Biochemical Characteristics of Recent Cholera Isolates in the Far East *

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Numerous tests (Pollitzer b) designed to differentiate classic strains of cholera vibrios from the biotype El Tor strains found predominating in the more recent cholera outbreaks have given inconsistent results (Mukerjee & Guha Roy e). Other tests, however, have appeared to be very reliable (Finkelstein & Mukerjee; d Mukerjee 9). Since extreme variability is one of the primary characteristics of the genus Vibrio, isolation and identification of pathogenic and other vibrios require continuous re-examination of the methods employed for differentiation. Serial examinations on representative isolates from the various cholera outbreaks occurring in the Far East from August 1961 through February 1964 have been performed to provide information on possible changes in selected biochemical activities.

Materials and methods

Source of cultures. The 503 El Tor strains examined were isolated from patients in cholera outbreaks occurring in various geographical areas throughout the Far East during the three years 1961-64. Many of the strains were obtained through the courtesy of Dr J. M. Mackenzie and Dr R. E. Alvarez, Hong Kong; Dr P. R. Aragon, Dr J. C. Azurin and Mrs C. Z. Gomez, Manila; Dr Gan Koen Han, Djakarta; Professor H. S. Maitland, Kuala Lumpur, Malaysia; and members of the 406 Army Medical Research Laboratory working in Pusan, South Korea. All other El Tor isolates were from cholera patients under study by United States Naval Medical Research Unit No. 2 (NAMRU-2) cholera teams conducting clinical research investigations.

The true cholera vibrios (80 strains) were received from several sources: 39 from the Pakistan-SEATO Cholera Research Laboratory (CRL), Dacca, East Pakistan; 10 from Dr C. H. Yen, then WHO Epidemiologist, Institute of Public Health, Dacca; and 10, originally isolated in India, from Dr G. K. Han, University of Indonesia, Djakarta. The remaining 21 were laboratory stock strains from the National Institutes of Health, Bethesda, Md., the Walter Reed Army Institute of Research, Washington, D.C., and Keio Gijuku University, Tokyo, Japan.

The non-agglutinable vibrios (38 strains) were from two sources: 17 strains, isolated in 1962 from patients suffering from diarrhoeal disorders, were received from Dr A. S. Benenson, Director of the CRL, Dacca. The remaining 21 strains were received from Mrs C. Z. Gomez, Bureau of Research and Laboratories, Department of Health, Manila, Philippines.

Chicken cells and sheep cells. Blood was collected aseptically in Alsever’s solution from one- or two-day-old chicks or mature sheep routinely used as the cell sources for all haemagglutination-inhibition and complement-fixation tests. Three washings were made in 0.85% sodium chloride and appropriate suspensions for testing were made from packed cells. For our purposes fresh cell suspensions were made at weekly intervals; however, older cells will give satisfactory results after several weeks’ storage under refrigeration.

Haemagglutination tests. Agar-grown vibrio suspensions and 2.5% chick red cell suspensions were employed according to the method of Finkelstein & Mukerjee. d Tests in the home laboratory were performed on spot plates and were read after one minute’s shaking on a constant-speed rotary-shaking apparatus. Field tests were performed on glass slides sectioned with a glass-marking pencil, and were read after one minute’s manual mixing.

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Negative or weak reactions were read under 10× stereoscopic magnification, the oblique-light method of Lankford\(^f\) being used.

**Haemolytic tests.** Haemolytic activity was determined by three different test procedures. The "Greig" test (Greig\(^g\)) was performed with alkaline peptone broth cultures (pH 8.5) after 72 hours of incubation, utilizing 5% sheep cell suspensions in place of goat cells as originally advocated. A modification of the method of Feeley & Pittman\(^h\) was employed, using anaerobically incubated nutrient broth cultures (pH 7.2) and testing with 1% sheep cell suspensions after 24, 48, and 72 hours' incubation. Beginning in May 1963, a more sensitive semi-solid agar overlay method\(^i\) was used in conjunction with the two other haemolytic test procedures. The method employed is a slight modification of the haemolytic test utilized by Freimer, Krause & McCarty\(^j\) when investigating the haemolytic activity of L forms and protoplasts of group A streptococci. More recently Clyde\(^k\) has adapted it to demonstrate the haemolytic activity of Eaton's pleuropneumonia-like organism. The test was performed by overlaying 18-24 spot cultures of the vibrio on nutrient agar plates with 20 ml of a melted and cooled 0.6% gelatin-saline medium to which sheep cells had been added in a final concentration of 0.4%. In all haemolytic tests, two hours' incubation at 35°C, followed by overnight refrigeration at 4°C, was employed after addition of the test cell suspension. Appropriate known haemolytic and non-haemolytic strains and both heated and normal broth controls were included in all tests.

**Cholera bacteriophages.** Fifteen cholera bacteriophages were used in the study. Seven of these were *Vibrio cholerae* phages and included Mukerjee groups I, II, III, and IV. The other eight were El Tor phages isolated from stool specimens or lysogenic strains from epidemics in Taiwan, 1962; Philippines, 1962-63; and Saigon, 1964. In testing the seven *V. cholerae* phages, it was evident that they were closely related and offered no additional information on strain differentiation. The eight El Tor phages, although obtained from three separate outbreaks over a period of three years, had similar lytic action patterns. At low titres they were quite specific since they lysed approximately 90% of the El Tor strains but were non-lytic against typical *V. cholerae* strains. However, at higher titres (RTD 10\(^4\) and greater) their specificity was lost and their lytic action could be compared to that of Mukerjee group III phage. Optimal results in cholera phage testing cannot be obtained unless consideration is given at all times to the different degrees of phage sensitivity.

