Vibrio cholerae Serogroup O1 in Northeast Thailand

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Strains of Vibrio cholerae serogroup O1 biotype El Tor that are susceptible to Mukerjee cholera bacteriophage group IV (S. Mukerjee, Bull. W.H.O. 28:333–336, 1963) were found. Cholera vibrios isolated from epidemics in northeast Thailand were characterized, and 57 of 60 strains isolated in 1986 were susceptible to cholera phage IV. However, all 113 strains isolated in 1988 were not susceptible to the phage. All isolates in both epidemics revealed behaviors typical of El Tor vibrios, except phage IV susceptibility in the 57 strains. Although the plaques of phage IV were generally translucent, plaques on some isolates looked transparent, just like those on classical vibrios. The organisms grown in the plaques were lysogenized. If this kind of strain is frequently isolated, the biotype of V. cholerae O1 should be reconsidered.

The seventh cholera pandemic due to biotype El Tor vibrios which began in 1961 shows no signs of declining. During the past 27 years, however, various changes in the epidemic cholera vibrios have been seen. Since around 1963, changes in hemolytic properties have been widely recognized through "loss of hemolytic property in routine test methods" (6). In 1982, however, Iwanaga et al. (9) reported that El Tor vibrios isolated in Kenya showed hemolytic activity with routine test methods, represented by the method of Feeley and Pittman (5). Subsequently, Nakasone et al. reported 31 El Tor vibrio strains isolated in Bangladesh in 1986 (12). They found that 8 strains were positive by the method of Feeley and Pittman and 21 were beta-hemolytic on blood agar plates (12). Otherwise, reports on hemolysis of epidemic El Tor vibrios have been rare in the past two decades.

All epidemic strains of El Tor vibrios were lysogenic until 1966, when "cured" strains suddenly appeared in Cambodia and Thailand (11). Thereafter, epidemics due to nonlysogenic El Tor vibrios have frequently been reported and these vibrios have become even more prevalent than lysogenic Celebes-type strains (9, 12, 13). Epidemics due to antibiotic-resistant strains were occasionally seen in, for example, Tanzania (18), Bangladesh (7), and Kenya (3). Fortunately, these resistant strains soon disappeared.

In 1963, El Tor vibrios invaded East Pakistan (now Bangladesh), where classical cholera had been endemic. Thereafter, the prevalence of El Tor cholera increased while that of classical cholera decreased (15). In 1973, classical cholera finally disappeared from Bangladesh, and it was not seen for nearly 10 years. In 1982, however, classical cholera suddenly reappeared and spread throughout Bangladesh (16). Some investigators have reported that classical vibrios isolated after 1982 were slightly different from those isolated before 1973 (12, 16).

Changes in epidemic cholera vibrios, such as those mentioned above, must be considered for ecology of the organisms and for epidemiology of the disease. Detailed characterization, therefore, of epidemic cholera vibrios at different places and at different times must be done. This paper describes the characteristics of Vibrio cholerae serogroup O1 strains isolated in northeast Thailand in 1986 and 1988.

MATERIALS AND METHODS

Bacterial strains. A total of 173 strains of Vibrio cholerae serogroup O1 isolated from cholera patients in northeast Thailand (Fig. 1) were used. Of them, 60 strains were isolated in 1986 in Khon Kaen province and 113 strains were isolated in 1988 in Udon Thani province. V. cholerae H218 and C154 (both classical serotype Ogawa), which had been stocked in freeze-dry vials, were used for the study of bacteriophages. The isolates were stocked in a butt of nutrient agar (Eiken Co., Tokyo, Japan) prepared with a double amount of water until use.

Characterization of isolates. The characteristics of each isolate were determined on the basis of serotype, biotype, prophage type, drug susceptibility pattern, and cholera toxin production. Although biotype should be decided essentially by hemolytic properties of the organisms (4, 14), hemagglutinating properties, Voges-Proskauer reaction, and susceptibilities to cholera phage group IV (10a) and polymyxin B were also examined.

Serotype was determined by the slide agglutination method using commercial anti-Ogawa and anti-Inaba sera (Denkaseiken Co., Tokyo, Japan).

Hemolytic properties were examined by the method of Feeley and Pittman (5) and on blood agar plates. The antigenic activity of El Tor hemolysin was assayed by the reversed passive latex agglutination method (8).

Hemagglutinating activity was examined with sheep erythrocytes. Organisms grown on agar plates were emulsified in a drop of normal saline to make a milky suspension. A drop of 2.5% washed sheep erythrocytes was then added, and the slide was tilted back and forth to mix the cells. Clumping of the erythrocytes was judged as a positive reaction.

