Eumycetoma, a chronic granulomatous disease characterized by a subcutaneous mass, multiple sinuses and purulent discharge containing grains, remains difficult to diagnose and treat. Madurella mycetomatis is the most common causative agent of eumycetoma. Using a serum pool from patients with active mycetoma, we screened a M. mycetomatis-specific λgt11 cDNA library which was shown to contain 8% of cDNA inserts encoding proteins involved in glycolysis. Two of these enzymes, fructose-bisphosphate aldolase (FBA) and pyruvate kinase (PK), were produced in vitro and their antigenicity was studied with bead-based flow cytometry. It appeared that both FBA and PK IgG antibodies were present in eumycetoma patient sera. However, only FBA antibody levels were found to be significantly higher in eumycetoma patient sera when compared to healthy Sudanese controls. Furthermore, FBA and PK were also found to be expressed on the hyphae present in the mycetoma grain. In conclusion, this study presents two new antigenic proteins of M. mycetomatis next to the translationally controlled tumour protein (TCTP): the glycolytic enzymes FBA and PK. These antigens might be useful as vaccine-candidates in the prevention of mycetoma.

Keywords  mycetoma, antigen, glycolysis

Introduction

Madurella mycetomatis is the most common causative organism of eumycetoma in man. Eumycetoma is a chronic granulomatous disease, characterized by a subcutaneous mass, multiple sinuses and purulent discharge containing grains. The infection is commonly found in the mycetoma belt, between latitudes of 15°S and 30°N of the equator. Countries within this region include Sudan, Somalia, Nigeria, Ethiopia, India, Mexico, Columbia and Argentina. Although multiple microorganisms are etiologic agents of mycetoma, in Sudan 70% of the mycetomas are caused by M. mycetomatis [1].

While everybody is exposed to M. mycetomatis in the endemic area, not everyone develops mycetoma. Patients are usually males between 20 and 40 years of age who work in the fields. Since these are the individuals who provide support to their families, the disease has severe socio-economic impacts [1,2]. The consequences are worsened by the tendency of patients to present late because of poor access to healthcare facilities in endemic areas and their fear of amputation. The most successful outcome of the disease is attained by a combination of early diagnosis and proper treatment consisting of surgery and prolonged antifungal therapy with either itraconazole or ketoconazole [3,4]. Presently, recurrence rates are high.

The presence of a subcutaneous mass, sinuses, and grains are diagnostic for mycetoma. In order to provide proper treatment, it is important to identify the causative agent. At the moment, identification is difficult and relies on histopathologic examination of material from surgical biopsies and by culture of portions of the same biopsy tissue [3,4]. A PCR test has been developed to identify M. mycetomatis, but is not appropriate for bedside use [5].

Serological testing could be useful, but is currently underexplored. Since it is known that eumycetoma patients develop antibodies against M. mycetomatis [6], several serodiagnostic techniques were devised. However, due to the use of non-standardized and poorly prepared crude
antigens, cross reactivity and false positive and negative results hamper the diagnostic value of these tests [7]. It was only recently that the first immunoreactive protein, the translationally controlled tumour protein (TCTP), and its used in an ELISA test for translationally controlled tumour protein (TCTP), and its groups according to lesion size, i.e., small (<5 cm), moderate (5–10 cm) and large (>10 cm), with 20 patients in each group [8,9]. The use of lesion size as the basis for creating the groups was made to determine if the extent of the lesion influenced the development of antibody levels. As controls, sera of 31 healthy Sudanese males from the same endemic region, 20 actinomycetoma patients and 20 healthy Dutch controls were used.

**Materials and methods**

**Sera**

Sera were collected from 60 male patients seen between 2007 and 2008 in the Mycetoma Research centre in Khartoum, Sudan. The patients were divided into three groups according to lesion size, i.e., small (<5 cm), moderate (5–10 cm) and large (>10 cm), with 20 patients in each group [8,9]. The use of lesion size as the basis for creating the groups was made to determine if the extent of the lesion influenced the development of antibody levels. As controls, sera of 31 healthy Sudanese males from the same endemic region, 20 actinomycetoma patients and 20 healthy Dutch controls were used.

