

ANTIBIOTIC SUSCEPTIBILITY AND ITS GENETIC ANALYSIS OF VIBRIO CHOLERAE NON-O1, NON-O139 FROM ENVIRONMENTAL SOURCES IN LAO PEOPLE'S DEMOCRATIC REPUBLIC

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Abstract: In order to determine the epidemiological features of cholera in Lao PDR, the presence of mobile genetic elements such as plasmid, class I integron and SXT element in *V. cholerae* isolated from surface water were examined. Among the 22 strains isolated from 13 distantly separated sampling sites, no mobile genetic elements associated with drug resistance were found reflecting the antibiogram of the strains. Nevertheless, cholera epidemics due to multiple drug resistant *V. cholerae* occurred repeatedly in those areas until 2000.

Vibrio cholerae is a natural inhabitant of aquatic environments and is known to be the causative agent of the acute gastrointestinal disease. Transmission of *V. cholerae* is associated with consumption of contaminated water and contaminated foods [1, 2]. In spite of the importance and success of rehydration therapy for cholera, antimicrobial therapy still plays a critical role because it can reduce the volume of diarrhea as well as shorten the duration of symptoms and the excretion of *Vibrios* in the stool [3]. The occurrence of antibiotic-resistant strains of *V. cholerae* is being reported with increasing frequency [2, 4, 5]. However, the antibiotic susceptibilities of organisms spatially and temporally fluctuate. These susceptibilities have to be examined in order to achieve a better understanding of the epidemiological features.

In *V. cholerae*, antibiotic-resistant genes have been found on transmissible plasmids [6-8]. Recently, in addition to plasmids, integron and SXT element have also been described as vehicles in the transport of resistance genes [9, 10]. The analysis of antibiotic resistance determinants on these vehicles is being used as an epidemiological tool.

In Lao People's Democratic Republic (Lao PDR), the antibiotic susceptibility pattern of *V. cholerae* O1 isolated from cholera patients had been monitored for 8 years (1993-2000) [11, 12]. Although the pathogens were sensitive to the therapeutic antibiotics as expected until 1996, the pattern of antibiotic susceptibility began to change in 1998 [11]. We found that strains isolated before 1997 were resistant to streptomycin and harbored class I integron, while strains isolated after 1997 were resistant to chloramphenicol,

tetracycline, streptomycin and sulfamethoxazole-trimethoprim and harbored SXT element [13]. In this study, antibiotic susceptibility and the presence of plasmid, class I integron and SXT element were examined in *V. cholerae* strains isolated from surface water in order to determine the epidemiological features of *V. cholerae* in Lao PDR.

A total of 13 surface water samples from branches of the Mekong River were collected in February 2003. This water is used routinely by people living near the river. The sampling sites were located in cholera epidemic areas until 2000, but no cholera patient was found in 2001, 2002 and 2003. Isolation of *V. cholerae* strains from the samples was accomplished by a modification of the method of Wai and colleagues [14]. About 100 ml of each sample water was taken into two sterile bottles containing 100 ml of 2% alkaline peptone water (pH ~ 9.0 ~). One bottle was supplemented with sodium pyruvate (final concentration at 0.1%) or catalase (final concentration at 50 U/ml) to stimulate viable-but-nonculturable (VBNC) *V. cholerae* O1. All samples were incubated for 24 h at 37 °C, and then cultured on thiosulfate-citrate-bile salt-sucrose (TCBS) agar for selective isolation of *Vibrio* species. After 24 h incubation at 37 °C, yellow colonies were extracted from each TCBS agar and identification of isolates was performed by biochemical tests. The colony-growth pattern of the sample cultured in the supplemented media showed no differences from that cultured in a plain alkaline peptone water, and *V. cholerae* O1 was not detected at all. O-antigen was determined by slide agglutination with polyvalent O1, mono-specific Ogawa-Inaba antisera and with specific anti-O139 antisera

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obtained commercially (Denka Seiken Co., Tokyo, Japan). A total of 22 strains of *V. cholerae* were isolated. All of these environmental *V. cholerae* isolates were non-O1, non-O139 serotypes, and cholera toxin non-productive.

The minimum inhibitory concentrations (MICs) of nine antibiotics were determined by agar dilution technique according to the specifications of NCCLS [15]. To examine the production of beta-lactamase by ampicillin resistance isolates, clavulanic acid was used at a constant concentration of 4 µg/ml in combination with ampicillin. The susceptibility profiles of *V. cholerae* isolates are given in Table 1. Of the 22 environmental isolates, two were resistant to polymyxin B alone; one was resistant to polymyxin B and ampicillin; and 15 were resistant to ampicillin alone. All isolates were highly sensitive to the other antibiotics examined. For all of the ampicillin resistant isolates, clavulanate reduced the level of MICs to the same as that for the susceptible isolates.

