TETRACYCLINE RESISTANT AND POLYMYXIN B SENSITIVE *VIBRIO CHOLERAE* O1 EL TOR ISOLATED FROM THE RECENT EPIDEMICS

MASAAKI IWANAGA¹, SITHAT INSISIENGMAI², NAOMI HIGA¹
AND LAY SISAVATH²

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Abstract: By examining 99 *Vibrio cholerae* O1 isolates from cholera epidemics in a variety of areas in Laos in 1998, we found two unusual characteristics of the organisms. All except 4 isolates were moderately resistant to tetracycline without having plasmids, and susceptibilities to the other drugs were as expected. Eleven isolates showed the same level of susceptibility to polymyxin B as classical *V. cholerae* O1. With the exception of 4 tetracycline-susceptible strains, all the isolates were resistant to or poorly sensitive to the vibriostatic agent O/129 (2,4-diamino-6,7-di-iso-propyl pteridine phosphate). All isolates, including those susceptible to polymyxin B, produced E1 Tor hemolysin which was neutralized by anti-E1 Tor hemolysin and were resistant to cholera phage IV, indicating that they were of the E1 Tor biotype.

Key words: *Vibrio cholerae* O1, tetracycline, polymyxin B, drug sensitivity, Laos

INTRODUCTION

The current cholera pandemic, which began in 1961 from a focus in South Sulawesi, Indonesia, shows no signs of declining. During the past 4 decades, there have been a variety of changes in the epidemic cholera vibrios; these changes include hemolytic properties of E1 Tor strains, drug susceptibilities, disappearance and reappearance of classical cholera vibrios and the appearance of new serovar *V. cholerae* O139. Tetracycline was originally the first choice for antibiotic therapy and prophylaxis of cholera. The first epidemic due to a tetracycline resistant strain was seen in Tanzania in 1977 (Mhalu *et al.*, 1979). Soon after this, a tetracycline resistant strain caused an outbreak in Bangladesh (Glass *et al.*, 1980). The vibrios isolated in both countries were plasmid-mediated multiple drug-resistant strains. The first outbreak in Tanzania was long-lasting and spread to Kenya around Lake Victoria (Ehara

et al., 1983), whereas the outbreak in Bangladesh was limited to the Matrab area and disappeared after 5 months. The emergence of drug-resistant strains in Tanzania was attributed to extensive use of tetracycline for prophylaxis (Towner *et al.*, 1980). In Bangladesh, however, prophylaxis was not common, and the source of outbreak could not be determined. Although a few strains of drug-resistant *V. cholerae* O1 have been reported (Kobari *et al.*, 1970; Threlfall *et al.*, 1993; Yamamoto *et al.*, 1995), there have been no large outbreaks other than these two episodes. In 1998, cholera outbreaks due to tetracycline-resistant strains occurred in a wide area of Laos. Here, we describe characterization of the *V. cholerae* O1 isolated in Laos in 1998.

MATERIALS AND METHODS

Bacterial strains: A total of 99 *V. cholerae* O1 strains isolated from patients in Laos in 1998 were

1 Department of Bacteriology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

2 Center for Laboratory and Epidemiology, Km3, Thadeua Rd., Vientiane, Lao People's Democratic Republic

Corresponding author: MASAAKI IWANAGA

Mailing address: Department of Bacteriology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

Phone: +81-98-895-3331 Ext. 2285

FAX: +81-98-895-2951

E-mail: [REDACTED]

Table 1 Characteristics of isolates and the places of isolation

Place	number of isolates	resistant to tetracycline	resistant to O/129*	sensitive to polymyxin B
Vientiane	82	78	77	3
Xaisettha	43	39	39	2
Xaithani	30	30	29	1
Sisathanak	5	5	5	0
Thaluang	2	2	2	0
Thourakham	1	1	1	0
Maxaythang	1	1	1	0
Khammouan	9	9	9	0
Savanakhet	4	4	4	4
Champasak	4	4	4	4
Total	99	95	94	11

*includes moderately resistant strains

examined. The strains were isolated during the period from May to November 1998 from a wide area in the Southern half of the country as shown in Table 1.

Identification of *V. cholerae* O1: The isolates were identified as *V. cholerae* O1 by routine laboratory tests. Serotype was determined by the slide agglutination method using commercial anti-Ogawa and anti-Inaba sera (Denkaseiken Co. Tokyo).

