Madurella mycetomatis grains within a eumycetoma lesion are clonal

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Abstract

Eumycetoma is a neglected tropical infection of the subcutaneous tissue, characterized by tumor-like lesions and most commonly caused by the fungus Madurella mycetomatis. In the tissue, M. mycetomatis organizes itself in grains, and within a single lesion, thousands of grains can be present. The current hypothesis is that all these grains originate from a single causative agent, however, this hypothesis was never proven. Here, we used our recently developed MynSTR assay, a highly discriminative typing method, to determine the genotypes of multiple grains within a single lesion. Multiple grains from surgical lesions obtained from 11 patients were isolated and genotyped using the MynSTR panel. Within a single lesion, all tested grains shared the same genotype. Only in one single grain from one patient, a difference of one repeat unit in one MynSTR marker was noted relatively to the other grains from that patient. We conclude that within these lesions the grains originate from a single clone and that the inherent unstable nature of the microsatellite markers may lead to small genotypic differences.

Lay Abstract

In lesions of the implantation mycosis mycetoma many Madurella mycetomatis grains are noted. It was unknown if grains arose after implantation of a single isolate or a mixture of genetically diverse isolates. By typing the mycetoma grains we showed that all grains within a single lesion were clonal and originated from a single isolate.

Keywords: mycetoma lesions, MynSTR assay, clonality, Madurella mycetomatis, black grains.

Introduction

Eumycetoma is a neglected tropical infection endemic in tropical and subtropical areas and is caused by several fungi. It is characterized by the formation of tumor-like lesions with multiple sinuses discharging grains.1,2 Many fungi can cause eumycetoma, however, Madurella mycetomatis is the most common causative agent.3–6 The transmission mode of eumycetoma is not clear, but it is hypothesized that the causative agent is introduced in the subcutaneous tissue via a traumatic inoculation.7 Once inside the lesion, the fungus will form a protective structure called a fungal grain, in which the fungus itself is embedded.8 Madurella mycetomatis forms black grains, which can be seen inside the tissue (Fig. 1), and in large lesions, thousands of grains can be present.9 The current hypothesis is that M. mycetomatis enters the site of infection via a small trauma, however, it is not known if only a single fungus or a mixture of fungi is introduced into the subcutaneous tissue during this trauma. The fungi that survive inside the host tissue will start to divide and grains will form. When the lesion progresses, multiple pockets with numerous grains are noted in the tissue.10 If the grains within these pockets did arise from the introduction of a single fungus, all grains within a lesion should be genetically identical. However, if the grains within these pockets did arise from the introduction of a mixture of fungi, then mixed genotypes would be encountered in a single lesion. We recently developed a short tandem repeat (STR) assay that can be used to study the genetic variation in M. mycetomatis (MynSTR). This assay consists of 11 STR markers specific for M. mycetomatis and when applied to a collection of 120 isolates assumed to be epidemiologically unrelated, resulted in a Simpson’s diversity index of 0.997.11 It can be performed on DNA isolated from cultures, but due to its high specificity and reproducibility, also on DNA isolated from M. mycetomatis grains embedded within clinical material.

In this study, we applied the MynSTR assay to DNA isolated from grains from single lesions to determine whether the grains are clonal and derived from the introduction of a single M. mycetomatis strain at the site of infection or if they are of a mixed genotype and derived from multiple genetically diverse M. mycetomatis strains. Since individual M. mycetomatis isolates differ in antifungal susceptibility towards itraconazole, the current drug of choice, it is important to know if the grains in a single lesion are clonal or did arise from multiple strains introduced into the wound simultaneously.12

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Table 1. Demographic data of 11 Eumycetoma patients from which grains were sampled.

