ISHAM
INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2018, 56, S153–S164
doi: 10.1093/mmy/myx061
Review Article

Review Article

Closing the mycetoma knowledge gap

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Received 14 April 2017; Revised 30 May 2017; Accepted 14 July 2017; Editorial Decision 26 June 2017

Abstract

On 28th May 2016, mycetoma was recognized as a neglected tropical disease by the World Health Organization. This was the result of a 4-year journey starting in February 2013 with a meeting of global mycetoma experts. Knowledge gaps were identified and included the incidence, prevalence, and mapping of mycetoma; the mode of transmission; the development of methods for early diagnosis; and better treatment. In this review, we review the road to recognition, the ISHAM working group meeting in Argentina, and we address the progress made in closing the knowledge gaps since 2013. Progress included adding another 9000 patients to the literature, which allowed us to update the prevalence map on mycetoma. Furthermore, based on molecular phylogeny, species names were corrected and four novel mycetoma causative agents were identified. By mapping mycetoma causative agents an association with Acacia trees was found. For early diagnosis, three different isothermal amplification techniques were developed, and novel antigens were discovered. To develop better treatment strategies for mycetoma patients, *in vitro* susceptibility tests for the coelomycete agents of black grain mycetoma were developed, and the first randomized clinical trial for eumycetoma started early 2017.

Key words: Mycetoma, Madura foot, Host factors, Epidemiology, Diagnosis, Therapy.

Introduction

Mycetoma is an implantation mycosis, characterized by large painless tumor-like subcutaneous swellings, the formation of sinuses, and discharge that contains grains. It is frequently located in the foot and hand; however, no body part is exempted.[1] Although mycetoma can be caused by either bacteria (actinomycetoma) or fungi (eumycetoma), the pathology and symptoms are similar. Mycetoma prevails in many tropical and subtropical countries and shares characteristics with many of the neglected tropical diseases:

- Mycetoma is a chronic, slowly developing condition that can become progressively worse if undetected and untreated.

- It can cause life-long disabilities and places a burden on the family members.
- Mycetoma patients are often stigmatized.
- Mycetoma afflicts the poorest people, often living in remote areas.[2]

Although mycetoma occurs in a wide range of countries, the prevalence is unknown and no control programs exist. Also, the management of mycetoma infections is not uniform and no guidelines exist. Therefore, there is still a long road ahead of us to be able to properly manage mycetoma patients and develop effective control programs.

The road to recognition

Before 2013, mycetoma was among the most neglected conditions in the field of tropical medicine. Worldwide, only a few centers reported research data mainly on basic science. Most clinical studies addressing treatment were restricted to case reports or small case series, leaving many unresolved issues in particular in the treatment of eumycetoma.

A landmark meeting was held on February 1, 2013, in Geneva by Drugs for Neglected Diseases *initiative* (DND*i*) to identify knowledge gaps and research priorities.[1] It appeared that there was a lack of proper data on incidence, prevalence, and mapping of mycetoma and the mode of transmission. Furthermore, there were no appropriate tools for early detection, and the current treatment, especially for eumycetoma, was particularly disappointing.

It was felt that advocacy was badly needed. Scientific symposia were organized at various international tropical medicine conferences, and a comprehensive review paper on mycetoma was published.[3] The PLoS Neglected Tropical Diseases journal agreed to a collection of papers on mycetoma. Other efforts included coverage on Al Jazeera TV channel of the Mycetoma Research Centre (MRC) in Sudan, and a video was produced by the World Health Organization (WHO). Furthermore, students from the University of Toronto won the Global Health Untold Stories Contest in 2015 that was launched by the Johns Hopkins Bloomberg School of Public Health website 'Global Health Now'.[4,5] This led to a three-part series by journalist Amy Maxmen on mycetoma.[6–8] DND*i* added mycetoma to their portfolio in 2015 in order to identify an effective, safe, and affordable treatment for eumycetoma. In collaboration with Eisai Ltd., Japan, and the MRC in Sudan, DND*i* designed the first ever randomized clinical trial in the treatment of eumycetoma. The MRC was designated a WHO Collaborative Center in 2015. In 2013, mycetoma was added to the list of Neglected Tropical Diseases from WHO, in the category of 'other' neglected conditions. Generally, it was felt that mycetoma should be added to the list of 17 NTDs to attract the interest of donors. The Sudan Ministry of Health proposed a resolution for which the support of many endemic and nonendemic countries was obtained. The resolution was adopted by the WHO Executive Board in January 2016 and endorsed by the World Health Assembly on 28 May 2016.

In this review, we will discuss the efforts of the past 4 years to close the knowledge gaps that had been identified.

Methods

Search strategy

Articles on mycetoma published in the years 2013, 2014, 2015, 2016, and early 2017 were searched using the electronic database PubMed, using the following search terms: Mycetoma, Madura foot, eumycetoma, and actinomycetoma. Studies published in languages other than English, French, Spanish, Portuguese, German, or Dutch were excluded. Studies were excluded when the article was not on mycetoma or was written solely as a review. Only data that had not been published before were included. Population figures were derived from IndexMundi (http://www.indexmundi.com/facts/indicators/SP.POP.TOTL/compare#country = ma), as described before.[9] If studies had data older than 1960, the population size of 1960 was used to calculate the prevalence.

