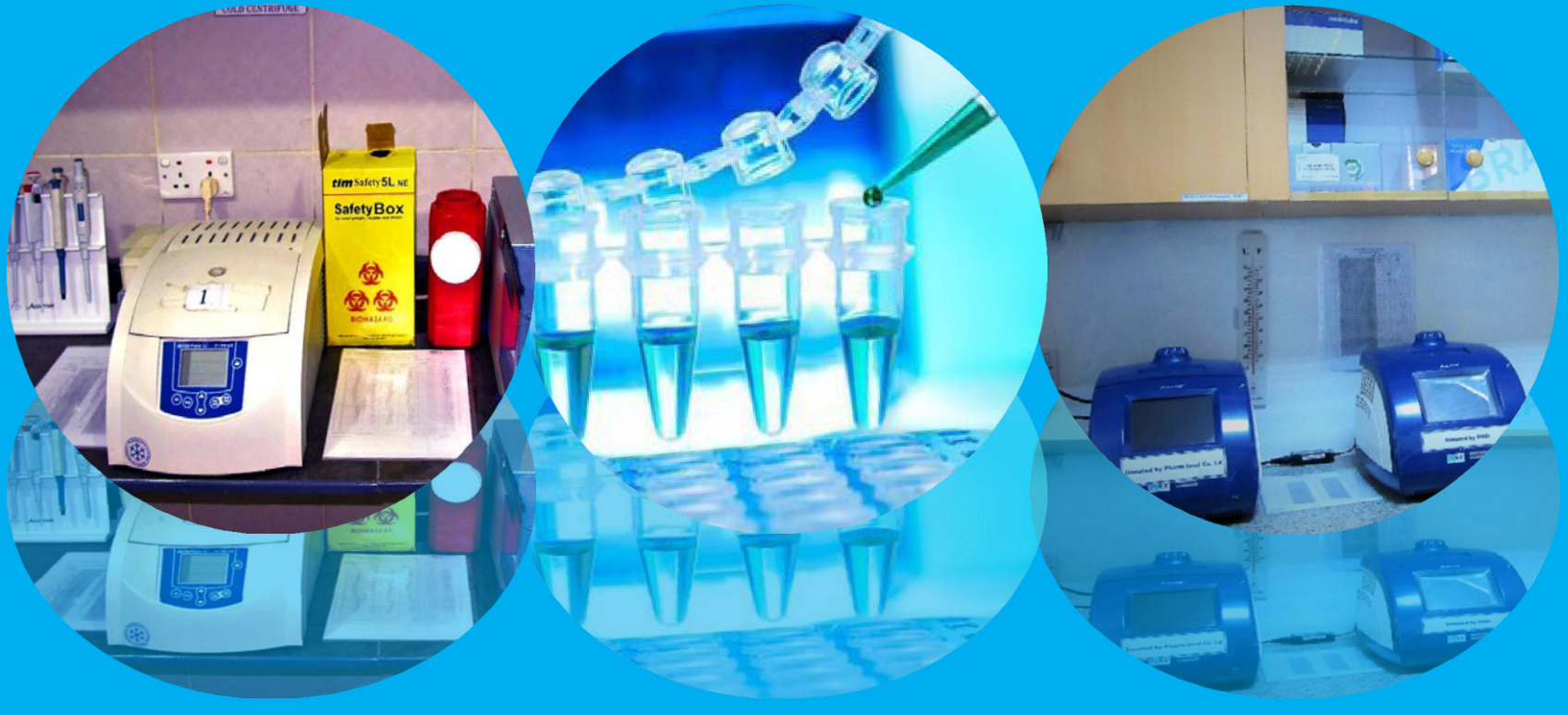

The Mycetoma Research Center,
University of Khartoum
WHO Collaborating Center
on Mycetoma & Skin NTDs

Standard Operating Procedures for *Madurella mycetomatis* Specific PCR Mycetoma Diagnosis

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Standard Operating Procedures

for *Madurella mycetomatis* Specific PCR Mycetoma Diagnosis

Introduction

This Standard Operating Procedure (SOP) outlines the steps for performing *Madurella mycetomatis*-specific Polymerase Chain Reaction (PCR) to diagnose mycetoma. Madurella mycetomatis is one of the most common causative agents of mycetoma. PCR is a sensitive and specific method to detect the presence of this pathogen in clinical samples.

