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Characterization of *Vibrio cholerae* O1 Isolated in Thailand in 1987

Mamoru IWAMI[1], Yoshio ICHINOSE[1], Masahiko EHARA[1]
Tatsuro NAITO[1] and Krongkeaw SUPAWAT[2]

[1] *Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, 12−4 Sakamoto-machi, Nagasaki 852, Japan*

[2] *Department of Medical Sciences, National Institute of Health, Nonthaburi 11,000, Thailand*

Abstract: A total of 100 strains of *Vibrio cholerae* O1 isolated from 95 diarrheal patients hospitalized in Thailand in 1987 and from 5 water sources were characterized biochemically. All the strains of *V. cholerae* O1 tested were serotype Inaba, biotype *El Tor* and Celebes original type in prophage typing. Hemolytic activity to sheep red blood cells was detected in 92% of isolates in heart infusion broth but all were positive in heart infusion broth containing 1% glycerol by the method of Barua and Mukerjee (1964). Ninty-seven of the 100 strains of *V. cholerae* O1 were positive in the chicken red blood cell agglutination test and all strains with haemagglutinin (HA) activity were sensitive to D-mannose except one resistant strain. Ninty-nine strains of *V. cholerae* O1 were sensitive to all five drugs tested (chloramphenicol, tetracycline, streptomycin, ampicillin, erythromycin and nalidixic acid). One strain was resistant to ampicillin with an MIC of more than 100μg/ml and resistant to 10μg/ml of 2,4−diamino−6,7−diisopropyl-pteridine phosphate (O/129). The MICs against tetracycline and chloramphenicol were 3.13 and 12.5μg/ml, respectively. No multiply drug-resistant strains of *V. cholerae* O1 were isolated.

Key words: Vibrio cholerae, Epidemiology

INTRODUCTION

Cholera outbreak due to *V. cholerae* O1, biotype *El Tor* in Thailand has not subsided yet since the current *El Tor* pandemic started in 1961. Hundreds of people are still suffering from diarrhea due to *V. cholerae* as well as in other countries. Biological characters of causative agents are changing such as reappearance of classical vibrio in Bangladesh

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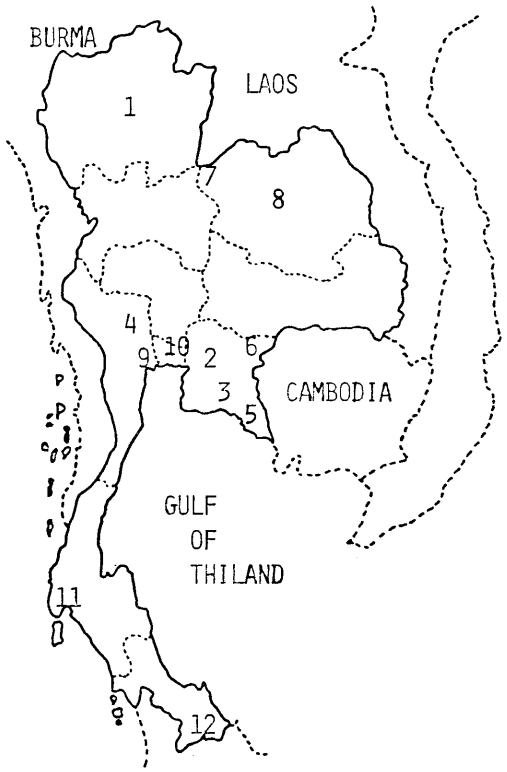
(Samadi *et al.*, 1983), emergence of multiply drug-resistant strains of *V. cholerae* in Bangladesh (Glass *et al.*, 1980) and African countries (Towner *et al.*, 1979, Ichinose *et al.*, 1986, Maimone *et al.*, 1986) and the hemolytic property of *El Tor* vibrio (Barrett & Blake, 1981).

Thus, we collected strains of *V. cholerae* and characterized them biochemically and bacteriologically. The present paper describes the biological characters of *V. cholerae* O1 isolated in Thailand in 1987.