**Construction of the cDNA expression library**

The *M. mycetomatis* mm55 strain was cultured for 4 weeks on Sabouraud dextrose agar (Difco Laboratories) at 37°C. The colony was excised from the agar, frozen in liquid nitrogen, and ground in a porcelain mortar. The resulting pulp was kept frozen in liquid nitrogen until the RLT buffer in the RNeasy Maxi system (Qiagen) was added. The sample was then incubated for 3 min at 56°C to fully disrupt the cell walls. Total RNA was isolated using the bacterial protocol of the RNeasy Maxi system (Qiagen). After the recovery of RNA, traces of DNA were removed with DNase I (Ambion) according to the manufacturer’s indications. Poly(A)+ mRNA was purified by binding it to Oligotex particles using the Oligotex mRNA spin protocol (Qiagen). The purified poly(A)+ mRNA was used to synthesize double-stranded cDNA with the Universal RiboClone cDNA Synthesis System (Promega Benelux BV). Finally, the double-stranded cDNA was ligated into the *AgtI/EcoRI* treated vector and packaged using the Gigapack III Gold-11 packaging extract (Stratagene Europe).

**Screening the cDNA expression library**

To determine the cDNA insert nucleotide sequence of 64 individual phages, purified phages were boiled for 10 min and 5 μl of this crude extract was amplified in a 50 μl reaction volume containing 1x Supertaq PCR buffer 1 (HT Biotechnocondury Ltd., Cambridge, UK), 0.2 mM PCR nucleotide mix (Amersham Life Sciences, Roosendaal, The Netherlands), 25 pmol forward primer (5’-GGTGCGGACGACTCTGAGGACCGC-3’), 25 pmol reverse primer (5’-TTGACCCAGACCACTGGTAAATG-3’), and 1.2 U Supertaq (HT Biotechnocondury Ltd.). The PCR consisted of a predenaturation step of 4 min at 94°C and 35 cycles, each of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of elongation at 72°C. This was followed by a postelongation step of 4 min at 72°C. PCR products were sequenced (BaseClear), and their insert sequences were compared with other sequences in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov/BLAST).

**Construction of the expression vector**

FBA and PK were discovered in a random screening of the *M. mycetomatis* cDNA expression library described above. In order to synthesize recombinant proteins, the corresponding cDNA sequences were cloned into vector pENTR/D-TOPO (Invitrogen, Breda, The Netherlands) according to the manufacturer’s instructions. The sequences were then transferred into the destination vector pDEST (Invitrogen) which was transformed into *Escherichia coli* BL21-AI according to the manufacturer’s instructions. The insert sequence was verified via sequencing.

**Expression and purification of the recombinant protein**

To express recombinant fructose bisphosphat e aldolase (MmFBA), pyruvate kinase (MmPK) and translationally controlled tumour protein (MmTCTP), a culture containing *E. coli* BL21-AI containing the recombinant plasmid was grown to an OD₆₀₀ of 0.25. Upon reaching OD₆₀₀ of 0.6, expression was induced by addition of isopropyl β-D-thiogalactoside (Fermentas, St. Leon-Rot, Germany) and L-arabinose (Sigma, Zwijndrecht, The Netherlands) to a final concentration of 1 mM and 0.2% w/v, respectively. The cells were pelleted after the OD₆₀₀ reached 1.0. The histidine-tagged proteins were purified using metal affinity chromatography (Ni-NTA technology; Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. The eluate was dialyzed against 2 M, 1 M, 0.5 M, 0.25 M urea in PBS and, finally, against PBS. The proteins were analysed for purity on a 12% SDS-PAGE gel stained with PageBlue Protein Staining Solution (Fermentas).

