Genetic Variability, Antigenicity and Antifungal Susceptibility of *Madurella mycetomatis*

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Genetic Variability, Antigenicity and Antifungal Susceptibility of *Madurella mycetomatis*

Genetische variabiliteit, antigeniciteit en antifungale gevoeligheid
van
Madurella mycetomatis

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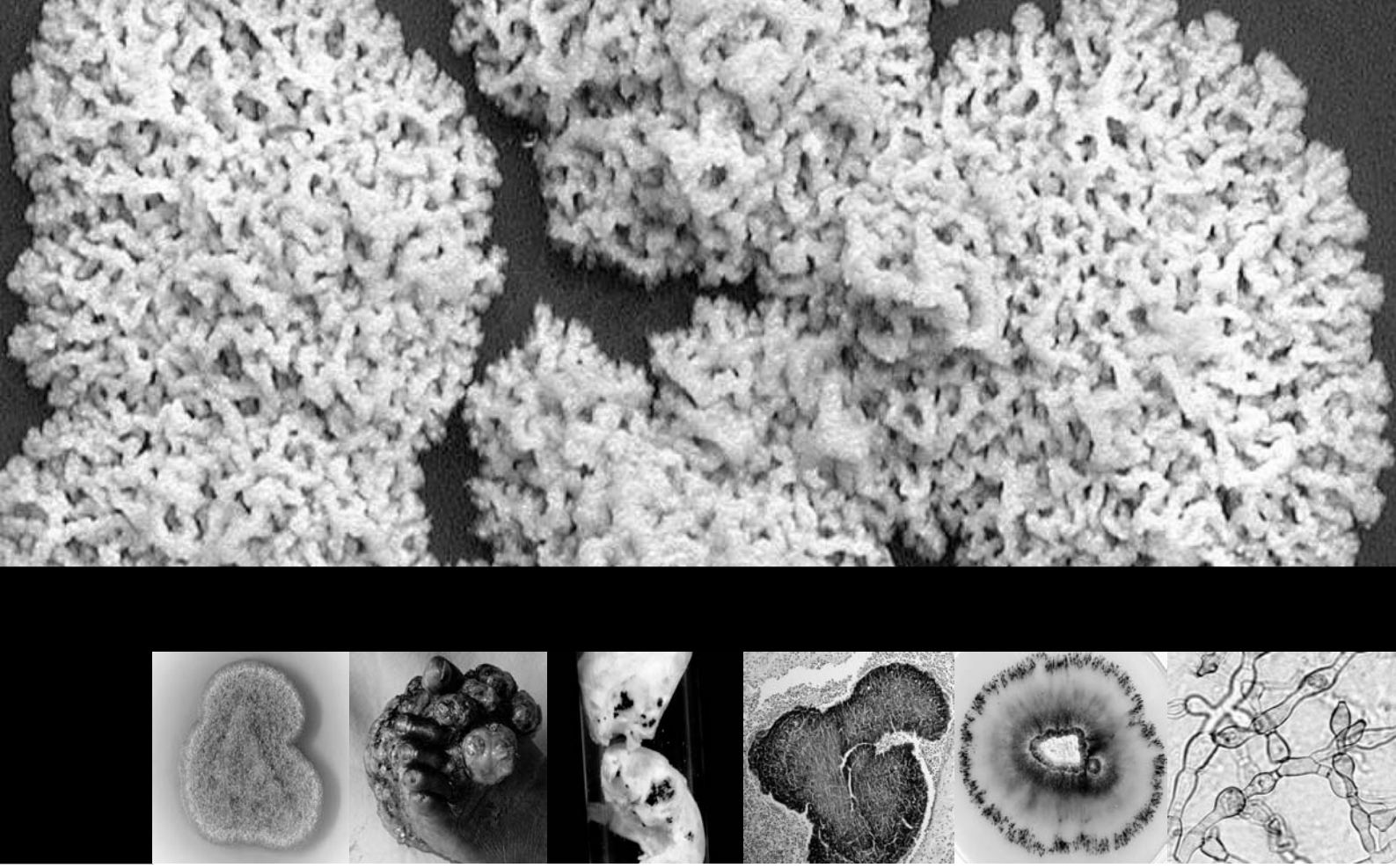
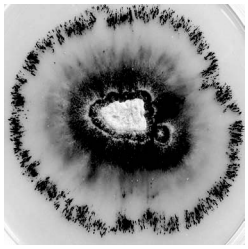


General introduction

Chapter 1



Scope of the thesis

Wendy W.J. van de Sande

Introduction

Mycetoma is a chronic, granulomatous, progressive inflammatory disease, characterized by a subcutaneous mass, tumefaction and the formation of sinus tracts. The sinuses discharge seropurulent material and fungal or bacterial grains. This disease, which can be caused by both bacteria and fungi, is encountered all over the world but is endemic in the so-called "mycetoma-belt", between 30 ^{o}N and 15 ^{o}S of the equator. Sudan is one of the countries in which mycetoma has a high incidence. In this country the most common causative agent is the fungus *Madurella mycetomatis.*

Although mycetoma was already described in 1842, still relatively little is known about this disease. Diagnostic assays and treatment are still suboptimal. Current diagnostic assays are time-consuming and not very reliable. Treatment, especially in eumycetoma, is difficult and usually involves both surgery and medical treatment. Adequate antifungal treatment may take years and is usually started without even knowing if the causative agent is actually susceptible to the chemotherapeutic agent applied.

Scope of the thesis

To understand the difficulties in diagnosing and treating mycetoma we first review this disease in **chapter 2.** The current knowledge about this disease, its prevelance, the diagnostic assays used, and the treatment given to the patients are highlighted. As will become clear in this chapter little is known about the disease. In the remaining of this thesis we will focus on three main themes:

1. Genetic variation of *M. mycetomatis* and its human host

In the chapters belonging to this theme it was assessed whether genetic variability of both pathogen and host could be related to the outcome of the disease. In **chapter 3** the selective amplification of restriction fragments (AFLP®) is used to devide 37 Sudanese clinical isolates of *M. mycetomatis* into two main clusters (clusters I and II) and one minor cluster (cluster III). The strains in cluster I mostly derived from patients in Central Sudan and were generally obtained from larger lesions than the strains found in cluster II. In **chapter 4** several human genes involved in neutrophil function were studied with PCR-restriction analysis. It appeared that patients usually had a genotype correlated with a higher CXCL8 expression and a lower nitric oxide expression. This was confirmed phenotypically.

2. Antigenicity of *M. mycetomatis*

In order to improve the diagnostic serology for mycetoma caused by *M. mycetomatis* the antigenicity of *M. mycetomatis* was assessed. In **chapter 5** the first immunogenic antigen, a protein homologous to the translationally controlled tumour protein (TCTP) was discovered using a *M. mycetomatis* cDNA expression library. Both IgM and IgG responses could be measured for this protein, with the highest antibody levels found associated with the largest

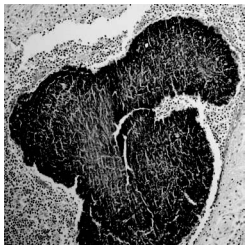
lesions. Unfortunately, not all patients formed antibodies against this protein. In **chapter 6** it was shown that although *M. mycetomatis* secreted significant amounts of galactomannan-like compounds into the culture medium, these compounds were not detected in the patient serum.

3. Antifungal susceptibility of *M. mycetomatis*

In order to improve the antifungal therapy in patients we tried to gain more insight in the *in vitro* susceptibility of *M. mycetomatis* against various antifungal agents. In **chapter 7** a CLSI (formerly NCCLS) based assay was developed for *M. mycetomatis*. To facilitate endpoint reading a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) assay for fungal viability was used. In **chapter 8** the assay described in chapter 7 was compared to a commercial Sensititre assay for the antifungal agents amphotericin B, ketoconazole, itraconazole, fluconazole and voriconazole. In **chapter 9** the *in vitro* susceptibilities for the less commonly used antifungal agents artemisinin and tea tree oil were determined. In **chapter 10** it was assessed what the effect of melanin produced by *M. mycetomatis* was on the *in vitro* susceptibilities to amphotericin B, ketoconazole, itraconazole, fluconazole and voriconazole.

Finally in **chapters 11 and 12** the findings reported in this thesis are summarized and discussed**.**

Chapter 2



Black grain mycetoma, a neglected infection

Wendy W.J. van de Sande

Adapted from:
Abdalla O.A. Ahmed, Willem van Leeuwen, Ahmed Fahal, **Wendy W.J. van de Sande**, Henri Verbrugh and Alex van Belkum**,** Mycetoma caused by *Madurella mycetomatis*: a neglected infectious burden*, The Lancet Infectious Diseases* 2004, 4: 566-574.
Abdalla O.A. Ahmed, **Wendy W.J. van de Sande**, Ahmed Fahal, Irma Bakker-Woudenberg, Henri Verbrugh and Alex van Belkum, Management of Mycetoma: Major Challenge in Tropical Mycoses with Limited International Recognition, *Current Opinion in Infectious Diseases* 2007, 20(7): 146-151.

Introduction

Mycetoma is a chronic infectious disease, which remains localized, involves cutaneous and subcutaneous tissues, the fascia and bones (95). The disease is characterized by tumefaction, draining sinuses and the presence of grains (95).

The oldest recorded case of possible mycetoma involves the skeleton found in Israel of an adult woman buried in the Byzantine period (300-600 AD) (95). This skeleton had morphologic changes in the bones of the distal portion of the lower extremities which are suggestive for mycetoma (95). The first written description of mycetoma was found in the Indian religious book *Atharva Veda,* where the disease was called "padavalmika", meaning foot-anthill (95). In 1842 the first case was described in western literature by Dr. John Gill, a physician of the Madras Medical Service of the British Army in India (112). His colleague Dr. Colebrook proposed the name "Madura foot" in 1848 (95, 112). The term mycetoma was first used by Vandyke Carter in 1860 (112). He was the first to believe that mycetoma could be separated into two varieties depending on the colour of the grains associated with the disease. *M. mycetomatis* probably caused the black variety of mycetoma Carter described, whereas one of the aerobic actinomycetes caused the light-coloured variety (95).

Epidemiology and causation

As was already apparent from this short history lesson, mycetoma has a worldwide but uneven distribution. The disease is endemic in tropical and subtropical regions but the African continent has the highest prevalence (figure 1). Mycetoma prevails in the mycetoma belt that stretches between the latitudes of 15° south and 30° north (18, 86). The belt includes Sudan, Somalia, Senegal, India, Yemen, Mexico, Venezuela, Columbia, Argentina, and other countries (18, 86, 89, 93). In Africa, mycetoma is most frequently seen in Sudan, Senegal, Mauritania, Kenya, Niger, Nigeria, Ethiopia, Chad, Cameroon, Djibouti, and Somalia with increased morbidity in poor people lacking access to essential health care facilities (2, 3, 13, 22, 37, 39, 85, 119).

In Sudan, Abbot (2) noted the admission of 1231 mycetoma cases to outpatient clinics throughout the country within a period of 2.5 years. In 1964, Lynch (85) gave an estimation of 300–400 new cases per year in Sudan. The disease has also been extensively reported in India (17, 119). Reports on mycetoma have also come from the USA, Ceylon (now Sri Lanka), Germany, Egypt, Turkey, Philippines, Japan, Lebanon, Thailand, Iran, Netherlands, and Saudi Arabia (25, 31, 45, 47, 55, 77, 122). Most of these imported cases involved immigrants who probably contracted the infection in their home countries. This complicates the clinical diagnosis since it is frequently unexpected (31).

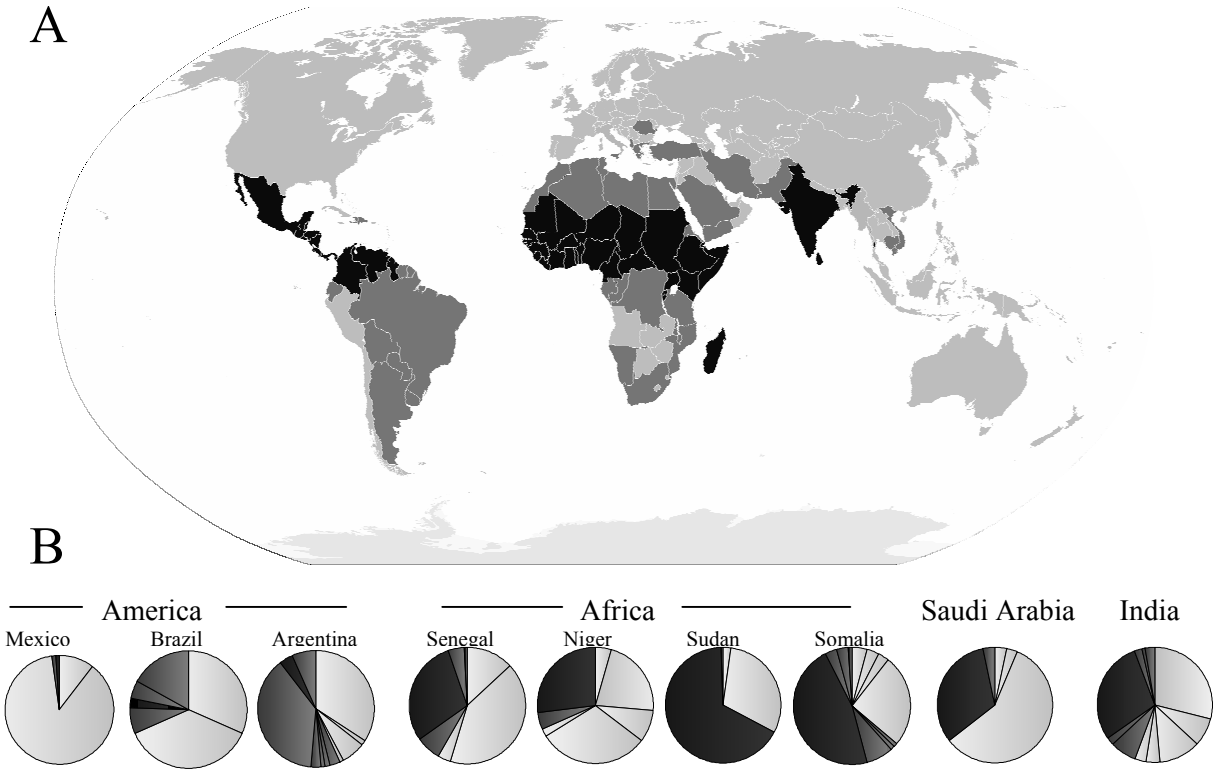


Figure 1: Mycetoma endemic areas. A: Dark grey coloured countries are countries with common and frequent incidence. Lighter coloured areas are countries with regular to moderately common incidence. B: Actinomycetoma (light grey) versus Eumycetoma (middle grey) in a selection of countries. To highlight the differences in etiological agents over the world *M. mycetomatis* is separately shown in dark grey (2, 15, 23, 36, 37, 40, 75, 83, 101, 112).

The geographical distribution of the mycetoma causative agents shows considerable variation that could be explained by environmental factors, especially lack of rainfall (18, 87, 93). Areas where mycetoma prevails are reasonably arid with a short rainy season of 4–6 months. Rainfall is 50–1000 mm per year, with a relative humidity of 60–80%, and fairly constant temperatures of 30–37°C throughout the day and night. The rainy season is followed by a dry season of 6–8 months with a relative humidity of 12–18%, and daytime temperatures rising to 45–60°C. Temperatures might fall to 15–18°C during the night (87). This extreme alteration in weather conditions might be a prerequisite to the survival of the causative organism in its natural niche.

Many micro-organisms are capable of causing mycetoma (table 1) and several of these organisms seem to have their natural habitat in soil or plant materials including thorns. The different micro-organisms form grains of different colours and morphology which may be of help in the taxonomic positioning and species identification of the causal organisms. The most prevalent causative agent of eumycetoma worldwide, and in Africa in particular, is *Madurella mycetomatis* (95). As is seen in figure 1 in some parts of central Africa, including Sudan, *M. mycetomatis* causes more than 70% of all mycetoma infections (18, 59).

Etiologic agents causing Mycetoma					
Eumycetoma			Actinomycetoma		
Agent	Grain colour	Occurrence	Agent	Grain colour	Occurrence
Acremonium falciforme	White	E	*Actinomadura madureae*	White/ Yellow/Pink	C
Acremonium kiliense	White	R	*Actinomadura latina*		E
Acremonium recifei	White	R	*Actinomadura pellitieri*	Red	O
Aspergillus flavus	Green	E	*Actinomyces israelii*	White/Yellow	O
Aspergillus nidulans	White	E	*Gordona terrae*	-	E
Cladophialophora bantiana	Black	E	*Nocardia asteroides*	White	R
Corynespora casiicola	Black	E	*Nocardia brasiliensis*	White	C
Curvularia geniculata	Black	E	*Nocardia caviae*	White/Yellow	R
Curvularia lunata	Black	E	*Nocardia farcinica*	White/Yellow	R
Cylindrocarpon cyanescens	White	E	*Nocardia mexicana*		E
Cylindrocarpon destructans	White	E	*Nocardia transvalensis*	White	E
Exophiala jeanselmei	Black	E	*Nocardia veterana*		E
Exserohilum rostrata	Black	E	*Nocardiopsis dassonvillei*	Cream	E
Fusarium monoliforme	White	E	*Streptomyces somaliensis*	Yellow/Brown	O
Fusarium oxysoprum	White	E			
Fusarium solani	White	E			
Hormonema spp.		E			
Leptosphaeria senegalensis	Black	R			
Leptosphaeria thompkinsii	Black	E			
Madurella grisea	Black	R			
Madurella mycetomatis	Black	C			
Microsporum canis	White	E			
Neotestudina rosatii	White	E			
Phialophora verrucosa	Black	E			
Plenodomus avramii	Black	E			
Polycytella hominis	White	E			
Pseudallescheria boydii	White	C			
Pseudochaetosphaeronema larense	Black	E			
Pyrenochaeta mackinnonii	Black	E			
Pyrenochaeta romeroi	Black	R			
Rhinocladiella atrovirens		E			
Scedosporium apiospermum		E			
Scytalidium dimidiatum	No grains	E			
Trichophyton spp	White	E			

Table 1: Etiologic agents causing Mycetoma. Agents are divided into fungal and bacterial species. Next to the species, the grain colour and the occurrence are given. C = common, O = occasional, R = rare and E = exceptional (1, 2, 10, 15, 23, 29, 33, 34, 36, 37, 40, 45, 47, 55, 59, 75, 77, 82, 85, 101, 119, 122, 130).

Clinical presentation

Mycetoma initially presents as a slowly progressive and painless subcutaneous swelling, sometimes in combination with a history of preceding trauma (18, 95). However, the incubation time before classic signs develop is not well-defined: the first symptoms might need several months to years to become palpable. Investigations are complicated by the fact that most patients tend to present late, by an absence of clinical symptoms, by lack of accessible health care facilities, or because of patients' fear of amputation (18, 95, 112).

The duration of the disease, the type of causative organism, the site of the infection and possibly the immune response of the host, can all affect the clinical presentation of

mycetoma (52). However, patients with a short disease history might present with massive lesions and severe destruction of deep tissues and even of bones (figure 2). By contrast, others might present with minor localised lesions that had already existed for a long time without any evidence of major progressive tissue destruction (Ahmed Fahal, unpublished data). The subcutaneous swelling is usually firm and rounded but it can also be soft and lobular. It is rarely cystic (50), and is often movable. The subcutaneous nodule increases in size and secondary nodules might evolve as well (52). The nodules might suppurate and drain through multiple sinus tracts, and these sinuses can close transiently after discharge during the active phase of the disease. Fresh adjacent sinuses might open whereas some of the old ones might heal completely. The nodules are connected to each other through deep sterile abscesses, and to the skin surface (18, 52).

Mycetoma can affect any part of the body, and lesions are not restricted to the limbs only (figure 2). Most cases are usually seen in the feet (70%), followed by hands (12%), then legs and knee joints (52, 95). In highly endemic areas, other parts of the body might become affected as well. These include the arm, head and neck, thighs, and the perineum. Rarely, the chest and abdominal walls, facial bones, mandible, paranasal sinuses, eyelid, vulva, orbit, scrotum, and old surgical incisions might also be affected (18, 52). Mycetoma spreads locally or through the lymphatic system, and, rarely, through the bloodstream. Secondary nodules can arise in the affected area, sometimes with more distant lymphatic metastases in some advanced cases. Secondary sites are seen more often in actinomycetoma than in eumycetoma, suggesting that bacteria might spread more easily. Mycetoma is usually painless, and the mycetoma lesion has been suggested to produce substances that have an anaesthetic effect (48). At a late stage of the disease, the absence of pain might be due to nerve damage by the tense fibrous tissue reaction or endarteritis obliterans, or alternatively, poor vascularisation of the nerves. In final stages of the disease, pain might be due to invasion of the bone or to secondary bacterial infections (6, 52). As the mycetoma granuloma increases in size, the skin may become smooth and shiny, and areas of hypopigmentation or hyperpigmentation can develop. Increased local hyperhydrosis has also been seen in some patients (18, 48).

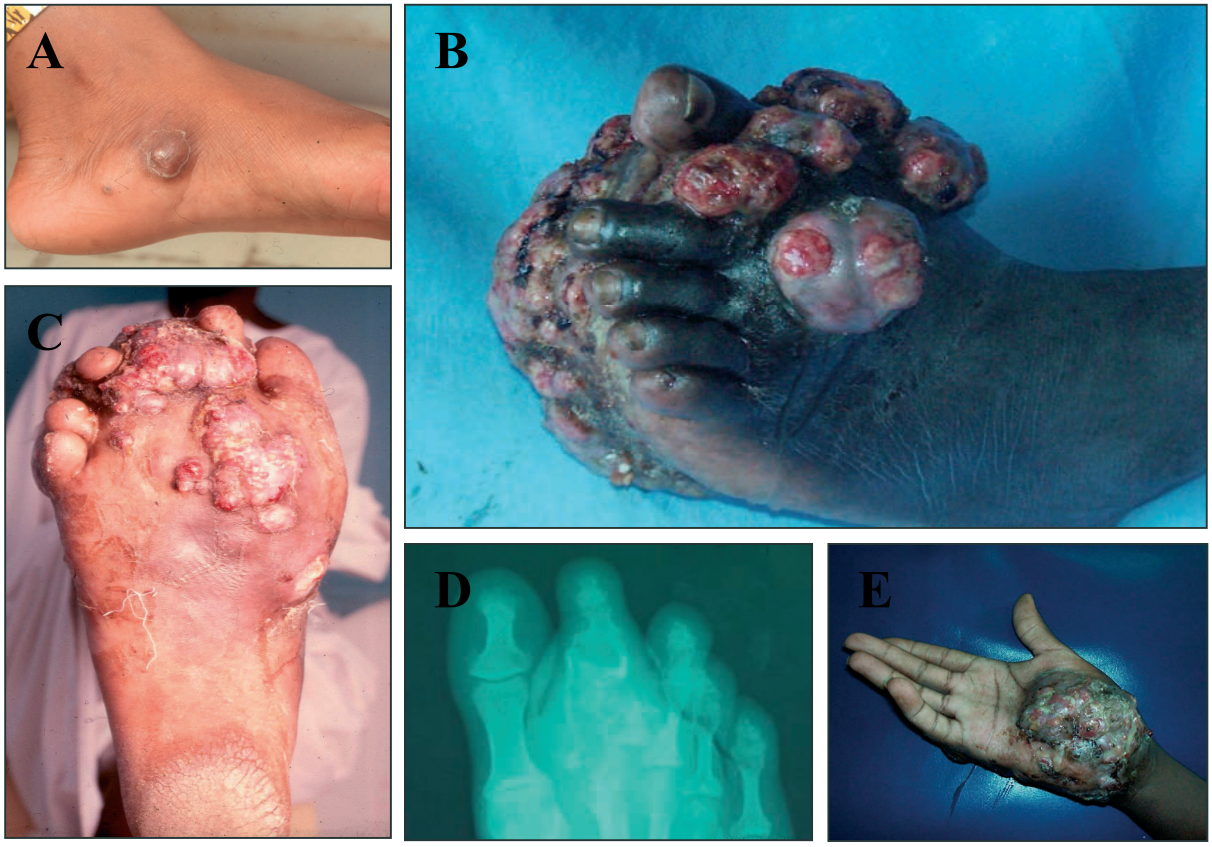


Figure 2: The clinical presentation of mycetoma. A: an early case of mycetoma of the foot; B and C: advanced cases of mycetoma of the foot; D: a X-ray showing bone destruction in mycetoma, E: advanced mycetoma of the hand, the second most common site for mycetoma.

Susceptible population and risk factors

Anyone living in an endemic area could become infected, but infection is more common in herdsmen, farmers, and other field laborers who are in frequent and direct contact with the field environment. Males are five times more often affected by mycetoma than females, even in areas where both sexes spend a lot of time outdoors (52, 95). Mycetoma is seen in all age groups, but it usually affects adults between 20 and 40 years old. The infection is not considered to be transmissible from person to person or from animal to human.

There are some conflicting reports about the role of the immune status of the susceptible population. Some investigators reported partial impairment of the cell-mediated immune-response (CMI) in patients severely infected or not responding to medical treatment (92). But overall, mycetoma is commonly seen in immunocompetent individuals. However, dissemination from the primary site is a common feature among immunocompromised individuals (46). For example HIV-infected individuals show a tendency towards accelerated mycetoma progression (58). The small number of case studies available and HIV patients presenting with various forms of mycetoma does not yet allow for definite conclusions to be drawn on the relation between immune status and mycetoma susceptibility and progression (24, 103). In animals, some evidence supports the impaired CMI hypothesis. For example mycetoma is more successfully induced in athymic mice than in immunocompetent mice (88).

The more rapid progression of induced mycetoma in immunosuppressed goats compared with healthy goats provided another important piece of evidence (60). By contrast, Bendl and co-workers (15) did not confirm this finding.

The humoral immune response has been evaluated by Wethered and his colleagues (131) using an ELISA system. High IgM levels were seen in most patients with mycetoma due to *M. mycetomatis,* whereas low levels of IgA were detected in some of the patients. Sera from patients did better in the ELISA as compared to western blots, indicating possible involvement of polysaccharide or fatty acid antigens in *M. mycetomatis* infection. IgM and IgG and complement factors have been identified on the surface of the grains, and on the filaments inside the grains in case of actinomycetoma (42).

Diagnosis

In endemic areas, mycetoma must be considered in the differential diagnosis of all subcutaneous swellings. Clinical scores should be developed for a more thorough definition of the different disease presentations. Presently, clinical evaluation is not very reliable. Therefore, it is advisable to send clinical specimens, preferably those containing grains, for adequate diagnostic microbiology laboratory testing. However, lack of experience, non-state of the art diagnostic facilities or improper clinical specimens complicate the identification of many of these relatively rare species (10, 31). Since different agents can cause mycetoma, identification of the causative agent is important to develop an appropriate plan of treatment (table 2). However, available strategies are few and have many limitations.

At the level of the individual patient, modern imaging methodology is considered diagnostically valuable. Magnetic resonance imaging, for instance, has been shown to be informative in the detection and even identification of fungal grains and for the assessment of therapeutic success (28, 68, 118). Additionally, magnetic resonance imaging facilitates the detection of specific morphological features of the fungal grains (so-called dot-in-circle signs) which are suggested to be highly specific for mycetoma lesions (116). However, regions where these types of diagnostic facilities are most urgently needed, are usually the ones where such facilities are lacking because of financial difficulties.

At the level of the fungus, direct examination of crushed grains might be useful in determining whether the mycetoma is caused by bacteria or fungi. Unlike fungal grains, crushed bacterial grains show fine filaments, which can be Gram-stained. Isolation of mycetoma agents can be difficult, especially in case of the obligate anaerobic actinomycetes. Culture needs deep surgical biopsies containing grains. Biopsies without grains are not suitable for diagnosis (44). The large numbers of causative organisms and the fact that many black-grain producing fungi do not produce conidia or do so only after long incubation periods, complicate the identification process (31, 105).

Histopathological techniques are useful for confirming the clinical diagnosis of mycetoma and in the differentiation of several bacterial causes of mycetoma since the immunopathology of actinomycetoma is known (114, 115) (table 1). However, correct identification of fungi causing mycetoma by histology only is difficult, if not impossible, and culture confirmation should always be pursued (105). With histopathological examination of

fungal mycetoma it is only possible to categorize some of the pigmented fungi to a certain extent.

Main clinical differences between eumycetoma and actinomycetoma		
	Eumycetoma	Actinomycetoma
Disease		
Causative agents	Fungi	Bacteria
Lesion characteristics	Well-encapsulated with a clear margin	Diffuse with no clear margin
Sinuses	Few	Many
Colour of grains	Different colours, but mostly white or black	Different colours, but not black
Course of infection	Slowly progressive	Inflammatory and rapid progression
Bone invasion	After a long time	Rapid
Cavities in radiograph	Small in number, but large in size with clear margins	Numerous, small in size (except in case of Actinomadura madurae with unclear margins)
Management		
Drug of choice	Ketoconazole Itraconazole	Dapsone+Streptomycin Rifampicin or sulfadoxine-pyrimethamine Amikacin+cotrimoxale
Management of choice	Both medical and surgical intervention required	Only medical treatment is sufficient (in some advanced cases in combination with surgery)
Surgery only	Might cure small, well-encapsulated lesions, up to 90% recurrence in most cases	Not indicated
Medical treatment only	Partial cure or improvement Recommended before surgery to prevent local spread Needed after surgery to prevent recurrence	Useful in most cases
Most successful outcome	Early diagnosis followed by adequate antifungal treatment in combination with surgery	Early diagnosis followed by medical treatment for a sufficiently long period

Table 2: Main differences between eumycetoma and actinomycetoma in disease characteristics and management.

Few studies have focused on serological methods for early detection and monitoring of patients under treatment (87, 98, 120). Immuno-electrophoresis, immunodiffusion and ELISA are developed for the extended diagnosis of mycetoma (61, 90, 120). The problem with such serological tests is the use of non-standardized and poorly prepared crude antigens, which are prone to cross reactivity, especially between actinomycetes, and false-positive and -negative results (10). Recently, the first genuine ELISA test for the detection of *M. mycetomatis* infection was presented (126). This test involved the detection of antibodies against the first protein antigen of *M. mycetomatis* that was serologically identified, cloned, sequenced and produced as a recombinant protein. The translationally controlled tumour protein (TCTP) was found to be a predictor for the size of the lesion and the duration of the infection. In cured patients the antibodies frequently became undetectable. The usefulness of this first assay should be evaluated in large serological screening tests in the endemic regions, preferably in a longitudinal study (126).

Fine-needle aspiration cytological methods for mycetoma have also been described, and are considered to be useful since they can be done with or without local anaesthesia (41).

To improve the quality of diagnosis of mycetoma, molecular tests have recently been developed for several agents of black-grain eumycetoma (8, 35). The availability of these molecular detection and identification tests will help microbiologists in species definition, which is necessary for proper management of patients. Ahmed et al. developed *M. mycetomatis*-specific PCR-based tests based on the sequence of the internal transcribed spacer in the ribosomal gene complex, a region which has been successfully utilized by many investigators for the development of diagnostic DNA amplification assays (8). Post-amplification restriction digestion facilitates correct species identification, and clearly differentiates *M. mycetomatis* from *Madurella grisea* (8). Similar results were recently reported from France, where large numbers of black-grain eumycetoma agents were studied (35). The test is not only useful for detection and identification of *M. mycetomatis* in patients (7), but can also be applied for the detection of fungal material in the environment. This approach led to successful amplification of *M. mycetomatis* DNA from soil samples, whereas attempts through culture efforts were negative for the same samples (4, 121).

Disease management and treatment

Mycetoma is difficult to treat and improper management is devastating, leading to poor social and economical perspectives. No cases of spontaneous cure have ever been reported. Proper management of mycetoma strongly depends on the identification of the causative organism. Knowing the mycetoma type (actinomycetoma or eumycetoma) is mandatory for the initiation of correct medical management. Actinomycetoma chemotherapy alone namely results in high cure rates (60% to 90%), and surgery is only required in advanced cases including those refractory to antimicrobial treatment (84). For eumycetoma surgery is still the most acceptable means of treatment (82, 84). The main aim of complementary surgery is complete excision of small, well-encapsulated lesions, or the reduction of the amount of infected tissue to be cured by chemotherapy. Surgery has major undesirable side-effects. Effective surgery needs aggressive excision or debridement under general anaesthesia, which usually cripples the limb or leads to permanent disability. Furthermore it's success rate is usually dependent on the stadium of the disease. Early cases, in which the disease process is still limited and localized, with well-encapsulated lesions and without bone involvement can be treated efficiently with surgery. Advanced cases have a higher recurrence rate. In mycetoma surgery done without a combination of effective chemotherapeutic treatment is not feasible due to its high rate of recurrence (up to 90%). In overall combination of medical treatment before and after surgery results in the most successful outcome (49, 95).

Chemotherapeutic treatment is dependent on the causative agent. If this agent is not known, patients are subjected to both empirical antifungal and antimicrobial treatment (63). In actinomycetoma the nature of the etiologic agent normally determines the choice of antimicrobial (combination) therapy (18). The choice of antimicrobial agent is usually based on personal, single-center experiences, since no double blind, placebo-controlled clinical studies into the efficacy of antimicrobial treatment are done (1, 21, 33, 46, 73, 74, 76, 79, 100,

102, 106, 107, 130). Consequently, there is no international consensus as to the nature of the optimal treatment regimen for mycetoma patients in general.

Eumycetoma is deemed to be relatively non-responsive to antifungal treatment by many and radical surgical amputation is the intervention option preferred, especially in areas with limited medical resources and where not much else than surgery can be offered. Even when access to antifungal agents can be provided, eumycetoma still responds variably, ranging between complete clinical cure, limited improvement to no improvement whatsoever. Poor responses to different antifungal agents even occur when these are given in combination and at high doses. This is in contrary to *in vitro* susceptibilities. Many reports showed that *M. mycetomatis* has a low MIC to the azoles, the polyene amphotericin B and the echinocandin anidulafungin (9, 127). Overall, early diagnosis followed by surgical excision of small lesions and antifungal treatment before and after surgery usually results in the most successful outcome (95). Local surgical excision alone is rarely successful and might in some cases even enhance the lymphatic spread (111).

Chemotherapy of eumycetoma by treatment with the new azole-class antifungals was piloted in the late 1980s. The initial trials showed a clear progress in healing of the lesions (20). Itraconazole in particular showed great promise in these trials and in equivalent *in vitro* tests (124). Currently, although showing varying degrees of clinical efficacy, itraconazole and ketoconazole are still the best treatment options (38, 110, 129). Within the Mycetoma Research Center (Khartoum, Sudan; http://www.mycetoma.org), both ketoconazole (400–800 mg daily) and itraconazole (400 mg daily) are recommended for first line use (48). The duration of treatment varies with the severity of the infection and the general health status of individual patients. Treatment may need to continue for 18-24 months or more and the liver function of the patients needs regular monitoring, especially in advanced mycetoma (49). However, long-term treatment may lead to antifungal resistance, which may in turn complicate patient management. Clinical follow-up has shown that itraconazole treatment has a better success and a lower recurrence rate than ketoconazole. In particular, lesions that eventually remain are well-encapsulated and distinctly localised. This renders final surgical treatment a likely option. But in advanced lesions, especially when bone tissue is involved, the response to chemotherapeutic treatment still remains very poor (111). The disadvantage of the azole drugs is their expensiveness, which limits immediate availability to patients in poor regions.

Hopes on effective medical treatment are increasing with the introduction of broad spectrum triazoles like voriconazole and posaconazole. However, lack of essential health care facilities, the absence of health insurance coverage, and high cost of these new azoles may limit their use in poor endemic regions in those developing countries where the agents are required most. In addition, solid antifungal efficacy data are desperately needed for the proper management of mycetoma. Only two recent papers are available for review. The first paper described a single-center open label efficacy and safety study of terbinafine in the treatment of eumycetoma. Twenty five percent of patients were cured and 55% showed clinical improvement. Although the study was limited by the small number of patients included (n=20), lack of randomization, missing control groups, lack of standardized cure or improvement criteria and the possible investigators bias, the study is still considered as one of

the unique ones done to evaluate the efficacy of antimycotic drugs used for treatment of mycetoma (100). In the second study, the effect of posaconazole was evaluated on 6 eumycetoma cases caused by *M. mycetomatis* (n=2), *M. grisea* (n=3) and *S. apiospermum* (n=1). Five patients were successfully treated, whereas one did not show improvement (102).

A difficult decision in case of mycetoma patients is when to stop treatment. The prevalence of the infection in poor patients living in remote areas renders continuation of treatment and regular follow-up rather difficult and usually even impossible. Some investigators tried to set some standards for clinical, serologic, or radiologic cure, but so far there is no consensus on these standards. Many patients in whom medical treatment was stopped without a well-validated clinical score suffered from recurrence after initial improvement. There is no clear definition of cure in mycetoma and there is no test of cure that enables the differentiation between infected and non-infected patients (84). The TCTP ELISA that was recently described (126) revealed a correlation between serological response and the severity or stage of eumycetoma. This requires further elaboration, preferably also using additional fungal antigens in the test system. In the end such systems could be used for prospective detection of early stages of infection or relapse after therapy.

The genus *Madurella* and its two species

Usually, *Madurella* species are slow-growing fungi that produce dark colonies composed of a dense, melanised, and mostly sterile mycelium. *Madurella* species are well known agents of black-grain mycetoma. Two species are recognised, *M. mycetomatis* and *M. grisea* (32). In the past *M. mycetomatis* has been known under different names. In 1905, Brumpt placed an organism described by Laveran in 1902 as *Streptothrix mycetomi* in the genus *Madurella*. Later the fungus was renamed as *Madurella mycetomi* and this name was used for more than seven decades. In 1977, the British Medical Research Council published memorandum number 23 (Nomenclature of fungi pathogenic to humans and animals) in which the name was corrected to *M. mycetomatis*. The name was corrected because the specific epithet must be in the genitive case (the *Madurella* of mycetoma). Since mycetoma is a Greek neuter noun, the correct citation is mycetomatis (78).

In different culture media, *M. mycetomatis* strains show various colony morphologies (figure 3). The growth rate is moderate, and the colonies are white and woolly at first, becoming olivaceous, yellow, or brown, generally producing a brownish, diffusing pigment. In poor media, sclerotia of 750 μm diameter can develop (105). Colonies are mostly sterile, composed of a dense melanised mycelium. No efficient sporulation has ever been seen. However, phialides with minute conidia in short chains and collarettes may be seen (32, 95, 105). Some investigators reported that better sporulation was noted when *M. mycetomatis* was cultured in straw extract agar, wheat extract agar, soil extract agar, or water agar (78, 108). In most cases, the microscopic appearance of *M. mycetomatis* and *M. grisea* is quite similar. Some isolates of *M. grisea* were reported to form pycnidia. Such isolates are indistinguishable from *Pyrenochaeta mackinnonii* (105). Species-differentiation of *M. mycetomatis* and *M. grisea* can be made by differences in sugar assimilation and optimal growth temperature. *M. mycetomatis* assimilates lactose but not sucrose, whereas *M. grisea* assimilates sucrose but not

lactose. *M. mycetomatis* grows well at 37 ^{o}C, while *M. grisea* does not grow at 37 ^{o}C (growth is seen at 30 ^{o}C) (32, 105). This finding might also explain the observed difference in virulence.

M. mycetomatis and *M. grisea* are both placed in the genus *Madurella* but recently molecular techniques showed that these fungi in fact do not have the same phylogenetic ancestors. Based on the ribosomal sequences, *M. mycetomatis* has been repositioned in the order of the ascomycetes, whereas *M. grisea* is now an acknowledged member of the order of the pleosporales (30). In the same study it appeared that a wide range of closely related agents can actually cause eumycetoma. These data might be essential to make a complete inventory of potential eumycetoma agents (30).

When different isolates of *M. mycetomatis* deriving from patients originating from endemic areas were genetically compared by large scale random amplification of polymorphic DNA (RAPD) they did not show major differences, despite the sometimes strongly differing phenotypes seen when cultured (5). Later this same set of strains was compared with a more differentiating technique, called amplified fragment length polymorphism (AFLP) analysis. From this study it appeared that the strains were indeed genetically diverse and that the isolates obtained from larger lesions were genetically closely related and clustered separately (125). Interestingly, a molecular marker segregating isolates originating from similar geographical focus was also associated with a higher MIC for amphotericin B (125).

Pathology of *M. mycetomatis* infection and ultrastructure of the lesion

In tissues, *M. mycetomatis* forms numerous black sclerotia (grains). Grains are vegetative aggregates of the fungal mycelia embedded in a hard brown matrix (figure 4) (44). This matrix consists of extra-cellular cement that seems to be 1,8-dihydroxynaphthalene melanin in combination with host tissue debris. This rigid matrix might act as a barrier protecting the fungus from the natural immunity of the host and from antifungal agents (95). Melanin-like pigments in *Cryptococcus neoformans* or the dimorphic fungal pathogen *Paracoccidioides brasiliensis* are detectable *in vitro* and *in vivo* and have an important role in the pathogenesis of various mycoses (56, 57). Melanins are thought to be protective in circumstances of host-induced oxidative stress (64, 69, 80).

The grains have different shapes and sizes. In stained sections, the grain is mostly rounded, oval, or trilobed. Two types of grains have been identified: filamentous and, less commonly, vesicular. The filamentous type of grain consists of brown, septate and branched hyphae that might be slightly more swollen and thick towards the periphery of the colony (44, 51, 95). The vesicular type of grain, which is less common, has a cement-like matrix in the cortex and a central area filled with numerous vesicles (6–14 μm in diameter) and light-coloured hyphae (105).

Figure 3: Various colony morphologies of *M. mycetomatis.* A: isolate mm44 on sabouraud agar, B: isolate mm55 on sabouraud agar, C: isolate mm55 melanised, on minimal medium agar

Triple layered tissue reaction zones have been described around the grains (51). An inner neutrophil zone immediately around the grain, an intermediate zone containing mainly macrophages, and an outer zone consisting of lymphocytes and plasma cells mainly can be seen under the microscope (figure 4) (42, 44, 51).

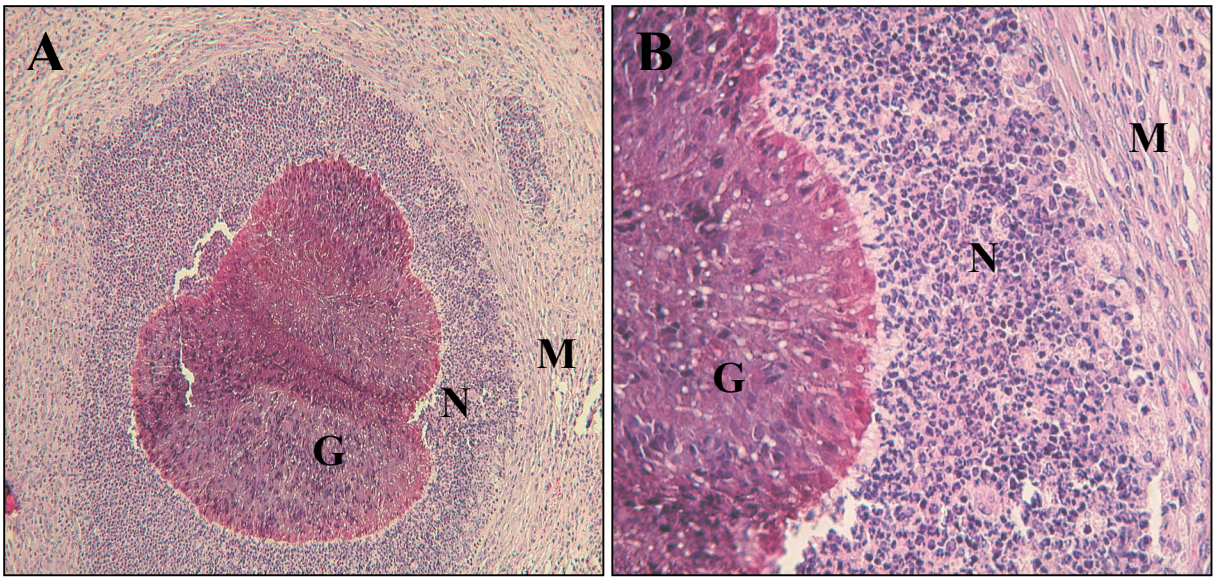


Figure 4: Triple layered tissue reaction around a *M .mycetomatis* grain 40x enlarged (left) and 400x enlarged (right). In both pictures the grain (G) is surrounded by the neutrophil layer (N) and the macrophage zone (M).

Studies on the ultrastructure of *M. mycetomatis* showed an elaborate development of mesosomes as the most striking ultrastructural feature (54). In tissue sections, the hyphae show a thick cell wall, which becomes thicker in the periphery of the grain (44, 48, 67). It seems that excessive production of polysaccharides in the fungal cell wall renders the fungus less susceptible to the action of antifungal agents and more resistant to the host immune system, owing to possible interference with leucocyte interaction with specifically recognised cell–wall antigens (66, 67, 132).

Fungal viability and antifungal susceptibility

The vegetative cells of *M. mycetomatis* die rapidly in conditions of moist heat (e.g., 60°C for 30 min) and are easily killed by 70% ethanol. Homogenised mycelia from fresh agar cultures survive well at 4°C, −20°, and −80°C for several weeks (unpublished data, A.O.A. Ahmed). Little is known about the *in vitro* and *in vivo* susceptibility of the fungus to different antifungal agents, especially to the new generation azoles and the echinocandins. In the past, some reports described the effectiveness of ketoconazole in treating mycetoma patients. Ketoconazole treatment usually leads to some degree of improvement (27, 91). Few investigators have described *in vitro* susceptibility testing results. Preliminary data have shown that azoles were capable of inhibiting the growth of at least 50% of all *M. mycetomatis* strains included in the studies. Unfortunately, the *in vivo* activity of azoles is often poor (14, 128). Still, long-term treatment with itraconazole seems to be the best therapeutic regimen at present. However, variable responses to itraconazole have also been described (14, 95). Eumycetoma due to *M. mycetomatis* responds poorly to medical treatment with ketoconazole, especially in late, advanced cases, but lesions of patients under ketoconazole treatment remain localised and well encapsulated (48).

