BACTERIOPHAGE TYPING OF *VIBRIO FLUVIALIS*

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Abstract. Six stable bacteriophages of *Vibrio fluvialis* were isolated from 44 surface water specimens collected in Thailand and Japan. Twelve different phage types were found among 109 *V. fluvialis* isolated from feces of diarrheal patients and the environment. Seventy-three percent (80/109) of these 109 isolates were typable with these phages. One phage type, designated as A (1) was predominant and accounted for 43% of the *V. fluvialis* examined. The six bacteriophages used in this typing scheme were stable for at least during a three-month storage at 4°C. This proposed bacteriophage typing scheme may be of valuable aid in tracing sources and routes of infection in outbreaks of *V. fluvialis* infection in man.

INTRODUCTION

Vibrio fluvialis (previously called group F vibrio, or EF 6 vibrio) is a halophilic vibrio species implicated as a cause of cholera-like diarrhea in humans (Furniss *et al,* 1977; Huq *et al,* 1980). This organism has been isolated from the feces of humans suffering with diarrhea as well as from the environment such as sewage, river water, esturine water, shellfish, crustacea in various countries (Brenner *et al,* 1983; Huq *et al,* 1980; Huq *et al,* 1985; Kudoh *et al,* 1983; Seidler *et al,* 1980; Suthienkul *et al,* 1985; Tacket *et al* 1982; Thekdi *et al,* 1982). The enteropathogenicity of this bacterium has not been clearly defined.

In 1983, Kudoh *et al* described 10 different O antigenic groups of *V. fluvialis,* and Shimada and Sakazaki (1983) defined 18 O antigenic groups. Later, O-serogroup 19 of this species was reported (Shimada *et al,* 1987). Presently, reports on this organism are limited and there are no reliable typing schemes to characterize isolates of *V. fluvialis* in epidemiological investigations. Therefore, the author attempted to develop a phage typing method for *V. fluvialis* for ecological and epidemiological use. This communication describes the typing scheme devised.

MATERIALS AND METHODS

Bacterial strains

A total of 109 strains of *V. fluvialis* was examined : 101 strains were isolated from feces of diarrheal cases or healthy persons in Thailand, Japan, Bangladesh, Singapore and India, 8 strains were from sewage outlets, rivers, canals, and estuary waters in Thailand. Strain 9554-78 (VL 2926), isolated from human feces used as a reference strain of *V. fluvialis,* was kindly provided by Dr Y Kudoh, Tokyo Metropolitan Research Laboratory of Public Health (TMRLPH), Tokyo.

The methods used for the isolation and identification of the organisms have been previously described in detail by Furniss *et al* (1977) and Lee *et al* (1981). All the stock strains used were maintained in semisolid nutrient agar at room temperature throughout the experiments.

Media

Agar base (BA) for the isolation of bacteriophages consisted of 1% polypeptone (Daigo Eiyo Co Ltd, Tokyo), 0.5% NaCl, and 1.3% Bacto agar (Difco), (adjusted to a final pH 7.8). All plates were dried at 37°C before used. The formula of the soft agar (SA) was the same as above, except 0.6% instead of 1.3% Bacto - agar was used and 0.2% of yeast extract was added. Peptone water (PW) contained the same composition as SA but Bacto-agar was excluded. This medium was used for culturing the host strains and also for diluting phage samples.

Water specimens

Twenty-nine water samples were collected in Thailand and 15 in Japan from estuarine waters, fish markets, sewage waters, and river waters. Twenty ml of each water specimen were collected in sterile screw-capped tubes by holding the tube 30 cm below the surface.

Cultivation of strains

Cultures of *V. fluvialis* used for phage isolation (or phage typing) were inoculated into 2 ml of PW and incubated without shaking at 37°C overnight or with shaking at 37°C for 4 - 6 hours. Inoculum size was 0.1 ml of a suspension containing approximately 10^8 - 10^9 bacterial cells/ml.

Isolation of the phage from water or sewage specimens

Ten ml of each water specimen was centrifuged at 17,300*g* for 10 minutes at 4°C. Supernatants were then filtered through a 0.45 μm membrane filter (Millipore). Initially, one ml of each filtrate was mixed with 0.1 ml of an overnight culture of ten selected strains of *V. fluvialis* in 10 ml of PW. After overnight incubation at 37°C, chloroform was added and the cultures were centrifuged at 17,300*g* for 10 minutes. The supernatant was collected, sterilized by filtration, and stored at 4°C.