Production of and susceptibility to phage: Susceptibility to cholera phage IV, E1 Tor phage 5, kappa-phage, fs-1 and fs-2 phage was examined by the following method. First, 10 ml of peptone agar (1% peptone, 0.5% NaCl, 1.5% agar) was poured into a Petri dish (diameter, 9 cm) and solidified. Overnight heart infusion broth culture of each isolate (0.2 ml) and 4 ml of soft agar (1% peptone, 0.5% NaCl, 0.5% yeast extract, 0.6% agar) kept at 43°C were mixed and poured on to peptone agar plates. Aliquot of 10 µl of phage solution at the routine test dilution were spotted on to the soft agar plates containing the organisms. After 24 hr incubation at 37°C, the vibrios showing plaque formation were regarded as susceptible strains. Production of kappa phage by the organisms was examined as described by Takeya and Shimodori (1963). Briefly, broth cultures of each isolate were treated with chloroform to kill the bacterial cells. The killed culture fluid (0.1 ml), a 0.2 ml broth culture of an indicator strain H218 (*V. cholerae* O1, serotype Ogawa, biotype cholerae) and 4 ml soft agar kept at 43°C were mixed and poured on to peptone agar plates. After overnight incubation at 37°C, plaque formation was observed. To examine productivity of filamentous phage fs-1 and fs-2, the organisms were cultured in HIB with shaking overnight and the cell-free supernatant was spotted on to the indicator strain UDT109 (*V. cholerae* O1 E1 Tor, Inaba).

Cholera toxin production: The organisms were cultured in AKI medium at 37°C for 20 hr in a stationary test tube, after which the cholera toxin in the culture supernatant was titrated using the reversed passive latex agglutination method (Iwanaga and Yamamoto, 1985).

Hemolysin production: The isolates were inoculated on to sheep blood agar plates, and hemolysis around the grown colonies was examined. The isolates were also cultured in heart infusion broth supplemented with 1% glycerol and incubated at 37°C for 24 hr. Washed sheep red blood cells at the final concentration of 0.5% were added to the culture supernatant, incubated at 37°C for 2 hr followed by overnight incubation in a cold room. Neutralization test was carried out by adding 1 to 20 volumes of anti-E1 Tor hemolysin serum to the culture supernatant.

Drug susceptibilities: Susceptibilities of the isolates to ampicillin (ABPC, Meiji), tetracycline (TC, Wako), erythromycin (EM, Dainihon), ofloxacin (OFLX, Daiichi) and polymyxin B (PmxB, Feizer) were determined by multipoint inoculation onto a series of heart infusion agar plates containing doubling dilutions of the drugs from 100 µg/ml to 0.0125 µg/ml. A 10-fold dilution of overnight broth culture was inoculated in to each plate using Microplanter (Sakuma Co. model MITP # 00257). The susceptibility was expressed as minimum inhibitory concentration (MIC) of each drug.

Isolation of plasmid and electrophoresis: Plasmid DNA was isolated by the method of Kado and Liu (1981) with slight modifications (lysing buffer was added and stand still at room temperature before phenol extraction) and electrophoresed in 0.8% agarose gel. The gels were stained with ethidium bromide and observed under a UV transilluminator.

RESULTS

All isolates revealed typical biochemical characteristics of *V. cholerae*, but 9 out of the 99 strains did not grow in peptone water without sodium chloride, and 19 strains were negative for Voges-Proskauer reaction. All isolates were toxigenic, and hemolytic on blood agar plates, and the hemolysis was inhibited by anti-E1 Tor hemolysin serum. All isolates belonged to serotype Ogawa.

Phage: Ninety-six of the 99 isolates were susceptible to E1 Tor phage 5, and none was susceptible to cholera phage IV. Kappa phage was produced by 96 isolates. The remaining 3 non-producers were susceptible to this phage; *i.e.* all belong to the Celebes type

Table 2 Production of and susceptibility to phages

phage	productive	sensitive	independent	Total
cholera IV	0	0	99	99
E1 Tor 5	0	96	3	99
kappa	96	3	0	99
fs-1	0	3	96	99
fs-2	0	7	92	99

Table 3 Drug susceptibilities of 99 isolates

$\mu\text{g/ml}$	TC	ABPC	EM	OFLX	Pmx-B
0.0125 $\geq$	0	0	0	86	0
0.025	0	0	0	13	0
0.05	0	0	0	0	0
0.1	0	0	0	0	0
0.2	1	0	0	0	0
0.39	3	0	0	0	2
0.78	0	0	0	0	8
1.56	0	1	0	0	1
3.13	54	26	38	0	0
6.25	41	72	61	0	0
12.5	0	0	0	0	0
25	0	0	0	0	0
50	0	0	0	0	7
100	0	0	0	0	80
100<	0	0	0	0	1

TC: tetracycline, ABPC: ampicillin, EM: erythromycin, OFLX: ofloxacin, Pmx-B: polymyxin B

including 3 cured Celebes. Three and 7 of the 99 isolates were susceptible to the filamentous phage fs-1 and fs-2, respectively. None of the strains produced fs-1 or fs-2 (Table 2).