**Antibody production**

Polyclonal antibodies against FBA and PK were raised in rabbits by Eurogentec. In short, two rabbits were immunized...
by injecting 100 μg of either recombinant FBA or recombinant PK in Freund’s Adjuvant, intradermal, and after 2, 4, and 8 weeks the immunization was boosted using 100 μg protein. Serum was taken before immunization and then 1 and 3 month after immunization.

**Bead-based flow cytometry**

To test the antigenicity of the *M. mycetomatis* proteins, the presence of antibodies against those proteins in patients was analyzed using bead-based flow cytometry (Luminex technology). The coupling of the purified proteins to SeroMAP beads (Luminex Corporation, Austin, TX, USA) and the multiplex antibody assays were performed according to the procedure described elsewhere [10]. In short, 25 μg of protein was added to 5.0 × 10^6 beads. The activation buffer consisted of 100 mM monobasic sodium phosphate (pH 6.2). To activate the carboxyl groups on the surface of the beads, 10 μl of 50 mg/ml N-hydroxy-sulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Pierce Biotechnology, Rockford, IL, USA) was used. As coupling buffer consisted of 50 mM 2-(N-morpholino)-ethanesulfonic acid, pH5.0 (MES) (Sigma) was employed in the studies. The final concentration of beads was adjusted to 3000 beads/μl with blocking-storage buffer. The microspheres were protected from light and stored at 4°C until further use. To determine non-specific binding, beads coated with bovine serum albumine (BSA) were included in the experiment. In case of non-specific binding, such median fluorescence intensity (MFI) values were subtracted from the antigen-specific results.

The different antigen-coated beads were mixed to a working concentration of 3000 beads per colour per reaction. Fifty microliters per diluted serum sample (1:100 in PBS-BN (PBS, 1% BSA, and 0.05% sodium azide pH 7.4)) were incubated with the beads in a 96-well filter microtiter plate (Millipore Corporation, Billerica, MA, USA) for 35 min at room temperature on a thermomixer plate shaker (Eppendorf, Hamburg, Germany). The plate was washed twice with PBS-BN that was aspirated using a vacuum manifold. The beads were resuspended in 50 μl PBS-BN and 50 μl of a 1:25 diluted R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-human IgG antibody (Jackson Immuno Resarch, Suffolk, UK). The plate was incubated on a plate shaker for 30 min at room temperature, washed and finally all samples were resuspended in 100 μl PBS-BN. Measurements were performed on a Luminex 100 instrument (BMD, Croissy Beaubourg, France) using Luminex IS 2.2 Software. The tests were performed in duplicate and MFI values, reflecting relative antibody levels, were averaged.

**Immunohistochemistry**

Grains obtained from three *M. mycetomatis* mycetoma patients were embedded in paraffin to prepare slides for histologic studies. The slides were rehydrated in PBS and boiled for 10 min in 10 mM sodium citrate (sigma). After 30 min incubation at room temperature, the slides were washed in 0.05% PBS-tween 20 and incubated for 30 min in a blocking solution (2% BSA, 5% sucrose in PBS). The primary antibody was diluted 1:100 in blocking solution and incubated for 30 min at room temperature. As primary antibodies, rabbit sera obtained six weeks after immunization against either recombinant *M. mycetomatis* FBA or recombinant *M. mycetomatis* PK were used. As negative control, preimmune sera from the same animals were employed under the same conditions. After washing, the slides were incubated with diluted goat anti-rabbit IgG HRP-conjugated Ab (1/50; Dako). The substrate 3-amino-9-ethyl-carbazole (AEC, Sigma) was used as the primary stain, and hematoxylin (Sigma) was used as counterstain.

**Statistical analysis**

IgG levels raised against the MmTCTP, MmFBA and MmPK antigens were compared between study populations by the Mann-Whitney U test (GraphPad Instat 3.00). A value of *P* < 0.05 was considered significant. The Kruskal-Wallis test (SPSS Inc 17) was used to test if there were significantly elevated antibody levels in patients with larger lesions compared to patients with smaller lesions, by including size (small, moderate, large) as the grouping variable.

