Bacteriophage Typing of Cholera

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A bacteriophage-typing scheme for Vibrio cholerae has been developed on the basis of the pattern of susceptibility of V. cholerae strains to four groups of freshly isolated cholera bacteriophages. Some 4066 strains of V. cholerae isolated in Calcutta during the period 1955-61 have been classified into seven types and subtypes. Less than 1% of the strains were untypable. No correlation was found to exist between the phage-types and serological types of V. cholerae. In lysogenic strains, however, a correlation was observed between the phage-types of V. cholerae and their lysogenic phages. Groups of infection deriving from a single source were found to be caused by single phage-types. The author also discusses the practical value of the phage-typing scheme for the epidemiological investigation of cholera.

The WHO Expert Committee on Cholera (1952) suggested that a vibrio reference laboratory be set up for the purpose of studying V. cholerae strains from the point of view of their epidemiological significance, using the technique of phage-typing. Accordingly, a study was undertaken by us in the Indian Institute for Biochemistry and Experimental Medicine, Calcutta, in 1955 and this is still being continued. Some of the findings have been published (Mukerjee et al., 1957, 1959, 1960; Mukerjee & Guha Roy, 1961). In the present paper the results of these studies over the last seven years are reviewed.

MATERIALS AND METHODS

Strains of Vibrio cholerae

Standard methods were followed for the isolation of V. cholerae strains from stools of cholera patients and for their maintenance in the laboratory. The vast majority of the strains were isolated in epidemics in and around Calcutta each year from 1955 to 1961. Strains of cholera vibrios were also obtained from epidemics in the different States of India, chiefly Bihar, Orissa, Uttar Pradesh and Madras, where cholera is endemic. In 1959 and 1960 strains were received also from cholera epidemics in Thailand.

Tests of V. cholerae strains used in phage-typing experiments

Each strain of V. cholerae was tested for the cholera-red and Voges-Proskauer reactions. Their biochemical characteristics were studied with five sugars—lactose, glucose, mannose, sucrose and arabinose. Haemolytic tests were done with each strain. Serological subtyping and S-R dissociation were tested by agglutination with monospecific Inaba, Ogawa and rough O sera.

Cholera bacteriophages

Standard types of cholera bacteriophages from earlier studies were obtained from the late Dr I. N. Asheshov of the Lister Institute of Preventive Medicine, London, in 1956. They were, however, found to be unsatisfactory for the phage-typing of V. cholerae as hardly any strain difference could be detected by their use. Attempts were therefore made to isolate fresh phage strains from local sources. In the course of the last seven years a large number of phages acting on V. cholerae have been isolated locally from stools of cholera patients, different water-sources and lysogenic strains of V. cholerae.

Test for lysogenicity of the vibrio strains

Each of the strains of V. cholerae was tested for lysogenicity by spotting on the lawn of a universally lysable strain of cholera vibrio. Some strains were also tested by culturing in groups of five in liquid media. The vibrio growths were then killed by chloroform and the broth was tested for evidence of any phage. The lysogenic phages were classified in the usual manner.

Lysis test of cholera phages

Tests for lysis of V. cholerae by cholera bacteriophages were carried out according to the

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methods followed by Craigie & Yen (1938) in the
growth of *Salmonella typhi*, but the results
were read after overnight incubation at 37°C. A
young culture of two-hour growth in nutrient broth
of the test strain of *V. cholerae* was used for phage-
susceptibility tests.

RESULTS

Grouping of cholera bacteriophages

In preliminary experiments, the old laboratory
collection of cholera bacteriophages belonging to
types B, C, D, E, F, G, H, M and R were tested for
differences in lytic activity using 154 strains of
cholera vibrio. However, differences in the sensitivi-
ties of vibrio strains to these phages were rarely
evident and the vibrios were lysable by all the phage
types.

The first series of 30 locally isolated cholera
bacteriophages from stools of cholera cases were
then tested with 200 strains of *V. cholerae*. These
phages could be classified into four distinct groups by
their patterns of lysis of these vibrio strains. This
grouping could be further confirmed by examining
other characteristics including plaque morphology
(Mukerjee, 1961a), thermal death-points (Mukerjee,
1961b), generation times (Mukerjee, 1961c) and
reciprocal cross-reaction on phage-resistant second-
ary growths of vibrios (Mukerjee, 1962a). When
tested antigenically the four groups of phages were
found to be quite distinct, with no overlapping of
their antigenicity (Mukerjee, 1962b).

