**SOP# 02.401.01 – *In vitro C. difficile* inhibition**

***Clostridium difficile in vitro* culturesupernatant inhibition assay**

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**Date of Rev.:**

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**Purpose**

The purpose of this SOP is to identify previously isolated fecal bacteria that exhibit growth inhibition of *Clostridium difficile* strains mediated by direct inhibition or nutrient competition.

**Scope**

The scope of this document is exploratory (to identify candidate organisms that inhibit growth of *Clostridium difficile*).

**Regulatory References**

NA

**Responsibility**

* Responsibility of experimentalist: understanding and performing this procedure as described; reporting any deviations or problems to area supervisor; adequately documenting the procedures and results.
* Responsibility of area manager or supervisor: ensuring that the analyst performing this procedure is qualified; ensuring that the procedure is followed, and updating the procedure as necessary.

**Definitions/Abbreviations**

* mL – milliliter
* μL – microliter
* μm – micrometer
* nm – nanometer
* OD600 – optical density, 600 nanometers
* M – molar
* BG – background
* v/v – volume/volume
* BSL2 – biosafety level 2
* BHIS – brain heart infusion supplemented
* *C. difficile* – *Clostridium difficile* 90556-M6S
* FDR – false discovery rate
* KOH – potassium hydroxide

**Related Documents**

* SOP# 03.001.01 – Anaerobic chamber operation and maintenance
* SOP # 03.003.01– Biosafety cabinet operation and maintenance

**Required Equipment and Materials / Reagents**

Equipment

* Spectrophotometer for optical density measurements: any will work as long as it can read OD600nm
* Coy Polymer Anaerobic Chamber (Coy 83070)
* Plate reader for 96-well optical density measurements (e.g. BioTek Synergy 2 Multi-Mode Reader): any will work as long as it can read OD600nm
* Microcentrifuge: any can be used as long as it reaches 10,000 x g
* Class II Type A2 Biosafety cabinet (e.g. Labconco Purifier Logic+): any manufactured biosafety cabinet may be used as long as it is Class II or higher

Materials/Reagents

* 5 mL polystyrene culture tubes (Fisher 1495940B)
* Disposable, sterile inoculation loops - 5 μl (VWR 12000-806)
* 3 mL luer lock syringes (BD 309628)
* 0.22 μm syringe filters (Millipore SLGS033SB)
* 1 M potassium hydroxide (KOH) (FLUKA 35113)
* 96-well tissue culture plates (CLS 3595)
* Optically transparent 96-well plate seal (Bio-Rad MSC1001)
* BHIS broth (pre-reduced in anaerobic chamber for 24 hours with 0.01% L-cysteine) (HiMedia MV210-500G)
* BHIS agar plates (pre-reduced in anaerobic chamber for 24 hours with 0.01% L-cysteine) (HiMedia MV210-500G)
* Sterile 1.5 mL microcentrifuge tubes (Sigma T9661)
* Frozen glycerol stock of *C. difficile* strain ATCC 9689 – lab stock with lab notebook designation
* Frozen glycerol stock of each isolate for assay
* pH strips (VWR BDH35309.606)

**Precautions**

* Personal protection equipment including gloves, lab glasses, and lab coat must be worn when executing this procedure.
* Potassium hydroxide (KOH) is highly corrosive. Consult the MSDS for KOH prior to handling it.

*Safety precautions for working with* C. difficile:

**Procedure**

Note: Prior to performing the assay, the operator should verify OD600 of stationary phase *C. difficile* and isolates by referring to lab notebooks/reports or by performing three sequential growth curves (n ≥ 3), measuring culture growth by OD600.

Day 0:

* Streak *C. difficile* strain (ATCC 9689) and isolates using sterile inoculation loops (VWR, 12000-806) for assay onto pre-reduced BHIS agar plates and incubate for 24 hours at 37**°**C under anaerobic conditions in anaerobic chamber (Coy 83070) (see SOP# 03.001.01 – Anaerobic chamber operation and maintenance).

