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*Research Paper*

# REAL-TIME PCR BASED ASSAY FOR THE DETECTION OF ACTINOMYCETOMA AMONG SUSPECTED PATIENTS IN SUDAN

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**Abstract**

The project aimed to estimate the efficiency and usefulness of real time PCR in diagnosis of actinomycetoma among Sudanese patients as Sudan is still classified as a part of mycetoma belt. In this study we collected 100 clinical samples from patients present with mycetoma lesion from different areas of Sudan during the period from November 2008 to August 2010. Pure isolates were obtained after incubation of the clinical samples at 37°C on different culture media. Identification scheme was carried out by using staining techniques and different biochemical reactions. Phenol Chloroform technique was used to isolate bacterial DNA. Then conventional PCR was used to amplify *strb-1* gene. Finally RT-PCR was used to amplify the same *strb-1* gene. Over the 100 clinical isolates (12%) were tentatively phenotyped as actinomycetes, three of which gave typical appearance of *Nocardia* spp. when using further confirmatory tests. Seven of the remaining nine isolates were identified as *Streptomyces* spp. when genotyped with PCR. Similar results were obtained by RT-PCR which provided further confirmation. The study concluded that RT-PCR assays gave significant improvement in the diagnosis since the time was taken is less and can work efficiently in management and controlling of the emergence this important neglected diseases.

Key words: Actinomycetoma, *Streptomyces* spp , *Nocardia* spp., *strb -1*, Sudan.

## INTRODUCTION

Madura is a diseases caused by either fungi or actinobacteria. The mycetoma caused by actinomycetes known as actinomycetoma. while fungal infection is known as eumycetoma. Mycetoma lesions are characterized by sinuses with pus and grains and by the mass of infection as well [1].

Geographically Madura etiological agents are variably distributed. The highest prevalence was found in Africa which is considered as a part of the tropical and subtropical regions where the disease is endemic. Worldwid e, mycetoma is known to be prevalent in a defined belt between the latitudes 30°North and 15° South [2] and includes India, Mexico, Senegal, Somalia, Yemen, Sudan and other countries [3]. Other

reports of mycetoma cases have been collected from temperate regions which are considered as dried with a limited rainy season and low rain rates with moderate to high humidity and an average temperature followed by dry humid season of 6 to 8 month [4].

In Africa, most mycetoma cases are eumycetoma due to *Madurella mycetomatis* but with few cases of actinomycetoma caused by Streptomyces, Nocardia, and Actinomadura [5].

Diagnosis of mycetoma is mainly dependant on the direct examination of grains from subcutaneous mass, sinus and discharges stained by Gram's for determining the type of mycetoma. It is not easy to gain pure cultures from the causative microorganisms, especially in case of actinomycetoma, as culture required deep surgical biopsy containing [6].

## MATERIALS AND METHODS

One hundred (n=100) clinical specimens (grains), were collected from patients with mycetoma attending different clinical centers in Sudan including Mycetoma Research Center, Soba Teaching Hospitals, Wad Madeni Teaching Hospital and Um Rawaba Hospital. A questionnaire was prepared and filled by the required data from all patients after being given their informed consents.

Grains were inoculated on LJ media, incubated at 37$^{o}$C and examined daily for the presence of any distinct growth. All suspected grown strains were again cultured on Glucose Yeast Extract Agar (GYEA) and Trypticase Soya Agar (TSA)**,** and then an identification scheme was adopted according to Quinn *et al*. [7] and Williams *et al*. [8]. Further confirmatory characterization was performed by applying the mycolic acids fingerprints as described by Minnikin [9].

### PCR-amplification and gel electrophoresis

Classical PCR was performed to all isolates that exhibited typical morphology of *Streptomyces* spp. DNA was extracted by phenol-chloroform method, primers used by Hunddleston *et al.* [10] were used to gain an ampilicon of 490 bp for *strb-1 gene*. The reaction was conducted with a 25 uL volume including 0.1% Triton X-100, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the dNTPs, 20 pmol of each primer with the following sequencing: F:5-TGAGCCTTGTAAGCGTCCAC-3 and R: 3-TTCATGCCGTGCTTCTCCAG-5, I U of *Taq* polymerase, and 3.0 uL of the tested DNA. *S. somaliensis* DSM 41607 was used as positive control. The negative control contained reaction mixture free from the tested DNA. PCR program was conducted with an initial 10 minutes denaturation step at 95°c for one cycle, followed by repeating cycles of denaturation (94°c for 30 seconds), annealing (30 seconds at 55°c) and extension (30 seconds at 72°c ) for 40 cycle, ended with 5 minutes as final extension at 72°c.

The ampilicons were separated on 2 % agarose gels with 5 µg ml of ethidium bromide.