Purpose

To provide a consistent, safe, and effective method for performing *Madurella mycetomati*s-specific PCR for the diagnosis and identification of the major causative agent of mycetoma.

Scope

This SOP applies to all laboratory personnel involved in the PCR diagnosis of patients with suspected or confirmed mycetoma caused by *Madurella mycetomatis*.

Responsibilities

Laboratory Technician/Technologist

• Prepares samples and performs the PCR.

Molecular Biologist/Pathologist

• Interprets the PCR results.

Laboratory Supervisor

• Ensures adherence to the SOP and quality control measures.

Infection Control Officer

• Ensures adherence to infection control protocols.

Equipment and Materials

- PCR thermal cycler
- DNA extraction kit
- *Madurella mycetomatis*-specific primers
- PCR reagents (e.g., Taq polymerase, dNTPs, PCR buffer)
- Sterile, nuclease-free tubes and pipette tips
- Microcentrifuge
- Agarose Gel electrophoresis equipment
- UV transilluminator or gel documentation system
- Personal protective equipment (PPE) (e.g., gloves, lab coat, eye protection)
- 100 b.p. DNA ladder
- Positive and negative control DNA samples

Procedure

Pre-Procedure Preparation

Sample Collection

- Collect clinical samples (e.g., grains, tissue biopsy) from the suspected mycetoma lesion using sterile techniques.
- Store and transport the samples at the appropriate temperature (usually 4°C) to the laboratory for processing.

Sample Identification

- Label each sample with patient details, date of collection, and unique sample identifier.
- Record the sample information in the laboratory log.

DNA Extraction

Preparation

- Wear appropriate PPE.
- Clean the work area and equipment with DNA decontaminant to prevent contamination.

DNA Isolation

Manual DNA Isolation Using (CTAP) Extraction Method

- Cetyl Trimethyl ammonium bromide (CTAB)
- Metal beads and 490uL from N- Cytyl- N, N, N Trimethyl Ammonium Bromide (CTAB) will be added to the sample in the Eppendorf tube.
- Tissuelyser will be used for mechanical destruction of the grain.
- 20pL from proteinase K will be added.
- The sample will be incubated in a water bath at 56°C for 45 minutes.
- 500uL from (chloroform (480uL) +isoamylalcohol (20L)) will be added to the sample.
- Then centrifuge for 10 minutes at 14000 rounds per minute (rpm).
- The supernatant will be transferred to a new Eppendorf tube.
- 34uL from ammonium acetate and 245uL from isopropanol will be added.
- Centrifugation for 5 minutes at 14000 (rpm) will be conducted.
- The supernatant will be discharged.
- Followed by washing with 70% ethanol (700uL).
- Then centrifuge for 5 minutes at 14000 (rpm)
- The pellet will be left to dry.
- 20uL of elution buffer will be added.
- Then quality check of the DNA will be done by the Nanophotometer.

• **DNA Extraction by Kits:**

- Follow the instructions of the DNA extraction kit to isolate DNA from the clinical samples.
- Ensure that the DNA extraction includes a positive control (known as *Madurella mycetomatis* DNA) and a negative control (no template DNA) to validate the procedure.

Quantification

- Quantify the extracted DNA using a spectrophotometer or fluorometer (DNA concentration should range between 1 to 10 ng/µL).
- Document the DNA concentration and purity (A260/A280 ratio of pure DNA is typically around 1.8).

PCR Amplification

PCR Reaction Setup

Prepare the PCR master mix according to the following components:

- DNA template (extracted sample DNA)
- *Madurella mycetomatis*-specific forward and reverse primers
- Taq polymerase
- dNTPs
- PCR buffer
- MgCl2 (if required by the buffer)
- Nuclease-free water to adjust the final volume

Thermal Cycling Conditions

Program the thermal cycler with the following conditions:

- Initial denaturation: 94°C for 3 minutes
- Denaturation: 94°C for 30 seconds
- Annealing: Optimal temperature for specific primers (e.g., 5560°-C) for 30 seconds
- Extension: 72°C for 1 minute
- Number of cycles: 3540-
- Final extension: 72°C for 5 minutes
- Hold: 4°C

Loading the Thermal Cycler

- Load the PCR tubes into the thermal cycler.
- Start the PCR run.

