

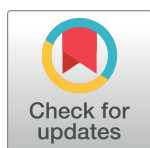
RESEARCH ARTICLE

# The Accuracy of Histopathological and Cytopathological Techniques in the Identification of the Mycetoma Causative Agents

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## Abstract

Mycetoma is a devastating neglected tropical disease, caused by various fungal and bacterial pathogens. Correct diagnosis to the species level is mandatory for proper treatment. In endemic areas, various diagnostic tests and techniques are in use to achieve that, and that includes grain culture, surgical biopsy histopathological examination, fine needle aspiration cytological (FNAC) examination and in certain centres molecular diagnosis such as PCR. In this retrospective study, the sensitivity, specificity and diagnostic accuracy of grain culture, surgical biopsy histopathological examination and FNAC to identify the mycetoma causative organisms were determined. The histopathological examination appeared to have better sensitivity and specificity. The histological examination results were correct in 714 (97.5%) out of 750 patients infected with *Madurella mycetomatis*, in 133 (93.6%) out of 142 patients infected with *Streptomyces somaliensis*, in 53 (74.6%) out of 71 patients infected with *Actinomyces madurae* and in 12 (75%) out of 16 patients infected with *Actinomyces pelletierii*. FNAC results were correct in 604 (80.5%) out of 750 patients with *Madurella mycetomatis* eumycetoma, in 50 (37.5%) out of 133 *Streptomyces somaliensis* patients, 43 (60.5%) out of 71 *Actinomyces madurae* patients and 11 (68.7%) out of 16 *Actinomyces pelletierii*. The mean time required to obtain the FNAC result was one day, and for the histopathological examinations results it was 3.5 days, and for grain it was a mean of 16 days. In conclusion, histopathological examination and FNAC are more practical techniques for rapid species identification than grain culture in many endemic regions.

## Author summary

In mycetoma endemic regions, the medical and health settings are commonly suboptimal, and only a few diagnostic tests and techniques are available. That had badly affected the patients' proper diagnosis and management and thus the late presentation of patients with advanced disease. In this retrospective study, the experience of the MRC on the common in use diagnostic tests in the period between 1991 and 2018 is presented. In this study, the sensitivity, specificity rates and diagnostic accuracy of grain culture, surgical biopsy histopathological examination and FNAC to identify the mycetoma causative organisms were determined. The histopathological examination appeared to have better sensitivity and specificity. Furthermore, the grain culture identification needs high experience, it is the tedious procedure, and cross-contamination is common hence misdiagnosis is frequent. It can be concluded that histopathological examination and FNAC are more practical techniques for rapid species identification than grain culture in many endemic regions with poor diagnostic setting.

## Introduction

Mycetoma is a chronic granulomatous subcutaneous inflammatory infection, endemic in subtropical and tropical regions, but it is reported globally [1, 2]. It is characterised by a painless subcutaneous swelling, multiple sinuses formation and a discharge that contain grains [3, 4]. The clinical presentation can give a clue to the diagnosis, but without further diagnostic testing it will lead to misdiagnosis and inaccurate treatment [5]. Mycetoma can be caused by different bacteria (actinomycetoma) or fungi (eumycetoma) [6, 7]. More than 70 different micro-organisms were reported to cause this infection, and hence it is essential to identifying the causative agents to the highest level of resolution which in turn will contribute to choosing appropriate treatment [8, 9]. In endemic regions, the most commonly used tools are culturing of the grains, surgical biopsy followed by histopathological examination and fine needle aspiration cytological (FNAC) examination [10, 11].

Currently, culturing the grains culture is still considered to be the golden standard for species identification in many centres [12, 13]. However, this technique is tedious, time-consuming due to the slow growth rate and it needs expert microbiologists to identify the causative agents based on the macroscopic appearance of the isolates. Furthermore contamination is common. Patients on medical treatment may have non-viable gains, and hence it is difficult to identify the causative organism [14, 15].

To overcome these difficulties, histological examination is often used complementary to culture. In a histopathological examination, it is easy to discriminate between fungal and bacterial causative agents [16, 17]. However, identification to the species level is more challenging and considered far from reliable [18, 19]. At the Mycetoma Research Centre (MRC), University of Khartoum, Khartoum, Sudan FNAC is a common tool to identify the causative organisms. It is less invasive and time-consuming compared to the histopathological and culture techniques [20, 21]. However, to the best of our knowledge there was no study performed in which the sensitivity and specificity of the two techniques for the identification of the mycetoma causative organisms were compared. With this background, this study was conducted at the Mycetoma Research Centre where 8500 confirmed mycetoma patients were seen and treated. In this retrospective study, the records of these patients were reviewed, and patients who undergone the three diagnostic tests were included.

