Use of immunoblotting in testing *Madurella mycetomatis* specific antigen

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Background: Though serodiagnosis of actinomycetoma is established, that of eumycetoma due to *Madurella mycetomatis* is limited because of lack of pure antigen. Reliable rapid tests are needed to make an accurate timely diagnosis. The purpose of this study is to detect antigen parts of *M. mycetomatis*, which act specifically with *M. mycetomatis* antibodies.

Methods: Cytoplasmic antigen was prepared from molecularly identified cultures of *M. mycetomatis* by sonication, ultracentrifugation, dried, weighed and appropriately reconstituted. *M. mycetomatis* cytoplasmic antigen were separated using 12% sodium dodecyl sulfate-polyacrylamide gel, and immunoblotting to detect the reactive ones.

Immunoblotting was carried out in nitrocellulose strips containing different molecular size. Sera from patients and co-patients as control were used.

Results: When stained with Coomassie brilliant blue R 250 seven molecular weights appeared but only three, 45, 60, 95 kDa reacted with *M. mycetomatis* patients few from control group, one from a malaria patient. No reactive band was observed with sera from actinomycetoma, *Aspergillus flavus*-associated aspergillosis, schistosomiasis, leishmaniasis, fungal sinusitis nor healthy controls.

Conclusions: Specific fractions of *M. mycetomatis* antigen which were demonstrated by immunoblotting showed 75% sensitivity and 95% specificity. The true negative tests were 14 patients (32.5%). This also means that immunoblotting is reasonably reliable in diagnosis and follow-up of eumycetoma patients.

Keywords: Immunoblotting, *Madurella mycetomatis* specific antigen, Mycetoma

Introduction

Mycetoma is a common neglected tropical disease, reported globally; it is endemic in tropical and subtropical regions with several medical and socio-economic impacts.¹ Its nature is a chronic specific granulomatous subcutaneous inflammatory disease characterised by the triad of painless subcutaneous mass, multiple sinuses and sero-purulent or purulent discharge that frequently contain grains.² It is caused by fungi or bacteria and hence it is classified as eumycetoma or actinomycetoma respectively.^{3,4} For eumycetoma, the commonest causative organism is *Madurella mycetomatis* and for actinomycetoma the causative organisms are *Streptomyces somaliensis*, *Actinomadura madurae*, *A. pelletierii* and *Nocardia brasiliensis*.^{5–7}

Management of mycetoma depends on correct diagnosis of the causative organism, extent of the disease and its spread along the different tissue planes.^{8,9} Imaging, cytological,

histopathological, phenotypic, molecular, culture and serological diagnostic techniques are available to achieve that.^{10–16} However, these tools are tedious, invasive, expensive and may not be available in areas endemic for mycetoma.¹⁷

For culture of grains and histopathological examination deep surgical biopsy under general or regional anaesthesia is needed.¹⁸ Grains culture is time consuming and false interpretation may occur.¹⁹ For proper histological diagnosis, the biopsy should include plenty of grains and special stains may be needed. The chance of local disease spread along the different tissue planes during the surgical biopsy procedure is high.²⁰

It is important to identify the causative organism to the species level in planning the therapeutic modality. This is done by different phenotypic and molecular diagnostic techniques which are expensive and available only in few research centres.^{21,22} The use of serodiagnosis in mycetoma is limited due to the

cross reactivity between different causative organisms. Antigen preparation is tedious and time consuming.^{23,24} In addition *M. mycetomatis* has different structures in both the mycelial and grain phase, which have different antigenic determinants.⁷

Due to the limitations and shortcomings of the currently available sero-diagnostic tools for mycetoma, this study set out to prepare a specific antigen of *M. mycetomatis* to differentiate between patients and healthy people and to develop a reliable and rapid method for accurate and timely diagnosis of eumycetoma.

Materials and methods

Study population

The study included 100 patients with confirmed eumycetoma due to *M. mycetomatis*, seen at the Mycetoma Research Centre (MRC), University of Khartoum, Khartoum, Sudan. The patients were classified according to the duration of the disease into four groups: newly diagnosed patients, patients on treatment, patients with recurrent disease and cured patients. The control group not showing symptoms of mycetoma were selected from co-patients and those with other infections. They included patients with confirmed actinomycetoma (n=10), malaria (10), aspergillosis (10), leishmaniasis (10), schistosomiasis (10) and *Aspergillus flavus*-associated aspergillosis, nasal polyps or fungal sinusitis (10). The control group included also healthy individuals from mycetoma endemic areas (n=25), and from non endemic areas (25).