### RT PCR of *strb-1*gene

A Real time PCR protocol was developed using bacterial DNA preparations as a template for the *Taq* polymerase [11]. A volume of 25 µl reaction was used containing the following: 0.2 mM of each of the four dNTPs (Promega), 0.1 µM of each primer, 10 µl of the template DNA, 0.5 U of *Taq* polymerase (Promega) buffer, and 2 µl of a 1:10,000 dilution of SYBR Green Dye I. Negative controls free from the tested DNA were used. Amplifications were achieved in a Techne, detection system (Quantica real time PCR machine) with the following program: 35 cycles of 30 seconds at 93°C, 30 seconds at 49°C, and 2 minutes at 72°C, ended by 10 minutes at 72°C. Positive results were monitored by sigmoid curves which were confirmed by the presence of a band of 490 bp in 2.0 % agarose gel.

## RESULTS

Clinical specimens were collected from mycetoma patients either from sinuses discharges or from incision biopsies. Colony morphology, different biochemical results and TLC were used to identify the isolates. The results showed that 12 (12%) reflect typical morphology of actinomadura among which 3 (3%) were identified as *Nocardia* spp. (Figure 1,2), while 9 (9%) of the clinical isolates showed streptomyces - like growth.

### Polymerase Chain Reaction

PCR detected *Streptomyces* spp in 7 (7%) of the nine suspected isolates with a band of (490 bp) compared with 100 bp DNA marker (Figure 3).

### RT PCR analysis

All of the nine suspected isolates were subjected to RT PCR. The results confirmed the presence of *stbr-1* gene in seven isolates which reflected typical findings to that proved by classical PCR ( Figure 4)**.**


**Figure 1.** Morphology appearance of *Nocardia* spp. obtained from suspected cases of actinomadura reflecting glabrous, rough, adherent colonies with different color ranging from white to orange.

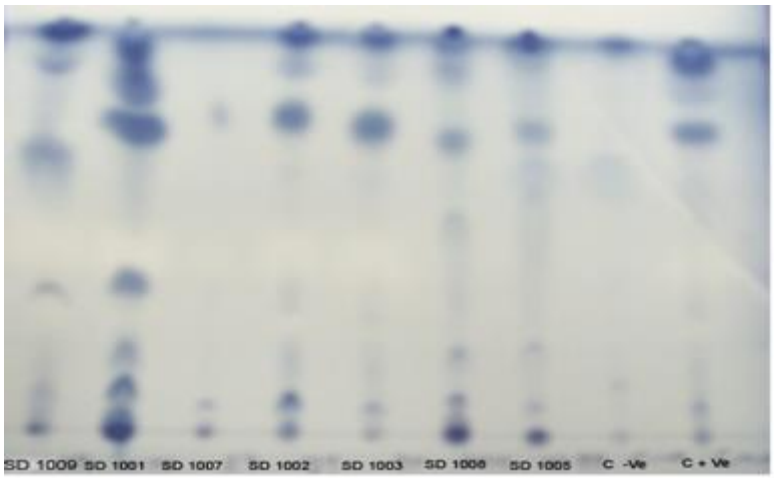


**Figure 2.** Mycolic acids profile of suspected nocardiae tested with thin layer chromatography (TLC).

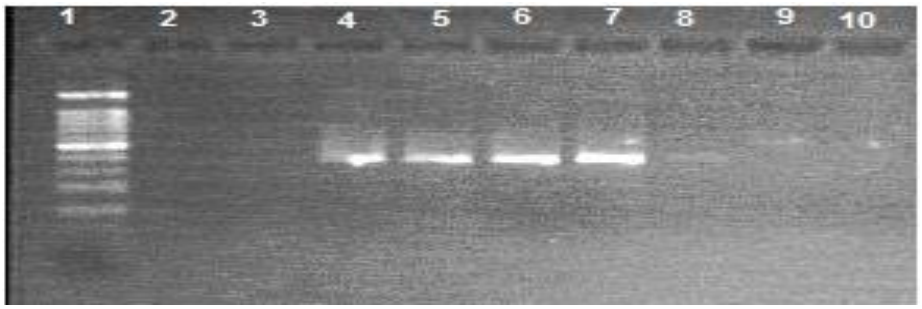


**Figure 3.** The amplicon of *stbr-1* on 2% agarose gel. Lane 1, 100 bp molecular weight ladder; Lanes 2, negative control; Lane 3,8,9,10 tested samples showing negative results for *stbr 1* gene; Lane 4,5,6, tested samples showing positive results; Lane 7, (490 bp) positive control (*S. somaliensis* DSM 41607).