## Materials and methods

### Ethics statement

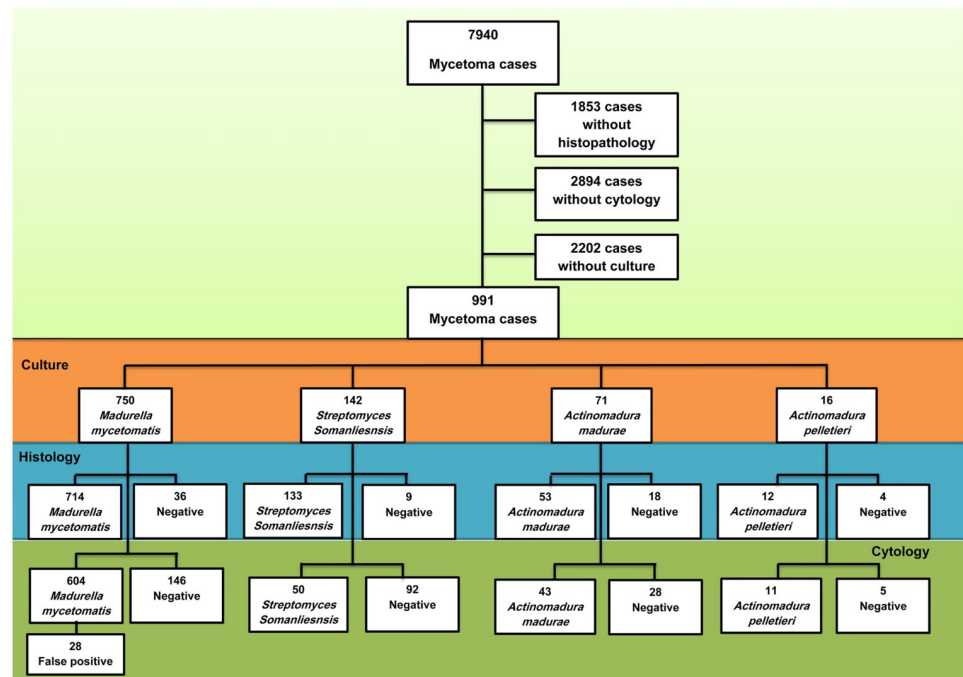
Following the Mycetoma Research Centre Institutional Review Board ethical approval, all the histopathological, cytological and microbiological reports of the patients seen in the Mycetoma Research Centre over a 27-year period (January 1991 to January 2018) were reviewed.

### Study cohort

The data were collected in the pre-designed data collection sheet. The patient demographic characteristics, results of the three techniques were collected.

In this study, only patients in whom the causative organisms were identified by culture and had undergone both a fine needle aspirate for cytological examination and deep-seated excisional biopsy for histopathological examination were included. (Fig 1).

The number of true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) test results was calculated for each technique and was compared to the culture which considered as our gold standard method. According to these results, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated for each test. Accuracy was calculated as the proportion of true results (both true positives and true negatives) among the total number of cases.



**Fig 1. identification of the causative agents:** All culture, histopathological and cytological identification reports of patients seen between January 1991 and January 2018 in the Mycetoma Research Centre were retrieved from our electronic database. Of these patients in 2202 cases no microbiological report was available, of 1853 cases no histopathological report was available and in 2894 cases no cytological report was available leaving 991 mycetoma cases which met our inclusion criteria. The culture identification was considered the golden standard. Of these patients 750 cases were caused by *M. mycetomatis*, 142 cases by *S. somaliensis*, 71 were caused by *A. madurae* and 12 by *A. pelletieri*. From these cases the histological and cytological reports were compared to the culture results. A histological report or cytological report was either identical to the culture identification or negative. Results for both histology and cytology reports are shown.

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### Grains isolation

The grains were obtained by surgical biopsy and/or FNAC. For the latter, a 25-gauge needle was inserted into the lesion, and aspirates were taken. The yield of grains was assessed visually by the number and size of grains obtained. If the yield was low, a second aspiration was taken with a 23-gauge needle. When excessive bleeding from the lesions was encountered, a 27-gauge needle was used. The obtained sample usual splited into two parts; one was transported immediately to the microbiology department for culturing, the other part was sent to the histology department for histopathological and cytological.

### Culture

The mycetoma grains were washed three times in sterile normal saline. When a fungus was expected based on the clinical data accompanied the request form and the grains color and consistency (fungal grains tend to be hard and are either white, yellow and black in color according to the causatives agent), the grains were cultured on Sabouraud dextrose agar with gentamicin sulphate (400 µg/ml) for fungal grains at 37 °C. When an actinomycete was expected according to the clinical data and ultrasound report as well as the grain color and consistency (actinomycetoma grains tend to be soft and smooth) grains were cultured on Blood agar, Colombia agar, Glucose yeast extract agar, Brain-heart infusion, Löwenstein—Jensen agar and Modified Sabouraud agar supplemented with 0.5% yeast extract. After that the plates were incubated at 37°C for 7 to 14 days.

### Identification of the eumycetoma causative agent by culture

When a fungus was grown on the Sabouraud plate, it was identified based on its macroscopic and microscopic morphology. Table 1 demonstrate the characteristic of different eumycetoma causatives agent for macroscopically and microscopically identification.

Macroscopically the appearance of *M. mycetomatis* colonies is quite variable. At the beginning the colonies tend to be white, and upon maturing they change to yellow or brown. Some strains of *M. mycetomatis* are able to produce a brown pigment in the culture. The texture varies from smooth, flat or heaped. *Madurella mycetomatis* is differentiated from *T. grisea* by its ability to grow at temperatures up to 40°C and its inability to assimilate sucrose (Table 2).

Table 1. Macroscopical and microscopical characteristic of different *Madurella* species.