Until recently, no rigorous study using a standard protocol had been done to assess the susceptibility of large numbers of *M. mycetomatis* clinical isolates. In general, most investigators agree that early diagnosis followed by both surgery and chemotherapy with appropriate antifungal agents results in the most successful outcome (12, 18, 49, 95). Mendez-Tovar and colleagues (96) have shown that progesteron can inhibit the growth of *M. mycetomatis* and *Pyrenochaeta romeroi*, which might contribute to the sex bias in the incidence of mycetoma (48, 95). Addition of hormone therapy to the standard chemotherapeutic regimen has not yet been assessed in clinical trials. Recently two antifungal susceptibility testing protocols were published (9). One was based on guidelines approved by CLSI (previously the National Committee for Clinical Laboratory Standards) for assessing culture base viability, whereas the other was based on XTT (2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) chemistry (65). Susceptibility data for a large number of clinical isolates were obtained for the antifungals amphotericin B and itraconazole (9). Most isolates were more susceptible to itraconazole, which might be the most obvious current choice for therapy of mycetoma. The notion is confirmed by recent clinical results (unpublished data, A.O.A. Ahmed).

Mycetoma in animals

In addition to humans, natural mycetoma has also been reported in dogs, cats, horses, cows, goats, and even dolphins (19, 43, 60, 62, 70, 71, 81, 94, 97, 104, 109, 113, 117, 123). Many investigators have reported reproducible animal models for bacterial mycetoma, but few have been successful in developing *M. mycetomatis* infection in small animals in the laboratory. Occasionally, the characteristic black grains due to *M. mycetomatis* have been reproduced in an animal. This was done either by inoculation of *M. mycetomatis* mixed with killed tubercle bacilli or by use of congenitally athymic nude mice. In immunocompetent mice such lesions

were sometimes seen after repeated intraperitoneal inoculation (26, 88, 99). The most reproducible current infection model for *M. mycetomatis* eumycetoma is the one described for BALB/c mice (11). Different routes of inoculation, various adjuvants, host immune status, and gender of mice were evaluated for effect on the infection rate. The infection was found to be inoculum-dependent with increasing infection rates for larger inocula. Adjuvants were essential for induction of infection and both autoclaved soil from an endemic region or Freund's incomplete adjuvant seemed to be very effective adjuvants. Small black grains were produced in the peritoneal cavity of infected mice, once more corroborating the validity of the mouse model. Establishment of infection in immunocompetent mice suggests that in human too, an intact immune system is unable to eliminate the infectious agent (11). The role of adjuvant in establishing the infection is not clear, but it might be favoring the infection by modifying the host immune response. *Scedosporium apiospermum* (teleomorph *Pseudallescheria boydii*) and *Scedosporium prolificans* can infect immunocompetent individuals causing eumycetoma or disseminated invasive infections in immunocompromised hosts. *S. prolificans* is resistant to virtually all commonly used antifungal agents, and therefore their infections are difficult if not impossible to treat. In an experimental murine model of *S. prolificans* scedosporiosis, the combination of amphotericin B and caspofungin increased mice survival when treatment started one day post inoculation. However, combination therapy of amphotericin B and caspofungin did not show synergy (16). In contrast, infections due to *S. apiospermum* have been treated successfully with different azoles including ketoconazole and itraconazole. Voriconazole chemotherapy together with surgical debridement was used to successfully treat nodular skin lesions due to *S. apiospermum* in kidney transplant patients (53).

Conclusions

The morbidity caused by eumycetoma is large, and local health-care facilities are usually insufficient. The clinical impact of mycetoma has essentially remained unchanged over the last decades. Clinicians can still offer limited help, which is mostly restricted to amputation and supporting chemotherapy for which the efficacy has not yet been unequivocally proven. Developing curative and preventive measures should be the prime focus of ongoing and future research activities in eumycetoma. Whether the simple distribution of shoes to prevent people being infected through direct contact with soil, or the use of a successful vaccine will fill this current vacuum remains to be seen.

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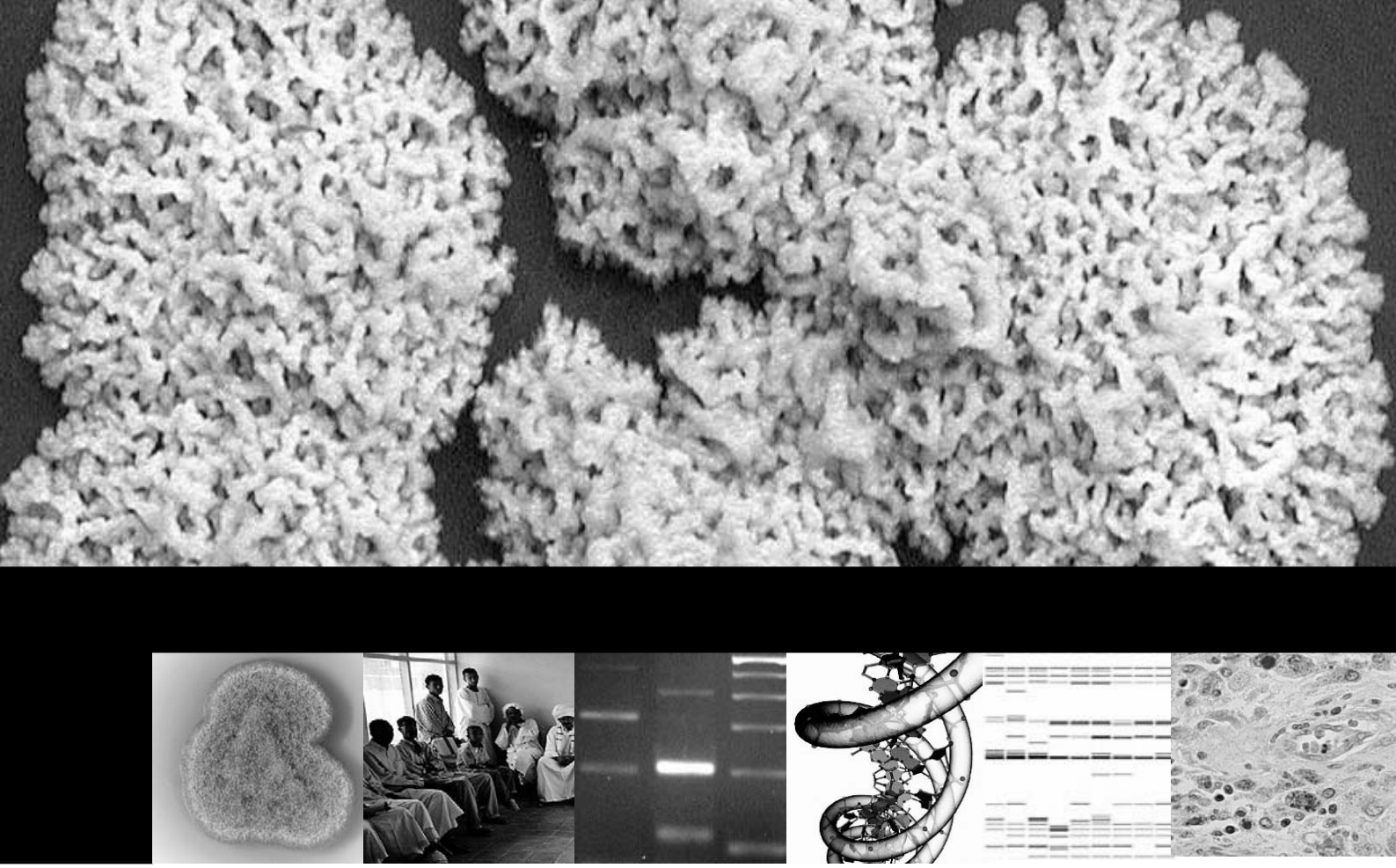
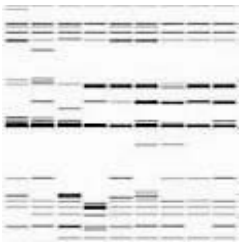


Genetic variation in Madurella mycetomatis and its human host

Chapter 3



Genotyping of Madurella mycetomatis by selective amplification of restriction fragments (AFLP®) and subtype-correlation with geographical origin and lesion size

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Abstract

One of the causative organisms of mycetoma is the fungus *Madurella mycetomatis*. Previously, extensive molecular typing studies identified Sudanese isolates of this fungus as clonal, and polymorphic genetic markers have not yet been identified. Here, we report on the selective amplification of restriction fragments (AFLP®) analysis of 37 Sudanese clinical isolates of *M. mycetomatis*. Out of 93 AFLP® fragments generated, 25 were polymorphic of which 12 were found in a large fraction of the strains. Comparative analysis resulted into a tree, composed of two main (clusters I and II) and one minor cluster (cluster III). Seventy-five percent of the strains found in cluster I originated from Central Sudan while in cluster II the origin of the strains was more heterogeneous. Furthermore, strains found in cluster I were generally obtained from larger lesions than the strains found in cluster II (Chi-Squared Test for Trend, p=0.03). From the 12 more commonly found polymorphisms four showed sequence homology with known genes. Marker A7 was homologous to an endo-1,4-beta-glucanase from *Aspergillus oryzae*, 97% identical markers A12 and B3 matched a hypothetical protein from *Gibberella zeae* and marker B4 was homologous to casein kinase I from *Danio rerio*. This latter marker seemed to be associated with strains originating from Central Sudan (p=0.001). This is the first report on a genotypic study where genetic markers were obtained which may be used to study pathogenicity in *M. mycetomatis*.

Introduction

In the 1840s, physicians of the Royal Army stationed in the Madura region in India reported an invasive disease which severely affected the foot. The foot degenerated into "one mass of disease of a fibrocartilaginous nature, with entire destruction of the joints, cartilages and ligaments". This disease is now known as mycetoma and can be differentiated into advanced cases and moderate to minor cases, with 80% of cases affecting the dorsal part of the foot (9, 17). However, it is also possible for other areas to become infected, including the hand, knee, arm, legs, head and neck (9). Since the first reports on mycetoma it appeared that mycetoma is not restricted to India but has a world-wide distribution (9). It is endemic around the Tropic of Cancer, between latitudes 15° South and 30° North (4). In these regions the climate is relatively arid and hot with alternating short rainy seasons and longer dry seasons (15). Countries where mycetoma has been reported include Argentina, Colombia, Venezuela, Yemen, Tunesia, Senegal, Somalia and Sudan (7-9). Sudan appears to have the highest number of cases per capita per year, which amounts to about 300-400 actual infections (17).

Mycetoma can be caused by a great variety of micro-organisms but these are not evenly distributed throughout the 'mycetoma belt' (17). In Sudan the most frequently encountered causative organism is *Madurella mycetomatis*. *M. mycetomatis* is a slow-growing fungus which forms a dark, sterile mycelium (4). Only two reports on genetic variability or lack thereof in *M. mycetomatis* isolates have appeared. In a report published by Lopes et al. it was shown that random amplification of polymorphic DNA (RAPD) and restriction endonuclease assays (REA) differentiated *M. mycetomatis* strains from different countries (14). In contrast, a large set of clinical *M. mycetomatis* isolates obtained from Sudan showed little genetic variation based upon classical high-throughput RAPD tests and PCR-RFLP tests and the species was identified as a clonal organism (2). Neither of these studies presented genetic markers, which could be used to generate epidemiologically relevant information or taxonomic frameworks. Another microbial DNA-based typing method is the AFLP® technique, a selective restriction fragment amplification method, which detects the absence or presence of DNA restriction sites by means of selective PCR (20, 21). Genomic DNA is completely digested with two restriction enzymes after which double-stranded adaptors are ligated to the resulting DNA fragments. The resulting fragments are then amplified using primers complementary to the adapter and restriction site sequences. To limit the number of amplified fragments, selective nucleotides can be added at the 3' ends of the primers (20, 21). AFLP® is a useful technique to differentiate strains within a species, even when clonal (20). The aim of the current study was to test whether the AFLP® technique could differentiate clinical *M. mycetomatis* isolates obtained from mycetoma patients in various regions of Sudan. Another important question addressed was whether genetic differences among *M. mycetomatis* isolates could be used to link isolates with demographic and clinical characteristics.

Materials and methods

Strains and patients

A total of 39 fungal isolates of black-grain eumycetoma patients were included. Thirty-eight of these strains were obtained from patients seen in the Mycetoma Research Centre, University of Khartoum, Sudan. One additional strain was obtained from a patient from Mali. The strains were isolated from biopsies and maintained on Sabouraud Dextrose Agar (Difco Laboratories, Paris, France). The strains were previously identified to the species level on the basis of morphology, PCR-based restriction fragment length polymorphisms (RFLP) and sequencing (1, 3). Thirty-seven of the isolates were identified as *M. mycetomatis*, one as a fungal species belonging to the *M. mycetomatis* cluster without appropriate species definition (strain mm27) and one as *Leptosphaeria senegalensis* (strain mm3) (6). For the patients the following characteristics were recorded: geographical origin, their lesion size, sex, age and duration of the disease. Assessment of the lesion size was by visual interpretation. Small lesions were those without sinuses and the volume of which was less than average. Large lesions clearly exceeded the average size and had multiple sinuses. No objective size parameters were developed.

Antifungal susceptibility

MICs were determined after 7 days using the colorimetric Sensititre® YeastOne® method (Trek Diagnostic Systems, Ltd, East Grinstead, England) as reported elsewhere (19).

DNA isolation

DNA was isolated as described before (2).

AFLP® analysis

AFLP® analysis was performed as described before (21). In short, DNA was restricted using endonucleases *Eco*RI and *Mse*I. After restriction, adaptors were ligated to the resulting fragments. The resulting fragments were pre-amplified with primers E: 5'-GACTGCGTACCAATTC-3' and M: 5'-GACGATGAGTCCTGAGTAA-3' after which a selective PCR was performed. The selective primers were identical to primers E or M but were extended with selective dinucleotides at the 3' terminus. Two primer combinations were used: E12/M12 and E20/M12. Primers E12 and M12 were extended with AC and primer E20 with GC. Primers E12 and E20 were radioactively labelled and the amplified material was analysed on 4.5% polyacrylamide slabgels. The presence or absence of markers is scored in a table, which could be transformed into a dendrogram with the program NTsys (Exeter Software, Sekautet, New York, USA).

Sequencing and primer design

Selected markers were excised from gel and reamplified with the following primers: 5'-AGCGGATAACAATTTCACACAGGACACACTGGTATAGACTGCGTACCAAT-3' and 5'-GACGATGAGTCCTGAGTAA-3'. These PCR fragments were sequenced, aligned and

compared to each other and to other sequences in the NCBI databank by BLASTN 2.2.8 and BLASTX 2.2.8 (5). Internal primers were designed for screening purposes (table 1).

Frag.	Accession number	Seq. Length (bp)	Present (%)	Homology	Primer	Sequentie (5' → 3')	Ann. temp. (°C)
A3	-	-	46.1	-	- -	- -	-
A4	AY918173	65	61.5	No homology	A4 fw A4 rv	CCT TCA TCG TGC CAC AGG CG CGC TGA GTA AAC GAG TTT TC	52
A5	AY918174	223	53.9	No homology	A5fw A5rv	TCA CAC TTA TCG AGG CAG AT GTA TGT CCA CGC AGG GCA TG	55
A6	-	-	51,3	-	- -	- -	-
A7	AY918175	161	15.4	endo-1,4-beta-glucanase	A7fw A7rv	ATG AGC TGG CTT GAT GGC GG TCC TGA GTA AAC GTC GAT CC	55
A8	-	-	35.9	-	- -	- -	-
A9	-	-	51.3	-	- -	- -	-
A10	AY918172	180	69.2	No homology	A10 fw A10 rv	GAG GAT GCG ACT TCG CCG CT CGC GAC GAG GGA CGT GAA TC	60
A11	AY918176	168	80.6	No homology	A11 fw A11 rv	GTA TAG TAC TAC GAC CAC CA CTT AGG ACT AGT ATA ACT AG	50
A12	AY918177	235	71.8	Hypothetical protein*	A12 fw A12 rv	CAG CGA AGC ACT AAT GAG GT CCA TGT TAG TAT CCT ACA AG	52
B3	AY918178	233	28.2	Hypothetical protein*	B3 fw B3 rv	AAG CAT CTA TGA GGT GAC CA CCA TGT TAG TAT CCT ACA AG	52
B4	AY918179	387	33.3	casein kinase 1, delta	B4 fw B4 rv	TTC GCC ACA TAC ATT GAC TA TTC TAA ATC GGC TGT AAG TC	50

Table 1: *Madurella mycetomatis* polymorphisms detected by AFLP® and the PCR primers at conditions designed for detecting each individual polymorphism. * The sequences obtained for these two fragments are 97% homologous. - no sequences determined

PCR analysis of the selected polymorphisms

The PCRs were performed in 50 µl reaction volumes containing 50 ng DNA, 1x Supertaq PCR buffer 1 (HT Biothechnology Ltd., United Kingdom), 0.2 mM PCR nucleotidemix (Amersham Life Sciences, Roosendaal, The Netherlands), 25 pmol forward primer, 25 pmol reverse primer and 1.2 U Supertaq (HT Biothernology Ltd., United Kingdom). The PCR consisted of a pre-denaturation step of 4 minutes at 94°C and 40 cycles each of 1 minute denaturation at 94°C, 1 minute annealing at variable temperatures and 1 minute elongation at 72°C each. This was followed by a post-elongation step of 7 minutes at 72°C. The annealing temperatures differ for each fragment and are stated in table 1. PCR products were visualised by electrophoresis on 3% agarose gels (Hispanagar, Sphaero Q, Leiden, The Netherlands).

Statistical analysis

Associations between fungal DNA polymorphisms, demographics and disease characteristics were tested for significance with the Fisher's Exact test (two-sided). The association of genetic features with geographic origin of the strain was studied by comparing each region with all others. The association with size of the lesion (small, medium, large) was tested with the Chi-Squared test for Trend. Correlations with age, disease duration, and MIC's to antifungals were tested with the Mann-Whitney test (two-tailed). All statistical calculations

were performed with GraphPad InStat version 3.00 (GraphPad InStat Software, Inc., San Diego, USA) except for the adjustment of the lesion size for disease duration. This latter was done by linear regression analysis with SPSS release 10.1.0 (SPSS, Inc., Chicago, USA).

Results

AFLP® fragment analysis

With the two primer combinations used for the 39 clinical black grain mycetoma isolates, 93 AFLP® markers were generated. Comparative analysis of the markers resulted into a score table and the phylogenetic tree shown in figure 1.

From the tree it can be concluded that strains mm3 and mm27 differ considerably from the other strains. Strain mm3 appeared to be the fungus *Leptosphaeria senegalensis* and strain mm27 is an as yet ill defined species but still a close relative of *M. mycetomatis* (see Materials and Methods and reference (6)) Consequently, mm27 is more closely related to the *M. mycetomatis* isolates than mm3. This was verified by comparisons of ITS sequences. In table 2 it can be seen that the ITS sequences obtained for strains mm39 and mm55 only differ in 0.6% from the already published ITS sequence for *M. mycetomatis* (GenBank accession number AF162133). The ITS sequence for mm27 (7.3% difference) and *L. senegalensis* (30.8% difference) differs considerably more.

Name	ITS difference (%)	Difference in bp	Identification at the species level
Reference	0	0	*Madurella mycetomatis*
Mm55	0.6	3	*Madurella mycetomatis*
Mm39	0.6	3	*Madurella mycetomatis*
Mm27	7.3	39	*Madurella species*
Mm3	30.8	164	*Leptosphaeria senegalensis*

Table 2: ITS sequence comparison of mm27 and mm3 with true *Madurella mycetomatis* isolates. ITS differences are stated as the percentage of base pair differences with the previously published sequence AF162133. Variability within the genus *M. mycetomatis* was noted at 8 positions (14).

In the *M. mycetomatis* cluster twenty-five markers were polymorphic. Thirteen of these markers were only incidentally seen in one to three strains while the rest of the markers were seen in at least 15% of the strains. This means that out of the *M. mycetomatis* markers, 26.9% were polymorphic, which is a relatively large fraction as compared to our previous RAPD data (2).

Two of the strains used in this study, strains mm72 and mm73 were isolated from a female patient with two independent large lesions, one on the sole of the foot and one in the knee. The lesion on the sole of the foot had been there for over 13 years while the lesion on the knee joint was just 4 years old. Figure 1 shows that both isolates are found in cluster I and are closely related but not identical. Strains mm33 and mm44 appeared to be 100% "AFLP® identical". Those strains originated from two different Central Sudanese patients (a 24-year-old male with a moderate lesion and a 28-year-old female with a large lesion) belonging to the same tribe. They were infected for two years, which could imply that they were infected with

the same strain originating from somewhere in that area. In cluster II strains mm46 and mm50 had exactly the same AFLP® banding pattern. Those two strains derived from Central Sudanese patients (a 28-year-old male and a 35-year-old male). Those two patients had both been infected for 1 year and had only small lesions.

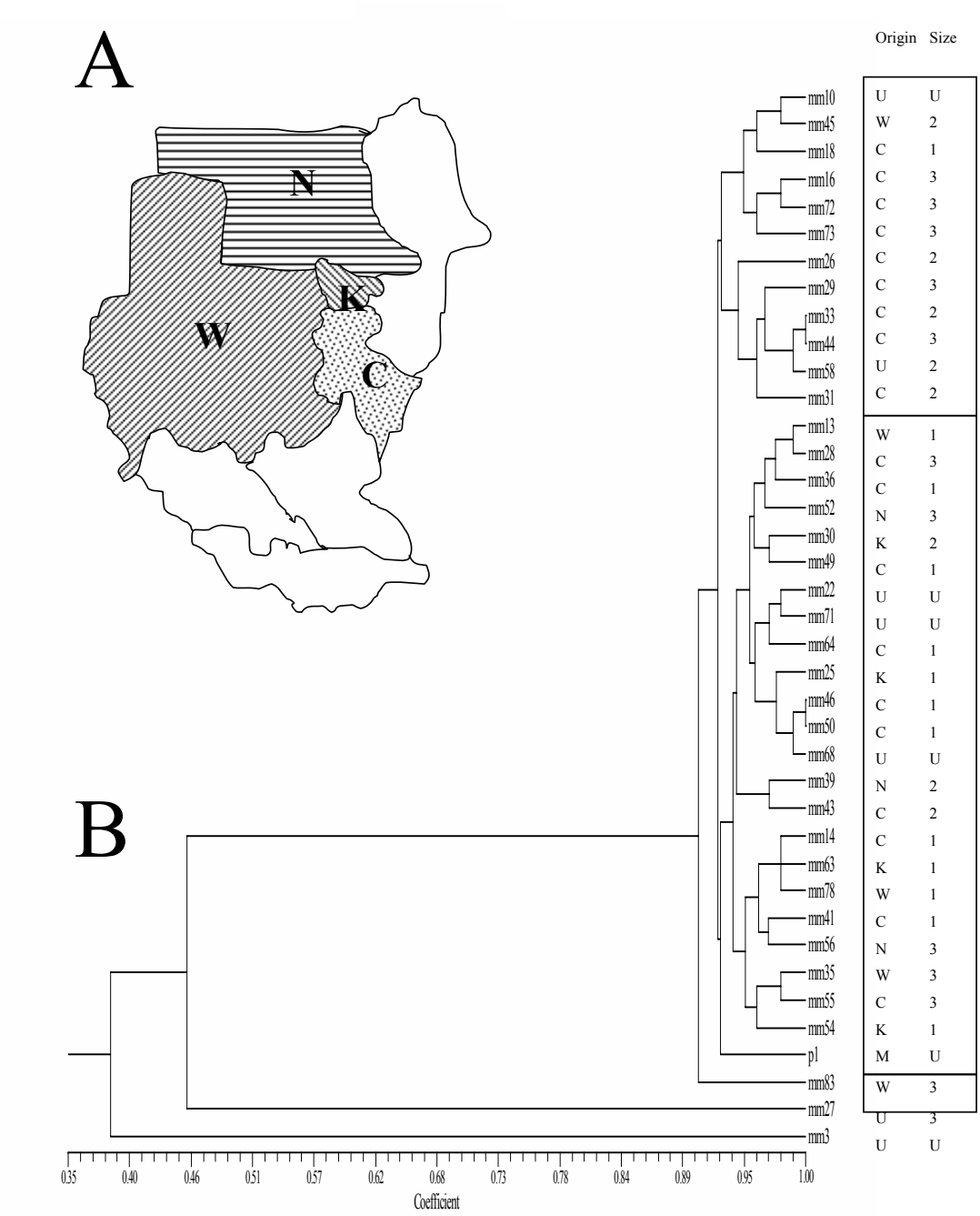

Figure 1: A: Sudanese sampling areas for clinical *Madurella mycetomatis* isolates, Sudan is divided into Central Sudan (C), Western Sudan (W), Northern Sudan (N) and Khartoum (K). B: Phylogenetic tree based on the AFLP® data obtained for 39 clinical black grain mycetoma fungal isolates. Thirty-seven of these isolates are *Madurella mycetomatis* isolates. Strain mm3 represents the fungus *Leptosphaeria senegalensis* and strain mm27 represents a fungal species belonging to the *M. mycetomatis* cluster without precise species definition. The *M. mycetomatis* strains are divided into clusters I, II and III. For each isolate the geographic origin and the size of the lesion are presented. Strains originated from Central Sudan (C), Western Sudan (W), Northern Sudan (N), Khartoum (K) and Mali (M). The lesion size is designated as small (1), moderate (2) and large (3). (U = unknown).

The *M. mycetomatis* strains were divided in two main clusters: I and II (figure 1). Minor cluster III consists of only one strain (mm83) which originated from Western Sudan and caused a large lesion. Cluster I and cluster II have several distinctive features but the most striking one is that in cluster I fragment A12 is largely absent and fragment B3 is largely present while in cluster II this is the other way around. Seventy-five percent of the strains found in cluster I originated from Central Sudan, while in cluster II only 45.8% of the strains originated from Central Sudan. The other 54.2% from the strains encountered in this cluster are divided as follows: 16.7% of the strains originated from Khartoum, 12.5% from North-Sudan, 8.3% from West-Sudan and 4.2% (n=1) from Mali. Data on lesion size are displayed next to the phylogenetic tree in figure 1 and it appeared that in cluster I only one strain caused a small lesion. All other strains in cluster I caused moderate (41.7%) or massive (41.7%) lesions. In cluster II, half of the strains caused small lesions while 12.5% and 33.3% caused moderate and large lesions, respectively. This may point to differences in virulence between the strains from the two main clusters.

DNA sequencing of polymorphic markers

Out of 93 amplimers, a total of 25 markers were polymorphic in the *M. mycetomatis* strains. Thirteen of these markers were only seen in one to three strains while the rest of the markers were seen in at least 15% of the strains. Eleven of these more common polymorphic markers were reamplified and sequenced. From four of these fragments no useful sequence could be obtained, probably due to the fact that mixtures of DNA fragments were excised from the gel. After BLASTN and BLASTX analysis, it appeared that the sequences obtained for fragments A12 and B3 were 97% identical. A tri-base substitution and two deletions were the only differences obtained, suggestive of gene duplication or heterozygostiy. Fragment A7 showed the highest homology (66% identity) with an endo-1,4-beta-glucanse gene from *Aspergillus oryzae*. Fragment B4 showed similarity with a casein kinase 1 isoform delta gene. The highest homologies for this gene were obtained with the species *Danio rerio*, namely 55%. The closely related fragments A12 and B3 matched a gene for a non-characterized hypothetical protein from *Gibberella zeae*. The other four fragments showed no significant homology with any other known sequence.

Development of PCR tests

Because no genome data are available for *M. mycetomatis*, internal PCR primer pairs were designed for all of the sequences obtained. PCR with the novel primers for fragments A4, A5, A7, A12 and B3 resulted in equally sized amplicons for all strains, except for mm3 and mm27. For fragments A10, A11 and B4 size-variable amplicons were obtained. As is stated in table 3, PCR for fragments A11 and B4 resulted only in positive PCR signals for those strains in which the original AFLP® fragment was present. For fragment A10, all strains gave a positive PCR signal but in the strains where the original AFLP® fragment was not present the PCR products were smaller in size. This size difference still allowed the discrimination of different types.

		Strain																																		
		10	13	14	16	18	22	25	26	28	29	30	31	33	35	36	39	41	43	44	45	46	49	50	52	54	55	56	58	63	64	68	71	72	73	78	83	p1
A10	A	0	1	1	1	1	1	1	2	1	1	0	0	1	0	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0
	P	1'	1	1	1	1	1	1	1'	1	1	1'	1'	1	1'	1	1	1	1	1	1'	1	1'	1	1	1'	1'	1	1	1	1	1	1	1	1	1	1'	1'
A11	A	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1
	P	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1
B4	A	0	0	1	1	0	1	0	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0
	P	0	0	1	1	0	1	0	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0

Table 3: Presence of AFLP® amplimers versus PCR amplicons A = AFLP, P = PCR, 0 = no amplicon present, 1 = amplicon present, 1' = amplicon present but slightly smaller, 2 = very faint amplicon present

Analysis of correlations

Strains from Central Sudan significantly more often belonged to cluster I (p = 0.049; table 4). With respect to the AFLP® markers tested we found the B4 fragment (a casein kinase delta homologue) in half of the strains originating from Central Sudan, but in none of the strains from the other regions (p = 0.001). Lesions caused by cluster I strains tended to be larger than those caused by other strains (p = 0.03). The size of the lesion appeared strongly associated with duration of disease (p=0.001, Pearsons's correlation coefficient). However, the association between lesion size and cluster did not decrease after adjustment for disease duration. No linkage with either cluster was found between genetic strain features and sex or age of the patients, or with duration of disease.

Trait	Clusters			Marker			
	I	II	P-value	marker	p-value	absent	present
Geographic origin							
Central Sudan versus the rest of Sudan	9/1	10/10	0.049	B4	0.001	8/12	11/0
West Sudan versus the rest of Sudan	1/9	3/17	NS		NS		
North Sudan versus the rest of Sudan	0/10	3/17	NS		NS		
Kharthoum versus the rest of Sudan	0/10	4/16	NS		NS		
Lesion Size							
Small versus Moderate versus Large	1/5/5	12/3/5	0.03		NS		
Sex							
Male verus Female	6/6	17/6	NS		NS		
Age of the patients in years (median)	19	21.5	NS		NS		
Duration of the disease in years (median)	3	3	NS		NS		
Antifungal Susceptibility (median MIC)							
Amphotericin B	1	0.5	NS	A4	0.02	1	0.5
	0.5	1		B4	0.02	0.5	1
Fluconazole	2	8	NS		NS		
Itraconazole	0.032	0.032	NS		NS		
Ketoconazole	0.032	0.064	NS		NS		
Voriconazole	0.125	0.125	NS		NS		

Table 4: Correlations of *Madurella mycetomatis* strains genetic clusters and polymorphic markers and various demographics, disease characteristics and antifungal susceptibility. P-values were obtained by Fisher's Exact test, Chi-Squared Test for Trend (size of the lesion), and Mann Whitney test (age, duration, and comparison of MICs).

Additionally, associations between genetics of the strains and antifungal susceptibility were studied. In figure 2A the distribution of the antifungal susceptibilities of cluster I and II are shown. MICs were obtained for the azoles itraconazole, ketoconazole, fluconazole and voriconazole as well for the polyenic amphotericin B, but not for 5-flucytosine.

As was already seen in the MIC distribution in figure 2A there is no obvious correlation between the antifungal susceptibility and strain clustering. This was confirmed with the Mann-Whitney test. For the individual markers the same analyses were performed. After statistical analysis of two of the polymorphic markers, namely A4 and B4, a correlation was found with susceptibility to amphotericin B. These data are shown in figure 2B. As is seen in this figure strains positive with A4 had lower MICs for amphotericin B (median MIC 0.5) than strains in which this fragment was not present (median MIC 1) (Mann-Whitney, p = 0.02). Strains for which fragment B4 could be amplified had higher MICs for amphotericin B (Median MIC of 1.0) than strains without B4 (Median MIC of 0.5) (p = 0.02). This difference is very small. However, all four strains with a MIC for amphotericin B of 2 or higher (usually implicated in therapy failure) were found in the group without fragment A4. Absolutely no association between the other markers and susceptibility for any of the antifungals tested was found.

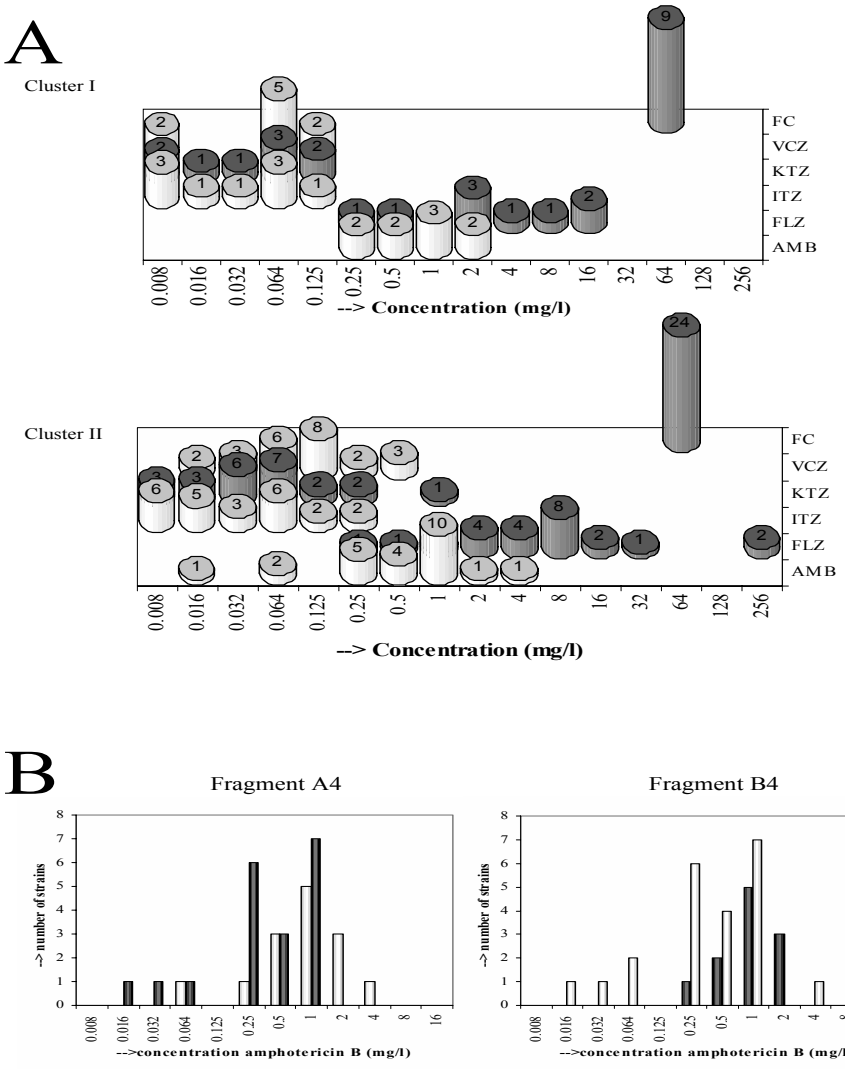

Figure 2: A: The antifungal distributions for both clusters I and II are shown for the following antifungal agents: amphotericn B (AMB), fluconazole (FLZ), itraconazole (ITZ), ketoconazole (KTZ), voriconazole (VCZ) and 5-flucytosine (FC). Each bar represents the number of strains (as shown on the top of the bar) with a MIC at the concentration listed on the x-axis for the antifungal shown on the y-axis. For example in cluster I there are three strains with an MIC of 1 mg/l for amphotericin B. B: the MIC distribution for amphotericin B for fragments A4 and B4. The number of strains for a certain MIC are shown for the two fragments. The strains for which a fragment is present are indicated with black bars (■), the strains for which this fragment is absent are indicated with white bars (□).

Discussion

Not much is known about genetic diversity in *M. mycetomatis*. Until now only two reports appeared in which genetic heterogeneity was investigated (2, 14). Lopes et al. collected 17 isolates from countries all over the world, ranging from the Netherlands Antilles to Argentina and from Djibouti to Morocco (14). By using both REA and RAPD it appeared that the 17 strains could be divided into 10 different groups (14). In contrast, the 38 strains from Sudan and 2 from Mali used in high throughput RAPD analysis by Ahmed et al. appeared to be highly clonal and all attempts to identify genetic markers failed (2). In the study performed by Lopes et al. the isolates obtained from Sudan could not be discriminated from each other as well, confirming regional clonality (14).

In the past AFLP® was demonstrated to be a valuable technique to type various fungal species such as *Aspergillus fumigatus* and *Cryptococcus neoformans* (12, 22). For *M. mycetomatis* we showed that AFLP® is as valuable for the to differentiation of this species as well. Despite the remarkable clonality found before with RAPD (2) AFLP® was able to discriminate these same strains into three clusters. This suggest that AFLP® is a much stronger technique than RAPD to discriminate *M. mycetomatis*. With AFLP® the *M. mycetomatis* isolates could be divided into two main and one minor clusters. Cluster I consisted mainly of strains originating from Central Sudan which caused moderate or large lesions while cluster II was more heterogeneous. Although there was an association with the lesion size and the duration of the disease the association found between cluster I and larger lesions still remained intact after adjustment for disease duration. As a matter of fact, the lesion size is probably defined on the basis of individual host-pathogen interactions. With the AFLP® conditions used it was also possible to discriminate between two *M. mycetomatis* isolates obtained from the same patient but from different lesions.

At least four of the 12 polymorphic markers were actually part of coding regions, two of which were not yet identified (A12/B3). Marker A7 was homologous to the gene for endo-1,4-beta-glucanase, an important enzyme involved in cellulose degradation (11, 18). Polymorphic marker B4, homologous to casein kinase 1 (CK1) delta, was primarily detected in strains isolated from Central Sudanese patients. CK1 is thought to play a physiological role in activating transcription of various DNA repair genes, in intracellular trafficking and in normal cell cycle progression (10, 13, 16, 23).

In the present study, MICs were determined by using the Sensititre system instead of using the modified NCCLS method for *M. mycetomatis*. It has been demonstrated that this system is in good agreement with the modified NCCLS method. Percentages agreement ranged between 91.2-100.0 %, depending on the antifungal tested (19). The reproducibility of this test system was also satisfactory ranging between 88.2 to 97.1 % (19). We found an association between susceptibility for amphotericin B and both markers B4 and A4. The distribution of MIC's of strains with and without the B4 and A4 marker differed significantly ($p=0.02$), but the difference was very small. The median of the groups differed only a single two-fold dilution, which is allowed as background variation in antifungal susceptibility testing. Furthermore, the median MICs for each group were in the treatable range and are therefore probably not clinically relevant. The association found in this study could be a

chance finding and not reproducible in other strain collections. Further study to the relevance of this finding is needed.

In conclusion, the AFLP® method differentiates clinical isolates of *M. mycetomatis*. AFLP® groups I and II are associated with different clinical presentation, with the group I strains apparently causing larger lesions. An AFLP® marker sequence with CK1 homology seemed to be associated with the geographical origin of the fungal isolate. We present the first pathogenicity markers for a fungal species that still has a devastating socio-economic effect on small communities in rural Sudan.

Nucleotide sequence accession numbers

The sequences reported here are deposited in the GenBank database under accession numbers AY918172 (fragment A10), AY918173 (fragment A4), AY918174 (fragment A5), AY918175 (fragment A7), AY918176 (fragment A11), AY918177 (fragment A12), AY918178 (fragment B3) and AY918179 (fragment B4).

Acknowledgements

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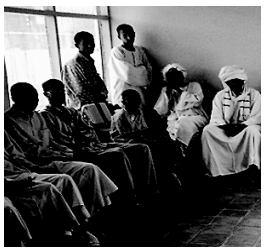
Chapter 4



Polymorphisms in genes involved in innate immunity predispose towards mycetoma susceptibility

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Abstract

Madurella mycetomatis is the main causative agent of mycetoma, a tumourous fungal infection characterized by infiltration of large numbers of neutrophils at the site of infection. In endemic areas the majority of inhabitants have antibodies to *M. mycetomatis,* though only a small proportion of individuals actually develop mycetomal disease. It, therefore, appears that neutrophils are unable to clear the infection in some individuals. To test this hypothesis, eleven single nucleotide polymorphisms (SNPs) involved in neutrophil function were studied in a population of Sudanese mycetoma patients versus geographically and ethnically matched controls. Significant differences in allele distribution for interleukin 8 (CXCL8), its receptor CXCR2, thrombospondin-4 (TSP-4), nitric oxide synthase 2 (NOS2) and complement receptor 1 (CR1) were found. Further, the $NOS2^{Lambaréné}$ polymorphism was clearly associated with lesion size. The genotypes obtained for CXCL8, its receptor CXCR2 and TSP-4 all predisposed to a higher CXCL8 expression in patients, which was supported by the detection of significantly elevated levels of CXCL8 in patient serum. The NOS2 genotype observed in healthy controls was correlated with an increase in NOS2 expression and higher concentrations of nitrate and nitrite in control serum. We present the first evidence of human genetic predisposition towards susceptibility to mycetoma, a neglected infection of the poor.

Introduction

Madurella mycetomatis is the most common fungal causative agent of eumycetoma in Sudan (2). This agent is abundantly present in the soil and on the vegetation in the endemic region (1). Chances for coming into contact with this fungus are, therefore, high for inhabitants of these areas. When using an ELISA system based on crude fungal extracts all individuals in the Sudanese endemic regions seemed to posses IgG antibodies against this fungus (2). With an ELISA based on a specific antigen of *M. mycetomatis,* the translationally controlled tumour protein (TCTP), antibody levels were found to be elevated in endemic control populations as well, although these levels were lower than for the patient population. No antibodies were found in Caucasian controls from Europe (38). This implies that most of the individuals living in endemic regions are regularly exposed to this pathogen, but that only a small percentage of these individuals actually develops the disease. A predisposing factor could be that the immune status of mycetoma patients is impaired (27). Using the tuberculin test, 2,4-dinitrochlorobenzene sensitization and lymphocyte proliferation induced by phytohaemagglutinin, deficiencies in cell-mediated immunity were previously documented among patients (27). Also, differences in blood group antigens between mycetoma patients and a matching healthy control population were investigated, but no correlation with blood group type and development of mycetoma was reported (12). Investigators did not note defects in HLA mediated presentation of pathogen-derived peptides to T-cells (12). It is currently not known if mycetoma patients suffer from substantial immune defects.

In previous reports it was shown that large numbers of neutrophils are present in the mycetoma lesion (10, 11, 17). Apparently, neutrophils are important in the early defence against mycetoma. Since the neutrophils are unable to clear the infection it was hypothesized that there might be genetic impairment in neutrophil function in mycetoma patients.

Neutrophils and monocytes are attracted to the site of infection by either antigens secreted by the invading micro-organism or by locally produced host-chemokines such as interleukin 8 (CXCL8), monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor α (TNFα). In mycetoma it has previously been shown that neutrophils are actively attracted by antigens secreted by *M. mycetomatis* in a complement dependent manner (40). Normally, when neutrophils arrive at the site of infection they will eliminate the pathogen through phagocytosis. In order to ingest pathogens, neutrophils are equipped to directly recognize either molecules on the surface of invading microbes or their opsonisation with serum host proteins including complement factors, mannose binding lectin (MBL) or antibodies. An example of such a receptor is complement receptor 1 (CR1), which is also the determinant for the Swain-Langley (Sl) blood group antigen and the McCoy (McC) blood group antigen (31, 36). After pathogen recognition, reactive oxygen species (ROS) including hydrogen peroxide, superoxide and nitric oxide are formed, which effectively kill ingested micro-organisms (21). This process is summarised in Figure 1.

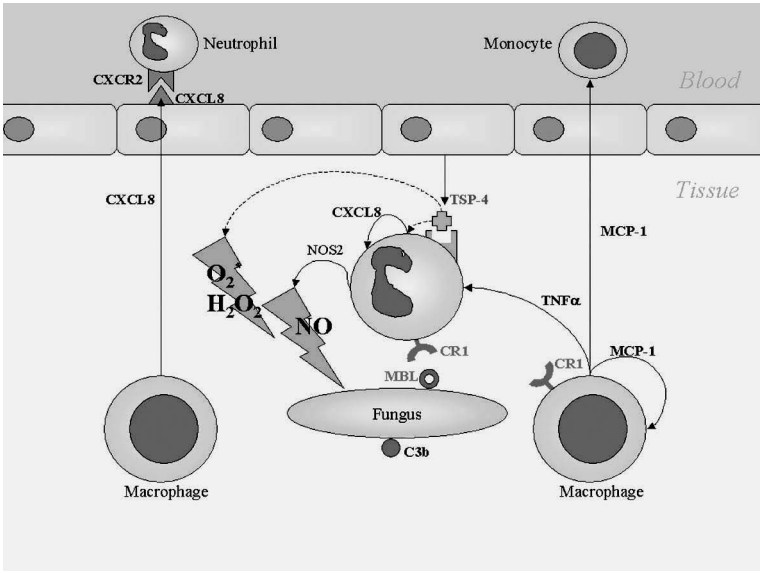

Figure 1: A simplified scheme of the innate immune response against fungi. When a fungus enters the body the innate immune system will be activated. Complement is activated via the alternative and lectin binding pathways by binding of C3 and MBL, respectively, to the fungal surface. Both pathways lead to the formation of the opsonin C3b on the fungal surface, which is recognized by complement receptor 1 (CR1) expressed by macrophages and neutrophils. MBL is also recognized by this receptor. Macrophages are already present in healthy tissue and will release a number of cytokines in order to kill the invading pathogen and attract more monocytes and neutrophils from the bloodstream to the site of infection. Monocytes are attracted by macrophage chemoattractant protein-1 (MCP-1) which will mature to macrophages into the tissue. Neutrophils are attracted by interleukin 8 (CXCL8). CXCL8 is recognized by neutrophils via receptors CXCR1 and CXCR2. Both chemokines also activate their target cells; in particular, CXCL8 and TNFα , which activate neutrophils to generate among others nitric oxide via nitric oxide synthase 2 (NOS2). Thrombospondin 4 (TSP4) is secreted by endothelial cells to stimulate the production of oxygen radicals and excretion of CXCL8.