Incidence, prevalence and mapping of mycetoma

Since there are no official prevalence and incidence numbers on the occurrence of mycetoma globally, an effort was made in 2013 to obtain a rough estimation of the global burden of mycetoma by a meta-analysis of cases published in the literature.[9] This meta-analysis was performed on 8763 cases published, and the prevalence was calculated by dividing the number of reported cases by the country population in each year.[9] Data retrieved from the literature search in the years 2013–2017 were added to update the number of cases published.[10–19] This doubled the number of cases recorded to 17607 cases,[10–14,17] and the previously published prevalence map was updated accordingly (Fig. 1). Most cases added to literature were from Sudan (6792 cases in 24 years)[14] and Mexico (3933 cases in 54 years),[10] countries from which many cases were already published in the past. Interestingly, also cases from countries, not previously included in the meta-analyses were reported. The first cases of the Comoro Islands[20] and Laos[21,22] were described. From Venezuela, nine cases were reported,[12] 21 cases from Brazil,[19] and even four autochthonous cases from Europe have been reported.[23,24] Of the four autochthonous cases

Table 1. Etiology of mycetoma in Argentina as presented at the Primeras Jornadas Argentinas de Micetomas, in Santiago del Estero, Argentina on 20–21 May 2016.

	Actimycetoma			Eumycetoma			
Hospital	*Actinomadura madurae*	*Nocardia brasiliensis*	*Streptomyces somaliensis*	*Trematosphaeria grisea*	*Madurella mycetomatis*	Others	Total Cases
Hospital Independencia (S del E) (1991–2014)	20	…	1	3	1	1	26
Lab. Anat. Pat. (Hosp. Indep) (2000–2015)	16	…	…	3	…	1	20
Hospital Público de Córdoba (1967–1987)	…	9	…	4	1	1	15
Hospital Señor del Milagro de Salta (Salta) (2014–2016)	…	3	…	…	…	…	3
Hospital F.J. Muñiz (Buenos Aires) (1984–2009)	33	8	2	33	4	15	95
Total	**69**	**20**	**3**	**43**	**6**	**18**	**159**

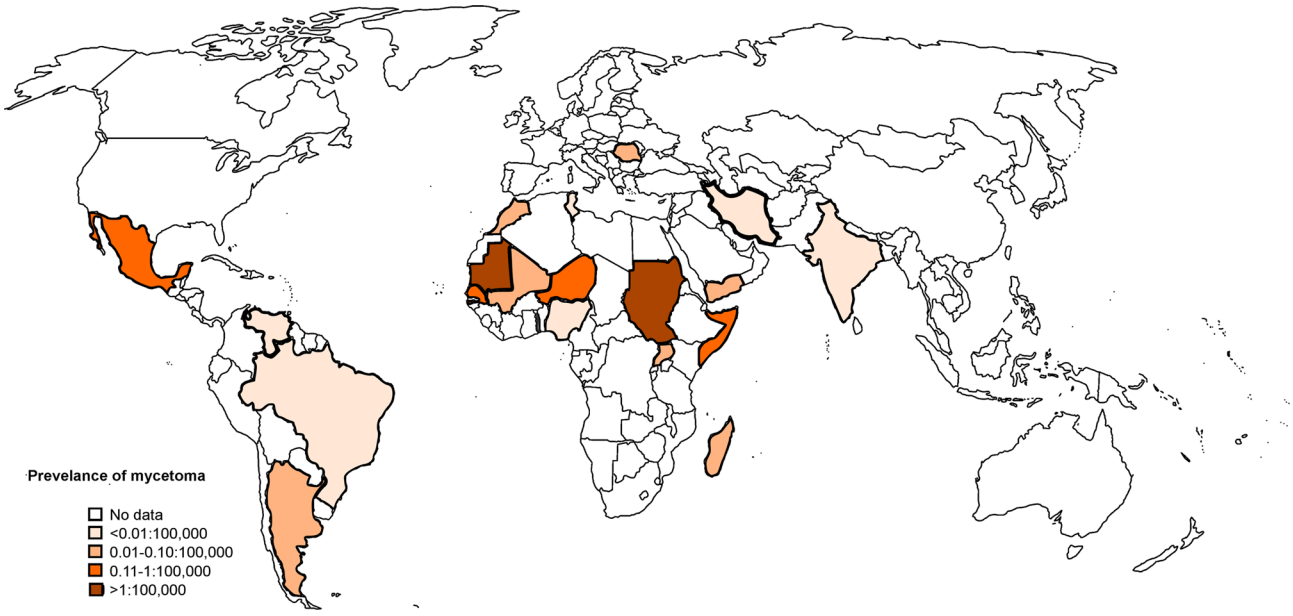

Figure 1. Average prevalence of mycetoma cases as calculated by the number of cases reported in a year in a certain country divided through the total population of that country of that same year as reported by www.indexmundi.com/facts/indicators/SP.POP.TOTL/compare.