Organism	Morphology on SDA	
	Morphology	Pigment Production
<i>Madurella mycetomatis</i>	dry, yellowish brown to gray; reverse, yellowish brown to gray.	variable
<i>Madurella tropicana</i>	dry, white brown to gray; reverse, white-brown to gray	orange-brown pigment
<i>Madurella pseudomycetomatis</i>	dry, brown to gray; reverse, brown to gray.	orange-brown pigment
<i>Madurella fahalii</i>	dry, yellowish brown to gray; reverse, yellowish brown to gray.	No pigment
<i>Trematosphaeria grisea</i>	greenish grey, becoming faint towards the margin; reverse dark brown to black.	no pigment
<i>Medicopsis romeroi</i>	greenish grey, becoming faint towards the margin; reverse dark brown to black.	No pigment
Organism	Microscopically appearance	
<i>Madurella mycetomatis</i>	Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Many Chlamydo spores are present.	
<i>Madurella tropicana</i>	Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Many Chlamydo spores are present.	
<i>Madurella pseudomycetomatis</i>	Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Chlamydo spores are present.	
<i>Madurella fahalii</i>	Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Many chlamydo spores are present.	
<i>Trematosphaeria grisea</i>	Hyphae branched, septate, hyaline or brown, thick-walled, verruculose	
<i>Medicopsis romeroi</i>	Hyphae branched, septate, hyaline or brown, thick-walled, verruculose	

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**Table 2. Carbohydrates Assimilation test for eumycetoma discrimination.**

Organism	Assimilation of Sugar			Growth at 37°c
	Glucose	Lactose	Sucrose	
<i>Madurella mycetomatis</i>	+	+	-	+
<i>Madurella pseudomycetomatis</i>	+	-	-	+
<i>Trematosphaeria grisea</i>	+	-	+	-
<i>Falciformispora senegalensis</i>	+	-	+	+
<i>Falciformispora tompkinsii</i>	+	-	+	+
<i>Nigrograna mackinnonii</i>	+	-	+	-
<i>Medicopsis romeroi</i>	+	-	+	+

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### Identification of the actinomycetoma causative agent by culture

When colonies are obtained, presumptive species identification is based on macroscopical and microscopical appearance of the species. Typical colonies of *Nocardia* spp and *Streptomyces* spp are dry to chalky in consistency, usually folded. The color will range from yellow to gray white. *A. madurae* and *A. pelletieri* strains produce cream- and red-pigmented mycelia respectively and lack aerial filaments on initial isolation. Ziehl-Neelsen staining is used to determine if the isolate is acid fast. *Nocardia* spp will stain positively and *Actinomadura* spp will stain negatively.

Different biochemical tests will be performed to identify the causative agent to the species level. These include the degradation of adenine, casein, and hypoxanthine; growth on adonitol; aesculin hydrolysis; glycerol; glycogen; D-raffinose; L-rhamnose; D-turanose; D-xylose; and L-aspartic acid (Tables 3 and 4).

### Cytological examination

The aspirate was allowed to air dry and was stained using Diff-Quick stain. The stained aspirates were examined by an expert histopathologist for the presence of the following cytomorphological features: smears cellularity, the host inflammatory tissue reaction, the presence and types of the causative organisms' grains. Species identification was based on species-specific criteria. In general, *M. mycetomatis* grains can be either small or large, are light to dark brown in colour and have irregular outlines and a crushing artefact when stained with hematoxylin and eosin (H&E) (Fig 2A).

*S. somaliensis* grains are difficult to see in H&E stained sections, they stain bright pink to hazy pink in colour, are often oval to irregular shaped and can be as aggregates (Fig 2C).

**Table 3. Different Biochemical test that used in discrimination between the actinomycetoma causatives agent.**

Organism	Acid Fast	Urease	Gelatin Hydrolysis	Decomposition of			
				Casein	Tyrosine	Xanthine	Hypoxanthine
<i>Nocardia asteroides</i>	+	+	-	-	-	-	-
<i>Nocardia brasiliensis</i>	+	+	+	+	+	-	+
<i>Nocardia otitidiscaviarum</i>	+	+	-	-	-	+	+
<i>Nocardia farcinica</i>	+	+	-	-	-	-	-
<i>Actinomadura madurae</i>	-	-	+	+	+	-	+
<i>Actinomadura pelletieri</i>	-	-	+	+	+	-	+
<i>Streptomyces somaliensis</i>	-	-	+	+	+	-	-

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**Table 4. Carbohydrates fermentation test for actinomycetoma discrimination.**

Organism	Utilization as a sole source of carbon						
	D- Galactose	D- glucose	I -myoinositol	D- manitol	L-ramnose	D-sorbitol	D-trehalose
<i>Nocardia asteroides</i>	V	+	-	-	V	-	V
<i>Nocardia brasiliensis</i>	+	+	+	+	-	-	+
<i>Nocardia otitidiscaviarum</i>	-	+	+	V	-	-	V
<i>Nocardia farcinica</i>	-	+	-	-	+	-	-
<i>Actinomadura madurae</i>	NA	NA	+	+	+	+	+
<i>Actinomadura Pelletieri</i>	NA	NA	+	-	-	-	+
<i>Streptomyces somaliensis</i>	NA	NA	+	-	-	-	-

V, variable; NA, not available

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*A. madurae* grains are small oval shaped, and it stained pink to red colour in H&E and tend to be as one mass without any fractures. *A. pelletierii* grains are small rounded to oval shaped, and they stained deep blue in H&E stained sections and tend to be fractured.