Many genetic polymorphisms that influence phagocytosis and killing by neutrophils have been described. For instance, a point mutation in the nitric oxide synthase type 2 promotor, namely NOS2^{Lambaréné}, has been shown to be associated with a 7-fold higher NOS activity (24). This genotype, and subsequently its higher nitric oxide levels, were shown to offer protection against severe malaria to a level similar to that of the sickle cell trait (24). Since neutrophils attracted to the mycetoma lesion apparently are unable to clear the infection, we investigated whether selected single nucleotide polymorphisms (SNPs) in genes involved in neutrophil function were more commonly found in the patient population than in an endemic reference population or vice versa.

Materials and methods

Study cohort

Blood samples were taken from patients and a matched control population in the Sudan endemic area between 2001 and 2004. Furthermore, from some of the patients biopsies were taken as well. No co-infections were recorded. Serum was stored at –20 ^{o}C until further use. For patients, the duration of disease, the size of the lesion and the site of infection were recorded. The mean age of the patients was 27.4 years (7-80 years), 72.8 % of the patients was male, which were figures comparable to those of the matched endemic control population (mean age 28.6, 73.8% male). For the patients, the mean duration of the disease was 6.98 years (<1 year-27 year). 89.6% of the patients had eumycetoma, 8% actinomycetoma and for 2.4% the type of mycetoma was not known. 78.4% of the patients had a lesion on the foot,

9.6% on the hand, 11.2% on the lower leg and 0.8% (1 patient) had a lesion on both foot and hand. 51.2% of the patients had a small lesion, 48.8% had a moderate to large lesion. Lesion size was measured in a comparable and standardized manner among mycetoma patients. A more clear definition of size was not possible since mycetoma lesions are diffuse, have a mass, and have ill-defined margins.

DNA isolation and genotyping

Genomic DNA was isolated from 265 blood samples (125 patients and 140 controls) with the large volume kit for the MagnaPure (Roche, Almere, The Netherlands) according to the manufacturer's descriptions. DNA was stored at –20 ^{o}C until further use. All PCR primers and amplification conditions are stated in Table 1. Genotyping of CXCR2, CXCL8 and TNFα was performed using a PCR tetra-primer amplification refractory mutation system (ARMS) (31). Genotyping of the other genes was performed with classical PCR-RFLP methods. Restriction enzymes used are also shown in Table 1 and were either obtained from New England Biolabs (Leusden, The Netherlands) or Fermentas (St. Leon-rot, Germany). All restriction endonucleases were used as described by the manufacturer.

Polymorphism		primer sequence (5'-->3')	PCR program 5'94^{o}C → …. → 7'72^{o}C	Restr. Enzyme	allele	length (bp)	Ref.
CR1							
Sl	24KnNde	ACCAGTGCCACACTGGACCAGATGGAGAAC-AGCTGTTTGAGCAT	44x(1' 94^{o}C+1' 58^{o}C+1' 72^{o}C)	MfeI	1 2	305 261+44	36
	25Rb	GGAGGAGTGTGGCAGCTTG					
McC	24KnNde	ACCAGTGCCACACTGGACCAGATGGAGAAC-AGCTGTTTGAGCAT	44x(1' 94^{o}C+1' 58^{o}C+1' 72^{o}C)	BsmI	a b	305 166+139	36
	25Rb	GGAGGAGTGTGGCAGCTTG					
CXCL8							
-251T→A	CXCL8-infw(T)	GTTATCTAGAAATAAAAAAGCATACAA	5x(1' 94^{o}C+1' 52^{o}C+1' 72^{o}C)→ 30x(1' 94^{o}C+1' 47^{o}C+1' 72^{o}C)	None	T A*	349+169 349+228	31
	CXCL8-inrv(A)	CTCATCTTTTCATTATGTCAGAG					
	CXCL8-outfw	CATGATAGCATCTGTAATTAACTG					
	CXCL8-outrv	CACAATTTGGTGAATTATCAAA					
CXCR2							
+785C→T	CXCR2-infw(C)	TCTTTGCTGTCGTCCTCATCTTCCTGAT	5x(1' 94^{o}C+1' 67^{o}C+1' 72^{o}C)→ 30x(1' 94^{o}C+1' 62^{o}C+1' 72^{o}C)	None	C T	451+226 441+281	31
	CXCR2-inrv(T)	AGGACCAGGTTGTAGGGCAGCCAGAAA					
	CXCR2-outfw	CTGCTTGTCTTACTTTTCCGAAGGACCG					
	CXCR2-outrv	TCTTGAGGAGTCCATGGCGAAACTTCTG					
MBL							
54	MBLfw	GTAGGACAGAGGGCATGCTC	35x(30s 94^{o}C+ 1' 58^{o}C+ 1' 72^{o}C)	BanI	w* m	245+84 329	39
	MBLrv	CAGGCAGTTTCCTCTGGAAGG					
57	MBLfw	GTAGGACAGAGGGCATGCTC	35x(30s 94^{o}C+ 1' 58^{o}C+ 1' 72^{o}C)	MboII	w* m	329 248+81	39
	MBLrv	CAGGCAGTTTCCTCTGGAAGG					
XY	MBLpromfw	GTTTCCACTCATTCTCATTCCCTAAG	35x(30s 94^{o}C+30s 60^{o}C+45s 72^{o}C)	BsaJI	X Y*	242+108 166+108+76	34
	MBLpromrv	GAAAACTCAGGGAAGGTTAATCTCAG					
MCP-1							
-2518	MCP-1fw	GCTCCGGGCCCAGTATCT	5x(1' 94^{o}C+1' 52^{o}C+1' 72^{o}C)→ 30x(1' 94^{o}C+1' 47^{o}C+1' 72^{o}C)	PvuII	A G*	236 182+54	14
	MCP-1rv	ACAGGGAAGGTGAAGGGTATGA					
NOS2							
Lambaréné	NOS-F	CATATGTATGGGAATACTGTATTTCAG	40x(30sec 94^{o}C+1' 60^{o}C+1' 72^{o}C)	BsaI	G C*	680 490	25
	NOS-4	TCTGAACTAGTCACTTGAGG					
TNFα							
-308G→A	TNF-308fwin	TGGAGGCAATAGGTTTTGAGGGGCAGGA	5x(1' 94^{o}C+1' 67^{o}C+1' 72^{o}C)→ 30x(1' 94^{o}C+1' 62^{o}C+1' 72^{o}C)	none	G* A	323 + 224 323 + 154	31
	TNF-308rvin	TAGGACCCTGGAGGCTGAACCCCGTACC					
	TNF-308fwout	ACCCAAACACACGCCTCAGGACTCAAC					
	TNF-308rvout	AGTTGGGGACACGCAAGCATGAAGGATA					
TSP							
29926	TSP4fw	AATTCCGCATCTTCACTTCAC	32x(40s 94^{o}C+30s 59^{o}C+40s 72^{o}C)	AvaII	C* G	143 + 78 221	42
G→C	TSP4rv	AACCGGTTCTGCTTTGATAAC					

Table 1: Primer sequences and PCR conditions for the different polymorphisms. All polymorphisms described here resulted not only in a nucleotide change but also in a functional amino-acid change, expect for CXCR2. For most genes difference of amino-acid resulted in differences in expression of the protein encoded. The allele which was indicated with higher expression of the corresponding protein is indicated with*.

CXCL8 expression
CXCL8 expression was measured in serum from 43 patients and 37 healthy controls with a CXCL8 ELISA (Diaclone, Amsterdam, The Netherlands) according to the manufacturer's instructions.

NOS production
The concentrations of nitrite and nitrate in serum from 43 patients and 37 healthy controls were determined as a reflection of NOS activity. Serum was diluted 4-fold in a solution containing 50 μM NAPDH (Sigma-Aldrich, Zwijndrecht, The Netherlands), 5 μM flavin adenine dinucleotide (FAD) (Sigma-Aldrich) and 200 U/l nitrate reductase (Sigma-Aldrich). To convert nitrate into nitrite the sample was incubated for 20 min at 37 ^{o}C. Excess NADPH was oxidised by adding 7760 U/l lactate dehydrogenase (Fluka Biochemicals, Buchs, Switzerland) and 10 mM sodiumpyruvate (Sigma-Aldrich) during a further incubation of 5 min at 37 ^{o}C. Finally, the sample was deproteinised by adding 10 μl of 300 g/l zinc sulphate. The sample was centrifuged for 10 min at 10,000 rpm and 100 μl of the supernatant was used to determine the nitrite concentration colorimetrically using the Griess Reagent System (Promega, Madison, WI). Concentrations of nitrite were estimated by comparing absorbance readings at 540 nm against those of standard solutions of sodium nitrite.

In situ analysis
Five mycetoma biopsies and one biopsy from the uninfected part of the foot of a mycetoma patient were embedded in paraffin. The mean age of the patients was 25.4 years (22-30 years), two of the patients were male. The mean duration of the disease was 5.3 years (2.5 year-9 year). All patients were infected with *M. mycetomatis* and had a lesion on the foot. One of the patients had a small lesion, the others had large lesions. Biopsies taken from the infected parts of the foot were chosen on the basis of the visible presence of grains to ascertain that grains were present in stained slides.

Slides were deparaffinized in xylene, dehydrated through a graded ethanol series and washed in distilled water. In order to retrieve the antigen epitopes, slides were heated for 10 min at 650 W in a microwave oven in 10 mM citrate buffer pH 6.4. Endogenous peroxidase was blocked by immersing the slides in 0.3% H_2O_2 in methanol for 30 min at room temperature. Non-specific bindingsites were blocked with 1:50 diluted normal goat serum (Vector Laboratory, Burlingame, CA) for 1 h at room temperature. Then the sections were incubated with the primary antibody at 4^{o}C overnight. Anti-CXCL8 (H-60, sc-7922, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used in a concentration of 4 μg/ml, anti-NOS2 (N-20, sc-651, Santa Cruz Biotechnology Inc.) in a concentration of 2 μg/ml. Both primary antibodies were rabbit polyclonal antibodies. Sections were further incubated with biotinylated goat anti-rabbit IgG (Vector) for 1 h at room temperature and another 30 min in ABC reagent (Vector). Peroxidase was developed with 3-amino-9-ethylcarbazole (Sigma-Aldrich) for 12 min. Development was stopped by washing 15 min in PBS-0.05% Tween. Sections were counterstained with haematoxylin (Sigma-Aldrich). For reference purposes some of the slides were also stained with haematoxylin and eosin or Grocott stain. As a control 2 *M. mycetomatis* isolates, *in vitro* cultured on Sabouraud agarose, and 1 *Candida*

albicans isolate were stained for endogenous CXCL8 like molecules, following the same procedure.

Statistical analysis

Verification of Hardy-Weinberg equilibrium was performed with Pearson's chi-square test . The effect of human polymorphisms in susceptibility to mycetoma was assessed with the logistic regression model (SPSS 11.0). Differences in allele frequency were analysed with the Fisher's exact test (GraphPad Instat software). The significance of differences in CXCL8 and nitrite/nitrate concentrations in serum were calculated with the Mann Whitney Test (GraphPad Instat software). P values lower than 0.05 were considered significant.

Results

Neutrophils are attracted to the site of infection

As is seen in figure 2 neutrophils are attracted to the site of infection. Around the fungal grain two main types of inflammatory reaction can be observed (11). The first was the type I reaction, where *M. mycetomatis* grains were surrounded by a large zone of neutrophils (Figure 2A) (11). The second reaction was characterized by the presence of histiocytes and multinucleated giant cells and a small number of neutrophils (Figure 2B).

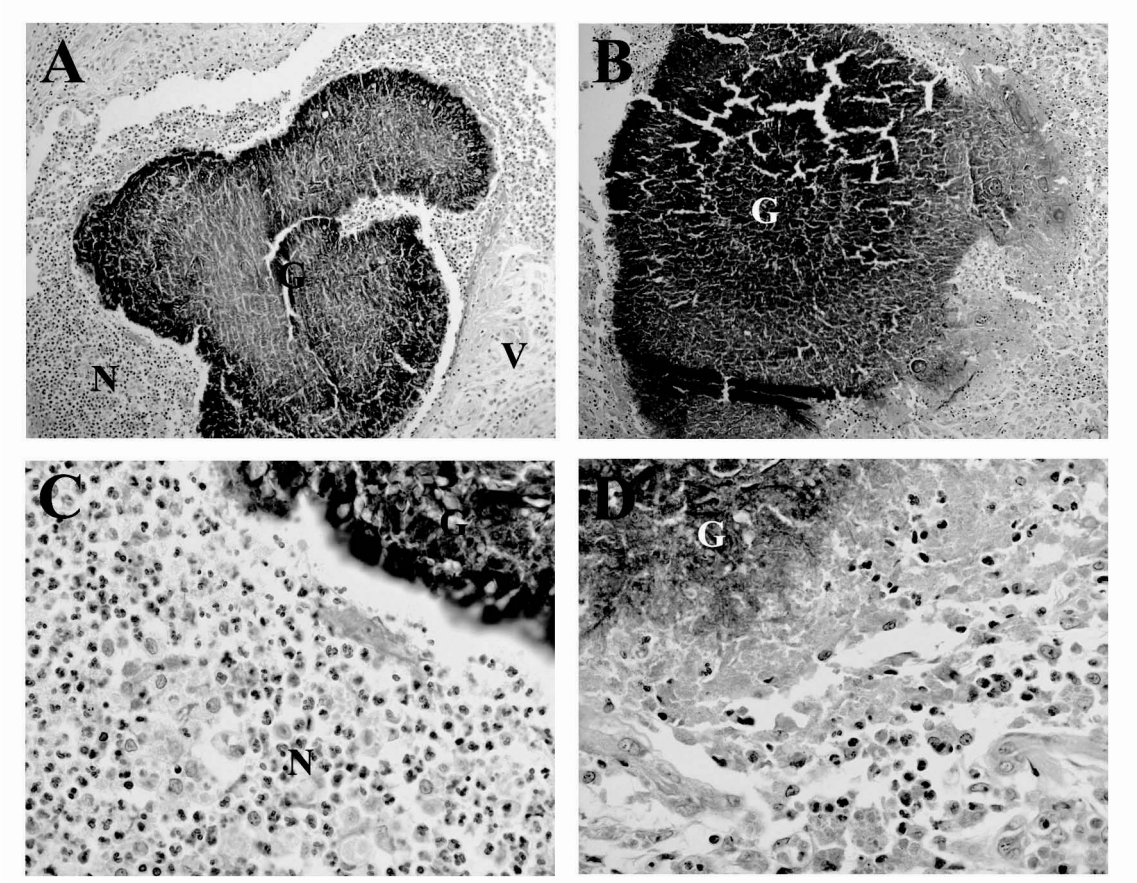

Figure 2: Two different inflammation reaction types in mycetoma (HE stained). A: type I inflammation reaction, characterized by an inner zone of neutrophils (N) surrounding the grain embedded in cementmaterial (G) and an outer vascular zone (V) (100x enlarged). B: type II inflammation reaction, in which the neutrophil zone is absent and is replaced by histiocytes and multinucleated giant cells (100x enlarged). C: type I inflammation reaction, 400 x enlarged. D: type II inflammation reaction, 400x enlarged.

CXCR2, CXCL8, TSP-4, NOS2 and CR1 polymorphisms are differentially distributed among mycetoma patients versus healthy endemic matched controls

To elucidate the possible deficiencies in neutrophil function among mycetoma patients, genotype and allele frequencies for the genes encoding for CXCR2, CXCL8, MCP-1, TNFα, MBL, MBL promoter, CR1, NOS2, and TSP-4 were determined. As shown in Table 2, genotype distributions for all SNPs reached Hardy Weinberg equilibrium except for CXCL8. Genotype distribution for CXCL8 in the control population was in disequilibrium (HWE, p = 0.003), but was in equilibrium in the patient population (HWE, p = 0.81). Differences in genotype distributions were found for CR1, CXCR2 and NOS2 (Table 2). Significant differences in allele frequencies were found for CR1, CXCR2, CXCL8, TSP-4, and NOS2 (Table 3). Obviously, the statistical significances of the CR1, CXCL8 and NOS2 polymorphisms are the most important ones, given the low p-values. No significant differences were found for MCP-1, TNFα, MBL, and MBL promoter SNPs.

Gene		Mycetoma genotype			
		Patients N=125 (%)	Controls N=140 (%)	Hardy-Weinberg Equilibrium	Binary logistic regression OR (95% CI)
CR1					
Sl	11	46 (36.8)	63 (45.0)	0.83	1
	12	53 (42.4)	61 (37.9)		2.23 (1.1-4.6)
	22	26 (20.8)	16 (11.4)		1.19 (0.7-2.0)
McC	aa	105 (84.0)	93 (66.4)	0.40	aa versus ab and bb
	ab	19 (15.2)	44 (31.4)		0.38 (0.21-0.68)
	bb	1 (0.8)	3 (2.1)		
CXCL8					
-251	AA	82 (65.6)	78 (55.7)	0.003	AA versus AT and TT
	AT	39 (31.2)	43 (30.7)		0.66 (0.4-1.0)
	TT	4 (3.2)	19 (13.6)		
CXCR2					
+785	TT	8 (6.4)	13 (9.3)	0.78	TT versus TC and CC
	TC	38 (30.4)	57 (40.7)		0.38 (0.21-0.68)
	CC	79 (63.2)	70 (50.0)		
MBL					
54	ww	113 (90.4)	132 (94.3)	0.73	Ww versus wm
	wm	12 (9.6)	8 (5.7)		1.76 (0.7-4.4)
	mm	0 (0)	0 (0)		
57	ww	95 (76.0)	104 (74.3)	0.06	Ww versus wm
	wm	28 (22.4)	30 (21.4)		0.91 (0.5-1.6)
	mm	2 (1.6)	6 (4.3)		
XY	XX	19 (15.2)	21 (15.0)	0.05	1
	XY	48 (38.4)	52 (37.1)		1.05 (0.5-2.1)
	YY	58 (46.4)	67 (47.9)		1.07 (0.6-1.8)
MCP1					
2518	AA	79 (63.2)	91 (65.0)	0.46	AA versus AG and GG
	AG	36 (28.8)	42 (30.0)		1.08 (0.7-1.8)
	GG	10 (8.0)	7 (5.0)		
NOS2					
L	GG	107 (85.6)	94 (67.1)	0.21	GG versus GC and CC
	GC	18 (14.4)	44 (32.9)		0.34 (0.2-0.6)
	CC	0 (0)	2 (0)		
TNFα					
-308	AA	106 (84.8)	117 (83.6)	0.29	AA versus AT and TT
	AT	17 (13.6)	23 (16.4)		0.91 (0.5-1.8)
	TT	2 (1.6)	0 (0)		
TSP4					
29926	GG	83 (66.4)	108 (77.1)	0.96	GG versus GC and CC
	GC	36 (14.4)	30 (21.4)		1.71 (1.0-2.9)
	CC	6 (2.4)	2 (1.4)		

Table 2: Genotype distributions of mycetoma patients in comparison to a matching healthy control population.

Gene	Allele	Mycetoma Allele frequency Patients N=125 (%)	Controls N=140 (%)	P value	OR (95% CI)
CR1					
Sl	1	145 (58.0)	187 (66.8)	**0.0390**	**0.68 (0.48-0.98)**
	2	105 (42.0)	93 (33.2)		
McC	a	229 (91.6)	230 (82.1)	**0.0014**	**2.37 (1.38-4.08)**
	b	21 (8.4)	50 (17.9)		
CXCL8					
-251	A	203 (81.2)	199 (71.1)	**0.0081**	**1.76 (1.17-2.65)**
	T	47 (18.8)	81 (28.9)		
CXCR2					
+785	T	54 (21.6)	83 (29.6)	**0.0372**	**0.65 (0.44-0.97)**
	C	196 (78.4)	197 (70.4)		
MBL					
54	w	238 (95.2)	272 (97.1)	0.2618	0.58 (0.23-1.45)
	m	12 (4.8)	8 (2.9)		
57	w	218 (87.2)	238 (85.0)	0.5306	1.20 (0.73-1.97)
	m	32 (12.8)	42 (15.0)		
XY	X	86 (34.4)	94 (33.6)	0.3988	1.04 (0.72-1.49)
	Y	164 (65.6)	186 (66.4)		
MCP1					
2518	A	194 (77.6)	224 (80.0)	0.5236	0.87 (0.57-1.32)
	G	56 (22.4)	56 (20.0)		
NOS2					
L	G	232 (92.8)	232 (83.6)	**0.0006**	**2.67 (1.51-4.72)**
	C	18 (7.2)	48 (16.4)		
TNFα					
-308	A	229 (91.6)	257 (91.8)	1.0000	
	T	21 (8.4)	23 (8.2)		
TSP4					
29926	G	202 (80.8)	246 (87.9)	**0.0301**	**0.58 (0.36-0.94)**
	C	48 (19.2)	34 (12.1)		

Table 3: Allele frequencies of mycetoma patients in comparison to a matching healthy as assessed with the Fisher's exact test. P-values and Odds ratio's are given. Significant p-values are highlighted in the bold lettertype.

Genotype	Lesion Size Massive N=41 (%)	Moderate N=20 (%)	Small N=64 (%)	Allele	Lesion Size Massive N=41 (%)	Moderate N=20 (%)	Small N=64 (%)	p-value (massive compared to small)	OR (95% CI)
CR1 SI									
11	14 (34.1)	8 (40.0)	24 (37.5)	1	44 (53.7)	25 (62.3)	76 (59.4)	0.4753	0.79 (0.45-1.39)
12	16 (39.0)	9 (45.0)	28 (43.8)	2	38 (46.3)	15 (37.5)	52 (40.6)		
22	11 (26.8)	3 (15.0)	12 (18.8)						
CR1 McC									
aa	36 (87.8)	17 (85.0)	52 (81.3)	a	77 (93.9)	36 (90.0)	116 (90.6)	0.4487	1.59 (0.54-4.70)
ab	5 (12.2)	2 (10.0)	12 (18.8)	b	5 (6.1)	4 (10.0)	12 (9.4)		
bb	0 (0.0)	1 (5.0)	0 (0.0)						
CXCL8									
AA	31 (75.6)	11 (55.0)	40 (62.5)	A	72 (87.8)	30 (75.0)	101 (78.9)	0.1368	1.93 (0.88-4.23)
AT	10 (24.3)	8 (40.0)	21 (32.8)	T	10 (12.2)	10 (25.0)	27 (21.1)		
TT	0 (0.0)	1 (5.0)	3 (4.7)						
CXCR2									
CC	2 (4.9)	1 (5.0)	5 (7.8)	C	64 (78.0)	30 (75.0)	102 (79.7)	0.8623	0.91 (0.46-1.79)
TC	14 (34.1)	8 (40.0)	16 (25.0)	T	18 (22.0)	10 (25.0)	26 (20.3)		
TT	25 (60.9)	11 (55.0)	43 (67.2)						
NOS2									
GG	31(75.6)	15 (75.0)	61 (95.3)	G	72 (87.8)	35 (87.5)	125 (97.7)	**0.0063**	**0.17 (0.05-0.65)**
GC	10 (24.3)	5 (25.0)	3 (4.7)	C	10 (12.2)	5 (12.5)	3 (2.3)		
CC	0 (0.0)	0 (0.0)	0 (0.0)						
TSP-4									
GG	28 (68.3)	12 (60.0)	43 (67.2)	G	67 (81.7)	30 (75.0)	105 (82.0)	1.0000	0.98 (0.48-2.01)
GC	11 (26.8)	6 (30.0)	19 (29.7)	C	15 (18.3)	10 (25.0)	23 (18.0)		
CC	2 (4.9)	2 (10.0)	2 (3.1)						

Table 4: Allele frequencies of mycetoma patients with massive, moderate and small lesions. Differences in allele frequencies in patients with large lesions compared to patients with small lesions were assessed with the Fisher Exact test.

Mycetoma patients do more often posses the Sl2 and McC^a genotypes of CR1

In the gene encoding for CR1 two different polymorphisms were determined, namely the Sl polymorphism and the McC polymorphism. These polymorphisms were previously shown to be associated with resistance to severe malaria (36). In the patient population the allele Sl2 was more often found than in the control population (Tables 2 and 3). The McC^b allele was more dominant in the control population. To assess if one of these polymorphisms in the gene

encoding CR1 were also associated with disease progression, the allele frequencies obtained for the patients were divided into three groups according to lesion size. Allele frequencies for the Sl allele and the McC allele were compared between the group with the largest lesions and the group with the smallest lesions. It appeared that in both groups no differences in allele frequencies for the Sl allele (p=0.47, Table 4) and the McC allele (p=0.45, Table 4) were found.

Mycetoma patients express high levels of CXCL8 during infection

Different allele frequencies were also found in the genes encoding for the neutrophil attractant CXCL8, its receptor CXCR2 and TSP-4. The genotypes for these genes that were more often encountered in the patient population were all correlated to phenotypes expressing high CXCL8 levels. When comparing the allele frequencies of the patients with large lesions with the allele frequencies of the patients with the small lesions, no correlation was found between these allele frequencies and the size of the lesion (Table 4).

To analyse whether the neutrophils present at the site of infection did indeed express CXCL8, lesion tissue was stained for CXCL8. CXCL8 producing cells were found in all samples. Grains surrounded by a so-called type I tissue-reaction (Figure 1) had only a few CXCL8 positive cells, neutrophils generally produced no CXCL8. More CXCL8 positive cells were noticed during a type II tissue reaction. Cells expressing CXCL8 were mainly macrophages, especially macrophages with hemosiderin deposits or cells surrounding them (Figure 3). Interestingly on about 50% of the hyphae within the grain CXCL8 was found (Figure 3A). This was not found when cultured *M. mycetomatis* was stained with antibody CXCL8. In contrast cultured *C. albicans* did stain with antibody CXCL8 which agrees with previously published data (3). CXCL8 only appeared to be present at the site of infection (n=5), since in control tissue from a non-infected part of the foot no CXCL8 expression was noted (n=1).

Because of the presence of CXCL8 at the site of infection it was presumed that it was also secreted in serum. Therefore, a CXCL8-specific ELISA was performed to measure the amount of CXCL8 present in serum. As is seen in Figure 4 serum CXCL8 levels were significantly elevated in mycetoma patients (mean=431.2 pg/ml). This increase was statistically highly significant when compared to the matched endemic population (Mann-Whitney, $p<0.0001$). To assess if the CXCL8 concentration was also an indication of the severity of the disease, it was analysed whether the CXCL8 serum concentrations found in patients with large lesions were higher than the concentrations found in patients with small lesions. No significant correlation with the size of the lesion and the amount of CXCL8 present in serum was found (Mann-Whitney, p=0.0973).

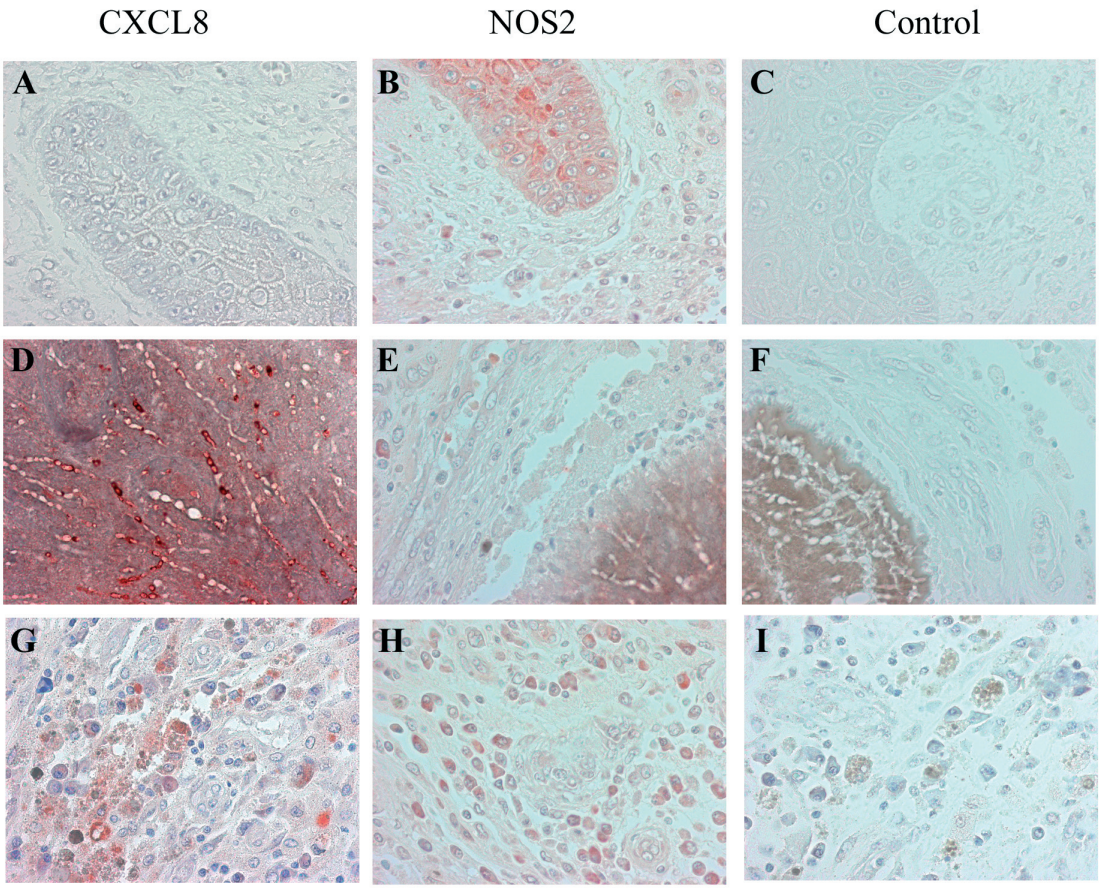

Figure 3: CXCL8 and NOS2 in *M. mycetomatis* mycetoma infected skin (400x enlarged). A: no CXCL8 is found in the epidermis. B: NOS2 expression in the epidermis. C: no expression is found in the control. D: CXCL8 expression on individual hyphae in the grain. E: No expression of NOS2 on hyphae, but some expression found in cells surrounding the grain. F: Control slide of the grain. G: high CXCL expression in activated macrophages and cells surrounding them in the vascular zone. H: NOS2 expression in the vascular zone. I: activated macrophages in the vascular zone of the non-stained control .

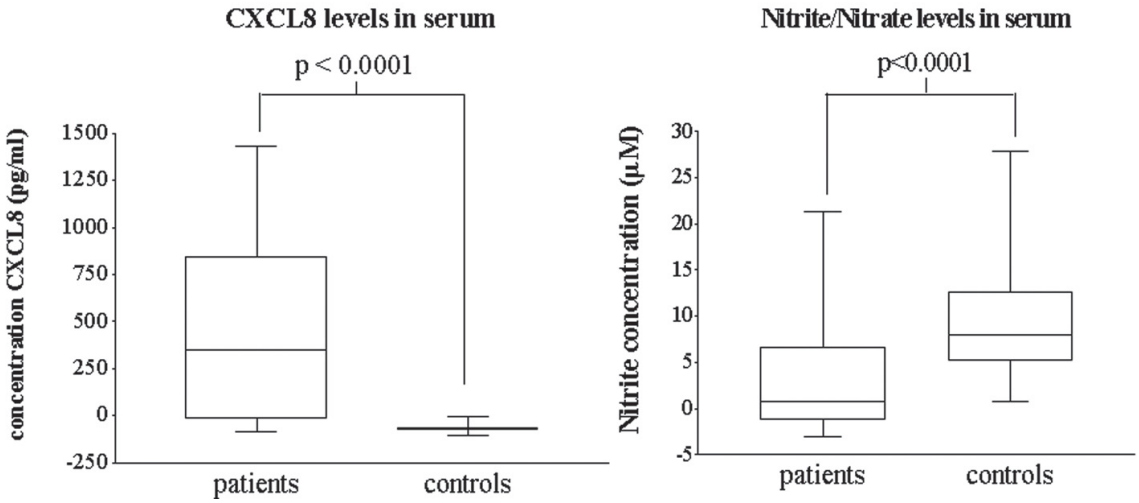

Figure 4: CXCL8 and nitrite/nitrate levels in serum. A: CXCL8 levels (pg/ml) determined in the serum of patients and a healthy Sudanese control population. B: nitrite/nitrate levels (µM) determined in the serum of patients and a healthy Sudanese control population. Significance was calculated with the Mann-Whitney test

NOS2 is expressed at the site of infection

The last polymorphism which was not equally distributed between patients and endemic control populations was in the gene encoding NOS2. The NOS2^{Lambaréné} polymorphism appeared to be more common in the control population as compared to the patient population. With immunohistochemistry it was shown that NOS2 was present at the site of infection (Figure 3). NOS2 was expressed throughout the entire dermis and epidermis. NOS2 production was found in the stratum corneum and stratum spinosum in both infected and uninfected parts of the foot and was therefore probably not specific for mycetoma (Figure 3B). NOS2 expression was also found in phagocytic cells. The number of NOS2 positive cells differed per patient and per grain but were clearly present in all 5 patients. The closer the grain was to the dermis, the more NOS2 positive cells were detected.

The frequency of the NOS2^{Lambaréné} polymorphism differed among sub-groups. Not only was the polymorphism more often found in the control population, but it was also more frequent among patients with the largest lesions. Only 3 out of 64 patients with small lesions displayed this polymorphism, whereas the frequency of this polymorphism was much higher in the patient group with the largest lesions (in 15 out of 61 patients ($p=0.0027$, Table 4)). Since the NOS2^{Lambaréné} polymorphism was more frequent in the control population we expected to find a higher NOS activity in the control sera. Therefore, the nitrite and nitrate concentrations in serum were determined. As is seen in Figure 3 the serum nitrite and nitrate concentrations were significantly lower among patients (mean=2.83 μM) than in the matched endemic control population (mean=9.28 μM). Since the NOS2^{Lambaréné} polymorphism was also found more often in patients with larger lesions it was determined whether the nitrite/nitrate concentration in serum of these patients were higher than in serum of patients with smaller lesions. Although the patients with the larger lesions did indeed have a higher nitrite/nitrate concentration in their serum (mean=4.36 μM versus mean=2.55 μM), the difference was not statistically significant (Mann-Whitney, $p=0.6028$).

Discussion

In this study it was shown that neutrophils are attracted to the mycetoma grains *in situ.* Two main types of inflammatory reaction were observed. Both reactions could be seen in the same lesion and are not unique to *M. mycetomatis.* They are also observed in mycetoma caused by *Petriellidium boydii*, *Neotestudina rosatii*, *Fusarium spp.* and *Acremonium* spp. (17). It has been suggested that the type I reaction is an early response to grain formation which is succeeded by the type II reaction (10). Apparently, neutrophils are important in the early defence against mycetoma. Some differences in genotype distributions between patients and a matched endemic population for some genes involved in neutrophil function were observed. The control individuals who were sampled were living in the same region as the patients and had a similar tribal and ethnic background. Unfortunately, at the time of collection of the samples we were not in a position to collect extensive amounts of demographic and health related data and we therefore cannot be totally sure that the control group was a complete match to the patient population. When the mycetoma patients were compared to the control population, differences were found for CR1, CXCL8, CXCR2, TSP4 and NOS2. No differences in distribution of SNPs in MCP-1, TNFα, MBL and MBL promoter were found.

In mycetoma patients the Sl2 and McC^a genotypes of CR1 were more common than in the endemic control population. The CXCL8, CXCR2 and TSP-4 genotypes correlated with a higher CXCL8 production, the NOS2 SNP correlated with lower NOS2 secretion. The latter were confirmed by physiological measurements of higher CXCL8 levels and lower nitrate/nitrite levels in patient serum.

Sl2 and McC^a genotypes of CR1 in mycetoma patients

From the data presented in the present paper it appeared that having a deviating complement receptor 1 could enhance the chance of developing a mycetoma infection. This observation was in agreement with our hypothesis that mycetoma patients have a genetic impairment in neutrophil function. Although CR1 is expressed on neutrophils, it is not unique for this cell type. In fact, CR1 is a receptor expressed by a whole range of other cells including follicular dendritic cells, macrophages, T- and B- lymphocytes, and erythrocytes. Two of the polymorhisms in the CR1 gene are responsible for the Swain-Langley (Sl) blood group antigen and the McCoy (McC) blood group antigen, both members of the Knops blood group typing system (32). Here we showed that the Sl2 and the McC^a alleles were more often found in mycetoma patients than in the matched endemic control population. This was unique since with other blood group typing systems such as the ABO blood groups and Rhesus factors no association was found with a predisposition to develop mycetoma (12). The McC^a allele associated with mycetoma was already described to be associated with severe cerebral malaria caused by *Plasmodium falciparum* (36). In contrast, the Sl2 allele offered some protection against this type of malaria (36).

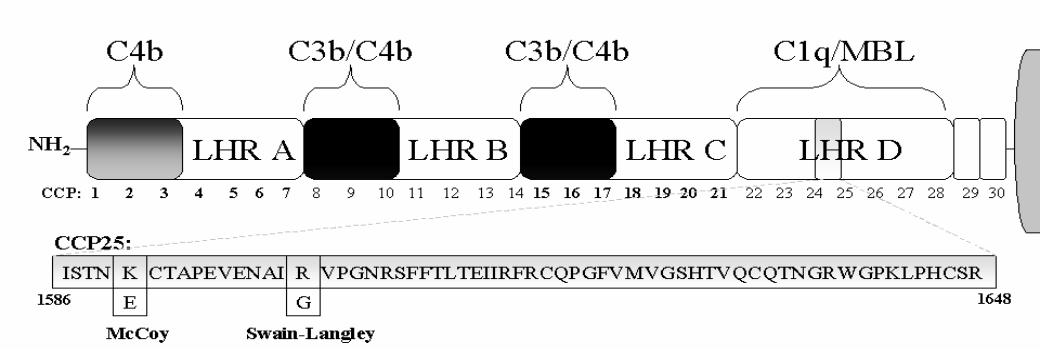

Figure 5: A schematic figure of complement receptor 1 (CR1). Complement receptor 1 is composed of 20 complement control protein repeats (CCPs), which are arranged in four long homologous regions (LHRs). CCP1-3 represent a C4b binding site, CCP8-10 and CCP15-17 represent two identical copies of a C3b/C4b binding site. In LHR D an additional bindingsite exists for C1q and MBL. The Knops blood group type polymorphisms are found in CCP25, on aminoacid positions 1590 and 1601

As is seen in Figure 5, the Sl and McC polymorphisms are present in the long, homologous repetitive D region of the CR1 gene. This is the region which codes for the binding structure of the protein in which MBL- and C1q binding occurs and could therefore cause conformational changes which could influence the function of the molecule, not only as executed on erythrocytes but also on other cell types (22, 36). On neutrophil surfaces, CR1 binds pathogens such as *Escherichia coli* and *Staphylococcus aureus* and presents them to phagocytic cells (15, 23). It is, therefore, conceivable that conformational changes in the

receptor also influence the efficacy of *M. mycetomatis* phagocytosis (15, 23). However, this remains to be determined since the effects of these polymorphisms on the function of the receptors has not yet been defined in full detail.

Mycetoma patients express high levels of CXCL8 during infection

Additional differences in allelic distributions were found for the genes encoding CXCL8, its receptor CXCR2 and TSP4, which implied that alterations in neutrophil attraction are associated with the development of mycetoma. In mycetoma patients the CXCL8 -251A allele, the CXCR2 +785C allele and the TSP4 29929C allele (also known as the 387P variant) were found more often than in the control population. These alleles are all associated with an increased production of CXCL8 (31). CXCL8 is produced by many cell types, including macrophages, as a chemokine to attract neutrophils to the site of infection. TSPs are also secreted at the site of injury and stimulate the chemotactic response of neutrophils (29). CXCR2 is activated by CXCL8 and this activation enhances the generation of ROS and the phagocytosis of pathogens (33). CXCL8 is usually barely detectable in the normal skin, but strong CXCL8 production can be observed in psoriasis, atopic dermatitis and in acute generalized exanthematous pustulosis (19). Here we show that in the mycetoma lesions CXCL8 is also abundantly present. The extent of CXCL8 expression appeared to be dependent on the inflammation type with more CXCL8 positive cells present during the type II reaction, a reaction characterized by a higher amount of histiocytes and giant cells. Macrophages with hemosiderin deposits and with high CXCL8 expression were found in the vascular zone (see Figure 3G). CXCL8 was also found on hyphae within the grain, suggesting that CXCL8 is bound to the *M. mycetomatis* hyphae in order to prevent either neutrophil attraction or activation. Another explanation could be that CXCL8 simply becomes trapped when the cement material is formed. Cement material is composed of remnants of fungal and host cells. It is not expected that *M. mycetomatis* forms CXCL8 analogs such as *C. albicans* does, since cultured *M. mycetomatis* did not react with anti-CXCL8 antibody (3).

CXCL8 was not only expressed in the skin, high amounts of CXCL8 were also detected in the serum of the mycetoma patients. CXCL8 concentrations normally found in infectious diseases are 5-10 times lower as reported here (18, 26, 37) except for very severe infections such as gram-negative bacteremia (20). The concentrations of CXCL8 found in the serum of the mycetoma patients were even 10 times higher as concentrations found in skin diseases like psoriasis (6). However, such elevated concentrations of CXCL8 are not exceptional since in skin diseases such as dermatitis herpetiformis, a skin condition characterized by the accumulation of neutrophils, comparable concentrations CXCL8 were found (16). Apparently, neutrophil accumulations are accompanied by high CXCL8 concentrations in serum, which supports the data suggesting that CXCL8 production is indeed important during the innate immune response to mycetoma.

NOS2 is expressed in lower amounts in patients than in controls

The fifth gene with SNP frequency differences between patient and controls was *NOS2*. This gene encodes a synthase involved in generating nitric oxide, a radical toxin to most micro-organisms (41). However, nitric oxide can play a dual role in infections. Nitric oxide defends

the host against various microbial agents, but sometimes the nitric oxide-mediated inflammation causes too much damage to host cells and thereby conversely supports microbial invasion (41). Comparing allelic distribution of the NOS2 gene variants, it appeared that the NOS2 G954C mutation was more common among Sudanese healthy controls than in mycetoma patients. This genotype was considered beneficial since the substitution from G to C results in a phenotype with a 7-fold higher baseline NOS activity (24). Indeed, a higher NOS activity in the control population was confirmed by an elevated nitrate/nitrite concentration measured in the sera as compared to the patients. Although for most infections nitrite and nitrate levels are increased, especially in active infections, reduced nitrite and nitrate levels were also found in patients with chronic hepatitis, tuberculosis or malaria (4, 5, 13). Apparently, a high concentration of nitric oxide in serum offers protection against mycetoma.

High CXCL8 production and low NOS2 expression delay wound healing

Both CXCL8 and NOS2 levels influence acute inflammation and repair of damaged tissues in the skin (27). By attracting neutrophils to the site of infection, CXCL8 co-determines efficient killing of invading microbes either by phagocytosis or by secreting oxygen- or nitrogen-radicals (30). This is confirmed by the high NOS2 expression during wound repair (28). If for some reason NOS2 expression is suppressed, wound repair is much slower (28). Too much nitrogen and especially oxygen radicals can also cause serious tissue damage (8, 9). This will hamper wound healing as was shown by improved wound-healing in neutrophil-depleted mice as compared to control mice (8). Mycetoma is thought to develop after traumatic inoculation of the causative agent by for instance a thorn prick. If too much CXCL8 is produced after this thorn prick at the site of entry, too many neutrophils are attracted which could result in additional tissue damage. Patients tend to have low levels of nitric oxide which could result in less efficient killing. NOS2 has shown to be of importance in another cutaneous infection, namely leishmania. For this infection it has been shown that inhibition of NOS2 results in non-healing cutaneous leishmanial lesions and even in reactivation of latent leishmania (7, 35).

In conclusion, functional expression differences in genes involved in neutrophil function were documented for mycetoma patients. The Sl2 and McC^a blood group antigens were more often found in the patient population than in the endemic Sudanese reference population. Deviations in genes encoding for CXCL8, CXCR2 and TSP-4 were found which resulted in a higher CXCL8 production in mycetoma patients. Altered allele frequencies in the *NOS2* gene resulted in a lower nitric oxide production in mycetoma patients. Higher CXCL8 production and lower nitric oxide production are both implicated in less efficient wound healing, which could be a significant risk factor for developing mycetoma.

