CHOLERAGENIC PROPERTY OF CERTAIN STRAINS OF EL TOR, NON-AGGLUTINABLE, AND WATER VIBRIOS CONFIRMED EXPERIMENTALLY

BY

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There are many examples (Mackie, 1929; Taylor *et al.*, 1937; Hisano, 1938; Lefrou *et al.*, 1945; Yajnik and Prasad, 1954) of patients suffering from choleraic symptoms not caused by the "true" cholera vibrios (Ogawa, Inaba, or Hikojima). The organisms closely associated with such a condition are non-agglutinable vibrios (NAG), or cholera-like organisms isolated from tanks, rivers, etc. (Yajnik and Prasad, 1954; Abou-Gareeb, 1959).

Clinically it has been shown that El Tor vibrios are responsible for causing outbreaks of cholera from time to time with varying degree of severity (Kraus, 1909; Hoppe-Seyler, 1916; Kovacs, 1926; de Moor, 1938, 1949; Mukherji, 1955; Felsenfeld, 1960; Felsenfeld *et al.*, 1961). The disease caused by NAG and El Tor vibrios, which may or may not produce appreciable mortality, is characterized by sudden onset, violent diarrhoea, vomiting, and dehydration. Yet to recognize these as true cases of cholera the isolated organisms should be agglutinable and non-haemolytic.

Experimentally it has not been possible to demonstrate clearly the pathological role of the above vibrios. It has also not been accepted that NAG and water vibrios could produce true cholera. Their association with "diarrhoeic" conditions was considered "accidental." Such views are not unlikely in the absence of experimental evidence.

Only a few workers have studied the role of El Tor, NAG, and water vibrios in the pathogenesis in laboratory animals. Kabeshima (1918) found El Tor lethal to mice when injected subcutaneously. Intraperitoneal injection of the passaged strain of El Tor had killed 18 out of 30 white mice within 24 hours (Koesoemadilaga, 1939). These indicated the toxic nature of the vibrios rather than their ability to produce cholera. It was pointed out before (Panse and Dutta, 1961) that the toxic and choleragenic properties of a vibrio were distinct entities. Many Gram-negative bacilli produce toxic effects in laboratory animals.

Gupta *et al.* (1956) injected cholera-like vibrios isolated during a mild "gastroenteritis" outbreak at Allahabad in 1954 into the ligated gut of the rabbit, and observed reactions similar to those injected with true cholera vibrios. The loop was distended with fluid, which was frankly blood-stained and often rice-water with a pinkish hue. This was confirmed by De (1957). The latter author further demonstrated similar reactions with El Tor and *Escherichia coli* strains. The reactions were rather non-specific and had little resemblance to clinical cholera. Further, the conditions of experiment in a closed loop were more artificial than natural.

Experimental evidence is provided here which would justify the assumption that some strains of El Tor, NAG, and water vibrios (cholera-like) are responsible for true attacks of cholera clinically. How far it is reasonable to exclude them from the category of true (not serologically) cholera vibrio needs serious consideration.

Two serotype strains of *E. coli* isolated from cases of gastroenteritis and one strain of *Salmonella typhi* isolated from a case of enteric were studied as controls using the same experimental model.

Griffitts (1944) has demonstrated that virulence of *Vibrio comma* in mice was enhanced if the organisms were inoculated with mucin. Virulence of *V. cholerae* is increased when passaged through the rabbit (Dutta and Habbu, 1955; Gallut, 1957).

Methods

Strains.—All the strains were maintained in a lyophilized state in a refrigerator. The NAG and the El Tor vibrios were isolated from cases having choleraic symptoms. The water vibrios were isolated from tanks or rivers. All these were received through the courtesy of Professor M. N. Lahiri, formerly of the All India Institute of Hygiene and Public Health, Calcutta. The *E. coli* O 55 and O 111 strains were kindly supplied by Dr. J. B. Srivastava, Director, Central Research Institute, Kasauli, and the two Ogawa strains were isolated by one of us (M. V. P.) during an epidemic of cholera in Calcutta in 1959. The Kumbh Mela strain (KM 1), a non-agglutinable strain, was isolated from the stool of a patient during an epidemic of "acute gastroenteritis" at Allahabad (Yajnik and Prasad, 1954). This was received through the courtesy of the late Professor V. S. Manglik. The *Salm. typhi* strain was locally isolated from a case of enteric fever. The identification of the strains was established by their agglutinating characters against specific antisera, wherever applicable, and their biochemical reactions.