Five milliliters of blood was taken after informed written consent was obtained. Sera were separated and stored in small aliquots of 1 ml each and stored at -20°C until used.

M. mycetomatis strains

Two *M. mycetomatis* strains ID mm72 and mm83 were obtained from MRC culture collection and used in this study. They were collected from surgical biopsies taken from patients seen at the MRC. Strains were cultivated in 500 ml of mycological peptone liquid medium for 10 to 15 days.¹⁴ Typical growth curves of the fungi were obtained then identified by morphological appearance and brown pigment production in agar and confirmed by PCR and sequencing using the primers 5'-AGCGGATAACAATTT CACACAGGACACACTGGTATAGACTGCGTACCAAT-3' and 5'-GACGAT GAGTCTGAGTAA-3'.

Antigen preparation

The antigens were prepared by reaping at the log phase. Cytoplasmic antigen was obtained from fungus growth in mycological peptone water at 37°C for 10 d. Growth was scraped off and homogenized in Griffith tubes using normal saline. Suspended particles were broken further by an ultrasonic disintegrator. The sonicated material was collected in dialysis bags and dialyzed against distilled water for 24 h, changing the water twice. The dialyzed fluid was then brought down to about half of its volume using polyethylene glycol. The concentrated residue in the dialyzed bag was centrifuged at 50 000 g for 30 min and the supernatant collected as cytoplasmic antigen, protein

was measured using Nano-drop and distributed aseptically in 2 ml quantities in lyophilisation tubes. They were freeze-dried and kept at -20°C . The dried material was used as antigen after reconstitution in sterile distilled water, to the concentration of 40 mg/ml.¹⁵⁻¹⁷

PAGE analysis of the immunodominant antigens

The proteins analytical electrophoresis was carried out in acrylamide gel as described by Laemmli.¹⁸ Proteins were dissociated into their individual polypeptide subunits. The cytoplasmic antigen sample was mixed with loading buffer as to have 200 $\mu\text{g}/\text{ml}$ a final concentration plus 10 μl of 2mercapto – ethanol (2ME) per gel and heated to dissociate the proteins before they were loaded on the gel.^{19,20}

Individual slots in the same gel were used to electrophorese the high and low molecular weight protein standards. The gel contains 8 to 18% gradient resolving gel and a 5% stacking gel. Electrophoresis was run at 80 V for 2 hours in SDS running buffer and the gels were stained with Coomassie blue R-250.¹⁸ The molecular weight standards included a mixture of purified proteins covalently coupled to a blue and orange dye that resolved to 10 bands between 7 and 240 kDa when electrophoresed.

Immunoblotting identification of the immunodominant antigens

Further characterisation of the *Madurella mycetomatis* cytoplasmic antigens isolated was carried out by an immunoblotting assay. In brief, after electrophoresis was completed, the proteins were transferred to nitrocellulose membranes (PROTRAN BA 85, 0.45-, μm -pore size) with a Trans-blot cell (Bio-Rad) for 60 min at 100 V; 25 mM Tris-192 mM glycine, (pH 8.3) buffer and 20% methanol were used as described by Towbin.¹⁹ The nitrocellulose strips were then blocked with 2% fish gelatin in pH 7.2 PBS for 60 min at 37°C .

Sera from patients and controls were diluted 1:25 with 1% gelatin in PBS-Tween 20 (1:1000) and individually incubated with the nitrocellulose strips with gentle agitation for 2 h. After five washes, an anti-human immunoglobulin G-(H+L) horseradish peroxidase conjugate (invetrogen) diluted 1:1000 was added. After five washes, the freshly prepared substrate solution containing 4-chloro- α -naphthol was reconstituted with 5 ml methanol and 100 μl of H_2O_2 , added to 25 ml PBS, then added with gentle agitation for 10 to 15 min. The reaction was stopped by adding tap water after observation of bands with suitable color intensity, then the strips were air dried to avoid the fading of color; the strips were sealed under a plastic sheet and stored in the dark. Plate band recognition frequencies and patterns were calculated. Sera that showed one or more of the diagnosis bands were considered positive.^{21,22}

Statistical analysis

The crosstabs χ^2 test was used to determine the Western blot test sensitivity and specificity, and $p < 0.1$ was considered statistically significant.

The sensitivity and specificity of the Western blot tested by this equation:

sensitivity = $\frac{a}{a + c}$

specificity = $\frac{d}{b + d}$

(a: true positive, b: false positive, c: false negative, d: true positive).