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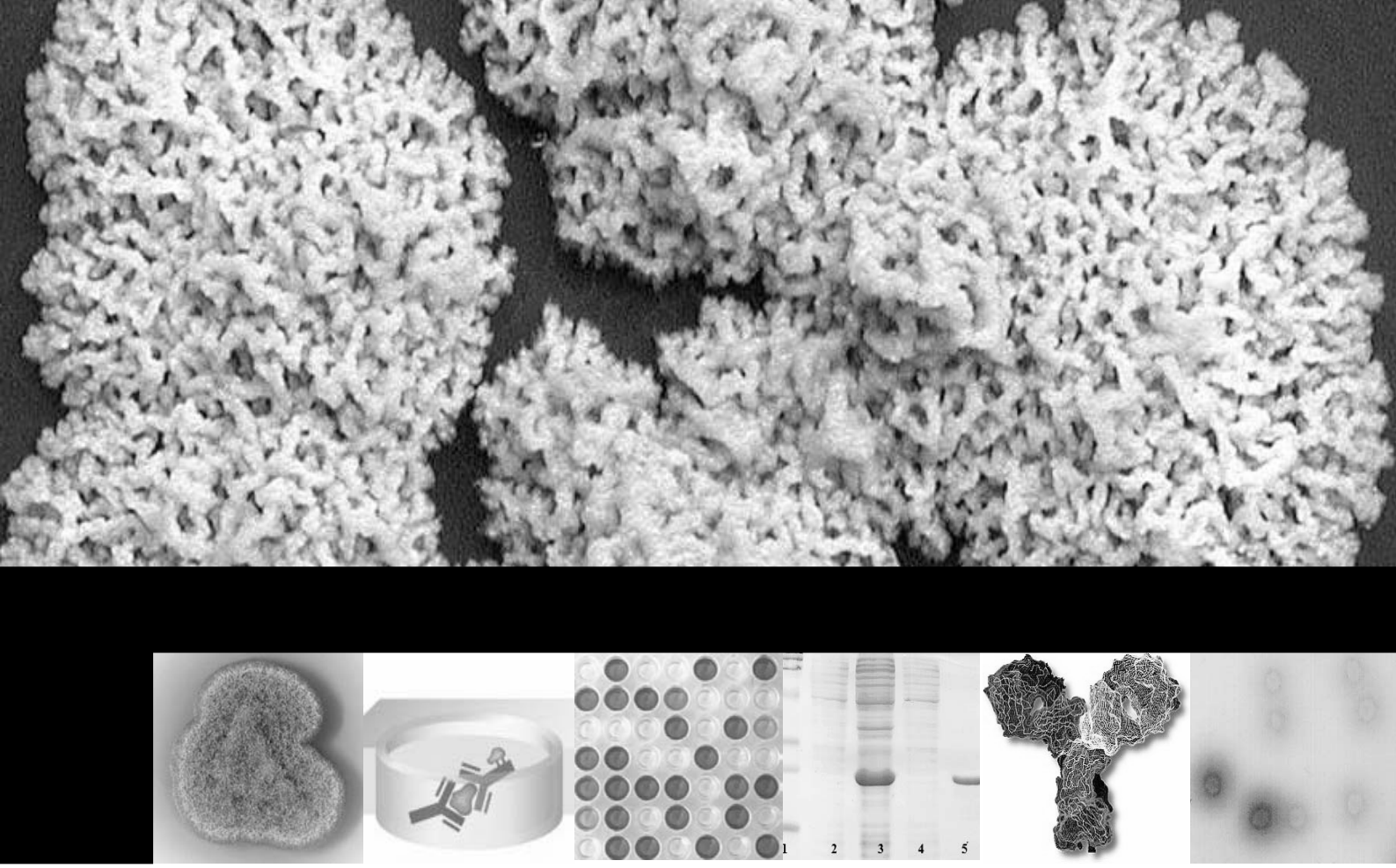
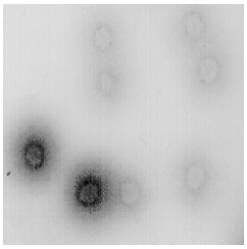


Antigenicity of Madurella mycetomatis

Chapter 5



Translationally controlled tumour protein from Madurella mycetomatis, a marker for tumorous mycetoma progression

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Abstract

About forty years ago antibodies against the fungus *Madurella mycetomatis* were first demonstrated to be present in eumycetoma patients, a disease characterized by tumourous swellings. To date nothing is known about the individual immunoreactive antigens present in this fungus. We here identify its first immunogenic antigen, a protein homologous to the translationally controlled tumour protein (TCTP), a well-conserved histamine release factor in a range of eukaryotes. The gene for this antigen was demonstrated to be present in two variants in *M. mycetomatis*, with 13% amino acid difference between the two proteins encoded. *In vitro*, TCTP was secreted into the culture medium. *In vivo*, it was found to be expressed on hyphae present in developing stages of the eumycetoma-characteristic black grain. Significant IgG and IgM immune responses, against the whole protein and selected *M. mycetomatis*-specific peptides, were determined. The antibody levels correlated with lesion size and disease duration. Overall, the patients with the largest lesions had the highest antibody level, which lowered with decreasing size of the lesion. After 6-15 years of disease duration the antibody levels were the highest. TCTP is the first well-characterized immunogenic antigen, simultaneously the first monomolecular vaccine candidate, identified for the fungus *M. mycetomatis*.

Introduction

Madurella mycetomatis is a fungus capable of inducing eumycetoma in humans (figure 1) (5). Eumycetoma is a major mycological health problem of severe morbidity in tropical and subtropical areas (20). The disease is characterized by extensive tumour-like subcutaneous masses and discharge of fungal grains, blood and pus and primarily affects the earning members of the society (5, 18, 20). Treatment of advanced cases usually implies amputation of the infected limb (17, 26).

Figure 1: An advanced case of eumycetoma caused by *Madurella mycetomatis* of the foot.

Almost nothing is known about the pathogenic routes used by this fungus. Both host and fungal factors involved in disease progression are essentially unknown. Identification of such factors is important for the development of prophylactic and therapeutic measures against eumycetoma. In addition, the diagnosis of *M. mycetomatis* as the primary cause of eumycetoma is troublesome. Recently developed PCR tests are useful but do not provide a simple tool for bedside use (3). There is an urgent need for the development of simple high speed and high throughput tests for use in the endemic tropical regions. The usefulness of serological tests is under-explored and ELISA tests need to be developed.

As early as in 1964 Mahgoub was able to demonstrate that eumycetoma patients developed antibodies against *M. mycetomatis* (27, 30). Counter immuno-electrophoresis (CIE), immunodiffusion and ELISA, were developed to detect antibodies raised against different mycetoma causative agents, using crude culture extracts as antigen (23, 29). This did not identify the type of antibodies produced or the nature of the antigens involved. It was not until the second half of the 1980s that it was experimentally demonstrated that the cytoplasm, organelles and, predominantly, the cell wall of *M. mycetomatis* were antigenic (40). About the same time it was also determined that IgM and IgG were the dominant immunoglobulins resulting from mycetoma (39). In 1991, the first attempts were made to characterize the nature of the epitopes present in the crude extracts used for the initial experiments (42). Cytoplasmic proteins were extracted from several eumycetoma agents and, although the different *M. mycetomatis* isolates had very heterogeneous protein profiles by SDS-PAGE, the antigenic make-up was quite similar within the species (42). The precise nature of the immunodominant proteins was not determined in this study.

Molecular biology techniques were previously used for the identification of antigens and allergens from various fungal species such as *Aspergillus fumigatus* and *Fusarium culmorum (24, 33)*. In analogy to these studies, we now searched a newly developed *M. mycetomatis* cDNA expression library for immunodominant antigens. Our aim was to identify

antigens present on *M. mycetomatis,* which might be used in serodiagnostic tools or as vaccines, both therapeutic and prophylactic. We present the full characterisation of the translationally controlled tumour protein (TCTP), the first immunoreactive protein of *M. mycetomatis,* its histopathological features, and its use in immunological monitoring of disease progression.

Materials and Methods

M. mycetomatis strains

M. mycetomatis strain mm55, isolated from the lesion of a 22-year-old patient seen in the Mycetoma Research Centre, University of Khartoum, Sudan, was used in this study. This strain was isolated by direct culture of the black grains obtained by a deep biopsy and identified by morphology, PCR-RFLP and sequencing of the ITS region (3). This strain is part of a well-defined strain collection deriving from Sudanese patients and is considered the type strain in phylogenetic and antifungal susceptibility testing as well (2, 4, 6, 37). The strain was maintained on Sabouraud Dextrose Agar (Difco Laboratories, Paris, France) at 37 ^{o}C. Passage to fresh medium was done on a monthly basis. Of this same collection four strains, strains mm30, mm45, mm46 and mm83, were used for comparative sequencing studies.

Sera and tissues

48 human sera were obtained from patients in various stages of mycetoma development (Mycetoma Research Centre, University of Khartoum, Sudan). For each patient age, sex, disease duration, relative size of the lesion and site of infection were recorded. The definition of small, moderate and large lesions were based on comparisons among mycetoma patient lesion. More accurate measurements were not possible, since some mycetoma lesions are diffuse, have a mass, and have ill-defined margins. As a control population, random sera from 39 healthy Sudanese individuals from the same region were collected. As a true negative control, sera from 6 healthy Dutch individuals were taken. Mice sera were obtained by infecting female BALB/c mice intraperitoneally with 140 mg sonicated wet weight mycelia of the strain mentioned above according to the previously published protocol (6). The sera from 10 infected mice were pooled and used in the experiments described here. Furthermore, grains and surrounding tissue derived from infected mice were obtained and preserved in buffered formalin. Permission for animal experiments was granted by the Animal Ethics Committee of Erasmus MC. Polyclonal anti-Translationally controlled tumour protein (TCTP)-antibodies were raised commercially in rabbits by Eurogentec (Eurogentec, Liège, Belgium). In short, rabbits were immunised by injecting 100 μg recombinant TCTP, and after 2, 4 and 8 weeks the immunisation was boosted using 100 μg per boost. Serum was taken before immunisation, one month after immunisation and three months after immunisation.

Construction of the cDNA library

The *M. mycetomatis* mm55 strain was cultured for 4 weeks on Sabouraud Dextrose Agar (Difco Laboratories) at 37 ^{o}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 from the RNeasy® Maxi system (Qiagen GmbH, Hilden, Germany) was added. The sample was incubated for 3 min at 56 ^{o}C to fully disrupt the cell walls. Total RNA was isolated using the bacterial protocol from the RNeasy® Maxi system (Qiagen). After total RNA isolation, traces of DNA were removed with DNAse I (Ambion Ltd., Huntingdon, United Kingdom) according to the manufacturers' 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 synthesise double-stranded cDNA with the Universal RiboClone® cDNA Synthesis System (Promega Benelux BV, Leiden, The Netherlands). Finally, the double-stranded cDNA was ligated into the λgt11/EcoRI/CIAP-treated vector (Stratagene Europe, Amsterdam, The Netherlands). The quality of the library was established by adding X-gal (Promega) in a final concentration of 2 mg/ml in topagar and blue-white screening for recombinant phages. The percentage of phages containing *M. mycetomatis* cDNA was 20%.

Immunological screening of the cDNA library

E. coli Y1090 cells (Stratagene Europe) were grown overnight in LB-broth supplemented with 0.2% (w/v) maltose, 10 mM $MgSO_4$ and 50 μg/ml ampicillin. The bacteria were pelleted for 10 min at 500 g and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Two hundred thousand recombinant phages were absorbed to the cells for 20 min at room temperature. Top-Agar medium (26 ml) was added to 2.4 ml of cells and poured on a pre-warmed 500 cm^2 LB agar plate. The plate was incubated at 37^{o}C until the plaques were clearly visible. Subsequently they were overlaid with a Protran® nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany) for 10 min at room temperature. The membrane was washed three times with Tris-Buffered Saline Tween-20 (TBST) (10 mM Tris-HCL, 150 mM NaCl, 0.05% Tween-20, pH 8.0) and blocked for 30 min with 1% BSA (Sigma-Aldrich, Zwijndrecht, The Netherlands) in TBST. Mouse serum obtained from *M. mycetomatis* infected mice was diluted 1:130 in TBST and pre-incubated with *E. coli* Y1090 extract to absorb non-specific antibodies. This extract was prepared by pelleting an overnight *E. coli* Y1090 culture, resuspending the pellet in a third of the original volume in EDTA buffer (50 mM Tris-HCl pH8, 10mM EDTA), freezing it 1 h at –80^{o}C and sonicating it 6 times for 15 s at 10 micron. Cell remnants were pelleted at 500 g and the supernatant was used as the *E. coli* Y1090 extract. The blot was incubated for 45 min with the treated mouse serum. After washing, the membrane was incubated for 30 min with anti-IgG alkaline phosphatase conjugate. Colour development was started by adding Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) (Roche Diagnostics Gmbh, Mannheim, Germany). Positive phages were selected and purified by repeated screening.

Sequence analysis

To determine the nature of the insert present in antigenic phages, purified phages were boiled for 10 min. Five μl of this crude extract was amplified in 50 μl PCR-mixture consisting of 1x Supertaq PCR buffer 1 (HT Biotechnology Ltd., United Kingdom), 0,2 mM PCR nucleotide mix (Amersham Life Sciences, Roosendaal, The Netherlands), 25 pmol primer gt11fw (5'-

GGTGGCGACGACTCCTGGAGCCCG-3'), 25 pmol primer gt11rv (5'-TTGACACCAGACCAACTGGTAATG-3') and 1.2 U Supertaq (HT Biotechnology Ltd.). The PCR was composed of a pre-denaturation step of 4 min at 94 ^{o}C and 35 cycles consisting of a 1 min denaturation at 94 ^{o}C, 1 min annealing at 50 ^{o}C and 2 min elongation at 72 ^{o}C. This was followed by a post-elongation step of 7 min at 72 ^{o}C. The PCR products were sequenced (BaseClear, Leiden, the Netherlands) and their insert sequences were compared with other sequences in the NCBI database (**http://www.ncbi.nlm.nih.gov/BLAST**).

Population analysis

We used the primers TCTPfw (5'-GCCGAGGAGGCTCTCGAGGA-3') and TCTPrv (5'-ATCGGGATCCTTAGACCTTCTCCTCCTTCAG-3') to determine presence of the TCTP sequence in the other 37 *M. mycetomatis* strains in our collection. The PCR program was identical to the one described in the next section for constructing the expression vector. The resulting PCR products were digested with the endonuclease *Alu*I to determine the variability within TCTP found in various strains.

Construction of a TCTP expression vector

The purified phages containing the complete TCTP coding region were boiled for 10 min and 5 µl of these extracts were amplified in 50 µl PCR-mixture consisting of 1x Supertaq PCR buffer 1 (HT Biothechnology Ltd., United Kingdom), 0.2 mM PCR nucleotidemix (Amersham Life Sciences, Roosendaal, The Netherlands), 25 pmol forward primer (5'-ACGTGCTAGCCATCACCATCACCATCACATTATCTACAAGGATATTAT-3'), 25 pmol reverse primer (5'-ATCGGGATCCTTAGACCTTCTCCTCCTTCAG-3') and 1.2 U Supertaq (HT Biotechnology Ltd., United Kingdom). The PCR was composed of a pre-denaturation step of 5 min at 92 ^{o}C and 10 cycles consisting each of 10 s denaturation at 92 ^{o}C, 30 s annealing at 45 ^{o}C and 1 min elongation at 68 ^{o}C followed by 15 cycles consisting each of 10 s 92 ^{o}C, 30 s 45 ^{o}C and 1 min at 68 ^{o}C which extended with 10 s per cycle. The PCR ended with a post-elongation step of 4 min at 68 ^{o}C. In a 2:1 molar ratio the PCR product and vector pET11c (New England Biolabs Inc, Ipswich, USA) were restricted for 2 h at 37 ^{o}C with 10 U *Nhe I* and 10 U *Bam HI* in buffer M (All Roche Diagnostics). After removal of excess restriction enzyme (Zymo Research, Orange, USA) the PCR product was ligated into the pET11c vector with 3U T4 ligase (Promega Benelux BV, Leiden, The Netherlands). The resulting plasmid was electroporated into electrocompetent *E. coli* BL21. To determine the absence of point mutations generated during the cloning process, the sequence of the insert was verified (BaseClear) by alignment with the sequence obtained from the original recombinant phages.

Expression and purification of the recombinant fusion protein

To express the His-tagged TCTP fusion protein, a culture containing the *E. coli* BL21 strain with the recombinant plasmid was grown to an OD_{600} of 0.6. To induce expression, IPTG (Promega Benelux BV) was added to a final concentration of 1 mM and the culture was grown for another 2 h. The culture was pelleted and resuspended in 20 mM phosphate, 0.5 M NaCl and 10 mM imidazole. The cell suspension was sonicated at 4 ^{o}C and cell remnants

were pelleted. The histidine-tagged recombinant proteins were purified using a HiTrap™ column (Amersham Life Sciences) and eluted (300 mM imidazole, 10 mM $Na_2HPO_4 \cdot H_2O$, 10 mM $Na_2HPO_4 \cdot H_2O$, 0.5 M NaCl). The eluate was dialysed against distilled water for 6 h. The size and purity of the recombinant protein were determined on a 12% SDS-PAGE gel stained with Coomassie® G250 (Bio-Rad).

Immunohistochemistry
Grains obtained from experimentally infected mice were embedded in paraffin and histological slides were prepared. The slides were rehydrated in PBS and incubated for 30 min in a blocking solution (1% BSA, 5% sucrose in PBS). The primary antibody was diluted 1:100 in blocking solution and incubated for 30 min. As primary antibody, rabbit serum raised against recombinant *M. mycetomatis* TCTP was used. This serum was obtained 6 weeks after immunisation. As negative control, pre-immune serum from the same animal was used under the same conditions. After washing, the slides were incubated with diluted goat anti-rabbit IgG horseradisch peroxidase conjugated antibody (1:50, Sigma-Aldrich). The substrate NovaRed™ (Vector, Burlingame, CA, USA) was as the primary stain, haematoxyline was used as counterstain.

Recombinant protein ELISA: The purified recombinant protein (12.5 μg per well) was coated onto Maxisorp plates (Nunc A/S, Roskilde, Denmark) overnight at 4 ^{o}C. After blocking for two h with 1% BSA (Sigma-Aldrich) and 5% sucrose (Mallinckrodt Baker, Deventer, The Netherlands) plates were washed and incubated for 1 h with 1:50 diluted patient serum. After washing, 1:5000 diluted goat anti-human IgM (Sigma-Aldrich) or 1:10000 diluted goat anti-human IgG (Sigma-Aldrich) was applied and incubated for 1 h. The reaction was developed for 15 min in 3,3', 5,5' tetramethyl benzidine (TMB) (Meddens Diagnostics) which was stopped with 2M H_2SO_4. Absorption was measured at 450 nm in a model 550 microplate reader (Bio-Rad).

Peptide ELISA
In order to bind the peptides shown in table I to Covalink ELISA-plates (Nunc), these plates were pre-incubated for 30 min at 37^{o}C with 10 mM SPDP (3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester) (Sigma). Each peptide (10 μM in 0,1 M Tris-HCl, pH 8.0), was coupled to the bivalent linker via the N-terminal cysteine residue for 60 min at 37^{o}C. Empty binding sites were blocked for 2 h at 37^{o}C with 6% low-fat milk (Similac 2, Abbott) before 1:50 diluted patient serum was allowed to bind (45 min at 37^{o}C). After 30 min of incubation with the secondary antibody (1:10000, goat anti-human IgG (Sigma-Aldrich)) the reaction was developed as described above.

Name	Variant I	Variant II
Peptide 1	VDCAMVVEDAVNA	Not determined
Peptide 2	EAEEALEDAAVKV	EAEEALEDQAIKV
Peptide 3	DAAVKVNNVVNSF	DQAIKVNDVIHYF
Peptide 4	SVKKALQDAGKSE	SVKKALQDAGKSE
Peptide 5	KSEDEVKEFETKA	KSEDEVKEFETKA
Peptide 6	TKAQAYVKDTVLP	TKAQAYVKDTILP

Table 1: Peptide sequences constructed on the *M. mycetomatis* specific TCTP domains. These sequences were based upon non-conserved sequences of variant I of the TCTP sequence when compared to other TCTPs. In this table the homologues for variant II are also shown. Differences between this variant and variant I are highlighted in the bold font in the sequence for variant II. The peptide 1 homologue in variant II could not be determined because no sequence data was available for this area. Peptides developed for variant I are the ones constructed and used in the peptide ELISAs.

Statistical analysis

IgG and IgM levels raised against the TCTP antigen or the various TCTP peptides were compared between study populations by the Mann-Whitney test (GraphPad Instat 3.00). A $p<0.05$ was considered significant. The Mantel-Haenszel Chi square test for trends was used to test if there was a significant trend of higher levels in bigger lesions, by including size (small, moderate, massive) as independent variables, and the log transformed antibody level as dependent variable.

Nucleotide sequence accession numbers

The sequences of the *M. mycetomatis* homologues of TCTP were deposited in the GenBank. GenBank accession numbers of the cDNA sequences for variants I of this gene are DQ218143 (strain mm55), DQ218146, (strain mm46) and DQ218147 (strain mm83). GenBank accession numbers for variants II of this gene are DQ218144 (strain mm30) and DQ218145 (strain mm45). The DNA sequences for the two variants in these strains are deposited under numbers DQ218148-DQ218152.

Results

Antigenic phages contain a M. mycetomatis translationally controlled tumour protein (TCTP) homologue

In our search for antigens of *M. mycetomatis,* an 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. A total of approximately 6×10^8 recombinant phages were screened with sera obtained from mice experimentally infected with *M. mycetomatis*. Twelve phage clones were found to significally bind antibodies and these were rescreened to purity. PCR amplification, restriction analysis and subsequent sequencing demonstrated that all 12 immunoreactive plaques harboured the same 692 base pair insert, encoding a single open reading frame (ORF) of 516 nucleotides. The ORF defined a 171 amino acid polypeptide sequence, which was followed by an untranslated sequence of approximately 156 bp including a poly A tail of 15 nucleotides (figure 2).

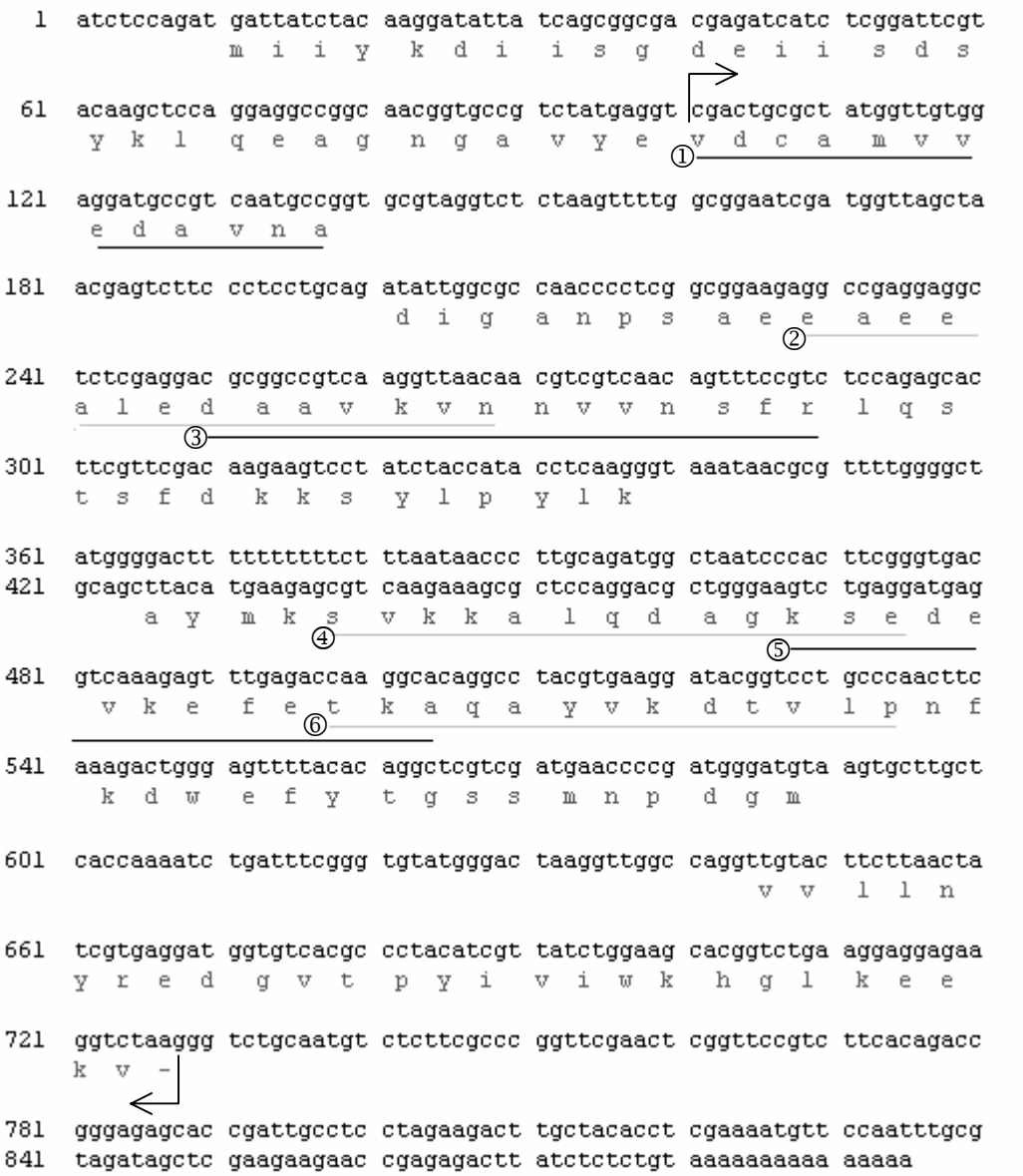

Figure 2: Nucleotide sequence and deduced amino acid sequence of the TCTP gene of *Madurella mycetomatis.* In this sequence at least four exons can be found: exon 1 (amino acids 99-137), exon 2 (amino acids 200-337), exon 3 (amino acids 423-585) and exon 4 (amino acids 644-727). These exons are shown translated and are followed by three introns: intron 1 (amino acids 138-199), intron 2 (amino acids 338-422) and intron 3 (amino acids 586-643). The sequence preceding the first arrow (amino acids 1-8) and after the second arrow (amino acids 728-895) are mRNA. For only the sequence between those arrows (amino acids 9-727) the DNA sequence is known. Peptides are underlined and numbered.

As seen in figure 3 the protein showed significant homology with the translationally controlled tumour protein (TCTP) gene of various organisms including fungi and yeasts. The highest degree of homology was observed for the TCTP gene of the fungus *Neurospora crassa* (GenBank™ accession number XP_326319), with 61% aminoacid identity and 71% aminoacid similarity. At the protein level, *M. mycetomatis* TCTP was less closely related to mammalian TCTP, with only 39% identity and 56% similarity.

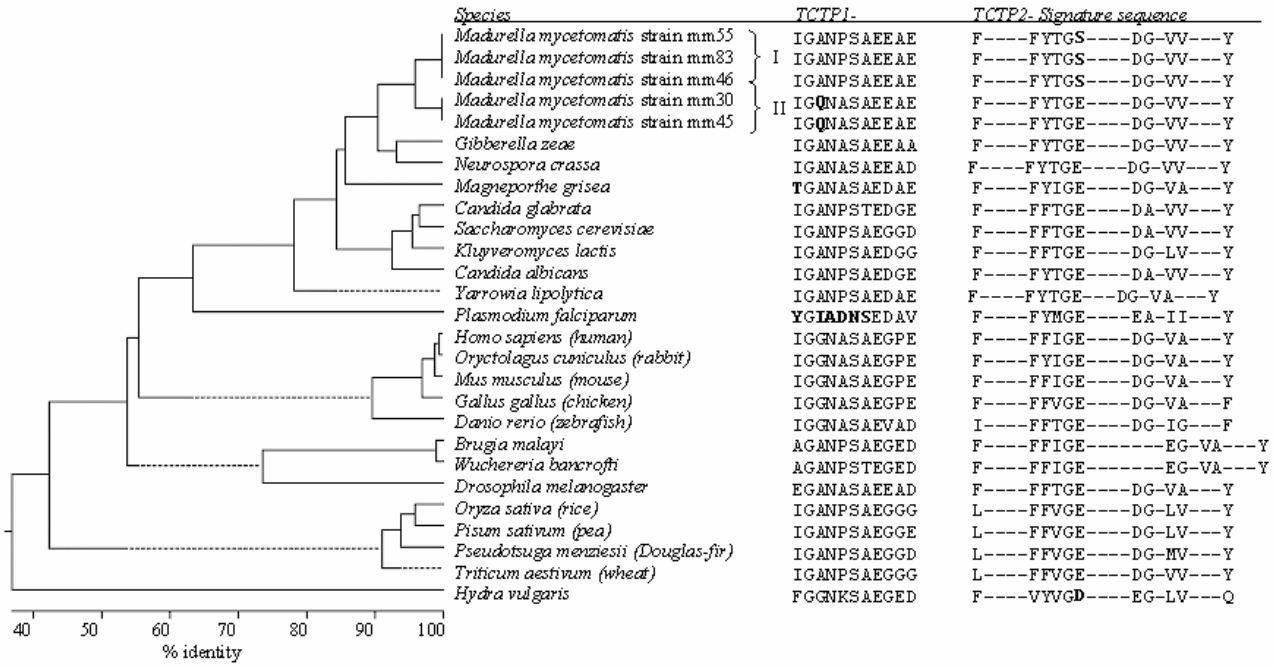

Figure 3: Phylogenetic tree analysis of TCTP protein sequences from different species. The tree distances were calculated using the Clustal V algorithm in the programm MegAlign (DNAstar). Both *M. mycetomatis* TCTP variants (I and II) (GenBank™ accession No. DQ218143-DQ218147), were compared to TCTP sequences obtained from *Brugia malayi* (GenBank™ accession No. AAC47622), *Candida albicans* (EAK99010), *Candida glabrata* (XP_449328), *Danio rerio* (NP_937783), *Drosophila melanogaster* (DmTCTP, NP_650048), *Gallus gallus* (NP_990729), *Gibberella zeae* (XP_382699), *Homo sapiens* (NP_003286), *Hydra vulgaris* (AAB18413), *Kluyveromyces lactis* (CAH01617), *Magnaporthe grisea* (EAA56278), *Mus musculus* (p63028), *Neurospora crassa* (XP_326319), *Oryctolagus cuniculus* (CAA12650), *Oryza sativa* (P35681), *Pisum sativum* (P50906), *Plasmodium falciparum* (NP_703454), *Pseudotsuga menziesii* (CAA10048), *Saccharomyces cerevisiae* (P35691), *Triticum aestivum* (AAM34280), *Wuchereria bancrofti* (AAK71499) and *Yarrowia lipolytica* (XP_504451). For each of the organisms the TCTP1 and TCTP2 signature sequences are given. In this sequence amino acids which do not correspond to the signature sequence (given in bold) and amino acids which do not belong in the signature sequence (-) are shown as well.

In order to establish whether this gene was ubiquitously found in all *M. mycetomatis* strains we screened 38 isolates for its presence. After PCR screening of genomic DNA of these isolates it appeared that all strains possessed the TCTP gene homologue (data not shown). PCR RFLP exploring the genes from these isolates revealed that there was not one isoform of this gene but two. Subsequently sequencing of these genes confirmed this finding. The isoforms as shown in figure 4 were named variant I and II. Variant I was found in 53% of the isolates while variant II was found in 47% of the isolates. Further PCR analyses showed that both TCTP genes contained at least three introns of 64 bp (intron 1), 161 bp (intron 2) and 56 bp (intron 3) and four exons (figure 2).

According to the PROSITE pattern database (http://www.expasy.ch/prosite/entry, PDOC00768) two signature patterns can be identified for TCTP amino acid sequences, namely TCTP1 and TCTP2. After comparing the MmTCTP amino acid sequences with the database entries it was noticed that amino acid positions 49-54 in the MmTCTP variant I were identical to the typical TCTP1 signature sequence, [**I**FAE]-[**G**A]-[G**A**S]-**N**-[**P**AK]-**S**-[GT**A**]-**E**-[GD**E**V]-[P**A**GEQV]-[D**E**QGAV]. In contrast, the TCTP2 sequence of this MmTCTP variant deviated from the known signature sequence, [**F**LIV]-**x4**-[**F**LVH]-[F**Y**]-[MIVC**T**]-**G**-E-**x(4**,7)-[**D**ENP]-[**G**AST]-**x**-[LI**V**M]-[GA**V**I]-**x3**-[F**Y**QW]. For the second variant this finding was reversed. This variant had a deviating TCTP1 signature sequence [**I**FAE]-[**G**A]-[GAS]-**N**-

[P**A**K]-**S**-[GT**A**]-**E**-[GD**E**V]-[P**A**GEQV]-[D**E**QGAV] and a canonical TCTP2 signature sequence [**F**LIV]-**x4**-[**F**LVH]-[F**Y**]-[MIVC**T**]-**G**-**E**-**x(4**,7)-[**D**ENP]-[**G**AST]-**x**-[LI**V**M]-[GA**V**I]-**x3**-[F**Y**QW]. Both deviating sequences each displayed only one amino-acid difference. In the TCTP2 sequence of variant I, glutamate was replaced by serine and in the TCTP1 sequence of variant II, glutamine was found instead of either glycine, alanine or serine.

Figure 4: Comparison of the two TCTP variants. A. Alignment of the two TCTP variants of *M. mycetomatis* based on the BLOSUM 62 algorithm. Identical amino acid residues are shown as a dot. B. Banding patterns obtained for each variant after treatment with the endonuclease *Alu*I.

Since we obtained a complete cDNA sequence for variant I we used this to express and purify the recombinant TCTP variant I gene product as a histidine-tagged fusion protein. The yield of this recombinant protein was 50 mg per litre of *E. coli* culture. SDS-PAGE gel analysis of the purified recombinant protein with the histidine tag revealed one clear band with a molecular weight of approximately 26 kDa (figure 5). This was slightly larger than the expected molecular weight but still in full accordance with the molecular weight of other cloned TCTPs (22). As seen in figure 5, the purified protein was essentially free of contaminating proteins.

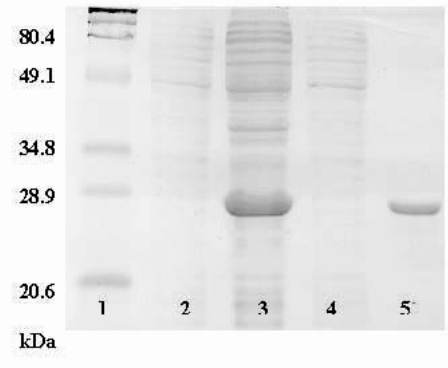

Figure 5: Twelve percent Coomassie stained SDS-PAGE of the recombinant his-tagged *M. mycetomatis* protein. Lane 1: SDS-Page broad range marker, lane 2: total *E. coli* extract before inducement of the TCTP expression, lane 3: total *E. coli* extract after inducement of the TCTP expression, lane 4: protein extract after binding the his-tagged recombinant TCTP protein to the HiTrap column, lane 5: eluted his-tagged recombinant TCTP protein

TCTP is expressed in the developing grain

To assess whether TCTP was expressed *in vivo* we isolated grains in their surrounding tissues from mice infected with *M. mycetomatis*. These grains were embedded in paraffin and antibodies raised against TCTP were used to demonstrate the presence of TCTP in these grains. The results obtained are shown in figure 6.

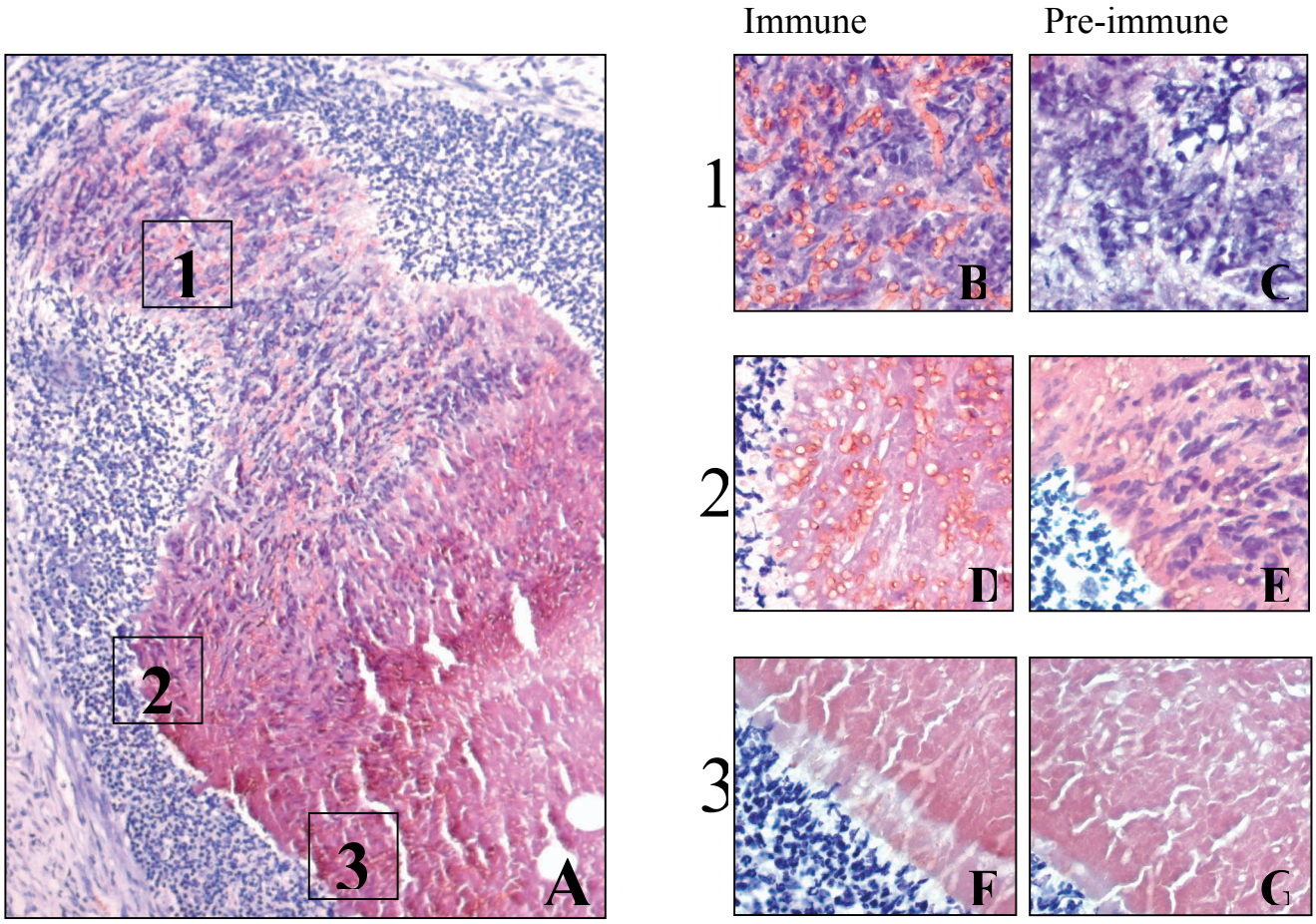

Figure 6: A. Stained histological section of a grain isolated from a mouse interaperitoneally inoculated with 120 mg fungal mycelia and soil adjuvant (100x). Immunohistochemistry with anti-TCTP antibodies, counterstained with haematoxylin. The grain is surrounded by a type 1 host reaction. The grain is surrounded by three zones of host inflammation cells. The first zone, closest to the grain is composed of neutrophils, the second zone is composed of histiocytes. The outer layer is the vascular layer. The grain itself is divided into three developmental stages. The first stage is the early developing stage (1), the second stage is the intermediate stage (2) and the third stage is the developed stage (3). B. TCTP is highly expressed in first stage of development. Immunohistochemistry performed with immune serum. C. First stage of development with pre-immune serum. D. TCTP expression in second stage of development, immune serum. E. Second stage of development, pre-immune serum. F. No TCTP expression in third stage of development, immune serum. G. Third stage of expression, pre-immune serum. Figures B-G are photographed at 400x. TCTP expression is stained with the substrate NovaRed (orange).

The grains in the intraperitoneal mouse 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 (16, 18, 19). The zone directly around the grain was composed mainly of neutrophils. This zone was followed by a region of histiocytes and an outer vascular zone. The fungal cells in the grain itself are mostly embedded in brown-pigmented cement material. It was clearly seen that

TCTP is not evenly expressed in the grain. In the grain itself, three stages of grain development are seen. The first stage (figure 6A, number 1) is the stage in which the grain is still forming. The brown-pigmented cement material in this part of the grain is not present yet (figures 6B and 6C) and the neutrophil zone surrounding the grain is not completely formed and only a few cell layers thick. This zone is invaded by the grain and fungal cells are in direct physical contact with the immune cells. The fungi are mostly seen as complete hyphae. In this part of the grain, TCTP was expressed abundantly (figure 6B). Most cells express the protein but some exceptions are noticed, even within hyphae where other cells do express the protein. In the second stage of the grain formation (figure 6A, number 2), the cement material is more developed but individual fungal cells are still seen (figures 6D and 6E). Some of the neutrophils are also still found between the fungal cells. The number of cells expressing TCTP was inversely related to the amount of cement material present. In the last stage (figure 6A, number 3) the grain is fully formed. The fungus is embedded into cement material and no neutrophils are seen within the grain (figures 6F and 6G). In this stage no visible TCTP expression was documented (figure 6F).

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

The phage containing the TCTP insert was selected because of its reactivity with sera from infected mice. Mice infected with *M. mycetomatis* had antibodies against the protein while healthy control mice did not (results not shown). To test the antigenicity of MmTCTP in humans, an ELISA was developed. The IgA, IgM and IgG levels against the protein were measured. None of the patients or controls displayed an IgA level against this protein, but clear IgM and IgG responses were detected. Since only comparative optical densities were measured, a prozone phenomenon could not be ruled out. In figure 7 IgM and IgG levels against TCTP are summarised. Both in the patient group as well as in the Sudanese control group, significant IgM and IgG levels were measured against the protein, however in a lower percentage of controls than patients, and not all patients developed elevated levels. All individuals in the Dutch healthy control group had low anti-TCTP levels overall. For some of the patients it was known whether the fungus causing the infection produced TCTP variant I or II. Patients infected with a type II variant had detectable antibodies in an ELISA coated with the variant I protein. No significant difference in antibody binding was noticed when serum of variant II patients was used (data not shown). It was seen that the IgM level in the *M. mycetomatis* infected people was significantly higher compared to the Sudanese controls (Mann-Whitney, $p=0.03$) or to the Dutch control group ($p=0.003$). The IgG levels of the *M. mycetomatis* infected people were only significantly higher compared to the Dutch control group ($p=0.002$). It was also noted that the patients with massive eumycetoma lesions had the highest absorbances and that patients with the smaller or cured lesions had lower IgG levels (MH test for trend, $p=0.030$). No significant trend was documented for the IgM immuneresponse. Furthermore, it was seen that after a disease duration of 6-10 years anti-TCTP levels were highest.

Since some individuals in our healthy control group from Sudan had raised levels of antibodies against the recombinant TCTP-protein we analysed whether these elevated levels were due to the *M. mycetomatis* homologue of this antigen or due to cross-reactivity. Cross-reactivity could occur with TCTPs from other infectious agents, such as *Plasmodium*

falciparum and *Leishmania spp.,* which are locally endemic pathogens in the Sudan region as well. In order to test this, known TCTP sequences for various species were compared and we designed six *M. mycetomatis* specific peptides based upon non-conserved sequences of variant I of the TCTP sequence (see figure 2 for the position, code and sequence of the peptides).

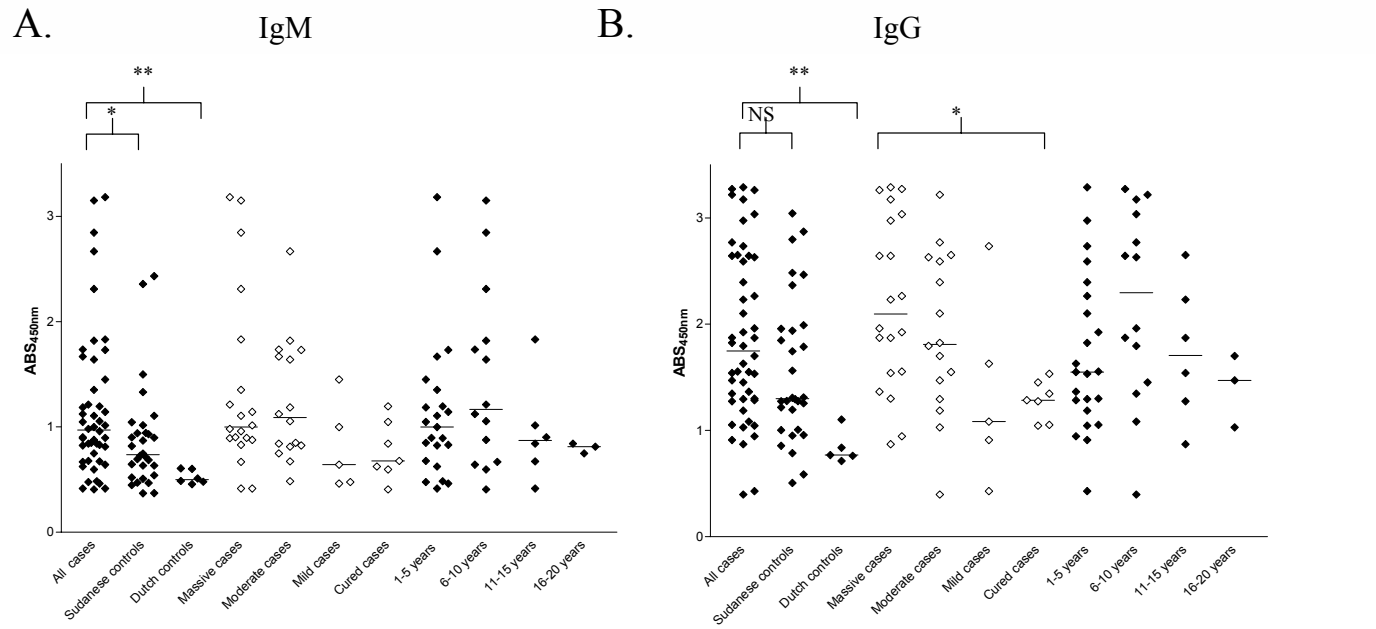

Figure 7: IgG levels against the purified recombinant his-tagged TCTP protein measured at a wavelength of 450 nm in an ELISA system. Each point represents the mean of three independent measurements per patient or a healthy control. A: IgM levels in correlation of the size of the lesion and the duration of the disease. B: IgG levels in correlation of the size of the lesion and the duration of the disease. The differences between the geometric means were tested for significance with the Mann-Whitney test. Furthermore it was tested if there was a significant trend or higher level in bigger lesions, by including size (small, moderate, massive) as independent variable, and log transformed level as dependent variable in a linear regression model. The p-values are stated as follows: $p \leq 0.0100$ (**), $p \leq 0.05$ (*), p = not significant (NS).

As a precaution we also compared the peptides designed to variant II of the *M. mycetomatis* TCTP protein. Five of the six peptides were conserved in *M. mycetomatis*, with a maximum of 2 amino acids difference. Only one peptide, peptide 3, was not conserved. For this peptide, 6 out of the 13 amino acids were different between the two variants. With these peptides, ELISAs were performed and both the patient and the control sera were retested. In figure 8 the antibody levels against the six individual peptides are shown. Peptides 1, 2, 3, 4 and 5 can clearly differentiate the patient population from the healthy Sudanese control population, but with peptide 6 the mean IgG levels of the patient population and the healthy Sudanese control population were not significantly different. When the patients were stratified according to massive lesions, moderate lesions, small lesions and cured patients the same picture was obtained for the peptides as with the whole TCTP protein (data not shown). Overall, the patients with the largest lesions had the highest antibody levels against the respective peptides, the level lowered with the decreasing size of the lesion. After 6-15 years of disease duration levels were the highest.

