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Diagnostic uses of cholera bacteriophages

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INTRODUCTION

As a rule other species of vibrios occur in conjunction with *Vibrio cholerae* in cholera endemic areas, and these different species are likely to be isolated from cholera patients as well as from environmental sources. Prompt and unequivocal identification of the isolated strains is of critical importance for precise determination of the causative agents.

Findings of earlier workers on the use of cholera bacteriophages for differentiating vibrio strains have been inconclusive. Reviewing these studies Pollitzer (1959) could not find sufficient justification for cholera bacteriophage tests being used in cholera laboratory work.

Recent work in these laboratories on the host ranges of cholera bacteriophages has, however, yielded interesting results which shed new light on the problem. During the course of investigations on phage-typing of *V. cholerae* four groups of cholera bacteriophages were isolated from recent cholera epidemics in Calcutta (Mukerjee, Guha & Guha Roy, 1957). The sensitivity patterns of a series of strains of *V. cholerae* as well as of vibrio strains belonging to other species have been tested with these phage groups (Mukerjee *et al.* 1957; Mukerjee, Guha Roy, Guha & Rudra, 1959; Mukerjee, Guha Roy & Rudra, 1960; Mukerjee, 1959). The results of these studies are considered in this paper in relation to the possible uses of cholera bacteriophages for diagnostic purposes.

METHODS AND MATERIALS

Cholera bacteriophages

The four groups of cholera bacteriophages used in this study had been recently isolated from cholera epidemics in Calcutta, and have been described in connexion with the investigations on the phage-typing of *V. cholerae* (Mukerjee *et al.* 1957, 1959, 1960). Isolation, purification, storage and determination of the routine test dilution of these phages, and the technique of phage-sensitivity tests of vibrio strains, were carried out according to methods described there.

Vibrio strains

(i) *V. cholerae*. Thirteen hundred and twenty-eight strains of *V. cholerae* isolated in the Calcutta epidemics between 1955 and 1959 have been tested.

(ii) *El Tor vibrios.* Forty-three strains of pathogenic El Tor vibrios were obtained from Mr S. J. W. Tanamal of Makessar, Indonesia. These strains had been isolated from cholera-like epidemics in Celebes islands. Seven El Tor vibrios isolated from water sources in Calcutta were supplied by Prof. M. N. Lahiri of the All-India Institute of Hygiene and Public Health, Calcutta. Seven strains of El Tor vibrios from cholera epidemics in Thailand were received from Lt.-Col. O. Felsenfeld, Executive Director, SEATO Cholera Research Laboratory, Bangkok, Thailand. Five of these strains had been isolated from cholera patients, one from a contact carrier and one from water. These strains were supplied for phage-typing work along with strains of *V. cholerae*, there being no indication of the presence of El Tor strains in the collection at the time of supply. Two El Tor vibrios were identified among a group of *V. cholerae* strains received in this laboratory from a bacteriologist who had isolated them from cholera patients in Calcutta in 1958.

(iii) *Non-agglutinating (NAG) vibrios.* Forty-eight strains of NAG vibrios originating from different sources have been tested. They were isolated from cholera patients and various environmental sources including food, drink and natural water supplies in cholera endemic areas in Calcutta and Bangkok. They were found to belong to groups I–V of Heiberg's classification.

Test for phage sensitivity

Circular spot or streak cultures of the test strains were made on marked areas in Petri dishes. With the help of a platinum loop of 3 mm. in diameter and delivering 0·01 c.c. volume at a time, one loopful of a phage preparation was superimposed on them. The results were read after overnight incubation at 37° C.

Haemolytic tests

Haemolytic test was done with sheep erythrocytes using 24 hr. culture in alkaline broth (pH 9·0). The results were read after 2 hr. incubation at 37° C. and then overnight in refrigerator.

Soda-serum-agglutination and soda-sublimate-precipitation tests

The methods used were essentially those of Tanamal (1959, and personal communication). For soda-serum-agglutination a high titre cholera O serum (free from preservative) was diluted in progressive twofold dilutions from 1 in 200 in 0·3 % $Na_2CO_32H_2O$ solution in distilled water. Thick suspensions of the test strains were made in distilled water. In about 0·5–1 c.c. of the serum dilutions in small tubes were added two or three drops of the vibrio suspensions. After mixing the tubes were incubated at 37° C. Prompt agglutination of most of the El Tor strains could be observed in tubes containing lower dilution of the serum. Agglutination nearly to the highest agglutination titre of the serum could be observed after overnight incubation with the El Tor strains but not with *V. cholerae.*

For sublimate-precipitation test a few drops of thick suspensions of the test strains were added in tubes containing 0·5 c.c. of 0·5 % $NaHCO_3$ solution. The

tubes were shaken and left for 15 min. Then 0·5 c.c. of 0·5 % $HgCl_2$ was added in each of the tubes, which was again shaken. *V. cholerae* immediately formed floculations while El Tor suspension remained stable.