from Europe, three were seen in Italy (two in Albanian forest workers[23] and one in an Italian florist[24]) and one from France in a gardener.[25] In all three Italian cases, the causative agent was considered to be *Actinomadura madurae*, but in the molecularly identified case only 99.1% identity with the 16S gene of *A. madurae* was found.[23,24] In the French case, *Nocardia boironii* was the causative agent.[25]

In Argentina, the first conference on mycetoma was organized in collaboration with the ISHAM working group on mycetoma (Primeras Jornadas Argentinas de Micetomas, in Santiago del Estero). A total of 159 thus far unpublished cases were presented from the following centers: Hospital Independencia (Santiago del Estero, period: 1991–2014) with 26 cases; Laboratorio de anatomía patológica de Hospital independencia (Santiago del Estero, period: 2000–2015) with 20 cases; Hospital Público de Córdoba (Córdoba, period: 1967–1987), with 15 cases; Hospital Señor del Milagro de Salta (Salta, period: 2014–2016), with three cases and Hospital F.J. Muñiz (Buenos Aires, period: 1984–2009), with 95 cases (Table 1). Of the 159 cases presented, 92 cases (57.8%) were actinomycetoma and caused by *A. madurae* (69 cases; 43.4%), *Nocardia brasiliensis* (20 cases; 12.6%) or *Streptomyces somaliensis* (3 cases; 1.9%). Sixty-seven eumycetoma cases (42.2% of the total

number reported) were described, and these were caused by *Trematosphaeria grisea* (43 cases, 27.0%), *Madurella mycetomatis* (six cases; 3.8%), and other fungi (18 cases, 11.3%) such as *Scedosporium* spp, *Acremonium* spp, and *Fusarium* spp. If we compare these data with the previous report of 2006,[26] the actinomycetoma cases due to *A. madurae* are now slightly more frequent. It is important to mention that these cases are predominantly accounting for 55–70% of actinomycetoma cases; this is different from what is reported in Mexico.[10,15]

In the past, identification of the causative agents was mainly performed by culture and/or histology. Particularly in the case of nonsporulating fungi, this has hampered accurate species identification.[27,28] In the last 5 years, molecular approaches have been used to unravel the taxonomic affiliations of eumycetoma causative agents archived in collections of the Westerdijk Fungal Biodiversity Institute (formerly, the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands),[28–30] the UK National Mycology Reference Laboratory (NCPF; National Collection of Pathogenic Fungi; Bristol, UK)[27] and the Institut Pasteur (UMIP; Collection de Champignons et Actinomycetes Pathogenes; Paris, France).[27] This has resulted to the renaming of various mycetoma causative agents and emphasized the need for molecular identification tools for final identification. *Pyrenochaeta romeroi* and *Pyrenochaeta mackinnonii* became *Medicopsis romeroi* and *Nigrograna mackinnonii, Leptosphaeria senegalensis,* and *Leptosphaeria tompkinsii* became *Falciformispora senegalensis,* and *Falciformispora tompkinsii* and *Madurella grisea* was renamed as *T. grisea*.[28,31] *M. grisea* actually appeared to be polyphyletic. This species was deposited 31 times in the NCPF and UMIP culture collections[27] and was renamed after molecular identification as *Nigrograna mackinnonii* (8 times), *F. senegalensis* (1 time), *M. romeroi* (12 times), *Rhytidhysteron rufulum* (4 times), *Emarellia grisea* (5 times), and *Emarellia paragrisea* (1 time).[27] Since this species also encountered 139 times in epidemiological studies,[10–18] one should wonder if all 139 isolates were identified correctly. Probably 139 is an overrepresentation of *T. grisea,* and several of these isolates should actually be either *E. grisea, E. paragrisea, M. romeroi,* or *N. mackinnonii*. It is therefore promising to note that the most recent epidemiological study has sequenced the ITS region and the D1/D2 domain of the 28S rDNA to identify the fungal causative agents and 16S rDNA region to identify the bacterial causative agents.[12]

