Preselection of Hemolytic Variants of El Tor Vibrios

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Historically, hemolytic activity in tube tests was regarded as the *sine qua non* in the differentiation of El Tor from classical cholera vibrios. However, in the recent outbreaks of cholera in Southeast Asia, many strains of agglutinable vibrios have been isolated which share a large number of properties ascribed to El Tor vibrios (and not the classical variety), but which lack hemolytic activity. These are now generally regarded as nonhemolytic El Tor vibrios, although there is some disagreement as to how they should properly be classified. J. C. Feeley (J. Bacteriol. **89**:665, 1965) suggested reclassification of the cholera vibrios into five "types" of a single species on the basis of their reactions in tube and plate hemolysis tests, phage IV sensitivity, the direct slide hemagglutination test with chicken erythrocytes, and the Voges-Proskauer (VP) reaction; he selected only these tests from the many (O. Felsenfeld, Bacteriol. Rev. **28**:72, 1964), including virulence for chick embryos (R. A. Finkelstein, Nature **202**:609, 1964), which have been used to differentiate El Tor from classical vibrios. The present communication is concerned with observations on strains received (courtesy of A. S. Benenson) from the recent outbreak in Teheran. These strains contained colonial variants, recognizable on meat extract-agar (MEA) with oblique illumination (R. A. Finkelstein and C. Z. Gomez, Bull. World Health Organ. **28**:327, 1963), which gave variable reactions on blood-containing media. The hemolytic activity could be predicted from the colonial form on MEA. Thus, if we were to employ Feeley's terminology, type 4 variants can be selected from initially type 5 strains. The picture is complicated by the observation that some tube tests resulted in "partial" hemolysis. This phenomenon may be related to the presence of the hemolysin-destructive factor of A. Wake and M. Yamamoto (J. Bacteriol. **91**:461, 1966).

Each of the 21 strains examined was of the Ogawa serotype, and each was hemagglutinative in the direct slide test of R. A. Finkelstein and S. Mukerjee (Proc. Soc. Exptl. Biol. Med. **112**:355, 1963) confirming their identity as El Tor vibrios. All of the strains and colonial variants tested exhibited enhanced virulence for chick

embryos (R. A. Finkelstein, Nature **202**:609, 1964), and each was resistant to polymyxin (50 units) in the disc technique. In fact, there has been complete correlation in the results of these three tests with reaction to Mukerjee's group IV phage, which also differentiates El Tor from classical cholera vibrios (Bull. World Health Organ. **28**:333, 1963), with all strains tested on primary isolation by the author in cholera epidemics in the Philippines, India, Thailand, and Viet Nam. Undoubtedly, exceptions occur in some stock cultures (J. C. Feeley, J. Bacteriol. **89**:665, 1965), and even in fairly recent isolates after a delay in testing coupled with transfer or maintenance in some artificial media (S. Rizvi, M. I. Huq, and A. S. Benenson, J. Bacteriol. **89**:910, 1965). With the opportunity for genetic interchange between classical and El Tor vibrios now simultaneously present in India, more exceptions may be expected in the future.

Eight pairs of colonial forms, most easily described as opaque (O) and translucent (T), were selected from eight strains for testing of hemolytic activity. Each of the paired substrains gave characteristic reactions on 5% sheep blood-agar, the translucent variety producing zones of hemolysis around isolated colonies, whereas the opaque forms manifested only hemodigestive action in crowded areas of the plates. With the exception of the isolated translucent variants, 17 of the 21 strains were nonhemolytic when received by us. Thus, it appears most likely that the translucent hemolytic variant arises in an initially nonhemolytic opaque population. This was subsequently borne out when cultures freshly isolated by the author in an El Tor Inaba outbreak in Korat, Thailand, were examined. All of the strains were of the opaque type and were nonhemolytic when first tested, but subsequently translucent hemolytic variants could be isolated from laboratory-maintained cultures. Testing of other cholera strains on blood-agar revealed that classical varieties were either totally nonhemolytic or produced hemodigestion in crowded areas, whereas El Tor strains from various sources ranged from markedly hemolytic, more so than the variants noted above, to nonhemolytic, usually with hemodigestion.

The paired substrains were also tested for hemolytic activity in Brain Heart Infusion broth and in Heart Infusion by tube dilution, as recommended by J. C. Feeley and M. Pittman (Bull. World Health Organ. **28**:347, 1963). In the former medium, the opaque types were not hemolytic, whereas the translucent types exhibited some hemolytic activity at 24 hr, usually not complete and usually with a prozone of no activity; the translucent types were not hemolytic when 48-hr cultures were tested. In Heart Infusion broth, neither opaque nor translucent types manifested any significant hemolytic activity when tested at 18 or 43 hr. Both opaque and translucent types were found to be choleraogenic in infant rabbits (R. A. Finkelstein et al., J. Infect. Diseases **114**: 203, 1964) inoculated intraintestinally with approximately 10^4 vibrios.

The significance of these observations and their relationship to the evolution and classification of cholera vibrios is not clear. Evaluation of the hemolytic activity of freshly isolated strains on agar plates may provide an additional useful

marker for epidemiological studies, but it should be recognized that this characteristic is unstable. Although El Tor and classical cholera vibrios are similar morphologically and antigenically, I cannot agree with those who would regard them as a single species, because there are so many tests which can be applied in which the two kinds of vibrios cluster into two separate groups with little overlapping. Now that both are unequivocally acknowledged to cause epidemic cholera, there would seem to be no more drawback to considering them as separate species of the same genus than there is in so regarding the salmonellae, shigellae, or other pathogens which are "speciated" on the basis of fewer differences than exist between the El Tor and classical vibrios. The present observations emphasize the importance of examining freshly isolated cultures and the need for prompt lyophilization if reliable and reproducible results are to be obtained in various laboratories.

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