Detection of (1→3)-β-D-Glucan in Eumycetoma Patients

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Mycetoma can be caused by bacteria (actinomycetoma) or fungi (eumycetoma). Here, we demonstrated in 45 eumycetoma patients, 30 actinomycetoma patients, and 30 healthy controls that (1→3)-β-D-glucan detection in serum cannot reliably be used to discriminate between the two types of mycetoma.

Mycetoma is a neglected chronic implantation mycosis, characterized by large tumor-like swellings and located mainly in the extremities. Mycetoma can be caused by more than 56 different pathogens (1), either of bacterial (actinomycetoma) or of fungal (eumycetoma) origin. To identify the causative agent, a deep-seated surgical biopsy is performed and histology, cytology, and culture assays are performed. As mycetoma can be caused by both bacteria and fungi, a rapid distinction between the two is necessary for initiation of proper treatment (2–4). Ideally, a rapid method would dispense with the requirement for a deep-seated surgical biopsy. A rapid diagnostic test, which can be performed in serum, is therefore to be preferred.

(1→3)-β-D-Glucan is a cell wall polysaccharide present in many fungal species. It is secreted during growth and disseminates from the site of infection. It can be detected in sera of patients with different fungal infections (5–9). There are currently no reports published on (1→3)-β-D-glucan detection in eumycetoma patients. In this study, we therefore set out to determine if (1→3)-β-D-glucan levels could be measured in eumycetoma patients and if measuring these levels would enable us to discriminate eumycetoma patients from healthy controls living in regions of endemicity or actinomycetoma patients.

Seventy-five human sera were obtained from Sudanese mycetoma patients (45 patients with eumycetoma and 30 patients with actinomycetoma) seen in the Mycetoma Research Centre in Khartoum, Sudan, and 30 control sera were obtained from healthy volunteers at the blood bank in Khartoum, Sudan. For each patient, age, gender, disease duration, relative size of lesion, and site of infection were recorded. For each control, age and gender were recorded. Lesion size was measured, and lesions with a diameter of <5 cm were defined as small, lesions with a diameter of 5 to 10 cm were defined as moderate, and lesions with a diameter of >10 cm were defined as large. The demographic data for the controls were matched to those for the mycetoma patients, and the data for each group are represented in Table 1. Written informed consent was obtained from patients and controls, according to guidelines from the medical ethical committee at Soba University Hospital, Khartoum, Sudan. The study protocol was approved by this medical ethical committee. Sera were sent to the research laboratories of Associates of Cape Cod, Inc., and (1→3)-β-D-glucan concentrations were determined according to the protocol of the instructions for use of the Fungitell kit. Based on the (1→3)-β-D-glucan values measured, a receiver operating characteristic (ROC) analysis was performed using GraphPad Prism 5.03. The area under the ROC curve was 0.90 (Fig. 1A). With the standard cutoff value of 80 pg/ml, in this population, a sensitivity of 68.2% (95% con-

fidence interval [CI], 52.4 to 81.4%), a specificity of 93.3% (95% CI, 77.9 to 99.2%), and a positive likelihood ratio of 10.2 were found. Increasing the cutoff value to 85 pg/ml had no effect on the sensitivity, but the specificity and the likelihood ratio increased to 96.7% (95% CI, 82.8 to 99.9%) and 20.5, respectively. Therefore, for this population, a cutoff value of 85 pg/ml was deemed appropriate.

The (1→3)-β-D-glucan concentrations measured in sera of eumycetoma and actinomycetoma patients and of healthy controls living in areas of endemicity are shown in Fig. 2A. As is seen in this figure, the median (1→3)-β-D-glucan concentration in the eumycetoma group was 208 pg/ml, which was significantly higher than the median (1→3)-β-D-glucan concentrations in the healthy control group (27.0 pg/ml; $P < 0.0001$ [Mann-Whitney test]) or in actinomycetoma patients (72.5 pg/ml; $P = 0.026$ [Mann-Whitney test]). Although the overall (1→3)-β-D-glucan concentration in sera in the eumycetoma patients was high, still 15 out of 45 patients had (1→3)-β-D-glucan levels below the cutoff value of 85 pg/ml. To determine if the size of the lesion played a role, the patients were grouped according to lesion size (Fig. 2B). As is seen in Fig. 2B, patients with large [median of 314.5 pg/ml of (1→3)-β-D-glucan] mycetoma lesions had overall higher (1→3)-β-D-glucan concentrations in serum than patients with moderate [median of 187 pg/ml (1→3)-β-D-glucan] or small [median of 97.5 pg/ml (1→3)-β-D-glucan] lesions, but this difference was not significant ($P = 0.23$ [Kruskal-Wallis test]).

To determine if the (1→3)-β-D-glucan was also able to discriminate eumycetoma patients from actinomycetoma patients, a second ROC curve, comparing these two groups, was made. As is seen in Fig. 1B, when actinomycetoma patients were used as a control group, the area under the ROC curve decreased to only 0.65 (Fig. 1B). This was due to the fact that 14 out of 30 actinomycetoma patients also had (1→3)-β-D-glucan levels in serum above the cutoff value of 85 pg/ml (Fig. 2A). Four of these patients had

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TABLE 1 Patient demographic and clinical characteristics

Characteristic	Value for:		
	Eumycetoma patients	Actinomycetoma patients	Controls
No. of subjects	45	30	30
Mean age (yr) (range)	29.7 (14–70)	37.7 (11–70)	31.7 (16–58)
No. male/no. female	35/10	24/6	24/6
Mean duration (yr) (range)	6.6 (0.5–20)	9.0 (1–28)	
No. with lesion on:			
Foot	39	30	
Hand	6	0	
No. with lesion size			
Small	14	9	
Moderate	15	10	
Large	16	11	
No. with causative agent			
<i>Madurella mycetomatis</i>	45	0	
<i>Actinomyadura madurae</i>	0	13	
<i>Actinomyadura pelletieri</i>	0	2	
<i>Streptomyces somaliensis</i>	0	15	

