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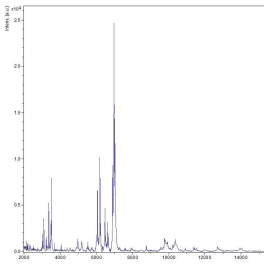
Absence of Candida africana in Ugandan Pregnant Women: Results from a Pilot Study



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We use this protocol and it's working

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Disclaimer

The information provided in this pilot study protocol is intended for research purposes only and should not be used for clinical decision-making without consulting appropriate healthcare professionals. The protocol is subject to modifications based on ongoing research findings and should be implemented with caution and adherence to ethical guidelines and institutional regulations

Abstract

Candida africana, an emerging variant identified since 2001 as a distinct species or variety within the *Candida albicans* complex, exhibits a global prevalence of 1.67%. This study outlines the methodology employed to investigate the presence of *C. africana* vaginitis in Uganda through a pilot study conducted in three hospitals in central Uganda, focusing on pregnant women receiving antenatal care. The protocol details the procedures implemented for the collection of vaginal swabs from pregnant women and the utilization of laboratory techniques including microscopy, fungal culture, and MALDI-ToF analysis. Notably, the prevalence of *C.africana* vaginitis was zero, establishing a baseline data tp inform future exploration into cryptic yeast species associated with vulvovaginal candidiasis in Uganda

Guidelines

Inclusion Criteria

Pregnant women who presented with clinical symptoms of either vaginal/vulva Itching, vaginal discharge, history of previous VVC infections, and those with on-going antifungal treatment for vulvovaginal candidiasis were included in the study

Exclusion Criteria

Pregnant women with signs of bleeding per vaginum were excluded from the study. This was due to the increased potential for interference with accuracy of test results, discomfort for the patient, and increased risk of introducing pathogens into the vaginal area

Materials

Specimen collection materials:

- Sterile cotton swabs
- High vaginal swab (HVS) tubes
- Biohazard Ziploc bags
- Microbiology request forms
- Consent forms

Personal Protective Equipment (PPE)

- Gowns
- Gloves
- Safety glasses

Laboratory equipment

- Biological safety cabinet
- Glass slides
- Pasteur pipette
- Slide heater block
- Microscope with 10 × and 40 × objectives
- Centrifuge

Wet preparation Microscopy

- 10% KOH

Gram stain

- Crystal violet
- Gram-iodine solution
- Acetone-alcohol solution
- Safranin

Culture media

- Sabouraud Dextrose Agar (SDA) supplemented with Chloramphenicol
- ChromAgar Candida Media
- Corn Meal Agar

MALDI-ToF

- 100 % Ethanol-Sterile Solution
- Sterile WFI Quality Cell Culture Grade Water
- Reusable polished steel MALDI target plate; 96 sample positions
- Bruker matrix HCCA (HCCA = α-Cyano-4-hydroxycinnamic acid)
- 70% Formic Acid
- Bacterial test standard (BTS)
- Bruker Standard Solvent
- 1.5 mL Microcentrifuge tubes
- 1.0 mm Zirconia/Silica Beads

Additional materials

- Human serum
- Microcentrifuge tubes

- Swabs for extraction procedure
- Ziploc bags for transportation
- Portable cooler box

Safety warnings

Standard personal protective equipment, including gowns, gloves, and safety glasses, should be consistently worn during all procedures to ensure the safety of personnel. Where appropriate, the set procedures must be conducted within a biological safety cabinet to minimize the risk of contamination. Precautions should be taken with substances such as formic acid, known for its flammable, toxic, and corrosive properties, and α-Cyano-4-hydroxycinnamic acid (HCCA), which poses risks of skin and serious eye irritation

Ethics statement

Ethical approval and consent to participate Ethical approval for this study under protocol reference number SBS-2022-253 was obtained from the Makerere University School of Biomedical Sciences Research Ethics Committee (SBSREC; IRB No 00007568) at their 124th convened meeting held on 15/12/2022. Informed consent was obtained from all study participants

Before start

Data on age, gestation period, clinical signs and symptoms were collected using a Research Ethics Committee approved structured questionnaire from all pregnant women included in the study. This proceeded the informed consent to participate in the study provided by the pregnant woman

Specimens collection

1 **High Vaginal Swab**

1.1 Explain the specimen collection procedure to study participant (Pregnant woman) and gain consent. Advise woman to pass urine if needed.