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Day 1:

Use sterile inoculation loops to inoculate single colonies of *C. difficile* or isolates (6 per 96 well plate) into separate tubes 3 mL liquid pre-reduced BHIS broth and incubate 16 hours overnight (do 3 replicates of isolates, 6 of *C. difficile*)

Day 2:

1. Confirm by measuring OD600 of liquid cultures that *C. difficile* and isolates have reached stationary phase.
2. Remove 3 replicate cultures of C. difficile from the polymer anaerobic chamber and 3 replicate cultures of each isolate and transfer immediately to the Biosafety Cabinet. (See SOP # 03.003.01– Biosafety cabinet operation and maintenance.)
3. Transfer 1.5 mL culture each to sterile 1.5 mL microcentrifuge tubes. Centrifuge at 10,000 x g for 3 minutes.
4. Using 3 mL sterile syringes with attached 0.22 μm filters (Millipore SLGS033SB) filter-sterilize supernatant from each culture and divide sterilized culture volume in half into sterile 1.5 mL microcentrifuge tubes (750 μL per tube).
5. To one half of tubes, measure pH by dropping 50 μL supernatant onto pH strips and add dropwise 1 M KOH to each culture supernatant until pH reaches 7.0.
6. Transfer culture supernatant aliquots back into anaerobic chamber and prepare three replicates each in a 96 well plate with 200 μL culture volume (BG == background):
   1. BHIS broth only blank (BG)
   2. BHIS broth + 1:100 (v/v) overnight *C. difficile* culture
   3. *C. difficile* supernatant only blank (BG)
   4. *C. difficile* supernatant + 1:100 (v/v) overnight *C. difficile* culture
   5. *C. difficile* supernatant (pH 7) only blank (BG)
   6. *C. diffiicle* supernatant (pH 7) + 1:100 (v/v) overnight *C. difficile* culture
   7. Isolate supernatant only blanks (BG)
   8. Isolate supernatant + 1:100 (v/v) overnight *C. difficile* culture
   9. Isolate supernatant (pH 7) only blanks (BG)
   10. Isolate supernatant (pH 7) + 1:100 (v/v) overnight *C. difficile* culture

Day 3:

1. At 24 hours, remove 96-well tissue culture plate from anaerobic chamber and measure OD600 for each culture by removing plate from anaerobic chamber and reading in BSL2 rated fluorescent plate reader (e.g. BioTek Synergy 2 Multi-Mode Reader or similar 96-well plate reader that can measure OD600. )
2. To determine which isolates show inhibition against *C. difficile*, compare background subtracted culture densities to the density of *C. difficile* grown on (a) its own filter-sterilized culture supernatant and (b) regular BHIS broth medium as a positive control.
   1. Isolates said to inhibit *C. difficile* have an OD600 within the 95% confidence interval of the OD600 of *C. difficile* grown on its own filtered cultured supernatant (i.e. fail to reject the null hypothesis that mean OD600 is the same using unpaired t test, with multiple hypothesis correction using Benjamini-Hochberg FDR q = 0.05)
   2. Isolates with slight inhibition of *C. difficile* have an OD600 below the 95% confidence interval of the OD600 of *C. difficile* grown in fresh BHIS broth (i.e. reject the null hypothesis that mean OD600 is the same, with alternative that mean OD600 for *C. difficile* in fresh broth is greater, with multiple hypothesis correction using Benjamini-Hochberg FDR q = 0.05)
   3. pH-dependent inhibition is said to occur when either (a) or (b) occurs only in the un-neutralized filtered culture supernatant
   4. pH-independent inhibition is said to occur when either (a) or (b) occurs both in the neutralized and un-neutralized filtered culture supernatant

**Version History**

This is version 1 of 1

**Worksheets**

NA

**Appendix**

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