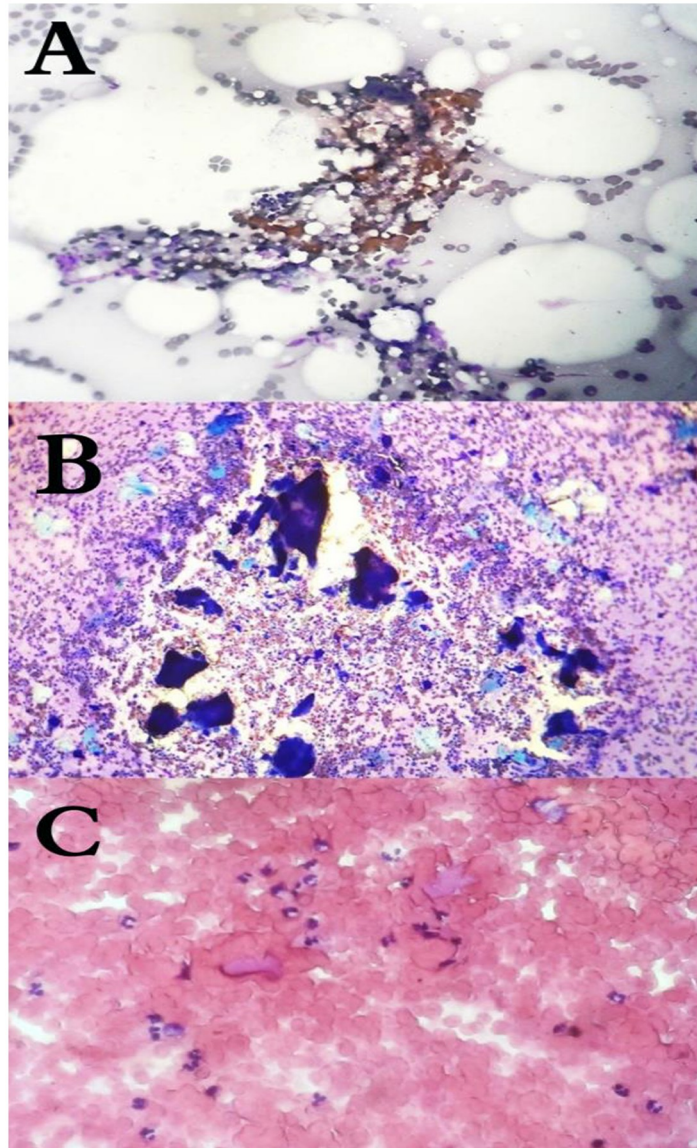
### Histopathological examination

All patients underwent surgical biopsy under anaesthesia, which was fixed in 10% formalin and processed further into paraffin blocks. 3-5-µm sections were obtained and stained with H&E. In our Histopathological laboratory the histopathologist issued the report with the species name according to the following criteria i.e. species specific criteria which have been used by all of them. *M. mycetomatis* grains tend to be large, light to dark brown in colour with irregular outlines. They tend to fracture when sections are cut. *M. mycetomatis* has two different types of grains, and these are the filamentous and vesicular. The filamentous type, is the most common type and consists of brown septated and branched hyphae that may be slightly more swollen towards the edges (Fig 3D).

*S. somaliensis* grains are rounded to oval in shape, with homogenous appearance in tissue sections. They appear faint yellow in unstained sections, and the grains are not well stained with H&E. Moreover, as a result of sectioning they may show longitudinal cracks, the filaments are fine (measured between 0.5–2 µm in diameter), closely packaged and embedded in cement matrix (Fig 3B).

*A. pelletierii* grains are small, round to oval in shape and semicircular and sickle like shapes have been observed as well. The filamentous structures are pretty difficult to be detected. However, a careful and meticulous examination of the periphery of the grains may show some of them. *A. pelletierii* grains stain deep violet with H&E, which is very characteristic and allows the definitive diagnosis without a need for culturing techniques (Fig 3C).

*A. madurae* grains ranged from yellow to white. Therefore, it can be difficult to discriminate them from the surrounding fat. Histologically the grain size ranges from small to large. The large grains have a characteristic variegated pattern. The periphery of the grain is opaque, homogenous and deep purple when stained with H&E stain, while the centre is less densely stained. Additionally, the periphery of the grains shows an eosinophilic material (Fig 3A). Smaller grains are more homogeneous and are difficult to distinguish from *A. pelletierii*. However, even the small grains of *A. madurae* have a more deeply stained purple fringe, which is not seen in *A. pelletierii*.

