Studies on Toxinogenesis in *Vibrio cholerae*

II. An In Vitro Test for Enterotoxin Production

MICHAEL L. VASIL, RANDALL K. HOLMES, AND RICHARD A. FINKELSTEIN

Departments of Microbiology and Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas 75235

Received for publication 26 July 1973

An Elek test for enterotoxin-producing strains of *Vibrio cholerae* is described. Thirty-five out of 37 strains of classical *V. cholerae* produced positive reactions, but only 1 of 69 El Tor vibrio strains was reactive. Tox(−) mutants of *V. cholerae* were also unreactive in the Elek test.

In 1948, Elek described a simple, in vitro technique for the detection of antigenic extracellular toxins produced by isolated strains of *Corynebacterium diphtheriae* and *Staphylococcus aureus* (2). The reliability and limitations of several modifications of this technique for identification of toxinogenic strains of *C. diphtheriae* were subsequently established by extensive comparisons of toxinogenicity tests performed in vitro and in experimental animals (7, 8, 10). We report here a modified Elek test to detect enterotoxin synthesized in vitro by *Vibrio cholerae*. The well-studied enterotoxin-producing strain 569B Inaba of *V. cholerae* has been used for determining optimal conditions for this test.

A previously prepared equine antiserum to cholera antigenoid was used throughout these studies (4). (Samples of this serum are available on request from Carl Miller, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014.)

In vitro tests for cholera enterotoxin were routinely performed on 100-mm-diameter plastic petri dishes containing agar media. Strips of sterile Whatman no. 1 filter paper (8 by 75 mm) were soaked in antiserum, drained, and applied to the surface of cooled but still molten agar in plates which were allowed to solidify at room temperature, and then dried for 2 to 4 h at 37 C prior to inoculation (2). After inoculation by surface streaking, cholera enterotoxin produced during incubation was detected by the formation of lines of toxin-antitoxin precipitate in the agar medium adjacent and at an angle to the bacterial growth (Fig. 1).

The following variables have been tested. Plates were prepared containing syncase broth (5), tryptose broth (Difco), meat extract broth, or brain heart infusion broth (Difco), and solidified with agar (Difco) or Noble agar (Difco) at concentrations of 1 and 1.5%. Duplicate plates were incubated at 30 and 37 C and observed daily for 3 days. After incubation, plates were placed at 4 C and examined again after 24 to 36 h. Volumes of 10, 15, 20, 25, and 30 ml/plate were tested with meat extract broth containing 1.5% Noble agar; and pH values of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 were tested on plates containing 10 ml of the same meat extract agar. Anti-cholera antigenoid serum was used undiluted and at dilutions of 1:2, 1:4, and 1:8. Inocula of *V. cholerae* 569B strain Inaba were from stationary and exponentially growing cultures in syncape broth and from overnight cultures on meat extract agar. The inocula were generally applied by streaking with a cotton swab.

The most easily visible toxin-antitoxin precipitin lines were observed after incubation at 37 C with undiluted antiserum and brain heart infusion solidified with 1.5% Noble agar (Fig. 1). Positive but weaker tests were observed on all media tested. The optimal pH range was 7.5 to 8.0. The precipitin lines were sharpest in plates containing 10 ml of agar medium. Weaker reactions were observed when the anti-cholera antigenoid serum was diluted to 1:2, 1:4, or 1:8. The medium from which the bacterial inoculum was prepared did not affect the results. All positive reactions could be detected after 24 h of incubation, but an additional 12 to 24 h of refrigeration enhanced some precipitin lines and aided in the scoring of weakly positive results.

Tests for enterotoxin production were conducted by the optimized technique on an additional series of 106 cholera vibrio strains, including both freshly isolated and old laboratory strains representing both classical *V. cholerae* and the El Tor biotype of both Inaba and
Ogawa serotypes from diverse sources. The strains, from the collection of R.A.F., had been maintained by lyophilization since shortly after their isolation or receipt. Of the classical V. cholerae strains tested, 11 out of 13 Inaba, 10 out of 16 Ogawa, and 4 of 8 untyped strains were positive. The proportion of positive strains was increased to 12: 13, 15:16, and 8:8 when the test was made more sensitive by diluting the serum 1:2. The two negative cultures were each old laboratory strains, although other old laboratory strains tested did give positive reactions. In contrast to virulent strains, these two strains failed to cause fluid accumulation in repeated tests in rabbit ileal loops with inocula of 10⁶ vibrios. Two serologically rough variants originally derived from a positive Ogawa serotype strain also gave positive reactions in the Elek test and are included in the above summary.

In striking contrast, only one Ogawa serotype El Tor vibrio strain of the 69 tested (24 Inaba, 25 Ogawa, and 20 untyped) was positive in the routine procedure. The positive strain, 3083 (Ogawa), was an isolate from the Viet Nam outbreak of 1964, and previously had been reported to be toxigenic (6). A more recent study (R. A. Finkelstein, M. L. Vasil, and R. K. Holmes, J. Infect. Dis., in press) indicated that its enterotoxin was not completely immunologically identical with that of (now) classical enterotoxin from strain 569B. This was confirmed by the present technique (Fig. 1). One additional El Tor strain, an isolate from the Celebes in 1936, produced a precipitin band only when a rabbit antiserum against purified 3083 toxin was used. This serum was equally as satisfactory as the equine anti-choleraoiden serum in recognizing classical toxigenic strains and strain 3083 but, with one exception noted, did not pick up any additional positive strains.

Tests were also performed with tox(−) mutants of V. cholerae 569B (R. A. Finkelstein et al., J. Infect. Dis., in press). These likewise failed to produce positive reactions in the Elek test (Fig. 1).

The present observations suggest that the Elek technique is a useful, simple, rapid, and inexpensive means of screening for enterotoxin production by classical V. cholerae strains and for use in studies on the genetics of toxigenicity employing mutants derived from toxigenic strains. The reason the majority of El Tor strains of unquestioned virulence failed to elaborate toxin detectable by the Elek test described here is not yet clear. It may be that El Tor strains produce less toxin than classical strains, and this is compatible with the observations of Bart et al. (1) that the El Tor biotype is considerably more prone to cause asymptomatic infections than is classical V. cholerae. Alternatively, it is conceivable that the El Tor biotype could elaborate an antigenically distinct toxin not recognized with the present antitoxins in a precipitin reaction. This, however, is less likely in the light of observations by Pierce et al. (9) that patients with El Tor cholera produce antibodies which neutralize 569B toxin and that immunization with purified 569B toxin protects rabbits against challenge with El Tor vibrios (3).

The differences between classical and El Tor vibrios in the Elek test adds to the growing list of characteristics which distinguish these two biotypes of cholera vibrios.
8. Ouchterlony, Ö. 1948. In vitro method for testing the