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Phage Specific for *Vibrio cholerae* O139 Bengal

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From the stool of a *Vibrio cholerae* O139 Bengal-infected patient, a phage that specifically lysed capsulated *V. cholerae* O139 strains only was isolated. The phage is useful for the confirmatory diagnosis of *V. cholerae* O139 infection and for the differentiation of variants that lack the capsule.

Since late 1992, *Vibrio cholerae* O139 Bengal has emerged as a second etiologic agent of cholera in the Indian subcontinent and neighboring countries (2). There are lytic phages specific for classical and El Tor biotypes of *V. cholerae* O1, the traditional causative agent of cholera (7, 10). Even though there are striking similarities between *V. cholerae* O1 and *V. cholerae* O139 strains, the latter is capsulated, unlike the former, and the phages of *V. cholerae* O1 do not lyse *V. cholerae* O139 strains (3). We have isolated a phage that was found to be specific for *V. cholerae* O139 isolates. We describe the characterization and utility of this phage in this report.

The various bacteria used in the study are listed in Table 1. The Argentine *V. cholerae* serogroup O139 strain ST2761 has an origin different from those of O139 Bengal strains, because it is cholera toxin negative and heat-stable toxin positive (8). Two Tn*phoA* mutants, mutants 6A and 3B, derived from a *V. cholerae* O139 Bengal strain, do not produce the O antigen or the capsular polysaccharide (4). *V. cholerae* cultures were maintained at −70°C in T1N1 medium (1% Trypticase, 1% sodium chloride [pH 7.4]) containing 15% glycerol. The other bacteria were maintained in sealed semisolid nutrient agar tubes at 22°C.

For isolation of phage, watery stools from cholera patients were cultured for *V. cholerae* O139 isolates by standard methods (3), and colonies were confirmed by slide agglutination with specific antiserum (9). The *V. cholerae* O139 isolates were then grown in nutrient broth (Difco, Detroit, Mich.) at 37°C for 4 h. A total of 0.1 ml of the culture was mixed with 5.0 ml of a 1:10-diluted homologous stool (from which the isolate originated) suspension in nutrient broth, and the mixture was incubated at 37°C for 20 h. The culture was centrifuged at 10,000 × *g* for 20 min at 4°C, chloroform (final volume, 1%) was added to the supernatant, and the mixture was left at 4°C for 20 h. Chloroform was evaporated from 1.0 ml of this supernatant by vortexing, and 0.05 ml each of 10^{-1} to 10^{-3} dilutions in phosphate-buffered saline (pH 7.2) were spotted onto a dried lawn of parent *V. cholerae* O139 (the isolate was obtained from the same stool sample from which the phage was isolated) on nutrient agar. The lawn was made from *V. cholerae* O139 isolates grown in nutrient broth at 37°C for 4 h. The inoculum was also spotted onto a lawn of *V. cholerae* O139 AI-1852 (a clinical isolate), which was subsequently used as the propagating strain. The plates were incubated at 37°C for 20 h, and plaques were looked for. Even though four phages were isolated from 40 stool samples, one phage, named JA1, was further studied. A single discrete plaque was purified five times by the soft agar (0.7%) overlay method (7) with strain AI-1852 until homogeneous plaques were obtained. The plaques were clear, with a diameter of approximately 1 mm. For growing phage JA1 in liquid medium, strain AI-1852 was grown in nutrient broth at 37°C for 4 h, and 0.1 ml of culture was inoculated into 10 ml of nutrient broth, which was then inoculated with phages from a single plaque. The bacterium-phage culture was incubated at 37°C for 24 h, when lysis of most of the bacteria occurred. The culture was centrifuged at 10,000 × *g* for 20 min, and to the supernatant, chloroform (final volume, 1%) was added to kill the unlysed bacteria. The supernatant was held at 4°C for 20 h, and chloroform was evaporated from an aliquot. The number of phage particles was determined by testing serial doubling dilutions by the soft agar overlay method with the propagating strain, AI-1852. The phage produced a titer of 10^8 PFU/ml. To obtain phage at a high titer, a plate lysis procedure was used. A 4-h nutrient broth culture of strain AI-1852 (approximately 10^8 CFU/ml) was mixed with phage at a multiplicity of infection of 0.1, and the mixture was plated as a soft agar overlay on a nutrient agar base. The plate was incubated at 37°C for 24 h, when a nearly confluent lysis of the bacteria occurred. The soft agar was scraped off and suspended in 5 ml of Tris-$MgCl_2$ buffer (0.05 M Tris-HCl, 0.02 M $MgCl_2$ [pH 7.5]). Chloroform containing 1% ethanol was added to constitute a 1% total volume. The phage preparation was centrifuged at 20,000 × *g* at 4°C to pellet the debris. The phage particles in the supernatant were pelleted at 30,000 × *g* for 2 h at 4°C in a Beckman SS-34 fixed-angle rotor. The phage pellet was resuspended in 1 ml of Tris-$MgCl_2$ buffer, filtered through a 0.2-μm-pore-size membrane filter (Gelman Sciences, Ann Arbor, Mich.), and maintained at 4°C. The titer of the phage, determined as described above, increased 1,000-fold to 10^{11} PFU/ml. This phage preparation was used to determine the morphology of the phage. It was negatively stained with 2% uranyl acetate and was examined under a Philips transmission electron microscope (model 420 T) (5). The phage particles possessed hexagonal heads and short tails, and thus belonged to the family *Podoviridae* (1) (Fig. 1). The same high-titer phage preparation was also used to determine the routine test dilution (RTD; the highest dilution that just failed to give confluent lysis) (7) by the soft agar overlay method. At the RTD the titer of the phage was 10^3 PFU/ml. For screening isolates for phage susceptibility, single colonies of test strains

