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AMBITION-*cm*
AMBIsome Therapy Induction OptimizatioN

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

Title of study	High Dose AMBISOME© on a Fluconazole Backbone for Cryptococcal Meningitis Induction Therapy in sub-Saharan Africa: A Phase III Randomized Controlled Non-inferiority Trial		
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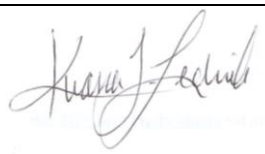
Author(s)	Kwana Lechiile Lab Scientist		02/08/2017
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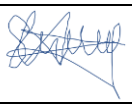
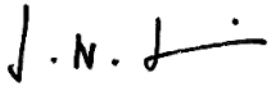
	Aude Sturny-Leclere Lab Technician, Institut Pasteur		02/08/2017
Approved by	Joseph Jarvis CI		02/08/2017

Revision History:		
Version Number	Effective Date	Reason for Change
1.0		First version

Laboratory Working Practice Document 2: Quantitative Cryptococcal Cultures

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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 microcentrifuge tubes
- Microcentrifuge tubes (1.5 -2 ml, sterile)
- Sabouraud Agar or Dextrose agar plates with chloramphenicol
- Inoculating loops

- Sterile distilled water

- 500µL unspun CSF (minimum)
- Biohazard bag

Procedures

A. Preparation

1. Put 11 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 "10"; "100"; "1000"; "10,000"
4. Mark patient's CSF with study number
5. Mark plates with dilution ("1", "10", "100", etc), dilution series (1st or 2nd), study day, date and study no.

For this protocol, the pure CSF will be called "1"
The 1:10 dilution will be called "10"
The 1:100 dilution will be called "100"
The 1:1,000 dilution will be called "1000"
The 1:10,000 dilution will be called "10000"

To ensure reproducibility of the data, dilutions will be done and plated twice. Therefore, make two tubes and two plates for each dilution ("10"x2, "100"x2, "1000" x2, "10000"x2). It is very important to make two independent dilutions rather than create duplicate plates of the same experiment.

B. Making Dilutions

1. Using 1000µL pipette place 900 µL distilled water in 4 microcentrifuge tubes
2. Take patient's CSF, from now on called "1"
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 1) and add it to the 900µL distilled water in tube 10. Discard pipette tip.

b. Vortex tube 10, take new pipette tip, pipette 100µL of vortexed tube 10 and add it to the 900µL distilled water in tube 100. Discard pipette tip.

c. Vortex tube 100, take new pipette tip, pipette 100µL of vortexed tube 100 and add it to the 900µL distilled water in tube 1000. Discard pipette tip.

d. Vortex tube 1000, take new pipette tip, pipette 100µL of vortexed tube 1000 and add it to the 900µL distilled water in tube 10,000. Discard pipette tip.