Materials and Methods

A) strains

A total of 100 strains of *V. cholerae* O1 were collected from the Department of Medical Sciences, National Institute of Health, Thailand. These strains were isolated from 95 cholera patients at the medical facilities in Thailand in 1987 and from 4 water sources as shown in Figure 1.



1. Chiang Mai (46)
2. Bangkok (12)
3. Chantaburi (8)
4. Nakhon Pathom (7)
5. Trat (6)
6. Chachoengsao (6)
7. Chaiyaphum (4)
8. Nakhon Ratchasima (4)
9. Samut Sogkhram (1)
10. Nonthaburi (1)
11. Phangnga (1)
12. Yala (1)

Fig. 1. Map of Thailand: showing the places and number of *V. cholerae* O1 strains isolated.

B) Biochemical properties

The strains stored in nutrient agar were isolated on to TCBS agar (Eiken) and Brom-Thymol-Blue media (Eiken) to check their purity. A colony from each stock was examined with the following media for identification, Kligler, SIM, Voges-Proskauer (VP), lysine and Simons citrate, and was simultaneously checked for the level of cytochrome oxidase. To determine the sensitivity to O/129, a single disc method containing 10μg/ml of 2,4-diamino-6,7-diisopropyl-pteridine phosphate was employed (Bauer *et al.*, 1966). The sodium chloride tolerance test, and sucrose, mannose and arabinose fermentation tests for Heiberg's grouping were also employed for further confirmation and classification.

C) Serological properties

Suspected strains were tested for agglutination with monospecific Ogawa and Inaba antisera (Denka Seiken, Janan).

D) Biotyping

All strains of *V. cholerae* O1 were tested for sensitivity to phage IV (Mukerjee, 1963) and polymyxin B (50IU) (Gangarosa *et al.*, 1967), chicken red blood cell agglutination (Finkelstein and Mukerjee, 1963) and sheep red blood hemolysis (Feeley and Pittman, 1963). The hemolysis test was also done with heart infusion broth containing 1% glycerol. Of each culture 0.2ml in peptone water (pH8.0) was mixed with 4ml of semi-solid agar (45℃) and mixture was overlayed onto a basal agar plate (15ml of nutrient agar). A loopful of phage IV suspension having 10^5 pfu/ml (1RTD) was spotted onto each culture, and a polymixin B disc (50IU) was also placed there. After overnight incubation the results were read. The chicken RBC agglutination test on a slide was done by using 2.5% chicken RBC suspension.

Techniques for quantitation of HA and HA inhibition with D-mannose were adapted from Jones *et al.* (1976). The hemolysis test was done by the slightly modified method of Feeley and Pittman (1963). Strains to be tested were cultured in 2ml of heart infusion broth and also in heart infusion broth containing 1% glycerol for 24h at 37℃. To each culture was added 0.2ml of 5% sheep blood cells in physiological saline. The degree of hemolysis was classified as follows: complete hemolysis (++), marked hemolysis with RBC sediments (+), weak hemolysis (+−), and no hemolysis (−).

E) Prophage typing

To determine the lysogenicity for kappa-phage, the method of Takeya and Shimotori (1963) was used. The strains to be tested were inoculated in peptone water (pH8.0) and cultured overnight at 37℃. A small amount of chloroform was then added to the culture and it was well shaken to kill the living cells. After low-speed centrifugation (3,000rpm, 30min), the supernatant was collected. A mixture of H218 culture (0.2ml) and semisolid agar (4ml) was overlayed onto the basal agar plate. The plate was then spotted with 0.1ml of the supernatant mentioned above. After overnight incubation at 37℃, the formation of

plaques or lysis zone was recorded. To determine the sensitivity to kappa-phage, 0.2ml of the same culture were mixed with 4ml of semi-solid agar at 45℃, and the mixture was overlayed on the basal agar plate. The kappa-phage suspension (15μl) was spotted onto the plate. Reading was done after overnight incubation at 37℃.