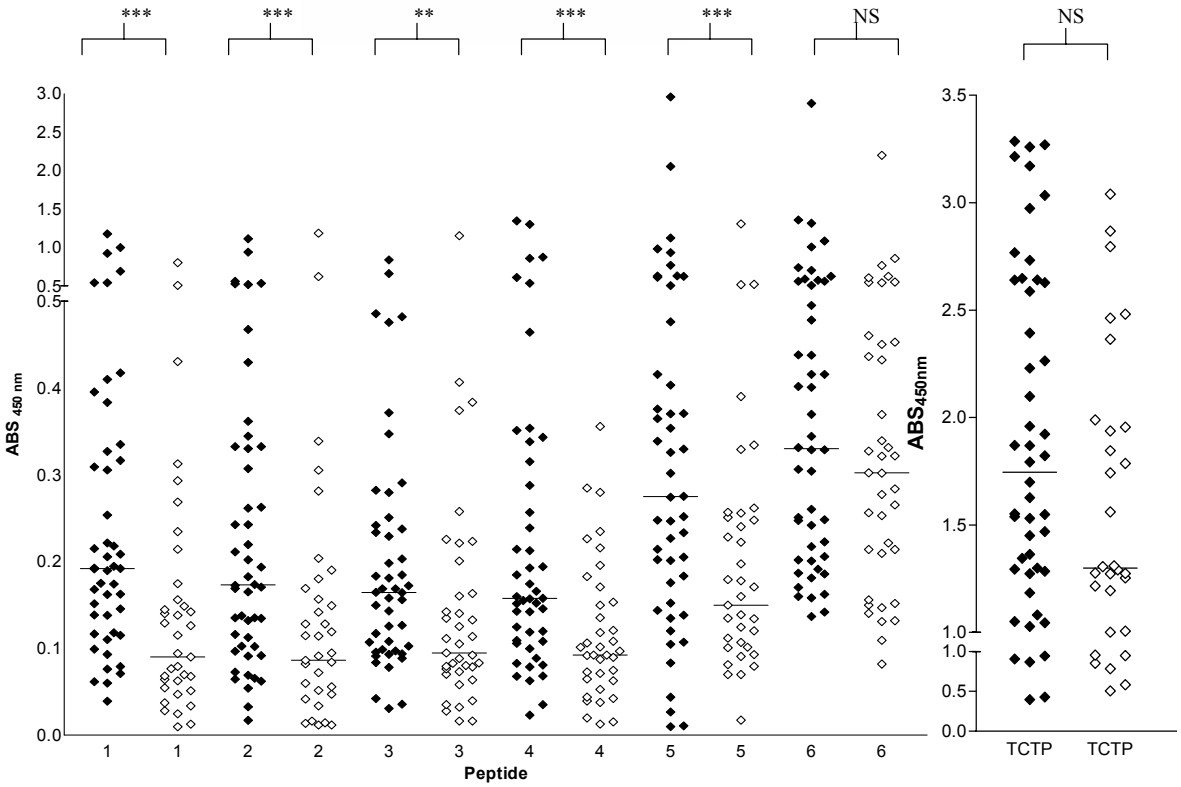

Figure 8: IgG level generated against the different TCTP peptides. In this graph the antibodies raised against the different peptides (numbers 1-6) in the *M. mycetomatis* infected patients (♦) are plotted next to antibodies found in the Sudanese healthy control group (◊). For reference the results obtained with the complete TCTP protein as shown in figure 7 are repeated in this figure as well (TCTP). The differences between the geometric means were tested for significance with the Mann-Whitney test. The p-values are stated as follows: $p \leq 0.0010$ (***), $p \leq 0.0100$ (**), $p \leq 0.05$ (*), p = not significant (NS).

Discussion

It has been demonstrated before that specific antibodies are raised against the agents of mycetoma. It was, however, never clear what the nature of the immunogenic epitopes was (23, 27, 29). In the present study we completely identify and characterise the first immunoreactive antigens from a *M. mycetomatis* isolate namely TCTP. Many functions have been recorded for TCTP. This protein appears to be involved in cell cycle control, stress responses, histamine release and interleukin production (12, 38). It is also known to bind the anti-malarial and anti-cancer drug artemisinin (7, 15). In our search for antigens, we constructed an expression library in phage λgt11, which was screened with sera obtained from *M. mycetomatis* infected mice. Screening the library resulted in 12 antigenic phages which all contained the same insert, namely the *M. mycetomatis* homologue of TCTP. This finding differs from results obtained for other pathogens such as *Mycobacterium tuberculosis* for which large numbers of independent and clearly different antigenic phages were found (9). The antigenicity of *M. mycetomatis* TCTP was confirmed by rabbit immunisation. Other parasites are also known to produce TCTP. Some examples are *P. falciparum*, *Brugia malayi, Wuchereria bancrofti* and *Schistosoma mansoni* (8, 22, 25, 32). These TCTPs have been documented to be antigenic as well (8, 22, 25, 32).

In the present study we found that the TCTP gene isolated from the expression library was one of two variants present in the species *M. mycetomatis,* which again further corroborates the genetic heterogeneity within this species (36). Although TCTPs have been found in a great variety of eukaryotic organisms no one has yet reported the presence of multiple variants of this gene within one species. In the mouse, rabbit and human genomes there are indeed pseudogenes of this protein known (21, 34, 35). But in the more ancient *Hydra vulgaris* and *Labeo rohita* no doublet TCTP mRNA is found (38, 41). This raises questions on the possibly different physiological findings of the TCTP variants.

To test its antigenicity in humans an ELISA was developed with variant I of MmTCTP. A significant number of the patients seen in the Mycetoma Research Centre had raised antibodies against mmTCTP variant I, but, unfortunately, not all the patients. Reasons for this restriction are currently unknown but subject to further study. It also appeared that some individuals of the Sudanese healthy control population had an elevated antibody level against TCTP as well. The most simple an also likely explanation would be that these individuals raised antibodies after environmental exposure to the antigen. It could also be possible, since mycetoma has a long incubation time, that some of these individuals had sub-clinical or early *M. mycetomatis* infections. Another plausible explanation is cross-reactivity with TCTPs from other endemic infectious organisms. Although Rao demonstrated that antibodies raised against filarial TCTPs did not react against recombinant *S. mansoni* TCTP, Gnanasekar showed that antisera raised against *B. malayi* could detect recombinant *W. bancrofti* TCTP and visa versa (22, 32). These latter filarial TCTPs were closely related to each other, with 98% sequence homology at the amino acid level (22). From the phylogenetic tree shown (figure 3), it can be easily seen that the closest homology was found with TCTP genes of other fungi. In order to determine whether cross reactivity with TCTPs from other species were causing the high levels in the healthy Sudanese control population, specific TCTP peptides which showed as little homology as possible with other TCTP sequences were developed. In the peptide ELISAs there was a clear difference between the mean IgG levels raised against the different peptides in the patient population and the healthy Sudanese control population. Only with peptide 6 no statistic significant difference in the mean IgG levels was detected. Apparently, peptides provide more specific ELISA targets than the full protein. This is biologically plausible and suggests that cross reactivity between TCTPs from various organisms is occurring in humans. Pathological effects of this cross-reactivity deserve additional research.
Our experiments showed that the TCTP gene is present in all *M. mycetomatis* strains tested. Also, the immunogenic epitopes of variants I and II of the protein seems to be overlapping. This overlap was proven by comparing sera obtained from patients of whom it was known with which TCTP variant of *M. mycetomatis* they were infected. No statistically significant difference in antibody level was detected between the two variants. By developing peptides specific for conserved *M. mycetomatis* specific regions between the two variants, antibody responses could be compared. Since no difference in response between patients infected with variant I or variant II were found in the ELISAs, we could conclude that differences found in antibody level were not caused by the variability in the TCTP gene. This difference must be

due to other factors involved in TCTP expression. For instance, it is possible that not all strains transcribe this protein *in vivo* (although they do so *in vitro*) and even if TCTP is transcribed it is likely that not all patients form antibodies against this protein to the same extent.

In order to investigate TCTP expression *in vivo*, grains in surrounding tissues from infected mice were isolated. In these tissues TCTP expression could be demonstrated. TCTP expression was very high in the first stage of *M. mycetomatis* grain development, almost all fungal cells in this stage of development expressed TCTP. For other TCTPs it is known that the expression is also dependent on the developmental stage of the organism (22, 32, 35). TCTP is highly expressed in mitotically active stages while in postmitotic tissue it is hardly expressed at all (22, 32, 35). In the developing stage of the *M. mycetomatis* grain the fungal cells are probably mitotically active. Expression was less in the second stage of grain development and almost non-existent when the grain was completely formed. In this latter stage the fungus is probably not mitotically active anymore and the fungal cells are completely embedded in cement material. This latter could also result in shielding of the antigen. In the first 15 years of infection, the antibody levels were elevated. This finding was seen both for the whole protein as for the individual peptides. Levels drop after prolonged infection (>15 yrs). Since eumycetoma is a chronic infection with a long incubation time and a slow progression, *M. mycetomatis* may well reside in the host for a prolonged period as a mitotically less active organism. Combining the *in vivo* TCTP expression and the serological data this could indicate that after establishing the infection the organism becomes less reproductively active and less TCTP is expressed. The same can be said for the size of the lesion. It was demonstrated that the antibody response was associated with the size of the lesion since the patients with the largest lesions had the highest antibody levels. In patients with large massive mycetoma, many subcutaneous lesions exist in different stages of development, involving elevated high expression of TCTP. In the largest lesions probably most mitotically active cells are found. In these lesions the highest anti-IgG levels were found. Patients with only small lesions had similar antibody levels as the Sudanese controls. A correlation between the size of the lesion and the immune response was also reported for the crude antigens used in the double immunodiffusion method developed by Murray (30). Murray found a correlation with the number of precipitin bands and volume of the tissue involved in the disease (30). The TCTP antibody levels were also correlated with the duration of the disease. After 6-10 years anti-TCTP responses were the highest, whereas no correlation with duration of the disease was observed by Murray when using crude cell extracts of *M. mycetomatis* (30).

Eukaryotic TCTPs are reported to respond to a wide range of extra-cellular signals and cellular conditions (11-13, 31). Growth factors, cytokines, starvation, heat shock, heavy metal stress, calcium stress but also viral infections: all have been reported to either induce or repress the formation of TCTP in various organisms (11-13, 31). Since TCTP is known to be expressed as part of a protective mechanism in many other eukaryotes, it could be involved in the pathogenesis of mycetoma caused by *M. mycetomatis*. *M. mycetomatis* DNA has been

found in Sudanese soil and vegetation (1). When the fungus enters the body it will have to re-adapt and TCTP expression might be part of the adaptation process. A similar phenomenon has been noted for the parasite *B. malayi* (22). In this parasite no TCTP is found in the pre-infective stages but upon entering the host, TCTP becomes strongly upregulated. TCTP was also found to be upregulated by higher temperatures (28). It was hypothesised by Gnanasekar et al. that the entry of the parasite *B. malayi* from a cold-blooded insect vector to a warm-blooded host could trigger higher expression of BmTCTP (22). However, for *S. mansoni* TCTP expression was significant in all developmental stages, including the pre-infective ones (32). In the developing stages of the *M. mycetomatis* grain, TCTP expression is high. This high TCTP expression is typical for mitotically active tissues and could be associated with stress conditions imposed by the host immune system or nutrient depletion during the invasive growth (10, 35, 41).

In conclusion, we discovered that *M. mycetomatis* TCTP is antigenic in mice, rabbits and humans. The TCTP protein 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,* and in an ELISA system, disease stage dependent immuneresponses were documented. Although TCTP may not be the best diagnostic tool, the ELISAs presented here could be useful in seroprevalence studies. In addition, studies into the influence of TCTP on mycetoma development or its use as a therapeutic vaccine or a vaccine in the prevention of infection are urgently warranted.

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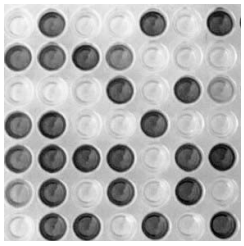
Chapter 6



Madurella mycetomatis compounds cross-reactive with galactomannan are detectable in culture supernatant but not in serum

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Abstract

Madurella mycetomatis, one of the causative agents of mycetoma, secreted significant amounts of galactomannan-like compounds into the culture medium. These compounds were not detected in the patient serum. It could be that the nature of the sclerotia in patients (cementmaterial, melanin, tight package) prohibits diffusion of the galactomannan.

Introduction

Madurella mycetomatis is a causative organism of eumycetoma, a disease characterized by tumourous masses, and formation of grains and purulent material (1). At present treatment of mycetoma is based on surgery and long-time antifungal therapy to prevent relapses. Ideally, continuous diagnostic monitoring of patients to determine the success or failure of antifungal therapy should be implemented. For the diagnosis of invasive aspergillosis, for instance, a sandwich enzyme-linked immunosorbent assay is widely used (5). Circulating galactomannan or galactomannan-containing proteins are detected by the assay through an antibody raised against the galactofuran epitopes present on these molecules (5). Immunological cross-reactivity has been described for *Paecilomyces variotii, Penicilllium spp, Geotrichum capitatum* and *Cryptococcus neoformans* (3, 7). In the present study such galactomannan cross-reactivity was investigated for *M. mycetomatis*.

Materials and Methods

M. mycetomatis culture supernatant and patient sera
Culture supernatant from 32 *M. mycetomatis* isolates was obtained by culturing a 70% transmission inoculum in RPMI-medium for 7 days at 37 ^{o}C (8). Serum was obtained from patients and healthy controls and frozen at –20 ^{o}C until further use.

Galactomannan assay
The platelia Aspergillus assay (Bio-Rad, Veenendaal, The Netherlands) was performed according to the manufacturer's implications. The amount of galactomannan was determined with a concentration curve of pure galactomannan.

Results

Strain-dependent amounts of *M. mycetomatis* galactomannan-like compounds were secreted into the culture medium (figure 1). According to the cut-off value of 1 ng/ml, six isolates secreted no galactomannan-like compounds. The other 24 strains secreted these compounds in concentrations up to 12.9 ng/ml. In only 2 of the 16 patient sera galactomannan levels above the cut-off value of 1 ng/ml were found (Figure 1). There was no correlation with either extent or duration of the disease. In none of the healthy volunteer sera galactomannan levels above 1 ng/ml were found.

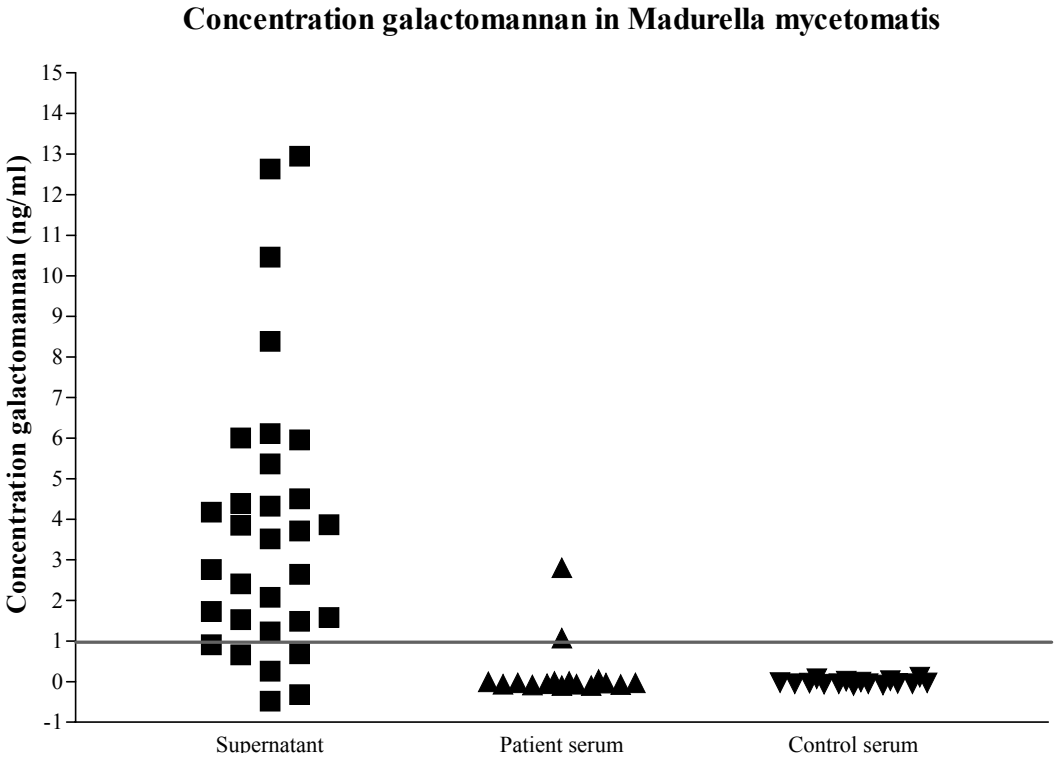

Figure 1: Galactomannan concentration in *M. mycetomatis.* Galactomannan concentrations were determined in the culture supernatant after culturing for 7 days in RPMI culture medium (■). Galactomannan concentrations were also determined in patient serum (▲) and serum from healthy Sudanese controls (▼).

Discussion

Significant amounts of galactomannan-like compounds were found to be secreted into the culture medium. Cross-reactivity in the galactomannan assay has already been described (3). The fact that galactomannan is secreted in the culture medium but not recovered from patient circulation can be caused by a variety of reasons. It could be that the structure of the sclerotia in patients (cementmaterial, melanin, tight package) prohibits diffusion of the galactomannan. For aspergillosis it has already been demonstrated that in some cases galactomannan could be detected in the bronchoalveolar lavage but not in the serum (2, 9). Furthermore, it could be that antifungal treatment of mycetoma hampers the performance of this test. All patients were treated with either high doses of itraconazole or ketoconazole. Marr et al. demonstrated that administration of antifungal agents on the day of test sampling decreased the sensitivity of the assay significantly (4). It could also be that the antigen is not secreted because of a lack of nutrients. It has already been shown for *Aspergillus niger* and *Penicillium fellutanum* that secreted antigens can be reused as carbon source when nutrients are deprived (5, 6). In nutrient-rich conditions, *Aspergillus spp.* release high amounts of galactomannan during growth, but these amounts can be reduced in a nutrient-poor environment such as serum. In conclusion, *M. mycetomatis* secreted compounds which are immunologically cross-reactive to Aspergillus galactomannan *in vitro* but not *in vivo* .

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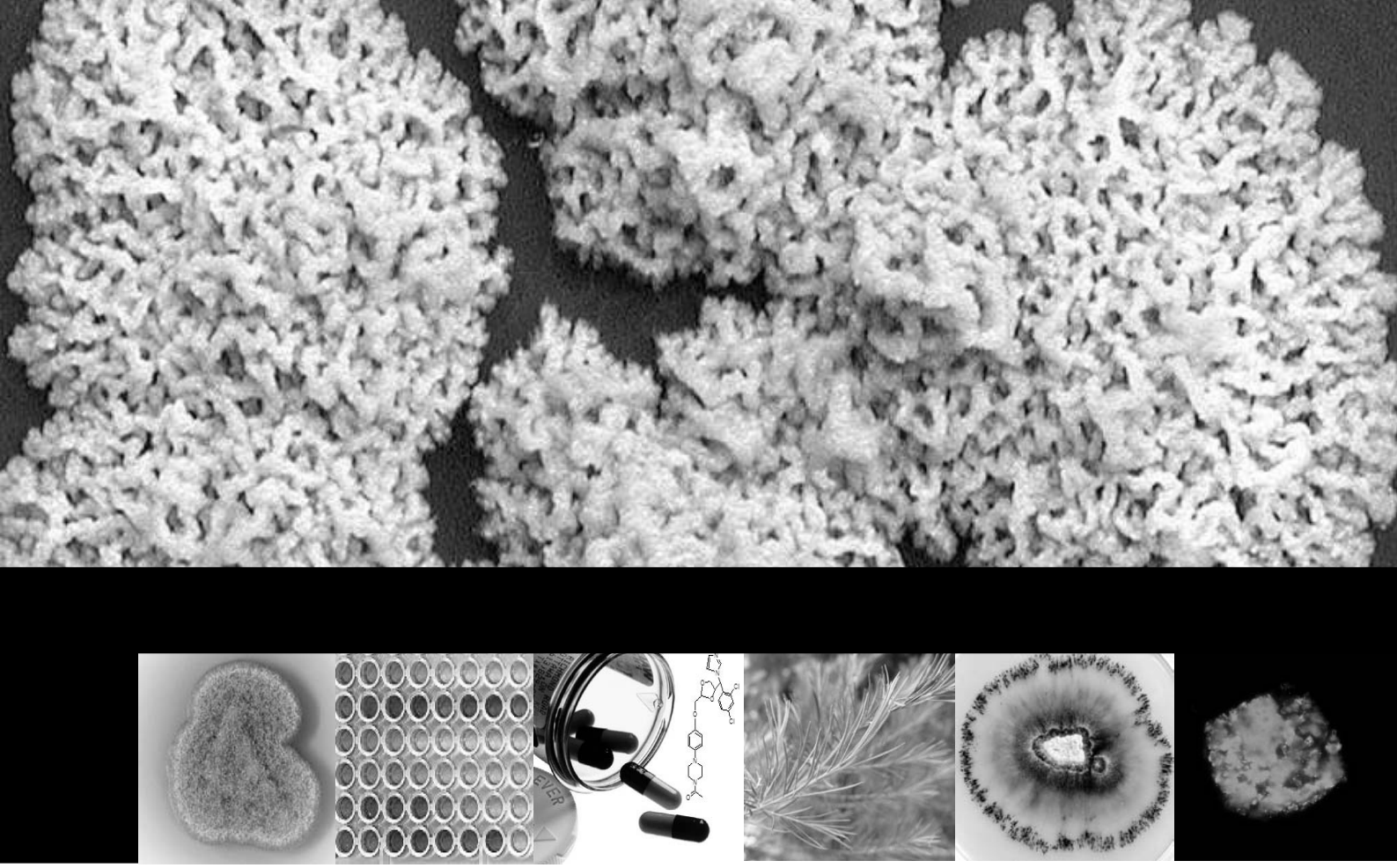
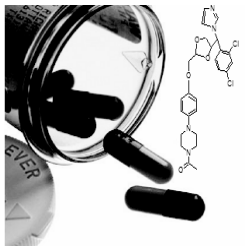


Antifungal Susceptibility of Madurella mycetomatis

Chapter 7



In vitro susceptibilities of Madurella mycetomatis to itraconazole and amphotericin B assessed by a modified NCCLS method and a viability-based 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay

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Abstract

Susceptibilities of *Madurella mycetomatis* against amphotericin B and itraconazole *in vitro* were determined by protocols based on NCCLS guidelines (visual reading) and a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide (XTT) assay for fungal viability. The XTT assay was reproducible and sensitive for both antifungals. Itraconazole (MIC at which 50% of the isolates tested are inhibited [MIC_{50}]) of 0.06 to 0.13 mg/liter) was superior to amphotericin B (MIC_{50} of 0.5 to 1.0 mg/liter).

Introduction

Little is known about the susceptibility of the fungus *Madurella mycetomatis*, the major cause of eumycetoma, to antifungal agents (6). In the past, ketoconazole was promoted as the drug of choice (4, 6, 8, 10, 15, 16), but clinical response to ketoconazole is often poor (5, 17, 18, 25, 26). Other studies show that early treatment with itraconazole seems to be optimal (5, 10, 17). Here we evaluate the *in vitro* activities of itraconazole and amphotericin B against 36 clinical isolates of *M. mycetomatis*.

Materials and Methods

MICs were determined visually by a method based on the NCCLS (approved standard M38-A) (20). In addition, a quantitative colorimetric method using the dye 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide (XTT) was used (12, 13, 24).

M. mycetomatis strains

Independent clinical isolates (n = 34) obtained from Sudanese mycetoma patients visiting the Mycetoma Research Clinic (University of Khartoum, Khartoum, Sudan) during the year 1999 were included. Two additional clinical isolates were derived from patients from Mali (2). Strains were cultivated on Sabouraud dextrose agar with or without 80 mg of gentamicin (Centrafarm, Etten-Leur, The Netherlands) per liter or on malt extract agar (Difco Laboratories, Paris, France). Species were identified as described previously (1, 9).

Antifungal agents

Itraconazole was obtained from Janssen Pharmaceutica Products, Ghent, Belgium, and amphotericin B was obtained from Bristol-Myers Squibb, Woerden, The Netherlands.

In vitro antifungal susceptibility testing

The protocol for susceptibility testing (broth macrodilution) was based on the NCCLS procedure for filamentous fungi (approved standard M38-A) [20]. To prepare inocula from cultures in RPMI 1640 with L-glutamine (0.3 g/liter) and 20 mM morpholinepropanesulfonic acid, mycelia were harvested by 5 min of centrifugation at 2,158 x g and washed once with sterile saline. After sonication (20 s at 28-μm maximum power; Soniprep, Beun de Ronde, The Netherlands) of the hyphal suspension, Tween 60 was added at 0.05% (vol/vol), and the transmissions were adjusted to 70 % at 660 nm (Novaspec II; Pharmacia Biotech). The inoculated tubes were incubated at 37°C for 7 days. Inoculum reading controls (hyphal suspension in saline without antifungals) were included, as were growth controls without antifungals.

The viable fungal mass was determined colorimetrically with XTT as the substrate as described previously (19). Tubes containing final concentrations of 250 μg of XTT/ml and menadione (58 μM) were incubated for 2 h at 37°C and for another 3 h at room temperature.

The tubes were then centrifuged, and the extinction coefficient of the supernatant was measured at 450 nm in a microplate reader.

Results

Figure 1 shows the reproducibility of antifungal susceptibility testing of an *M. mycetomatis* strain (mm55) according to the XTT assay. For amphotericin B, a sudden switch to full reduction in viable fungal mass was observed. Exposure to itraconazole resulted in a concentration-dependent gradual decrease.

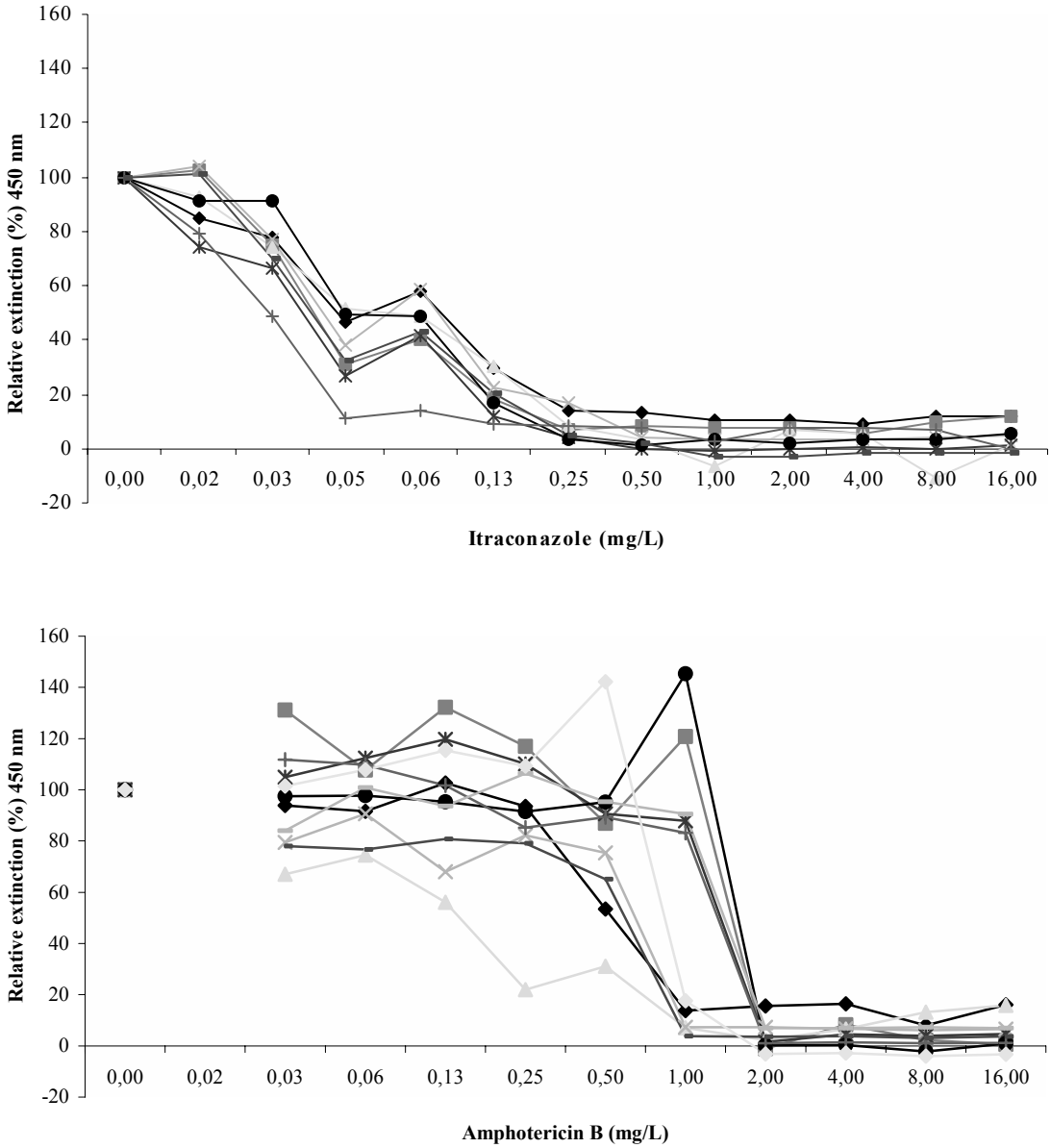

Figure 1: Reproducibility of susceptibility testing of *M. mycetomatis* strain mm-55 against amphotericin B and itraconazole by the XTT method. Curves represent the relative extinction at 450 nm for each drug concentration compared to the growth control (100%). Assays were repeated eight times.

In Figure 2, the results for the XTT assay and the modified NCCLS method were compared for *M. mycetomatis* strain mm55. A concentration-dependent pattern of antifungal activity, each being different for amphotericin B and itraconazole, was observed. The MICs of amphotericin and itraconazole for *M. mycetomatis* mm55 were 1 to 2 and 0.25 to 0.5 mg/liter, respectively. The MICs of the other isolates are shown in table 1.

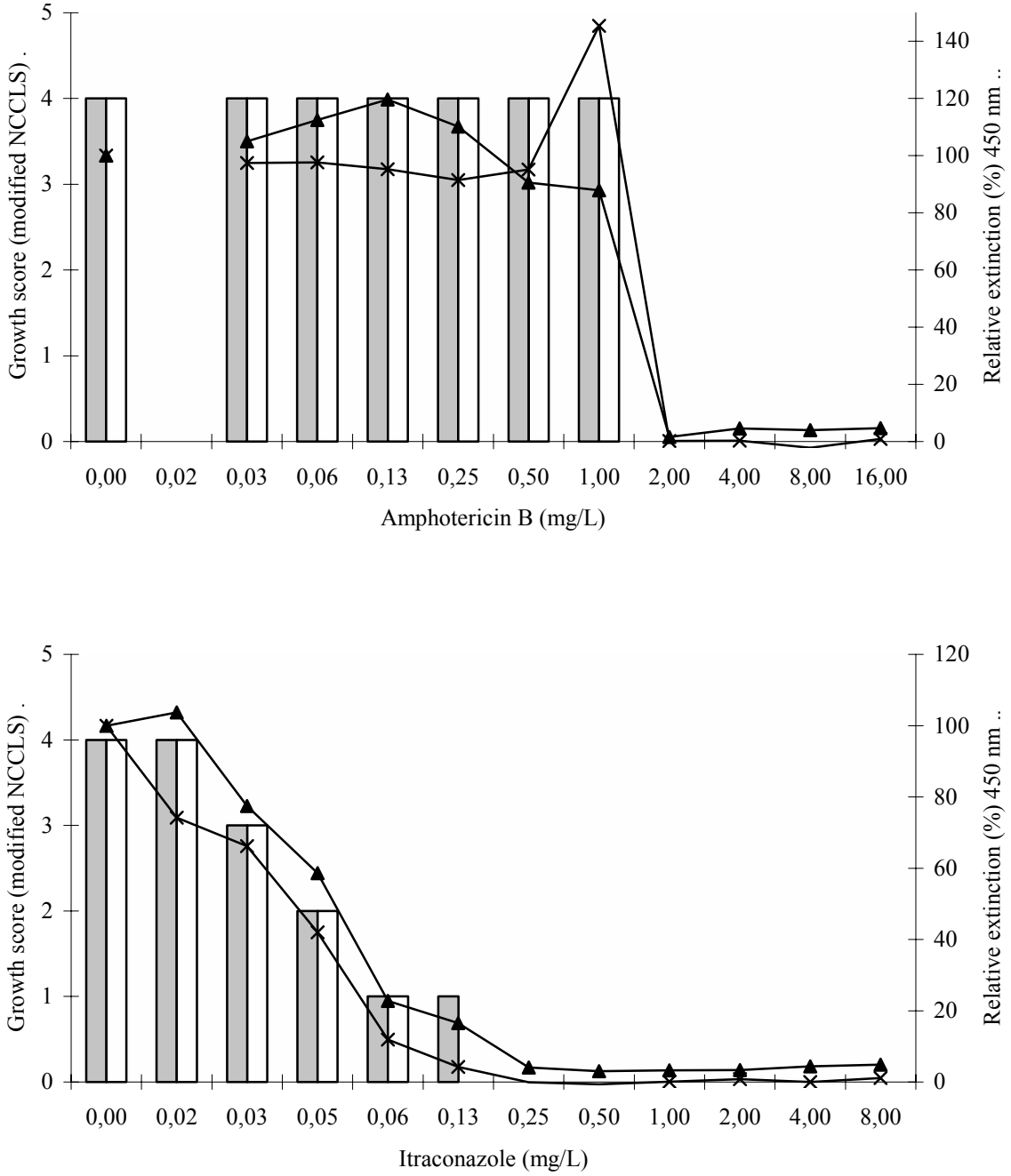

Figure 2. Antifungal susceptibility testing of *M. mycetomatis* strain mm-55 against amphotericin B and itraconazole in duplicate. Curves represent the relative extinction at 450 nm for each drug concentration compared to the growth control (100%) obtained by the XTT assay. Bars represent the growth levels obtained by the modified NCCLS method as determined in a separate experiment.

Isolate	MIC (mg/liter)					
	Amphotericin B			Itraconazole		
	XTT (>50 %)	XTT (>80 %)	NCCLS	XTT (>50 %)	XTT (>80 %)	NCCLS
Mm71	0.125	0.125	0.125	0.03	0.06	0.125
P2	0.125	0.125	0.125	0.06	0.125	0.25
Mm46	0.125	0.125	0.25	0.01	0.01	0.01
Mm54	0.125	0.25	0.5	0.01	0.01	0.03
Mm30	0.125	0.5	0.5	0.125	1	0.5
Mm49	0.125	0.5	0.5	0.01	0.01	0.06
Mm58	0.125	0.5	1	0.06	0.125	0.06
Mm63	0.25	0.25	0.25	0.03	0.03	0.06
Mm39	0.25	0.5	0.25	0.06	0.06	0.06
Mm36	0.25	0.5	0.5	0.06	0.06	0.06
Mm41	0.25	0.5	0.5	0.06	0.125	0.125
Mm83	0.25	0.5	0.5	0.03	0.06	0.06
Mm22	0.25	0.5	1	0.06	0.125	0.125
Mm14	0.5	0.5	1	0.06	0.125	0.125
Mm28	0.5	0.5	0.5	0.06	0.125	0.06
Mm10	0.5	0.5	1	0.25	1	1
Mm29	0.5	0.5	1	0.03	0.06	0.06
Mm25	0.5	1	0.5	0.06	0.125	0.06
Mm18	1	1	1	0.01	0.03	0.01
Mm55	1	1	1	0.06	0.25	0.25
Mm50	1	1	2	0.01	0.03	0.03
Mm52	1	1	4	0.06	0.5	0.5
Mm26	1	2	1	0.06	0.25	0.25
Mm72	1	2	1	0.06	0.06	0.03
Mm78	1	2	1	0.06	0.06	0.06
Mm43	1	2	2	0.125	0.25	0.25
Mm64	1	2	2	0.03	0.03	0.06
Mm68	1	2	2	0.06	0.5	0.05
P1	1	2	2	0.25	0.5	1
Mm45	1	4	2	0.25	0.5	0.5
Mm73	2	2	1	0.06	0.06	0.25
Mm56	2	2	2	0.03	0.06	0.06
Mm13	2	4	4	0.5	1	0.5
Mm35	4	4	4	0.5	1	0.5
Mm16	4	8	4	0.25	0.5	0.5
Mm31	4	4	4	0.125	0.5	0.25

Table 1: Antifungal susceptibilities of 36 *M. mycetomatis* clinical isolates. For the XTT assay, the concentrations resulting in more than 50 % or 80 % reduction in viable fungal mass are shown. For the NCCLS modified method, the concentrations resulting in complete inhibition of fungal growth (MIC) are shown. Data are median values for three determinations.

Discussion

The filamentous nature of *M. mycetomatis* frustrates the straightforward use of the standardized NCCLS protocols since a conidial suspension is usually used as an inoculum (7, 11, 20). Preparing a standardized inoculum for the poorly differentiating and nonsporulating fungal species is problematic (9). To standardize the inoculum, the harvested hyphae were first homogenized. These inocula were found to be within the recommended range of 0.4 x 10^4 to 5 x 10^4 CFU per ml (20).

As the initial hyphal suspension already shows significant turbidity, which complicates visual reading of the antifungal activity, the XTT assay was also used. It generated reproducible data and showed good agreement with the MIC data for amphotericin B. This overall agreement was also documented for various other fungal species (19).

The antifungal effect of itraconazole is superior to that of amphotericin B. Approximately 45% of the 36 *M. mycetomatis* isolates showed susceptibility to itraconazole concentrations of less than 0.13 mg/liter, whereas amphotericin B was not effective at those concentrations. Prevention of growth of all isolates was obtained with itraconazole at 1 mg/liter or less and with amphotericin B at 8 mg/liter or less; these results are in agreement with earlier findings for other filamentous ascomycetes (14, 18, 20). Activities of itraconazole against dermatophytes (11) and agents of hyalohyphomycosis, phaeohyphomycosis, chromoblastomycosis, and mycetoma were also demonstrated (18). Recently, itraconazole has been effectively used for the treatment of a case of fungal mycetoma due to *Fusarium solani* (27). Compared to itraconazole, voriconazole showed comparable or increased *in vitro* activity against a number of emerging and less common mold pathogens (21). The high *in vitro* susceptibility of the *M. mycetomatis* isolates may nominate itraconazole as the drug of choice for treatment. About 33% of the *M. mycetomatis* isolates included in this study had an amphotericin B MIC that is higher than the average peak level in plasma of 1.5 mg/liter (22). In addition to the relatively low antifungal activity, the requirement for long-term treatment of mycetoma patients together with the potential toxic side effects of amphotericin B further limits its use as a first-line therapeutic agent.

In conclusion, the XTT assay is optimal for antifungal susceptibility testing of *M. mycetomatis* since it avoids visual reading. It also provides additional insight into the dynamics of killing. The assessment of antifungal susceptibility may be particularly useful for patients not responding to initial medical treatment.

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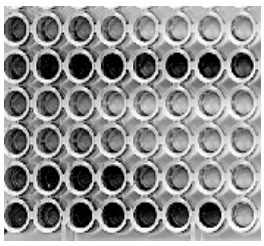
Chapter 8



In vitro antifungal susceptibility testing of Madurella mycetomatis for six antifungal agents by the Sensititre system in comparison with a viability-based XTT assay and a modified NCCLS method

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Abstract

The *in vitro* susceptibilities of 36 clinical isolates of *Madurella mycetomatis*, prime agent of eumycetoma in Africa, for ketoconazole, itraconazole, fluconazole, voriconazole, amphotericin B and 5-flucytosine were determined by the Sensititre system. This system appeared to be a rapid and easy test, and by using hyphal suspensions it generated results comparable to those of a modified NCCLS method. After 10 days of incubation the antifungal activity of ketoconazole (MIC_{90} of 0.125 μg/ml), itraconazole (MIC_{90} of 0.064 μg/ml) and voriconazole (MIC_{90} of 0.125 μg/ml) appeared superior to that of fluconazole (MIC_{90} of 128 μg/ml) and amphotericin B (MIC_{90} of 1 μg/ml), MIC values being in the clinically relevant range. All isolates were resistant to 5-flucytosine (all MICs above 64 μg/ml). Based on the relatively broad range of MICs for the antifungal agents obtained, routine antifungal susceptibility testing for *M. mycetomatis* isolates seems to be relevant for adequate therapeutic management.

Introduction

Eumycetoma is a chronic disease endemic in many tropical and subtropical countries and is characterized by tumefaction, draining sinuses and the presence of grains (8, 9, 15, 21 24). The lesion initiates in the subcutaneous tissue and spreads to destroy the surrounding skin, the muscles and the bones (8, 15). Eumycetoma can be caused by a variety of micro-organisms, but the causative organism most frequently encountered in Sudan is the fungus *Madurella mycetomatis* (9, 10, 15, 24). Mycetoma caused by *M. mycetomatis* is usually treated with extensive surgery, combined with prolonged antifungal therapy (10, 15, 21). Surgery for mycetoma ranges from local excision of distinct lesions to amputation of the infected limb (8,10). In clinical trials a number of antifungals including itraconazole and ketoconazole are being used for the treatment of eumycetoma with various degrees of success (9, 16, 29). Limited data are available regarding the antifungal susceptibility of *M. mycetomatis* (2, 25, 31). Determination of the *in vitro* susceptibility of the non-sporulating *M. mycetomatis* is troublesome. In the method for susceptibility testing of spore-forming filamentous fungi according to the NCCLS guidelines (M38-A) a conidial suspension is used as an inoculum (28). For non-sporulating fungal species a hyphal suspension has to be used (2). In a recently published study by Ahmed *et al.* the hyphal inocula of *M. mycetomatis* were standardized by sonicating the fungus to get a homogeneous suspension (2). Using the modified NCCLS method the authors obtained reproducible amphotericin B data for 90% of the *M. mycetomatis* isolates tested (2). However, for itraconazole visual reading of the gradually decreasing antifungal activity was not always accurate. That is why Ahmed *et al.* used the XTT-assay to ease endpoint reading. In this assay the antifungal activity can be quantified by relating the viable fungal mass to levels of fungal mitochondrial dehydrogenase activity (18,26). The XTT assay appeared appropriate and accurate for determination of antifungal susceptibility of *M. mycetomatis* (2). Unfortunately, the modified NCCLS method combined with the viability based XTT assay are both time consuming and laborious and therefore not easily implemented to screen *M. mycetomatis* isolates routinely for their antifungal susceptibility against a large number of antifungals.

A less laborious method, which became recently available, is the Sensititre system (Trek Diagnostic systems, Ltd., East Grinstead, England). This system is a commercial microdilution method that uses the oxidation-reduction indicator Alamar blue to determine the *in vitro* susceptibilities to amphotericin B, fluconazole, itraconazole, ketoconazole, 5-flucytosine and voriconazole. The test has been successfully used to assess the susceptibilities of various yeasts and filamentous fungi including *Aspergillus* spp. (4, 7, 23, 27). In the present study we evaluated the *in vitro* activities of a number of antifungal agents against 36 clinical isolates of *M. mycetomatis* by using the Sensititre commercial system. In addition we compared this assay with the modified NCCLS method and the viability based XTT assay.

Materials and Methods

M. mycetomatis strains

A total of 36 clinical isolates of *M. mycetomatis* were included in this study. Thirty-four of these strains were obtained from patients seen in the Mycetoma Research Centre, University of Khartoum, Sudan. Two additional strains were obtained from patients originated in Mali. The strains were isolated from biopsies and maintained on Sabouraud Dextrose Agar (Difco Laboratories, Paris, France). The strains were previously identified by morphology and PCR-RFLP (2, 3, 5).

Antifungal agents

For the modified NCCLS method and the XTT assay, ketoconazole and itraconazole were obtained from Janssen Pharmaceutical Products, Belgium, fluconazole and voriconazole were obtained from Pfizer BV (Capelle aan de IJsel, The Netherlands) and amphotericin B from Bristol-Myers Squibb (Woerden, The Netherlands). 5-Flucytosine was obtained from ICN Pharmaceuticals Holland BV, Zoetermeer, The Netherlands.

In vitro antifungal susceptibility testing

MICs were determined independent in duplicate with the colorimetric Sensititre® YeastOne® method (Trek Diagnostic Systems, Ltd, East Grinstead, England), the XTT assay and the modified NCCLS method (2). *M. mycetomatis* was cultured for 10 days at 37^{o}C in RPMI-1640 medium supplemented with L-glutamine (0.3 g/L) and 20 mM MOPS. The mycelia were harvested by 5 min centrifugation at 2158 g and washed with sterile saline. To homogenise the inoculum, the mycelia were sonicated for 20 sec at 28 micron (Soniprep, Beun de Ronde, The Netherlands). For performing the Sensititre test the final inoculum was prepared from the homogenised fungal suspension mixed with the YeastOne® broth (Trek Diagnostic systems) to obtain a transmission of 70 % at 660 nm (Novaspec II, Pharmacia Biotech). Hundred microlitres of this suspension were applied to the Sensititre YeastOne® plate and the test was incubated for 7 days at 37 ^{o}C. MIC endpoints were determined visually at the first blue well for amphotericin B and the first purple well for the other antifungals. Drug concentrations used in this test ranged from 0.008 μg/ml to 16 μg/ml for itraconazole, ketoconazole, voriconazole and amphotericin B and from 0.125 μg/ml to 256 μg/ml for fluconazole and from 0.032 μg/ml to 64 μg/ml for 5-flucytosine.

