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Preparation of inocula for experimental infection of blood with *Streptococcus pneumoniae*

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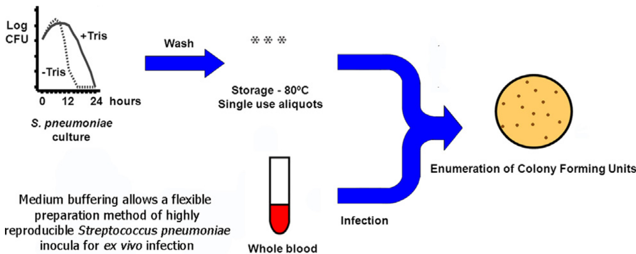
GRAPHICAL ABSTRACT



ABSTRACT

Experimental infections of either cells or animals require the preparation of good quality inocula. Unfortunately, the important pulmonary pathogen *Streptococcus pneumoniae* is a fastidious microorganism that suffers an autolysis process when cultured in vitro. Supplementation of Todd–Hewitt broth with a biological buffer (20 mM Tris–HCl, pH = 7.8) promotes a six hours delay in the beginning of the autolysis process. Additional improvements include washing bacteria before freezing, avoiding manipulations after thawing, and the use of glycerol (<18%) as a cryoprotectant, instead of reagents like skimmed milk that may affect cell cultures. With the proposed protocol >70% of the frozen bacteria was viable after 28 weeks at −80 °C, and aliquots were highly homogeneous. We have tested their utility in a whole blood infection model and have found that human plasma exhibits a higher microbicidal activity than whole blood, a result that we have not found previously reported. Additionally, we

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have also observed significant variations in the antimicrobial activity against different strains, which might be related to their virulence.

- Media culture buffering extends *S. pneumoniae* viability for 6 h.
- Washing before freezing of single use aliquots minimizes manipulation after thawing.
- Experimental infection with the frozen inocula has shown that plasma has higher bactericidal activity than blood.

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Method details

1. Seed the whole surface of a blood agar plate with *Streptococcus pneumoniae* from stock and incubate overnight at 37 °C. Other media that allow the growth of the bacterium, like Todd–Hewitt agar plates are also appropriate.
2. Harvest the bacteria and inoculate 40 ml of buffered Todd–Hewitt broth. Composition: Todd–Hewitt broth supplemented with 2% yeast extract and 20 mM Tris(hydroxymethylaminoethane (Tris, $pK_a = 8.1$). Adjust pH with HCl to 7.8 before sterilization.
3. Incubate the culture for 6–10 h at 37 °C in an incubator at 200 r.p.m. The viability of the bacteria remains high for this period of time in buffered Todd–Hewitt broth.
4. Centrifuge culture at 10,000 × *g* at 6 °C for 5 min in sterile conditions and discard supernatant. From this point, bacteria should always be below 6 °C, until they are frozen at −80 °C.
5. Wash bacteria twice with cold phosphate buffered saline (PBS) by centrifuging at 10,000 × *g* at 6 °C for 5 min. Bacterial wash after freezing of the stock results in a higher variability in the number of recovered viable bacteria.
6. Suspend bacteria in 1200 μl of cold RPMI-1640/15% glycerol.
7. Freeze single use aliquots at −80 °C. The stock concentration usually is above 10^9 bacteria/ml. Quantify the colony forming units (CFU) as described below one week after freezing because the bacterial death rate is high in the first days.

Experimental infection of whole blood

A modification of the direct bactericidal test of Lancefield [1] was performed.

8. Collect blood from healthy donors, following informed consent and approval of the protocol by the institution Clinical Research Ethics Board. Clotting was inhibited by K_3-ethylenediaminetetraacetic acid (EDTA). We have not tested other inhibitors.
9. Dilute biological samples (blood, plasma or serum, 40%) with RPMI-1640 medium (60%) and place 200 μl in 2 ml microtubes.
10. Infect diluted samples with 10^3 bacteria/100 μl and incubate at 37 °C in a rotator (20 r.p.m.) for the indicated times. Slightly different rotator speeds do not influence the outcome of the experiment.
11. After incubation, dilute 10 μl of infected blood in 90 μl of water and lyze cells in an ultrasonic bath for 3 min. We use a 35 kHz ultrasonic bath (half wave mode).
12. Inoculate 50 μl of the released bacteria in 55 mm diameter agar plates (Todd–Hewitt broth with 0.5% yeast extract and 1.5% agar). After absorption, add an agar overlay (Todd–Hewitt broth containing 0.5% yeast extract/0.75% agar) supplemented with 100 mg/ml 2,3,5,-triphenyltetrazolium chloride, a dye indicative of cellular metabolism that stains the colonies deep red

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