Rabbit-passaged Strain.—Group of rabbits 8 to 10 days old were inoculated intra-intestinally with a heavy dose of live organisms. In a few instances the animal died, while in others they were sacrificed at the end of 24 hours. The vibrios were recovered from the large intestinal contents using alkaline peptone water. The recovered strains isolated as a pure culture were referred to as "rabbit-passaged." They were preserved in a lyophilized state.

Preparation of Infective Dose.—The freeze-dried vibrios were regenerated in broth, plated on agar slants, and incubated overnight. A 1-ml. loopful of the culture was suspended in 10 ml. of the nutrient broth and again incubated for three hours. At the end of this period the broth contained about 10^9 organisms per ml. Then 3 ml. of the culture was put in a 10-ml. ampoule and sealed. It was shaken in an electric shaker for one minute. One or two drops of octyl alcohol was added to remove the froth. The culture was suitably diluted in broth before use.

Administration of Vibrios with Mucin.—A 5% mucin (British Drug Houses) suspension was made in distilled water. This was strained through muslin and sterilized

by heating at 100° C. for half an hour for three successive days. The solution of mucin was mixed with vibrios just before infecting animals in the proportion of 4 ml. of mucin and 1 ml. of vibrio suspension.

Choleragenic Property of Strains.—Ten-day-old rabbits of either sex weighing 100 to 150 g. were used (Dutta and Habbu, 1955). The litters were kept with their respective mothers in separate cages. Suitable numbers of live vibrios (normal or rabbit-passaged) were injected into the small intestine either with or without mucin. In the case of an attack of cholera the animal lost weight, suffered from profuse diarrhoea (rice-water stool), dehydration, anuria, etc.

Results

Infection With Unpassaged Strain

In Table I are summarized the results of experiments in which the young rabbits were inoculated intra-intestinally with 10^9 organisms per 100 g. body weight together with mucin. The strains of Ogawa 82 and 89 produced characteristic signs and symptoms of cholera with death in four out of the six animals taken. Out of

TABLE I.—*Infection of 10-day-old rabbits with 10^9 micro-organisms per 100 g. of body weight*

Strains	Animals Used	Diarrhoea	Mortality	Signs and Symptoms
Ogawa 82	6	5/6	4/6	Typical cholera
,, 89	6	4/6	4/6	,,
El Tor 5/61	4	4/4	4/4	,,
,, ,, 34/62	4	4/4	4/4	,,
,, ,, 208	4	1/4	0/4	—
NAG 31	4	0/4	0/4	—
,, 37	4	1/4 (slight)	0/4	—
,, 45	5	0/5	0/5	—
,, 91	6	0/6	0/6	—
,, X 12	6	1/6 (slight)	0/6	—
,, X 19	4	0/4	0/4	—
Water vibrio S-121/4 ..	6	2/6	0/6	—
,, ,, W-655/1 ..	4	2/4	0/4	—
Kumbh Mela (KM 1) ..	3	0/3	0/3	—
E. coli O 55	4	0/4	0/4	—
,, ,, O 111	4	0/4	0/4	—
Salm. typhi	4	0/4	0/4	—

the four animals infected with El Tor 208, one suffered from diarrhoea and to some extent became dehydrated; it ultimately recovered. The remaining three animals did not show any visible signs of cholera. El Tor 5/61 and El Tor 34/62 caused typical cholera with death in all the animals infected.

Twenty-nine animals were used for testing six strains of NAG vibrios. None of the rabbits suffered from cholera, though NAG 37 and NAG X 12 caused slight diarrhoea in one rabbit from each group. The Kumbh Mela strain (KM 1) had no effect.

The water vibrios S-121/4 produced mild diarrhoea in two out of the six animals infected. The second strain also produced a similar effect. However, these animals recovered. The *E. coli* O 55 and O 111, and *Salm. typhi* had no ill effect.