During the period of observation over 600 strains
of cholera bacteriophages were isolated from stools
of cholera patients, lysogenic strains of cholera
vibrios and water-sources. All but a few of them were
found to belong to the above four groups (Mukerjee,
1962c). The few unclassified phages showed a very
wide range of lysis of cholera vibrios and did not
prove to be of value in phage typing.

Phage-types of *V. cholerae*

Strains of *V. cholerae* isolated in Calcutta epi-
demics were found to belong to five phage-types
definable by their patterns of susceptibility to
the four groups of bacteriophages. The patterns of lysis
of the vibrio types are given in Fig. 1. Type I vibrios
are universally lysable by the four phage-groups.
Types 2, 3 and 5 vibrios are resistant to the action of
one phage-group each, namely, group I, group II and
group III respectively. Type 4 vibrios are resistant
to two of the phage-groups, I and II.

During 1955 to 1961, 4066 strains of cholera
vibrios isolated in the Calcutta area were phage-
typed. The results are summarized in the table.

Untypable strains

All strains of *V. cholerae* were tested for phage-type
soon after isolation from patients, and there was
little likelihood of their being denatured. But 37
strains, amounting to less than 1% of all the strains
examined, were found untypable. They fell into
three groups. The first group of untypable vibrios
consisted of strains which were sensitive to group I,
III and IV phages but were not lysable by group II
phages. From their sensitivity to typing phages they
should have been classified as type 3 vibrios. But
antigenically they were rough strains. The group II
phages are "smooth-specific" ones, failing to lyse
strains of *V. cholerae* in which the smooth somatic
antigen is absent (Mukerjee, 1959). The rough
strains of *V. cholerae* isolated from cholera patients
showing the phage-sensitivity pattern of type 3
vibrios and not carrying any marking phage have
been classified as "untypable" as any strain,
irrespective of its original type, is likely to become
insensitive to group II phages on undergoing rough
dissociation. In the second group of untypable
vibrios there were three strains which showed phage-
susceptibility patterns different from those of the
known phage-types of *V. cholerae*. The third group
of untypable cholera vibrios showed only partial
sensitivity to the routine test dilution of one or more
typing phages even on repeated tests.
**Subtypes of V. cholerae**

Cholera vibrios belonging to types 2, 3, 4 and 5 were tested for evidence of subtypes by phage adaptation. Of these only type 2 vibrios showed the existence of subtypes—namely, 2a, 2b and 2c. Type 1 vibrios, which formed the predominating type occurring in the Calcutta area until 1960, are now being tested for the presence of biochemical subtypes. Although there are slight differences in sugar-fermenting properties among strains belonging to this type, there is no evidence so far to show that subtyping of type 1 vibrios by this method may be of epidemiological value.

It has also been found recently that type 1 vibrios can be classified into three or four subtypes by their patterns of susceptibility to a series of El Tor phages. But as more than one of these subtypes occurred in type 1 strains from single isolated epidemics, this method of subtyping does not appear to be of value for epidemiological work.

**Phage-types and serological subtypes of V. cholerae**

It has been observed throughout this series of experiments that no correlation exists between phage-type and serological subtypes of *V. cholerae*. All the phage-types were present in each of the serological subtypes.

**Pathogenicity of the different phage-types**

No special clinical feature could be observed in groups of persons suffering from a particular phage-type of cholera vibrio. Nor did the toxic manifestations and fatality rates among persons with different phage-types exhibit any marked differences.

**Phage-types and lysogenicity patterns of V. cholerae**

The universally lysable type 1 vibrios usually showed no evidence of phage in their culture. On very rare occasions, type 1 strains showed phage contamination which was readily eliminated after one or two subcultures. Some strains of type 2 and 4 vibrios showed the presence of group I phages in their cultures, while group II phage was present in some of the type 3 vibrios and group III phage in type 5.

