Visualising virulence factors: *Trichophyton benhamiae* subtilisins demonstrated in a guinea pig skin ex vivo model

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**Abstract**

**Background:** Dermatophytoses rank among the most frequent communicable diseases in humans, and the zoonotic transmission is increasing. The zoophilic dermatophyte *Trichophyton (T.) benhamiae* is nowadays one of the main causes of *tinea faciei et corporis* in children. However, scientific data on molecular pathomechanisms and specific virulence factors enabling this ubiquitous occurrence are scarce.

**Objectives:** To study tissue invasion and the expression of important virulence factors of *T. benhamiae*, isolates that were recovered from two groups of hosts (humans vs. guinea pigs (GP)) using an ex vivo skin model.

**Methods:** After confirmation of species identity by ITS sequencing, CFU suspensions of dermatophyte isolates (*n* = 20) were applied to the skin infection model and cultured. Employing specific immunofluorescence staining techniques, the expression of subtilisin 3 and 6 and metallocarboxypeptidase A was analysed. The general mode of invasion was explored. Results were compared with biopsies of naturally infected GP.

**Results:** All isolates were successfully recovered and proliferated well after application to the infection model. Progressive invasion of hyphae through all skin structures and destruction of explants were observed with early events being comparable to natural infection. An increasing expression of the examined virulence factors towards the end of culture was noticed but no difference between the two groups of isolates.

**Conclusions:** For the first time, important in vivo markers of dermatophytosis were visualised immunohistochemically in an ex vivo skin infection model and in skin biopsies of GP naturally infected with *T. benhamiae*. More research on the underlying pathomechanisms of dermatophyte infection is urgently needed.

**Keywords**
ex vivo model, guinea pig skin explants, immunofluorescence, metallocarboxypeptidase A, subtilisins, Trichophyton benhamiae, virulence factors, zoonotic dermatophytes
### INTRODUCTION

Fungal infections of skin, nail and hair caused by dermatophytes are common worldwide and represent a growing health burden in animals and man. The high prevalence of dermatophytoses is connected to the dermatophytes’ extraordinary ability to degrade keratins. These insoluble fibrous proteins represent major constituents of the aforementioned host structures and are degraded into short peptides and free amino acids to be used as nutrients. The thereto necessary proteolytic activity is exerted through synergistically acting endo- and exoproteases secreted by dermatophytes. However, an ‘ecological switching’, that is a differential expression of these secreted proteases depending on the host species or experimental system, was noticed. For Trichophyton (T.) spp., major endoproteases expressed during in vitro growth were subtilisins (Sub) 3 and 4 and fungyalins Mep 3 and 4; major exoproteases were aminopeptidases Lap1 and 2, dipeptidyl-peptidases DppIV and DppV and metallocarboxypeptidase A (MCPA). In contrast, the most abundant protease found during experimental animal infection was Sub6. It was also found in numerous patient samples of dermatophyte nail infections but not with trauma or non-dermatophyte infections. Hence, Sub6 is considered a robust marker of in vivo trichophytosis and onychomycosis caused by Trichophyton spp.

The aforementioned findings suggest distinct functions for different proteases of the same protein family during in vitro growth and in vivo infection and demand for further research integrating results from different experimental approaches.

Recently, we developed an ex vivo infection model based on guinea pig skin explants (GPSE) for the investigation of dermatophytoses induced by T. benhamiae. Ex vivo models comprise the physiological 3D microenvironment with crucial features, for example hair follicles, which is an important advantage compared with other in vitro or (2D) cell culture systems.

The zoonotic dermatophyte T. benhamiae was only recently categorised as an emerging pathogen due to frequent misdiagnosis and increased incidences. Its main reservoir and the most common source of human infection are guinea pigs (GP), which are often held as household pets. Asymptomatic carriage (mainly GP) but also a wide range of symptoms (erythematous eczema, itch, scales, crusts, alopecia and scars), immunologic responses and localisations of tinea are described for both companion animals and human patients.

Since the aforementioned experimental GPSE set-up was proven a suitable model for the early stages of T. benhamiae skin infection, it was now employed to analyse the expression of important virulence factors on protein level. Therefore, Trichophyton isolates derived from two distinct groups of hosts (human patients vs. infected GP) were applied, immunofluorescence (IF) stainings were carried out, and the obtained expression patterns were semi-quantitatively evaluated. Additionally, the general mode of fungal invasion was examined and compared with skin biopsies of naturally infected GP.