Bacteriophages in the filtrate were sought by the agar-layer method. Equal volumes (0.1 ml) of filtrate and bacterial culture of the host strains were spread on the surface of BA plates, and 3 ml of melted SA supplemented with 1% of 0.1 M $CaCl_2$ were added. After thorough mixing, the plates were incubated at 37°C overnight. If no phage plaque was seen with the first ten indicators, the water specimen was tested with ten additional indicator bacteria. This process was repeated with arbitrarily chosen 77 among 109 strains.

The bacteriophages were purified by repeated single-plaque isolations as follows. A single plaque with surrounding medium was transferred by sterilized loop to 2 ml of PW and incubated at 37°C for 4 hours. The broth was centrifuged at 17,300*g* for 10 minutes at 4°C. The supernate was the ten - fold serially diluted and plated by the agar - layer method as described above. The second and the third single - plaque isolations followed the same procedure.

Propagation of *Vibrio fluvialis* bacteriophages

After the third single - plaque isolation, phage suspensions were stored at 4°C for later propagation. A modification of tha agar - layer method of Anderson and Williams (1956) was used. Plates of phage inoculum containing the highest dilution that would yield confluent lysis were frozen at -70°C for at least 4 hours and subsequently thawed at 37°C. The liquid part and soft agar were subsequently mixed, and after centrifugation, the filtered supernate was used as a phage stock.

The routine test dilutions (RTDs) were determined for each phage preparation by loop - spotting ten - fold dilutions of phage stocks onto a lawn of freshly inoculated host - propagating cells on BA plates. The dilution producing nearly confluent lysis was used as the RTD. Reading and interpretation of results followed the method of Anderson and Williams (1956). Briefly, all drop areas having a clear or nearly clear zone were recognized as confluent lysis (CL); an intermediate degree of lysis was recorded as semi-confluent lysis (SCL); 81 - 120 plaques was recorded as 3 +, 41 - 80 plaques as 2 +, 20 - 40 plaques as 1 +, 6 - 20 plaques as ± and 0 - 5 plaques as negative.

Phage typing procedure

One hundred and nine host strains were tested. The RTDs of the reference phages were determined by loop - spotting the phage preparation onto well - dried plates, freshly inoculated with 0.1 ml of a test culture and 1 drop of 0.1 M $CaCl_2$. After spotting, the plates were dried at room temperature before incubation at 37°C for 18 - 24 hours. The reactions were read and recorded in the same way as for the determination of RTD. A hand lens was used for inspection of the plates.

RESULTS

The development of a phage typing scheme

Twenty-five out of 31 bacteriophages isolated from either water samples in Thailand or from fish markets in Japan were not stable and easily lost their lytic activity after the first or second plaque isolation. Six phages which persistently produced identical clear plaques when tested on their host strains were used as reference phages. These six phages which were isolated from water samples collected either in Japan and/or Thailand were used to develop a phage typing scheme for 109 isolates of *V. fluvialis*. These reference phages designated as 1 - 6 are given in Table 1.

Successive single - plaque isolation and propagation of these six phages were carried out in order to obtain the optimal RTD titer. Phage typing of *V. fluvialis* was conducted by spotting the RTD of the 6 reference phages on the 109 host

strains and by recording the lytic patterns of the susceptible strains. A proposed phage typing scheme is shown in Table 2. The typing scheme employs 6 phages giving 12 lytic patterns. The lytic patterns are classified alphanumerically in the usual way so that new letters and numbers may be added as desired if new phage types are discovered.

The percentages of different phage types using the 6 reference phages with 109 *V. fluvialis* strains are shown in Table 3. Seventy-three (80/109) percent of these strains were phage - typable. Type A (1) was mostly seen (43%), followed by types A (1, 4), A (1, 3), A (1, 2) and D (4), respectively. The other types were found at low frequency, and only one strain was lysed by a single phage.