Since plasmids are known to encode resistance to beta-lactams, the presence of plasmid was examined by the modified method of Kado and Liu [16]. Cells were grown on nutrient agar plates overnight at 37 °C. Colonies were

scraped from the plates and suspended in 100 µl of a solution containing 50% glucose, 25 mM EDTA and 1 M Tris (pH 8.0). Then 200 µl of lysing solution containing 1% SDS and 0.2 N NaOH was added. After lysis, 300 µl of phenol-chloroform-isoamylalcohol (25: 24: 1) was added, and the samples were mixed well. After centrifugation, a sample of the supernatant was electrophoresed on a 0.5% Seakem ME agarose. *Shigella flexneri* 2a YSH 6000 [17] harboring approximately 230 kb plasmid was used as the control strain. None of the 16 ampicillin resistant isolates harbored any plasmids (data not shown).

Next, we investigated the presence of class I integron, which has been reported to contain a gene cassette that conferred resistance to beta-lactams [18]. To identify class I integron, primers inDS-F (5'-CGGAATGGCCGAGCAGATC-3') and inDS-B (5'-CAAGGTTTGACCAGTTGCG-3'), which are specific for the 5' conserved segment containing integrase gene (*intI 1*) [19], were used to amplify this region by PCR. PCR with inDS-F and inDS-B primers did not yield amplicons for any of the 22 environmental isolates but yielded an 871 bp amplicon for the clinical isolate 93LA5 as a positive control (Fig. 1).

Table 1. Antibiotic susceptibilities of *V. cholerae* isolates

Strain *	MIC (µg/ml) [§]										<i>intI 1</i>	<i>intSXT</i>
	PMX	TC	AMP	AMP-CLA	EM	NA	CP	ST	SM			
3p-4	0.4	0.8	>100	0.4	3.13	0.1	0.8	10.0	12.5	-	-	
3p-7	>100	0.2	6.25	1.6	12.5	0.2	1.6	10.0	6.25	-	-	
3p-8	0.4	0.2	>100	0.4	12.5	0.1	0.8	5.0	12.5	-	-	
3p-9	0.4	0.2	>100	0.8	12.5	0.1	0.8	5.0	12.5	-	-	
3p-10	0.4	0.2	>100	0.8	12.5	0.2	0.8	5.0	12.5	-	-	
3p-11	0.4	0.2	>100	0.4	12.5	0.2	0.8	5.0	12.5	-	-	
3p-13	0.4	0.2	>100	0.4	12.5	0.2	0.8	5.0	12.5	-	-	
3p-14	0.4	0.2	>100	0.8	12.5	0.2	0.8	5.0	12.5	-	-	
3p-15	>100	0.4	6.25	3.13	12.5	0.2	1.6	10.0	6.25	-	-	
8p-2	0.8	0.4	>100	0.8	12.5	0.2	1.6	2.5	12.5	-	-	
11pw--2	0.8	0.4	>100	0.8	12.5	0.2	1.6	2.5	12.5	-	-	
12pw-2	3.13	0.4	>100	0.4	12.5	0.2	1.6	10.0	12.5	-	-	
12pw-3	3.13	0.4	>100	0.8	6.25	0.2	1.6	5.0	12.5	-	-	
12pw-4	3.13	0.4	>100	0.8	6.25	0.2	1.6	5.0	12.5	-	-	
12pw-6	3.13	0.4	>100	0.8	6.25	0.2	0.8	10.0	12.5	-	-	
12pw-7	3.13	0.4	>100	0.8	6.25	0.2	0.8	5.0	12.5	-	-	
12cpw-2	1.6	0.2	>100	0.8	6.25	0.2	0.8	5.0	6.25	-	-	
13pw-2	1.6	0.4	6.25	1.6	25	0.2	0.8	40.0	12.5	-	-	
13pw-3	0.8	0.4	6.25	3.13	25	0.2	1.6	40.0	6.25	-	-	
13pw-4	1.6	0.8	6.25	3.13	25	0.2	1.6	40.0	6.25	-	-	
13pw-5	1.6	0.8	6.25	3.13	25	0.2	1.6	40.0	12.5	-	-	
13pw-7	>100	0.8	>100	1.6	3.13	0.2	0.8	5.0	6.25	-	-	

* p, strain recovered from peptone water with pyruvate; pw, strain recovered from peptone water without supplement; cpw, strain recovered from peptone water with catalase.

§ Abbreviations: PMX, polymyxin B; TC, tetracycline; AMP, ampicillin; CLA, clavulanic acid; EM, erythromycin; NA, nalidixic acid; CP, chloramphenicol; ST, sulfamethoxazol-trimethoprim; SM, streptomycin.

