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AMBITION-*cm*
AMBIsome Therapy Induction OptimizatioN

Laboratory Working Practice Document: 2 (All sites excluding Botswana): Quantitative Cryptococcal Cultures

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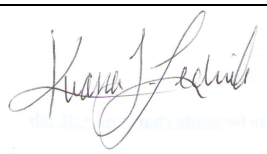
Author(s)	Kwana Lechiile Lab Scientist		10/09/2019
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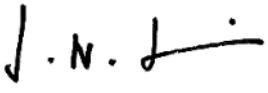
Approved by	Joseph Jarvis CI		10/09/2019

Revision History:		
Version Number	Effective Date	Reason for Change
1.0	02/08/2017	First version
1.1	10/09/2019	Addition of a new recommendation (page 6)

Laboratory Working Practice Document 2: Quantitative Cryptococcal Cultures

Introduction and Purpose

Colony-Forming Units (CFUs) is one of the most crucial tests for assessing virulence of *Cryptococcus neoformans*. For the optimum results, unspun CSF should be processed immediately for CFUs whenever possible, BUT after hours, over the weekends and public holidays, can be refrigerated at 4°C until processed.

Supernatant from the spun sample or the deposit from the CSF shouldn't be processed for CFUs, because supernatant will give false low CFUs and deposit will give false high CFUs.

Application Scope

This SOP describes how to perform quantitative cultures by serial dilution of CSF, and calculate colony forming units (CFU) per milliliter (mL).

Personnel Responsibilities

This SOP applies to all Laboratory Personnel performing CFU counts of *Cryptococcus* isolates. Standard precautions should be used for HIV-positive samples, and vortexing the sample may create aerosol with Cryptococci.

References

Brouwer et al. Combination antifungal therapies for HIV-associated cryptococcal meningitis: a randomised trial. Lancet. 2004 May 29;363(9423):1764-7.

Safety requirements

- Wear appropriate Personal Protective Equipment (PPE)
- Process samples inside Biological Safety Cabinet (BSC)

Materials required

- Vortex
- Incubator 30°C
- Pipette 1000µL
- Pipette 100µL
- 100µL and 1000µL pipette tips, sterile
- Rack for test tubes
- 5 Test tubes (sterile)
- 5 Sabouraud Agar or Dextrose agar plates with chloramphenicol
- Sterile distilled water (5 x 900 µL = 4.5ml)
- 300µL unspun CSF (minimum)
- Biohazard bag

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Procedures

A. Preparation

1. Put 5 plates in the 30°C incubator to dry

   Turn plates upside-down, rest them slanted on upside-down lids

2. Turn class 2 safety cabinet on
3. Mark tubes "1"; "2"; "3"; "4"
4. Mark patient's CSF with study number and study day
5. Mark plates with dilution ("0", "1", "2", etc), study day, date and study no.
6. Divide plates into half with marker

For this protocol, the pure CSF will be called "0"
The 1:10 dilution will be called "1"
The 1:100 dilution will be called "2"
The 1:1,000 dilution will be called "3"
The 1:10,000 dilution will be called "4"

To ensure reproducibility of the data, dilutions will be plated twice.

B. Making Dilutions

1. Using 1000µL pipette place 900 µL distilled water in test tubes 1-4
2. Take patient's CSF, from now on called "0"
3. Vortex undiluted CSF (**always** keep lid on)

4. Using the 100µL pipette **WITH A NEW TIP EACH TIME**, pipette:

   a. 100µL of vortexed undiluted (tube 0) and add it to the 900µL distilled water in tube 1. Discard pipette tip.

   b. Vortex tube 1, take new pipette tip, pipette 100µL of vortexed tube 1 and add it to the 900µL distilled water in tube 2. Discard pipette tip.

   c. Vortex tube 2, take new pipette tip, pipette 100µL of vortexed tube 2 and add it to the 900µL distilled water in tube 3. Discard pipette tip.

   d. Vortex tube 3, take new pipette tip, pipette 100µL of vortexed tube 3 and add it to the 900µL distilled water in tube 4. Discard pipette tip.