5. **Repeat the same procedure for dilution series 2 (see Figure 1)**

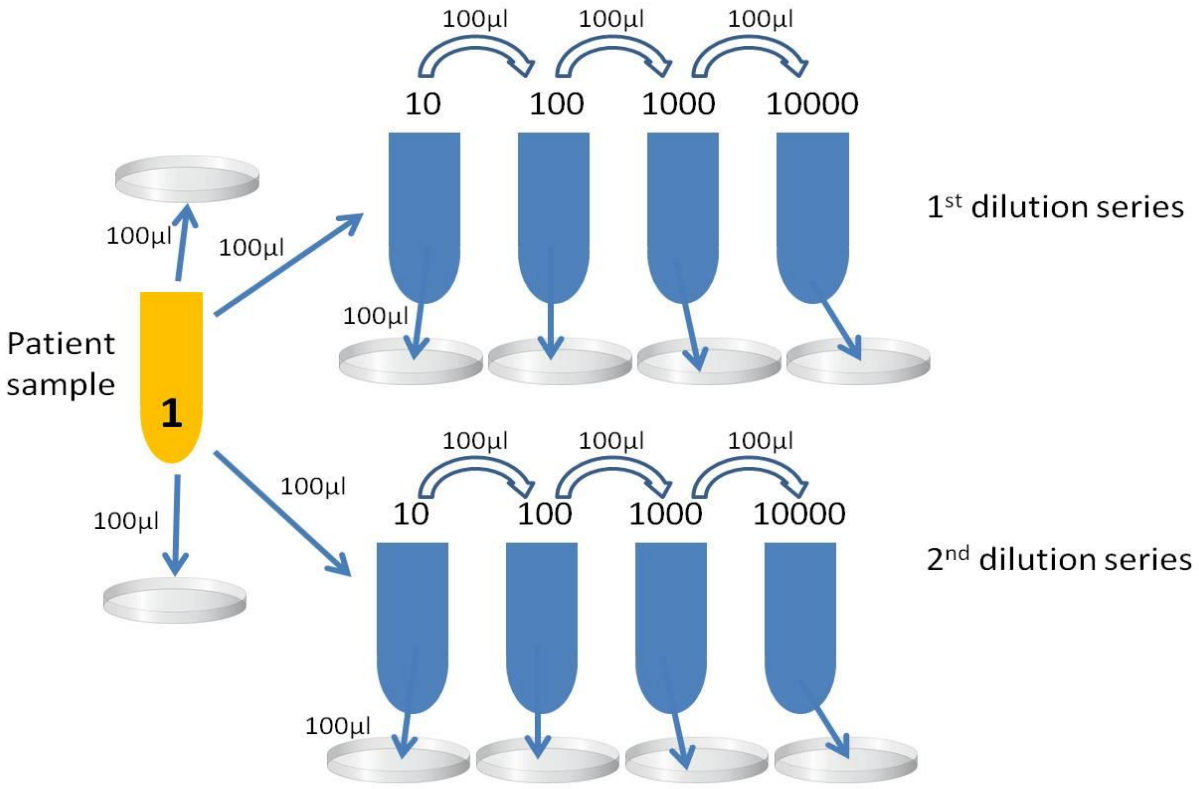

Figure 1: Making Serial Dilutions. (Please note that two independent dilution series are made)

C. Spreading Dilutions on SDA Plates

1. Vortex undiluted CSF
2. Pipette 100 µl of vortexed undiluted CSF (tube 1) into the center of plate "1"
3. Disperse CSF by rotating the plate in small angles and spreading with the inoculating loop. Spread until all liquid is absorbed into the agar
4. Repeat this for tubes 10, 100, 1000, 10,000 and the corresponding plates
5. **Remember to vortex tubes before taking aliquots for plating**
6. Wrap each individual plate with parafilm around the opening to reduce the risk of contamination
7. **Repeat the procedure for 2nd dilution series**

D. Cleanup

1. Discard tubes 10, 100, 1000, 10,000, and pipette tips in biohazard bin.
2. Turn plates upside down and transfer them into the 30°C incubator.
3. **Save the remaining patient CSF and put it into storage (see WPD 3)**

E. Counting Colonies

1. Plates should be incubated for 5-7 days before counting. Final counts occur on day 14.
2. Select the plate that contains more than 20 but less than 200 colonies.
3. Turn the plate upside down and use a permanent marker to put a dot below each colony
4. Record the total number of colonies (dots) on the plate.
5. There is no need to count plates that contain too many or too few colonies. However, as a quality control, estimate (eyeball) the number of colonies on the plates obtained from one dilution up and down from the one you are counting. There should be approximately 10 times more/less colonies on the plates with nearest dilutions.

   For example, if you counted 50 colonies on plate "100", you would expect to have approximately 500 colonies on plate "10" and approximately 5 colonies on plate "1000". Make a note, if there are any discrepancies.
6. Count plates for 1st and 2nd dilution series.

F. Recording Data

1. Calculate CFU by multiplying the number of colonies on the plate by the dilution factor.

Example: if 50 colonies were counted on plate "1", the CFU number will be 50x1 = 50
If 50 colonies were counted on plate "10", the CFU number will be 50x10 = 500
If 50 colonies were counted on plate "100", the CFU number will be 50x100 = 5000

2. Calculate and record CFU number for both 1st and 2nd dilution series.

3. Take average of the highest counts of two of the same dilutions from the series and multiply by 10 to get CFU/ml units

4. Results should be recorded and reported to the study doctor

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