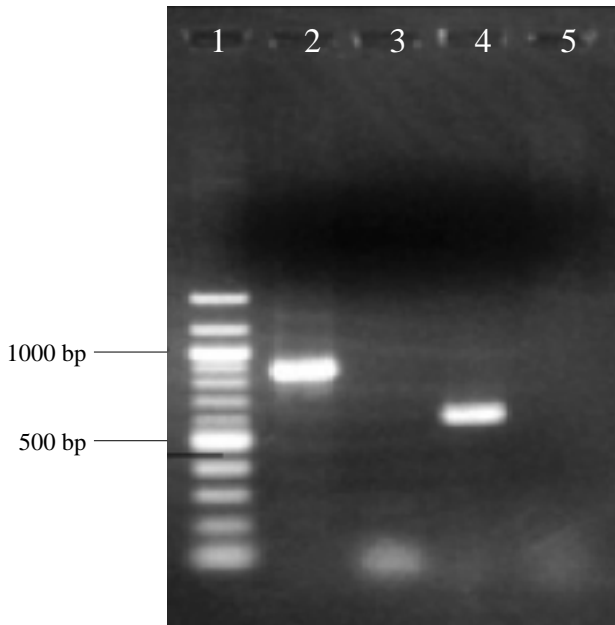


Fig. 1. Examples of PCR for the presence of class I integron using the primers inDS-F and inDS-B (lanes 2 and 3) and SXT element using primers INT1 and INT2 (lanes 4 and 5) among *V. cholerae* non-O1, non-O139 strains isolated from Lao PDR. Lanes: 1, 1,100-bp molecular mass standard; 2, strain 93LA5 (clinical isolate in 1993 harboring class I integron); 3 and 5, strain 3p-8 (environmental isolate); 4, strain 00LA1 (clinical isolate in 2000 harboring SXT element).

SXT element is responsible for resistance to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim in *V. cholerae* [20]. Recently Hochhut et al. reported that deletion of the antibiotic resistance genes located on SXT element rendered these strains sensitive to the antibiotics [20]. We also noticed that *V. cholerae* O1 harboring SXT element were sensitive to all antibiotics examined, and in these strains, antibiotic resistant gene cluster was absent from the SXT element. All of the 22 isolates were sensitive to these four antibiotics, but we analyzed the possible presence of the SXT element backbone by PCR. Primers INT1 (5'-GCTGGATAGGTTAAGGGCGG-3') and INT2 (5'-CTCTATGGGCACTGTCCACATTG-3'), which are specific for SXT integrase gene (*ints_{sxt}*) [20], were used. However, the expected 592 bp amplicon was not obtained from any of the environmental isolates (Fig. 1).

V. cholerae O1 and O139 are known to enter a viable-but-non-culturable (VBNC) state in the environment, and recovery of the non-culturable cells from samples relies on culture methods [21]. In this study, all environmental isolates were non-O1, non-O139 serotype isolated from samples by treatment with catalase or sodium pyruvate which enhance the recovery of non-culturable cells [14]. The fail-

ure to detect *V. cholerae* O1 may have been due to their small population among a large number of non-O1 Vibrios. Therefore, further trials, such as immuno-magnetic separation technique using antibody against *V. cholerae* O1, are recommended.

The environmental non-O1, non-O139 isolates in this study showed resistance to polymyxin B and/or ampicillin. Resistance to these antibiotics was found to be common among the environmental non-O1, non-O139 isolates from Malaysia, Thailand and South India [2, 22, 23]. This result illustrates the occurrence of antibiotic-resistant environmental non-O1, non-O139 *V. cholerae* strains worldwide. However, it should be noted that environmental non-O1, non-O139 isolates clearly showed resistance to fewer antibiotics in comparison with clinical *V. cholerae* O1 isolates from Lao PDR [13]. In addition, there was a clear difference in distribution of class I integron and SXT element among clinical O1 isolates (unpublished observations) and environmental non-O1, non-O139 isolates from Lao PDR, as none of the environmental isolates harbored class I integron or SXT element. Thungapathra and colleagues [24] showed that multiple antibiotic resistances were more common among clinical isolates. Dalsgaard and colleagues reported that distribution of class I integron was associated with therapeutic use of antibiotics [25]. Thus, it seems unlikely that environmental non-O1, non-O139 isolates from Lao PDR are under antibiotic stress. In other words, the environmental waters examined are not contaminated with the organisms from a clinical origin.

The majority of ampicillin resistant *V. cholerae* contained plasmids and showed resistance to multiple antibiotics [8, 9, 23-25]. However, in Lao PDR, environmental isolates were resistant only to ampicillin without harboring plasmid, and *V. cholerae* O1 isolates from cholera patients were sensitive to ampicillin [12]. Class I integron and SXT element are occasionally lacking the drug resistance gene, but, they can acquire the resistance gene later. Therefore, Class I integron and SXT element should be monitored even in drug susceptible *V. cholerae*.

In the background of the present study, there is a fact that *V. cholerae* O1 in the world is simultaneously gaining resistant to a variety of anti-microbial drugs. There is a possibility that the transmission of SXT element takes place in the environmental water where *V. cholerae* consists of a flora, but this seems unlikely because we failed to find SXT element in the environmental *V. cholerae*. The transmission route of SXT element remains unclear.

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