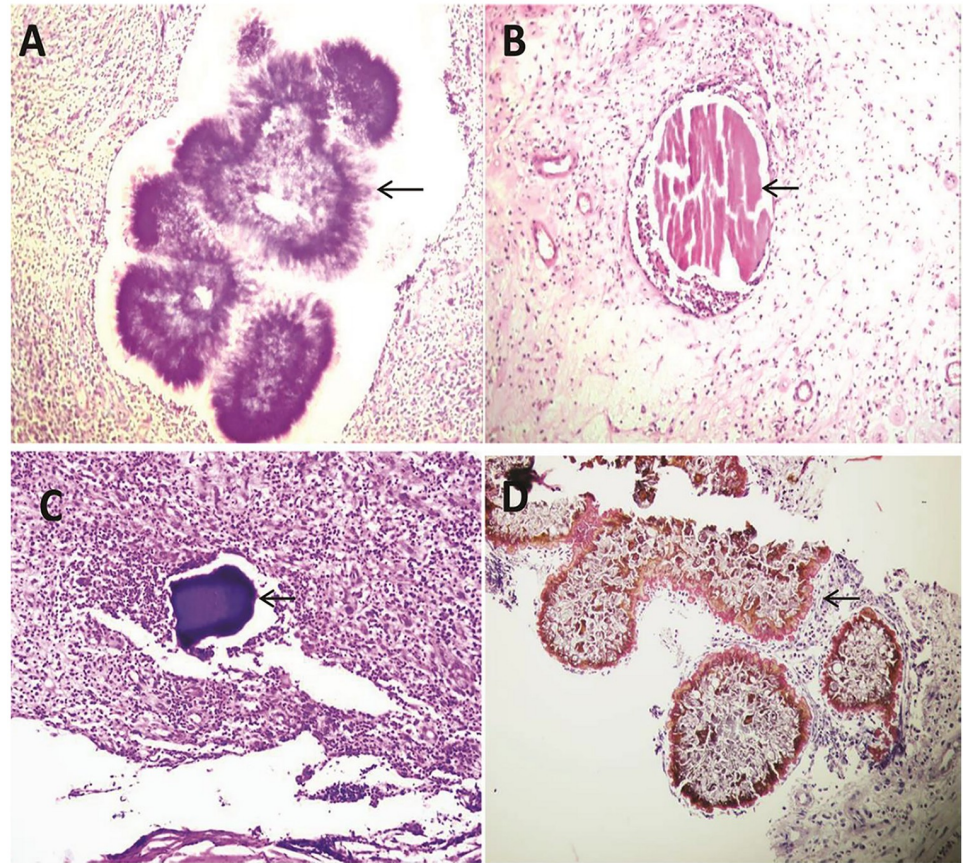


**Fig 2. Cytology of mycetoma causative agents.** (A): The cytological appearance of *M. mycetomatis*, (B): *A. pelletierii*, (C): *S. somaliensis* in fine needle aspirates stained with HE (magnification 10 times).

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## Results

In this study, 991 patients out of 7940 patients were eligible and were included in the analysis. Their ages ranged between 5 and 75 years old. The majority were males 737 (74.3%), and most of them were students 327 (32.9%) and farmers 167 (16.8%). The majority of the patients (837 out of 991), gave a history of discharge that contained grains and the majority of these grains were black (565; 57%) followed by yellow (104; 10.5%), white (60; 6.1%) and red grains (14; 1.4%). In this cohort, the majority of patients, (72.6%) had no history of local trauma, only 191 (19.3%) patients did recall a local trauma and the remaining 73 (7.4%) patients were not certain.



**Fig 3. Histopathological appearance of mycetoma causative agents.** The histopathological appearance of *A. madurae* (A), *S. somaliensis* (B), *A. pelletierii* (C) and *M. mycetomatis* (D) in HE stained sections (Magnification 10x).

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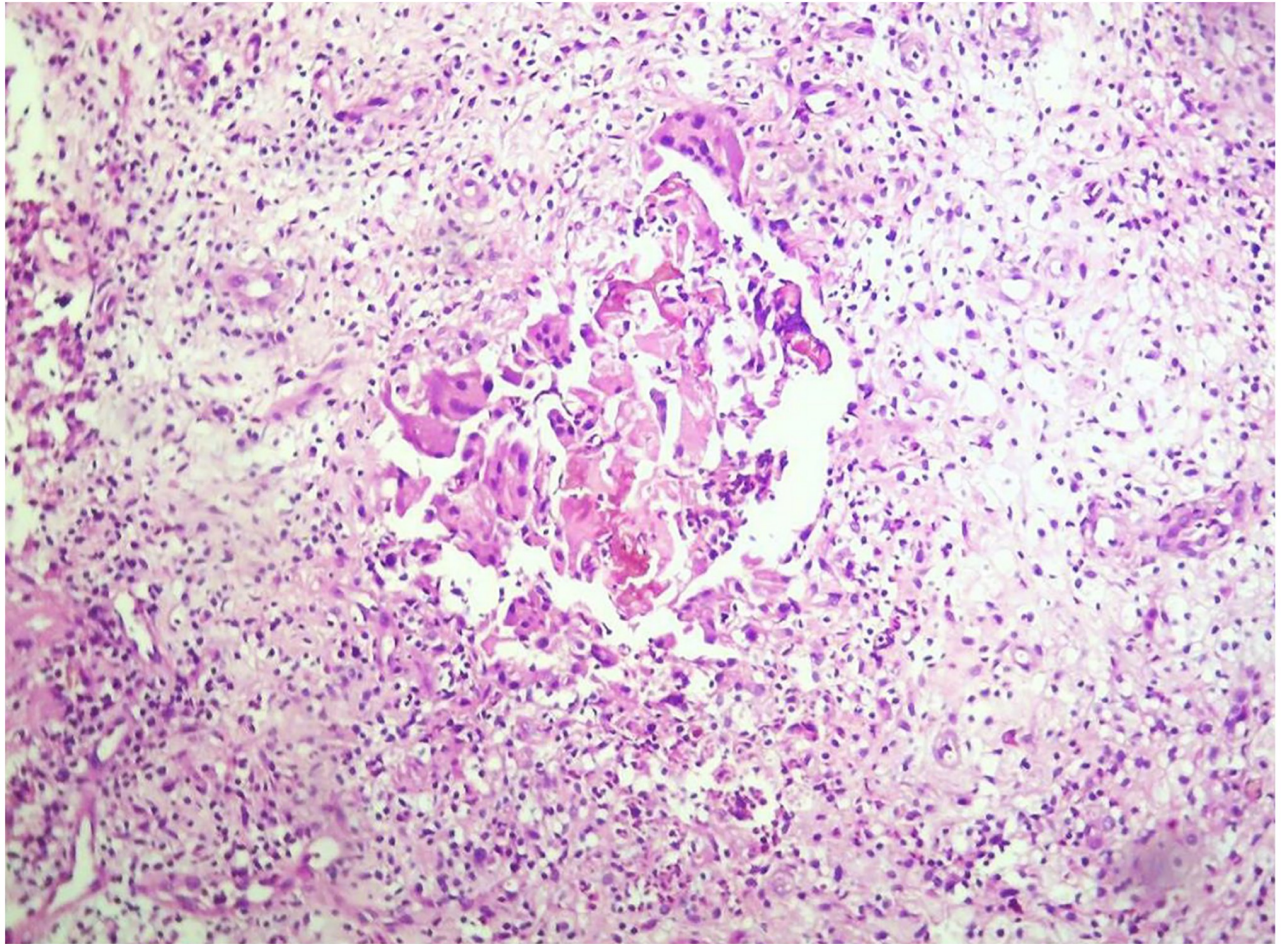
Based on the culture reports of the grains, in 750/991 (75.6%) of the patients the mycetoma was caused by *M. mycetomatis*, in 142/991 (14.4%) it was caused by *S. somaliensis*, in 71/991 (7.16%) it was caused by *A. madurae* and in 16/991 (1.6%) it was caused by *A. pelletieri*. In 11 patients no growth was reported from the grains obtained during the sample collection. The time to growth differed case by case and ranged between 5 and 28 days.