1.2 Place the pregnant woman in a lithotomy position (lying on her back with her legs flexed and spread apart). Ensure privacy is maintained

1.3 Put on sterile gloves, and gently separates the vaginal lips (labia) to access the vaginal canal. Inserts a sterile cotton swab into the vaginal canal until some slight resistance is felt. Rotated the swab 8 times in one direction to collect a sample from the vaginal walls

1.4 Remove the swab carefully to avoid contamination and place it in its plastic casing. Collect two swabs from each participant. Label each HVS tube with the participants unique identification number, the time and date of collection

1.5 Complete microbiology request form, and attach it to the signed consent form from the participant

1.6 Place all the 2 high vaginal swabs from a participant in biohazard Ziploc bag with accompanying forms, and store at 2 - 8^{O}C until they are transported to the Microbiology Laboratory to testing

Specimen Transportation

2 Vaginal swabs in Ziploc bags were placed in a portable cooler box along with the questionnaire, request form, consent form, and transported to the analysis laboratory within 8 hours of collection

Specimen processing

3 **Physical inspection of the specimen**

3.1 The sample swabs were first carefully examined to enable selection of the correct part of the sample likely to contain the fungus. Caseous or bloody areas were selected. Attention was paid to the color and thick consistency of the vaginal secretion

4 Direct gram Stain

4.1 Using one of the swabs, prepare a thin smear of the sample (discharge) on a clean, dry glass slide

4.2 Fix and dry on a slide heater block at 37^{o}C

4.3 Add crystal violet to cover the smear and wait for 1 minute

4.4 Drain off the crystal violet by rinsing with clean water, being careful not to let the flow of water fall directly on the smear

4.5 Add gram-iodine solution to cover the smear and leave for 1 minute

4.6 Rinse with water in the same manner as in 9.4 above

4.7 Flood the slide with 50% acetone - alcohol for 5 seconds

4.8 Rinse again with water

4.9 Counter stain with safranin for 1 minute and rinse in water. Dry on slide warmer at 37^{o}C

4.10 Examine at low power (10-40x), then under oil immersion (100x) for the presence of gram organisms. Yeast cells are gram positive. Note the size, shape and arrangement of the fungal elements

4.11 Perform a Nugent scoring by by evaluating each Gram-stained smear for large gram-positive rods (*Lactobacilli* morphotype), small Gram-variable rods (*Gardnerella vaginalis* morphotypes), small gram-negative rods (Bacteroides species morphotypes), and curved gram-negative rods (*Mobiluncus* spp. morphotypes).

4.12 Determine the number of organisms and the N-scores for each morphotype as shown in *Table I* below

	A	B	C	D
	Score	Lactobacillus(Large gram-positive rods)	Gardnerella and Bacteroides	Curved gram-negative rods
	0	4+	0	0
	1	3+	1+	1+ or 2+
	2	2+	2+	3+ or 4+
	3	1+	3+	
	4	0	4+	

0 = no morphotype, 1+ = < one morphotype, 2+ = 1–4morphotypes, 3+ = 5-30 morphotypes, and 4+ = > 30 morphotypes

4.13 A total Nugent score ≤ 3 indicated that the smear was negative for BV, 4-6 indicated altered vaginal flora, 4–6 with polymorphonuclear cells indicated altered vaginal flora with inflammatory conditions, and ≥7 indicated that the smear results were consistent with BV

5 10% Potassium Hydroxide (10% KOH) wet preparation examination

5.1 Take a clean, glass slide free of grease

5.2 Using a Pasteur pipette, place a large drop of 10% KOH solution on the slide. Gently mix the swab with a drop of 10% KOH

5.3 Cover the sample with a coverslip. Leave the sample on damp cotton wool for 2 to 5 minutes to allow the clearing process to take place

5.4 Examine the clear sample at low magnification (10x). Examine the whole coverslip from corner to corner in a zigzag pattern

5.5 Turn to a high magnification (40x). Adjust the light entering the condenser as you inspect at high power.

5.6 Examine if present, type of branching of hyphae, budding, non-budding cells, septation and thickness of hyphae

5.7 Look for and evaluate the types and numbers of the yeast cells (round, elongated, ovoid, single and multiple budding). True pseudohyphea crisscross epidermal cells in a random fashion and the strands are usually of a uniform diameter

5.8 quantifying fungal elements in a wet preparation examination using the following scale: 1+ (Scanty): Few fungal elements observed; rare hyphae or spores. 2+ (Moderate): Moderate number of fungal structures; hyphae and spores present. 3+ (Abundant): Numerous fungal elements; densely distributed hyphae and spores. 4+ (Very Abundant): Extremely high fungal load; overwhelming presence of hyphae and spore