Infection With Rabbit-passaged Strain

When the rabbits were inoculated with the passaged strains the virulence was enhanced. The infective dose was reduced from 10^9 to 10^6. The results are given in Table II. Both the Ogawa 82 and 89 now killed all the infected animals. El Tor 208 produced diarrhoea and death in three out of the four animals inoculated. With the exception of NAG 45 and NAG 91, the remaining four strains of NAG produced typical cholera but were less virulent than the Ogawa, as evidenced by the lower mortality rate. The animals that died suffered from profuse diarrhoea, extreme dehydration, anuria, etc. The post-mortem picture was identical with that produced by Ogawa.

TABLE II.—*Infection of 10-day-old rabbits with 10^6 micro-organism with rabbit-passaged strains per 100 g. of body weight*

Strains	Animals Used	Diarrhoea	Mortality	Signs and Symptoms
Ogawa 82	6	6/6	6/6	Typical cholera
,, 89	6	6/6	6/6	,,
El Tor 5/61	4	4/4	4/4	,,
,, ,, 34/62	4	4/4	4/4	,,
,, ,, 208	4	3/4	3/4	,,
NAG 31	6	3/6	3/6	,,
,, 37	6	5/6	4/6	,,
,, 45	5	0/5	0/5	—
,, 91	4	0/4	0/4	—
,, X 12	4	4/4	4/4	Typical cholera
,, X 19	6	6/6	5/6	,,
Water vibrio S-121/4 ..	5	3/5	3/5	,,
,, ,, W-655/1 ..	6	4/6	4/6	,,
Kumbh Mela (KM 1) ..	3	3/3	3/3	Not characteristic
E. coli O 55	4	2/4	2/4	,,
,, ,, O 111	4	2/4	1/4	,,
Salm. typhi	4	0/4	0/4	—

The water vibrios, which had earlier caused mild diarrhoea only, now brought about 60 to 65% mortality with the typical picture of experimental cholera when the strains were passaged through the rabbit.

Both the coliform organisms (O 55, O 111) caused diarrhoea and death in some animals, but the picture was not characteristic of cholera. The diarrhoea was mild and there was no anuria. The stools were greenish and thick. On post-mortem examination both the small and the large intestines appeared normal to the naked eye. In contrast to true cholera vibrios, there was hardly any transudate in the large intestine.

The Kumbh Mela strain (KM 1), which was innocuous before, brought about the death in all the animals when the strain was rabbit-passaged. The picture had no resemblance to experimental cholera. The diarrhoea was mild and there was no congestion of the intestines or accumulation of any fluid in the large intestine. Both intestines appeared normal. The stools were greenish in appearance.

No untoward effect was observed with the *Salm. typhi* strain even after rabbit-passage.

Discussion

Previously it was shown by Dutta and Habbu (1955) that it was necessary to increase the virulence of a vibrio (Ogawa or Inaba) if 100% mortality with a typical picture of cholera in the rabbits was desired. This was confirmed while using the strains of Ogawa 82 and 89. In the course of our investigations during the last eight years it was observed that some vibrios (Ogawa or Inaba) recently isolated from clinical cases retained sufficient virulence to cause the disease, with death (100%), in the rabbit. It was therefore not always essential to passage the vibrios.

The two pathogenic strains of *E. coli* in massive doses failed to produce any effect in the animals. After they were rabbit-passaged, their virulence was no doubt increased: they caused diarrhoea and death in a certain percentage of the animals, but the ante-mortem and post-mortem pictures did not simulate experimental cholera. The infant rabbits were not susceptible to *Salm. typhi* in the doses used.

In the Kumbh Mela, Allahabad (Yajnik and Prasad, 1954), NAG vibrios gave rise to an epidemic of "acute gastroenteritis." The mortality was only 2.7% of the 339 cases recorded. Yajnik and Prasad concluded that the general epidemiological and clinical pictures were not suggestive of a true cholera outbreak of the usual type. One of the vibrios isolated from the epidemic and tested here (KM 1) did not produce any ill effect on rabbits. After animal passage it caused mild diarrhoea but none of the true symptoms of cholera. Experimentally, too, the picture was more in line with "gastroenteritis" than with true cholera. The disease produced by the "true" vibrio (Ogawa) in the infant rabbit appeared characteristic and specific in nature.