In this study, out of the 991 mycetoma cases, the correct species identification was obtained for 912 cases using histopathological examination. Using FNAC, the correct diagnosis was obtained in 708 cases. The histopathological examination confirmed the diagnosis of *M. mycetomatis* in 714 of 750 cases with 95.2% sensitivity, 95.4% specificity and diagnostic accuracy of 95.3%. For FNAC only 604 out of 750 *M. mycetomatis* cases were identified, resulting in a sensitivity of 80.5%, a specificity of 88.4% and a diagnostic accuracy of 82.4%.

Out of 142 *S. somaliensis* cases, 133 were also identified with histopathological examination with 93.7% sensitivity, 98.9% specificity and diagnostic accuracy of 98.2%. With FNAC only 50 out of 133 *S. somaliensis* cases were identified, resulting in a sensitivity of 35.2%, a specificity of 99.3% and a diagnostic accuracy of 90.1%.

53 out of 71 cases with *A. madurae* identification were identified by histopathological examination, with a sensitivity of 74.7%, 99.5% specificity and diagnostic accuracy of 97.7%. FNAC identified 43 out of 71 cases with a sensitivity of 60.6%, specificity of 94.4% and diagnostic accuracy of 91.9%.





**Fig 4. Histopathological section from patient diagnosed as *M. mycetomatis* based on the culture results; Histopathology report was been negative for fungus and when we retrieved the slide we can see adherence of histiocytes at the center with sparse amount of the grains (H and E, X 10).**

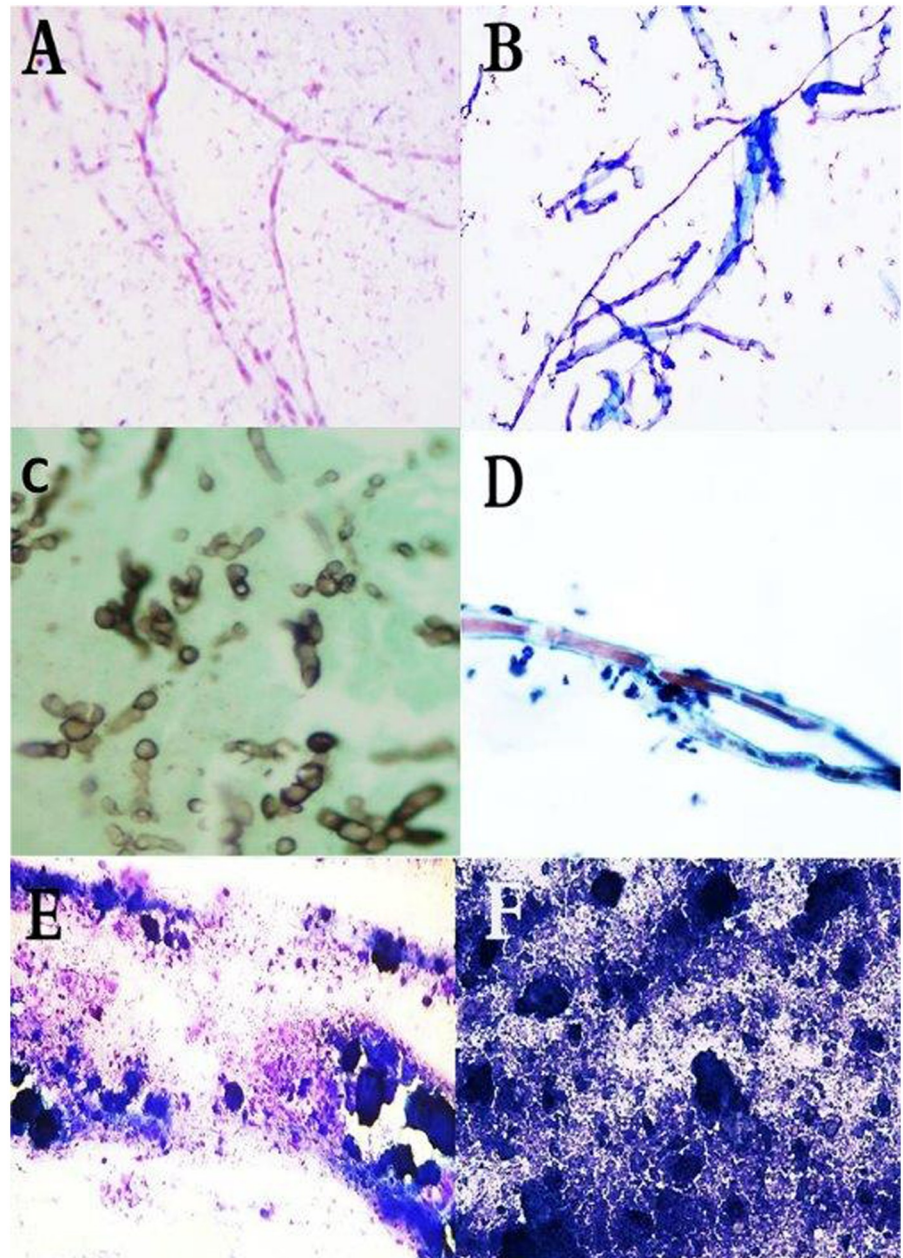
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For *A. pelletierii* out of 16 cases; 12 were also identified with histological examination with 75.0% sensitivity, 100% specificity, and diagnostic accuracy of 99.6%. For FNAC a sensitivity of 68.8%, a specificity of 99.7% and a diagnostic accuracy of 99.2% were obtained.