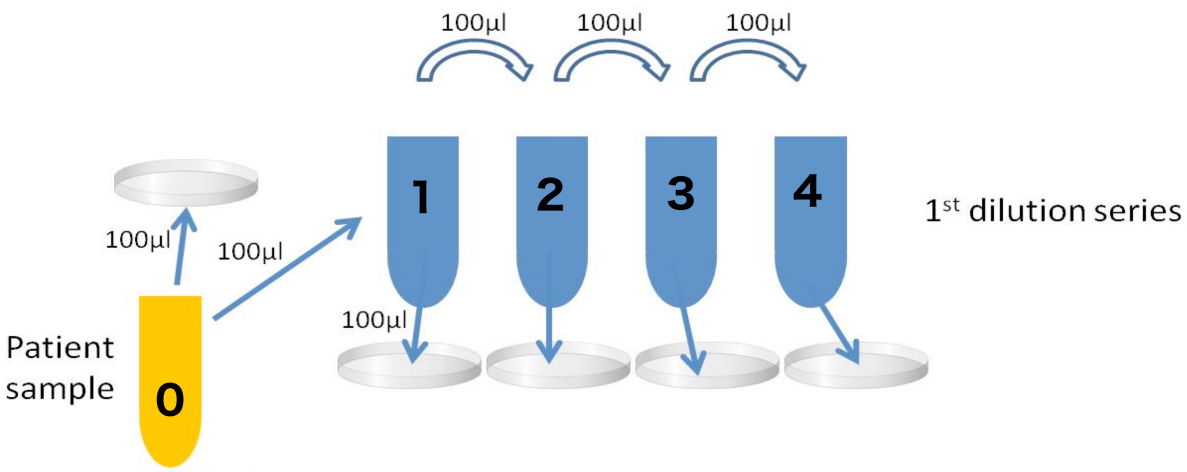

Figure 1: Making Serial Dilutions.

C. Spreading Dilutions on SDA Plate

1. Use **100 µl** pipette to spread CSF dilutions onto plate.
2. Vortex tubes *each time* before removing fluid.
3. Use **new pipette tip** each time.
4. Vortex undiluted CSF
5. Pipette 100 µl of vortexed undiluted CSF and distribute in 15-20 drops on *one half* of plate 0.

NOTE: Do not touch the agar with the pipette tip. Ensure drops are not too close to edge of plate or over any area you have written on with marker pen (difficult to count colonies later). Try not to "stab" the agar with the pipette tip.

6. Squeeze out any small volume remaining by gently touching the agar with the pipette tip.
7. Re-vortex undiluted CSF
8. Pipette 100 µl of vortexed undiluted CSF and distribute in 15-20 drops on *the other half* of plate 0. See Figure 2.
9. Repeat this for tubes 1-4 / plates 1-4.

