PHAGE RESISTANT MUTANTS OF THE CHOLERA VIBRIO AND ISOLATION FROM IT OF TEMPERATE PHAGE

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Discovery of phage resistant forms of the cholera vibrio among the strains isolated during the latest cholera epidemics in the endemic areas requires that several aspects of these agents be studied in depth. The purpose of the present study was to determine the number of phage resistant mutants of the cholera vibrio in relation to the typical phages, to be used as a possible indicator of their activity.

METHODS AND RESULTS

The classical method of Luria and Delbruck was used for demonstration of the phage resistant mutants. The average data obtained in determination of the concentration of mutants for 2-8 samples of each phage type are presented in Table 1. The average number of viable parental cells of the strains of cholera vibrio used in the study varied from $9 \cdot 10^7$ to $57 \cdot 10^7$/ml. We considered the strains which did not allow development of stable colonies on plates saturated with phage, as being sensitive to phage. The strains which formed not more than 50 phage-resistant colonies were classified as weakly resistant; those forming 50-100 colonies as moderately resistant, and those with over 100 colonies as highly resistant.

The data show that the tested samples of typical phages are poorly active even on stock cultures of cholera vibrio, kept for a long time on nutrient media. In addition, attention is directed to a comparatively large number of strains highly resistant to specific phages (about 60%), isolated during the latest cholera epidemics (Pakistan, 1958; Afghanistan(1960); India, 1962).

The next problem consisted in studying the cell content and basic properties of phage resistant mutants of the cholera vibrio. Purification of phage-resistant mutants from phage was carried out by means of alternating transfers in broth and on agar. A hundred individual colonies were transferred to test tubes with 1 ml of Martin's medium. After $3^1/_2$ h of growth at 37° a drop of culture was spread with a dolly on a section of an agar plate. The inoculum was dried and then phage was placed on it with a loop. The results were observed after 18 h of incubation at 37°. In broth the experiment was conducted in a similar manner: one drop of a broth culture and 2 drops of complete phage were placed in a test-tube with 4.5 ml of broth. Inocula of cultures without phage served as control. The results were recorded after $3^1/_2$ h of cultivation at 37°.

It has been established that mutants obtained from poorly resistant strains were not pure, and those from strains having a high resistance to phage were pure, consisting of cells resistant to bacteriophage.

The nature of growth of phage resistant mutants in broth and in semi-solid agar differed from that of the stock strains. Most of phage resistant mutants produced slight turbidity, while the stock strains formed a pellicle, leaving the medium clear. The phage resistant mutants have a relatively slow rate of growth. The growth of phage resistant mutants of cholera vibrio on agar was, as a rule, very slight; formation of a number of G-colonies was observed.

Changes in cell morphology in phage resistant mutants were absent. The mutants were motile to the same degree as the stock strains, not adapted to phage. It is possible that the diffuse grwoth in semisolid agar is related to the facultative anaerobiosis of the mutants.

TABLE 1. Relation of Different Strains of Cholera Vibrios to Typical Phages

Strain of phage	Number of strains							
	stock collection				Isolated during the last cholera epidemics			
	Sensitive to phage	Phage resistant mutants			Sensitive to phage	Phage resistant mutants		
		I	II	III		I	II	III
D*	3	2	0	2	4	9	4	14
A Saratov	1	2	1	3	1	2	4	24
C "	0	0	0	7	2	0	0	29
Ogava "	1	4	0	2	0	5	1	25
1 Mukerdzhi	7	0	0	0	3	2	1	25
Total no. of strains studied	7				31			

* Type D contained a small concentration of other types.

Legend: I) weekly stable strains; II) of average resistance; III) highly resistant.

The results of the biochemical properties, agglutinability and certain other properties of phage resistant mutants are given in Table 2, from which it is seen that there was no change in carbohydrate fermentation by the cholera vibrio after aquisition of resistance to phage; the ability to produce indole remained. H_2S was not produced neither by the stock nor by phage resistant cultures. A larger number of mutant strains coagulated milk than did the stock strains. Slight hemolytic properties shown by 3 phage resistant mutants 1666—D/633, 1135—FA/9609_1 and 1135—FA/9609_2 are of some interest. The parent strains of the cholera vibrio (1666 and 1135) were not hemolytic.