| Patient | Site     | No. of grains | Gender | Duration of infection (years) | Location       | Duration of treatment at the time of surgery (months) | Duration of treatment at the time of writing the manuscript (years) | Clinical outcome |
|---------|----------|---------------|--------|-------------------------------|----------------|-----------------------------------------------------|-------------------------------------------------------------------|------------------|
| 1       | Left foot| 10            | Male   | 3                             | Sennar          | 6                                                   | 2                                                                 | On treatment     |
| 2       | Left foot| 9             | Male   | 4                             | Sennar          | 6                                                   | 3                                                                 | Cured            |
| 3       | Left foot| 5             | Male   | 3                             | Sennar          | 6                                                   | 2                                                                 | On treatment     |
| 4       | Right foot| 7             | Male   | 2                             | Sennar          | 6                                                   | 1                                                                 | On treatment     |
| 5       | Left foot| 5             | Male   | 3                             | White Nile      | 6                                                   | 1.5                                                               | Cured            |
| 6       | Left foot| 5             | Male   | 2                             | White Nile      | 6                                                   | 1                                                                 | Cured            |
| 7       | Left foot| 7             | Male   | 2                             | White Nile      | 6                                                   | 1.5                                                               | Cured            |
| 8       | Left foot| 3             | Male   | 3                             | White Nile      | 6                                                   | 3                                                                 | Cured            |
| 9       | Right foot| 3             | Male   | 4                             | White Nile      | 6                                                   | 1.5                                                               | Cured            |
| 10      | Left foot| 3             | Male   | 5                             | White Nile      | 6                                                   | 3                                                                 | Cured            |
| 11      | Right foot| 5             | Male   | 5                             | White Nile      | 6                                                   | 3                                                                 | Cured            |

No = Number.

Materials and methods

Patients

Eleven Sudanese patients with eumycetoma of the foot who presented at the Mycetoma Research Center, Khartoum, Sudan, were included in this study. All patients were male, five were from the Sennar State and six were from the White Nile state (Table 1). The mean duration of the disease was 3.3 years and ranged from 2 to 5 years. All patients were on itraconazole treatment, the current treatment of choice. The duration of the treatment differed per patients and ranged from 1 to 3 years, with a mean duration of treatment of 2 years (Table 1). As part of the standard mycetoma treatment, after six months of itraconazole therapy, mycetoma lesions are surgically removed. From the removed tissue, grains were sampled from different pockets within a lesion (Fig. 1(A)). This was done under sterile conditions using a different sterile surgical blade, each blade was used once to extract the grains from each pocket. On average, six grains per patient were sampled (range 3 to 11) (Table 1). Informed consent was obtained from each patient preoperatively. The study was approved by the Mycetoma Research Center, Khartoum, Sudan, ethical committee.

DNA isolation

Before DNA isolation, grains were washed with saline (0.90% w/v NaCl) twice. After individual grains were picked from the tissue, DNA from single grains was isolated using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Irvine, California, USA) according to the manufacturer’s instructions with modifications. Metal beads (3 mm Ø) were used instead of ZR BashingBead™ as earlier described to ensure a high yield.13 The DNA quality and quantity were measured using the Nanodrop (Thermo Scientific, USA).

Real-time PCR for optimization of grain DNA for MmySTR genotyping

The MmySTR assay was optimized for use with 1 ng of M. mycetomatis DNA which generates an easy to interpret MmySTR profile. Since mycetoma grains often contain a mixture of M. mycetomatis DNA, bacterial DNA from secondary bacterial infections14 and human DNA, we aimed to standardize the concentration of the M. mycetomatis DNA within the mixed DNA sample before MmySTR genotyping. For this, we used a real-time PCR targeting a M.
mycetomatis specific 123 bp intergenic fragment between the genes MMYC01_203 016 encoding for the transmembrane and coiled-coil domain-containing protein 4 and MMYC01_203 017 encoding for the V-type proton ATPase subunit H to approximate the concentration of M. mycetomatis DNA within the DNA sample. This was done by comparing C_t values of the DNA isolated from grains to those obtained using DNA isolated from a pure M. mycetomatis culture. Briefly, the 20 μl reaction consisted, 0.25 μM of both forward (5′-AAGAGAAGAGGACCGACT-3′) and reverse (5′-GTGGGCTTTCGGTGAGTGAT-3′) primers, 2 μM of SYTO™ 82, and 1 ng of genomic DNA in 1x LightCycler® 480 probes master (Roche Diagnostics). Each sample had a technical replicate. The 40 cycle reaction amplification consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles with denaturation at 95°C for 0.5 s, and annealing/extension at 60°C for 30 s. The C_t values were automatically generated and analyzed with the LightCycler software. The C_t values obtained for grain samples (C_p of sample) were compared to those of a pure culture of M. mycetomatis isolate MM55 (C_p of control). To obtain a DNA concentration in which ~1 ng/μl of M. mycetomatis DNA is present, the following formula was used 2(C_p of control-C_p of sample). In this formula, the efficiency of M. mycetomatis specific PCR was assumed to be 2, C_p control was the average C_p value of 1 ng/μl of DNA isolated from a M. mycetomatis culture and C_p of the sample was the average C_t value of the grain sample. Finally, the normalized DNA concentrations were subsequently used for MmySTR genotyping.