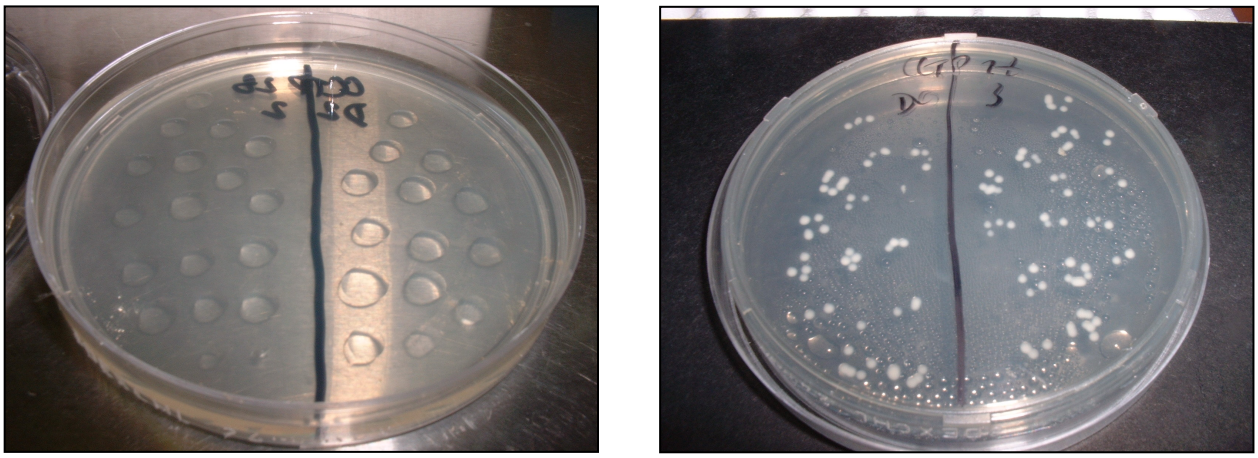

Figure 2. Labelled Plate with 100µL CSF dropped onto each half (L). After incubation at 30 degrees for 48 hours the distinct cryptococcal colonies are visible.

D. Cleanup

1. Discard tubes 1, 2, 3, 4, and pipette tips in biohazard bin.

2. Gently transfer upright agar plates stacked into pile into 30°C incubator. Incubate inoculated agar side face up for approximately 24 hrs to allow drops to dry, then turn over and start daily colony counts after 48 hours.

3. **Save the remaining patient CSF and put it into storage (see WPD 3)**

4. Turn off biosafety cabinet after use, and thoroughly clean work area.

E. Counting Colonies

1. Count only once the colonies are well-formed yet can still be distinguished from each other (rather than merging into one another). This is unlikely to occur before 48 hours. Choose a plate where distinct colonies are visible (and count dilution up or down from this as a check of good tenfold dilution technique).

2. Turn on the class 2 safety cabinet.

3. Count the plate with the lowest dilution with distinct colonies, with a minimum of 15 distinct colonies on each half (30 total). Plates with more than 100 colonies per half become difficult to count. See figure 2.

4. Keep the plates agar up (i.e. no need to open lids) and mark, on the outside of the plate, the colonies using a marker pen.

5. Count both halves of the plates and record the value on the plate and in the lab book.

6. Re-incubate the plates and perform daily counts (using different coloured pens) until such time when no new colonies have appeared for 3 consecutive days. At this time perform the final count (QC) and enter into lab book. Discard the plate .

7. If a plate shows no growth, incubate for 7 days and then discard and record QC as 0.

NB:

In cases where there has been low or no growth on previous cultures, you may choose to save resources by not plating up a full five plates for subsequent QCCs.
For example, if there is only growth on the neat and 1:10 plate then on the next culture you may prefer to only plate up the neat, 1:10 and 1:100.
Similarly, if there is no growth you may prefer to only plate the neat and 1:10.
The approach to this can be determined by each individual site.

F. Recording Data

To calculate the absolute number of colonies (colony forming units or CFU) per milliliter of CSF:

For example:

You count 80 colonies on one half and 86 colonies on the other half of plate 3.

Plate 0= 1:1

Plate 1= 1:10

Plate 2= 1:100

Plate 3= 1:1,000

Plate 4= 1:10,000

Take the average of the two halves (80+86)/2= 83.

Multiply by the dilution i.e. 83 x 1,000 (as plate 3)= 83,000.

This is for a volume of 100 µl - but you want colony forming units/ml, so you need to multiply by 10 (as 1ml= 1000 µl). Thus 83,000 x 10= 830,000 CFU/ml. Enter this number into the CSF results lab book under QC.

All results should be recorded in the lab results book and results to be communicated to trial doctors

Training

Each staff member receives or has direct access to applicable Working Practice Documents (WPDs).

Each staff member reviews the applicable WPDs once a year.

All WPD training is documented and tracked in the training log located in the Investigator Site File (ISF)

New staff is trained on applicable WPDs within 30 days of employment and all WPDs within 90 days of employment.

Staff members whose duties fall within this WPD scope are retrained within 14 days of the approval of each WPD revision.

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Staff signatures: (signing below indicates that you have read this SOP and understand the material contained in it)

Date	Name (Please print)	Signature