The XTT assay and the modified NCCLS method were performed according to Ahmed *et al.* (2). In short, MIC endpoints in the XTT assay were determined by adding the substrate 2,3-Bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide} which is enzymatically converted to the coloured formazan in viable cells (18). The extinction of the supernatant was measured spectrophotometrically at 450 nm. The MIC endpoints were defined as the lowest concentration resulting in 80 % or more reduction in viable fungal mass (2). The MIC endpoints using the modified NCCLS method are determined visually as the lowest concentration that prevented any discernable fungal growth (2,28). Drug concentrations used in both tests ranged from 0.016 μg/ml to 8 μg/ml for

itraconazole, ketoconazole, voriconazole and amphotericin B and from 0.25 μg/ml to 128 μg/ml for fluconazole and from 0.125 μg/ml to 64 μg/ml for 5-flucytosine.

Results

For a total of 36 *M. mycetomatis* isolates the susceptibilities for ketoconazole, itraconazole, fluconazole, voriconazole, amphotericin B and 5-flucytosine were determined by using the Sensititre system, the NCCLS method and the XTT assay. For two strains MICs could not be determined using the Sensititre system because these strains did not grow in the presence of Alamar blue. The MIC distribution for the other 34 isolates is shown in figure 1. The MICs for the different antifungals appeared variable and dependent on the *M. mycetomatis* isolate. The majority of the *M. mycetomatis* strains showed high susceptibility to ketoconazole, itraconazole and voriconazole. For ketoconazole MICs ranged from <0.016-1 μg/ml and 0.125 μg/ml ketoconazole was needed to inhibit 90 % of the isolates. The isolates were even more susceptible to itraconazole. For this antifungal an MIC-range of <0.016-0.5 μg/ml was found and only 0.064 μg/ml was needed to inhibit 90 % of the strains. The antifungal activity of voriconazole was similar to ketoconazole, with MICs ranging from <0.016-1 μg/ml, and a concentration of 0.125 μg/ml was needed for inhibition of 90 % of the isolates. The only azole for which a high range of MICs (0.25- >128 μg/ml) was found was fluconazole. A concentration of 16 μg/ml fluconazole was needed to inhibit 90 % of the isolates. For the non-azoles the isolates were less susceptible. For amphotericin the MICs were between <0.016 μg/ml and 4 μg/ml and a concentration of 2 μg/ml was required to inhibit 90 % of the strains. All *M. mycetomatis* isolates were resistant to 5-flucytosine.

To determine the accuracy of the Sensititre test, all *M. mycetomatis* strains were investigated with three methods: the modified NCCLS method, the XTT assay and the Sensititre method. The percentage agreement in experimental outcome for each antifungal is shown in table 1. As is concluded from these data the reproduciblity of the Sensititre test was good. Reproducible results, differing no more than a 1 step dilution were obtained in more than 90% of the strains for all the antifungals except for ketoconazole (Table 1). For this antifungal only a reproducibility of 88.2% was found, which is still very high. When the Sensititre system was compared to the NCCLS method in 88.2% to 100% of the cases identical MICs or MICs differing a single dilution step difference were obtained. This was comparable to the agreement obtained between the NCCLS method and the XTT assay (85.3% to 100.0%). A somewhat lesser agreement was found between the Sensititre system and the XTT assay. In this case the agreements for the non-azole antifungals were still high (82.4 % for amphotericin B and 100.0% for 5-flucytosine) but marginally lower for the azoles. For fluconazole, the agreement was even as low as 67.6 %, while for the other azoles this varied between 70.6% and 91.2%. Overall, the XTT assay resulted in relatively higher MICs compared to the Sensititre method, being a two-step or three-step dilution difference.

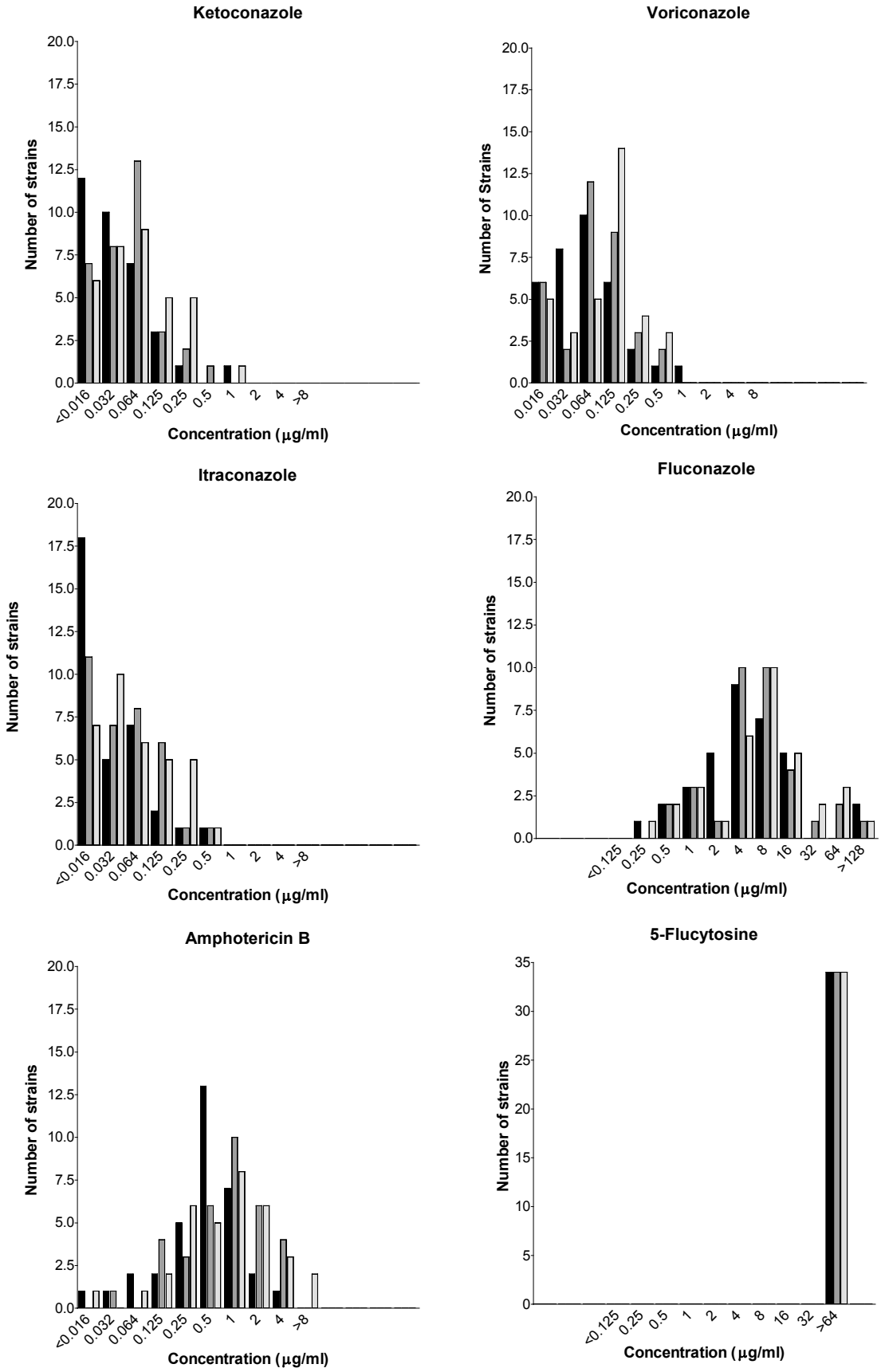

Figure 1: Antifungal susceptibility (MIC) of 34 *Madurella mycetomatis* isolates for various antifungal agents according to the Sensititre system (black), the NCCLS method (dark grey) and the XTT assay (light grey)

Antifungal	Reproducibility[1] of the Sensititre system (%)	Sensititre system versus NCCLS method (%)[2]	Sensititre system versus XTT assay (%)[2]	NCCLS method versus XTT assay (%)[2]
Ketoconazole	88.2	100	79.4	97.1
Fluconazole	91.2	91.2	67.6	85.3
Itraconazole	97.1	88.2	70.6	91.2
Voriconazole	91.2	97.1	91.2	94.1
Amphotericin B	91.2	100	82.4	94.2
5-Flucytosine	97.1	100	100	100

Table 1: Reproducibility and accuracy of the Sensititre method in comparison to the NCCLS method and the XTT assay.
[1] The reproducibility of the Sensititre system was determined by calculating the percentage agreement between tests. The sensititre system was considered reproducible for a certain isolate when the duplicate testresults differed not more than a single dilution in MIC value.
[2] The MICs obtained in each test were compared to MICs in the other tests. To validate the Sensititre system, data were compared with both those of the NCCLS method and those of the XTT assay. In order to compare the different MICs, percentage agreement was calculated. MICs were considered to be in agreement when not more than a single dilution difference in MIC was obtained. For comparison, the percentage agreement between the NCCLS method and the XTT assay is stated in the fifth column.

Discussion

Recently Ahmed *et al.* reported two reproducible assays to measure antifungal susceptibility of *M. mycetomatis* isolates to antifungal agents, being an adapted protocol based on the NCCLS (M38-A) guidelines and a viability-based XTT assay for facilitating endpoint-reading (2). Both test systems appeared reproducible and sensitive, but were also time-consuming. For routine use a test system for antifungal susceptibility should be cheap, fast and easy to interpret as well. Recently, the Sensititre system for determination of antifungal susceptibility of several yeast and fungal species like *Candida spp., Crytococcus spp. a*nd *Aspergillus spp.* has been introduced (4, 6, 20, 23, 27, 30). In this system, the MIC endpoints can be determined visually because of the dye Alamar blue that is converted from blue to red when fungal growth occurs (4, 6, 20, 23, 27, 30). Various studies show that the MICs obtained for several yeasts in the Sensititre system were in good agreement with MICs obtained with the NCCLS method (4, 6, 20). However, for *Aspergillus spp.* there was less agreement between both methods (23, 27). To investigate the value of the Sensititre system for *M. mycetomatis* isolates in the present study, this system was compared to the modified NCCLS method and the XTT assay (2). Good agreements were found between MICs obtained with the Sensititre method and modified NCCLS method. Overall, the MICs obtained with the Sensititre method were equal to or one-step lower than the MICs obtained with the modified NCCLS method. The fact that we found lower MICs with the Sensititre system was also reported for other fungal species like *Cryptococcus neoformans* and *Aspergillus spp.* (6, 27). Although we found lower MICs, the difference was not statistically significant. The discrepancies between the Sensititre system and the XTT-test were higher. This may be explained by the indicator systems being different in both test systems (17, 21). The discrepancies between the Sensititre system and the XTT-test were highest for the azoles. This suggests that the trailing endpoint effect, usually obtained with azoles, is measured more effectively with XTT than with Alamar blue.

In the present study the antifungal susceptibilities of 36 *M. mycetomatis* isolates against ketoconazole, itraconazole, fluconazole, voriconazole, amphotericin B and 5-flucytosine were determined using the Sensititre method. For two of the strains MICs could not be determined because these strains did not grow in the test medium in the presence of Alamar blue. Jahn *et al.* encountered the same problem when testing isolates of *Aspergillus fumigatus*. They found strain dependent differences which could not easily be explained (17). In the present study both ketoconazole and itraconazole appeared to be very effective in inhibiting the *M. mycetomatis* strains. For both antifungals only low concentrations were needed to inhibit 90 % of the clinical isolates, being 0.125 μg/ml and 0.064 μg/ml, respectively. The MICs found for both antifungals correlate with attainable serum levels (5). Ketoconazole was one of the first antifungal agents used in the treatment of eumycetoma caused by *M. mycetomatis*, later itraconazole has been used as well (21, 22, 24). Although some clinical studies showed that ketoconazole and itraconazole resulted in complete cure, the clinical response to both agents is often poor (2, 21, 22, 24). This may partially be explained by the observed variation in MICs for the *M. mycetomatis* isolates. Fluconazole was less effective than ketoconazole and itraconazole in inhibiting the fungal growth. Two isolates had high MICs of >64 μg/ml fluconazole while the other isolates had MICs ranging from 0,125 μg/ml to 16 μg/ml. Although these MICs are high, they still correlate with physiologically attainable serum levels (5, 11). Voriconazole, a relatively new azole which is highly effective against aspergillosis, showed similar high antifungal activity towards the *M. mycetomatis* strains compared with ketoconazole and itraconazole (13). Amphotericin B appeared to be less effective than ketoconazole, itraconazole and voriconazole in inhibiting *M. mycetomatis.* This observation is in accordance with the study performed by Ahmed et al. (2). They also found that amphotericin B was less effective than itraconazole to inhibit *M. mycetomatis*, 33% of the isolates had MICs for amphotericin B, which exceeded the maximum plasma peak levels (2). Of all the antifungals tested in the present study 5-flucytosine was the least effective. Even at high concentrations no fungal inhibition was noticed. The *M. mycetomatis* isolates appeared to be resistant against 5-flucytosine, which is also the case for many other filamentous fungi (1, 12, 14, 19, 28).

In conclusion, the Sensititre system is an appropriate system for the determination of the antifungal susceptibility of *M. mycetomatis* strains. The fungus was highly susceptible to ketoconazole, itraconazole and voriconazole, moderately susceptible to fluconazole and amphotericin B and resistant to 5-flucytosine. The variation in MICs observed for the different *M. mycetomatis* isolates suggests that the introduction of routine antifungal susceptibility testing for *M. mycetomatis* isolates is important for adequate therapeutic management.

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Chapter 9

In vitro susceptibility of Madurella mycetomatis, prime agent of madura foot, to tea tree oil and artemisinin

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Abstract

Eumycetoma caused by *Madurella mycetomatis* is treated with surgery and high doses of itraconazole and ketoconazole. The latter agents are toxic and new therapies are required. MICs were determined for artemisinin and tea tree oil (TTO), two natural herbal compounds. *M. mycetomatis* was not susceptible to artemisinin but TTO did inhibit its growth. Since TTO's prime component easily penetrates the skin, TTO could be a useful agent in the treatment of eumycetoma.

Introduction

Eumycetoma is a subcutaneous disease caused by a variety of micro-organisms, both bacteria and fungi. The most common fungal causative agent is *Madurella mycetomatis*. Eumycetoma is usually treated for extended periods of time with high doses of either itraconazole or ketoconazole. Prolonged treatment with high doses of azoles can result in hepatic toxicity. In order to identify alternative antifungal therapies, the susceptibility of *M. mycetomatis* to other frequently used antifungal agents (amphotericin B, 5-flucytosine and voriconazole) have been determined before. *M. mycetomatis* remained most susceptible towards the azoles; no activity was seen with 5-flucytosine (27). In search of new antifungal agents, research is shifting to more traditional anti-infective folk medicines. An example of such a natural compound is, artemisinin, which is isolated from the plant *Artemisia annua* and used in traditional Chinese medicine (8, 16). In the early 1970s it was established that artemisinin was the compound in this plant extract with antiparasitic activities against *Plasmodium falciparum* (16). *A. annua* was also resistant to common plant pathogenic fungi (19). This resistance was due to substances produced by endophytes present on *A. annua*, however, artemisinin has antifungal activities too (19). *Cryptococcus neoformans* and *Saccharamyces cerevisiae* were inhibited by artemsinin, but *Candia albicans* was not (10, 18). Another example of a natural compound which appears to be successful in curing infections is tea tree oil (TTO), extracted from *Melaleuca alternifolia*. This oil was used by the Australian Bundjalung Aborigines of New South Wales for its anti-inflammatory and antimicrobial properties (6, 11).
In vitro MICs have been determined for a wide variety of micro-organisms and clinical studies indicated that treatment with TTO could improve the course of acne, dandruff, onychomycosis, oral candidiasis and tinea pedis (6). Since both artemisinin and TTO appear to be successful in clearing certain fungal infections, the *in vitro* susceptibilities of a collection of strains of *M. mycetomatis* were compared to both agents and itraconazole, the agent most widely used in eumycetoma treatment.

Materials and Methods

M. mycetomatis strains
In this study MICs, for a total of 34 clinical isolates of *M. mycetomatis* were determined (32 obtained from the Mycetoma Research Centre, University of Khartoum, Sudan, 2 from Mali) for itraconazole (Janssen Pharmaceutical products, Berchem, Belgium), artemisinin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and TTO (Novasel, Skarup, Denmark). The strains were isolated from biopsies and maintained on Sabouraud Dextrose Agar (Difco Laboratories, Paris, France). The strains were previously identified on basis of morphology and PCR-RFLP (1, 25).

In vitro antifungal susceptibility testing
MICs were determined independently in triplicate using the previously reported 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H-Tetrazolium-Hydroxide

(XTT) assay (2, 27). Drug concentrations used ranged from 0.016 μg/ml to 16 μg/ml for itraconazole (ITZ) and artemisinin (ART) and 0.016 % (v/v) to 1% (v/v) for TTO. All dilutions were prepared in DMSO, the final concentration DMSO per inoculum was as described by the CLSI criteria (20).

Results

In concordance with previously published MICs for *M. mycetomatis*, all strains were highly susceptible to itraconazole, the drug of choice to treat eumycetoma in Sudan (figure 1). MICs for itraconazole were evenly distributed in concentrations ranging from <0.002 μg/ml to 0.06 μg/ml (figure 1). A concentration of 0.03 μg/ml was needed to inhibit the growth of 90% of the isolates (table 1).

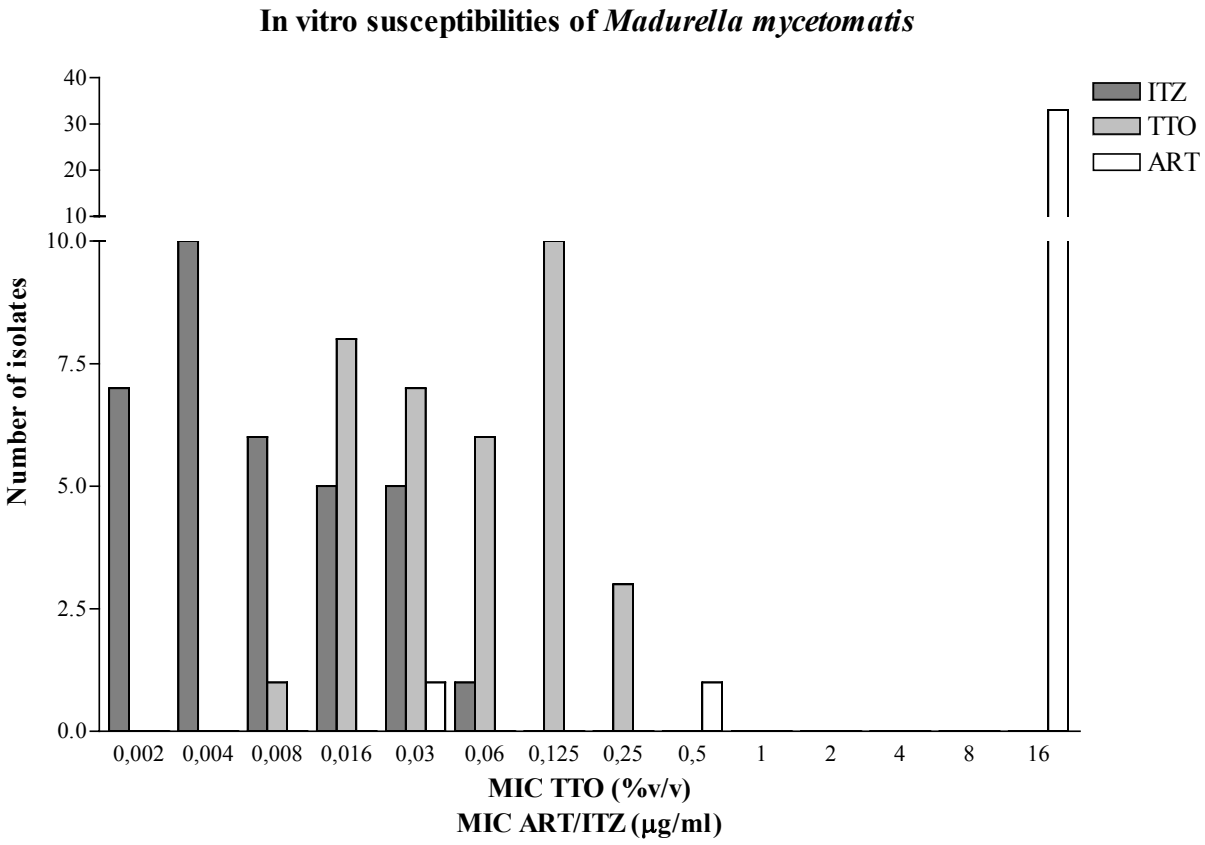

Figure 1: Antifungal susceptibilities (expressed as MICs) of 34 *M. mycetomatis* isolates to itraconazole (ITZ), Tea Tree Oil (TTO) and artemisinin (ART) according to the XTT assay. Although in this graph *M. mycetomatis* seems to be the most susceptible to itraconazole, comparisons with TTO should be made with care. Itraconazole concentrations are shown in μg/ml while TTO concentrations are in % v/v.

Isolates were less susceptible to artemisinin. Only two isolates were inhibited in growth by artemisinin, with MICs of 0.03 μg/ml and 0.5 μg/ml, respectively. The other 32 isolates were not inhibited, not even by 16 μg/ml artemisinin. In 19 of the 34 strains there was a marginal

inhibition in growth visible. Inhibition ranged from 20% to 50%. In the other 15 strains no inhibition and sometimes even stimulation of growth was noted.

In contrast to artemisinin, TTO did inhibit *M. mycetomatis* growth. As seen in figure 1, MICs ranged from 0.008 % (v/v) to 0.25 % (v/v). A concentration of 0.06 % (v/v) was needed to inhibit 50% of the isolates, a concentration of 0.25 % (v/v) was needed to inhibit 90% of the isolates (table 1).

Antifungal agent	MIC		
	Range	50%	90%
ITZ (µg/mL)	<0.002 - 0.06	0.008	0.03
ART (µg/mL)	0.03 - >16	>16	>16
TTO (% v/v)	0.008 - 0.25	0.06	0.25

Table 1: Susceptibility of *M. mycetomatis* to Itraconazole, Artemisinin and Tea Tree Oil. The *in vitro* antifungal susceptibilities of *M. mycetomatis* to itraconazole (ITZ), artemisinin (ART) and tea tree oil (TTO). In this table the range of MICs found, the concentration at which 50 % op the isolates are inhibited and the concentration at which 90% of the isolates are inhibited are shown.

Discussion

The most common antifungal agents used in the treatment of mycetoma caused by *M. mycetomatis* are ketoconazole and itraconazole (3, 9). Success rates with these drugs are variable though and toxic side effects occur. Therefore, the antifungal susceptility of *M. mycetomatis* to a variety of other antifungal agents should be analysed. *In vitro* antifungal susceptibilities to amphotericin B, itraconazole, ketoconazole, fluconazole, voriconazole and 5-flucytosine have already been described (27). In the present study less common antifungal agents are explored, namely TTO and artemisinin, both representatives of traditional folk medicines. For artemisinin some antifungal effect against *Cryptococcus neoformans* and *Saccharamyces cerevisiae* but not against *Candida albicans* were noted previously (10, 18). *M. mycetomatis* was not inhibited by this drug. Interestingly very high MICs for *S. cerevisiae* were obtained when this species is cultured in medium with a fermentable carbon source such as glucose, while low MICs were found when nonfermentable carbon sources, such as glycerol or ethanol, were used (10, 18). In *S. cerevisiae* the inhibitory effect of artemisinin was shown to be caused by disrupting the depolarization of the mitochondrial membrane potential (10, 18). Another striking feature of artemisinin is its binding to the translationally controlled tumour protein (TCTP). Generally, the higher the TCTP expression, the more susceptible the cells were to artemisinin (4, 8). Although *M. mycetomatis* expresses TCTP both *in vivo* and *in vitro*, this did not result in growth inhibition by artemisinin (26).
More interestingly, *M. mycetomatis* was found to be highly susceptible to TTO. Concentrations below 0.25 % (v/v) resulted in full inhibition of fungal growth. This is comparable to the susceptibilities found for the yeasts *Candida spp* and *Malassezia spp* and the filamentous fungi *Alternaria spp, Cladosporium spp, Fusarium spp* and *Penicillium spp* (13-15, 28). The susceptibilities of *Aspergillus spp* were variable, with Vazquez reporting high MICs exceeding 2% (v/v) while Hammer et al. reported MICs in the range 0.12 % (v/v).

A. niger required concentrations as high as 8% (v/v) to be inhibited (14, 28). In *C. albicans*, TTO alters the permeability of the cells and inhibits respiration in a dose-dependent manner (7, 12). TTO has also been used in clinical studies. It has been tested in the treatment of onychomycosis, refractory oral candidiasis and tinea pedis (5, 17, 22-24, 29). Success rates differ in each study. Some researchers reported improvement with TTO while others did not. For example, Tong et al. found that treatment with 10% (v/v) TTO in patients with tinea pedis did not give significant improvement while Satchell et al. did find significant improvement with 25% (v/v) TTO (22, 24). In order for a topical treatment with TTO to be effective in the treatment of subcutaneous mycetoma, the antifungal components of the agent must penetrate the skin. Nielsen and Nielsen, showed that the least lipophilic constituents of TTO could penetrate the skin (21). Among these less lipophilic agents were eucalyptol, α-terpineol and terpinene-4-ol (21). For some of the constituents of TTO the antifungal activity has been determined against a range of fungi, both yeast and filamentous fungi. Terpinene-4-ol, α-terpineol, α-pinene, aromandendrene and β-pinene all had antifungal activities (11). The antifungal activities against *C. albicans* of the first four components were comparable to the antifungal activities found with TTO itself but much lower MICs for β-pinene were found (11). TTO consists of 40% terpinen-4-ol, which is an active antifungal agent and able to penetrate the skin. Considering that *M. mycetomatis* is in growth inhibited by TTO, would suggest that TTO might be useful as a topical agent in the treatment of eumycetoma.

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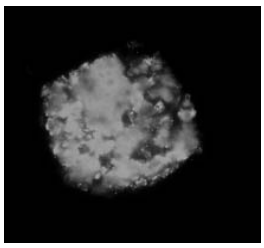
Chapter 10



Melanin biosynthesis in Madurella mycetomatis and its effect on susceptibility to itraconazole and ketoconazole

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Abstract

One of the hallmarks of eumycetoma is the formation of fungal grains, which are secreted by multiple sinuses in infected tissues. *Madurella mycetomatis* grains are black. This black colour was shown to be due to the presence of melanin. Melanin can be produced through various biochemical pathways. It appeared that *M. mycetomatis* melanisation could be blocked by inhibitors of the pyo- and dihydroxynaphthalene (DHN)-melanin pathways but not by inhibitors of the dihydroxyphenylalanine (L-DOPA)-melanin pathway. Melanin isolated from *M. mycetomatis* cells provides *in vitro* protection against the killing effects of the oxidant permanganate and several antifungals. When melanin was added to the culture medium, MICs were found to be 16-fold elevated in the case of itraconazole and 32-fold for ketoconazole. MICs for amphotericin B, fluconazole and voriconazole were not affected. Since itraconazole and ketoconazole are the main antifungal agents used to treat mycetoma, the clinical relevance of the *in vitro* rise in MIC should be studied further.

Introduction

One of the main characteristics of eumycetoma lesions is the formation of grains that are secreted through draining sinuses. Grains are encountered in different colours and textures which are all dependent on the nature of the causative agent (1). Grains can be green, yellow, red or pink but the most frequently encountered "colours" are white or black (1). The grains of the most common causal fungus, *Madurella mycetomatis,* are black (Figure 1). *M. mycetomatis* grains consist of densely packed fungal mycelia embedded in a hard, brown matrix composed of extra-cellular cement material (1, 31). The cement material seems to be composed of an extra-cellular pigment and host tissue debris (1, 31). It is presumed that the black compound in the grain is melanin produced by *M. mycetomatis*.

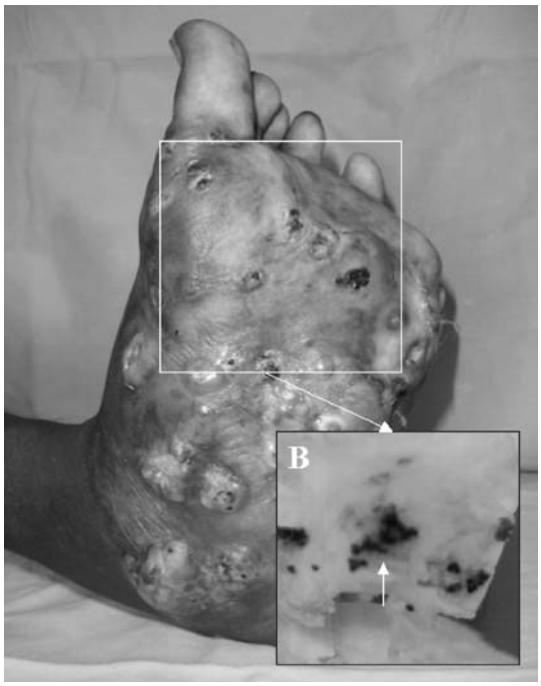

Figure 1: An example of a mycetoma-foot. The black grains within the lesions are shown in the figure B.

Melanins are negatively charged, hydrophobic, macromolecular pigments formed by oxidative polymerization of phenolic or indolic compounds (3, 14, 18). Melanin has been shown to protect micro-organisms against UV-radiation, enzymatic lysis, oxidants and killing by alveolar macrophages (3, 14, 18). It has also been shown to chelate metal ions, to function as a physiological redox buffer, to provide structural rigidity to cell walls and to help store water and ions (18). Melanins may also play a role in protecting against antimicrobial drugs (3). The ability of certain microbes to produce melanin has been linked to virulence and pathogenicity in their respective animal or plant host species (19). Notably, albino strains of *Cryptococcus neoformans* are less potent in establishing an infection than melanised strains (17, 20, 24). Also, for the plant pathogens *Magnaporthe grisea* and *Colletotrichum lagenarium* appresorial melanin is necessary for generating appropriate turgor and, hence, invasive power when infecting plant tissue (18).

Fungi may biosynthesise melanin by a variety of pathways. One of the most extensively studied melanised fungal pathogens, *C. neoformans,* forms its melanin from diphenolic compounds such as 3,4-dihydroxyphenylalanine (L-DOPA) (19). Other fungi including *Aspergillus fumigatus*, *Exophiala* spp., and *Alternaria alternata* biosynthesize melanin from polyketide compounds such as 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (19). A third pathway has only recently been described in the cheese spoiling yeast *Yarrowia lipolytica,* the human pathogen *C. neoformans* and in bacterial species such as *Shewanella algae* (2, 9, 26). Use of this pathway leads to melanin formation from tyrosine precursors but not from L-DOPA (2, 26). The main difference between this latter pyomelanin and the other

two melanins is that this pyomelanin is soluble. It is secreted by the fungus while DOPA – and 1,8-dihydroxynaphthalene (DHN-) melanin are solid, insoluble and usually bound to the cell wall.

The aim of the present study was to identify the pathways used by *M. mycetomatis* to form its melanin and to investigate possible physiological roles of melanin during mycetoma infections.

Material and methods

Immunohistochemistry

Deep tissue biopsies were taken from advanced mycetoma lesions from 3 different patients and embedded in paraffin. Slides were rehydrated and boiled for 1 hour in 6M HCl to detect possible melanisation of the grains. After boiling only the grain structures were left, whereas the surrounding tissue had dissolved. Non-boiled slides were used to determine if melanin was found outside the grains as well. All slides were blocked by 2% BSA and 5% sucrose. Slides were incubated with melanin-specific antibody 6D2 (provided by Dr. Nosanchuk Albert Einstein College of Medicine, New York) for 1 hour at room temperature at a concentration of 5 μg/ml. Monoclonal antibody 6D2 (IgM) was generated against melanin derived from *C. neoformans,* this antibody was not specific for *C. neoformans* melanin since other types of melanin could also be detected. Non-melanised fungi were not bound by this antibody (21). Substitution of the primary antibody with buffer was used as a negative control. Binding of the primary antibody was detected by a FITC-labelled goat-anti-mouse antibody (CLB Biotechnology Services, Amsterdam, The Netherlands). The slides were mounted in DAPI-containing Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and examined with a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss, Göttingen, Germany).

M. mycetomatis strains and culture conditions

For this study 8 isolates of *M. mycetomatis* were used. These strains are part of a large culture collection from the Sudan Mycetoma Research Centre (Khartoum, Sudan). Cultures were maintained on Sabouraud dextrose agar (Difco Laboratories, Paris, France) by repeated subculture. For melanin isolation, strains were subcultured on minimal medium, which was composed of 2% agar (Difco Laboratories, Paris, France) 15 mM glucose (Baker, Deventer, The Netherlands), 10 mM $MgSO_4$ (Merck, Haarlem, The Netherlands, 29.4 mM KH_2PO_4 (VWR, Leuven, Belgium), 13 mM glycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 40 mg/ml gentamicin (Centrafarm, Etten-Leur, The Netherlands). To determine which precursors *M. mycetomatis* uses to form its melanin, the melanin-enhancers L-tyrosine and L-DOPA were added to minimal medium in a concentration of 0.1 mM and 1 mM, respectively. Inhibition of melanin synthesis was tested by adding the following inhibitors to the minimal medium: sulcotrione (Riedel-deHaën, Seelze, Germany), niacin (Sigma-Aldrich, Zwijndrecht, The Netherlands), glyphosate (Luxan, Elst, The Netherlands), tricyclazole (Riedel-deHaën) and phthalide (Acros Organics, Geel, Belgium). Concentrations tested were: 0 mg/l, 5 mg/l,

25 mg/l, 50 mg/l, 75 mg/l, 100 mg/l and 200 mg/l. Strains were incubated for two months at 37 ^{o}C. After such an incubation period, all control cultures were clearly melanised.

Melanin isolation

Melanin for *M. mycetomatis* strain mm55, clinical isolate *C. neoformans* strain 106 and *A. fumigatus* ATCC 204305 was isolated as described by Youngchim et al (33). In short, *M. mycetomatis* hyphae or *C. neoformans* yeast were first enzymatically lysed to form protoplasts. The protoplasts were incubated in the denaturant 4.0 M guanidine thiocyanate to generate dark particles, which were treated with proteinase K to remove residual proteins. Finally, the pellet was boiled in 6.0 M HCl for 1 hour to obtain pure melanin. Melanin concentration was measured by weighing dried mass. As a control, the dark particles were spotted onto glass slides and were probed with antibody 6D2 as described above.

Anti-oxidant activity

The anti-oxidant activity of the melanin was determined by a simple and rapid method (7). This method was based on the ability of a scavenger to inhibit the oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5.5'dithio-2-nitrobenzoic acid (DTNB). To oxidize 60 μM TNB, 6.4 mM permanganate was used. To scavenge this reaction, different amounts of melanin were added ranging from 80 μg/ml to 2.5 mg/ml melanin. As a control, oxidation was also spectrophotometrically assessed by the decrease of the permanganate concentration in solution at a wavelength of 525 nm.

Antifungal susceptibility

Antifungal susceptibilities were tested in duplicate using the Sensititre system (Trek Diagnostic Systems, Ltd.) as described by Van de Sande et al. (27). Since *M. mycetomatis* does not form melanin in RPMI medium, 250 μg/ml of purified melanin was added to each culture. Melanin was isolated from *M. mycetomatis* strain mm55. The experiments were repeated with 250 μg/ml melanin purified from *C. neoformans*, 250 μg/ml melanin purified from *A. fumigatus* and 250 μg/ml bovine serum albumin (BSA) (Sigma-Aldrich, Zwijndrecht, The Netherlands). MICs determined for isolates incubated without melanin were compared to those obtained in the presence of melanin. A difference in the MIC was deemed significant if there was at least a two-fold difference between MICs obtained in the presence or absence of melanin for the same strain.

Binding of antifungal agents to melanin

A volume of 200 microlitre of 800 μg/ml of amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (Janssen Pharmaceutical Products, Beerse, Belgium), ketoconazole (Janssen Pharmaceutical Products, Beerse, Belgium), fluconazole (Pfizer BV, Capelle aan de Ijsel, The Netherlands) or voriconazole (Pfizer BV, Capelle aan de Ijsel, The Netherlands) was incubated with various concentrations of *M. mycetomatis* melanin (25 mg/ml, 2.5 mg/ml, 250 μg/ml, 25 μg/ml, 2.5 μg/ml) for 3h at 37^{o}C. Melanin particles were removed by centrifugation and the supernatant was used to determine the concentration of the antifungal compound as was left in solution. Concentrations of antifungal agents were

assessed with a standard large plate agar diffusion procedure with diagnostic sensitivity test agar (Oxoid, Basingstoke, UK) for amphotericin B, voriconazole and fluconazole and with yeast-peptone agar (7g/l yeast-nitrogen base, 7 g/l trypticase peptone, 15 g/l glucose and 15 g/l agar) for itraconazole and ketoconazole. As test organism *Candida albicans* (amphotericin B) and *Candida pseudotropicalis* (itraconazole, ketoconazole, fluconazole and voriconazole) were used. Concentrations for the melanin-incubated samples were compared to a standard series for each antifungal agent ranging from 0.25 to 4 μg/ml.

Results

M. mycetomatis is melanised in the human host

M. mycetomatis produces black grains in the human host (Figure 1). In order to determine whether the black colour of these grains is due to melanin, antibody 6D2 was used to detect melanin in tissue infected with *M. mycetomatis*. In figures 2A and 2B, a *M. mycetomatis* grain is seen, surrounded by neutrophils. When such a tissue section was stained with antibody 6D2 it was seen that not the entire grain but essentially its rim and some individual hyphae within the grain were reactive and, consequently, considered melanised. Obviously, native human melanin was also found in the melanocytes present in the patient's skin (results not shown). Surprisingly, melanin was also detected inside the neutrophils surrounding the fungal grain, which could suggests that fungal melanin, melanised fungal particles or non-polymerized precursors of melanin are phagocytosized. The precise role of this finding should be further studied. Melanisation of the grain itself was only seen when tissue sections were boiled in 6M HCl (Figures 2D and 2E). This implies that melanin exposure needs to be enhanced prior to immunodetection, as was also described for *Coccidioides posadasii* where the lipid-rich spherule outer wall blocked reactivity of antibody 6D2 (21).

M. mycetomatis produces melanin via the pyo- and DHN-melanin pathways

M. mycetomatis was cultured in the presence of either the melanin inducers L-tyrosine and L-DOPA or the different melanin-inhibitors. L-DOPA appeared to be toxic to *M. mycetomatis*, since no growth was noted when it was added to the medium, even at low concentrations. This implied that L-DOPA was no useful precursor for *M. mycetomatis* melanin biosynthesis. This was confirmed by cultivation in the presence of both inhibitors of the L-DOPA-pathway (niacin and glyphosate). When either of these inhibitors was added to the culture media, melanisation was similar as for the non-inhibited controls (Figure 3). When examined by light-microscopy, the fungal cell walls showed clear melanisation. No inhibition of melanin synthesis was noted. These observations confirmed that *M. mycetomatis* does not use the L-DOPA-melanin biosynthesis pathway to form its melanin. In contrast, cultivation in the presence of additional L-tyrosine (precursor of both the L-DOPA- and the pyo-melanin pathway) resulted in a light-brown colony showing melanised particles and a secreted pigment. This secreted pigment turned black in the presence of ironsulfate.

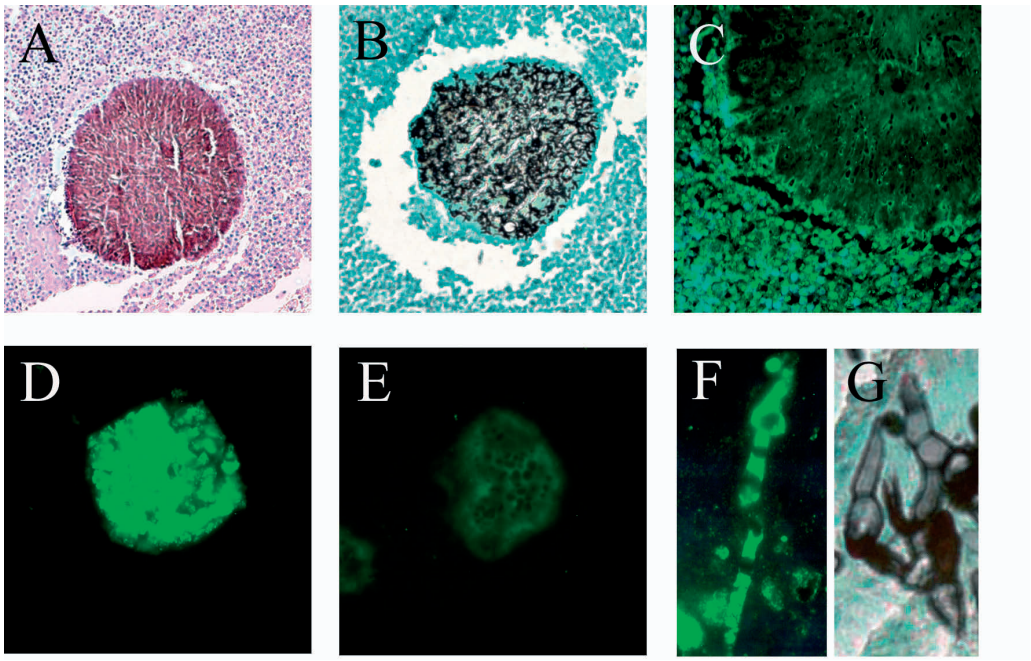

Figure 2: Melanisation of M. mycetomatis grains *in vivo* . All figures represent grains found in human subcutaneous tissue of the foot. In figures A and B, grains are stained with HE and Grocott respectively. In figure C the same tissue is stained with melanin-specific antibody 6D2. In this figure melanin was shown to be present in individual cells in the neutrophil zone but the grain itself was hardly stained (magnification 630x). To detect melanisation in the grain, slides were pre-treated by boiling 1 hour in 6M HCl to remove all other cellular compounds and afterwards stained with antibody 6D2. In figure D melanisation is seen within the grain itself. No melanisation is seen when the primary antibody was replaced by PBS only (figure E). Within the grain individual hyphae are melanised (F). Figure G represent a single hyphae within the grain (Grocott)

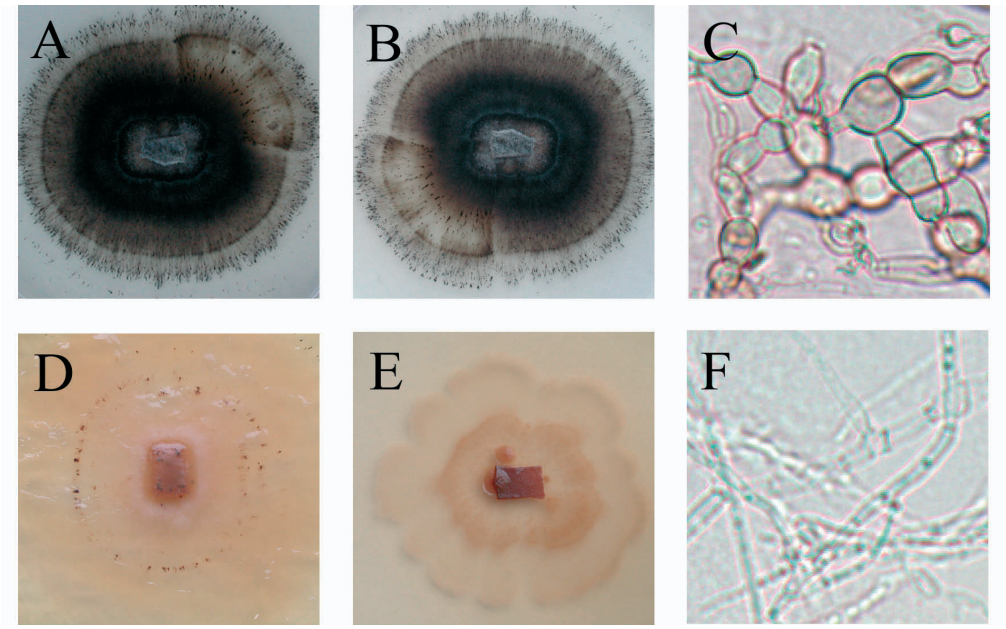

Figure 3: Melanin inhibition. In order to establish which pathway is used by *M. mycetomatis* inhibition experiments with 5 melanin-inhibitors are performed. In figure A the wildtype melanised isolate is shown. No difference between a non-inhibited isolated (figure A) and a niacin-inhibited or glyphosate inhibited isolates was noted (50 mg/l glyphosate, figure B). A non-melanised phenotype was obtained when 50 mg sulcotrione was added to the culture medium (figure D). When sulcotrione was added a brown pigment was secreted into the culture medium. No melanin was formed when either 50 mg/l phthalide or 5 mg/l tricylazole was added to the culture medium (5 mg/l tricyclazole, figure E). The melanised phenotype was microscopically also different from the non-melanised phenotype. On figure C a 400x magnification of the melanised phenotype is seen. The non-melanised phenotype is shown in figure F. This photo is made at a 400x magnification. The cells are longer, less branched and slimmer than the melanised phenotype.

Cultivation in the presence of an inhibitor of the pyo-pathway (sulcotrion) or with inhibitors of the DHN-pathway (phthalide and tricyclazole) resulted in non-melanised phenotypes (Figure 3). Melanisation of the cell wall vanished and the fungal hyphae appeared to be elongated (Figure 3). Inhibition of melanin synthesis was noted at concentrations of 25 mg/l sulcotrion or higher. Tricyclazole was an even more potent inhibitor of the DHN-melanin pathway than phthalide was. Only 5 mg/l tricyclazole was needed to completely block melanin synthesis while 50 mg/l phthalide was needed to establish the same effect. This shows that in case of *M. mycetomatis* tricyclazole is a stronger DHN pathway inhibitor than phthalide.

M. mycetomatis melanin provides protection against oxidants

The purified melanin particles of *M. mycetomatis* strain mm55 resembled the particles seen in culture. The amount of melanin was culture- and strain-dependent, meaning that some phase-variation was noted in the amount of melanin production in each isolate. In order to establish whether melanin protects *M. mycetomatis* against reactive oxygen species produced by the host, we investigated whether it scavenged one of the strongest oxidants known, permanganate (Figure 4). When melanin was added to the permanganate it was noted that the more melanin was present, the less permanganate was left. If less permanganate was present the oxidation of TNB was less efficient since TNB was still measurable in the solution. From this experiment it appeared that 200-250 µg melanin could effectively scavenge the oxidation of TNB by permanganate.

