SHORT ARTICLES

OBSERVATIONS ON *VIBRIO ELTOR*

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THE differentiation of species within the genus *Vibrio* is difficult, but from the standpoint of human disease the essential problem is to identify with certainty and speed the exclusively human pathogens *Vibrio cholerae* and *Vibrio eltor*. From 1906, when Gotschlich first isolated *Vibrio eltor* at the Tor quarantine station in Sinai, this organism has been responsible for numerous epidemics throughout Bengal and India. In recent years it spread in South-East Asia and Africa and has reached pandemic proportions. The recent outbreak due to this organism in Australia in passengers in an aircraft travelling from Bahrain (*British Medical Journal*, 1972) appears to have been food-borne rather than water-borne, and underlines the necessity for rapid identification of this organism. This paper describes the findings on 174 isolates of *Vibrio eltor* in the hope that it may be useful as a guide to bacteriologists who may not have extensive experience with this pathogen.

MATERIALS AND METHODS

Faecal specimens from 518 patients, who had been admitted to the Infectious Diseases Department of our hospital, were examined before any antibiotic or other drugs were administered (Barua, 1970). In most cases rectal swabs were taken and transported to the laboratory in peptone water, pH 7.5. In a few cases a sample of stool was sent in a container. Initial isolation of the cultures was carried out in the local hospital laboratory. The specimens were inoculated into alkaline peptone water, pH 8.5, and incubated at 37°C for 6 hours, when a surface pellicle was seen to develop. The cultures were then plated on to Monsur (1963) and TCBS media (Kobayashi *et al.*, 1963; Felsenfeld, 1967). Smooth, convex colonies on Monsur agar, grey to black in colour and surrounded by a clear halo, were further tested, and so were colonies on TCBS agar that fermented sucrose and appeared as smooth yellow colonies 2 to 3 mm in diameter. The colonies were examined microscopically, and subcultured to nutrient-agar slopes and Kligler's medium. When required for phage typing, 24-hour agar slope cultures were inoculated into nutrient broth, pH 7.5, and incubated at 37°C for 2 hours, when a faint turbidity became evident. This culture was used for phage typing as described below. Altogether 174 strains of *Vibrio eltor* were isolated and studied.

Slide agglutination with polyvalent *Vibrio cholerae* O antiserum and specific tests for Inaba and Ogawa biotypes were performed from the agar slope cultures or from Kligler's medium, after less than 24 hours' incubation. All media were prepared from Difco dehydrated powders.

Biochemical tests. The isolates were tested for oxidase production; for the utilisation of glucose in Hugh and Leifson's O-F medium (1953); by the Voges-Proskauer test (Barritt's method, 1936) and for the production of indole. Mannose, sucrose, arabinose and mannitol were tested for acid production.

Haemolysins. Haemolytic activity was determined by the tube method on 24-hour cultures in heart-infusion broth, as recommended by Feely and Pittman (1963); 0.5 ml of culture was added to 0.5 ml of 1% (v/v) washed sheep erythrocytes resuspended in physiological saline. A control tube with broth replacing the culture was included in each series. The tubes were incubated at 35–37°C for 2 hours and placed in the refrigerator overnight before reading.

Received 31 Jan. 1973; accepted 15 Mar. 1973.

Plate haemolysis tests were also carried out on blood-agar plates incorporating 5% (v/v) fresh sheep RBC. The plates were examined after 24 hours' incubation. Haemolysis was accepted when a zone of clearing at least 4 mm in diameter appeared.

Chick red-cell agglutination. Washed suspensions of chick RBC were used at a concentration of 1.5% (v/v). Macroscopic slide agglutination was observed after a few minutes. Different degrees of agglutination were designated, + + + +, + + +, + +, +, 0.

Sensitivity to polymyxin B. The agar plate sensitivity method was used, with disks containing 50 units of polymyxin B (Han and Khie, 1963). The strains were also tested with colistin-sulphate disks (10 µg).

Phage typing. Bacteriophages were obtained from the Indian Institute of Biochemistry and Experimental Medicine. Phage typing was performed by the method of Mukerjee (1963*a* and *b*, 1965). Each phage suspension was filtered through a 0.22-µm Millipore filter, and five 10-fold dilutions were made in nutrient broth. The routine test dilution of the

TABLE
Results of phage typing of 174 strains of *Vibrio eltor* by Mukerjee's method

Phage type	Sensitivity to phage						Number of strains belonging to type
	<i>V. eltor</i>					<i>V. cholerae</i> IV	
	I	II	III	IV	V		
1	+	+	+	+	+	—	0
2	+	+	+	—	+	—	0
3	+	+	—	+	+	—	0
4	+	+	—	—	+	—	120
5	+	—	—	—	+	—	53
6	—	+	—	—	+	—	0
Untypable	—	—	—	—	—	—	1

+ = Sensitive; — = insensitive.

phage (RTD) was the highest dilution that gave confluent lysis of a spot culture of a sensitive vibrio after overnight incubation. This dilution of phage was used for subsequent tests. The sensitive vibrio was *Vibrio eltor*, no. 757, (type 1, Macassar). Nutrient-agar plates were marked with six circles of 1.2 cm diameter. One loopful (3-mm diameter) of the culture under test was transferred to each of the marked areas. A loopful of each of the five typing phages for *Vibrio eltor* was then placed in one of the circles. The sixth circle received a loopful of *V. cholerae* phage IV as a control. Strains were designated sensitive or resistant according to Mukerjee's criteria. In case of doubt the experiment was repeated until consistent results were obtained. Mukerjee's (1970) system comprises six phage types as shown in the table, and this scheme was used for the identification of the phage types of our strains.