Culture

6 **Sabouraud Dextrose Agar (SDA) supplemented with Chloramphenicol**

6.1 Streak the sample onto the surface of the medium by gently rolling the swab over a small area of the surface at the edge, and then streak from that area with a loop. Include a positive control plate inoculated with control strains of *C.albicans* ATCC 10231 in the batch

6.2 Incubate the plates at 25–30°C for 24hours

6.3 Note the growth, color, elevation, size, margins, texture, of the colonies. Report No fungal growth after incubation for 10 days

7 **ChromAgar Candida Media**

7.1 Obtain an isolated colony from the SDA and inculcate it on a subdivided ChromAgar plate. Incubate for 24hrs at 35 - 37^{O}C

7.2 Note the color of the colony and identify the yeast species based on colony color, according to the manufacture's guidelines

8 **Germ Tube Test**

8.1 Suspend a very small inoculum of the yeast cells obtained from an isolated colony in 0.5mL of Human serum. Incubate at 35 - 37^{O}C for no longer than 3 hours

8.2 After incubation, remove a drop the suspension and place it on a microscope slide. Cover with a cover slip and examine under low power magnification for the presence of germ tubes.

8.3 A germ tube is defined as an appendage that is half the width and 3 - 4 times the length of the yeast cell from which it arises, without a constriction at the point of origin of the germ tube from the cell

9 **Corn Meal Agar Morphology**

9.1 Obtain an isolated colony from the primary culture plate (SDA)

9.2 Inoculate a plate of corn meal agar containing 1% Tween 80 and trypan blue by making two lines at right angle to each other. Put a cover slip at the "x" intersection of the lines

9.3 Incubate the corn meal agar plate at 30^{O}C for 72hours

9.4 After 72hours, examine the areas under the coverslip for the presence of blastoconidia, arthroconidia, pseudohyphae, hyphae, or chlamydospores

9.5 Record the presence or absence of chlamydospores, and any other features seen

Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry

10 **Extraction procedure**

10.1 Perform extraction procedure in duplicate

10.2 Label two 1.5 mL microcentrifuge tubes for each sample, including QC strains

10.3 Add 500 µL of 100% Ethanol to each.

10.4 Add approximately 50 µL of 0.1 mm diameter silica beads to each tube, using the scaling on the microcentrifuge tube

10.5 Wet a sterile swab using the ethanol from the suspension (from step 15.3) or sterile deionized water. Press swab against the side of the tube to remove excess fluid

10.6 Collect approximately a circle of 1-2 cm diameter of mold from the agar plate using the swab, selecting spores (conidia) and hyphae if possible. Be careful not to pick any agar when picking up the colonies.

10.7 Suspend the collected material in the 1.5 mL tube. Centrifuge for 3 minutes at 14,000g

10.8 Remove the Ethanol supernatant

10.9 Re-suspend the pellet in 20µL of 70% formic acid. Vortex briefly

10.10 Add 20µL of acetonitrile. Vortex briefly and Centrifuge for 3 minutes at 14,000g

10.11 Add 1 µL of the supernatant, taking care not to disturb the pellet, and spot, in duplicate, to a 96-spot reusable stainless steel target plate

10.12 Add 1 µL of the Bacterial test standard (BTS) suspension to two spots on the 96-spot reusable stainless steel target plate. Simply allow to air dry

10.13 As soon as spots are dry, immediately overlay with 1 µL of HCCA matrix (HCCA = α-Cyano-4-hydroxycinnamic acid). Do not delay the addition of HCCA matrix as it will decrease the quality of your run.

10.14 Air dry for two minutes.

10.15 As soon as spots are dry, subject them to MALDI-TOF analysis. NOTE: Target must be completely dry before it is inserted into MALDI BioTyper.

10.16 Be sure that the location of each specimen on the 96-spot reusable stainless steel target plate is recorded on a sample key spreadsheet. Verify the spreadsheet numbers to make sure they match the original sample numbers.

10.17 Interprete the resulting spectra using Bruker database

11 **Performance specification**

11.1 Bruker allows for any score above 2.0 to be acceptable for a species identification

12 **Interpreting results**

12.1 At least one of the duplicates for each control strain must generate a score of ≥ 2 with a correct identification (no conflicting results between the duplicates)

12.2 If the control strains generate scores < 2 or incorrect identification, the results should not be interpreted, and the extraction/submission process must be repeated.

12.3 If no species identification is made, repeat extraction/submission process using a new subculture

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