RESULTS

Cholera bacteriophages showed marked variations in lytic affinities for different species of vibrios as well as for different strains belonging to the same species. The host ranges of cholera phage groups are summarized in Tables 1–3.

Cholera vibrios

The susceptibility of different strains of cholera vibrios to the four groups of cholera bacteriophages have been tested in course of phage-typing of these strains. The results are briefly presented in Table 1.

Table 1. *Lysability of* V. cholerae *by cholera bacteriophages (at routine test dilution)*

Phage group ...	I	II	III	IV	Total no. of *V. cholerae* strains tested
No. of strains of *V. cholerae* lysable	1260 (94·9 %)	1113 (83·8 %)	1317 (99·2 %)	1328 (100 %)	1328

It may be seen from the above table that the four groups of cholera phages acted on a wide range of the host strains but they differed in their lytic spectra for the susceptible cholera vibrios. Thus the group IV phage could lyse all the *V. cholerae* strains tested while the group II phage lysed only 83·8 % of them.

Detailed records of the phage sensitivity of each of the 1328 strains of *V. cholerae* have been published (Mukerjee *et al.* 1957, 1959, 1960). On the basis of their sensitivity patterns to the four groups of phages the *V. cholerae* strains have been classified into five types. One of the types has been further classified into three subtypes by phage adaptations. Following this, the bacteriophages have thus been utilized for strain identification of *V. cholerae* in epidemiological studies.

It has also been reported (Mukerjee, 1959) that the group II cholera phages of the present series are 'smooth-specific' lysing only the smooth elements of *V. cholerae* in a culture and having no lytic affinities for the rough ones. It has been found possible to utilize this phage group for determining the degree of S–R dissociations of strains of *V. cholerae* by following lysis by this phage group spectrophotometrically.

El Tor vibrios

Fifty-nine strains of El Tor vibrios have been tested. Their sensitivity patterns to the four groups of cholera phages are given in Table 2.

It may be seen from Tables 1 and 2 that at its routine test dilution the group IV bacteriophage was universally lytic for strains of *V. cholerae* but failed to lyse any of the El Tor strains from different sources, even when used undiluted. This group

of phage therefore appeared to be a valuable aid for differentiating the two species of agglutinable vibrios. Though El Tor strains were also insensitive to the group-II phage the restricted lytic range of this phage group for *V. cholerae* rendered it unsuitable for differentiation of the vibrio species.

Of the seven El Tor strains from Bangkok three were non-haemolytic. Their correct identity would have been missed and they could have passed as *V. cholerae*, but for their non-susceptibility to cholera phage groups, particularly the group IV phage. Their identification as El Tor strain was confirmed by soda-serum-

Table 2. *Lysability of El Tor vibrios by cholera bacteriophages*

Type of vibrio	Total no. tested	No. of strains found sensitive to cholera phage group							
		I		II		III		IV	
		Undil.	RTD	Undil.	RTD	Undil.	RTD	Undil.	RTD
El Tor, Makessar	43	37	1	Nil	Nil	43	37	Nil	Nil
El Tor, water	7	7	Nil	Nil	Nil	7	6	Nil	Nil
El Tor, case Calcutta	2	Nil	Nil	Nil	Nil	2	2	Nil	Nil
El Tor, case Bangkok	7	5	Nil	Nil	Nil	7	1	Nil	Nil

Undil. = undiluted phage; RTD = routine test dilution of cholera phages.

Table 3. *Lysability of NAG vibrios by cholera bacteriophages*

Total number of strains tested	No. of strains found sensitive to cholera phage groups							
	I		II		III		IV	
	Undil.	RTD	Undil.	RTD	Undil.	RTD	Undil.	RTD
48	4	Nil	Nil	Nil	13	11	2	1

agglutination and sublimate-precipitation tests. On maintenance in laboratory and repeated haemolysis tests all but one strain developed haemolytic properties.

One non-haemolytic El Tor vibrio from a water source in Calcutta has also been identified in a similar manner.

Non-agglutinating vibrios

The phage sensitivity of the non-agglutinating vibrios are given in Table 3.

The NAG vibrios showed much lower susceptibilities to cholera bacteriophage than *V. cholerae*. None of the NAG strains was lysable by a group II phage. Similar observations have been recorded by the earlier workers (Asheshov, Asheshov, Khan, Lahiri & Chatterjee, 1933; Pasricha, De Monte & Gupta, 1936; White, 1936) with type A cholera phages. The other three groups of phages were lytic for only a small proportion of the NAG strains tested. The degrees of

non-susceptibility to cholera phages, particularly for group II, may be used to differentiate between these strains from *V. cholerae* even in the absence of cholera O serum.