**Results**

**Glycolytic enzymes of *M. mycetomatis***

In search for antigens of *M. mycetomatis*, a cDNA expression library in phage λgt11 was constructed. This library consisted of 6.0 × 10^4 independent clones with an average insert length of 1500 bp. After randomly sequencing 64 phages it appeared that the inserts were of a diverse nature (Table 1). Many of the inserts encoded either for proteins involved in translation and transcription (23%) or metabolism (19%). It appeared that five of these phages encoded for single open reading frames (ORF) which showed significant homology with proteins involved in glycolysis (Table 1). Based on similarity searches in GenBank it was concluded that three of these phages encoded FBA, and the other phages encoded for PK. For FBA, the highest degree of homology was observed with the FBA gene of the fungus *Verticillium albo-atrum* (GenBank accession number XP_003000775), with 78%
Table 1  Nature of cDNA the inserts in 64 randomly selected phages from the λgt11 library.

<table>
<thead>
<tr>
<th>Nature of insert</th>
<th>Number of inserts</th>
<th>Percentage</th>
</tr>
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<tbody>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycolysis</td>
<td>5</td>
<td>7.8</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>4</td>
<td>6.3</td>
</tr>
<tr>
<td>Secondary metabolism</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>3.1</td>
</tr>
<tr>
<td>Transcription and translation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosome-associated</td>
<td>5</td>
<td>7.8</td>
</tr>
<tr>
<td>Histone-associated</td>
<td>3</td>
<td>4.7</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>10.9</td>
</tr>
<tr>
<td>Signal-transduction</td>
<td>5</td>
<td>7.8</td>
</tr>
<tr>
<td>Transport</td>
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</tr>
<tr>
<td>Transporter channels</td>
<td>6</td>
<td>9.3</td>
</tr>
<tr>
<td>Carriers</td>
<td>3</td>
<td>4.7</td>
</tr>
<tr>
<td>Cell wall associated</td>
<td>2</td>
<td>3.1</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>21</td>
<td>32.8</td>
</tr>
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</table>

The number of phages which can be classified in the given categories is presented together with the percentage.

amino acid identity and 88% amino acid similarity. For PK the highest degree of homology was observed with the PK gene of the fungus Chaetomium globosum (GenBank accession number XP_001227720), with 93% amino acid identity and 95% amino acid similarity (Fig. 1). At the protein level, M. mycetomatis glycolysis proteins were less closely related to their human variants, with no significant homology for FBA, and only 52% identity and 66% similarity for PK (Fig. 1).

Since glycolysis proteins are considered essential for fungal survival it was assumed that all M. mycetomatis isolates possessed these genes. In order to verify this, we screened 12 isolates for their presence. After sequencing it appeared that, all strains possessed homologous sequences and without any sequence variation (data not shown).

According to the PROSITE pattern database (http://www.expasy.ch/prosite/entry) there are two signature sequences known for class II FBAs. After comparing the MmFBA amino acid sequence with the database entries it was noticed that the amino acid positions 102-113 in the MmFBA sequence were identical to the FBA class II signature I [FVVM]-x(L3)-[LIVMH]-[APNT]-[LIVM]-x(1,2)-[LIVM]-H-x-D-H-[GACH] and that the amino acid positions 173-184 were identical to signature II [LIVM]-E-x-E-[LIVM]-G-x(2)-[GM]-[GSTA]-x-E. The MmPK amino acid sequence possessed the pyruvate kinase active site signature [LIVAC]-x-[LIVM]-[LIVM]-[SAPCV]-K-[LIV]-E-[NKRST]-x-[DEQHS]-[GSTA]-[LIVM] on amino acid positions 9-21.