With the histopathological examination, false negative result was reported in 36/750 *M. mycetomatis* cases, 9/142 *S. somaliensis* cases, 18/71 *A. madurae* cases and 4/16 *A. pelletieri* cases. To determine the false negative results reasons, the histopathological slides were re-examined. There were various reasons for the false negative, and that included the absence of mycetoma histopathological architecture resulted in overlooking the causative agent (Fig 4).

Furthermore, in some blocks, the grains were absent; either because the tissue was not homogeneously infected by the causative agent and that the part which was taken for histology or the section contained no grains. This latter might be overcome by examining multiple sections at different depths of the histology blocks especially when inflammation and necrosis are noted.

False positive results were obtained in 28 of the cases. This was attributed to the presences of numerous structures that can mimic the appearance of *M. mycetomatis* and that included vegetables, synthetic fibres and algae which can resemble fungal hyphae and calcification (Fig 5).



**Fig 5. False positive identifications in cytology.** Showing cytological smears of (A) *M. mycetomatis* hyphae stained with Giemsa stain, (B) Synthetic Fibers stained Wright-Giemsa stain. (Giemsa stain, X40). (C) Smear showing hyphae of *M. mycetomatis*. (D) Smear showing elongated structures of the Oedogoniales order. The chloroplasts form a chain interrupted by clear zones (X40). (E) Smear showing *M. mycetomatis* grains after being crushed on the smear. (F) smear with abundant calcific debris without intact cells taken from patient with tumoral calcinosis (Diff Quick, X10).

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In overall, using histology correct species identification was obtained in the majority of cases. The mean time to identify the culture isolates was 16 days (range 5 to 28 days), for histology it was 3.5 days (range 2 to 5 days), and for cytology, it was one day (range 1 to 2 days). This demonstrated that reliable species identification using histology was obtained in 92.0% of cases within an average time reduction of 13.5 days, for cytology this was 71.4% of cases with

time reduction of 15 days, indicating that adding histology or cytology to the diagnostic techniques used for species identification resulted in an earlier start of treatment.

## Discussion

The accurate identification of mycetoma causative agents is considered the cornerstone for the initiation of appropriate therapy. Hence a rapid and accurate diagnostic tool to achieve the definitive species identification is considered a critical part in patient treatment and management [21–23]. Different laboratory techniques for species identification are in use, including culture, histopathology, [7, 25], FNAC [8, 24], serological assays and imaging [26–29] as well as different molecular diagnostic tools [30–35]. However, not all these assays are available in endemic regions. In the Mycetoma Research Centre, culturing of the grains, histopathology and FNAC are routinely performed and have been used for the past 27 years. In this communication we have used the data collected for the last 27 years to assess the sensitivity, specificity and diagnostic accuracy of histopathology and cytology in the identification of mycetoma causative agents in comparison to the current golden standard: culturing.

This study showed that the histopathology was more accurate to FNAC in terms of species identification. Our results are in line with that reported previously by Yousif and colleagues [36]. They reported 90.9% agreement when histopathology was compared to FNAC for the diagnosis of *M. mycetomatis* (90.9%) while for actinomycetoma causative agents it was only 60%. The lower diagnostic agreement of actinomycetoma causative agents could have been caused by morphological similarities of these microorganisms. Furthermore, both techniques are operator dependent and need intensive training and experience which could have its reflections on the accuracy.

Mycetoma can be caused by more than 70 different causative agents [37], but the distribution of these species is not everywhere the same which could cause differences in diagnostic accuracy in different regions. In some of the mycetoma endemic regions, mycetoma is caused by closely related species. Morphologically these organisms may look similar which could cause a challenge in the identification of these organisms. In Mexico, the most common causative agents are *Nocardia brasiliensis* and *Nocardia asteroides* [37], two closely related species which are difficult to differentiate from each other based on histopathology [38, 39]. In Senegal, the most common causative agents of eumycetoma are *M. mycetomatis* and *Falciformispora senegalensis* which both can cause black grain mycetoma [37, 40]. In the black grains of *F. senegalensis*, the centre is non-pigmented, and the cement is absent, whereas at the peripheries the grains are dark coloured and brown cement is present. However, this is also seen in black grains of *Trematosphaeria grisea* and certain grains of *M. mycetomatis*. Hence an expert pathologist is needed to differentiate between these organisms [41].