The Voges-Proskauer reaction was examined by using Voges-Proskauer soft agar (Eiken Co.). Susceptibility to polymyxin B was examined with a plate
dilution technique to determine the MIC of the drug. Agar plates containing the drug at concentrations ranging from 100 to 0.2 μg/ml were prepared, and organisms which grew with a drug concentration greater than 15 μg/ml were regarded as resistant to polymyxin B.

Susceptibility to cholera phage IV was examined as follows. 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. An overnight broth culture of the organisms (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 overlaid on the peptone agar plate. Phage solution (15 μl) at the routine test dilution was spotted on the soft agar plate containing the organisms.

Prophage typing was done basically as described by Takeya and Shimodori (17) using kappa phage and its host strain H218 (V. cholerae serogroup O1 biotype cholerae serotype Ogawa). Susceptibility of the organisms to kappa phage was examined with the method used for the cholera phage IV susceptibility test mentioned above. Production of kappa phage by the organisms was examined as follows. The organisms cultured in broth were killed with chloroform. The killed culture fluid (0.1 ml), a broth culture of strain H218 (0.2 ml), and soft agar (4 ml) were mixed and overlaid on a peptone agar plate. After overnight incubation at 37°C, plaque formation was regarded as kappa phage production. The organisms were classified into three groups; group 1 produced kappa phage and was not susceptible to the phage, group 2 did not produce kappa phage but was susceptible, and group 3 did not produce kappa phage and was not susceptible. The groups were regarded as Celebes type, cured type, and Ubol type, respectively.

**Cholera toxin production.** The organisms were cultured in AKI medium (Bacto-Peptone, 1.5%; yeast extract, 0.4%; sodium chloride, 0.5%; sodium bicarbonate, 0.3%) at 37°C for 20 h in a stationary test tube, after which the cholera toxin in the culture supernatant was titrated by using the reversed passive latex agglutination method (10).

**Drug susceptibilities.** Susceptibilities of the isolates to ampicillin and tetracycline were determined by using multipoint inoculation onto a series of agar plates containing doubling dilutions of the drugs. A 10-fold dilution of overnight broth culture was planted by using a microplanter. The media used were heart infusion agar and heart infusion broth (Eiken Co.).

## RESULTS

All the isolates belonged to serotype Inaba. The biotype-specific reactions are shown in Table 1. Hemolytic activity was negative for all isolates when they were examined by the method of Feeley and Pittman (5). However, 92 of 173 strains revealed beta-hemolysis on blood agar plates (sheep erythrocytes). Antigenic activity of El Tor hemolysin (19) was detected in all the isolates. The amount of hemolysin produced by the isolates ranged from 8 to 4,000 ng/ml, as determined by the reversed passive latex agglutination method (8). Hemagglutination was positive in 155 of 173 strains (90%). The Voges-Proskauer reaction was positive in 135 strains (78%). The cholera phage IV susceptibility test was positive for 57 of 60 isolates isolated in Khon Kaen, while all 113 strains from Udon Thani were not susceptible. The MIC of polymyxin B against these cholera vibrios was more than 50 μg/ml, and they were regarded as resistant.

Prophage typing classified the isolates into 110 Celebes-type, 58 cured-type, and 4 Ubol-type isolates. The strains from Khon Kaen consisted of 57 cured-type isolates (95%), 2 Celebes-type isolates, and 1 Ubol-type isolate. Those from Udon Thani consisted of 108 Celebes-type isolates (96%), 3 Ubol-type isolates, and 1 cured-type isolate, while 1 strain proved untypeable. Cured-type strains in Khon Kaen were all susceptible to cholera phage IV, but one cured-type strain in Udon Thani was resistant. The plaques of cholera phage IV on the isolates are presented in Fig. 2.

The isolates were examined for ampicillin and tetracycline susceptibilities. The MIC of ampicillin ranged from 1.56 to 12.5 μg/ml, while that of tetracycline was consistently lower than 0.2 μg/ml.

The isolates generally produced high levels of toxin. More than 80% of the isolates produced cholera toxin at concentrations from 125 to 1,000 ng/ml. Nine strains did not produce detectable amounts of cholera toxin (less than 2 ng/ml).

## DISCUSSION

All V. cholerae serogroup O1 strains mentioned in this paper were positive for the production of El Tor hemolysin.
Iwanaga and Yamamoto previously reported that cholera toxin production by El Tor vibrios in AKI medium usually ranged from 20 to 100 ng/ml (10). In the present study, however, more than 80% of the isolates produced from 125 to 1,000 ng of the toxin per ml. We also possess data for 31 strains of El Tor vibrios isolated in Bangladesh in 1986 (12), all of which produced cholera toxin within the range of 125 to 1,000 ng/ml (data not published). The question of whether this increased productivity is a general tendency of El Tor vibrios worldwide or just indicates a dispersion of the parameters will be answered only when epidemic strains are examined at different places and at different times.

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LITERATURE CITED


