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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1038/JID.2015.369.

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Sub6 (Tri r 2), an Onychomycosis Marker Revealed by Proteomics Analysis of *Trichophyton rubrum* Secreted Proteins in Patient Nail Samples

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TO THE EDITOR

Onychomycosis, the most prevalent nail disease, is mainly caused by two dermatophyte species, *Trichophyton rubrum* and *Trichophyton interdigitale*, with a frequency in the range of 80% and 20%, respectively (Monod et al., 2002). To determine if the proteases secreted by *T. rubrum* in vitro during keratin digestion were involved in nail degradation, we investigated the fungus secretome in onychomycosis by proteomics analysis.

In a first experiment, mass spectrometry analyses were performed using a pool of extracts from 12 donors infected by *T. rubrum* as described in the Supplementary Materials and Methods online. Patient consent for experiments was not required because French laws consider human tissue leftover from surgery as discarded material. Proteins were extracted from each sample using a nonionic acid labile surfactant (ALS-400). The secretion of the following four secreted proteases of *T. rubrum* proteins extracted from nail beds was identified after subsequent SDS-PAGE separation and *in-gel* digestion coupled to mass spectrometry analysis: subtilisin-like protease 6 (Sub6, Q9UW97), subtilisin-like protease 7 (Sub7, Q8NID9), dipeptidyl-peptidase 5 (DppV, Q9UW98), and leucine aminopeptidase 2 (Lap2, Q5QHG6) (Table 1 and Supplementary Table S1 online). In particular, 12 unique peptides were found for Sub6, suggesting that this protease was abundantly secreted during nail infection. No *T. rubrum* proteins were detected in the collected samples from abnormal nails with trauma but without fungal infection (data not shown). No additional proteins were identified from a sequential second extract, suggesting that all soluble secreted proteins were already extracted in the first extraction (Supplementary Materials online). The high amount of Sub6 secreted by *T. rubrum* in onychomycosis and the presence of DppV were confirmed by Western blot analysis and by a shotgun protein identification experiment in SDS-PAGE gels using the same pooled extract for mass spectrometry analysis and specific antisera (Supplementary Table S2 and Figure S1 online). Surprisingly, most proteases secreted by the fungus during its in vitro growth in a keratin medium including subtilisin-like protease 3 (Sub3, B8XGQ6), subtilisin-like protease 4 (Sub4, A7UKV6), leucine aminopeptidase 1 (Lap1, Q5QHG5), dipeptidyl peptidase 4 (DppIV, Q5J6J3), and metallocarboxypeptidase (M14A, A6XGK3) (Giddey et al., 2007; Zaugg et al., 2008) were not detected either by mass spectrometry or by Western blot analysis (Supplementary Table S3 and Figure S1 online).

Abbreviations: MRM, multiple reaction monitoring

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Table 1. *Trichophyton rubrum* proteins identified by LC-MS/MS in infected nail beds samples

Accession	Description	Score	Coverage	No. of proteins	No. of unique peptides	Band
Q9UW97	Subtilisin-like protease 6	205.96	46.12	1	12	1, 2, 3, 4, 5, 6, 7
F2SX42	Polyubiquitin	119.92	70.74	1	0	
F2SEJ4	Ubiquitin	62.22	26.8	1	0	
Q6QUM1	Actin	29.27	18.4	1	0	
Q8NID9	Subtilisin-like protease 7	18.22	22	1	4	5
F2SH28	Enolase	8.34	12.56	1	2	
Q9UW98	Dipeptidyl-peptidase 5	6.48	7.16	1	3	1
F2SUU6	Glucan 1,3-beta-glucosidase	6.24	18.16	1	3	
F2SLY0	Putative uncharacterized protein	6.13	34.25	1	3	
F2SZI9	Beta-glucosidase	4.59	7.79	1	2	
F2SN23	Superoxide dismutase [Cu-Zn]	3.95	25.32	1	2	
F2SU38	Hsp70-like protein	2.26	6.88	1	1	
Q5QHG6	Leucine aminopeptidase 2			1	3	3

Proteins were extracted from infected nail beds samples using nonionic surfactant ALS-400. Proteins were separated by SDS-PAGE, and then digested using trypsin before peptide identification by mass spectrometry (Supplementary Table S1). Proteins of *T. rubrum* were identified in the protein grouping mode. Similarly proteases of *T. rubrum* proteins were also identified by mass spectrometry analysis after SDS-PAGE from pieces of gel corresponding to positive immunostaining observed in Western blot analysis as indicated in Supplementary Figure S1. In the right column, the numbers of bands in which the proteases were identified are indicated. Lap2 was identified by SDS-PAGE/LC/MS/MS in the second analysis coupled to Western blot. Twelve peptides of Sub6 could be identified by LC/MS/MS after separation of proteins using SDS-PAGE with an Orbitrap velos (Thermo Scientific, Bremen, Germany) as a mass spectrometer. In crude extracts from infected nail samples, only four Sub6 peptides could be identified with high confidence without extensive sample preparation and SDS-PAGE using TripleTOF 5600 System (ABSciex, Redwood Shores, Canada), Orbitrap EliteTM (Thermo Scientific, Bremen, Germany), and MaXis impact (Bruker, Bremen, Germany) mass spectrometers (data not shown). No additional proteins of *T. rubrum* were identified from crude extracts without SDS-PAGE separation.

Abbreviations: LC, liquid chromatography; MS, mass spectrometry.