F) Drug sensitivity test

Minimum inhibitory concentration (MIC) was determined by the agar plate dilution method recommended by Japan Society of Chemotherapy (1981), using Mueller-Hinton agar containing two-fold dilutions of standardized antibiotics from 100 to 0.2μg/ml.

Hundred-fold dilutions of overnight broth culture of the test strains (10^6/ml) were inoculated onto the plates containing antibiotics. The MIC was taken to be the lowest concentration (μg/ml) in which bacterial growth was completely inhibited. The antibiotics tested were tetracycline (TC), nalidixic acid (NA), ampicillin (ABPC), streptomycin (SM) and chloramphenicol (CP) (Sigma).

V. cholerae O1 (H218) was used as a control. To check the sensitivity to O/129, a single disc method containing 10μg/ml of 2,4-diamino-6,7-diisopropyl-pteridine phosphate was employed (Bauer *et al.*, 1966).

RESULTS

A total of 100 strains were confirmed as *V. cholerae* O1 by the following properties: motile, ferment glucose without gas and sucrose but not lactose, produce oxidase but not hydrosulfate and indole pyruvic acid, utilize citrate and lysine, grow in alkaline peptone water without sodium chloride. In the sugar fermentation test for Heiberg's grouping, all strains were revealed to be positive in sucrose and mannose and negative in arabinose. Therefore, all of them belong to Heiberg's group 1. All strains were Inaba serotype. As shown in Table 1, all strains of *V. cholerae* O1 were resistant to phage IV and polymyxin B. In the case of the chicken RBC agglutination test, 97 strains were positive and 3 were negative. All the strains with HA activity were sensitive to D-mannose except for one resistant strain. In the Voges-Proskauer reaction, 85 strains were positive and 15 strains were negative. Eighty seven strains showed complete hemolysis, 5 strains marked hemolysis with RBC sediments, 3 strains weak hemolysis, and 5 strains no hemolysis. On the other hand, all strains showed a positive reaction in heart infusion broth containing 1% glycerol although 6 strains showed marked hemolysis with RBC sediments (Table 2). Therefore, all strains were classified as biotype *El Tor*. Table 3 shows the results of prophage typing for kappa-phage. All the strains were lysogenic for kappa-phage and not sensitive to kappa-phage. Therefore, all the strains were identified as Celebes original type. Table 4 shows the MIC for 100 strains of *V. cholerae* O1. Strains giving an MIC of 50μg/ml or more for tetracycline and 100μg/ml or more for streptomycin and ampicillin were considered resistant. Ninty-nine strains of *V. cholerae* O1 were sensitive to all drugs tested (CP, TC, SM, ABPC, EM and NA). Nalidixic acid showed the strongest anti-vibrio

activity. The growth of all isolates of *V. cholerae* O1 was inhibited at a concentration of less than 0.2μg/ml. One strain was resistant to ABPC, with an MIC of more than 100μg/ml and resistant to 10μg/ml of 2.4−diamino−6.7−diisopropyl-pteridine phosphate (O/129). The MICs against TC and CP were 3.13 and 12.5μg/ml, respectively.

Table 1. Biotyping Test

Properties	No. of strain
Phage IV sensitivity	
−	100
+	0
Polymyxin B sensitivity	
−	100
+	0
Chicken RBC agglutination	
+	97*
−	3
Voges-Proskauer reaction	
+	85
−	15

*: All strains with HA activity were sensitive to D-mannose except one strain resistant to D-mannose.

Table 2. Haemolysis Test

Hemolysis	HIB*	HIB (1% glycerol)
++	87	94
+	5	6
+−	3	0
−	5	0

*: Heart infusion broth

Table 3. Prophage typing

Type	Lysogenicity	Sensitivity	No. of strain
Celebes			
original	+	−	100
cured	−	+	0
Ubol	−	−	0

Table 4. Number of strains showing each MIC

μg/ml	CP	TC	SM	ABPC	EM	NA
0.2>		47			7	100
0.2		52				
0.39	31					
0.78	68			1	1	
1.56				72	74	
3.13		1		26	18	
6.25						
12.5	1		27			
25			73			
50						
100						
100<				1		

Discussion

All the strains of *V. cholerae* O1 isolated in Thailand in 1987 were Inaba serotype, biotype *El Tor*. *V. cholerae* O1, biotype *El Tor* was commonly observed in other countries, although replacement of classical vibrio by *El Tor* has occurred exceptionally in Bangladesh in 1982 (Samadi *et al.*, 1982).