The mode of transmission

Another knowledge gap concerns the way people become infected with mycetoma. During the Primeras Jornadas Argentinas de Micetomas conference, in Santiago del Estero, it was noted that most of the cases reported came from the northwest of Argentina, which presents a tropical climate, with a diversity of areas, as it is influenced by a mountain range that crosses it. From this zone, the region with the highest number of mycetoma reports is Santiago del Estero (latitude: 27°47′42″S and longitude: 64°15′41″O) with an altitude of 188 m above sea level. With an annual average temperature of 27.4°C (exceeding 45°C in summer), and a limited rainfall (400–600 mm, annually), it represents a semi-arid region with xerophytic vegetation where the shrub vinalopó is prevalently found. In this region, agricultural and forestry activities and the production of charcoal in ovens are the main economic activities. Like other parts of Latin America, women are equally involved in agricultural activities and are therefore similarly exposed to mycetoma causative agents as men are. Most of the cases were reported after a thorn prick of "vinal" or "viñal" (*Prosopis ruscifolia*) plants. As in Morocco and in Southeast India, regions with the same ecological conditions,[9] most cases of this region were caused by *A. madurae* and *T. grisea*. In the provinces of Tucumán and Salta, regions with a climate similar to Mexico, predominantly mycetoma cases caused by *N. brasiliensis* were reported.[10,15] Eumycetoma cases were only seen sporadically.[10,15] In Tucumán an average temperature of 20°C is seen with an annual rainfall of 900–1000 mm. Sugar cane and citrus plantations are common. In Salta, the average temperature is 17°C with an annual rainfall of 690 mm. Plantations of tobacco, legumes, soy, and potatoes are found in this region. Although mycetoma was most predominant in the northwest of Argentina, the highest number of reports were from Buenos Aires. The majority of these cases originated from Santiago del Estero, Chaco and Formosa, among others, and also include patients from Bolivia and Paraguay.[26]

Although the primary reservoir of the causal agents is still not known, based on the above observations it seems that each causative agent has its own ecological niche. The route of transmission is not completely clear. While for each of the regions, thorny shrubs have been identified, and thorn pricks occur frequently, not everyone seems to develop mycetoma, suggesting a role for the immune system and possibly, genetic susceptibility, or a critical inoculum.[1]

In the past 5 years, only limited data have become available on the mode of transmission. We identified only four articles in which some novel data on the natural habitat of mycetoma causative agents could be identified. These included two case studies,[32,33] a phylogenetic analysis,[34] and a study using ecological niche modelling.[35] One patient obtained an injury in a swamp in Virginia, United States, and developed a *N. brasiliensis* actinomycetoma lesion within 2 weeks.[32] The other patient developed an

Exophiala jeanselmei eumycetoma 1 year after an injury by a sea urchin spine.[33] Although the causative agents differed, in both cases a water-rich environment was implicated. Another natural habitat was proposed for *M. mycetomatis* based on a phylogenetic analysis. *M. mycetomatis* appeared to be a nonsporulating asexual *Chaetomium* species, and many *Chaetomium* species have mammal dung or dung-enriched soil as the prime ecological niche.[34] Therefore, dung could also be a natural niche for *M. mycetomatis*, although up until now no *M. mycetomatis* has been recovered from dung. Samy and associates computed the ecological niche of mycetoma causative agents based on data from 44 mycetoma cases from Sudan, land surface temperature, monthly normalized difference vegetation index, and soil data for two different depths.[35] It appeared that this niche overlapped with that of *Acacia* trees and therefore these trees might be implicated in the development of mycetoma.[35] *Acacia* trees have large thorns that might help with introducing the causative agent to the subcutaneous tissue. Furthermore, DNA from *M. mycetomatis* was found to be present on thorns,[36] and in many surgically excised lesions, thorns are found embedded in the lesion, which makes it plausible that indeed these trees could be involved in the introduction of the causative agent to the subcutaneous tissue.

The role of the immune system in the development of mycetoma was also studied.[37–40]. To study the genetic background associated with the susceptibility of mycetoma infection, several single-nucleotide polymorphisms (SNPs) of genes either associated with the innate immune system or cytokine production were studied.[40–44] Verwer et al. and Geneugelijk et al. concentrated on chitinase and collagenase production by the host.[40,41] Verwer demonstrated that in the *M. mycetomatis* grain, fungal hyphae contained chitin and that the human chitinases chitrotriosidase and AMCase bound to the hyphae within the grain.[40] Genetically seen, mycetoma patients more often had a 24-bp insertion in one of the copies of their chitotriosidase gene compared to healthy endemic controls (Table 2).[40] This genotype results in decreased enzyme activity.[40] Geneugelijk demonstrated that the grain itself was surrounded by collagen and that metalloproteases 2 (MMP-2) and 9 (MMP-9), responsible for collagen deposition, were expressed by immune-cells surrounding the grain.[41] Furthermore, active MMP-9 was detected in serum of mycetoma patients.[41] No genetic differences were found for MMP-2 and MMP-9 between patients and healthy controls (Table 2), but in males a difference was noted in tissue inhibitor of matrix metalloproteinase 1 (TIMP-1).[41] This could indicate, that especially in males, a more extensive collagen capsule is formed and thus protects the grain from influences of the immune system or antifungal agents. To study the role of genetic SNPs in cytokine production, Mhmoud et al. looked at chemokine CCL5 and interleukin-10 (IL-10).[42] It appeared that two SNPs in the CCL5 gene and one in the IL-10 promotor were associated with mycetoma development (Table 2).[42] Furthermore, serum levels for both CCL-5 and IL-10 were elevated in patients compared to healthy endemic controls.[42] More cytokine-levels were determined by Nasr et al.[37] In their study the cytokines in blood of 70 eumycetoma patients caused by *M. mycetomatis* were compared with the cytokines in blood of 70 healthy endemic controls.[37] It appeared that significantly higher levels of interferon gamma (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), IL-4, IL-5, IL-6, IL-10, and IL13 were obtained in mycetoma patients compared to healthy endemic controls.[37] No significant difference was found for IL-1β.[37] However, these cytokines were measured in patients with a mycetoma infection and compared to healthy controls so a marked difference is to be expected. More interesting is to know if immune cells of healthy individuals might react differently when exposed to *M. mycetomatis* antigens. To answer this question Elegab et al. stimulated peripheral blood mononuclear cells (PBMC) of 27 mycetoma patients and 21 healthy individuals from the same endemic regions with an uncharacterized culture filtrate of *M. mycetomatis*.[38] PBMCs of *M. mycetomatis* infected patients produced statistically significant higher concentrations of IL-10 and lower concentrations of IFN-γ, indicating that PBMCs of mycetoma patients tend to react with a Th2 response and PBMCs of healthy controls with a Th1 response.[38] The importance of a Th2 response for mycetoma patient also indirectly found by van Hellemond et al. who demonstrated that mycetoma patients more often had a schistosomiasis coinfection than healthy endemic controls.[39] Chronic schistosomiasis is associated with a Th2 cytokine environment.[39]