Since we obtained complete cDNA sequence for FBA and the second half of the PK gene (when compared to the PK amino acid sequence of P. anserina, the first 249 amino acids were missing, while the last 279 amino acids were present) we used these sequences to express and purify the recombinant gene products as histidine-tagged fusion proteins. SDS-PAGE gel analysis of the purified recombinant protein with the histidine tag revealed one clear band for each protein, with molecular weights 40 kDa and 31 kDa for FBA and PK, respectively (data not shown).

Association between lesion size, duration of the disease and antibody levels

To test the antigenicity of the FBA and PK in humans, the recombinant proteins were coupled to luminex beads. The IgG levels against these proteins were measured in M. mycetomatis infected patients, actinomycetoma patients and healthy controls. Beads without protein coupled to them, but blocked with BSA served as a negative control. In Fig. 2 IgG levels against TCTP (as a positive control), FBA and PK are summarized. Clear IgG responses were detected against TCTP, FBA and PK. Overall, it appeared that the antibody levels in eumycetoma patients are higher than those in actinomycetoma patients or healthy Sudanese controls. This difference was only significant for FBA (Fig. 2: Mann-Whitney, \( P = 0.0025 \)). The IgG levels in actinomycetoma patients were similar to the levels in healthy individuals. All eumycetoma patients had significantly higher IgG levels against all three M. mycetomatis proteins measured compared to the healthy Dutch controls (Mann-Whitney, \( P < 0.0001 \)). Moreover, even the Sudanese healthy controls had significantly higher IgG levels against TCTP, FBA, and PK than the Dutch healthy controls (Mann-Whitney, \( P < 0.0001, P = 0.0161 \) and \( P = 0.0028 \), respectively). As is shown in Fig. 3, it was also noted that the patients with massive eumycetoma lesions had the highest MFI values than those with smaller lesions although this difference was not significant (Kruskal-Wallis, \( P = 0.34 \) for TCTP, \( P = 0.54 \) for FBA, \( P = 0.16 \) for PK).

FBA and PK are expressed in the fungal grain

From the presented results, it appeared that FBA and PK were antigenic in the patient population. Therefore we assessed whether these proteins were expressed locally in the grain or in the surrounding tissue in patients infected with M. mycetomatis. For this purpose immune serum from rabbits immunized with either FBA or PK was employed (see Fig. 4). The grains in the human tissues are typically filamentous and are surrounded by three zones of inflammatory cells, a type I tissue reaction as described by Fahal and El Hassan [2,11,12]. The zone directly around the grain was composed mainly of neutrophils (Fig. 4A) which was followed by a region of histiocytes and an outer vascular zone at the perimeter. The fungal cells in the grain itself are mostly embedded in brown-pigmented cement material (Fig. 4B). As is seen in Figs. 4C and 4D, FBA and PK were detected against TCTP, FBA and PK. Overall, it appeared that the antibody levels in eumycetoma patients are higher than those in actinomycetoma patients or healthy Sudanese controls. This difference was only significant for FBA (Fig. 2: Mann-Whitney, \( P = 0.0025 \)). The IgG levels in actinomycetoma patients were similar to the levels in healthy individuals. All eumycetoma patients had significantly higher IgG levels against all three M. mycetomatis proteins measured compared to the healthy Dutch controls (Mann-Whitney, \( P < 0.0001 \)). Moreover, even the Sudanese healthy controls had significantly higher IgG levels against TCTP, FBA, and PK than the Dutch healthy controls (Mann-Whitney, \( P < 0.0001, P = 0.0161 \) and \( P = 0.0028 \), respectively). As is shown in Fig. 3, it was also noted that the patients with massive eumycetoma lesions had the highest MFI values than those with smaller lesions although this difference was not significant (Kruskal-Wallis, \( P = 0.34 \) for TCTP, \( P = 0.54 \) for FBA, \( P = 0.16 \) for PK).

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PK expression were mainly found in the hyphae embedded in the cement material of the fungal grains. Neither FBA nor PK expression were observed outside the grain. No colouration of the hyphae was noted when the slides were stained with pre-immune sera (Fig. 4B).