### ANIMALS, MATERIALS AND METHODS

#### 2.1 Fungal isolates and infection experiments

Isolates of T. benhamiae were recovered from human patients (n = 10) and infected GP (n = 10). Species identity was analysed by sequencing of the internal transcribed spacer (ITS) region of the fungal rDNA from cultures grown on Sabouraud-Dextrose Agar (4%; Sifin Diagnostics GmbH; 14 days, 28°C). Total DNA was extracted according to the manufacturer’s instructions of the QIAamp® DNA Mini Kit (Qiagen) with an additional overnight Proteinase K digestion at 56°C and 600 rpm agitation. The ITS region was amplified using the universal primers LSU266 and V9D (for primer sequences and thermal profile see ref.16) and the Red HS Taq Master Mix (Biozym). PCR products were purified with the QIAquick® PCR Purification Kit (Qiagen), and Sanger sequencing was performed with Microsynth Seqlab. Dermatophytes were identified by similarity search using the Basic Local Alignment Search Tool (BLASTn; https://blast.ncbi.nlm.nih.gov/Blast), and their sequences were edited with Chromas 2.6.6 software (Technelysium, South Brisbane, Australia). Alignment and a phylogenetic analyses with isolates of T. mentagrophytes and T. interdigitale as outgroups were conducted in MEGA X.

Preparation of conidia suspensions for inoculation and GPSE, and infection experiments were carried out as previously described. Briefly, skin explants were prepared from the flank region of disinfected and clipped GP after euthanasia with state approval and in accordance with the ethical policies of the journal. Explants were transferred to cell culture inserts, supplied with growth medium and directly inoculated with 1 × 10^3 CFU in 2 µL PBS of one of the above-mentioned T. benhamiae isolates. A T. mentagrophytes isolate was prepared and employed equally; 1 × 10^3 cells of Geotrichum candidum being a yeast-like fungus were counted and directly applied. Inoculated GPSE and controls were incubated for 10 days at 30°C, 5% CO₂ and 95% relative humidity. Fixation in parafomaldehyde (4%) and paraffin-embedding of samples according to standard protocols ensued at days 3, 5, 7 and 10.

#### 2.2 Histological and IF stainings

Histological analyses of infected GPSE were conducted using 1-µm thin skin sections subjected to the PAS reaction (standard protocols) and an upright Olympus BX 51 microscope (Olympus Deutschland GmbH). Skin biopsies of naturally infected GP were obtained from feed animals from the Zoo Leipzig after euthanasia; skin sections thereof were prepared and evaluated identically.

To visualise Trichophyton isolates in selected samples, a DyLight 594-coupled anti-Trichophyton antibody was produced and applied as previously described (note: different fluorophores). To assess hyphal invasion of GPSE, double IF stainings of the desmosomal cadherin desmoglein-1 (Dsg1) and fungal elements were carried out in selected samples. Therefore, dewaxed and
rehydrated GPSE sections were subjected to heat-induced epitope retrieval in Citrate Buffer (pH 6, 10 x Antigen Retriever [Merck KGaA, Darmstadt, Germany], 20 minutes, steam cooker) and subsequently cooled to room temperature (RT; 20 minutes). After permeabilisation (0.5% Triton X-100 [AppliChem GmbH, Darmstadt, Germany] in phosphate-buffered saline [PBS], 5 minutes, RT), unspecific binding sites were blocked using 5% normal goat serum in PBS (Jackson ImmunoResearch by Dianova GmbH; 30 minutes, RT). Then, the sections were incubated with an anti-Dsg1 antibody overnight (cat. no. MABT118 [Merck], 1:50 in PBS, 4°C). Three washing steps with PBS ensued; subsequently, a DyLight 488-coupled secondary goat anti-mouse antibody (1:500 in PBS with 0.001% bisbenzimide 33342 trihydrochloride [Hoechst, Merck]) was applied (4 hours, RT). The sections were washed with PBS and incubated with the anti-Trichophyton antibody as previously mentioned (4 hours, RT).11

