pNON 1	16	1	0.25	0.5	0.02	0.02	4	4	0.5
pNON T 1	32	4	0.5	0.25	0.02	0.06	4	4	0.5
pELT T 1	32	8	0.5	0.5	0.02	0.06	4	4	0.5
pELT R 1	32	8	0.5	0.5	0.13	0.06	4	4	0.5
pELT P 1	64	8	0.5	0.25	0.06	0.06	4	4	0.5

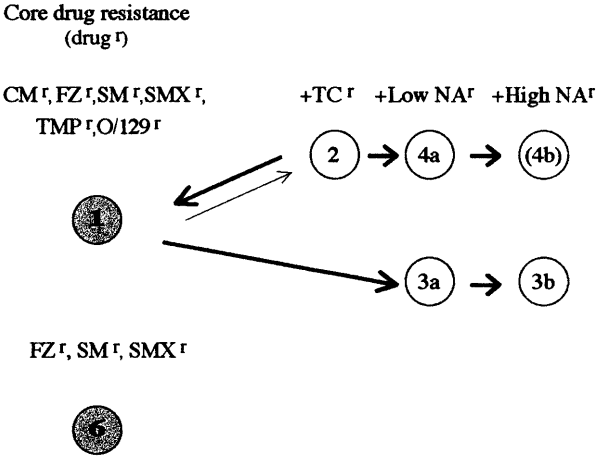

Fig. 2. A possible model of VC MAR transition. VC MAR types are from Table 3. Thick lines represent recent changes in Asian countries such as Bangladesh.

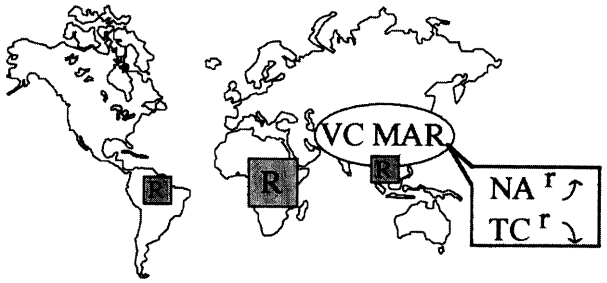

Fig. 3. World distribution of VC MAR and R plasmids of *V. cholerae* O1 and O139. R indicates the confirmation of a drug resistant (R) plasmid. The incidence of resistance to nalidixic acid and tetracycline has been markedly changing recently (text).

Mutations in outer membrane proteins such as porins also result in cross resistance to chloramphenicol, trimethorim, and nalidixic acid in some gram-negative bacteria[45]. Such mutations in outer membrane proteins of *V. cholerae* O1 and O139 may also be responsible for VC MAR.

Still another possibility exists that the VC MAR phenotypes are encoded by a cluster of drug resistance genes and such drug resistance genes are components of the gene cassettes within the integron[46-48]. Integrons are unable to move themselves (unlike transposons), but can be mobile by site-specific recombination between bacterial DNAs[46-48]. The gene encoding resistance to streptomycin and spectinomysin of *V. cholerae* O1 from Vietnam has been shown to be located on a gene cassette within class I integron[49], although *V. cholerae* O139 did not contain class I integrons[50].

Waldor et al. have shown that three-drug resistance to streptomycin, sulfamethoxazole, and trimethoprim of *V. cholerae* O139 is encoded by the chromosomally integrating genetic element (conjugative transposon), and can be transferred to *V. cholerae* O1 and *E. coli* by bacterial conjugation, followed by integration into the recipient chromosome in a site-specific manner independent of recA[51]. The marked appearance of VC MAR in *V. cholerae* O1 and O139 as demonstrated in this study calls for a more accurate understanding of the precise mechanisms.

In some regions or strains, the multiple drug resistance was due to R plasmids. In this study, we isolated an R plasmid (pNOIN T1) from an *V. cholerae* O139 strain from Thailand. This was the second report of R plasmid isolation from *V. cholerae* O139 after the isolation of pNON1 from Indian strains[22]. The plasmid pNON T1 had unique features: first, in contrast to previous R plasmids from *V. cholerae* O1 and O139, it did not belong to IncC; second, it had a very big molecular size corresponding to ca. 6% of the *E. coli* genome size. pNON T1 must have derived from a distinct origin and been acquired by *V. cholerae* O139.

In Africa, R plasmids have been more frequently found in multiple drug resistant *V. cholerae* O1 strains (Fig. 3). The isolated R plasmids belonged to IncC, like many other R plasmids of *V. cholerae* O1[27]. An IncC-belonging R plasmid (pELT P1) from Peruvian *V. cholerae* O1 had a surprisingly high ability to spread among bacteria.

In summer 1997, many cases of cholera in patients who had never been abroad were reported in Japan. Most were incidences of sporadic infection and various transmission routes were suggested, but the infection sources were not identified in any of the cases[52]. Many of those *V. cholerae* O1 strains (58.8%) showed three-drug resistance to furazolidone, streptomycin, and sulfamethoxazole (VC MAR6), which was consistent with the three-drug resistance (VC MAR6) detected in the strains isolated from travelers to Indonesia (Bali) in 1995. Based on the drug-resistance markers, it is possible that the *V. cholerae* O1 strains isolated in 1997 from Japanese patients who had never been abroad are epidemiologically related to the strains from Indonesia (Bali) isolated in 1995.

V. parahemolyticus with a new serotype (O3 : K6) emerged in Japan in 1996[53], and the first large outbreak occurred in Niigata (manuscript in preparation

on a molecular epidemiological study). *V. parahemolyticus* O3 : K6 has been recognized as an important emerging pathogen in South East Asia[54]. Therefore, there must be an infection route(s) which greatly contributes to the transmission of the vibrios among Asian countries, e. g. via travelers or seafood which the vibrios likely colonize[55] (such as lobsters, crabs, and possibly the zooplankton, i.e., copepods they consume). The vibrios may survive in coastal waters around Japan in a climate-linked situation (as a coccoid form?)[55,56], and contaminate zooplankton as well as seafood such as fish, shell fish, or crabs. Thus there remains a risk for sporadic cases (or outbreaks) due to the vibrios.

Multiple drug resistance (VC MAR) is concentrated in Asian countries such as India, Bangladesh, and Thailand (Fig. 3). In recent years, the tetracycline-resistance of *V. cholerae* has tended to decrease. In Bangladesh, 50-60% or more of strains were tetracycline-resistant in 1992[5], but this decreased to about 40% in 1994, with none detected in 1999. In contrast, the frequency of resistance to nalidixic acid increased and the resistance level was elevated. This was considered to be due to a decrease in the use of tetracycline and the extensive use of quinolones. Strains that became highly resistant to nalidixic acid tended to be slightly resistant even to newer quinolones such as norfloxacin. Resistance to quinolones may increase in the future. Based on the survey of in vitro susceptibilities of *V. cholerae* O1 and O139, it might be practical to use tetracycline (or doxycycline) for preventive medication. Azithromycin is also a drug to be considered, because all types of *V. cholerae* O1 and O139 including multiple drug-resistant strains are highly susceptible to azithromycin.

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