Results

A total of seven bands were present in the two lyophilised *M. mycetomatis* cytoplasmic antigens when stained with Coomassie brilliant blue R 250 staining on 12% polyacrylamide gels.

Five bands at molecular masses of 15, 32, 45, 60, and 95 kDa were consistently more heavily stained than the other bands; antigen prepared from stir culture and from solid culture showed the same bands (Figure 1).

Nitrocellulose paper strips containing antigen were tested against 20 control sera (10 positive and 10 negative) to evaluate the validity of the test.

Figure 1. SDS-PAGE and Coomassie brilliant blue R 250 staining patterns of *M. mycetomatis* cytoplasmic antigen bands from (A) mm1 broth media; (B) mm2 broth media; (C) mm1 cytoplasmic antigens prepared from broth and solid culture; (D) mm2 cytoplasmic antigens prepared from broth and solid culture. L is a molecular weight standard (iNtRON Biotechnology). This figure is available in black and white in print and in color at Transactions online.

Table 1. The reactivity of the different patients groups with antigens using the Western blot test

Patient groups	n	Positive		Negative	
		n	%	n	%
New cases	12	7	58	5	42
On follow-up	41	22	54	19	46
Recurrent	29	24	83	5	17
Cured	18	4	22	14	78
Total	100	57	57	43	43

The reactions of 195 different sera tested by immunoblotting using the purified antigens are shown in Table 1. Out of the 100 sera from patients with eumycetoma caused by *M. mycetomatis*, 57 sera reacted with the molecular weight 45, 60 and 95 kDa for both strains (Table 1).

Only eight sera (24%) from the control groups reacted with 45 kDa and 60 kDa. One serum from a patient with malaria, sera from two healthy control reacted with the 45 kDa antigen while six sera from healthy persons from endemic areas had reacted with the 45 and 60 kDa antigen (Figure 2 and Table 2).

The sensitivity of the Western blot test for eumycetoma diagnosis was 75%, and its specificity was 95%. The p value was significant (0.24).

Figure 2. Immunoblot analysis showing the patients and control groups sera reactivity. A: Actinomycetoma; As: aspergillosis; C: control negative; E: endemic area people; L: molecular weight standards mark; M: malaria; MM: Eumycetoma patients. This figure is available in black and white in print and in color at Transactions online.

Table 2. Reactivity of patients and control groups sera to 45, 60 and 95 kDa molecular weight of antigen using Western blot test

Groups	Sera no.	Positive sera with 45 kDa	Positive sera with 60 kDa	Positive sera with 95 kDa
Eumycetoma	100	57	57	57
Endemic area healthy persons	25	6	4	0
Malaria	10	1	0	0
Actinomycetoma	10	1	0	0
Aspreglosis	10	0	0	0
Schistosomiasi	10	0	0	0
Leishmaniasis	10	0	0	0
Non endemic area healthy persons	25	0	0	0

Discussion

More than 50 micro-organisms were reported to cause mycetoma and hence the clinical presentations, response to different treatment modalities and prognosis differ in mycetoma. The diagnosis of the causative organism to the species level is mandatory to recommend the appropriate treatment. The current diagnostic tools for mycetoma, culture and histopathology, are invasive, time consuming and not field friendly. Serodiagnosis though proved reliable in diagnosis of actinomycetoma and monitoring patients in this group, yet they are of low sensitivity, and specificity. Thus there is a pressing necessity to develop novel, simple, rapid and economical diagnostic tools for the diagnosis of mycetoma and to monitor the therapeutic outcome.

Counter immuno-electrophoresis (CIE), immunodiffusion (ID) and ELISA tests were developed to detect antibodies against different causative agents, using crude culture extracts or cytoplasmic antigen. In the past, ID was a popular test to diagnose mycetoma, however, CIE was found to be quicker and more sensitive than ID.^{4,5,16} The ELISA used by McLaren and colleagues⁵ Taha⁶ and Salinas-Carmona²⁸ showed that both mycetoma patients and healthy controls from the endemic areas had antibodies against mycetoma agents. This reduced the specificity of ELISA and hence its suitability for diagnostic purposes.