El Tor can cause cholera. In 1937 it was responsible for an outbreak in Celebes in which the mortality rate reached 75% (de Moor, 1938). There is now evidence that El Tor vibrios have invaded other parts of Indonesia and the Philippines, and have reached as far as Hong Kong in the north (Mukerjee and Guha Roy, 1962). Tanamal (1959) had reported about paracholera outbreaks which were associated with a lower mortality. Clinically, therefore, the patients can have cholera and the death rate can be high. The experimental results reported here confirm the clinical findings.

Maslennikowa (1927) isolated strains of vibrio from cases of suspected cholera. These strains were non-agglutinable. Gardner and Venkataraman (1935) suggested that a large number of non-agglutinable vibrios could give rise to cholera-like diarrhoea which could be mistaken for true cholera. Russell (1936), in Calcutta, found that during the interepidemic period only non-agglutinable strains could be isolated from cases of clinical cholera, while during the epidemic stage only agglutinable vibrios were isolated. Doorenbos (1937) concluded that the clinical syndrome of cholera could be produced by vibrios which were agglutinable with specific sera as well as those which were not agglutinable with the same sera. Read (1937), in Calcutta, had isolated only NAG vibrios from a considerable number of cases with choleraic symptoms during the height of an epidemic. Cholera-like vibrios were responsible for an outbreak of nine cases of "enteritis" among the crew of a ship (Hisano, 1938). Lefrou *et al.* (1945) described an epidemic of "cholerine" in Sudan in 1945. Bruneau (1947) isolated non-agglutinable vibrios from a patient suffering from typical but non-fatal cholera. Clinically, therefore, an attack due to NAG vibrios could be sporadic or in an epidemic form, but with low morbidity rates. Presumably, many cases of cholera produced by NAG vibrios have not been reported as these vibrios had no status as true vibrios for the purpose of official recognition. While not all the non-agglutinable strains may be pathogenic, there are some which under favourable circumstances could cause the disease.

The variability of the organisms which produce clinical cholera has been reported periodically. Pollitzer (1959) quoted many instances of loss of agglutinability of true vibrios or acquisition of agglutinability by NAG vibrios. The variability may be due to environmental conditions, mutation, or dissociation. Whatever may be happening in nature, the possibility of such transformation cannot be ruled out. Lal (1953) pointed out that "actually all descendants of a vibrio which has caused cholera in man and is capable of doing so are entitled to that name irrespective of their size, shape, culture, and biochemical reactions, chemical and antigenic constituents and epidemic behaviour, unless they have undergone permanent mutation." Experimentally, cholera can be produced with El Tor, NAG, and even with water vibrios.

The vibrios have been classified as "true cholera vibrios" or "non-cholera vibrios," depending on their antigenic character. However, it has been shown that in addition to the true cholera vibrios—that is, of Inaba and Ogawa serotypes—other vibrios, such as El Tor, NAG, and water isolates, are capable of choleragenesis in the infant rabbit as well as in humans. On this basis the term "true cholera vibrios" should perhaps be broadened to include those strains other than the recognized serological types that are capable of producing clinical cholera. This concept has recently been recognized in the case of the El Tor vibrios.

It would not be inappropriate to quote Gupta *et al.* (1956) that "many of the so-called 'inagglutinable' strains are capable of producing clinical cholera. But the antigenic structure and choleragenicity of vibrios are independent attributes and may be dissociated. Hence to define 'true' cholera vibrio, a search for the mechanism and the test for choleragenicity alone would provide the decisive answer." This is what has been attempted here.

Summary

Ogawa and Inaba strains of *V. cholerae* are known to cause cholera-like disease in infant rabbits. The disease resembles human cholera. The method is shown to be specific, since no other intestinal organisms, such as *E. coli* and *Salm. typhi*, could cause such a malady.

Infant rabbits infected with El Tor and non-agglutinable vibrios suffered from profuse diarrhoea (rice-water stool), dehydration, anuria, etc. The post-mortem picture, too, resembled that of an attack due to Ogawa or Inaba. Experimental evidence supports the clinical observation that cholera can be caused by some strains of El Tor and non-agglutinable vibrios.

Some of the cholera-like vibrios isolated from water sources could under favourable circumstances produce cholera-like disease in the infant rabbit. It indicates the potential danger of their causing cholera in either sporadic or epidemic form.

Apart from the serological classification, cholera vibrios could be classified on the basis of clinical manifestations of the disease. The strains of El Tor, some strains of non-agglutinable vibrios, and water vibrios come into this category.

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