Development of methods for early diagnosis

It has also become apparent that it is very difficult to determine the true burden of mycetoma, to identify its primary reservoir and the monitor therapeutic response because there are currently no safe, reliable, fast, and cheap diagnostic tools to determine mycetoma causative agents.[1,45] It was hoped that isothermal DNA amplification techniques, and serological-based procedures would be developed for mycetoma causative agents identification.[1] In the past 5 years efforts have been made to develop or test such assays.

One of the easily used, noninvasive techniques to assist in the diagnosis of mycetoma was the use of dermoscopy. White/yellow grains became clearly visible with the use of this technique,[46] but it did not assist with the

Table 2. Single nucleotide polymorphisms (SNP) determined in mycetoma patients and healthy controls in Sudan.

Gene	SNP	rs-number	*P* value (association with mycetoma)	Reference
3β-HSD		rs6203	.710	43
AMCase	A50G	rs61756687	.647	40
	A290G	rs41282492	.717	40
	10bp insertion 5'UTR	rs143789088	.720	40
CCL5	−403	rs2107538	.250	42
	−25	rs2280788	<.0001	42
	Int1.1.	rs280789	<.0001	42
Chitotriosidase	24bp insertion	Rs3831317	.004	40
COMT		rs4680	.006	43
CR1	Sl	rs17047661	.039	44
	McC	rs17047660	.001	44
CXCL8	−251	rs4073	.008	44
CXCR2	+785		.037	44
CYP1B1		rs1056836	1.000	43
CYP17		rs743572	.846	43
CYP19		rs700518	.004	43
IL-10	−819	rs1800871	.0005	42
	−592	rs1800872	.780	42
MBL2	54	rs1800450	.262	44
	57	rs1800451	.531	44
	XY	rs7096206	.399	44
MCP1	2518	rs1024611	.524	44
MMP-2	−1306		.39	41
MMP-9	−1562		1.00	41
NOS2	L		.0006	44
TIMP-1	+372		.0004 (males); .53 (females)	41
TNFα	−308	rs1800629	1.000	44
TSP4	29 926		.030	44

Single nucleotide polymorphisms (SNPs) determined in mycetoma patients and healthy controls in Sudan. This is an update of a previously published table.[82] Published genetic association studies performed in mycetoma patients and healthy controls in the Sudan are summarized. For each gene, the SNP determined is indicated, including the rs-number assigned to that specific SNP. Associations in all studies were determined using Fisher's exact test and the corresponding *P* values are given together with the reference for the study in which that association was found.

Table 3. Comparison of DNA amplification methods for identification of *Madurella mycetomatis.*

	PCR	RCA	LAMP	RPA
Culture required	No	Yes	No	no
Pre-amplification of ITS region needed with PCR	No	Yes	No	No
PCR thermal cycler required	Yes	Yes	No	No
Reaction temperature	94°C → 58°C → 72°C	65°C	65°C	65°C
Time to identification	6 h	6 h	2 h	40 min
Detection limit		0.003 ng	0.5 ng	0.2 ng
Specificity	100%	100%	100%	100%
Estimated cost ($)	7.7		<1–5.3	4.25
Reference	47,83	48	47	47

identification of the causative agent. To rapidly identify species molecularly, three isothermal amplification techniques for the determination of *M. mycetomatis* have been developed, namely, Recombinase Polymerase Amplification (RPA), Loop-mediated isothermal AMPlification (LAMP) and Rolling Circle Amplification (RCA).[47,48] RCA has also been developed for *Madurella pseudomycetomatis, Madurella tropicana, Madurella fahalii, F. senegalensis, F. tompkinsii, M. romeroi, T. grisea.*[48] Although all three isothermal amplification techniques appeared to perform well, RPA and LAMP especially seemed interesting, since no thermocycler was needed in the procedure (Table 3).