Drug susceptibilities: Almost all isolates were moderately resistant to tetracycline, with MIC of 3.13 or 6.25 $\mu\text{g/ml}$. Only 4 isolates from an area of Vientiane were sensitive to tetracycline with MIC of 0.2 or 0.39 $\mu\text{g/ml}$. Most of the isolates showed polymyxin B MICs of 100 $\mu\text{g/ml}$, but 11 showed MICs of 0.39 to 1.56 $\mu\text{g/ml}$. Polymyxin B-susceptible strains were isolated from Vientiane (3 of 80 isolates), Savanakheth (4 of 4) and Champasak (4 of 4), areas separated by fairly large distance. The susceptibilities to ampicillin, erythromycin, and ofloxacin were similar to the expected values (Tables 2 and 3).

Susceptibility to O/129: The size of the zone of growth inhibition around the disk containing 150 mg of the vibriostatic agent O/129 (2,4-diamino-6,7-di-isopropyl pteridine phosphate) was variable, therefore, the susceptibilities were defined as follows. Resistance was defined as an inhibition zone of 0 to 1 mm, and susceptibility was defined as a zone of more than 10 mm. Other strains were expressed as intermediate. According to

these criteria, 31 strains were resistant and 5 (including 4 tetracycline-sensitive strains) were sensitive to O/129.

Plasmids: No plasmids were detected in 20 randomly selected strains, whereas the tetracycline-resistant strains isolated in Kenya in 1982 examined as positive controls contained plasmids.

DISCUSSION

The present study revealed 2 novel findings in the drug susceptibility pattern of epidemic *V. cholerae* O1. Among the 99 *V. cholerae* O1 E1 Tor isolated in these epidemics, 11 were sensitive to polymyxin B with the same MIC as classical cholera vibrios, and 95 were moderately resistant to tetracycline without harboring any plasmids as far as 20 isolates examined are concerned. The MIC of polymyxin B against *V. cholerae* O1 E1 Tor is 50 to 200 $\mu\text{g/ml}$, whereas that of classical *V. cholerae* O1 is 0.4 to 1.56 $\mu\text{g/ml}$. Although we found a strain against which the MIC of polymyxin B was 6.25 $\mu\text{g/ml}$ in the epidemic in 1994 in Laos (Higa *et al.*, 1995), epidemic E1 Tor strains showing the same susceptibilities as classical strains (0.4 to 1.56 $\mu\text{g/ml}$) were found in the present study for the first time. In Southern Laos, all isolates (8 strains) were polymyxin B-sensitive, whereas 3 of 80 isolates from Vientiane, the capital of Laos. *V. cholerae* O1 E1 Tor were distinguished from classical *V. cholerae* O1 by their productivity of E1 Tor hemolysin. However, the susceptibilities of *V. cholerae* O1 to polymyxin B and cholera phage IV have been reported to correspond to the productivity of E1 Tor hemolysin (Nakasone *et al.*, 1987). Therefore, these 2 biotypes are usually distinguished by testing susceptibility to polymyxin B. However, the present study suggested that biotype should be determined by analysis of E1 Tor hemolysin productivity and susceptibility to cholera phage IV.

All strains of tetracycline-resistant *V. cholerae* O1 reported to date were plasmid-dependent, multi-drug resistant, with a tetracycline MIC of 25 to 200 $\mu\text{g/ml}$ (Coppo *et al.*, 1995; Glass *et al.*, 1980; Towner *et al.*, 1980), except a report by Yamamoto *et al.* (1995). In contrast, the organisms described here did not harbor any plasmids, and were moderately resistant to tetracycline with MICs of 3.13 or 6.25 $\mu\text{g/ml}$. MICs of EM, ABPC and OFLX remained within the expected range. These isolates were generally resistant to the vibriostatic agent O/129. Only 5 isolates including 4 tetracycline-sensitive strains were sensitive to O/129. These observations do not, however, necessarily indicate a close correlation between resistance to O/129 and tetracycline, as that *V. cholerae* O139 is resistant to the former but susceptible to the latter (Albert *et al.*, 1997; Higa *et al.*, 1995).

Recently, 83 of 93 isolates (89%) of tetracycline sensitive *V. cholerae* O1 E1 Tor collected from a variety

of areas throughout the world were reported to be lysogenized by the phage fs-2 (Ikema and Honma, 1998). However, in the present study, 92 of 99 isolates of tetracycline resistant *V. cholerae* O1 E1 Tor were not associated with fs-2. Considering the phages as the gene vector, the relation between fs-2 and drug sensitivities is now under investigation.

The phenotypic changes in these isolates must have been based on chromosomal mutations as seen in the case of sulfamethoxazole-trimethoprim-resistant *V. cholerae* O139 in which susceptibility was shown to be dependent on chromosomal self-transmissible genetic elements (Waldor *et al.*, 1996), since no plasmid was detected in the examined 20 isolates. The phenotypes based on chromosome would be more stable than that associated with plasmids, therefore, careful monitoring of these mutant organisms is required.

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