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Spore Preparation Protocol for Enrichment of Clostridia from Murine Intestine

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[Abstract] In recent years, many spore-forming commensal Clostridia found in the gut have been discovered to promote host physiology, immune development, and protection against infections. We provide a detailed protocol for rapid enrichment of spore-forming bacteria from murine intestine. Briefly, contents from the intestinal cecum are collected aerobically, diluted and finally treated with chloroform to enrich for Clostridia spores.
Keywords: Spores, Clostridia, Bacteria, Mammalian, Murine, Intestine, Colonization resistance

[Background] Chloroform kills vegetative bacterial cells but not spores and thus is a useful treatment for enriching Clostridia, the dominant spore-forming group in the mammalian intestine. Experimental procedures for chloroform treatment of mouse feces exist (Momose *et al.*, 2009; Yano *et al.*, 2015). However, they utilize specialized equipment including an anaerobic chamber. We realized that several brief exposures to oxygen occur during experimental manipulation of intestinal contents in preparation for and after chloroform treatment. Therefore, we reasoned the sufficient recover of murine spore-forming bacteria could be obtained without the use of an anaerobic chamber. Since spore-forming Clostridia are a dominant species in the mammalian intestine, this protocol could potentially be used for isolation of spores from the intestines of other mammalian organisms, including larger rodents, primates, and humans.

Materials and Reagents

1. Sterile 1.5 ml microcentrifuge tubes (Eppendorf, catalog number: 022363204)
2. 15 ml tubes
3. Female (male mice may be used if necessary) C57BL/6 mice aged 8-12 weeks (THE JACKSON LABORATORIES)
4. Compressed CO_2 gas in cylinder (AirGas)
5. Chloroform (Sigma-Aldrich, catalog number: 288306)
6. Sterile PBS pH 7.4 (Reference 2)

Equipment

1. Sterile necropsy instruments (operating scissors, tweezers and forceps) to avoid contamination

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2. Shaker with 200 rpm capacity and 37 °C setting (or inside 37 °C room)

Procedure

1. Without pre-charging the chamber, place the animal(s) in the chamber and introduce 100% carbon dioxide (whenever possible euthanize animals in their home cage). Open the CO_2 tank or valve regulator to initiate flow of gas. A fill rate of about 10% to 30% of the chamber volume per minute with carbon dioxide, added to the existing air in the chamber should be appropriate to achieve a balanced gas mixture to fulfill the objective of rapid unconsciousness with minimal distress to the animals. Wait approximately 3-5 min for animal to stop moving or breathing. Maintain CO_2 flow for a minimum of 1 min after respiration ceases. Death was confirmed by cervical dislocation.
2. Open the abdominal cavity using operating scissors to remove the cecum. Using operating scissors, keep the cecum intact by carefully excising to not lose any contents.
3. Open the cecum with operating scissors and add the cecal contents to a 1.5 ml microcentrifuge tube on ice.
4. Take cecal contents and dilute 1:10 in PBS (w/v). Add chloroform in a final concentration of 3% (v/v).
5. Incubate the cecal contents/chloroform mixture by shaking at 200 rpm at 37 °C for 30 min.
6. Allow the chloroform to settle to the bottom of the tube at room temperature (approximately 20 min).
7. Remove top aqueous layer to recover the spores and add to a sterile 1.5 ml tube. Be careful not to take any chloroform that has settled to the bottom of the tube.

Data analysis

Real-Time PCR using Clostridia specific primers were used for subsequent analysis of log CFU of Clostridia in feces of mice that received spores isolated by this protocol (Rivera-Chávez *et al.*, 2016). Fold changes of ratios (mRNA levels) were transformed logarithmically prior to statistical analysis. An unpaired Student's *t*-test was used to determine whether differences in fold changes between groups were statistically significant ($P < 0.05$).

Notes

1. Expect 100 to 200 mg of cecal contents per mouse.
2. It is very important to collect the cecal contents as quickly as possible once mouse has been euthanized to ensure maximum recovery of spores.
3. Use sterile technique to not contaminate cecal contents during transfer into a sterile 1.5 ml tube.
4. If pooled cecal contents from multiple mice will be prepared, then 15 ml tubes could be used to

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accommodate greater volumes.

5. Isolated spores should be used fresh (same day of isolation) to ensure maximum viability of spores. We do not recommend storing spores.

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