Agar Diffusion Method for the Differentiation of
Bacillus anthracis

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A method was developed for identification of Bacillus anthracis based on elaboration of protective antigen by individual colonies and its detection by double-diffusion precipitation in agar plates.

The main difficulty in identification of Bacillus anthracis is in distinguishing it from nonmotile forms of B. cereus. The antigenic and biochemical similarities between these organisms have led some investigators (9) to propose that B. anthracis is a pathogenic variant of B. cereus. A variety of methods were proposed for their differentiation, some of the more recent of which include specific lysis by phage gamma (3, 4), differential toxicity of sterile culture filtrates for mice (2), and the inability of B. anthracis to grow on media containing 0.3% phenethyl alcohol or 0.25% chloral hydrate (7).

Animals may be rendered immune to anthrax by immunization with live avirulent spores or with sterile filtrates of cultures of B. anthracis grown under particular conditions. This acquired resistance is apparently based primarily on immunity to protective antigen (10), an extracellular antigen of B. anthracis elaborated in vivo during infection or in vitro under carefully controlled conditions of growth (1, 6, 12). Other Bacilli were not reported to induce resistance to anthrax nor to produce protective antigen (5, 6); accordingly, production of this antigen is potentially useful for identification. The present report describes a practical method of differentiation based on detection of protective antigen produced by individual colonies on solid medium.

Test colonies were obtained by inoculating the surface of peptone agar with overnight nutrient broth cultures of B. anthracis, B. cereus, B. cereus var. mycoides, B. circulans, B. thuringiensis, B. megaterium, B. subtilis, B. polymyxa, B. sphaericus, and B. licheniformis. The peptone agar was prepared by suspending the following constituents in 890 ml of water and autoclaving for 15 min at 120 C: Albimi Brucella broth (Pfizer Diagnostics), 28 g; Noble agar, 20 g; CaCl2·2H2O, 0.0076 g; MnSO4·H2O, 0.0009 g; MgSO4·7H2O, 0.0099 g; KH2PO4, 0.068 g; K2HPO4, 0.087 g. After the medium had cooled to 48 C, 100 ml of 10% (w/v) NaHCO3 and 10 ml of 20% (w/v) glucose were added aseptically. The latter solutions were sterilized by filtration. The final pH of the medium was 7.5. The medium was transferred in 12-ml volumes to plastic petri plates (15 by 120 mm) and inoculated on the day of preparation.

The inoculated plates were sealed with tape, enclosed in plastic bags, and incubated in an inverted position at 37 C for 16 to 18 hr. Discs of agar bearing single colonies 3 to 5 mm in diameter were cut from the agar plate with a no. 5 cork borer and transferred with an inoculating loop to matching outer wells of agar diffusion plates (11). The center wells of the agar diffusion plates were precharged 16 to 18 hr previously with anthrax antiserum. The antiserum was obtained by repeated immunization of a pony with aluminum hydroxide-adsorbed culture filtrate of B. anthracis (8). It contained antibodies for at least 23 antigens, including the protective antigen. The plates were incubated at room temperature in a humid atmosphere and observed for lines of precipitation after 24 and 48 hr.

Determination as to whether the resulting lines of precipitation were produced by diffusion of protective antigen was based on the type of interaction with lines produced by purified protective antigen in adjacent wells. Concentration of the control antigen was adjusted to give clear lines of precipitation of sufficient intensity to permit interaction with lines developing near the transferred colonies. Preparation and purification of protective antigen will be described in a forthcoming paper.

Precipitate lines that exhibited a pattern of identity with the control antigen were obtained with all 14 of the test strains of B. anthracis, whereas no such lines were obtained with the 17 strains representing nine other species (Table 1). Bacillus cereus HBA 248 originally received as B. anthracis and subsequently reclassified as B.
TABLE 1. Growth and protective antigen formation by Bacillus strains on peptone agar

| Species and strains | Growtha | Pattern of identity with protective antigenb | Macoid growthb |
|---------------------|---------|--------------------------------------------|----------------|
| B. anthracis         |         |                                            |                |
| Avirulent           |         |                                            |                |
| V770-NP1-R, 116NP1-R2, 107-NP2-R1, 1133-NP1-R3, Sterne | ++++     | +                                      | −              |
| Virulent            |         |                                            |                |
| 107, Vlb, Volume, NH, M36, Ohio, S. Dakota, Kansas, 116-NP2-F | ++++     | +                                      | +              |
| B. cereus           |         |                                            |                |
| ATCC 6464, 7064, HBA 248 | ++++     | −                                      | −              |
| B. megaterium       |         |                                            |                |
| ATCC 8245, ATCC 6458, 9985, 11561 | +++     | −                                      | −              |
| B. subtilis         |         |                                            |                |
| ATCC 943, ATCC 9466, 9524, 9860 | ++      | −                                      | −              |
| B. cereus var. mycoides | ++++   | −                                      | −              |
| ATCC 6462 |         |                                            |                |
| B. circulans        |         |                                            |                |
| ATCC 9966 |         |                                            |                |
| B. thuringiensis    |         |                                            |                |
| NR 996 |         |                                            |                |
| B. polymyxa         |         |                                            |                |
| ATCC 8526 |         |                                            |                |
| B. sphaericus       |         |                                            |                |
| ATCC 7054 |         |                                            |                |
| B. licheniformis    |         |                                            |                |
| ATCC 9945 |         |                                            |                |

* No growth, −; increasing relative size of colonies, ++, ++++, ++++.  
* Presence of character, +; absence of character, −.

cereus (7) failed to produce a protective antigen line. Antigens of B. cereus were capable of precipitating antibodies found in anthrax antiserum, but the lines failed to exhibit a pattern of identity with the control antigen (Fig. 1A). Interpretation of results may be facilitated by absorption of the equine hyperimmune antiserum with vegetative cells of B. anthracis and lyophilized culture filtrate of B. cereus. This treatment removes nonspecific lines of precipitation without affecting the protective antigen line (Fig. 1B).

Comparable results were obtained with hyper-immune antisera obtained from burros that had been immunized with live anthrax spores. Interpretation of results is easier because this antiserum primarily contains antibody against protective antigen, and absorption to remove cross-reacting antibodies is not required as with the pony antiserum. Satisfactory results were also obtained in limited trials with antiserum produced in rabbits by immunization with gel-adsorbed antigen (8).

The peptone agar, in addition to allowing elaboration of protective antigen, promoted capsule formation of known virulent anthrax strains and inhibited the growth of B. megaterium, B. cereus (7) failed to produce a protective antigen line. Antigens of B. cereus were capable of precipitating antibodies found in anthrax antiserum, but the lines failed to exhibit a pattern of identity with the control antigen (Fig. 1A). Interpretation of results may be facilitated by absorption of the equine hyperimmune antiserum with vegetative cells of B. anthracis and lyophilized culture filtrate of B. cereus. This treatment removes nonspecific lines of precipitation without affecting the protective antigen line (Fig. 1B).

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subtilis, B. polymyxa, and B. sphaericus. The inhibitory effect on growth of these strains is the result of NaHCO₃ in the medium. These results show that identification of B. anthracis appears feasible by use of a simple in vitro assay for protective antigen. The method may be useful for practical identification of B. anthracis in clinical or environmental specimens.

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