

NOTES

Radial Passive Immune Hemolysis Assay for Detection of Heat-Labile Enterotoxin Produced by Individual Colonies of *Escherichia coli* or *Vibrio cholerae*

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A rapid screening test has been developed to detect heat-labile enterotoxin produced by individual colonies of *Escherichia coli* or *Vibrio cholerae* growing on media solidified with agar. The applicability of this method for isolating *tox* mutants of *E. coli* and *V. cholerae* has been demonstrated.

A variety of sensitive in vitro assays for cholera enterotoxin (CT) or for the immunologically related heat-labile enterotoxin (LT) of *Escherichia coli* has been reported, including tissue culture bioassays (3, 11), hemagglutination tests (8, 15), passive immune hemolysis tests (4), enzyme-linked immunosorbent assays (17), and radioimmunoassays (2, 10, 14). Although these tests are suitable for quantitating CT or LT in individual bacterial cultures or culture supernatants, they are not ideal for screening very large numbers of bacterial clones for toxigenicity. Visually scorable immunological tests have been developed to detect the CT produced by colonies or streaks of selected toxigenic strains of *Vibrio cholerae* (9, 18, 20), and these methods have been used successfully for identifying *tox* mutants of *V. cholerae* for genetic studies (1, 9, 15, 16, 18, 20, 21). In contrast, attempts to develop visually scorable screening tests for LT produced by single colonies of *E. coli* have not been successful, and the use of laborious bioassays has been a significant technical limitation for genetic studies of LT (12, 13, 19). In the present communication we report the successful application of a radial passive immune hemolysis method for detecting production of LT and CT by single colonies of selected toxigenic strains of *E. coli* and *V. cholerae*.

Erythrocytes from defibrinated sheep blood stored at 4°C were collected by low-speed centrifugation (3,000 × *g* for 10 min), washed four times at 22°C with syncase broth (5), and resuspended in a volume of syncase broth equal to the original blood volume. For all experiments with *V. cholerae*, syncase medium contained 5 g of sucrose per liter, and for all experiments

with *E. coli*, 2.5 g of glucose per liter was substituted for sucrose. Syncase agar contained 10 g of Difco Noble agar per liter of syncase broth, and syncase soft agar contained 5 g of Difco Noble agar per liter of syncase broth. Syncase blood agar was prepared by adding 1 volume of washed sheep erythrocytes in syncase broth to 5 volumes of molten syncase agar at 55°C. The molten syncase blood agar was divided into 3-ml samples, and each sample was inoculated with 0.1 ml of syncase broth containing 100 to 200 viable bacteria. The samples of molten syncase agar were then poured as overlays on the surface of 10-ml samples of solidified syncase agar in petri dishes (15 by 100 mm). The plates were incubated for 18 to 24 h at 30°C for *V. cholerae* or for 48 to 72 h at 37°C for *E. coli* to permit formation of bacterial colonies, elaboration of enterotoxin, and spontaneous binding of enterotoxin to plasma membrane receptors on the erythrocytes. Each plate then received an additional 2-ml overlay of syncase soft agar containing hyperimmune goat antiserum prepared in our laboratory against highly purified CT (15) (1:400 was the final dilution of antiserum for experiments with *V. cholerae* and 1:200 for experiments with *E. coli*), reconstituted guinea pig complement (Grand Island Biological Co., 1:8 final dilution), and sodium azide (2 mg/ml). Optimal dilutions of the hyperimmune goat anti-CT serum and of guinea pig complement were determined empirically by testing serial dilutions of each component in a checkerboard fashion. After incubation for 1 h at 37°C, the plates were examined for the development of hemolytic halos surrounding enterotoxinogenic colonies.

Figure 1 demonstrates the results obtained

with the highly toxinogenic *V. cholerae* strain 569B Inaba (9), the toxinogenic *E. coli* strain KL320(pCG86) (19), harboring the LT + ST plasmid pCG86, and the nontoxigenic *E. coli* strain KL320 (19). The specificity of this radial passive immune hemolysis test for enterotoxin was demonstrated by control experiments showing that: (i) the development of hemolytic halos was dependent on the presence of both goat anti-CT serum and guinea pig complement; (ii) no hemolytic halos were observed when an equivalent dilution of preimmune goat serum was substituted for the hyperimmune anti-CT serum; (iii) no hemolytic halos were observed when the assay was performed with the severely hypotoxinogenic mutant strains of *V. cholerae* designated M5, M13, M27, M32, and RV31 (15); and (iv) no hemolytic halos were observed with the isogenic but nontoxigenic *E. coli* strain KL320. Variable results were obtained when the radial passive immune hemolysis test was performed with other well-characterized LT-producing strains of *E. coli*. Visible hemolytic halos were obtained with strains H10407 (6) and Throop D (6), but no halos were observed with strains 339t5 (6) and 711(p307) (13). The radial passive immune hemolysis assay described here is thus less sensitive for detecting LT or CT than tissue culture bioassays and radioimmunoassays (2, 3, 10, 11, 14).