Table 2. Detection of *Trichophyton rubrum* Sub6 in clinical samples by multiple reaction monitoring (MRM)

Mycology positive	Sub6 positive (total sample)
Trichophyton rubrum	98 (108)
Trichophyton rubrum (+ melanine, black pigmentation)	3 (3)
Trichophyton rubrum (africanis)	0 (1)
Trichophyton rubrum (+ Scopulariopsis brevicaulis)	0 (1)
Trichophyton rubrum (+ pyocyanique)	1 (1)
Trichophyton rubrum (Mycelium observed with negative mycology)	3 (4)
	105 (118)
Trichophyton mentagrophytes var. interdigitalis	0 (19)
Trichophyton soudanense	0 (1)
Epidermophyton floccosum	0 (1)
Mycology positive (molds and yeasts)	
Scytalidium dimidiatum	0 (6)
Scytalidium hyalinum	0 (2)
Fusarium	0 (4)
Aspergillus	0 (6)
Scopulariopsis brevicaulis	0 (2)
Acremonium sp	0 (1)
Candida albicans	0 (3)
	0 (24)
Mycology negative	
Psoriasis Onychopathy	0 (2)
Onychogryphosis	0 (1)
Trauma	0 (10)
Others	1 (42)
	1 (55)

Quantifications of Sub6 across 218 clinical samples using an MRM assay including samples infected by *T. rubrum,* negative controls and other nail diseases (for details see Supplementary Table S5).

We developed multiple reaction monitoring (MRM) assays to quantify *T. rubrum* Sub3, Sub4, Sub6, Sub7, DppIV, DppV, Lap1, Lap2, and M14A directly from nail crude protein extracts as described in the Supplementary Material. MRM is a powerful method for sensitive quantitative measurement of target proteins (Castro-Gamero et al., 2014; Keshishian et al., 2007). Sub6 was detected in all infected nail bed samples but not in the control samples (trauma) or in the blank sample (heavy peptides only). Sub7 and DppV were detected in few samples (Supplementary Table S4 online). This may be related to the abundance and/or the stability of these proteins. Lap2 was detected rarely and at very low intensity in agreement with the faint detection of Lap2 using Western blot analysis, suggesting that Lap2 is discreetly produced in onychomycoses (Supplementary Table S2 and Figure S1). No other proteases secreted by the fungus during its growth in vitro in a keratin medium (i.e., Sub3, Sub4, Mep3, Mep4, Lap1, DppIV, and M14A) could be detected in the protein extracts from infected human nails despite the sensitivity of the method and therefore did not appear to be involved during the establishment of

onychomycosis. Quantifications of Sub6, Sub7, and DppV were subsequently performed across 218 clinical samples using MRM assay including negative controls and other nail diseases (Supplementary Table S5 online). Sub6 was detected in almost all samples infected by *T. rubrum* (105 of 118 samples) with one or more peptides, but not in the other samples including nail trauma, psoriasis, and nail beds infected by other pathogens (molds and yeasts) (Table 2). However, a restricted number of samples (13%) identified as positive after mycology culture and/or direct observation were not identified as positive in the MRM assay. Four of 118 of these samples after extraction from nail beds showed a very low concentration of proteins—less than 0.08 mg/ml—which may explain the negative response in the MRM assay. In addition, three samples (mycological culture negative) were identified as positive by MRM and direct mycological examination. The comparison between the clinical evaluation from direct mycological examination and culture results and our mass-spectrometry-based assay showed a good correspondence, as indicated by the high scores of the sensitivity (0.89) and the specificity (0.99) of the assay. Positive and negative predictive values showed important scores: 0.99 and 0.88, respectively. From these results, Sub6 can be considered as a marker of *T. rubrum* nail infection. The identification and quantification of Sub6-specific peptides using MRM were restricted to *T. rubrum* (Table 2). Putative *T. interdigitale* Sub6 could not be identified using this MRM assay because the amino acid sequences of the peptides used for MRM are different in the *T. interdigitale* corresponding peptides (Supplementary Table S6 online).

The gene encoding Sub6 was previously revealed by microarray analysis to be the most upregulated protease gene during skin infection in guinea pigs with the dermatophyte *Arthroderma benhamiae* (Staib et al., 2010). As in the present investigations on onychomycosis, none of the *A. benhamiae* genes encoding specific endo- and exoproteases involved in keratin digestion in vitro was found to be upregulated during skin infection. In contrast to *T. rubrum*, which is anthropophilic, *A. benhamiae* is zoophilic and causes highly inflammatory cutaneous infections in humans and rodents. As a general conclusion, the proteases secreted in vitro during protein degradation and in vivo during infection are different whatever the dermatophyte species and the tinea.

Sub6 and DppV were first described as the major allergens Tri r 2 and Tri r 4, respectively, in *T. rubrum* (Woodfolk, 2005; Woodfolk et al., 1998). These antigens were found to induce dual immune responses and elicit either immediate hypersensitivity or delayed-type hypersensitivity skin test reactions in different individuals. Exposure to *Trichophyton* proteins may result in bronchial sensitization and symptomatic asthma that can be controlled with systemic antifungal therapy (Ward et al., 1989, 1999; Woodfolk, 2005; Woodfolk et al., 1998). There are also eczematous skin reactions to dermatophytes that are distant of the area of the dermatophytes. These skin reactions, called dermatophytids, are generally reported in patients with tinea pedis, most often with *T. interdigitale* but also with *T. rubrum* (Ilkit et al., 2012; Veien et al., 1994). The dermatophytids go away once the dermatophyte infection has been cured. In cases of eczematous skin reaction of unknown origin, it could be relevant to perform skin test reactions using both Sub6 and DppV antigens. A positive reaction could be indicative of a nondetected dermatophyte infection and suggest further clinical investigations for antifungal treatment.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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