Agglutinability of some strains of phage resistant mutants of cholera vibrio was less pronounced than in parent strains prior to phage action. Trypoflavin test was positive in 25 phage resistant mutants, and 7 were also positive in 25 phage resistant mutants, and 7 were also positive in the parent strains.

In correlating the increase of resistance to phage with lysogenization, we attempted to isolate phages from phage resistant mutants. It must be pointed out that no evidence of lysogenization was found in all phage mutants used by us in the course of this study.

The tested mutants of the cholera vibrio were inoculated in test tubes with Martin's broth (pH 7.8) which were incubated at 37° for $3\frac{1}{2}$ h. Phage sensitive strains of the cholera vibrio used for production of cholera phage were inoculated simultaneously in other test tubes with broth. Then, according to the recommendation by R. I. Pikovski and M. G. Gelashvili (1), we heated the $3\frac{1}{2}$ h broth cultures of phage resistant mutants at 56-58° for 30 min to inactivate the bacteria.

Bacteriophage sensitive strains of the cholera vibrio were inoculated on sectors of agar cups and, after drying, heated cultures of phage resistant mutants were added with a loop on the surface of "indicator" strains, to demonstrate the presence of phage in the mutants. The inoculated dishes were left at room temperature (20-25°) in order to have a less pronounced growth and allow better visualization of the phage.

Using this method we were able to obtain 9 latent phages from 148 tested phage resistant mutants of the cholera vibrio. When no heated broth cultures of phage resistant mutants were placed on the indicator strain, lysis due to phage was not observed.

Phages demonstrated on agar cultures readily multiplied in sensitive strains of the cholera vibrio in Martin's broth. Completely stable lysis was usually observed after 0.5 ml of the heated culture of phage resistant mutant, producing lysis of bacteria spread on agar, was placed into a flask with 25 ml of Martin's broth to which was added 5 drops of broth culture of a sensitive strain of the cholera vibrio. After multiplication phage was treated with khinosol and passed through a Seitz filter. Phages isolated from mutants did not lyse homologous and other phage resistant mutants either on agar or in broth.

Phage resistant mutants of the cholera vibrio were preserved either in semisolid agar or lyophilized in ampules at 5°. The latent phages were repeatedly isolated from the mutants for 3-4 months, after which time it was impossible

TABLE 2. Properties of Phage Resistant Mutants of the Cholera Vibrio

Fermentation			Indol	H_2S	Coagulation of milk	Lysis of sheep erythrocytes	Agglutinability	Trypoflavine test
mannose	sucrose	Arabinose						
Parent strains								
148	148	0	148	0	121—, 27+	0	134 to titer, 14 to $^3/_4$ titer	141 —, 7+
Phage resistant mutants								
148	148	0	148	0	113—, 35+	145—, 3+	120 to titer, 10 to $^1/_2$ titer, 8 to $^1/_4$ titer	123—, 25+

Note. 148 strains were tested; the number of strains having particular properties is noted.

to obtain phage from some of the mutants using the above described procedure. It is interesting to note that cured mutants 1135-FA/9609 also lost their hemolytic property and that mutant 1666-D/633, repeatedly yielding bacteriophage, retained the property of lysing a 5% suspension of sheep erythrocytes in broth culture.

These data confirm the relationship [2, 3] present between the hemolytic properties and the action of bacteriophage on the cholera vibrio.

LITERATURE CITED

1. R. I. Pikovskaya and M. G. Gelashvili, Zh. Mikrobiol. (1963), No. 6, p. 91.
2. W. Doorenbos, Ann. Inst. Pasteur (1932), v. 48, p. 457.
3. Idem, C. R. Soc. Biol. (1936), v. 121, p. 130.