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TABLE 1. Phage susceptibility test results for various bacteria

Organism	No. of isolates	Source of isolates	No. of isolates susceptible to phage JA1
V. cholerae O139 Bengal	86	Clinical and environmental Bangladesh[a]	85
V. cholerae O139 Bengal	19	Clinical, India[b]	18
V. cholerae O139 Bengal	13	Clinical, Thailand[c]	13
V. cholerae O139 Bengal	4	Clinical, China[d]	3
V. cholerae O139 Bengal, Tn*phoA* mutant 6A	1	CVD, United States[e]	0
V. cholerae O139 Bengal, Tn*phoA* mutant 3B	1	CVD, United States	0
V. cholerae O139 ST2761	1	Clinical, Argentina[f]	0
V. cholerae serogroups 2-138 and 140-155	1[g]	NIH, Japan[h]	0
V. cholerae O1 biotypes classical and El Tor, serotypes Inaba and Ogawa	10	ICDDR,B[i]	0
Vibrio parahaemolyticus	2	ICDDR,B	0
Vibrio fluvialis	2	ICDDR,B	0
Vibrio mimicus	2	ICDDR,B	0
Aeromonas sobria	2	ICDDR,B	0
Aeromonas hydrophila	2	ICDDR,B	0
Aeromonas caviae	2	ICDDR,B	0
Aeromonas trota	2	ICDDR,B	0
Plesiomonas shigelloides	2	ICDDR,B	0
Salmonella spp.	5	ICDDR,B	0
Shigella spp.[j]	15	ICDDR,B	0
Escherichia coli	4	ICDDR,B	0
Klebsiella spp.	4	ICDDR,B	0
Enterobacter spp.	4	ICDDR,B	0
Pseudomonas spp.	4	ICDDR,B	0
Proteus spp.	4	ICDDR,B	0
Providencia spp.	4	ICDDR,B	0

[a] Cultures isolated through the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), laboratories from field outbreaks, surface water, and hospitalized patients between 1992 and 1995.
[b] National Institute of Cholera and Enteric Diseases, Calcutta, India.
[c] Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand.
[d] Chinese Academy of Preventive Medicine, Beijing, People's Republic of China.
[e] CVD, Center for Vaccine Development, University of Maryland.
[f] Through the University of Göteborg, Göteborg, Sweden.
[g] One strain of each serogroup.
[h] NIH, National Institute of Health, Tokyo, Japan.
[i] From the culture collection of ICDDR,B.
[j] Includes some serotypes of all four species.

were inoculated into 3 ml of nutrient broth and incubated at 37°C for 4 h. A lawn was made with this culture on a well-dried nutrient agar plate. The phage at the RTD was spotted (0.05 ml each) onto the lawn, and the plate was incubated at 37°C for 20 h. The presence or absence of lysis was recorded. *V. cholerae* O139 AI-1852 was included as a positive control.