Although isothermal amplification techniques will enhance the access to molecular identification tools in the endemic countries, a more important improvement was made to really implement molecular diagnostic tools in the identification of mycetoma causative agents. In the past, molecular identification tools were only used to identify fungi to the species levels using DNA obtained from grown cultures. This meant that only after culturing for several weeks, final identification was given by polymerase chain reaction (PCR). Recently, a DNA isolation method for grains was developed that allowed direct PCR, LAMP and RPA to be performed on direct clinical biopsy material.[47] One study showed, from the 10-black grain surgical biopsy specimens taken, all were positive for *M. mycetomatis*. The two clinical samples that were not suspected for mycetoma were negative.[47]

Also, efforts have been made to develop novel serodiagnostic tools.[49] In a cytoplasmic extract of two *M. mycetomatis* isolates, three proteins with molecular masses of 45, 60, and 95 kDa reacted consistently with sera from eumycetoma patients.[49] Immunoblots of these antigens were prepared, and 100 eumycetoma sera were tested. Of these 100 sera, 57 tested positive for the 45 kDa protein, 57 with the 60 kDa protein, and 57 with the 95 kDa protein.[49] The 95 kDa protein seemed to be the most discriminative since none of the 25 healthy endemic controls or the 25 nonendemic controls reacted to this protein. Also, none of the 10 actinomycetoma patients or 40 patients with other diseases reacted to this protein.[49] Overall, the immunoblot reached a sensitivity of 75% and a specificity of 95%.[49] Unfortunately, the nature of the identified antigens is currently still not known, but it would be interesting to test this immunoblot in a clinical setting.

Currently it is unclear to what extent smaller, localized lesions or more extensive lesions may be diagnosed with peripheral blood tests (whether dependent on antigen or antibody detection, or detection of fungal or actinobacterial DNA by PCR). Mycetoma is a primarily focal disease and systemic illness with fungaemia or bacteremia is not a feature; only in exceptional cases lymphatic or hematogenous spread occurs. Alternatively, a test aimed on detecting the organisms on an FNA sample using for example LAMP may be more sensitive.

Also the (1→3)-β-D glucan assay was assessed in eumycetoma patients.[50] And (1→3)-β-D glucan is a cell wall polysaccharide that is present in many fungal species and secreted during growth.[50] It was demonstrated that median (1→3)-β-D glucan concentrations in sera of eumycetoma patients were significantly higher than median (1→3)-β-D glucan concentrations in sera of healthy controls, but still 15 out of 45 patients had (1→3)-β-D glucan levels below the detection limit of 85 pg/ml.[50] Furthermore, actinomycetoma patients also had detectable levels of (1→3)-β-D glucan in their sera, which made it impossible to discriminate between eumycetoma and actinomycetoma patients.[50] Therefore, measuring (1→3)-β-D glucan might not be relevant in the discrimination between eumycetoma and actinomycetoma patients.

Since in many laboratories histology is still the only technique used for the identification of the eumycetoma causative agent, efforts were made to improve this technique. Batran showed that henna extract could be used to stain mycetoma grains.[51] Frickman and associates demonstrated that isolating DNA from histology slides and perform a panfungal PCR with sequencing on mycetoma patients, resulted in noninterpretable results. The panfungal PCRs appeared to readily react with DNA from contaminating spores of environmental fungi, and only species-specific PCRs were reliably used on DNA isolated from histology slides.[52] Ahmed and colleagues used an antibody against *Aspergillus* species to demonstrate with immunohistochemistry that the grain found in the mycetoma lesion was indeed an *Aspergillus* spp., thus introducing a new method to affirm the species identification on histology slides.[53]

Treatment

Since mycetoma can be caused by bacteria and fungi, treatment options differ per causative agent. The current recommended regimen for actinomycetoma is combination therapy in 5-week cycles. Patients are treated 3 weeks with amikacin sulphate intramuscularly together with 5 weeks of oral trimethoprim-sulfamethoxazole.[54] This regimen has a cure rate of about 90%.[54] For eumycetoma, currently, a combination of itraconazole therapy with surgery is recommended.[54] In moderate (5–10 cm in size with bone involvement) and massive (>10 cm in size with bone involvement and secondary bacterial infections) lesions, 400 mg/day itraconazole is started 6 months prior to surgery.[55] For smaller lesions, wide local excision is indicated.[55] After surgery, 400 mg/day itraconazole is given for 3 months in case of small lesions or for six months for moderate to massive lesions.[55] Unfortunately, out of 1013 patients who underwent this regiment, 276 patients (27.2%) developed a recurrence.[56] The overall response rate for eumycetoma is therefore disappointing. To improve the overall therapeutic outcome, one could focus on the identification of novel and more efficient antifungal agents but also on the improvement of the surgical procedure itself.