(1→3)-β-D-glucan concentrations exceeding 200 pg/ml, and the highest concentration measured in this group was 3,420 pg/ml. These elevated levels of (1→3)-β-D-glucan could be the result of the disease management for actinomycetoma. Actinomycetoma is treated with antibiotic agents, and it is known that in certain antibiotic agents (1→3)-β-D-glucan is detected (10); however, the antibiotics usually given for actinomycetoma (trimethoprim-sulfamethoxazole and amikacin) are not associated with positive reactions in (1→3)-β-D-glucan assays (10). As mycetoma is a subcutaneous disease, many patients use dressings on their lesions. As already demonstrated by others, patients exposed to gauze or sponges during surgery often test false positive in (1→3)-β-D-glucan detection assays (11–13). Another reason could be the causative agents themselves. Certain bacteria have been described to react positively in the Fungitell assay (14, 15). As actinomycetoma in Sudan is usually caused by either *Actinomyadura madurae*, *Actinomyadura pelletieri*, or *Streptomyces somaliensis*, we evaluated

if these elevated levels were related to the bacterial causative agent (Fig. 2C). We also grew seven *Streptomyces somaliensis* isolates in RPMI 1640 medium supplemented with L-glutamine and morpholinepropanesulfonic acid (MOPS) (Lonza, Breda, The Netherlands) and incubated for 3 weeks at room temperature (RT). Nonincubated medium was used as a control. The median (1→3)-β-D-glucan concentrations after 6 days, 2 weeks, and 3 weeks were all below the cutoff values, indicating that no (1→3)-β-D-glucan is formed by *S. somaliensis* when cultured in RPMI 1640 medium at room temperature. This finding does not exclude the possibility that (1→3)-β-D-glucan is not formed *in vivo*. First, *in vivo* grains are formed, which are not produced under *in vitro* culture conditions. Second, RPMI medium is a synthetic medium which might not resemble the nutrients available in the subcutaneous lesions, and therefore, different sugar components might be formed *in vitro* relative to those formed *in vivo*. Third, *S. somaliensis* was cultured at room temperature, not body temperature, which

FIG 1 ROC curves. (A) Receiver operating characteristic (ROC) curve analysis of (1→3)-β-D-glucan levels in sera of eumycetoma patients compared to those in sera of healthy controls living in areas of endemicity. In the right lower corner of the graph, the calculated area under the curve (AUC) is shown. (B) ROC analysis of (1→3)-β-D-glucan levels in sera of eumycetoma patients compared to those in sera of actinomycetoma patients. In the right lower corner of the graph, the calculated AUC is shown.

FIG 2 Measured (1→3)-β-D-glucan levels in sera and culture supernatants. (A) (1→3)-β-D-Glucan in sera of eumycetoma patients, actinomycetoma patients, and healthy controls living in areas of endemicity. Each dot indicates the mean (1→3)-β-D-glucan concentration for each patient, and the horizontal lines represent the median (1→3)-β-D-glucan concentrations for each group. The red line represents the cutoff value as determined from the ROC curve in Fig. 1A. (B) (1→3)-β-D-Glucan in sera of eumycetoma patients with large, moderate, or small mycetoma lesions. Each dot indicates the mean (1→3)-β-D-glucan concentration for each patient, and the horizontal lines represent the median (1→3)-β-D-glucan concentrations for each group. The red line represents the cutoff value as determined from the ROC curve in Fig. 1A. (C) (1→3)-β-D-Glucan in sera of actinomycetoma patients infected with *Actinomyces madurae* (AM), *Actinomyces pelletieri* (AP), or *Streptomyces somaliensis* (SS). (D) Culture supernatants of *Streptomyces somaliensis* grown in colorless RPMI medium after 6 days, 2 weeks, and 3 weeks. As a control, colorless RPMI medium was taken. Each dot indicates the mean (1→3)-β-D-glucan concentration for each patient or culture supernatant, and the horizontal lines represent the median (1→3)-β-D-glucan concentrations for each group. The red line represents the cutoff value as determined from the ROC curve in Fig. 1A.

might also influence the growth characteristics. Although we did not demonstrate the production of (1→3)-β-D-glucan in *S. somaliensis* *in vitro*, for *Nocardia* spp. it has been reported. *Nocardia* spp. are implicated as actinomycetoma-causative agents. Accordingly, these results suggest that the Fungitell assay may not be suitable for discrimination of these types of actinomycetoma from eumycetoma.

Furthermore, mycetoma patients usually also seek medical advice with traditional healers (16). In Sudan, 42.4% of all eumycetoma patients used herbal medicine (16); for actinomycetoma, no numbers are known, but it is envisioned the numbers are similar. These herbs are not sterilized before topical administration, and superinfections are common (16). One could imagine that some fungi will be present in these ointments as well, which could cause false-positivity in a (1→3)-β-D-glucan detection assay. Furthermore, plants themselves also contain small amounts of (1→3)-β-D-glucan, named callose (17), which in its turn could interfere with the (1→3)-β-D-glucan detection assay.

In conclusion, it is possible to measure (1→3)-β-D-glucan levels in sera of eumycetoma patients. As (1→3)-β-D-glucan levels were detected in a large proportion of the actinomycetoma patients, the Fungitell assay is probably not suitable for discrimination of eumycetoma patients from actinomycetoma patients. Further studies are needed to determine if this assay can be used to

determine therapy efficacy when patients are treated with antifungal agents.

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