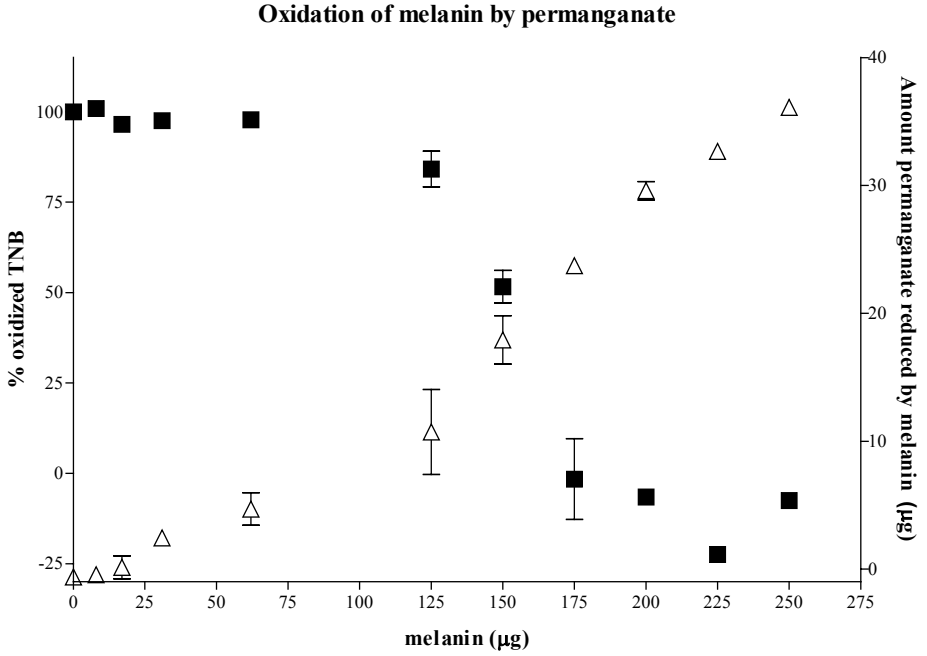

Figure 4: Antioxidant activity of melanin. Fungal melanin in different concentrations was incubated with 6.4 mM permanganate. After 10 minutes incubation TNB was added and measured. TNB oxidation was calculated by the following formula: (OD_{412} of sample)/(OD_{412} of oxidized TNB)*100% (■). The concentration permanganate reduced by melanin was stated as well (Δ). Overall, the more permanganate was reduced by melanin the less TNB was oxidized.

M. mycetomatis melanin provides protection against itraconazole and ketoconazole

Since *M. mycetomatis* melanin was shown to protect against permanganate, it might also protect against other agents that are detrimental to the fungus, including antifungal agents. MIC measurements, with or without addition of melanin extracted from *M. mycetomatis,* were performed for six antifungal agents, including the polyene amphotericin B, the antimetabolite 5-flucytosine and the azoles ketoconazole, itraconazole, voriconazole and fluconazole (see table 1). The same experiments were repeated with L-DOPA melanin extracted from *C. neoformans* (Figure 5), DHN melanin isolated from *A. fumigatus* (results not shown) and albumin (results not shown). No significant differences (meaning less than one two-fold dilution step) in MIC were noted for amphotericin B, 5-flucytosine, fluconazole and voriconazole upon preincubation with either type of melanin or albumin. However, a significant difference in the MICs for itraconazole and ketoconazole was documented in the presence of DHN-melanin isolated from either *M. mycetomatis* (Figure 5) or *A. fumigatus* (results not shown). This effect was not documented when assays were supplemented with L-DOPA melanin or BSA. Due to the effect of *M. mycetomatis* melanin, itraconazole MICs were 16-fold increased, an even more significant increase was noted for ketoconazole (32-fold). These elevations in MIC were comparable to the elevations found when *A. fumigatus* melanin was added. When taking into account that azole MICs of 2 μg/ml or higher may be associated with failure of therapy, this implies that upon melanin supplementation 6 out of 8 and 5 out of 8 strains should be considered clinically resistant to ketoconazole and itraconazole, respectively (23). Without melanin supplementation, only a single strain should be considered resistant to ketoconazole.

	Antifungal agents											
	Ketoconazole		Itraconazole		Voriconazole		Fluconazole		Amphotericin B		5-Flucytosine	
	No Mel	Mel	No Mel	Mel	No Mel	Mel	No Mel	Mel	No Mel	Mel	No Mel	Mel
Mm46	<0.008 (<0.008)	0.25 (0.125-0.5)	<0.008 (<0.008)	0.125 (0.06-0.25)	<0.008 (<0.008-0.016)	<0.008 (<0.008-0.016)	1 (0.5-2)	1 (1-2)	0.25 (0.125-0.5)	0.5 (0.5-1)	>64 (>64)	>64 (>64)
Mm30	0.016 (0.016-0.03)	1 (0.5-1)	<0.008 (<0.008-0.016)	0.25 (0.25)	0.06 (0.06-0.125)	0.06 (0.06)	16 (8-16)	16 (16)	0.25 (0.125-0.5)	0.25 (0.25)	>64 (>64)	>64 (>64)
Mm50	0.03 (0.03-0.06)	2 (1-2)	0.016 (<0.008-0.032)	1 (0.5-1)	0.06 (0.06-0.125)	0.06 (0.06)	4 (4-8)	8 (4-8)	1 (0.5-1)	1 (0.5-1)	>64 (>64)	>64 (>64)
Mm68	0.06 (0.06)	2 (1-4)	0.125 (0.06-0.25)	4 (2-8)	0.06 (0.06)	0.125 (0.125)	8 (8)	16 (8-16)	1 (0.5-1)	2 (1-2)	>64 (>64)	>64 (>64)
Mm14	0.06 (0.06-0.125)	2 (2-4)	0.125 (0.06-0.125)	2 (2-4)	0.032 (0.032-0.064)	0.064 (0.032-0.064)	4 (4)	4 (4)	0.5 (0.5)	0.5 (0.5)	>64 (>64)	>64 (>64)
Mm55	0.125 (0.06-0.125)	2 (2-4)	0.125 (0.06-0.25)	2 (1-4)	0.06 (0.03-0.125)	0.06 (0.06-0.125)	8 (4-16)	8 (8)	1 (0.5-2)	2 (1-2)	>64 (>64)	>64 (>64)
Mm35	0.25 (0.25-0.5)	16 (8-16)	0.25 (0.125-0.5)	2 (2)	0.5 (0.25-0.5)	0.25 (0.125-0.25)	256 (128->256)	256 (256->256)	4 (2-4)	4 (2-4)	>64 (>64)	>64 (>64)
Mm13	2 (1-2)	16 (16)	0.5 (0.25-1)	>16 (>16)	2 (1-4)	2 (1-2)	>256 (>256)	>256 (>256)	0.5 (0.25-1)	0.5 (0.5)	>64 (>64)	>64 (>64)

Table 1: MICs determined for 8 *M. mycetomatis* isolates for ketoconazole, itraconazole, voriconazole fluconazole, amphotericin B, 5-flucytosine. Alle MICs were determined with the Sensititre assay and MICs are shown without the addition of melanin (no mel) or with the addition of melanin (mel).

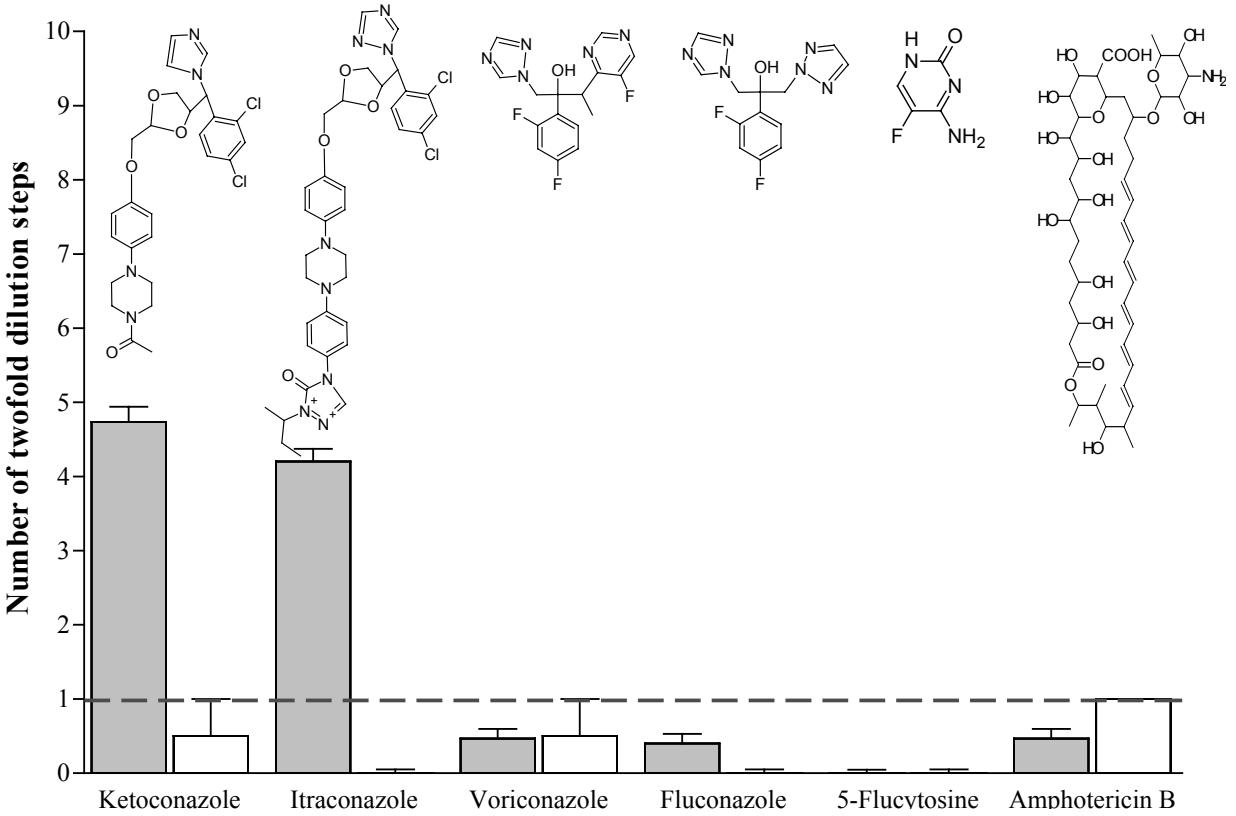

Figure 5: MIC shift of DHN-melanin supplemented isolates (grey) and L-DOPA-melanin supplemented isolates (white) in comparison with non-melanin supplemented isolates. The absolute difference in MIC was calculated by extracting the MIC for a certain strain without melanin supplementation from the MIC of that same strain with melanin supplementation. Differences were stated as the number of two-fold dilutions in difference. The dashed line in this figure shows the difference allowed between tests for the same strain. Furthermore in this figure the structure of the different antifungal agents are shown.

M. mycetomatis melanin binds amphotericin B, itraconazole and ketoconazole

Since *M. mycetomatis* MICs for itraconazole and ketoconazole were increased with supplementation of melanin, it was investigated if DHN- and L-DOPA-melanin bound amphotericin B, itraconazole, ketoconazole, fluconazole and voriconazole. In figure 6 it is seen that amphotericin B is bound by both DHN-melanin and L-DOPA melanin. Binding occurred in a dose-dependent manner, the more melanin present, the lower the concentration of amphotericin B left in solution was after 3 h of incubation at 37^{o}C. DHN-melanin was also able to bind ketoconazole in a dose-dependent manner, although binding was not as effective as seen for amphotericin B. After 3h of incubation with 25 mg of DHN-melanin still 46.8 % of ketoconazole was left in solution. Binding was only found for DHN-melanin, since still 90.5 % of ketoconazole was present after incubation with 25 mg of L-DOPA melanin. The same effect was noticed for itraconazole although to a lesser extent. Fluconazole and voriconazole were not bound by either type of melanin.

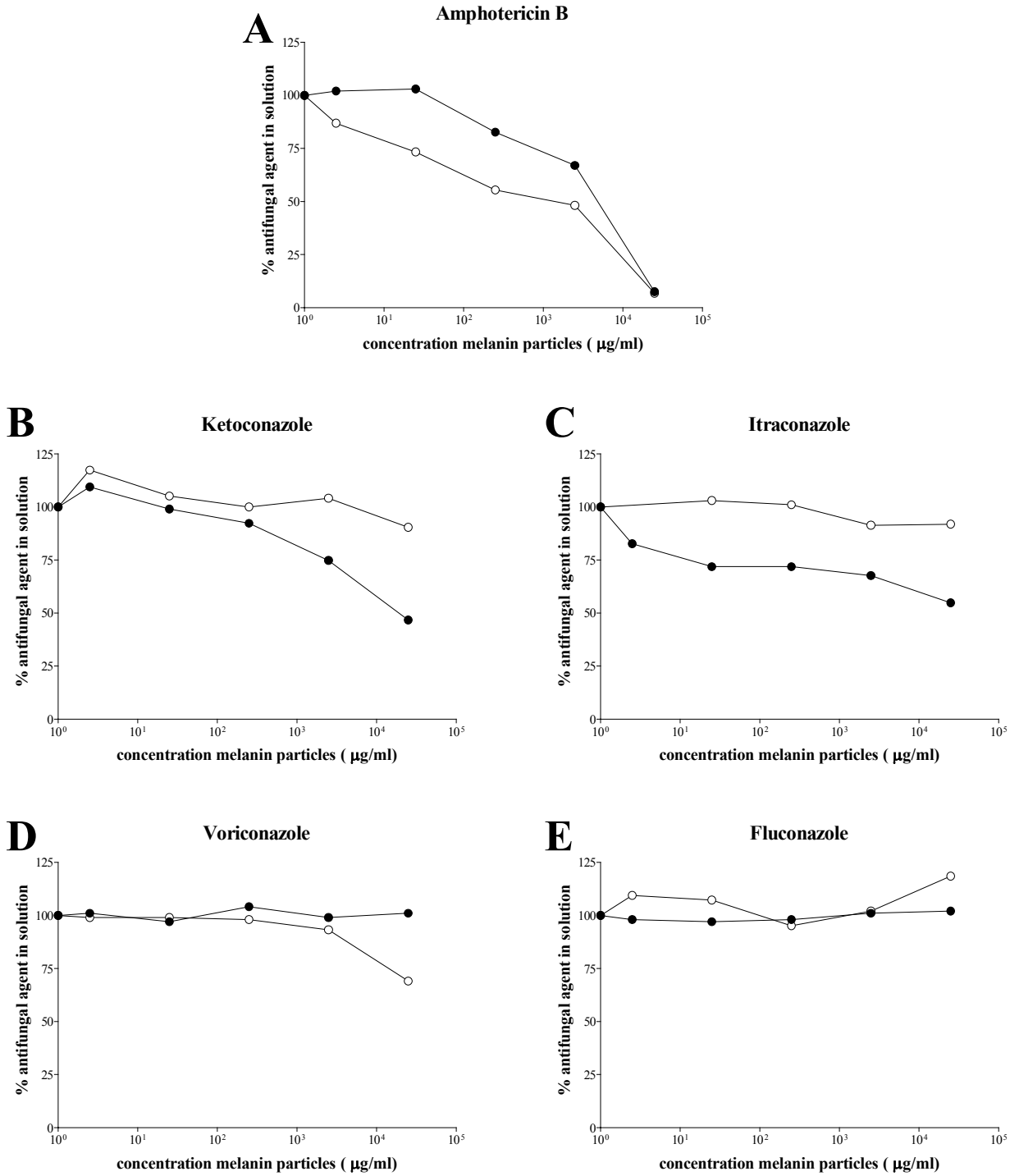

Figure 6: Antifungal binding capacity of DHN-melanin isolated from *M. mycetomatis* (•) and L-DOPA-melanin isolated from *C. neoformans* (o). Fungal melanin in different concentrations was incubated with 800 µg/ml of amphotericin B (A) ketoconazole (B), itraconazole (C), voriconazole (D) or fluconazole (E) for 3 h at 37°C. After incubation melanin was pelleted and the concentration of each antifungal agent left in solution was determined with agar diffusion. Curves represent the concentration antifungal agent left in solution after incubation with various concentrations of melanin compared to the same antifungal agent without melanin.

Discussion

M. mycetomatis actively melanised *in vivo*. Melanin is found within the fungal grain as well as inside phagocytes surrounding the grains. *In vitro* melanisation of *M. mycetomatis* can be blocked with inhibitors of the pyo- and the DHN-pathways but not with inhibitors of the DOPA-pathway. The DHN-melanin inhibitor tricyclazole was a stronger inhibitor than phthalide. A similar difference has already been described for DHN-melanin-producers such as *Aspergillus* spp and *Penicillium* spp. (32).

DHN-melanin and L-DOPA melanin are dark insoluble phenolic or indolic compounds, whereas pyomelanins are mostly brown, soluble pigments derived from homogentisic acid (HGA) (19). Since pyomelanins are brown soluble pigments it was expected that inhibition with sulcotrion would result in a phenotype lacking the secreted pigment in the culture medium, resembling sulcotrion-treated pyo-melanin producing micro-organisms such as *Mycosphaerella graminicola* (15). The black melanin would not be affected by sulcotrione because this was probably synthesized through another melanin biosynthesis pathway. However, the phenotype obtained was opposite, suggesting that although sulcotrion inhibits melanin synthesis it does not inhibit synthesis of the secreted pigment, which, therefore, is probably not a classical pyomelanin. Since sulcotrione did inhibit synthesis of the cell wall-associated melanin, the pigments produced could be precursors of the solid melanin type. For instance, inhibition of DHN-melanin synthesis in certain fungal species (e.g. *Pyricularia oryzae* and *Wangiella dermatitidis*) resulted in a carmine red pigment in the culture media consisting of flaviolin and 2-hydroxyjuglone (10, 16). Another option is that the pigment is pyo-melanin but the conversion of *p*-hydroxyphenylacetaldehyde (HPAD) into hydroxyphenylacetic acid (HPAA) is catalysed by an enzyme not inhibited by sulcotrion. Interestingly, the pigment secreted by *M. mycetomatis* has some features, which have also been documented in pyomelanins. For instance, both pigments turn black in the presence of iron (11). Another option is that *M. mycetomatis* simply forms two types of melanin simultaneously. For *C. neoformans* it has recently been demonstrated both L-DOPA melanin and pyomelanin can be synthesized by the same organism, depending on the availability of the substrate (9).

The DHN-melanin isolated from *M. mycetomatis* was able to scavenge permanganate, one of the strongest oxidants known to modern chemists. During a mycetoma infection, grains are usually in close contact with a layer of neutrophils which actually secrete reactive oxidant species such as hypochlorous acid, hydrogen peroxide and reactive oxygen (5, 8). The fact that permanganate was effectively melanin-scavenged implicates a strong protective function for the melanin produced by *M. mycetomatis* during infection.

Supplementation of DHN-melanin in *in vitro* antifungal susceptibility assays rendered *M. mycetomatis* less susceptible to the antifungal agents itraconazole and ketoconazole, the prime agents used in clinical settings (1). Not much has been published on the effect of melanin on antifungal agents. Most of these studies were performed with melanised yeast cells versus non-melanised ones (6, 29). The fungi were cultured in chemically defined media to acquire a melanised phenotype and were transferred afterwards into adjusted culture media to determine the MICs. Interestingly, the usually melanised isolates of *C. neoformans* and *Histoplasma capsulatum* were not able to produce melanin in these test media, not even when additional substrate was added (13, 28). During cultivation the melanised phenotype

disappeared and all resulting offspring was non-melanised (13). This could explain why no differences in MICs were found. Similar to other fungi used in melanin protection studies thus far, *M. mycetomatis* does not form melanin in RPMI culture media, the medium used in the M38A protocol from the Clinical and Laboratory Standards Institute (CLSI) during drug susceptibility testing (4). In order to establish the effect of melanin on the MIC we chose to add purified *M. mycetomatis* melanin to the culture medium as an *in vitro* simulation of the *in vivo* situation during infection. Furthermore, it was not feasible to determine MICs on minimal medium. Melanisation took at least one month and when melanised cells were inoculated on a fresh minimal medium agar plate, the resulting colony was not melanised. Only after a month of cultivation, melanised phenotypes were seen.

Although for other fungi no MIC shift was described, it was shown that the time required for killing of melanised cells was much longer (13, 29, 30). This was thought to be the result of binding of antifungal agents to melanin (13, 28-30). We here demonstrate that co-incubation of DHN-melanin with either itraconazole or ketoconazole during susceptibility testing results in an MIC shift. However, itraconazole and ketoconazole are two azoles with high protein binding capacity. *In vivo* serum protein binding is more than 90% for both azoles. *In vitro* protein binding has been described to affect MICs for ketoconazole, fluconazole and amphotericin B but not for itraconazole (25). When we performed our antifungal assay with 250 μg/ml albumin, no shift in MIC was observed and we therefore do not expect that the MIC shifts we observed with melanin are confounded by low protein amounts. The effect appeared melanin-specific since this increase was also noted when *A. fumigatus* DHN-melanin was added to the culture medium but no increase in MIC was noted when L-DOPA melanin was added. The fact that we did not document an amphotericin B MIC shift for L-DOPA melanin was unexpected since there is evidence that binding of amphotericin B or caspofungin to L-DOPA melanin significantly reduced its efficacy in time-kill assays (28). In our own binding-assay we also found that amphotericin B is bound by both L-DOPA- and DHN-melanin. This binding did not result in a MIC shift when our antifungal susceptibility assay was supplemented with either type of melanin. In our assay the melanin remains in the solution during the whole experiment, in the time-kill assays performed by others melanin was removed after a period of incubation prior to MIC determination. Apparently, when leaving melanin in the solution, the amphotericin B bound by melanin might still be able to inhibit *M. mycetomatis* growth. In previous studies no binding of the azoles voriconazole, fluconazole and itraconazole and the antimetabolite 5-flucytosine to L-DOPA melanin was noted (28, 29). This was in agreement with the results we obtained in our binding-assay. Only DHN-melanin was able to bind the azoles itraconazole and ketoconazole, no evidence of binding to L-DOPA melanin was found in either the binding assay or the MIC assay. This suggests that binding of itraconazole and ketoconazole was specific for DHN-melanin, which could explain that the only other MIC shift for itraconazole under influence of melanin was described in a DHN-melanin-producing fungus (*Wangiella dermatitidis*) (22). Since all azoles have a similar working mechanism, the differences in melanin binding should be traceable to the molecular structure of the azoles. Itraconazole and ketoconazole, which are structurally similar, differ considerably from fluconazole and its derivate voriconazole (12). All azoles possess an azole-ring, the active domain of the molecule. The azole-ring of the

imidazole ketoconazole is slightly different from the other three, harbouring only two nitrogen atoms instead of three. The difference in MIC effect is probably not functionally associated with the presence of an azole-ring. Interaction of melanin with itraconazole and ketoconazole is probably mediated by the dichlorobenzene ring or the long polyaromatic side chain or a combination of the two (see Figure 5). Binding of the azoles to melanin *in vitro* results in a decrease in MIC because of the lack of accessibility to the fungus. However, by binding the azole to the grain an artificial "slow release" capsule might be created, resulting in a constant local release of the antifungal agent. This implies that the effect of the binding of itraconazole and ketoconazole to DHN-melanin *in vitro* is not easily translated to its effect *in vivo*. Further studies are needed to establish the precise physiological role of melanin in the fungal grain.

In conclusion, we demonstrated that *M. mycetomatis* is melanised *in vivo* and we identified the biochemical pathways leading to this melanisation. Melanisation of *M. mycetomatis* offers protection to strong oxidants and the antifungal agents itraconazole and ketoconazole *in vitro*. Its protective role *in vivo* still needs to be established.

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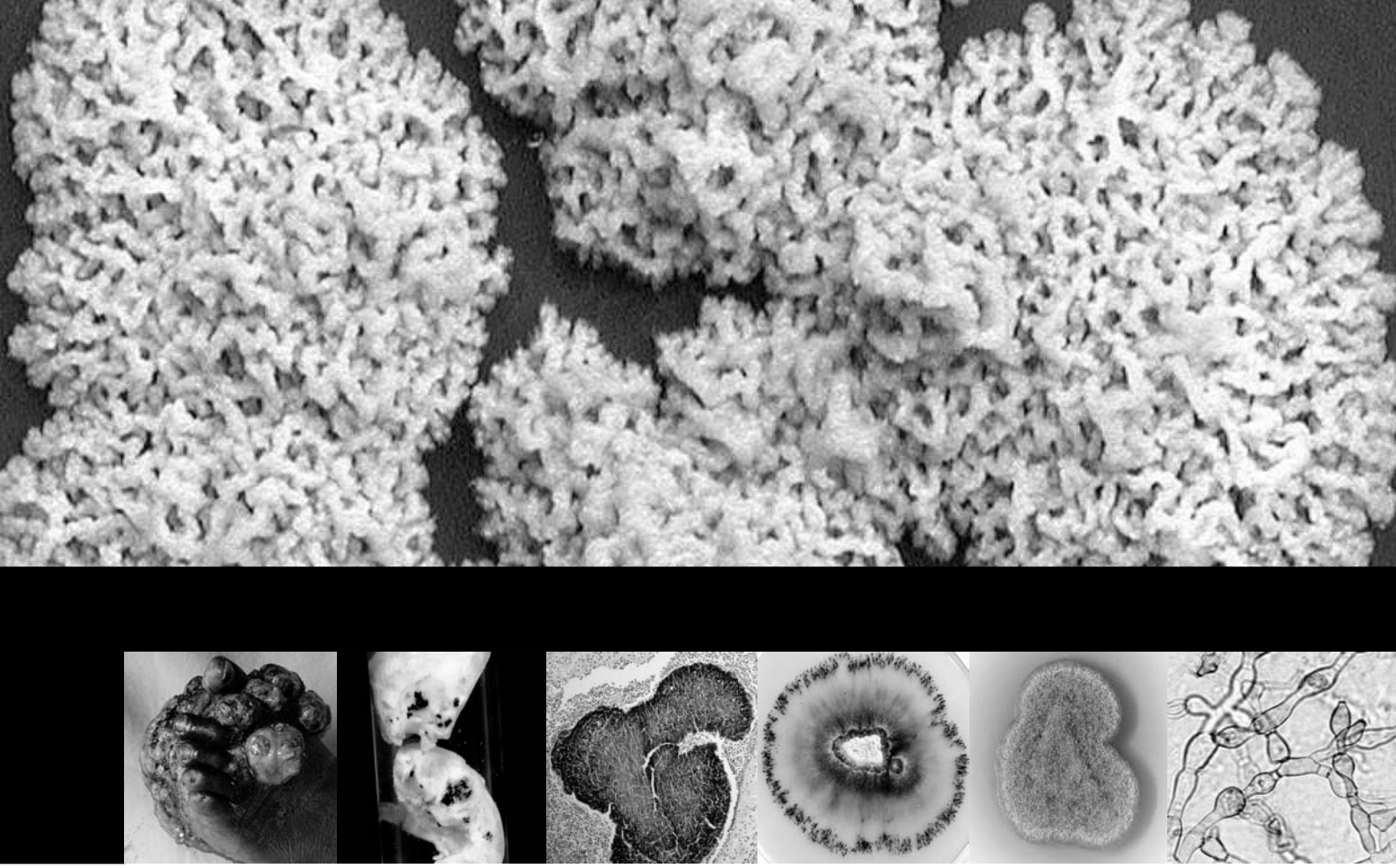
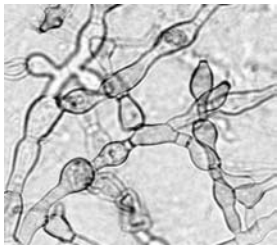


General discussion and concluding remarks

Chapter 11



Summarizing discussion

Wendy W.J. van de Sande

Mycetoma is one of the less-studied infections occurring in the developing world. This infection, characterized by its deforming subcutaneous mass, its sinuses and the discharge of grains and purulent material still poses a hard-to-treat disease in many tropical and subtropical areas (7, 23, 24). The disease usually affects young men between 20 and 40 years of age and, therefore, it has a huge effect on local economy (7, 23, 24). One of the countries with the highest incidence is Sudan, with an estimated number of ~500 new cases seen in hospitals in 1955 (1). The incidence that year was about 4.6 cases per 100.000 inhabitants (1, 57). Still, this is an estimation, the real incidence probably is higher, because patients tend to go to hospital only when the disease is in an advanced stadium and not all hospitals were invloved in the study of Abbott (1). Nevertheless, the incidence calculated is about the same as the one for meningococcal-C disease in The Netherlands before the vaccination campaign (15). Despite this relatively high though regional incidence and its deforming, debilitating nature, little attention has been paid to mycetoma so far. When searching PubMed for publications on this disease, 1631 hits can be found of which 723 are case reports and 148 are reviews. In total 88 of those articles are about mycetoma caused by *Madurella mycetomatis*. As seen in **chapter 2** research has focused mainly on the epidemiology, the pathology, the diagnosis and the treatment of patients. In this thesis research was focused on the genetic variation in both pathogen and host, the antigenicity of *M. mycetomatis* and the antifungal susceptibility of *M. mycetomatis*. Topics which were only meagerly explored before.

Genetic variation in *Madurella mycetomatis* and its human host

Little is known about the risk factors associated with *M. mycetomatis* infections. *M. mycetomatis* is thought to reside in the soil in endemic areas. This was at least hypothesised because a large fraction of the patient population suffered from previous trauma, such as thorn pricks, before developing the infection (24). Although *M. mycetomatis* has never been successfully cultured from soil samples, Ahmed *et al*. were able to demonstrate that *M. mycetomatis* DNA can be found in the soil and vegetative material in the endemic areas in Sudan (3). When becoming infected with *M. mycetomatis*, the clinical presentation is quite similar in most patients, although there can be individual variations. For instance, the *M. mycetomatis* collection used in all the studies in this thesis, is derived from 40 myetoma patients. Some of the patients had large lesions, while others had only small lesions (**chapter 3**, reference 61). These differences were associated with the duration of the disease, since some of the patients with large lesions were only infected for about a year and others with small lesions were infected for more than 10 years. The outcome of any infection is usually the result of both genetic predisposition of the patient and virulence factors of the pathogen. In order to establish if some of the isolates in our collection were genetically predisposed to forming large lesions, we typed our collection with AFLP (amplified fragment length polymorphism) as described in **chapter 3**. It appeared that strains originating from Central Sudan, which had formed moderate to large lesions, were genetically distinct from strains which had formed small lesions only, and which primarily originated from other parts of Sudan. Interestingly, strains originating from central Sudan had a marker homologous to a casein kinase I gene from *Danio rerio*, which was not found in strains originating from other

parts of Sudan. This same marker was also found to be associated with a higher MIC for amphotericin B. The study described in **chapter 3** was the first to monitor genetic diversity in a large collection of *M. mycetomatis*. In a previous study, RAPD (random amplification of polymorphic DNA) was used to type this collection (4). Unfortunately RAPD proved not to be a good technique for subtyping *M. mycetomatis* because even after using 20 different primer species the authors had to conclude that the isolates were clonal (4). This clonality was confirmed by sequencing the ITS region of *M. mycetomatis* (5). This was contradicted by the AFLP technique used in **chapter 3**, implicating that AFLP is a more suitable technique for typing *M. mycetomatis* than RAPD is. Differences in *M. mycetomatis* genotypes have also been described by others (16, 18, 42). Lopes *et al.* used RAPD to type *M. mycetomatis* isolates originating from different countries (42), while Desnos-Ollivier *et al.* used ITS sequencing to show differences between isolates (18). The studies could not link genetic polymorphisms to fungal virulence traits in the way we did in **chapter 3**.

Showing that *M. mycetomatis* isolates may have a genetic make up for inflicting large lesions was a first step in understanding the pathogenesis of the disease. Usually the outcome of a disease is not only dependent on the pathogen itself, but also on the ability of the human host to clear the infection. In the past some studies have been done to determine why some develop mycetoma and others not. As is stated before, *M. mycetomatis* was shown to be present in the soil and vegetative material in the endemic area (3). The presumption that all inhabitants come into close contact with *M. mycetomatis*, was confirmed by various antibody and antigen tests (31, 53, 58, 62, 67). These serological diagnostic assays revealed that people living in the endemic areas all had certain amounts of antibodies against the causative agents. This implied that, although all people apparently contacted *M. mycetomatis,* only a small percentage developed this disease. It was therefore hypothesized that genetic impairment in the immune system was involved. This hypothesis was not new. In the past it had already been established that mycetoma patients probably had a deficiency in their cell-mediated immune response (46). This finding was never elaborated upon. Other genetic traits which could be of influence were investigated as well. For instance, a lack of differences in the ABO blood group types or Rhesus types between patients and the healthy Sudanese population was demonstrated (25). Since mycetoma lesions are usually characterized by a large zone of neutrophils surrounding them, we investigated if there was genetic impairment in one of the genes involved in neutrophil function (**chapter 4**). Out of eight genes investigated, different allele distributions were found for five of them. It appeared that mycetoma patients more often had a mutation in the complement receptor 1 gene (CR1). This receptor is not only found on neutrophils but also on many other cell types. The polymorphisms investigated here are responsible for the Knops blood group antigens Swain-Langley (Sl) and McCoy (McC). It appeared that the Sl2 and the McC^a genotypes were more frequently encountered in the patient population than in the control group. Genetic differences were also found in the genes encoding CXCL8, CXCR2 and TSP4. All genotypes documented were correlated with a higher CXCL8 production. CXCL8 is the interleukin responsible for attracting neutrophils to the site of infection. Higher CXCL8 production in patients was also demonstrated phenotypically in the sera of these patients. Neutrophils participate in the acute inflammatory response and repair of damaged tissues (48). In wounds, neutrophils have the task of killing

invading microbes either by phagocytosis or excreting oxygen or nitrogen radicals (48). The latter effect has a downside, since the oxygen radicals not only kill invading microbes but can also cause serious tissue damage (19, 20). In neutrophil-depleted mice it was shown that wounds actually healed better than in control mice (19). If depletion of neutrophils accelerates tissue repair, then a overdose of neutrophils at the site of infection could hamper wound healing. This was shown to be true for certain pulmonary diseases as well as for some autoimmune diseases as reviewed by Dovi (20). Delay in wound healing and extension of tissue damage in turn can be favorable for the pathogen, in deriving nutrients and in establishing infection. By producing too much CXCL8, and therefore attracting more neutrophils to the site of infection, the patient might unintentionally support the infection. The higher oxygen or nitrogen excretion caused by those neutrophils appear to be not harmful to *M. mycetomatis*. In **chapter 4** we showed that the production of H_2O_2 by neutrophils can be counteracted by the production of catalases by *M. mycetomatis*. In **chapter 10** we added that the melanin produced by *M. mycetomatis* is able to scavenge permanganate, one of the strongest oxidants known to modern chemists. The fact that permanganate was effectively scavenged implies a strong protective function for melanin during infection.

The nitric oxide synthase NOS2 was also differently distributed between patients and healthy controls. Most micro-organisms are susceptible to nitric oxide and are killed by it. In this case the mutation explored was more often found in the control population than in the patient population. The mutation, known as the $NOS2^{lambaréné}$ genotype, is associated with a higher nitric oxide production (38). This was phenotypically confirmed. Apparently, patients displayed lower nitric oxide production, which could impair killing of the micro-organisms. NOS2 has also been demonstrated to be prominently expressed and stimulatory during wound repair (47). Apparently, it is important that the wound heals slowly for establishing a mycetoma infection.

In summary, it appeared that genetic predisposition of the human host is involved in susceptibility to mycetoma infection. This could be a starting point for further research in this direction. Still, little is known on the genetic variability of the host or even the immune response triggered by a *M. mycetomatis* infection. Epidemiological, genetic, microbiological and demographic studies in endemic villages could be highly valuable in further establishing both the role of the human host and the pathogen in the development of mycetoma infections. By establishing why some people become infected and why others seem to be immune to the disease, could help develop future prevention campaigns and generate new opportunities in treatment. For instance, wounds in diabetic patients have been shown to heal better if neutrophils were repressed. By repressing or stimulating certain parts of the immune system, a *M. mycetomatis* infection might in the end be cleared by the host itself. The mouse infection model, developed by Ahmed *et al* could also be very helpful in developing this concept further (8). In the mouse model progression of the infection can be followed in both the fungus and the host. Upon entering the host, the fungus has to adapt itself. What genes will it activate? Why are some of the isolates more prone to give an extended infection, and why are "other genotypes" less likely to inflict an infection? What about additional host factors? What genes are essential to clearing this infection? How is the immune system responding to *M. mycetomatis*? These are all questions which still need to be answered.

Antigenicity of *M. mycetomatis*

As recorded in **chapter 2**, many different options are available for diagnosing mycetoma. Establishing a correct diagnosis is important for the further treatment of this deforming disease. However, culture, pathology, serodiagnostic tools, molecular diagnosis, they all have their downsides. The ideal diagnostic assay would be an assay which would be cheap, easy to handle in the developing world and give a 100% conclusive positive or negative result. This is not easy if not impossible to establish, especially in case of serological assays. We therefore set out to gain insight in the antigenic make up of *M. mycetomatis* on which surprisingly little was known. The fact that mycetoma patients developed antibodies against *M. mycetomatis* was used in the past to develop serodiagnostic tests by using crude culture extracts as antigen (31, 50). The only attempt to characterize the nature of the epitopes present in these crude extracts was published in 1991 (68). In that report cytoplasmic proteins were extracted from several eumycetoma agents and separated by SDS-PAGE. It appeared that the protein profiles were similar for all isolates tested (68). The precise nature of the most immunodominant proteins could not be determined in this study. No other attempts had been published. In **chapters 5 and 6** we attempted to identify antigens expressed by *M. mycetomatis* in order to clarify pathogenesis of this disease and, if possible, to develop a new diagnostic test with these antigens. In **chapter 5** we identified the translationally controlled tumour protein (TCTP) as the first major antigen of *M. mycetomatis* by developing a cDNA library. In **chapter 6** it was demonstrated that *M. mycetomatis* also secretes galactomannan-like antigens *in vitro*, but not *in vivo*.

TCTP is not unique to *M. mycetomatis*. TCTPs from other pathogens were already demonstrated to be antigenic (9, 28, 43, 56). The protein appeared to be present in two isoforms in *M. mycetomatis*, namely variant I (found in 53% of the isolates) and variant II (found in 47% of the isolates). *M. mycetomatis* TCTP (mmTCTP) antigenicity in humans was established by measuring the IgG and IgM responses by an ELISA developed with variant I of mmTCTP. Most patients had elevated antibody levels against mmTCTP variant I, but, unfortunately, not all. It also appeared that some individuals of the Sudanese healthy control population had an elevated antibody level against TCTP as well. Those individuals probably raised antibodies upon environmental exposure to the antigen which was confirmed by other serodiagnostic assays. Cross-reactivity with TCTPs from other endemic infectious organisms could also have occurred, as was already demonstrated for filarial TCTPs (28, 56). In order to determine whether cross reactivity with TCTPs from other species were the cause of the high antibody levels in the healthy Sudanese control population, six ELISAs with specific TCTP peptides were developed. In five of those ELISAs there was a clear difference between the mean IgG levels found in the patient population and those found in the healthy Sudanese control population. Only with “peptide 6” no statistically significant difference in the mean IgG levels was detected. IgG and IgM immune responses against the whole protein and selected *M. mycetomatis*-specific peptides were found to be correlated with lesion size and disease duration. Overall, the patients with the largest lesions had the highest antibody level. After 6-15 years of disease duration the antibody levels were highest.

During infection TCTP seemed to be expressed mainly in developing grains. TCTP expression in the developing grain could be high because of several reasons. Firstly, when the fungus grows in the human host, it will be mitotically active and will encouter stress conditions imposed by the host immune system or nutrient depletion during invasion which upregulate TCTP expression as was shown in infections with other microbes (28, 56, 59). Also adaptation to new environmental conditions in general have been shown to upregulate TCTP. In the parasite *Brugia malayi* no TCTP expression in the parasite is found in the pre-infective stages, but upon entering the host TCTP becomes strongly upregulated (28).

The discovery of antigens expressed by *M. mycetomatis* during infection could give insight in the pathogenesis of this infection. Furthermore, it could eventually result in a vaccine candidate. In future experiments, cDNA libraries can be developed from strains grown in various culture media or in *in vivo* animal models. The first cDNA library we developed originated from a strain grown in standard culture medium, which results in a totally different phenotype of the fungus as seen during *in vivo* infection. By varying culture conditions, different proteins will be expressed, and the likelihood of finding different antigens increases. Developing an expression library from mRNA isolated from the grains present in infection is an interesting option. Although this might give practical difficulties in extracting mRNA, but when succeeding novel insights in disease progression might be expected. New antigens could not only be used as vaccine-candidates but also for the development of new diagnostic tools. The latter are still desperately needed, since the diagnostic tools available at the moment are either time-consuming (culturing) or prone to false positive or negative results (many of the serodiagnostic assays). Antigens can also be used to establish clinical cure. The galactomannan assay used in Aspergillosis is such an example. In that assay, galactomannan secreted by *Aspergillus fumigatus* can be detected in human serum. If galactomannan disappears this could indicate clinical cure. If such an antigen could be found for *M. mycetomatis* this could help in developing good criteria for determining clinical cure or improvement upon antifungal therapy.

Antifungal susceptibility of *Madurella mycetomatis*

At present, the treatment of eumycetoma caused by *M. mycetomatis* is usually based on local clinical experience rather than double blind placebo controlled clinical trials. In Sudan the most common treatment regime is 300-400 mg/day ketoconazole or 400 mg/day itraconazole for extended periods of time (23). Both treatment regimes alone can not cure mycetoma, but the lesions will become more localised and encapsulated which makes subsequent surgery much easier to perform (23). After surgery the patients are treated for several months or years with high doses of antimycotics. Still, recurrent infections are not uncommon. It was never shown that *M. mycetomatis* was actually susceptible to both agents *in vitro*. The reason for this is that *M. mycetomatis* does not sporulate, which makes it not suitable for classical NCCLS based *in vitro* antifungal susceptibility testing (13). In **chapter 7** an alternative method for non-sporulating *M. mycetomatis* was developed on the basis of the NCCLS criteria. *M. mycetomatis* hyphae were disrupted by sonication to obtain a homogeneous suspension, which could be exposed to various concentrations of antifungal agents. For

endpoint-reading, XTT was added after 7 days of incubation. XTT can be used to quantify the antifungal activity by relating the viable fungal mass to levels of fungal mitochondrial dehydrogenase activity (37, 51). This method appeared to be reproducible (**chapters 7 and 8**, references 6 and 63) and it could also be used to determine the antifungal susceptibilities against less common antifungal agents, such as the natural compounds artemisinin and tea tree oil (**chapter 9**). Artemisinin is isolated from the plant *Artemisia annua* and it is used in traditional Chinese medicine (21, 33). It is also a well-known agent used in malaria treatment. Tea tree oil is extracted from the tree *Melaleuca alternifolia* and is still used by the Australian Bundjalung Aborigines of New South Wales for its anti-inflammatory and antimicrobial properties (11, 32).

The down-sides of the NCCLS-based method were its time consumption and its laboriousness. In **chapter 8** a less laborious method was therefore adapted for determining the antifungal susceptibility of *M. mycetomatis*. This method was the Sensititre system (Trek Diagnostic systems, Ltd., East Grinstead, England). In this assay, the *in vitro* susceptibilities to amphotericin B, fluconazole, itraconazole, ketoconazole, 5-flucytosine and voriconazole were determined. Endpoint reading was facilitated by the oxidation-reduction indicator Alamar Blue. *In vitro* susceptibilities of various yeasts and filamentous fungi were already successfully assessed by this system (14, 22, 52). For *M. mycetomatis* this system was shown to give reproducible results for 90% of the isolates. The Sensititre assay was in agreement with the visually based NCCLS assay and to a lesser extend with the XTT assay. The latter can be explained by the indicator systems being different in both test systems. Since the discrepancies between both assays were the highest for the azoles, this could suggest that the trailing endpoint obtained with azoles is measured more effectively with XTT than with Alamar Blue. Furthermore, XTT is measured spectrophotometrically and Alamar blue is read visually. The spectrophotometer is usually more sensitive and specific than the eye in measuring small differences in colour.

By combining the data obtained with both assays used in **chapters 7, 8 and 9** the first *in vitro* antifungal susceptibilities for *M. mycetomatis* were determined for a large collection of isolates. *In vitro M. mycetomatis* appeared to be most susceptible to the azoles ketoconazole, itraconazole and voriconazole with MIC_{90}'s of 0.125 µg/ml, 0.064 µg/ml and 0.125 µg/ml, respectively. These MICs correlate with attainable serum levels (17). Fluconazole was the least effective azole, inhibiting the fungal growth at a MIC_{90} of 16 µg/ml. Although these MICs are high, they still meet physiologically attainable serum levels (17, 27). Amphotericin B appeared to be less effective than ketoconazole, itraconazole and voriconazole in inhibiting *M. mycetomatis* (MIC_{90} 2 µg/ml). Interestingly, *M. mycetomatis* was also very susceptible to tea tree oil, with a MIC_{90} of 0.25 % v/v. *M. mycetomatis* isolates appeared to be resistant to 5-flucytosine and artemisinin. Resistance to 5-flucytosine was already demonstrated for many other filamentous fungi (2, 13, 29, 30, 41). Resistance against artemisinin was noted for *Candida albicans* but not for *Cryptococcus neoformans* and *Saccharomyces cerevisiae* (26, 36).