Post-PCR Analysis

Gel Electrophoresis

- Prepare an agarose gel (1-2%-) with an appropriate DNA stain (e.g., ethidium bromide or SYBR Safe).
- Load the PCR products, DNA ladder, positive control, and negative control into the gel wells.
- Run the gel at an appropriate voltage (e.g., 100-120-V) until the dye front has migrated sufficiently.

Visualisation

- Visualise the gel using a UV transilluminator or gel documentation system.
- Compare the bands in the sample lanes with the positive control and DNA ladder to confirm the presence of *Madurella mycetomatis*-specific bands.

Interpretation and Reporting

Result Interpretation

- A positive result is indicated by the presence of a specific band at the expected size corresponding to the *Madurella mycetomatis* DNA.
- A negative result shows no specific band in the sample lane, while the positive control must show the specific band, and the negative control should show no bands.

Documentation

- Record the results in the laboratory log and the patient's medical record.
- Report the findings to the requesting physician with an interpretation of the PCR results.

Quality Control

Controls

- Ensure that each PCR run includes positive and negative controls.
- Repeat the test if controls do not perform as expected.

Contamination Prevention

- Use separate areas for DNA extraction, PCR setup, and post-PCR analysis.
- Change gloves frequently and use filtered pipette tips to avoid cross-contamination.

Quality Assurance and Safety

- Perform regular maintenance and calibration of the PCR thermal cycler and other equipment.
- Adhere to biosafety guidelines and proper waste disposal methods for biological samples and reagents.
- Participate in external quality assessment programs to ensure the reliability of PCR results.

References

• Ahmed AO, Mukhtar MM, Kools-Sijmons M, Fahal AH, de Hoog S, van den Ende BG, Zijlstra EE, Verbrugh H, Abugroun ES, Elhassan AM, van Belkum A. Development of a species-specific PCR-restriction fragment length polymorphism analysis procedure for identification of Madurella mycetomatis. J Clin Microbiol. 1999 Oct;37(10):31758-. doi: 10.1128/ JCM.37.10.31753178.1999-. PMID: 10488173; PMCID: PMC85521.

• Lim W, Siddig E, Eadie K, Nyuykonge B, Ahmed S, Fahal A, Verbon A, Smit S, van de Sande WW. The development of a novel diagnostic PCR for Madurella mycetomatis using a comparative genome approach. PLoS Negl Trop Dis. 2020 Dec 16;14(12):e0008897. doi: 10.1371/journal.pntd.0008897. PMID: 33326425; PMCID: PMC7743967.

• Arastehfar A, Lim W, Daneshnia F, van de Sande WWJ, Fahal AH, Desnos-Ollivier M, de Hoog GS, Boekhout T, Ahmed SA. Madurella real-time PCR, a novel approach for eumycetoma diagnosis. PLoS Negl Trop Dis. 2020 Jan 15;14(1):e0007845. doi: 10.1371/journal.pntd.0007845. PMID: 31940343; PMCID: PMC6986762.

• Ahmed SA, van de Sande WW, Desnos-Ollivier M, Fahal AH, Mhmoud NA, de Hoog GS. Application of Isothermal Amplification Techniques for Identification of Madurella mycetomatis, the Prevalent Agent of Human Mycetoma. J Clin Microbiol. 2015 Oct;53(10):32805-. doi: 10.1128/JCM.0154415-. Epub 2015 Aug 5. PMID: 26246484; PMCID: PMC4572522.

• Clinical guidelines on the molecular diagnosis of mycetoma.

• Institutional protocols for PCR and DNA extraction.

• Manufacturer's instructions for PCR reagents and equipment.

Real-time PCR

The setup of molecular diagnosis of mycetoma

QIAGEN QIAcube Automated DNA, RNA Purification System

Approval

This Standard Operating Procedure has been prepared, reviewed and approved by:

Miss Nema Ahmed Alfaki	Technologist	Nema
Dr Abdella Osman Ahmed	Senior Microbiology Scientist	Abdella Osman
Prof Ahmed Fahal	Center Director	Fahal

On May 8 2024.

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