Secondly, all the isolates were Celebes original type, which is regarded as the epidemic strains in the current seventh cholera pandemic and no Ubol strain, originating from Thailand in 1960, was detected in this study. Thirdly, the proportion of hemolytic strains isolated in Thailand was 95% and all the strains were hemolytic by using glycerol-containing broth as described by Sakazaki *et al.* (1971). The hemolytic property of *El Tor* vibrio, which has been used for differentiation from the classical strain, has been changing geographically and chronologically. According to Gallut (1971), the strains of *V. cholerae* isolated when the seventh cholera pandemic invaded into the African continent were non-hemolytic but Iwanaga *et al.* (1982). reported that 77% of the strains of *V. cholerae* isolated in Kenya in 1980 and 1981 were hemolytic by the method of Feeley and Pittman (1963). The proportion of the hemolytic strain isolated in Kenya in 1983 was 75.5% (Ichinose *et al.*, 1986).

On the other hand, the rate of hemolytic strain isolated in the Philippines has decreased gradually being 13.3, 5.0 and 0.0% in 1973−78, 1982 and 1984, respectively (Ichinose, 1985). It is significant epidemiologically to monitor the hemolytic property of *V. cholerae* O1. Finally, ninty-nine strains of *V. cholerae* O1 isolated in Thailand in 1987 were sensitive to all five antimicrobial agents (CP, TC, SM, ABPC, EM and NA). Only one strain was resistant to ABPC. No multiply drug-resistant strains of *V. cholerae* O1 as has occurred in Tanzania, Bangladesh and Kenya were found, probably because an extensive prophylactic chemotherapy to close and family contact as a control measure has not been done and intravenous replacement of fluid therapy and electrolyte loss is the basis for the treatment of cholera patients. In this study, we did not examine the sensitivity test to sulfamethoxazole-trimethoprim. Further investigation should be done about one resistant strain to O/129, because the O/129 resistance is closely associated with that of trimethoprim because of their common chemical structure.

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1987年タイで流行したコレラ菌の生物学的性状

岩見　守，一瀬休生，江原雅彦，内藤達郎（長崎大学熱帯医学研究所病原細菌学部門）
Krongkeaw SUPAWAT（タイ国立衛生研究所，ノンタブリ，タイ）

1987年タイで流行したコレラの入院患者から分離されたコレラ菌100株について生化学的性状検査を行った．分離されたコレラ菌100株の血清型はすべて稲葉型で，生物型はエルトール，プロファージ型別では，Ubol 型は見い出されず，すべてセレベス原型であった．ヒツジ赤血球溶血性試験では，ハートインフュージョンブロースの Feeley and Pittman の方法では95％の株が溶血性を示し，1％グリセリンを加えたハートインフュージョンブロースを用いた Barua

and Mukerjee の方法では弱溶血6株を含めると，100%の株が溶血性を示した．100株のコレラ菌のうち97株がニワトリ赤血球凝集能を有し，そのうち1株を除くすべての株が D－マンノースに感受性であった．

薬剤感受性試験では，アンピシリンに対する最小発育阻止濃度（MIC）が 100μg/ml 以上を示す1株の耐性株を除き99株は，クロラムフェニコール，テトラサイクリン，アンピシリン，エリスロマイシン，及びナリディキシン酸の5薬剤に対して感受性を示した．アンピシリン耐性の1株は 10μg/ml の 2,4－diamino－6,7－diisopropyl-pteridine phosphate で発育阻止されず，O/129 耐性株であった．この株のテトラサイクリン及びクロラムフェニコールに対する MIC は，それぞれ 3.13，12.5 であり，やや高い MIC を示したが，多剤耐性菌は検出されなかった．

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