Reproducibility of the phage typing scheme for *Vibrio fluvialis*

To determine whether susceptibility to a given phage type was a stable characteristic of a *V. fluvialis* strain, repeated tests were performed using the 6 reference phages (Table 2). The examinations were thrice repeated. Most of the pilot strains were stable for the lytic pattern. Exceptions were strains No. 15B, 790746, 15A, NT-806-008, and EF-6 9554-78 that belonged to type A (1), type B (2), type B (2, 4), and type F (6), respectively. Strain No. 15B usually showed the CL lytic reaction to phage 1, but an additional lytic reaction to phage 3 with the degree of + + + was found in the third repeated test. Strain No. C 790746 lost its lytic reaction to homologous phage 2 once during the three tests. Strain No. 15A usually had a lytic reaction to phage 2 and phage 4, but sometimes it failed to be lysed by phage 4. Strain No. NT-806-008 usually had a lytic reaction to phage 3, but it was sometimes also susceptible to phage 1. It may be due to its very small hazy plaque size. The reaction was very difficult to observe with the naked eye or even with the aid of a magnifying lens. Strains No. EF6 9554-78 was lysed by phage 6 but had additional reactions to phage 3 to the degree of + + + or +.

Stability of phages

In order to develop a usable phage - typing system for *V. fluvialis*, it was necessary to determine whether the reference phages were stable at 4°C (Table 4). All reference phage stocks were retested at 1 week intervals for one month, at 2-week intervals for a succeeding month, and once at the end of third month. All phage stocks were stable enough to maintain their original titer after storage for 3 months as determined by using appropriate RTD doses on respective host strains.

Stability of host bacteria

Further studies were carried out to ascertain whether clones of reference strains would lose their lytic characteristics after 5 successive subculturing. In each subculture, 5 - 10 clones of reference strains were pooled for the test. The 6 reference phages at appropriate RTDs were spotted on plates already inoculated with test strains. Most strains tested remained stable over the test period. Those showing variations were the same as those which gave varying lytic patterns in previous tests (*ie*, C790746, 15A and 15B). However, even those strains showing variations could be used as reference strains by choosing the clones which gave the constant lytic reactions.

Distribution of phage types

The phage type distribution according to the origin is shown in Table 3. Seventy-three (67%), and 7 (6%) of the 109 *V. fluvialis* from humans and environment, respectively, were typable with the 6 reference phages. Strains of type A (1), and A (1, 3) originated from both human, and environmental sources. A strain in type B (2, 4) was only from water sources and strains of the remaining phage types were from human sources.

DISCUSSION

Though *V. fluvialis* can be identified by a series of biochemical tests as studied by many workers (Furniss *et al*, 1977; Lee *et al*, 1981), it gives no information on the epidemiology of the disease. Typing of bacteria by using bacteriophage has been widely used in the epidemiological study of many infectious diseases. In the present study, the author succeeded in isolating stable bacteriophages of this organism from sewage and estuarine samples in Japan and Thailand.

The six reference bacteriophages were stable after storage at 4°C for at least 3 months. Up to 73% (80/109) of the *V. fluvialis* isolates could be typed by these six phages. Further efforts to identify

Table 1

Vibrio fluvialis phages used in the experiments : their source and plaque character.

Phage	Source	Propagation strain (source)	Plaque character
1	Sewage in Tokyo	Strain C790748 (Feces of diarrheal case in Thailand).	Turbid plaque of small size.
2	Canal water in Bangkok	Strain C790746 (Feces of diarrheal cases in Thailand).	Turbid plaque of medium size.
3	River water in Bangkok	Strain NT 806-008 (Feces of traveller's diarrheal case in Tokyo).	Turbid plaque of medium size.
4	Canal water in Bangkok	Strain 79-484 (Feces of Healthy person in Tokyo).	Large clear plaque with turbid halo.
5	Sewage in Tokyo	Strain ZIN 63 (Feces of diarrheal cases in Bangladesh).	Clear plaque of medium size.
6	Canal water in Bangkok	Strain 9554-78 (Received from Dr Y Kudoh, TMRLPH, Tokyo).	Large clear plaque with turbid halo.

Table 2

Phage typing scheme for *Vibrio fluvialis* and reference strain of each type.

Phage type	Reference strain	Reaction to typing phage					
		1	2	3	4	5	6
A (1)	15B	CL/OL - SCL/SOL	-	-	±	-	
A (1, 2)	C810232	CL/OL - SCL/SOL	CL - SCL	-	+	-	-
A (1, 2, 3)	C780267	CL	CL	CL	-	-	-
A (1, 3)	15C	CL	-	CL – + + +	-	-	-
A (1, 4)	79-484	CL - SCL	-	-	CL – + + +	-	-
A (1, 5)	C790743	CL	-	-	-	CL	-
B (2)	C790746	-	CL	-	-	-	-
B (2, 4)	15A	-	CL	-	+ + +	-	-
C (3)	NT 806-008	-	-	CL	-	-	-
D (4)	C810100	-	-	-	CL – + + +	-	-
E (5)	ZIN 63	-	-	-	-	CL	-
F (6)	9554-78	-	-	-	-	-	CL