The search for novel antifungal agents usually starts *in vitro*. For *M. mycetomatis* an *in vitro* susceptibility assay was developed in the past,[57] and *in vitro*

susceptibilities were determined for amphotericin B,[57,58] ketoconazole,[58] itraconazole,[57,58] fluconazole,[58] voriconazole,[58] posaconazole,[59] isavuconazole,[60] terbinafine,[59] 5-flucytosine,[58] caspofungin,[61] anidulafungin,[61] and micafungin.[61] In general, *M. mycetomatis* was most susceptible towards the azole class of antifungal agents. In 2014, Ahmed and associates tested the *in vitro* susceptibility of *M. mycetomatis* against ravuconazole, and this azole appeared to be the most potent azole against *M. mycetomatis* so far.[62] Minimal inhibitory concentrations (MICs) ranged from <0.002 μg/ml to 0.031 μg/ml, and the MIC_{90} was 0.016 μg/ml. This was much lower than the MIC_{90} of ketoconazole (0.25 μg/ml) and itraconazole (0.25 μg/ml).[62] Furthermore, for the first time to our knowledge, *in vitro* interaction studies of two classes of antifungal agents were performed. The azoles itraconazole and ketoconazole in combination with terbinafine did not result in synergy for *M. mycetomatis*.[63] Since *M. mycetomatis* is not the only fungus causing eumycetoma, efforts were also made to study the *in vitro* antifungal susceptibility of coelomycete agents of black grain eumycetoma. The *in vitro* antifungal susceptibility of *M. romeroi, F. senegalensis, F. tompkinsii, T. grisea, P. larense,* and *N. mackinnonii* were determined for amphotericin B, fluconazole, voriconazole, ketoconazole, itraconazole, posaconazole, 5-flucytosine, and caspofungin.[64] It appeared that most species had high MICs toward fluconazole, 5-flucytosine, and caspofungin and relatively low MICs for the azoles.[64] This was comparable with the results obtained for *M. mycetomatis*.[58,61] Only *M. romeroi* appeared to be different.[64] This species had very high MICs for ketoconazole and especially itraconazole, which might be a concern since the current therapy is based on itraconazole.[64] *In vitro* results might not always necessarily predict clinical outcome. Therefore, preclinical animal models are used as well. As demonstrated by Van de Sande and colleagues in a mouse model of *M. mycetomatis* mycetoma, amphotericin B and itraconazole were used to determine therapeutic efficacy.[65] In general, *M. mycetomatis* has relatively high MICs for amphotericin B and low MICs for itraconazole.[58] Surprisingly, in the mouse model, only amphotericin B was able to prevent grain formation when treatment was started 1 hour after initiation of infection and continued for 14 days.[65] Grains were still formed when itraconazole treatment was given.[65]

Although in endemic countries itraconazole is currently the drug of choice, in many nonendemic countries other azoles are preferably used. In 2013 and 2014, two papers were published in which a total of 16 eumycetoma patients seen in Paris were presented.[66,67] Furthermore, four case reports appeared: three from France[68–70] and one from New York.[71] The patients originated mainly from West Africa (14 patients), and the causative agents included *Madurella* spp. ($n = 5$),[66,67,69] *Fusarium solani* ($n = 3$),[66] *Falciformispora* spp. ($n = 2$),[67] *Acremonium recefei* ($n = 1$),[67] *Exophiala jeanselmei* ($n = 2$),[66,70] *Scedosporium apiospermum* ($n = 1$),[68] and unidentified fungal species ($n = 6$).[66,67,71] In this group of patients, one received no antifungal treatment, 12 received voriconazole for at least 6 months, and eight received posaconazole for at least 18 months.[66–69,71] In seven patients the triazole therapy was combined with either caspofungin (two patients), terbinafine (three patients), or 5-flucytosine (two patients).[66,67,69] Triazole treatment was given for 6–70 months (median 18.25 months) and resulted in cure in seven patients, failure in four patients, and seven patients were still under treatment.[66–70] In two patients paradoxical responses to posaconazole treatment were noted.[68,69] In both patients, inflammation, pain, and discharge of grains increased after starting posaconazole treatment[68] or a combination of posaconazole with 5-flucytosine.[68,69] In one patient, the condition dramatically improved within two weeks on morphine.[68] In another patient, diclofenac was given as a palliative measure.[69] A dramatic improvement was observed within 1 week and within 2 months clinical examination was normal.[69] Since these patients were suffering from mycetomas with different etiologies and were receiving different types of treatment, it is difficult to draw conclusions, but in general, the newer generation of azoles did not seem to perform better than itraconazole given in endemic regions.