An absolute value for the sensitivity of the radial passive immune hemolysis assay was determined for CT. Assay plates were prepared as described above for *V. cholerae*, but the bacterial inoculum was omitted and sodium azide (0.2 mg/ml) was added to the syncase blood agar overlay. Wells (3-mm diameter) were punched in the agar, and 5- μ l samples containing purified CT diluted in phosphate-buffered saline solution with 10% fetal calf serum were placed in the wells. The plates were incubated for 18 h at 30°C before treatment with goat anti-CT serum and guinea pig complement as described above. Figure 2A illustrates these assays, and Fig. 2B demonstrates the linear relationship that was observed between the diameter of the hemolytic zone and the logarithm of the concentration of CT. This assay can detect as little as 625 ng of CT per ml (3 ng of CT per well). Purified samples of the spontaneously formed toxoid choleraenoid produced zones of hemolysis comparable to those obtained with equimolar concentrations of choleraen. The radial passive immune hemolysis assay is significantly more sensitive than Mancini-type radial immunodiffusion assays for choleraen and choleraenoid (7).

The radial passive immune hemolysis assay described here provides a convenient method to test many thousands of colonies of appropriate strains of *V. cholerae* or *E. coli* for enterotox-

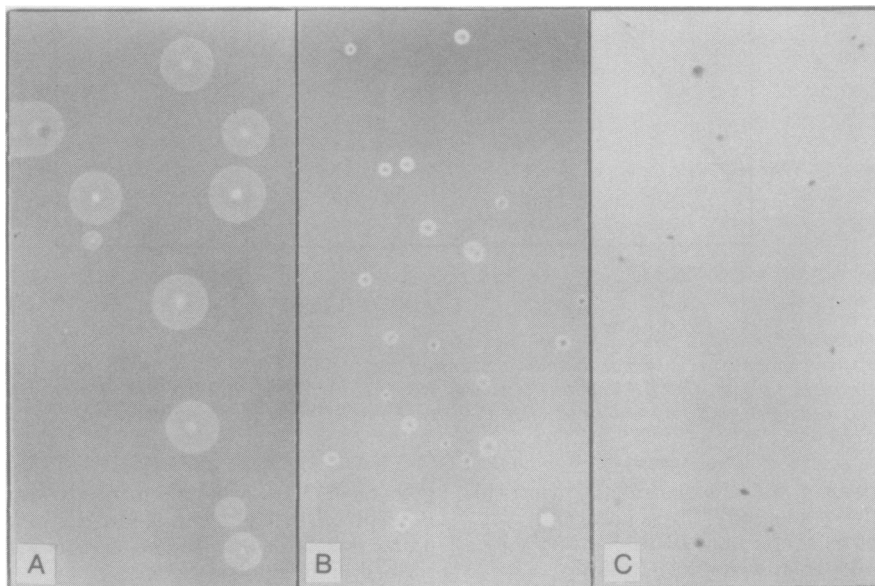


FIG. 1. Radial passive immune hemolysis assay for LT. Individual colonies of the enterotoxinogenic strains *V. cholerae* 569B Inaba (A) and *E. coli* KL320(pCG86) (B) are surrounded by halos, due to lysis of enterotoxin-sensitized sheep erythrocytes in the presence of anti-CT and complement. In contrast, colonies of *E. coli* KL320 (C) lack halos because they do not produce LT and are not able to sensitize erythrocytes for lysis by anti-CT and complement.