**Phage lysability tests.** Standard bacteriophage typing tests were performed (Mukerjee\(^l\)). Depending upon the titre, one loopful of cholera phage at the routine test dilution (RTD), 1000 RTD, or undiluted was spotted on the test vibrio and lysis read after overnight incubation at 37.5°C.

**Results**

The results of chicken cell agglutination, haemolytic and phage susceptibility tests on biotype El Tor vibrios, *V. cholerae* and non-agglutinable vibrios are shown in the accompanying table.

When the chicken cell agglutination and phage sensitivity tests are compared, we find remarkable agreement in the results. All 503 strains gave positive agglutination and none of the 463 strains examined for phage sensitivity were found to be lysed by Mukerjee group IV phage. The reverse is true for the 80 classic cholera strains tested. The results obtained with phage No. 7040 are also in good agreement. Of the 463 strains tested, 415 or 89.6% were found to be susceptible to lysis. The insensitivity of *V. cholerae* to phage No. 7040, as well as their negative chicken cell agglutination reactions, is also consistent. The chicken cell agglutination test for non-agglutinable vibrios (NAG's) is variable; however, the NAG's were insensitive to all phages tested against them.

The results show that all isolates from Hong Kong and Kowloon in August 1961, from Manila, Philippines, in September 1961 and July-August 1962, and from Taiwan in July and August 1962 were haemolytic both by the Greig test and, at all sampling intervals, by the method of Feeley & Pittman. The first isolates which failed to produce a demonstrable haemolysin with any test method were received from cholera cases in Malacca, Malaysia.

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\(^i\) First suggested by Taylor, J. (1941) *Cholera research in India 1934-1940 under the Indian Research Fund Association*, Cawnpore, Job Press.


in May 1963. This possibility had been anticipated, however, since as early as 1937-38 during the outbreak in the island of Celebes (Sulawesi), one strain, Mak No. 757, isolated by Dr C. E. de Moor, produced a non-haemolytic variant on serial laboratory transfer (Finkelstein & Mukerjee). Furthermore, Mukerjee reported two additional isolates from New Guinea as being non-haemolytic. The New Guinea and the Malacca isolates were found to be identical with isolates from other areas considered to be infected with El Tor cholera.

In 1963 the majority of isolates from all areas tended to be non-haemolytic, except for the isolates obtained during the short outbreak in Pusan, South Korea, in October. These latter strains were all found to be haemolytic, with the exception of two that gave negative Greig test reactions. The November-December Philippine isolates were almost all found to be non-haemolytic by the Greig and the Feeley & Pittman tests, but very weak haemolytic reactions were obtained with the semi-solid overlay method. Further, this very sensitive method was the only one with which a few positive haemolytic reactions were obtained in the case of the isolates from cholera patients in Saigon, South Viet-Nam, in January-February 1964. A recent personal communication from Mrs C. Z. Gomez states that from January through 31 March 1964, out of 271 isolates only 10 (3.6%) were found to be haemolytic. These were all derived from scattered areas and Mrs Gomez doubts if any correlation can be made between the place of origin and the haemolytic reaction.

In general, the relative effectiveness of the three haemolytic tests employed may be evaluated on the basis of the last two years' examinations. As expected, the Greig test detected the least number of haemolytic isolates. The method of Feeley & Pittman detected additional haemolytic isolates, especially at the 24- and 48-hour intervals of testing. The semi-solid agar overlay method also detected an additional number of strains that produce haemo-

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Lysin slowly or in very small amounts. This is indicated particularly well in comparing the results on the last Philippine and Saigon isolates where 25 and 9 strains, respectively, were found to have a demonstrable haemolysin undetected by other methods.

Discussion

The pronounced change in haemolytic activities observed on examining cholera isolates from different geographical areas in the Far East during 1961-64 makes it apparent that this single biochemical activity is not a reliable criterion for the separation of a biotype or subspecies of vibrio. Furthermore, the measurement of haemolytic activity is a problem of degree and may vary with different laboratories depending upon the availability or selection of media and adherence to the physical requirements of the test procedures employed. Also, it appears doubtful that any reliable epidemiological data can be derived because of the variable results obtained from serial cholera admissions occurring in the same epidemic area.

The complete agreement obtained throughout the past three years with the haemagglutination and phage sensitivity tests provides two excellent methods for the rapid differentiation between the classic cholera vibrios and the vibrios isolated recently in the Far East. The rapidity and simplicity of the chicken cell agglutination test make it suitable for field work or for use by laboratories with limited facilities. The use of the phage lysability procedures depends upon the availability of properly propagated and titred phage preparations for testing.

Broader experience with these aberrant vibrios may show that it is more realistic to consider them as biotypes of *V. cholerae* than as biotypes of the so-called "El Tor" vibrio. They may or may not possess haemolytic activity, but they do have a predictable reaction towards specific cholera bacteriophages. On the other hand, if these strains are simply designated as being *V. cholerae* serotype Ogawa or Inaba, certain distinctive characteristics are omitted which may provide useful information in epidemiological studies. Further examination of strains from different epidemics and geographical areas may lead to the development of a more reliable system for a subspecies classification.

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