MmySTR genotyping

The DNA samples were typed using MmySTR as previously described. Briefly, in each multicolor-multiplex 25 μl amplification reaction consisted of 0.5 μM concentrations of each specific primer and approximately 1 ng of M. mycetomatis DNA in 1x FastStart™ PCR Master (Roche Diagnostics). The amplification consisted of 35 cycles with 4 min initial denaturation at 94°C, 30 s denaturation at 94°C, 30 s annealing at 55°C, 30 s extension at 72°C, and a final extension of 7 min at 72°C. The obtained PCR products were diluted 1:40 in PCR-grade water and 2 μl of the diluted product was added to 0.1 μl of GeneScan 600 LIZ (Applied Biosystems) size marker and 18 μl of HiDi formamide (Applied Biosystems). The samples were heated at 94°C for 1 min, then 4°C and injected onto an ABI 3730 XL (Applied Biosystems) following the manufacturer’s instructions. The electropherograms were imported into Bionumerics software v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed using the Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA), plug-in. STR numbers were assigned relative to that of the reference sequence of strain MM55 which was included as a control.

Results

From the 11 patients included in this study, on average six (range 3–10) grains were isolated per patient, making a total of 62 grain samples from which DNA was isolated. This DNA was assumed to consist of both fungal DNA and DNA originating from the human host. In an average grain, 2.7 μg (range 0.23–9.75 μg) total DNA was present. To approximate the amount of M. mycetomatis DNA present, a M. mycetomatis specific RT-PCR was performed on the grain DNA and on DNA isolated from a cultured M. mycetomatis isolate. On average, each grain sample was found to have a mean of 0.2 μg of M. mycetomatis DNA (range 0.03–1.59 μg) (Fig. 1B). Low concentrations of M. mycetomatis DNA were observed in grains obtained from patients 1, 4, 5, and 6. More variation in M. mycetomatis DNA concentration was observed in grains obtained from patients 2, 3, 7, 8, 9, 10, and 11 (Fig. 1B). Based on these values, on average, M. mycetomatis DNA made up 10% of the total DNA isolated from the grain, while 90% was likely from human nature. Furthermore, there was no correlation between DNA load and patient outcome as low or high amounts of DNA were more or less the same in the ‘cured’ and ‘on treatment’ groups.

After diluting the grain samples to contain 1 ng/μl M. mycetomatis DNA, MmySTR typing was performed. Based on the MmySTR genotypes obtained, it was evident that the genotypes differed per patient. Ten different genotypes were found among the 11 patients. The genotype from grains obtained from patient 5 and patient 7 was identical (Fig. 2(A)). The genotypes encountered for the grains included in this study differed from those obtained in our previous study11 (Fig. 2(B)).

Per patient, multiple grains obtained from a single lesion were analyzed. The genotypes obtained from grains within a lesion were identical for 10 out of 11 patients (Fig. 2(A)). Only for patient 2, a difference was noted. From the nine grains analyzed for this patient, only one grain differed in a single STR marker. That single grain had 13 repeats for marker 3C, while the other eight grains had 14 repeats for that marker. The genotypes found within a single lesion were identical for all other patients.

Discussion

Using the MmySTR assay, we demonstrated that within a single lesion all grains share the same MmySTR genotype indicating that a single M. mycetomatis isolate is introduced in the lesion during traumatic inoculation. This isolate will grow and give rise to numerous grains within the lesion. Apart from patients 5 and 7, who were infected with a M. mycetomatis isolate sharing the same MmySTR genotype, all the other genotypes appeared genetically distinct, supporting our previous observation that M. mycetomatis is genetically divergent. Furthermore, these new genotypes were novel as they were not observed or identified previously. It is unclear why patients 5 and 7 were infected with genetically similar M. mycetomatis isolates. These patients originated from two different states, but these two states share a common boundary. It could be that they contracted the infection at a common site, however, since the patients were lost to follow up, we could not investigate this further.