Discussion

The diagnosis and treatment of *M. mycetomatis* mycetoma are difficult as the diagnostic tools and antifungal agents available at the moment are inadequate. Increasing our knowledge of the fungus and especially of the host-pathogen interactions could lead to new options for treatment and diagnosis. By randomly screening our expression library it appeared that 8% of the phages screened possessed inserts which encoded for proteins involved in glycolysis. This is not surprising since mRNAs encoding glycolytic enzymes can constitute up to 13% of the total mRNAs present in *Saccharomyces cerevisiae* [13]. Next to being abundant in the fungal cell, glycolytic enzymes also appear to be highly immunoreactive [14]. Therefore, we tested in this study two glycolytic proteins from *M. mycetomatis* for their antigenicity. Two proteins, FBA and PK, appeared to be able to induce an antibody response in rabbits and humans.

Antigenicity to glycolytic proteins can be expected based on the strong expression of these proteins in the cell, and the fact that multiple glycolytic proteins, including PK and GAPDH, have recently been found in the cell wall of various pathogens [15–19]. Furthermore, it has also been shown in *S. cerevisiae* that the glycolytic protein GAPDH is secreted when the cell wall is regenerating [19]. Some of these proteins have alternative functions in the cell wall. For instance, GAPDH also binds lysozyme, fibronectin, laminin, actin and myosin [17,20]. The presence of these enzymes in the cell wall suggests that the antigenic determinants are naturally exposed and, therefore, accessible to the host immune system during infection. This is demonstrated by the immunohistochemical detection of MmPK, MmFBA and CaGAPDH in human tissue [16]. Based on these observations, it is not surprising that next to MmPK and MmFBA, glycolytic proteins of *Aspergillus* *fumigatus*, *Candida albicans*, *S. pneumoniae*, *Schistosoma japonicum* and *Schistosoma mansoni* have been proven to be antigenic [21–25].

In this study, we showed that not all patients generated antibodies against FBA and PK, which is not unusual in cases of fungal mycetoma. In addition, differences have been found in patients with systemic candidiasis differences in the recognition of CaFBA, CaGAPDH and CaPK.
Fig. 2  Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG for recombinant Madurella mycetomatis his-tagged TCTP, FBA and PK in 60 eumycetoma patients (P), 20 actinomycetoma patients (AP), 31 healthy Sudanese controls (C) and 40 healthy Dutch controls (DC). Each symbol represents a single patient or control. Horizontal lines indicate median levels of anti-madurella antibodies. Significance was calculated with the Mann-Whitney test. Antibody levels measured for TCTP, FBA and PK were higher in eumycetoma patients compared to healthy Dutch controls ($P < 0.0001$). When compared to healthy Sudanese controls, only for FBA MFI values of eumycetoma patients were higher ($P = 0.0025$).

Swoboda et al. found that of the 22 patients with oesophageal and/or oral Candida infections only five had formed antibodies against CaPK [24]. Pitarch et al. confirmed this in four additional patients with invasive candidiasis, where only three had antibodies against CaFBA and CaPK [14]. The reasons behind these differences in antigen recognition could be pathogen or host-related. For instance, the TCTP antigen has been demonstrated to exist in two variants in

Fig. 3  Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG for recombinant Madurella mycetomatis his-tagged TCTP (A), FBA (B), and PK (C) in 60 eumycetoma patients (all patients), 20 eumycetoma patients with lesions larger than 10 cm in size (large lesion), 20 eumycetoma patients with lesions larger than 5 cm but smaller than 10 cm (moderate lesion), 20 eumycetoma patients with lesions smaller than 5 cm (small lesion) and 31 healthy Sudanese controls (C). MFI values were highest in patients with massive eumycetoma lesions and lowest in patients with small eumycetoma lesions. The difference in size was not statistically significant (Kruskal-Wallis, $P = 0.34$ for TCTP, $P = 0.54$ for FBA, $P = 0.16$ for PK).
Two novel immunogens in *Madurella mycetomatis*