It is clear that these serodignostic tests have many limitations which include tedious and lengthy antigens preparation, the antigens are crude and not standardized and cross reactivity between different mycetoma causative organisms.^{4,5} Cytoplasmic antigens were used previously by Mahgoub and Gumaa in serodiagnosis of both actinomycetoma and eumycetoma, but they showed broad cross reactivity between different organisms.^{4,5,26} Wethered reported on extra cellular antigenic material (MCFA) derived from the culture filtrate of *M. mycetomatis* in vitro and suggested using it to replace the cytoplasmic antigens.¹⁶ This study by Wethered and colleagues focused on detecting immunoglobulins and determining specific antibodies where IgM was found to be the dominant immunoglobulin. While the strains used were selected on morphological basis, in this study strains were selected on molecular criteria. The proteins in the previous study ranged from 31 to 97 while in this study they ranged from 15 to 100 kDa. However the most antigenic size was 95 kDa which is not different from the previous study. The basic difference between these two studies is that the former concentrated on using immunoblotting to identify antibodies while the latter concentrated on identifying a specific and sensitive antigen.

A recombinant antigen based sero-diagnostic test would be the preferred one. Currently only a single recombinant antigen has been prepared for *M. mycetomatis* and that is the translationally controlled tumor protein (TCTP). It was found mainly at the cell surface of the fungus and primarily in the developing part of the grain. TCTP has two variants in the species *M. mycetomatis*, however, it was not useful as a diagnostic tool as not all patients produced antibodies against it and in contrast some of the healthy controls did.²⁷ Also this antigen is known to be bound to some anti-malarial and anti-cancer drugs.^{8,9}

A purified antigen was prepared using immunoblotting for the diagnosis of nocardiosis with a specific immunodominant antibody and it was found to be useful.¹¹ This method was also used for diagnosis of chromoblastomycosis, and characterisation of *Fonsecaea pedrosoi* antigens.^{12,13}

In the present study, three molecular weight bands reacted with sera of patients with eumycetoma with limited cross reaction which increases the specificity of this antigen. In addition the positive results increased in the patients with recurrent disease than with new patients and those on treatment which also increased the test specificity.

Cured patients had increased negative results, which makes the test more reliable for patients follow-up and monitoring of treatment. It is interesting to note, patients on treatment had variation in the test results. This can be explained by differences in patients' responses to treatment and by differences in the expression of antigen by the infecting strains of *M. mycetomatis*. Also low or undetectable antibody responses could be the result of saturation of specific antibodies by excess mycetoma fungal antigens as demonstrated before by Wethered and colleague.¹⁷ It may be concluded that not all negative results indicate a disease free state and confirmation by other tests is needed to ensure the negativity of results.

Some normal individuals from mycetoma endemic areas had positive results: the explanation of that is multifactorial. Such individuals may have been exposed to *M. mycetomatis* antigens and had developed a specific humoral immune response but not disease, some had subclinical infection or had an early mycetoma infection.¹¹ Another explanation is cross reactivity with *M. mycetomatis* specific antigen from other endemic infectious organisms as some reports showed the association between mycetoma and schistosomiasis.²⁷

The main advantage of this test is that potentially it may lead to earlier diagnosis. This is critical because mycetoma is painless and develops over a long period both of which means that diagnosis is often missed until late in the evolution of the infection. In most patients with this it is possible to detect a positive serological reaction at early stages and in addition it may play a role in monitoring treatment. The negative results still require explanation but early diagnosis is the key to early institution of treatment and better results.

Conclusions

The Western blot technique showed a high degree of specificity and sensitivity in the diagnosis of mycetoma. A 95 kDa seemed to be the more specific part in the cytoplasmic *M. mycetomatis* antigen as it showed reaction with eumycetoma patients' sera only. The two other bands, 60 and 45 kDa, showed reactions with eumycetoma patients' sera and had limited cross reaction with sera from patients with no evidence of mycetoma, which reduces their sensitivity and specificity.

Extraction of specific protein fraction (26 and 24 kDa) from crude *N. brasiliensis* which was used in ELISA has been reported by Salinas-Carmona.²⁸ ELISA helped in the routine clinical laboratory confirmation of *N. brasiliensis* infection and used to monitor response to treatment. The purified antigen prepared in this study, if prepared on commercial scale, can be used in other serological test such as ELISA and CIE. The immunoblotting

technique is reasonably reliable in diagnosis and follow-up of patients with eumycetoma due to *M. mycetomatis*.

Further research is needed to determine methods to prepare protein 95 kDa in commercial amounts.

Author's contributions: HE, EM, AF conceived the idea; AF, EM took care of patients; HE and NM did the laboratory work; HE, EM wrote the first draft; all authors revised the paper. All authors read and approved the final manuscript. HE, EM are guarantors of the paper.

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