The phage-types of *V. cholerae* in the present typing scheme were determined by the resistance patterns of the strains to the typing phages. Immunity-conferring lysogenic phages have been isolated from all phage-types of *V. cholerae* other than type 1. The presence of phage-group I in types 2 and 4, of II in type 3 and of III in type 5 vibrios may be taken as their natural type-determining characteristic. But as a significant proportion of the strains were not lysogenic and lysogenicity in some strains was also not a stable character, typing of *V. cholerae* on the basis of lysogenicity cannot be considered satisfactory. However, identification of carried phages in strains of *V. cholerae* does provide a valuable confirmation of their classification by the present scheme. Carried phages, whenever isolated, were invariably found to be associated with strains belonging to specific phage-types.

**Stability of cholera phages and phage-types of V. cholerae**

The stability of the phage-types of *V. cholerae* and of the typing phages was studied by testing strains maintained under conditions of storage and cultiva-
tion in the laboratory for several years. The phages were found stable and all but a few strains of cholera vibrios also showed type-stability. Occasional changes in types of *V. cholerae* were, however, noted in the laboratory (Mukerjee et al., 1960). In some, such change was accompanied by loss of lyo-
genicity. Type 1 vibrios were perfectly stable.

The uniformity of phage-types of *V. cholerae* strains in successive isolates during convalescence was also tested in a number of cases. Strains isolated from the same patient on different dates always belonged to one type.

*Distribution of phage-types of V. cholerae in the Calcutta area*

The relative incidence of the different phage-types in Calcutta was analysed. The results are illustrated according to their distribution in the different corporation wards and police zones of the city in Fig. 2 and 3.

*Variations in the prevalent phage-types of V. cholerae in Calcutta*

Type 1 vibrios were found to form the most prevalent type in Calcutta from 1955 to the third quarter of 1960, when this type formed between 80% and 90% of all strains tested. In the last quarter of 1960, however, there was a sudden and marked change in the relative incidence of the phage-types of *V. cholerae* and type 3 vibrios became the predominant type. This changed pattern of incidence has persisted up to the time of writing. The change-over of predominant phage-types could be traced back to a group of infections by type 3 which occurred in corporation ward No. 2 in October and November 1960. Infections due to this type gradually spread in other parts of the city and got mixed with those due to other phage-types. This variation of type is illustrated in Fig. 4 and 5.

*Epidemiological studies of groups of patients origina-
ting from single sources*

Although in the present series a fairly large number of strains of *V. cholerae* have been tested from cholera patients in Calcutta, it has not been possible to identify groups of patients originating from single sources, because during an epidemic patients are greatly intermingled and a number of phage-types may be isolated in any particular locality. This is well illustrated by the following observations. In 1961 out of the 19 patients admitted to hospital from an area under one health unit in Chetla in Calcutta, 13 were found to be infected by phage-type 1 and 6 by type 3 *V. cholerae*. These were also the prevalent phage-types in other parts of the city. In another series, 105 strains of cholera vibrios were isolated in 1961 from patients in Howrah, a town situated on the other side of the river Hooghly from Calcutta but with free communications with it. These 105 strains consisted of three phage-types—types 3, 1 and 4 in order of frequency, which was parallel to that found in Calcutta during the same period. Further, most of the stool samples from patients in the Calcutta area were collected from hospitalized patients, and in hospital there was always the possibility of cross-
infection. It was therefore considered necessary to study the epidemiological value of the present phage-
typing scheme in localized epidemics at places remote from Calcutta where groups of patients could be traced to a common origin with reasonable accuracy. So far, three such epidemics have been investigated in Chanditala, Ranaghat and Bankura, where the origin of the epidemics could be traced to single sources of infection carried from Calcutta. The strains isolated in each of these epidemics were of uniform phage-type (Mukerjee et al., 1959). Strains of *V. cholerae* isolated in cholera epidemics of 1959 and 1960 in Bangkok, Thailand, were also found to belong to a single phage-type (Mukerjee & Guha Roy, 1961).