M. *mycetomatis*. Roughly half of the *M. mycetomatis* isolates possessed variant I, while the other half possessed variant II [8]. Of the glycolysis proteins, TCTP is considered to be an essential protein. Therefore, it was determined through sequencing if there were differences in the gene sequences of FBA and PK in *M. mycetomatis*. However, this was not the case. Next to differences in gene sequence, different splice variants of the mRNA could also result in different protein variants. In the analysis by 2D-electrophoreases of the cellular *A. fumigatus* proteome it was found that there were multiple forms of AfGAPDH. Of the five different forms, it appeared that two of these isoforms were highly immunogenic and three only moderately [21].

Host-based differences in antigen recognition have been noted when the serologic response to systemic *C. albicans* infections was studied in a murine model. It appeared that mice with different genetic backgrounds reacted differently to CaPK, CaFBA and CaGAPDH. The more resistant BALB/C mice only reacted weakly to CaPK and CaFBA and no antibodies were formed against CaGAPDH. In contrast, the more susceptible CBA/H mice reacted weakly against CaPK but strongly against CaFBA and CaGAPDH [26]. These three *Candida* antigens were also included in a large panel of antigens which were used to predict the clinical outcome of invasive candidiasis. It was found that patients with a good clinical outcome had higher overall levels of the protective antibodies, including...
those against CaFBA. Patients with a poor clinical outcome had lower levels of these antibodies and higher levels of non-protective antibodies such as those to CaGAPDH and CaPK [27]. To determine if MmFBA, MmGAPDH or MmTCTP could also predict the clinical outcome of mycetoma patient cohorts. These results may be easily explained for the Sudanese healthy controls and actinomyetoma-patients. These results may be easily explained for the Sudanese healthy controls and actinomyetoma-patients. Some of these individuals might have encountered M. mycetomatis without developing eumycetoma as indicated by the higher IgG levels measured in the Sudanese healthy control group and the actinomyetoma control group. Anti-M. mycetomatis antibodies were also detected in the Dutch controls which was unexpected since M. mycetomatis is not endemic in The Netherlands. An explanation for the higher antibody-levels found in some of the Dutch controls could be the high degree of conservation of the glycolytic enzymes across all fungal species (Fig. 1), causing cross-reactivity with some epitopes of these proteins shared by M. mycetomatis and other fungi present in the Dutch environment.

Based on the results presented here, FBA and PK of M. mycetomatis do not seem to be useful for diagnostic purposes. Although antibodies are formed, there is no clear difference between the IgG levels of healthy controls and patients. Perhaps the discriminatory power can be enhanced by measuring antibodies against epitopes that are specific for M. mycetomatis instead of the whole proteins, as was previously done for the antigen mmTCTP [8].

Due to their immunogenic properties, FBA and PK could be considered vaccine candidates. In the past few years, several antigenic glycolytic proteins of other pathogens have been tested in mouse models. For C. albicans, S. mansonii, Onchocerca volvulus and Streptococcus pneumoniae immunization with these proteins raised a protective immune response [22,25,28,29]. Furthermore, for S. mansonii vaccination with SmFBA resulted in a significant decrease in granuloma formation [22]. Based on the results presented here, mmFBA might be the best choice to use as a vaccine candidate. Antibody levels against FBA were significantly elevated in the eumycetoma patient group compared to the healthy Sudanese controls. Furthermore, unlike PK, FBA has low amino acid homology with the human host. This lack of homology is because FBA proteins can be found in two different classes. Animals express the class I variant of the protein and fungi express FBA class II. The functional properties are similar, but the two variants are sequentially and mechanistically distinct [30].

In conclusion, this study presents two new antigenic proteins of M. mycetomatis next to TCTP: the glycolytic enzymes FBA and PK. These antigens might be useful as vaccine-candidates in the prevention of mycetoma.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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