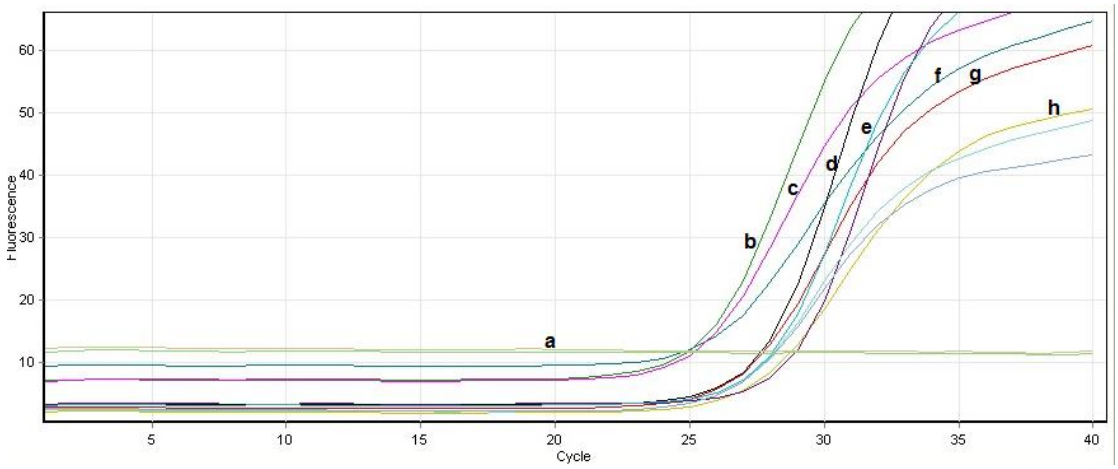


**Figure 4**. Results of real time PCR showing positive results for the presence of *Streptomyces* infections among patients with actinomadura. a; non template control, b; positive control (*S. somaliensis* DSM 41607), c,d,e,f,g and h are samples with positive results for *stbr-1* gene.

## DISCUSSION

Actinomycetes are responsible for variety of disease with actinomycosis being the most important, in addition to other opportunistic infections, hence the rapid and precized identification of these pathogens is becoming an increasingly vital goal for clinicians and researchers.

The accurate diagnosis of the type of mycetoma and detection of the depth of the lesion are both important for the successful medical treatment or surgical excision which is not easy since the available methods of diagnosis are neither sensitive nor specific [12].These include staining, cultivation, and serology in addition to aspiration cytology. However, the presence of grains in all these laboratory tests is of great importance [13]. Histopathological examination is less reliable than a positive culture [14] and since a deep surgical biopsy is required, it carries the risk of spread of infection [15]. Furthermore, it is so difficult to differentiate between the most important species of actinomycetes with histopathology and color of the grain [16]. Using conventional techniques in this project, our findings suggested the presence of actinomycetoma among 12% of the enrolled patients. Out of these, 3% were confirmed as *Nocardia* spp. while the other remaining isolates were tentatively classified as *Streptomyces* spp.

Similar results have been proved by many authors worldwide [1,3,5,19,21]. Characterization of aerobic actinomycetes by classical techniques needs high skills and will not allow novel discovery as new species will not be easy to differentiate from other known related species or genera [17]. Since these bacteria are slow growers, few days to several weeks are needed for genus stage characterization. When shifting to classical PCR and Real Time PCR we were able to confirm the existence of *Streptomyces* spp. among seven out of the nine isolates but we failed to amplify the *stbr1 gene* in the remaining samples (2/9). In this concern, different characterization techniques which increase the specificity could be used such as high-HPLC and molecular assays [18]. Thus, it is important to develop species-specific identification tools. Recently, Rolling Circle Amplification (RCA) for identification of etiology of eumycetoma was applied with 100% specificity and with the advantage of low-cost, high accuracy, rapidness and simplicity [19].
A recent review have concluded that one method of diagnosis is not sufficient to detect the causative agent and its method of treatment but two or more methods have to be combined for accurate treatment decision [20].

## CONCLUSION

Diagnosis, as well as treatment evaluation, are difficult tasks in mycetoma. Thus, PCR and RT-PCR as concluded from the results of this study, are of great importance to facilitate both accurate, rapid diagnosis and effective follow up as much as contamination is avoided, which is an tremendous improvement compared to the current phenotypic identification methods. However, the effectiveness of such techniques is limited by the fact that they should be undertaken in highly equipped centers, which may not be available in the far rural areas where the disease is prevalent. Moreover, a future perspective could be the application of real time PCR for the determination of etiological agent's DNA directly from clinical samples without the need for culture.

## Impact and Significant of the Project

Quick and accurate identification and characterization of the causative agents of mycetoma will provide essential information to clinicians and researchers for disease management and control. This is so crucial because management strategies for this disease are very different.

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## Conflict of interests

None declared.

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