From the nine grains analyzed for patient 2, one grain differed in a single STR marker by one repeat, suggesting that in massive lesions instability events can take place. Either during the four years the patient had this lesion or during therapy, this micro-evolution event in STR marker 3C occurred. Variation in the number of tandem repeats can occur by strand-slippage of the DNA polymerase during replication or recombination. Micro-evolution within fungal infections has been reported before and is often at random locations within
the genome. However, mutation rates in tandem repeats are usually a factor of 10 or more frequent than point mutations elsewhere in the genome. For *Aspergillus fumigatus*, a similar short tandem repeat assay (STRAf) was already developed in 2005. In this STRAf assay, similar micro-evolutionary events were also noted in two STR markers. They occurred in a single marker after five generations for two isolates and 36 generations for the third isolate. The other seven markers remained stable within 473 generations. Similar to the micro-evolutionary event we noted in our *Mmy*STR assay, all three micro-evolutionary observed with the STRAf assay occurred in a trinucleotide repeat marker with a relatively high copy number. Both features have been known to affect STR stability. Regarding unit-length, the effect of the length of the repeat is not always clear. Some studies demonstrate that a shorter unit length leads to more instability, while other studies demonstrate that a higher unit length leads to more instability. It is striking that for both *A. fumigatus* and *M. mycetomatis* the trinucleotide repeats were involved in micro-evolutionary events. With regards to the length of the repeat for *A. fumigatus*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Homo sapiens* it was demonstrated that instability occurs more often if the number of repeats is higher. However, for *M. mycetomatis*, marker 3C had 14 repeats before this micro-evolutionary event showing which is shorter compared to the unstable trinucleotide markers in the STRAf assay and also denotes a deletion contrary to STRAf but similar to what was observed in other studies.

Since *Mmy*STR determines genetic variation by analyzing 11 selected STR markers covering only a tiny fraction of the fungal genome, we will not detect all genetic variation occurring between the individual grains. The agreement between *Mmy*STR and Whole Genome Sequencing (WGS) has not been studied for *M. mycetomatis* due to the lack of available genomes. At this moment, only five *M. mycetomatis* genomes are available in the NCBI Genbank. However, for other fungal species such as *Candida auris*, a comparison between STR typing and WGS has been performed and demonstrated that the *C. auris* STR assay consisting of 23 STR markers could differentiate *C. auris* isolates which differed by ≥ 30 single nucleotide polymorphisms (SNPs), per genome. However, when strains only differed by 20 SNPs or less, they would be not be differentiated by the STR assay. This indicates that STR assays could be comparable to WGS.

The treatment of eumycetoma is characterized by recurrences, and it is not clear what the role of antimicrobial resistance is at the start or during treatment. The finding that mycetoma lesions are formed after the introduction of a single *M. mycetomatis* strain suggests that at the start of treatment all individual grains have an equal susceptibility profile for the drug given. This indicates that susceptibility testing can be performed on a single isolate and there is no need to test
multiple isolates from the same lesion. A limitation of the current study is that we sacrificed the complete grain for DNA isolation which prevented us to grow the isolate as well and perform in vitro susceptibility testing. We therefore could not verify that all grains within the lesion would have indeed an equal susceptibility profile. We did demonstrate that micro-evolution within the lesion can occur. For C. albicans it has been demonstrated that this micro-evolution can also occur in the azole target gene ERG11 and in the transcriptional activator of the multidrug efflux transporter CDR. It is, therefore, important to note that micro-evolution can occur during the infection and that the ManySTR assay will not pick up micro-evolutionary events outside the 11 selected STR markers. To identify these events, sequencing of the azole target gene and genes associated with azole resistance in other fungi can be performed by classical Sanger sequencing or by WGS and the rise of resistance can be determined by in vitro susceptibility testing.

Based on the data presented here, we can conclude that a eumycetoma lesion originates from the introduction of a single M. mycetomatis strain in the subcutaneous tissue and that all grains will have the same susceptibility for antifungal therapy. Micro-evolution can occur during the long time before and during treatment and further research is warranted whether this will result in selection of resistance against antifungal drugs. Furthermore, it will be interesting to sample and analyse grains obtained from lesions in different parts of the body in the future.

**Author contributions**

BN: formal analysis, data collection, validation, visualization, writing–original draft preparation and review/editing.
EES, SB, and AHF: patient sampling, original draft preparation and review/editing. MK: writing and review/editing.
CHW: Formal analysis, supervision, validation, visualization, original, review/editing. AV: supervision, validation, visualization, original draft preparation, and review/editing.
WWJvdS: conceptualization, project administration, formal analysis, supervision, validation, visualization, original, draft preparation, and review/editing.

**Declaration of interest**

None.

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