Haemophilus influenzae type b serum bactericidal assay (colony counting method)

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Supplies and Solutions

- Microtiter plate (round bottom)
- Cryovial
- Chocolate II agar plates (Becton Dickinson Cat # 21169-21267) – need to be fresh (<60 days)
- Fildes enrichment (BBL, Sparks, MD Cat # 220810) (Note 1)
- Brain heart infusion (BHI) broth
- BHI Broth with 2% Fildes Enrichment (3)
- Hanks' buffer with Ca^{++} and Mg^{++} (Life Technologies)
- dilution buffer: Hanks' buffer with Ca and Mg and 2% Fildes enrichment.
- Bacteria:
  - *H. influenzae* type b strain Eagan (4) or GB3291 (2)
- New born rabbit serum for complement (Pel-Freez, Brown Deer WI)
- CBER standard serum (lot 1983). A serum standard from FDA with 70 µg of Ab/ml.
- Serum PSAB-90 (Dana Farber Cancer Institute) or Gammaglobulin (Bayer, Elkhart, IN) for QC purpose.

Procedure for preparing bacteria

1. Inoculate hemophilus bacteria on a chocolate II agar plate and incubate the plate overnight (16 hours) at 37°C in a 5% CO_2 atmosphere.
2. Transfer about 10 isolated bacterial colonies to 20 ml of BHI broth with 2% Fildes enrichment in a 50-ml glass vial and incubate at 37°C , 5% CO_2 until the OD_{600} becomes 0.4-0.5. (Note 2)
3. Add 3 ml of sterile glycerol to the bacterial culture (20 ml). Mix well. Dispense 0.5 ml into each cryovial.
4. Quickly freeze all cryovials (except one) in 95% ethanol at -70ºC. Once frozen, store the vials at -70°C until use. The non-frozen vial will be used in step 5.
5. Determine the bacterial recovery from frozen vials. (It should be greater than 80%.)
   a. Thaw a vial of frozen bacterial aliquot (step 4).
   b.. Dilute both the unfrozen and thawed bacteria (step 4) 10^{-6}, 10^{-7}, 10^{-8}-fold in dilution buffer.
   c. Plate 100 µl from each dilution on a chocolate II agar plate.
   d. Incubate the plates overnight at 37°C in a candle jar.
   e. Count the colonies.
   f. Determine the ratio of thawed bacterial number to unfrozen bacterial number. The ratio should be >0.8.

6. Determine the dilution necessary to get about 1000 CFU per 20 µl.
   a. Prepare 6 tubes with 0.9 ml of dilution buffer
   b. Rapidly thaw an aliquot of bacteria.
   c. Add 100 µl of thawed bacteria to 1 ml of dilution buffer. Perform 10 fold serial dilutions by transferring 100 µl.
   d. Plate 10 µl in triplicate on a chocolate II agar plate.
   e. Incubate the plates overnight at 37°C in a candle jar.
   e. Count the colonies and determine the average.
   f. Determine the dilution factor required to yield 1000 CFU/20 µl.

Procedure (Note 4)

1. Perform twofold serial dilutions (8 or 10 dilutions) of antisera with dilution buffer.
2. Add 10 µl of diluted antiserum to duplicate wells of a microtiter plate.
3. Thaw an aliquot of bacteria.
4. Dilute the thawed bacteria in dilution buffer to prepare 1000 CFU/20 µl.
5. Add 20 µl of the diluted bacteria suspension.
6. Incubate at 37 ^{o}C for 15 min in a 5% CO_2 incubator.
7. Add 25 µl of baby rabbit complement (Note 3).
8. Add 25 ul of dilution buffer.
9. Incubate the plates at 37 °C for 60 min in a 5% CO_2 incubator.
10. Plate 5 µl of the reaction mixture on a chocolate II agar plate.
11. Incubate the plates at 37 °C in 5% CO_2 for 16 h.
12. Count the number of surviving bacteria.
13. Determine the serum dilution that kills $\geq$ 50% of the bacteria.

Assay Notes

Note 1: Fildes enrichment is peptic digest of sheep blood. It is rich in hemin and NAD. 5% supplement is usually used (3), but we have found that 2% is sufficient for the bactericidal assays.

Note 2: Bacteria will be in the exponential phase of growth. It takes about 2-3 hours. The broth acquires amber color.

Note 3: Complement lots should be qualified prior to use in the assay. Both active and heat inactivated baby rabbit complement is used to show that no bacterial killing is found during a 1 hour incubation period.

Note 4: Serum growth controls (all reagents except complement source) should be included when the serum source is unknown or it is suspected to contain antibiotics or any other inhibitory substances such as antibiotics.

References:

1. Romero-Steiner, S., J. Fernandez, C. Biltoft, M. E. Wohl, J. Sanchez, J. Feris, S. Balter, O. S. Levine, and G. M. Carlone. 2001. Functional antibody activity elicited by fractional doses of Haemophilus influenzae type b conjugate vaccine (polyribosylribitol phosphate-tetanus toxoid conjugate). *Clin Diagn Lab Immunol 8:1115*.

2. Romero-Steiner, S., W. Spear, N. Brown, P. Holder, T. Hennessy, P. Gomez De Leon, and G. M. Carlone. 2004. Measurement of serum bactericidal activity specific for Haemophilus influenzae type b by using a chromogenic and fluorescent metabolic indicator. *Clin Diagn Lab Immunol 11:89*.
3. Bergeron, M. G., P. Simard, and P. Provencher. 1987. Influence of growth medium and supplement on growth of Haemophilus influenzae and on antibacterial activity of several antibiotics. *J Clin Microbiol 25:650*.
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