CL = Confluent lysis
OL = Confluent opaque lysis
SCL = Semiconfluent lysis
+ + + = 81 - 120 plaques
+ + = 41 - 80 plaques
+ = 21 - 40 plaques
± = 6 - 20 plaques
- = 0 - 5 plaques
SOL = Semiconfluent opaque lysis

additional phages that can lyse the remainder are needed. Although some pilot strains showed variable lysis with their respective homologous phages as well as with others, those strains could be used as reference strains by a single-colony selection of re-isolating the specific type strain as mentioned in the past (Bernstein and Wilson, 1963). The reasons for this variation in lytic activities were unknown, but may be due to the period of storage time, or environmental conditions of the test, such

Table 3

The occurrence of different phage types of *Vibrio fluvialis* from human and environmental origins.

Phage types	No. (%) of isolates from		
	Human	Environment	Total
A (1)	42 (41.6)	5 (62.5)	47 (43.1)
A (1, 2)	5 (4.9)	-	5 (4.6)
A (1, 2, 3)	1 (1.0)	-	1 (0.9)
A (1, 3)	4 (4.0)	1 (12.5)	5 (4.6)
A (1, 4)	11 (10.9)	-	11 (10.1)
A (1, 5)	1 (1.0)	-	1 (0.9)
B (2)	1 (1.0)	-	1 (0.9)
B (2, 4)	-	1 (12.5)	1 (0.9)
C (3)	1 (1.0)	-	1 (0.9)
D (4)	5 (4.9)	-	5 (4.6)
E (5)	1 (1.0)	-	1 (0.9)
F (6)	1 (1.0)	-	1 (0.9)
Untypable	28 (27.7)	1 (12.5)	29 (26.6)
Total	101 (100)	8 (100)	109 (100)

Table 4

Stability of typing phage preparations.

Duration of storage at 4°C (weeks)	Titer (RTD*) of phage preparation					
	1	2	3	4	5	6
0	1×10^3	3×10^3	3×10^3	1×10^5	1×10^4	1×10^3
1	1×10^3	1×10^4	1×10^3	3×10^4	1×10^4	1×10^3
2	1×10^3	3×10^3	3×10^3	1×10^5	3×10^4	3×10^3
3	1×10^3	3×10^3	1×10^4	1×10^5	1×10^4	3×10^3
4	3×10^3	1×10^4	3×10^3	1×10^5	3×10^4	1×10^4
6	1×10^3	3×10^3	1×10^3	3×10^4	3×10^4	1×10^3
8	3×10^2	3×10^3	3×10^3	3×10^4	3×10^4	1×10^3
12	3×10^3	1×10^4	3×10^3	3×10^4	3×10^4	3×10^3

* Routine test dilution

as lot variation of the nutrient medium, quality or concentration of agar, or incubation temperature and so on, which may be similar to those of some *Salmonella typhi* phages (Adams, 1959; Bernstein and Wilson, 1963).

Although *V. fluvialis* bacteriophage typing scheme was tested on a limited number of strains, a little epidemiological information had been known. Type A (1) was the most predominant in the phage - typing scheme accounting for 43% of the isolates tested. This accounts for more than 50% of the typable strains. The sensitive strains of this type were from both human origin isolated from feces of patients in Bangladesh, India, Japan, Singapore, and Thailand, and environmental sources in Thailand. Type A (1) may be common among these countries. The susceptible strains of type A (1, 3) were also isolated from both human and environmental sources. The remainder of the

phage types were all from human sources, except for type B (2, 4) which was from water. Because of the small number of strains in the other phage types, it is not known whether they may be epidemiologically related or indigenous to sources, or characteristic of some countries (and not of others), as are found with certain typhoid Vi - phage types, staphylococcus or *Yersinia enterocolitica* phages (Anderson and Felix, 1953; Anderson and William, 1956; Bergan, 1978). More isolates of *V. fluvialis* obtained from world - wide distributed sources should be further typed. In addition, the specificity of these phages should be further typed with other species of vibrios (Shimada and Sakazaki 1983; Shimada *et al,* 1987). However, the phage typing scheme for *V. fluvialis* may be suited to routine diagnostic work, being both simple and rapid. When this method is combined with origin it may provide useful information for ecological and epidemiological studies and for further advanced studies of *V. fluvialis.*

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