Of the 122 isolates of *V. cholerae* O139 Bengal tested, all except three (one each from Bangladesh, India, and China) were lysed by the phage. The Argentine strain of *V. cholerae* O139 was not lysed. All of the *V. cholerae* O1 strains, the 153 different serogroups of non-O1, non-O139 *V. cholerae* strains described so far (9), and other bacteria also were not lysed (Table 1). Serum resistance is a marker for the presence of the capsular polysaccharide in *V. cholerae* O139 Bengal, as in other capsulated bacteria (11). The three *V. cholerae* O139 Bengal strains and the Argentine O139 serogroup strain that were not lysed by the phage, three of the Bengal strains lysed by the phage, and two *V. cholerae* O1 strains were tested for serum resistance by using guinea pig serum (11). The Bengal strains susceptible to the phage were resistant to serum (100% survival), the Bengal strains resistant to the phage, including Tn*phoA* mutants 6A and 3B, were susceptible to serum (0% survival), the Argentine strain was resistant to serum (100% survival), and the *V. cholerae* O1 strains showed some resistance (0.02% survival).

The phase variation in the colony morphology of *V. cholerae* O139 strains (opaque and translucent forms) was studied on L

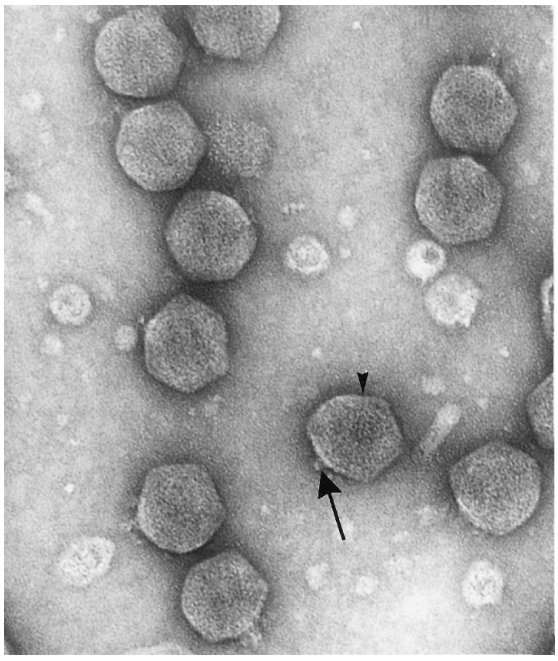

FIG. 1. Morphology of *V. cholerae* O139 phage JA1. Note the hexagonal head (indicated by the arrowhead) and short tail (indicated by the arrow). Magnification, ×180,000.

agar as described previously (6). The phage-susceptible *V. cholerae* O139 Bengal strains and the phage-resistant Argentine O139 serogroup strain produced opaque colonies, and the phage-resistant O139 Bengal strains produced translucent colonies. One of the phage-susceptible *V. cholerae* O139 Bengal strains, the phage-resistant Tn*phoA* mutant 6A, and a *V. cholerae* O1 strain were examined for the presence of a capsule (13). Only the phage-susceptible *V. cholerae* O139 strain possessed a capsule (data not shown).

It has been shown previously that *V. cholerae* O139 Bengal strains that have a capsule produce opaque colonies and are serum resistant (4, 11). Phage JA1 lysed *V. cholerae* O139 Bengal strains that were encapsulated, produced opaque colonies, and were serum resistant. These data suggested that the capsular polysaccharide of O139 Bengal strains is the receptor for the phage. Thus, the phage susceptibility test is also useful for finding out whether a biochemically and serologically confirmed *V. cholerae* O139 strain is capsulated or not.

The Argentine *V. cholerae* O139 serogroup strain possesses a capsule (12). Although it produced opaque colonies and was serum resistant, it was not phage susceptible. This is probably due to its different lineage from O139 Bengal strains. Thus, the phage susceptibility test also seems to confirm the independent origin of the Argentine O139 strain.

Studies on phages of *V. cholerae* O1 have been of historical interest. Among other things, phages have been used for the confirmatory diagnosis of *V. cholerae* O1 infection (7, 10) and for the differentiation of classical and El Tor biotypes of *V. cholerae* O1 (10). Phage JA1 should at least be useful for the confirmatory diagnosis of *V. cholerae* O139 Bengal infection and for the differentiation of *V. cholerae* O139 Bengal strains that have lost the capsule.

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