RESULTS

Of the 518 specimens examined, 174 were found to contain vibrios. All strains were H₂S negative, indole and oxidase positive, and fermentative by Hugh and Leifson's method. Acid was produced from mannose, sucrose and mannitol but not from arabinose. The Voges-Proskauer test proved positive in 135 of the 174 strains tested.

The tube and plate tests for haemolysis gave different results. By the former method only 10 isolates were lytic, whereas the plate method showed 172 strains to be lytic.

All the strains agglutinated chick RBC and all showed resistance to polymyxin B and to colistin. All the strains were agglutinated by polyvalent antiserum and Inaba-specific antiserum, but not by Ogawa antiserum. Hence, by this method all 174 strains were identified as *Vibrio cholerae*, serotype Inaba. Some of the features of our strains suggested *Vibrio*

eltor, but 164 of them lacked haemolysin by the tube method and 39 gave a negative Voges-Proskauer test. Such strains have been reported before as "El Tor biotypes". Furthermore, de Moor (1949) found that true cholera vibrios may show haemolytic properties and that the haemolytic activity of some freshly isolated El Tor strains may be deficient.

Phage typing yielded the results shown in the table. It should be noted that all strains were resistant to *V. cholerae* phage IV. Difficulty was encountered with nine of the strains, which on first isolation failed to fall into any of Mukerjee's six types. However, after two or three subcultures, eight of these fell into type IV or type V, the ninth remaining untypable. Although Takeya and Shimodori (1963) have suggested a relationship between lysogeny and virulence of *Vibrio eltor*, lysogeny was not investigated in the present study. We have not found it possible to develop a typing scheme based on the lysogenicity patterns of *Vibrio eltor*. In fact, such strains isolated from the stools of cholera patients have often been found to be non-lysogenic (Mukerjee, 1970).

DISCUSSION

In the identification of *Vibrio eltor* it would appear that none of the methods in general use are entirely reliable. The traditional haemolysis tests are unreliable. Clearing of the blood-agar plates has been reported in classical cholera strains (Bernard, Guillermin and Gallut, 1937). Although this clearing is said not to be due to a true haemolysin (Liu, 1959), it may lead to confusion.

The Voges-Proskauer test was negative in 39 strains, even when Barritt's method, which is generally rather sensitive, was used. We found that all the strains were resistant to polymyxin B, but the results of this test may apparently vary under different experimental conditions, as reported by Abe (1966). It is of interest that sensitivity tests with polymyxin B and colistin sulphate give similar results, thus confirming the findings of Abe (1966). The agglutination of chick RBC and resistance to Mukerjee's *Vibrio cholerae* group-IV phage confirmed identification as *V. eltor*. Two distinct phage types were obtained, and this would suggest that the outbreak originated from more than one source. However, *V. eltor* has a high mutation rate, and Mukerjee has found that some strains show divergent sensitivity patterns when tested repeatedly with the same set of phages. Non-haemolytic, chick-RBC-agglutinating El Tor vibrios have been described before amongst strains isolated in Iran in 1965 (Moureaux, 1970). In a small outbreak of cholera in Ahmedabad in 1969, all the strains isolated were resistant to *V. cholerae* phage IV and to polymyxin and agglutinated chick RBC. They were all non-haemolytic and were regarded as *Vibrio eltor* in spite of this finding. Bacteriophage typing revealed that they were mostly of types IV and VI, but one strain was of type V (Shah, Kanvinde and Patel, 1970).

The subdivisions of the cholera vibrio by Feeley (1965) and by Sen (1969) into several biotypes were made by means of the tube-haemolysis test and the Voges-Proskauer reaction. In the light of the current pandemic these biotypes require reappraisal. On the basis of our findings "*Vibrio cholerae* biotype *eltor*" would be a preferable designation to "*Vibrio eltor*".

SUMMARY

Stools from 518 patients, suspected of having cholera, were examined. From 174 of these patients *V. cholerae* biotype *eltor*, serotype "Inaba" was isolated. All 174 were resistant to Mukerjee's *V. cholerae* group-IV phage and to polymyxin B and colistin. They all agglutinated chicken RBC. Only 10 strains were positive by the tube haemolysis test with sheep RBC, but 172 were positive by the plate method. One hundred and twenty of the strains belonged to Mukerjee's *V. eltor* phage-type IV and 53 to phage-type V; one strain was untypable. It is suggested that these strains may have originated from more than one source.

We would like to thank Professor F. Shafa, the Head of the Department of Bacteriology and Immunology of the University of Tehran, for his help and guidance.

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