DISCUSSION

Cholera bacteriophages have been used in the laboratory for strain identification of *V. cholerae* and determination of their smooth-rough dissociation. The present results suggest that they may be further utilized to differentiate *V. cholerae* from other species of vibrios.

The cholera bacteriophages may be useful in screening out the NAG strains, particularly the auto-agglutinable ones and also those showing partial agglutination with a cholera O serum.

The presence of El Tor vibrios in cholera endemic areas makes it necessary to test for them separately. For this, there is need for a sensitive method suitable for routine application. And it has been found that sensitivity to the group IV phage can be utilized to differentiate between the two O-group I vibrios.

The non-pathogenic El Tor vibrios, first isolated by Gotschlich (1906) and subsequently found in different parts of the world as well as the pathogenic ones from the Celebes islands, closely resemble *V. cholerae*. They possess the same general characteristics of the vibrios and belong to Heiberg group I by sugar fermentation and to the O-group I of Gardner & Venkatraman (1935), being agglutinable by cholera O serum. The El Tor vibrios are usually differentiated from *V. cholerae* by their haemolytic action on sheep or goat erythrocytes in the Greig test. But as reported by Moor (1949) cholera vibrios may acquire haemolytic properties and the haemolytic power of El Tor vibrios in some newly isolated strains is retarded and may therefore be overlooked. In fact, he found some El Tor strains giving alternately positive and negative results with Greig test. Tanamal (personal communication) on the basis of his long experience with El Tor vibrios also found the haemolytic tests as unreliable advisers, in particular with freshly isolated El Tor strains. On the other hand, Doorenbos (1932) reported the acquisition of haemolytic properties by lysogenized strains of *V. cholerae*. Due to the above considerations and also for the variable results obtained in haemolysis test by using different methods it has been thought unwise to attach too much weight on the results of the Greig test (Wilson & Miles, 1955). Other tests like soda-serum-agglutination and soda-sublimate-precipitation (Tanamal, 1938) and tests for heat or chloroform inactivation of the agglutinability of *V. cholerae* (Gipsen, 1938; Meyer, 1939) have been developed to solve the problem. Some of these tests have been found to give inconsistent results, and none of these tests is used to differentiate between the two species of agglutinable vibrios in routine diagnostic work.

In many of the smaller laboratories engaged in bacteriological diagnosis of cholera even the haemolysis test is not done routinely and the agglutinable strains of vibrios isolated from cholera-like patients are classified as the cholera vibrios. But as can be seeen from the results in the present paper this test also fails to identify the non-haemolytic El Tor vibrio.

So far little attention has been paid to the possible existence of El Tor vibrios without haemolytic properties both in cholera endemic areas and also in regions free from cholera. Venkatraman, Krishnaswami & Ramkrishnan (1941) isolated non-haemolytic agglutinable vibrios, 'indistinguishable from *V. cholerae*' in open water sources in certain rural areas in Tanjore districts in South India in the absence of cholera. Read, Pandit & Das (1942) have also reported such strains from 'non-contact' water as well as from 'contact' water sources in India. It would appear from the present studies that the strains belonging to the former group, as well as some in the latter group, could possibly have been non-haemolytic El Tor vibrios. One such strain from a water source in Calcutta is reported in this paper. Analysis of agglutinable vibrios from Bangkok further indicated the possible presence of such strains in cholera patients and chances of their being wrongly identified as *V. cholerae*.

Cholera bacteriophages of the present series have been found to be stable under conditions of storage and maintenance in the laboratory. The phage-sensitivity tests are simple, rapid and easy to perform. For routine use the testing phages may conveniently be supplied to laboratories either in the form of phage filtrates or absorbed on filter-paper disks (Watanabe, 1958).

SUMMARY

1. Apart from strain identification of *V. cholerae* for epidemiological studies by phage-typing and determination of their S–R dissociation cholera bacteriophages may be utilized for other valuable diagnostic aids in the laboratory.

2. The universal susceptibility of *V. cholerae* strains to group IV cholera bacteriophage and universal lyso-resistance of El Tor vibrios to it provide a specific method for differentiating between the two species of vibrios. This test is simple and convenient for routine diagnostic use and should be of special value for identifying non-haemolytic El Tor vibrios, which are otherwise liable to be missed.

3. The marked insensitivity of the NAG vibrios to cholera bacteriophages, particularly their total insusceptibility to the phage of group II, enables these strains to be readily distinguishable from *V. cholerae*.

4. Cholera bacteriophages which are stable on maintenance and storage in the laboratory can conveniently be supplied absorbed on filter disks for the use of the smaller laboratories.

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