*Evidence of the spread of cholera infection from Calcutta to a neighbouring State from data on phage-types*

Only type 1 *V. cholerae* strains were isolated in Bihar State for several years until 1960. But in the first part of 1961 both type 3 and type 1 vibrios were isolated in the Patna-Mungher area of Bihar. This variation in type ran parallel to that in the Calcutta epidemics in the last quarter of 1960, mentioned earlier, with type 3 *V. cholerae* forming the pre-
dominant type. There is a large Bihari labour population serving in the industrial area of Greater Calcutta, members of which are likely to have carried the infection due to phage-type 3 vibrios on returning to their homes in Bihar. The infections due to this type of vibrio persisted there for some time but failed to become established, all strains isolated in the beginning of 1962 having been found to belong to type 1.

In another area of the same State, the Rajgir-Bihar-
Shariff area, an epidemic started following a fair in Raigir. The health authorities believed that the infection in Rajgir was carried from another fair in
FIG. 2
PREVALENCE OF PHAGE-TYPES OF V. CHOLERAE IN CALCUTTA, 1955-59

REFERENCES

CORPORATION WARD BOUNDARIES
1 TO 80 CORPORATION WARD No

○ PHAGE TYPE 1
△ PHAGE TYPE 2
□ PHAGE TYPE 3
X PHAGE TYPE 4
○ PHAGE TYPE 5
FIG. 3
PREVALENCE OF PHAGE-TYPES OF V. CHOLERAE IN CALCUTTA, 1960-61

The police zones from which the type 5 patients were brought to hospital could not be ascertained.
illness as well as in the incubation period of about a week and the convalescent stage of a week or two.

In the absence of fresh contamination, cholera vibrios can seldom be isolated from natural water-sources and authentic cholera vibrios are incapable of a saprophytic existence in nature. There is no evidence that the mutation of NAG vibrios to agglutinating types of *V. cholerae* forms the starting-point of an epidemic, although occasional mutations in stock cultures in laboratories or under the influence of antiserum and bacteriophage have been reported (Pasricha et al., 1933).

Cholera vibrios survive in actual cholera patients throughout the year in some endemic foci independently of transportation from outside. In Calcutta, for example, examination of stools of patients admitted in the cholera wards shows that *V. cholerae* can be isolated in every month of the year. The possibility that subclinical infections occurring during the inter-epidemic period in an endemic area may form the link between acute infections must also be taken into consideration. Favoured by the influence of seasonal factors and repeated passages through susceptible hosts, cholera vibrios may be maintained in reservoir in infections of a milder type, and may spread cholera. It is understandable that when such a mobile intermediate link in the chain of spread of the infection is lost sight of, the sudden onset of an epidemic remote from the source of origin in an endemic area may appear to have originated from nowhere.

It has been observed that the cholera epidemics of the Calcutta area are of mixed origin. With the seasonal changes an epidemic starts and cases are reported simultaneously from widely scattered areas, from which a number of phage-types of *V. cholerae* are isolated. The infecting strains soon get mixed up. Persons working at places away from their residence may carry the infection either way. In an interesting study, one such small epidemic in a cholera-free area could be traced as having originated in an infected area a long distance away, from which the first victim used to get his meals. Other avenues of infection, such as river or canal water, accidental contamination of municipal water supply lines, etc., also have their roles in spreading the disease. The large population living on boats in the River Hooghly pass their stools into the river and maintain a potent riverine source of infection.

In Calcutta there are several "black spots" from which a large number of patients are reported every year. The water supply for domestic use in

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**DISCUSSION**

**Epidemiological considerations**

It is generally accepted that there are no chronic carriers of cholera. The sources of infection are actual patients showing acute manifestations of the

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**FIG. 4**

VARIATIONS IN THE PREVALENT PHAGE-TYPES OF *V. CHOLERAE* IN CALCUTTA, 1955-61

**FIG. 5**

VARIATIONS IN INCIDENCE OF *V. CHOLERAE* PHAGE-TYPES 1 AND 3 IN CALCUTTA, 1955-61
these areas usually comes from open tanks or canals which are exposed to repeated contamination from acute cases. In Calcutta over 10 000 cholera patients are admitted to the hospitals every year, of whom about 1000 die. It was in view of the excellent opportunity thus available for epidemiological studies of cholera that the development of the phage-typing scheme described here was undertaken in our laboratories. The large numbers of strains of cholera vibrios and cholera phages used in this work were readily available. But, as pointed out earlier, the strains get freely mixed up in Calcutta epidemics and it is difficult to identify groups of patients deriving from a single common source. The phage-typing data for Calcutta have, however, been of value in indicating the wide distribution of cholera and the scattered prevalence of the phage-types in the area. The limited field surveys of localized epidemics outside the city originating from a single source, as well as the observations on the Bihar epidemics, have borne out the epidemiological value of cholera phage-typing.