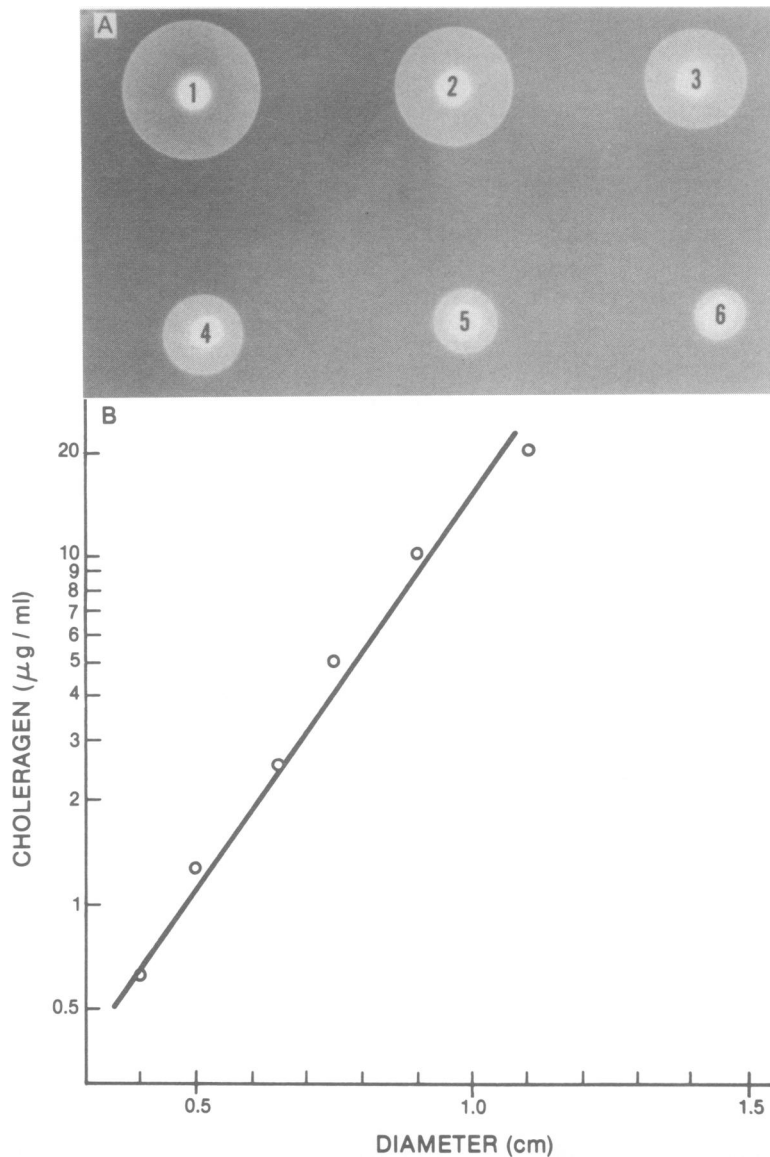


FIG. 2. Quantitative radial passive immune hemolysis assay for cholera enterotoxin. (A) Hemolytic halos surrounding wells inoculated with 5- μ l samples containing purified CT at the following concentrations: (1) 20 μ g/ml, (2) 10 μ g/ml, (3) 5 μ g/ml, (4) 2.5 μ g/ml, (5) 1.25 μ g/ml, and (6) 0.625 μ g/ml. (B) Quantitative relationship between halo diameter and concentration of CT. Each point represents the average value of duplicate determinations.

inogenicity in a single experiment. Using this assay we have succeeded in isolating: (i) *ts-tox* mutants of *V. cholerae* 569B Inaba that have a temperature-sensitive defect in their capacity to produce CT, and (ii) hypertoxinogenic mutants of *E. coli* that produce much larger halos in the radial passive immune hemolysis assay and yields of extracellular enterotoxin in glucose-synase liquid medium that are at least 20-fold

greater than the yield from the parental strain KL320(pCG86). The characteristics of these *tox* mutants of *V. cholerae* and *E. coli* will be described in detail elsewhere.

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ADDENDUM IN PROOF

E. coli KL320(pCG86) is an auxotrophic mutant that requires tryptophan, histidine, methionine, and proline for growth in minimal medium (19). In the radial passive immune hemolysis assay, KL320 (pCG86) produces small colonies surrounded by narrow hemolytic halos (Fig. 1B). We have noted that KL320(pCG86) forms large colonies lacking hemolytic halos when tryptophan (20 µg/ml) is added to the syncase blood agar used for the assay. In contrast, hypertoxinogenic mutants of KL320(pCG86) that produce greatly increased yields of extracellular LT form colonies that are surrounded by wide hemolytic halos in the radial passive immune hemolysis assay both with and without added tryptophan, although such hypertoxinogenic mutants usually remain auxotrophic for tryptophan.

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