These susceptibilities indicate that mycetoma is extremely susceptible to the azoles ketoconazole and itraconazole, which are currently used in the medical treatment of eumycetoma caused by *M. mycetomatis* (44, 45, 49). According to the low *in vitro* MICs

found in **chapters 7 and 8**, none of the isolates would be considered resistant. Surprisingly, although some clinical studies showed that ketoconazole and itraconazole treatment resulted in complete cure, the clinical response to both agents is often poor (6, 44, 45, 49). Taken into account that the appearance of *M. mycetomatis in vitro* differs from the black grains produced *in vivo* there is probably a factor involved in the *in vivo* situation which offers *M. mycetomatis* some protection against the antifungal agents used. In **chapter 10** our working hypothesis was that the black compound in the grain is melanin produced by *M. mycetomatis* and that this melanin may protect the fungus from the immune system and antifungal agents. Melanins are negatively charged, hydrophobic, macromolecular pigments formed by oxidative polymerization of phenolic or indolic compounds (12, 35, 40). Melanin has been shown to protect micro-organisms against UV-radiation, enzymatic lysis, oxidants, killing by alveolar macrophages, and antimicrobial drugs (12, 35, 40). Fungal melanin can be biosynthesised by three pathways. In the first pathway, melanin is formed from diphenolic compounds such as L-tyrosine and 3,4-dihydroxyphenylalanine (L-DOPA) (54). In a second pathway, melanin can only be derived from L-tyrosine, not from L-DOPA. This type of melanin, called pyomelanin differs from L-DOPA melanin by being a soluble brown pigment rather than an insoluble black pigment (10, 60). In the third pathway, melanin is biosynthesized from polyketide compounds such as 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (54).

We demonstrated that *M. mycetomatis* was actively melanised *in vivo*. Melanin was found within the grain as well as inside phagocytes surrounding the grains. *In vitro* melanisation of *M. mycetomatis* could be blocked with inhibitors of the pyo- and the DHN-pathways but not with inhibitors of the DOPA-pathway. Since pyo-melanins are soluble, the black pigment produced in the grain is probably DHN-melanin.

To establish if melanin did protect *M. mycetomatis* against antifungal agents, the *in vitro* susceptibilities to ketoconazole, itraconazole, fluconazole, voriconazole, amphotericin B and 5-flucytosine, with or without adding isolated melanin to the culture medium, were determined by using the adapted Sensititre assay (**chapter 8**). It appeared that melanin did protect *M. mycetomatis in vitro* against the antifungal agents itraconazole and ketoconazole (the prime agents used in clinical settings (7)) but not against the other antifungal agents. The spectacular 32-fold rise in MIC was unique for the melanin isolated from *M. mycetomatis*, since *Cryptococcus neoformans* melanin did not produce a rise in MIC. The limited number of experiments done in determining the effect of melanin on MICs of other melanised fungi did not show a MIC shift as was seen here. Melanin protected the fungi by extension of the killing-time (34, 65, 66). However, the problem with these time-kill experiments was that the fungi could not melanise in the culture medium used, and that, over time, isolates lost their melanised phenotype. Extension of the killing-time was thought to be the result of binding of amphotericin B and caspofungin to L-DOPA melanin (34, 64-66). In our assay no MIC shift for amphotericin B was noted when cryptococcal melanin was added. This suggests that, although amphotericin B is bound to cryptococcal melanin, its antifungal properties remain unaffected. No evidence was found that L-DOPA melanin bound azoles (64, 65). There was only one other publication in which the effect of DHN melanin on antifungal susceptibility was explored. A MIC shift for itraconazole was demonstrated, indicating that depletion of antifungal agents is dependent on the type of melanin formed (55). Surprisingly, the

spectacular MIC rises for *M. mycetomatis* was only documented for itraconazole and ketoconazole, not for voriconazole and fluconazole. Since all azoles possess the azole-ring, melanin probably does not bind to this ring. Instead, interaction of melanin with itraconazole and ketoconazole is probably mediated by the dichlorobenzene ring, the long side chain or a combination of the two, since those structures are not found in voriconazole and fluconazole.

As was already mentioned, the present antifungal treatment for mycetoma caused by *M. mycetomatis* is based on usage of either itraconazole or ketoconazole. This seemed justified by the low MICs found in **chapters 7, 8 and 9**. Taken the spectacular 32-fold rise in MIC for these agents when tested in the presence of melanin in combination with their toxicity, thus could indicate that these agents might not be the most optimal choice in treating mycetoma. Interestingly, voriconazole with equally low MICs, did not give a rise in MIC when melanin was added. Furthermore, voriconazole has a higher safety profile, with low toxicity. Favourable results have been obtained with voriconazole in the management of *M. mycetomatis* eumycetoma without bone involvement (39).

Through the development of assays for determining the *in vitro* susceptibility of *M. mycetomatis* against a variety of antifungal agents, the road has now been cleared for implementation of antifungal susceptibility testing in the clinical setting. By correlating *in vitro* antifungal susceptibility data with *in vivo* outcome of treatment, a standardised treatment regime must be developed. At the moment, each clinical institute applies its own regime. At the moment randomly trials in antifungal treatment are started. Terbinafine and posaconazole have been used in mini-trials in order to establish their therapeutic potential of those agents in mycetoma treatment. In the future such trials, with other new agents including voriconazole and the echinocandins such as caspofungin and anidulafungin could improve antifungal therapy. Good clinical trials should include large numbers of patients, have standardized criteria for cure or improvement and should include adequate matched control groups.

In conclusion

The work described in this thesis was geared towards gaining insight in the genetic diversity of *M. mycetomatis* and its human host in relation to the antigenicity and the antifungal susceptibility of *M. mycetomatis*. We can conclude that both *M. mycetomatis* and its human host are “collaborately defining” the outcome of a mycetoma infection. Some *M. mycetomatis* isolates have the capacity cause larger lesions and patients do more often have a genotype encoding for a higher CXCL8 production and a lower nitric oxide excretion, both implicated in delay of wound healing. It was demonstrated that *M. mycetomatis* excretes galactomannan-like antigens and a TCTP antigen *in vitro*. Only the latter is detectable in patient serum and could be used in serological studies. Three antifungal susceptibility assays (the NCCLS-based assay, the XTT assay and the Sensititre assay) were developed for determining the antifungal susceptibilities of *M. mycetomatis* against a great variety of antifungal agents. *M. mycetomatis* appeared to be susceptible to itraconazole, ketoconazole, voriconazole, fluconazole, amphotericin B and tea tree oil and resistant to 5-flucytosine and artemisinin. Furthermore, it was seen that the DHN-melanin produced by *M. mycetomatis* protects the fungus *in vitro*

against the azoles itraconazole and ketoconazole, resulting in 32-fold rises in MIC for those agents. This could suggest that the current therapy based on those agents might need revision.

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Chapter 12

Nederlandse samenvatting

Wendy W.J. van de Sande

Mycetoma is één van de "vergeten" infecties van de ontwikkelingslanden. Deze infectie wordt gekarakteriseerd door een misvormende subcutane massa, drainerende sinussen en uitscheiding van grains en purulent materiaal. In de endemische tropische en subtropische gebieden waar mycetoma hoofdzakelijk gevonden wordt, is de ziekte nog steeds erg moeilijk te behandelen (7, 23, 24). Over het algemeen worden vooral jonge mannen, in de leeftijd van 20 tot 40 jaar, geïnfecteerd. Zij vormen vaak de werkende bevolking van het land en mycetoma kan daarom een groot effect hebben op de lokale economie (7, 23, 24). Soedan is één van de landen waar deze ziekte het meest voorkomt. In 1955, werden ~500 nieuwe mycetoma patiënten opgenomen in ziekenhuizen (1). De incidentie was dat jaar ongeveer 4.6 gevallen per 100.000 inwoners (1, 57). Dit is echter slechts een schatting omdat de werkelijke incidentie waarschijnlijk hoger zal liggen. Patiënten in derdewereldlanden gaan over het algemeen pas laat naar een ziekenhuis, wanneer de ziekte al in een vergevorderd stadium is. Verder waren niet alle ziekenhuizen betrokken bij de studie van Abbott (1). De incidentie is echter relatief hoog, zelfs vergelijkbaar met de incidentie van het aantal meningococcen type C gevallen in Nederland voordat hiertegen gevaccineerd werd (15). Ondanks deze relatief hoge incidentie en de ernst van de infectie, was er slechts weinig aandacht voor mycetoma in de wetenschap. Als in Pubmed (een database voor medische literatuur) naar artikelen over mycetoma gezocht wordt, blijkt dat er slechts 1631 artikelen (van de 16 miljoen artikelen die in deze databank te vinden zijn) gevonden worden. Van deze 1631 artikelen zijn 723 artikelen zogenaamde case reports, waarin enkele patiënten besproken worden en 148 artikelen zijn reviews, samenvattingen van al eerder gepubliceerde bevindingen. In totaal zijn 88 artikelen verschenen over mycetoma veroorzaakt door de schimmel *Madurella mycetomatis*. Het onderzoek naar deze ziekte heeft zich tot nu toe geconcentreerd op de volgende onderwerpen: de epidemiologie van de ziekte, de pathologie, de diagnose en de behandeling van mycetoma patiënten (**hoofdstuk 2**).

In dit proefschrift is het onderzoek verdeeld in drie thema's. Het eerste thema betrof de genetische diversiteit van zowel de ziekteverwekker *M. mycetomatis* als de humane gastheer. Het tweede thema betrof de antigeniciteit van *M. mycetomatis.* De belangrijke onderzoeksvraag hier was welke factoren de schimmel bezit die een immuunrespons in de mens opwekken. Het derde en laatste thema betreft de antifungale gevoeligheid van *M. mycetomatis*. Een antifungaal middel is een antibioticum gericht tegen schimmels. Niet alle schimmels zijn even gevoelig voor deze middelen. In dit thema werd gekeken hoe gevoelig *M. mycetomatis* is voor de verschillende antifungale middelen die op dit moment beschikbaar zijn. Dit spectrum aan onderwerpen was tot nu toe maar weinig aan bod gekomen in de internationale wetenschappelijke literatuur.

Genetische diversiteit in *Madurella mycetomatis* en z'n menselijke gastheer

Er is slechts weinig bekend over de risicofactoren gerelateerd aan besmetting met de schimmel *M. mycetomatis*. Over het algemeen wordt gedacht dat *M. mycetomatis* zich in de bodem bevindt, en dan alleen in de endemische gebieden. Deze hypothese kwam tot stand doordat bij veel mycetoma patiënten ook bij gevorderde infecties nog stukjes Acacia doorn in de laesie aanwezig waren. Zij hadden vaak voordat ze een mycetoma-infectie opliepen een

wondje aan hun voet gehad waardoor de schimmel mogelijk binnen heeft kunnen komen (24). *M. mycetomatis* is echter nog nooit uit grondmonsters gekweekt maar het DNA van deze schimmel is wel in de grond en op planten in de endemische gebieden aangetroffen (3). Als men geïnfecteerd wordt door *M. mycetomatis* verloopt het ziektebeeld in de meeste patiënten vergelijkbaar. Er zijn echter wel individuele verschillen aantoonbaar. De *M. mycetomatis* collectie die in alle experimenten beschreven in dit proefschrift gebruikt werd, was afkomstig van 40 verschillende mycetoma patiënten. Zo hadden sommige patiënten erg grote laesies, terwijl anderen slechts kleine gelokaliseerde mycetoma-laesies hadden (**hoofdstuk 2**, referentie 61). Deze verschillen waren echter niet te wijten aan de duur van de ziekte, omdat sommige patiënten met grote laesies slechts één jaar geïnfecteerd waren met deze schimmel en anderen, die al meer dan 10 jaar geïnfecteerd waren, nog steeds kleine laesies hadden. Het verloop van infecties, wordt bepaald door zowel de genetische opbouw van de gastheer als door de virulentiecapaciteit van het pathogene micro-organisme. Virulentie factoren zijn vaak eiwitten of suikers die een schimmel in staat stellen infectie te veroorzaken of het immuunsysteem van de gastheer te omzeilen. De vraag of er genen in onze isolaten aanwezig waren die gerelateerd waren aan de ontwikkeling van grote of kleine laesies hebben we proberen te beantwoorden door de collectie te typeren. Dit werd gedaan met de zogenaamde AFLP (amplified fragment length polymorphism) typeer methode (**hoofdstuk 3).** Het bleek dat de isolaten die uit Centraal Soedan kwamen en die middelgrote tot grote laesies veroorzaakt hadden genetisch afweken van isolaten die slechts kleine laesies veroorzaakten. Deze laatste groep kwam ook niet speciaal uit één gebied in Soedan, maar bevatte isolaten uit zowel Centraal Soedan, West- en Noord-Soedan als ook uit de hoofdstad Khartoum. Wat opvallend was dat alle stammen die uit Centraal Soedan kwamen, een specifieke merker (een DNA-fragment) hadden die de andere stammen niet hadden. Deze merker vertoonde homologie met het caseine kinase I gen van de zebravis *Danio rerio.* Deze merker bleek ook nog eens geassocieerd te zijn met een hogere MIC voor amphotericine B. De studie zoals beschreven in **hoofdstuk 3** was de eerste die er ooit in slaagde om de genetische diversiteit van een grotere *M. mycetomatis* stammen-collectie vast te stellen. Het was echter niet de eerste studie waarbij genetische diversiteit werd onderzocht. Eerder was bijvoorbeeld RAPD (random amplification of polymorphic DNA) gebruikt (4). Het bleek echter dat RAPD een minder geschikte techniek was om *M. mycetomatis* te typeren. Zelfs na 20 verschillende primers gebruikt te hebben, konden de auteurs niets anders doen dan concluderen dat de Soedanese *M. mycetomatis* isolaten klonaal waren (4). De klonaliteit werd bevestigd door de constante DNA volgorde van de ITS regio (5). Dit duidt erop dat AFLP een hogere resolutie heeft dan RAPD. Dit geldt in ieder geval voor isolaten uit één geografische regio. Genotypische verschillen tussen *M. mycetomatis* stammen zijn ook door anderen beschreven (16, 18, 42). Zo vond Lopes bijvoorbeeld dat RAPD gebruikt kon worden om *M. mycetomatis* isolaten uit verschillende landen te typeren (42). Desnos-Ollivier liet zien dat er door de sequentie van de ITS regio te bepalen verschillen tussen de isolaten aangetoond konden worden (18). Geen van deze studies kon echter de genetische achtergrond van de schimmel in verband brengen met virulentie factoren. Dit hebben wij wel laten zien in **hoofdstuk 3.**

Sommige *M. mycetomatis* isolaten hebben blijkbaar een genetische blauwdruk die tot grote laesies leidt. Dit levert meer inzicht in de pathogenese van mycetoma. Maar zoals al

eerder gezegd, het ziekteverloop is niet alleen afhankelijk van de ziekteverwekker, maar ook van de gastheer. In het verleden zijn er een aantal studies uitgevoerd waarin werd onderzocht waarom sommige mensen wel myetoma krijgen, terwijl anderen dit niet krijgen. *M. mycetomatis* komt voor in de grond en op planten in het endemisch gebied en er is al aangetoond dat een groot gedeelte van de mensen die in dat gebied wonen in contact komen met de ziekteverwekker (3). Dit werd aangetoond toen verschillende antilichaam and antigeen testen ontwikkeld werden (31, 53, 58, 62, 67). Via die serologische testen kwamen onderzoekers erachter dat de meeste mensen die in het endemisch gebied leven, antilichamen gevormd hadden tegen de mycetoma veroorzakende micro-organismen. Dit duidde erop dat hoewel de meeste mensen blijkbaar in contact komen met *M. mycetomatis*, er slechts een klein percentage daadwerkelijk de ziekte ontwikkelde. Daarom werd de hypothese opgesteld dat de mensen die wel ziek werden een gebrek in hun immuunsysteem zouden hebben. Deze hypothese was niet nieuw, in het verleden was al aangetoond dat mycetomapatiënten een potentieel gebrek in de cel-gemedieerde immuunrespons hadden (46). Na dat eerste onderzoek is er echter nooit vervolgonderzoek uitgevoerd. Andere genetische karakteristieken die van invloed konden zijn op het ontwikkelen van de ziekte zijn ook onderzocht, ondermeer het ABO bloedgroepen systeem. Er werden echter geen verschillen tussen de ABO bloedgroepen of de Rhesus factoren gevonden tussen patiënten en een gezonde controlegroep (25). Omdat mycetoma lesies vaak gekarakteriseerd worden door een grote zone met neutrofielen (bepaalde cellen van het immuunsysteem) rondom de schimmel, hebben we gekeken of er een genetisch defect te vinden was in één of meerdere van de genen die betrokken zijn bij het functioneren van deze neutrofielen (**hoofdstuk 4**). Van de acht genen die onderzocht zijn werd er slechts in vijf verschil in allelverdeling tussen patiënten en controles gevonden. Het bleek dat mycetoma patiënten vaker een genetische mutatie in de zogenaamde complement receptor 1 (CR1) hadden. Deze receptor komt niet alleen voor op neutrofielen, maar ook op vele andere celtypen. De polymorfismen die wij bepaalden, zijn verantwoordelijk voor de Knops bloedgroep antigenen Swain-Langley (Sl) en McCoy (McC). Het bleek dat de Sl2 en McC^a genotypen vaker gevonden werden in de patiëntenpopulatie dan in de Soedanese controle groep. Genetische verschillen werden ook gevonden in de genen CXCL8, CXCR2 en TSP4. De genotypes die bij patiënten gevonden werden, waren allemaal gecorreleerd aan een hogere CXCL8 productie. CXCL8 is het interleukine dat verantwoordelijk is voor de aantrekking van neutrofielen naar een infectiehaard. Hoge CXCL8 productie werd ook fenotypisch in het serum van de patiënten vastgesteld. Neutrofielen zijn onder andere betrokken bij de ontstekingsreactie en het genezen van beschadigde weefsels (48). In wondjes hebben neutrofielen vaak de taak om de binnendringende micro-organismen, zoals bacteriën, te doden. Dit doen zij door middel van fagocytose en uitscheiden van zuurstof- en stikstof radicalen (48). Deze radicalen doden niet alleen de micro-organismen maar kunnen ook voor additionele weefselschade zorgen (19, 20). In muizen, waarbij neutrofielactiviteit onderdrukt werd, bleken wondjes zelfs sneller en beter te genezen dan in normale, gezonde muizen (19). Als het onderdrukken van neutrofielen het genezen van wondjes versneld, kan het tegenovergestelde ook waar zijn. Als te veel neutrofielen in de infectiehaard aanwezig zijn, kan dit genezing remmen. Dit is al eerder aangetoond in bepaalde long- en autoimmuunziekten (20). Het vertragen van de wondgenezing, of het veroorzaken van extra

weefselschade kan voor de schimmel voordeel opleveren. Zo kan het pathogeen makkelijker aan voedingsstoffen komen en kan het zich in de infectiehaard beter ontwikkelen. Door te veel CXCL8 te produceren en daardoor te veel neutrofielen naar de infectiehaard te sturen, kan de patiënt zelf onbewust de infectie helpen handhaven. De productie van zuurstof- en stikstof radicalen die hiermee gepaard gaan lijken niet erg schadelijk te zijn voor *M. mycetomatis*. In **hoofdstuk 4** bleek dat de productie van H_2O_2 door neutrofielen tegen gegaan kan worden door de productie van katalases door *M. mycetomatis*. In **hoofdstuk 10** werd vervolgens gezien dat *M. mycetomatis* melanine vormt. *M. mycetomatis* melanine kon permanganaat onschadelijk maken. Permanganaat is een van de sterkste oxidatoren. Het feit dat permanganaat onschadelijk gemaakt kon worden laat zien dat melanine mogelijk een efficiënte beschermer is voor de schimmel.

Het laatste gen waarin verschillen tussen de patiënten- en de controlepopulatie gevonden werden was het gen NOS2. Dit gen codeert voor een stikstof synthase. Stikstof oxide is een van de stikstof radicalen die door neutrofielen uitgescheiden wordt om micro-organismen te doden. De meeste micro-organismen zijn hier dan ook gevoelig voor en worden snel gedood. De NOS2 mutatie werd vaker in de controle populatie gevonden dan in de patiëntenpopulatie. De mutatie staat ook wel bekend als de $NOS2^{lambaréné}$ mutatie en wordt in verband gebracht met een hogere stikstof oxide productie (38). Dit werd fenotypisch bevestigd. Blijkbaar produceren patiënten een lagere hoeveelheid stikstof oxide, wat ervoor kon zorgen dat de *M. mycetomatis* minder effectief gedood werd. Ook NOS2 is betrokken bij de genezing van wondjes. Als NOS2 expressie onderdrukt wordt, geneest de wond veel minder snel (47). Blijkbaar is het belangrijk voor de schimmel dat de wond niet snel geneest. Mensen die een genotype hebben waarbij de wond snel geneest, hebben blijkbaar extra bescherming tegen een mycetoma-infectie.

Samenvattend blijkt dat genetische predispositie betrokken is bij het ontstaan van een mycetoma infectie. Ons werk vormt een goed startpunt voor meer onderzoek in deze richting. Van de immuunrespons die optreed nadat *M. mycetomatis* het lichaam binnen gedrongen is, is nog weinig bekend. Onderzoek in "endemische dorpjes" zou een goed startpunt zijn om de rol van zowel de schimmel als van de gastheer in het ontstaan en in de verdere ontwikkeling van een mycetoma-infectie te onderzoeken. De constatering dat sommige mensen wèl een infectie ontwikkelen en anderen ogenschijnlijk immuun zijn, kan helpen bij het voorkómen van deze ziekte. Meer inzicht in de rol van het immuunsysteem tijdens de ontwikkeling van een mycetoma-infectie kan ook nieuwe behandelingsmethoden opleveren. Bij mensen met diabetes bijvoorbeeld, blijkt dat als de neutrofielen onderdrukt worden, wondjes sneller genezen. Door bepaalde delen van het immuunsysteem te onderdrukken of door andere delen juist te stimuleren kan een *M. mycetomatis* infectie misschien door de gastheer zelf geklaard worden. Het muismodel, ontwikkeld door Ahmed, zou de basis voor zulke studies kunnen vormen (8). In een muismodel kan de infectie vanaf het begin gevolgd worden. Zowel de schimmel als de gastheer kunnen dan in detail bestudeerd worden. Als *M. mycetomatis* het gastheerlichaam binnendringt zal aanpassing plaats moeten vinden. Welke genen worden er aangezet? Zijn bepaalde *M. mycetomatis* isolaten beter in staat om een infectie te ontwikkelen dan andere isolaten? En wat voor een rol speelt fysiologie en de genetica van de gastheer? Welke routes zal die gebruiken om de infectie te klaren? Hoe reageert het immuunsysteem op

M. mycetomatis? Deze vragen zijn niet beantwoord. Met de studies beschreven in **hoofdstukken 3 en 4** is slechts een begin gemaakt.

Antigeniciteit van *M. mycetomatis*

Zoals in **hoofdstuk 2** al was beschreven, zijn er verschillende mogelijkheden om de diagnose mycetoma te stellen. Het stellen van de juiste diagnose is van belang voor de behandeling van deze ernstige ziekte. Microbiologisch kweken, pathologie, serodiagnostiek en moleculaire diagnostiek hebben allemaal hun gebreken. Een ideale diagnostische test moet makkelijk te gebruiken zijn in ontwikkelingslanden en een niet te weerleggen positieve of negatieve uitslag geven. Dit is niet gemakkelijk, zeker in het geval van serodiagnostische testen. Daarom hebben we geprobeerd om de antigeniciteit van *M. mycetomatis* in kaart te brengen. Het feit dat mycetoma patiënten antilichamen tegen *M. mycetomatis* opwekken, wordt gebruikt in het ontwikkelen van zulke serodiagnostische testen. In het verleden zijn er verschillende testen gemaakt met behulp van een ruwe antigeen suspensie, gemaakt uit cultuurextracten (31, 50). De enige poging om de epitopen in deze ruwe antigeen extracten te identificeren dateert uit 1991 (68). In die publicatie werden cytoplasma-eiwitten uit verschillende mycetoma veroorzakers geïsoleerd en gescheiden door middel van SDS-PAGE. Het bleek dat de verschillende *M. mycetomatis* isolaten allemaal dezelfde antigenen tot expressie brachten (68). Wat deze antigenen nu precies waren, werd niet bepaald. Ook later zijn er geen pogingen meer gedaan om antigenen die door *M. mycetomatis* geproduceerd worden te karakteriseren. In **hoofdstukken 5 en 6** hebben we gekeken welke antigenen door *M. mycetomatis* tot expressie gebracht worden in relatie tot de pathogenese van de ziekte en om een nieuwe serodiagnostische test met deze antigenen te ontwikkelen. In **hoofdstuk 5** werd het translationally controlled tumour protein (TCTP) ontdekt met behulp van een cDNA expressiebank. In **hoofdstuk 6** werd gedemonstreerd dat *M. mycetomatis* ook galactomannan-achtige antigenen *in vitro* uitscheidt; echter dit gebeurt niet *in vivo*.
TCTP is een eiwit dat niet uniek is voor *M. mycetomatis*. TCTPs van andere ziekteverwekkers bleken ook antigeen te zijn (9, 28, 43, 56). TCTP bleek echter in twee vormen in *M. mycetomatis* voor te komen: variant I komt in 53% van de isolaten voor en variant II komt in 47% van de isolaten voor. De antigeniciteit van TCTP in mensen werd vastgesteld met IgG en IgM antilichaam bepaling met een ELISA. De meeste patiënten hadden antilichamen ontwikkeld tegen mmTCTP variant I, maar helaas niet allemaal. Het bleek dat enkele gezonde Soedanese controles ook antilichamen tegen TCTP ontwikkeld hadden, mogelijk na blootstelling aan *M. mycetomatis* uit de omgeving. Kruisreactiviteit met TCTPs van andere endemische infectieveroorzakers kan ook opgetreden hebben. Dit was namelijk al bepaald voor filariële TCTPs (28, 56). Om te bepalen of kruisreactiviteit inderdaad het probleem was, werden zes peptiden ontworpen die zeer specifiek waren voor bepaalde delen van het *M. mycetomatis* TCTP eiwit. In vijf van de hier ontwikkelde ELISAs bleek er een duidelijk onderscheid te maken te zijn tussen patiënten en gezonde controles. IgG en IgM niveaus opgewekt tegen zowel het hele eiwit als de *M. mycetomatis* specifieke peptiden, bleken gerelateerd te zijn aan de grootte van de laesie en de duur van de ziekte. In het algemeen bleken de patiënten met de grootste laesies de hoogste antilichaam niveaus te hebben. Deze

niveaus werden lager naar mate de laesie kleiner werd. De hoogste antilichaamniveaus werden verder ook gevonden als patiënten al zo'n 6-15 jaar geïnfecteerd waren.

Tijdens de mycetoma-infectie, bleek TCTP hoofdzakelijk in de zich ontwikkelende grain tot expressie te komen. Dit kan verschillende redenen hebben. Zo blijkt dat wanneer een schimmel zich in menselijk weefsel vermenigvuldigt, deze schimmel mitotisch actief is. Verder zal het dan fysiologische stress ervaren, voorbeelden hiervan zijn het immuunsysteem van de gastheer of een gebrek aan voedingsstoffen. Al deze factoren zijn betrokken bij hoge TCTP expressie in andere organismen (28, 56, 59). Ook het zich aanpassen aan nieuwe omstandigheden blijkt van invloed op de TCTP expressie. Zo blijkt dat er geen TCTP tot expressie gebracht wordt in de parasiet *Brugia malayi* wanneer deze zich in een pre-infectief stadium bevindt. Wanneer de parasiet echter z'n gastheer binnendringt wordt TCTP ineens sterk tot expressie gebracht (28).

Zoals is **hoofdstukken 5 en 6** te lezen was, zijn in dit proefschrift de eerste specifieke antigenen voor *M. mycetomatis* beschreven. Dit is een eerste stap naar verbeterd inzicht in de antigeniciteit van *M. mycetomatis.* Door *M. mycetomatis* antigenen te beschrijven, vooral die antigenen die tijdens infectie tot expressie gebracht worden, kan meer inzicht verkregen worden in de pathogenese van deze infectie. Verder kan de ontdekking van zulke antigenen, zelfs leiden tot het ontwikkelen van een vaccin tegen de ziekte. In toekomstige experimenten kunnen cDNA expressiebanken ontwikkeld worden vanuit verschillende kweek- en infectiecondities. De cDNA expressiebank beschreven in **hoofdstuk 5**, werd vanuit een standaard kweekmedium geproduceerd. Op standaard kweekmedium heeft de schimmel een totaal ander fenotype gedurende infectie. Onder verschillende kweekcondities zullen verschillende eiwitten tot expressie gebracht worden. De kans is dan groot dat verschillende antigenen gekarakteriseerd zullen worden. Nieuwe antigenen kunnen niet alleen als vaccin-kandidaten beschouwd worden maar ook voor de ontwikkeling van goede serodiagnostische testen. Dit laatste is nog steeds nodig omdat de diagnostische testen die op dit moment gebruikt worden ofwel erg veel tijd kosten, ofwel te veel valspositieve en valsnegatieve resultaten geven. Verder zou het erg interesssant zijn als er een antigeen gevonden werd, wat uitgescheiden werd in de bloedstroom van de patiënten. Zo'n antigeen kan namelijk gebruikt worden om vast te stellen of iemand genezen is van de infectie. Op dit moment zijn er namelijk geen goede criteria om te bepalen of een patiënt genezen is of dat de patiënt goed reageert op therapie. Als een uitgescheiden antigeen verdwijnt uit het serum van een patiënt kan dit een indicatie zijn dat de patiënt klinisch genezen is.

Antifungale gevoeligheid van *Madurella mycetomatis*

De behandeling van eumycetoma veroorzaakt door *M. mycetomatis* is op dit moment vooral gebaseerd op de klinische ervaring van de behandelend arts. In Soedan worden patiënten meestal behandeld met ofwel 300-400 mg/dag ketoconazole ofwel 400 mg/dag itraconazole. Deze behandeling kan maanden tot zelfs jaren duren (23). Zelfs met dergelijke hoge doses antifungale middelen is de ziekte echter niet te genezen. De laesies zullen wel inkapselen en er zal geen verdere verspreiding plaats vinden waardoor de daaropvolgende operatie een stuk makkelijker uit te voeren is (23). Na de operatie worden de patiënten vaak nog maanden tot

jaren nabehandeld met antifungale middelen. Toch komt de infectie vaak terug, mogelijk omdat nog nooit wetenschappelijk bewijs is geleverd dat *M. mycetomatis in vitro* überhaupt gevoelig is voor deze antifungale middelen. De standaard antifungale gevoeligheidstesten zoals beschreven door de NCCLS maken gebruik van sporen waardoor deze testen niet voor de niet-sporulerende *M. mycetomatis* stammen gebruikt konden worden (13). In **hoofdstuk 7** werd een alternatieve methode, gebaseerd op de NCCLS criteria, voor *M. mycetomatis* ontwikkeld. In deze methode werden de hyfen van *M. mycetomatis* gehomogeniseerd door sonicatie. De homogene oplossing die dan ontstaat kan vervolgens aan verschillende concentraties antifungale middelen bloodgesteld worden. Om nauwkeuriger te kunnen bepalen wanneer de groei van de schimmel geremd wordt, werd XTT na 7 dagen kweken toegevoegd. XTT kan gebruikt worden om in de nog levende schimmelmassa de mitochondriale dehydrogenase activiteit te bepalen (37, 51). Deze methode bleek erg reproduceerbaar en nauwkeurig te zijn (**hoofdstukken 7 en 8**, referenties 6 en 63) en kon ook gebruikt worden om de antifungale gevoeligheid voor minder bekende antifungale middelen te bepalen. Voorbeelden hiervan zijn artemisinine en theeboom olie zoals beschreven in **hoofdstuk 9.** Artemisinine wordt geïsoleerd uit de plant *Artemisia annua* en wordt gebruikt in de traditionele Chinese geneeskunst (21, 33). Het is echter ook een bekend antimalaria middel. Theeboom olie wordt gewonnen uit de boom *Melaleuca alternifolia* en wordt gebruikt door de Australische Bundjalung Aboriginals van New South Wales omdat het ontstekingsremmende en antimicrobiële eigenschappen bezit (11, 32).

Het grootste nadeel van deze methode gebaseerd op de NCCLS-criteria is dat het erg veel tijd en werk kost om de test uit te voeren. Dit zorgt ervoor dat de test niet snel in de routine gebruikt zal worden om de antifungale gevoeligheid van *M. mycetomatis* te bepalen, zeker niet in de "endemische laboratoria" die vaak minder goede apparatuur hebben. In **hoofdstuk 8** werd dan ook een minder bewerkelijke methode ontwikkeld. Deze methode was de Sensititre methode (Trek Diagnostic systems, Ltd., East Grinstead, England). Met deze test kunnen de *in vitro* gevoeligheden voor amphotericine B, fluconazole, itraconazole, ketoconazole, 5-flucytosine en voriconazole bepaald worden. Om te bepalen of bij een bepaalde antifungale concentratie de schimmelgroei geremd wordt is de redox indicator Alamar blauw toegevoegd. Voor andere gisten en schimmels waren de *in vitro* gevoeligheden met dit systeem al eerder bepaald (14, 22, 52). Ook voor 90% van de *M. mycetomatis* isolaten bleek dit systeem reproduceerbare resultaten te geven. De gevoeligheden gevonden met de Sensititre test waren in overeenstemming met de klassieke NCCLS test en in iets mindere mate met de XTT test. Dit laatste kan verklaard worden doordat de indicators in beide testen verschillen in gevoeligheid en specificiteit. Een ander verschil was dat de XTT omzetting met de spectrofotometer gemeten werd, Alamar Blauw kleuring werd met het oog afgelezen. In het algemeen kan een spectrofotometer kleinere verschillen in kleur nauwkeuriger meten dan het oog.

Door de antifungale data te combineren (**hoofdstukken 7, 8 en 9)** konden de eerste *in vitro* gevoeligheden voor *M. mycetomatis* bepaald worden. *M. mycetomatis* bleek *in vitro* het meest gevoelig te zijn voor de azolen ketoconazole, itraconazole en voriconazole met een MIC_{90} van respectievelijk 0.125 μg/ml, 0.064 μg/ml en 0.125 μg/ml. Deze MICs zijn vergelijkbaar met concentraties die in serum bereikt kunnen worden (17). Fluconazole was de

minst effectieve azole, met een MIC_{90} van 16 μg/ml. Hoewel deze MICs erg hoog zijn, kunnen deze concentraties nog steeds effectief in serum bereikt worden (17, 27). Amphotericine B bleek minder effectief te zijn dan ketoconazole, itraconazole en voriconazole. Voor dit middel werd een MIC_{90} van 2 μg/ml gevonden. *M. mycetomatis* bleek ook erg gevoelig te zijn voor de theeboom olie. Hiervoor werd een MIC_{90} van 0.25 % v/v bepaald. *M. mycetomatis* was ongevoelig voor 5-flucytosine en artemisinine. Andere filamenteuze schimmels zijn ook resistent tegen 5-flucytosine (2, 13, 29, 30, 41). De gist *Candida albicans* bleek ook resistent te zijn tegen artemisinine, terwijl de gisten *Cryptococcus neoformans* en *Saccharomyces cerevisiae* wel gevoelig voor dit middel waren (26, 36).

In vitro is *M. mycetomatis* erg gevoelig voor de azolen ketoconazole en itraconazole, de middelen die op dit moment gebruikt worden voor therapie (44, 45, 49). Aan de hand van de lage MICs die in **hoofdstukken 7 en 8** gevonden werden voor deze middelen, zou geen van de isolaten resistent beschouwd worden. Toch is het opvallend dat in de klinische situatie veel patiënten terugkerende infecties krijgen (6, 44, 45, 49). Mogelijk zijn er in de *in vivo* situatie factoren die *M. mycetomatis* enige bescherming bieden tegen deze middelen. De schimmel groeit *in vitro* anders dan *in vivo*. In **hoofdstuk 2** zijn een aantal voorbeelden van de *in vitro* koloniemorfologie van *M. mycetomatis* te zien. De kolonies zijn bruin van kleur en zacht. In de patiënt vormt de schimmel zogenaamde grains. Deze zijn zwart en erg hard. In **hoofdstuk 10** was onze werkhypothese dan ook dat de zwarte component van deze grain melanine is dat door *M. mycetomatis* tijdens infectie gevormd wordt. Dit melanine zou de schimmel kunnen beschermen tegen het immuunsysteem van de gastheer en de antifungale middelen waar deze mee behandeld wordt. Melanines zijn negatief geladen, hydrofobe, macromoleculaire pigmenten die gevormd worden door middel van oxidatie van fenolische of indolische componenten (12, 35, 40). Het is al eerder aangetoond dat melanine micro-organismen beschermt tegen UV-straling, enzymatische lysis, oxidanten, de dodende werking van macrofagen en antimicrobiële middelen (12, 35, 40). Schimmelmelanine kan gevormd worden via verschillende routes. Via de eerste route wordt melanine gevormd uit difenolische componenten zoals bijvoorbeeld L-tyrosine en 3,4-dihydroxyfenylalanine (L-DOPA) (54). Via de tweede route wordt melanine uitsluitend uit L-tyrosine gevormd worden. Dit type melanine wordt pyomelanine genoemd en verschilt van het L-DOPA melanine doordat het oplosbaar is en bruin van kleur (10, 60). Via de derde route wordt melanine gevormd uit polyketide componenten, zoals 1,3,6,8-tetrahydroxynafthalene (1,3,6,8-THN) (54).

In **hoofdstuk 10** lieten we zien dat *M. mycetomatis in vivo* gemelaniseerd is. Melanine werd zowel in de grain als in de fagocyten rondom de grain gevonden. *In vitro* kon de melanineproductie in *M. mycetomatis* geblokkeerd worden met remmers op de pyo- en de DHN-biosynthese, maar niet met remmers van de L-DOPA biosynthese. Omdat pyomelanines oplosbaar zijn, is het zwarte pigment wat door *M. mycetomatis* gevormd wordt waarschijnlijk DHN-melanine.

Om te bepalen of dit melanine *M. mycetomatis* beschermt tegen de activiteit van antifungale middelen werden de *in vitro* gevoeligheden voor ketoconazole, itraconazole, fluconazole, voriconazole, amphotericine B en 5-flucytosine met de Sensititre test bepaald (zie **hoofdstuk 8**). De MIC werd bepaald in aan- of afwezigheid van zuiver melanine. Het bleek dat het melanine *M. mycetomatis in vitro* beschermt tegen de antifungale middelen

itraconazole en ketoconazole, de middelen die gebruikt worden in de kliniek (7). Melanine beschermde niet tegen de andere antifungale middelen. De spectaculaire 32-voudige stijging in MIC was uniek voor het melanine wat vanuit *M. mycetomatis* geïsoleerd was, omdat melanine geïsoleerd vanuit *C. neoformans* geen stijging in MIC te zien gaf. De beperkte aantallen experimenten die uitgevoerd zijn om het effect van melanine op de MICs van andere gemelaniseerde schimmels te bepalen hebben nooit eerder een zo duidelijke MIC verschuiving laten zien. Melanine beschermde de schimmels alleen door de tijd waarin een schimmel gedood wordt door een antifungaal middel te verlengen (34, 65, 66). In deze experimenten had men er echter geen rekening mee gehouden dat de schimmels geen melanine vormen in het kweekmedium waarin de test uitgevoerd werd. Tijdens het experiment verloren de gemelaniseerde isolaten hun melanine. De verlenging van de tijd waarin een schimmel gedood werd, werd toegeschreven aan het binden van amfotericine B en caspofungine aan L-DOPA melanine (34, 64-66). In onze test werd echter geen MIC shift voor amfotericine B waargenomen wanneer cryptococcenmelanine toegevoegd werd. Dit is te verklaren als cyptococcenmelanine wel amfotericine B bindt, maar ervoor zorgt dat de antifungale eigenschappen niet veranderen. In het verleden is geen bewijs gevonden dat L-DOPA melanine ook azolen kan binden (64, 65). Er is slechts één andere publicatie bekend waarin het effect van DHN melanine op de antifungale gevoeligheid onderzocht werd. In dat artikel werd wel een MIC verschuiving beschreven voor itraconazole. Dit kan dus betekenen dat de binding van antifungale middelen aan melanine afhankelijk is van het type melanine dat gevormd wordt (55). De MIC stijging werd voor *M. mycetomatis* alleen gevonden voor de azolen itraconazole en ketoconazole, maar niet voor de azolen voriconazole en fluconazole. Al deze azolen hebben een zogenaamde azole-ring. Hierdoor is het niet erg waarschijnlijk dat melanine aan deze ring bindt. De binding gebeurt waarschijnlijk via de dichlorobenzeenring of de lange zijketen, of door een combinatie van de twee. Deze structuren zijn uniek voor itraconazole en ketoconazole en zijn afwezig bij voriconazole en fluconazole.

Zoals hierboven al een aantal keer genoemd is, is op dit moment de antifungale behandeling voor *M. mycetomatis* mycetoma gebaseerd op de middelen itraconazole en ketoconazole. Dit leek aanvaardbaar wanneer gekeken werd naar de lage MICs die gevonden werden (**hoofdstukken 7, 8 en 9**)**.** Maar wanneer gekeken wordt naar de spectaculaire 32-voudige stijging in MIC onder invloed van melanine en het feit dat zowel itraconazole als ketoconazole erg toxisch zijn, is het de vraag of deze middelen nog wel de juiste keuze zijn. *M. mycetomatis* was net zo gevoelig voor voriconazole en er werd geen MIC stijging voor dit middel gevonden in aanwezigheid van melanine. Een ander voordeel van dit middel is dat het veel minder toxisch is dan de oudere azolen. Ook in de klinische situatie is al gebleken dat dit middel goed in staat is *M. mycetomatis* eumycetoma te genezen met name wanneer de botten nog niet geïnfecteerd zijn (39).

Door de ontwikkeling van testen waarmee de antifungale gevoeligheid voor *M. mycetomatis* bepaald kan worden ligt de weg nu open naar antifungale gevoeligheidsbepalingen in de routinediagnostiek. Door de *in vitro* gevoeligheidsdata te correleren aan de *in vivo* behandeling en de uitkomst hiervan, kan het huidige therapieschema verbeterd worden. Nu worden er nog willekeurige proeven met verschillende nieuwe antifungale middelen gedaan. Zo zijn de middelen terbinafine en posaconazole in mini-

experimenten gebruikt om te kijken of ze enige therapeutische effectiviteit hebben in de behandeling van mycetoma. In de toekomst kunnen zulke experimenten met nieuwe middelen, waaronder voriconazole en de echinocandines caspofungine en anidulafungine de antifungale therapie wellicht verbeteren. In goede klinische studies zou een groot aantal patiënten geïncludeerd moeten worden. Verder zouden er gestandaardizeerde criteria voor totale genezing of verbetering opgesteld moeten worden en natuurlijk zouden er controlegroepen meegenomen moeten worden. In de klinische studies die tot nu toe uitgevoerd zijn, bleken deze controles en criteria nogal eens te ontbreken.

In conclusie

Voor dit proefschrift hadden we als doel gesteld om meer inzicht te krijgen in de genetische diversiteit van zowel de schimmel *M. mycetomatis* als z'n humane gastheer. Tevens bepaalden we de antigeniciteit van *M. mycetomatis* en de antifungale gevoeligheid van *M. mycetomatis.* Uit **hoofdstukken 3 en 4** blijkt dat zowel *M. mycetomatis* als z'n humane gastheer bepalend zijn bij het ontstaan en het verder ontwikkelen van de infectie. Sommige *M. mycetomatis* isolaten zijn genetisch voorgeprogrammeerd om grotere laesies te veroorzaken, patiënten hebben vaker een genotype waardoor ze meer CXCL8 produceren en minder stikstof oxide uitscheiden, factoren waardoor wondjes minder gemakkelijk genezen en infectie wordt vergemakkelijkt. In **hoofdstukken 5 en 6** hebben we gezien dat *M. mycetomatis in vitro* TCTP en galactomannan-achtige antigenen uitscheidt. Slechts antilichamen tegen het TCTP antigeen zijn aantoonbaar in het serum van patiënten en kunnen daardoor gebruikt worden in serologische screenings. In **hoofdstukken 7, 8 en 9** werden drie antifungale gevoeligheidstesten (de NCCLS-gebaseerde test, de XTT test en de Sensititre test) ontwikkeld en vergeleken in de bepaling van de antifungale gevoeligheid van *M. mycetomatis* tegen een groot aantal antifungale middelen. *M. mycetomatis* bleek gevoelig te zijn voor itraconazole, ketoconazole, voriconazole, fluconazole, amfotericine B en theeboom olie en resistent tegen 5-flucytosine en artemisinine. Verder bleek in **hoofdstuk 10** dat het DHN-melanine, wat door *M. mycetomatis* geproduceerd wordt de schimmel *in vitro* beschermt tegen de azolen itraconazole en ketoconazole. Dit resulteerde in een 32-voudige stijging in MIC voor beide middelen en kan betekenen dat de huidige therapie gebaseerd op deze middelen misschien niet de juiste is.

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Chapter 13

Dankwoord

Wendy W.J. van de Sande

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Chapter 14

List of publications and curriculum vitae

Wendy W.J. van de Sande

List of publications

1. **van de Sande, W. W. J.**, W. van Vianen, M. Tavakol, and I.A.J.M. Bakker-Woudenberg. 2007. The antifungal effect of amphotericin B, itraconazole, voriconazole and caspofungin on conidia versus hyphae of *Aspergillus fumigatus.* Antimicrob Agents Chemother *Submitted*

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Curriculum vitae

Wendy Wilhelmina Johanna van de Sande werd op 1 februari 1980 geboren te Breda. In 1998 behaalde zij haar VWO examen op het Willem II College te Tilburg. Datzelfde jaar begon ze met de opleiding Biologie en Medisch Laboratoriumonderwijs aan Hogeschool Brabant in Etten-Leur. Deze studie rondde zij met lof af. Tevens bepaalde de Nederlandse Vereniging van bioMedisch Laboratoriummedewerkers (NVML) dat haar scriptie de tweede beste scriptie van dat afstudeerjaar was. Na haar stage op de afdeling Medische Microbiologie en Infectieziekten van het Erasmus MC te Rotterdam werd haar op deze afdeling een onderzoeksbaan aangeboden. Het eerste jaar werkte ze aan het promotie-onderzoek van haar voorganger waarna ze onder begeleiding van Prof. Dr. Dr. Alex van Belkum aan haar eigen promotietraject begon. Tijdens haar promotietraject werd ze gevraagd om mee te werken aan het *Aspergillus fumigatus* project. Na haar promotie zal Wendy zich verder gaan richten op dit project.