Due to the disappointing cure rates with itraconazole and the high costs of this drug for the patients, many patients look for other ways to treat their infection, and herbal treatment with local medicinal plant preparations are considered an alternative. Elfadil and colleagues demonstrated that chemical extractions of *Acacia nubica, Nigella sativa,* and *Boswellia papyrifera* at least had some *in vitro* antifungal activity against *M. mycetomatis*.[72] These plants differed however from the plants mostly used by eumycetoma patients.[73] Of the 311 mycetoma patients questioned at The Mycetoma Research Centre (MRC), University of Khartoum, Sudan, 42.4% had used herbal remedies in the past.[73] Commonly used herbs were *Moringa oleifera, Acacia nilotica, Cuminum cyminum, Citrullus colocynthis* of a mixture thereof.[73] Complications with herbal treatment were common (91 out of 132 patients) and included burns, skin necrosis, and infection.[73] Secondary bacterial infections with *Staphylococcus aureus* are common in eumycetoma patients, especially in lesions with open sinuses. The outcome of ketoconazole ($n = 102$ patients) therapy could be improved when the secondary *S. aureus* infection was treated with amoxicillin/ clavulanic acid

($n = 142$ patients).[74] Treatment with ciprofloxacin ($n = 93$ patients) did not show this effect.[74]

However, the prescription of next generation triazoles was not the only method to improve the current therapy for eumycetoma. Eumycetoma is often treated with a combination of antifungal therapy and surgery out of necessity as the lesion still persists after prolonged medical treatment. During the surgical procedure, the operative field is irrigated first with normal saline, povidone iodine solution, and hydrogen peroxide to remove and destroy any missed grains and hyphae.[55] However, other sterilizing solutions such as taurolidine ringer solution and chlorhexidine are also possible to be used. Looking at minimal fungicidal concentrations, 1% H_2O_2 seemed most effective, but the killing time needed was 2h,[75] A lower dilution of 1% Povidone-iodine was needed to kill *M. mycetomatis,* but the killing time was <5 min.[75] An *in vivo* comparison has not been done.

Currently, the treatment of eumycetoma is based on personal experience or on the few published case reports or case series. The first ever double-blind clinical trial on eumycetoma treatment is currently conducted at the MRC. The study is sponsored by DNDi, Geneva, in collaboration with Eisai Ltd., Japan. The trial is titled "A randomised, double-blind phase II proof-of-concept superiority trial of fosravuconazole 200 mg or 300 mg weekly dose versus itraconazole 400 mg daily dose, all three arms in combination with surgery, in patients with eumycetoma in Sudan."

Presently, there is no preventive or control measurements in mycetoma, and most patients present late with advanced disease where massive surgical excisions or amputation will be the only available treatment modalities. Most of the mycetoma patients are of low socioeconomic status, low health education and reside in remote areas with poor health facilities and hence the late presentation. To overcome all these constraints, the MRC in Sudan has established a regional mycetoma center in one of the endemic villages. The objectives of the center are early case detection, medical and surgical management of patients locally at the village, conducting community-based services and health education activities. The regional center is also a field research unit for the MRC. A considerable number of patients were seen during 2016; most of them underwent ultrasound examination, and 220 patients underwent surgery in the center.

Summary

Since the first meeting in Geneva in 2013, we were able to raise awareness for mycetoma. Mycetoma is now recognized as a neglected tropical neglected disease. Furthermore, mycetoma is no longer neglected amongst the stakeholders including DNDi, WHO, and others. The journey to recognition has raised world-wide interest and stimulated many of our working group members also to report more extensively on their experience in mycetoma. The number of cases reported to literature doubled, novel diagnostic tools have been developed and antifungal agents have been tested not only against the most common causative agent *M. mycetomatis* but also against other fungal causative agents.

Recognition by the WHO as a neglected tropical disease was only the starting point, and efforts should be made to keep the momentum. In terms of epidemiology, we need to collect basic epidemiological data to estimate the prevalence and incidence in endemic areas and any help to do this is welcome. Together we should prospectively collect clinical data on all mycetoma cases occurring worldwide. To assess which species are involved in mycetoma, we now should no longer rely solely on morphological identification but always include molecular identification especially in epidemiological surveys. We also should study the mode of transmission and translate the outcome of these studies to intervention studies leading to control.

As of now, genomes have been published for the most common causative agents of mycetoma including *N. brasiliensis*,[76,77] *S. somaliensis*,[78] *A. madurae*,[79] *M. mycetomatis*,[80] and *N. mackinnoni*.[81] We should use these genomes to gain insight in mycetoma pathogenesis but also in the development of novel diagnostic tools. Validation of isothermal amplification tools and PCR-based techniques should be done in clinical settings and compared to conventional techniques. One of the large drawbacks is that the current diagnostic tools are not suitable for field use and will not allow proper species identification in resource-limited laboratories. Efforts should be made in the development of point-of-care tests that can at least identify the most common causative agents. Since there are so many different causative agents for this disease, working group members should combine their forces and make a joint effort out of this.

The first double-blind and randomized study for mycetoma will give us for the first time the proper data on treatment response in a large group of eumycetoma patients. But this study focuses only on one class of antifungal agents, the azoles. What happens if resistance will occur? We need to establish a drug discovery pipeline to identify novel drugs with a mode of action different from the azoles. If these drugs are identified we also need to study the possibility of combining drugs with different modes of action. Could combination treatment shorten the duration of treatment? Could it prevent drug resistance? Are these treatments effective in *M. mycetomatis* but also in the other fungal causative agents?

In short, there are still many unsolved questions that need to be addressed to design effective control programs in each region.

Declaration of interest

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

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