Advantages and limitations of the phage-typing schemes

From the studies made on the various aspects of the present scheme of phage-typing of cholera vibrios, it may be concluded that it satisfies the following requirements for use in epidemiological studies.

(1) The typing phages are stable in respect of their lytic patterns.

(2) The phage-types of strains of *V. cholerae* are also found to be stable. Alteration in phage-types has been observed only in a very small percentage of strains during maintenance in the laboratory.

(3) The numbers of types and subtypes identified are adequate for a scheme of practical utility.

(4) The technique of the tests is simple and the results are reproducible and quickly available.

The following inherent limitations in the application of the phage-typing scheme for cholera should, however, be pointed out.

(1) There is no chronic carrier in cholera to which groups of epidemiologically related patients can be traced and control of outbreaks of the epidemic is not possible through isolation and "de-infection" of the carrier.

(2) Although a number of phage-types and subtypes of *V. cholerae* have been identified, the vast majority of the cholera vibrios of the Calcutta epidemics belonged to types 1 and 3, and those in other areas mostly to type 1.

Establishment of a central reference laboratory

The present studies have shown the existence of a number of phage-types of cholera vibrios and also the possibility of using the phage-typing scheme for epidemiological investigations.

In order to extend its scope and practical utility, however, it is necessary to establish a central reference laboratory for phage-typing of all strains of *V. cholerae* isolated in different areas and mapping out the foci of infection of each type. This would provide valuable aid in identifying the lines of spread of the infection.

RÉSUMÉ

L'auteur présente une revue des travaux effectués de 1955 à 1961 à l'Institut indien de Biochimie et de Médecine expérimentale de Calcutta et consacrés à la détermination par les phages des divers types de vibrios cholériques.

N'ayant pas réussi à établir de différences entre les souches de vibrios en se servant de phages d'anciennes collections de laboratoire, l'on s'est adressé à des chloraphages isolés sur place. Plus de 600 souches fraîches de chloraphages ont été testées. Elles appartiennent à 4 groupes différents. Quant aux 4066 souches de *V. cholerae* isolées sur des malades de Calcutta, elles peuvent être classées en 5 lysotypes sur la base de leur sensibilité à ces groupes de phages. Le lysotype 1 est lysable par tous les groupes de phages. Les lysotypes 2, 3 et 5 ne sont pas lysés respectivement par les groupes I, II et III. Les souches de type 4 sont insensibles aux phages des groupes 1 et II. Les vibrios de type 2 ont pu être par la suite repartis en 3 sous-groupes par des épreuves de lyso-adaptation. Trente-sept souches (soit 0,9% du nombre total des souches) n'ont pu être typées. Il n'existe aucune corrélation entre les lysotypes et les sous-types sérologiques de *V. cholerae*. Aucun signe clinique particulier, aucune manifestation toxique, aucune différence notable dans le taux de mortalité n'a pu être attribuée à un lysotype déterminé de vibrio cholérique.

Les souches de *V. cholerae* sont loin d'être toutes lysogènes et l'on ne peut donc établir le typage de *V. cholerae* sur la base de la lyso-génëité. Mais les phages isolés sur un type déterminé de vibrios sont toujours les mêmes et
de ce fait leur identification est très utile pour confirmer le classement de ces souches. Les souches de *V. cholerae* isolées à des dates différentes pendant la convalescence d'un même sujet appartennent toujours au même lysotype.


Parallèlement à la variation de prédominance de tel ou tel lysotype enregistrée à Calcutta au dernier trimestre de 1960, une variation similaire des souches de vibrios a été observée dans la région de Patna-Mungher, située dans l'Etat de Bihar. Ceci est dû à la propagation de l'infection par les ouvriers habitant l'Etat de Bihar mais travaillant dans la zone industrielle du grand Calcutta.

L'application de la méthode de typage par les bactériophages à l'établissement d'enquêtes épidémiologiques est discutée.

**REFERENCES**