The study performed here was a retrospective study, looking back at the records of the Mycetoma Research Centre for the past 27 years. During that time molecular identification of the causative agents was not performed and culture was considered the golden standard. Recently in the study conducted by Borman and colleagues demonstrated that using morphological identification, misidentifications occurred in many cases [42]. Out of 28 previously identified *Trematosphaeria grisea* isolates, 22 were, other fungal species [42]. For actinomycetoma causative organisms, misidentifications also have been described. In 2008, Quintana and associates demonstrated that half of the *S. somaliensis* isolates obtained from Sudan appeared to be *Streptomyces sudanensis* [43]. Furthermore, next to *A. madurae* and *A. pelletieri* also *Actinomyces madurae* was described [44]. Therefore a current ongoing study is including molecular diagnosis to determine the true etiology and predictive value of culture, FNAC and histology.

With the introduction of molecular diagnosis in our centre we already made the first step in this respect.

In this study, the sensitivity of histopathological technique was superior to that FNAC for all species tested. In that study they studied the performance of FNAC in comparison to histology in 19 different mycetoma patients. Out of these 19 patients, five patients had to be excluded due to inadequate aspirated materials. From the 14 remaining patients, 10 were diagnosed as *M. mycetomatis* with histopathology, and 4 were actinomycetoma. With this limited number of patients they could conclude that FNAC could identify the causative agent in 9 out of 10 *M. mycetomatis* patients. One patient identified by histology could not be identified with cytology, again confirming that histology was superior to FNAC in respect to species identification [15]. A result confirmed in our current study, as in our study 146 patients with *M. mycetomatis* mycetoma were missed with FNAC. However, of the three different identification methods used, FNAC was the most rapid and resulted in species identification within 1 day, instead of 3.5 days for histology or 16 days for culture. FNAC is a simple and rapid diagnostic technique which can be used at the one-stop diagnosis clinic and in epidemiological and field surveys. However, it has many limitations: it is an operator dependent technique can be painful and can lead to deep-seated bacterial infections. FNAC is less invasive than a deep-seated biopsy, as only a small puncture hole is obtained. With a deep-seated biopsy a larger area of the lesion is removed thereby also exposing a larger part of the lesion to secondary bacterial infections and creating a bigger risk for dissemination of the infection. Currently, deep-seated biopsies are only performed to obtain a diagnostic sample, not to reduce the burden of infection at the site of the lesion. This, because the lesion can be extensive, even when on the outside only a small lesion is seen. At the moment the fine needle aspirate is often taken blindly without guidance of ultrasound imaging which creates a risk that the operator might miss the pockets which contains grains. With the use of the ultrasound-guided aspiration, the diagnostic yield of the technique will improve which in its turn could enhance the number of cases in which positive species identification might be obtained.

The grains culture remains in many centres the cornerstone for the diagnosis of mycetoma. moreover, morphological identification of mycetoma causatives agent may be some times be difficult to achieved due to the overlapping and similarities encountered between different species as demonstrated in Table 1, However culture is a time-consuming procedure and an experienced microbiologist is needed to identify the organisms to the species level [45]. Cross-contamination is a common problem.

Recently an identification scheme of eumycetoma causative agents has been published which was based on the physiological properties of the causative agent, indicating that it is possible to use physiological properties to identify eumycetoma causative agents more reliably [1]. These include culturing at 37°C and growth on actidione, L-sorbose, glycerol, potassium 2-keto-gluconate, methyl-D-glucopyranoside, inositol and D-sorbitol. However, these tests are more time consuming and delay the identification of the causative agent which will potentially lead to delay in patient treatment. However, by complementing culturing with histology or FNAC a preliminary identification might be obtained earlier. Especially, since the treatment of mycetoma infections is dependent on the causative agent. Actinomycetoma is treated differently than eumycetoma. [22].

Correct identification to the species level will influence clinical decision making. The first diagnostic discrimination needed is the distinction between actinomycetoma causative agents and eumycetoma causative agents, since this would implicate either antibacterial treatment or a treatment based on surgery and antifungal treatment. In this situation fine needle aspiration cytology and histopathological examination were found to be highly sensitive in the discrimination between actinomycetoma from eumycetoma based on the morphological

characteristics; therefore, and based on our experience we recommended the used of FNAC in a low resources centers like in the rural centers where there is no Histopathology laboratories.

For the actinomycetoma causative agents, it is currently not known if the choice of antibacterial agent is dependent on the causative agents and studies are needed to evaluate if different treatment regimens are needed for each of the different bacterial causative agents. When this appears to be the case, identification to the species level becomes essential. For eumycetoma causative agents, surgery is always combined with itraconazole. However, *Medicopsis romeroi* and *Madurella fahalii*, were not susceptible towards itraconazole *in vitro*. This would indicate that discrimination between these species and the susceptible species would be mandatory and could potentially effect clinical management. Clinical evaluation of such cases and the effect on the therapeutic success rates are needed. However, before such a study can be performed proper species identification needs to be obtained.

In summary, the Cytopathologist/Histopathologist need to be aware of the many mimics that can look like the mycetoma causatives agent, and we highly recommended that the pathologists after issuing the diagnosis should recommend correlation with microbiology or provide a cautionary statement to advise clinicians of the limitations of identifying organisms with histopathologic/cytopathologic examination.

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