The expression of the virulence factors Sub3, Sub6 and MCPA was assessed immunohistochemically using customised anti-peptide antibodies and a modified catalysed reporter deposition (CARD) detection system.21 Peptide synthesis and rabbit immunisation were carried out by BioGenes GmbH (Berlin, Germany; Sub3: rvshkapgnkdfvy (131-144), Sub6: larvgskqaggtty (138-151), MCPA: r/wrknqpmnpr (233-247)). Antibodies were isolated from the obtained serum by affinity chromatography using peptide-sepharose according to the manufacturer’s instructions (CNBr-Sepharose 4B; GE Healthcare Company; instructions 71-7086-00 AF). Afterwards, horseradish peroxidase (HRP; Roche, Mannheim, Germany) was covalently coupled to these antibodies as described elsewhere.22 Fluorescein tyramide serving as HRP substrate was prepared according to Hopman and colleagues.23 Endogenous peroxidase present in dewaxed and rehydrated skin sections was blocked by incubation in 0.3% H2O2 and 0.1% NaN3 in 100 mmol/L Tris-HCl buffer (pH 8, 1 hour, RT). Afterwards, sections were washed and enzymatically digested for 1 hour (Sub3, MCPA) or 3 hours (Sub6), respectively, at 37°C with 10 mg/mL lysozyme (Merck; in 50 mmol/L Tris-HCl, pH 9 and 20 mmol/L EDTA). Subsequently, the respective HRP-coupled antibody was applied (1 µg/mL in PBS with 5% normal rabbit serum and 0.05% Tween-20, 1 hour, RT). After sequential washing in tap water, 0.9% NaCl with 0.05% Tween-20 and aqua dest., incubation in 4 µg/mL tyramide-FITC in amplification buffer followed (50 mmol/L imidazole/Tris-buffer, pH 7.6, 2 mol/L NaCl, 0.1% blocking reagent [cat. no. 11096176001; Roche by Merck], 0.0015% H2O2; 15 minutes in the dark). Another washing step and the nuclear counterstain with Hoechst ensued as outlined above. All sections were screened and documented using a Nikon Eclipse Ni microscope equipped with a ProRes CF cool camera and the ProRes Capture Pro 2.8.8 software (all from Jenoptik, Jena, Germany). The expression of the virulence factors was assessed semiquantitatively according to the following scoring system: 0 — no signal, 1 — intermediate staining, 2 — intense staining (analysis was drawn from photographs taken uniformly at an exposure time of 1 s and defined filter sets for Sub6 and Sub3/MCPA, respectively).

### 2.3 Statistics

Data were analysed using SigmaStat 2.03 and SigmaPlot 7.0 software (Systat Software GmbH, Erkrath, Germany). The Mann-Whitney U test was employed to detect statistically significant differences between the two groups of isolates and time points, respectively. P-values of <0.05 (*; **0.05<P<0.02) were considered significant, and results were presented as mean ± SD.

### 3 RESULTS

All of the 20 recovered dermatophytes were identified as *T. benhamiae* (identical to GenBank accession nos. KU257463.1 (white colony phenotype (w), n = 1) and MF614429.1 (yellow colony phenotype (y), n = 19)). The phylogenetic analysis included all isolates used during this study and closely related *Trichophyton* spp. (in total 29 sequences). The evaluated species form distinct and very robust clusters (bootstrap values all >77%). All *T. benhamiae* isolates were found in one clade, which was subdivided according to their colony phenotypes; the different origin of isolation was not reflected.

Figure 1 summarises the growth kinetics of different *Trichophyton* spp. (Figure 1A-C), details the interaction of hyphae and epidermal keratinocytes (Figure 1D) and depicts the striking growth differences between control isolates (Figure 1E,F).

Initially, fungal elements of all *Trichophyton* isolates were mainly found in the epidermis (Figure 1A). By no later than d7, hyphae were found in the dermis and all skin structures were destroyed. Digestion of hair remnants (Figure 1B) and sporulation (Figure 1C) were frequently observed by the end of culture. A preference for intra- or intercellular invasion of the skin could not be determined since fungal elements were found inside cells and also in-between cells, that is, co-localised to Dsg1-stained structures (Figure 1D). An isolate of the non-dermatophyte *Geotrichum candidum* served as non-invasive control (Figure 1E): this geophilic fungus proliferated well in culture but did not invade the tissue. The observed morphological alterations of GPSE (acantholysis, pyknotic nuclei) were common changes as expected during long-term tissue culture.11 The applied *T. mentagrophytes* isolate formed a massive mycelium on top of and around GPSE (Figure 1F); invasion and destruction were accelerated but basically similar to that caused by the *T. benhamiae* isolates.

Skin biopsies of naturally infected GP (n = 18) served as references for experimentally infected GPSE. Fungal presence in sections of those biopsies was verified histologically using the PAS reaction (Figure 2A,B) and IF stainings specific for *Trichophyton* spp (Figure 2C). Obviously, the causative agent was isolated and subjected to routine mycological culture and diagnostics. Species identity as *T. benhamiae* was confirmed through ITS sequencing or MALDI-TOF-MS (data not shown). Interestingly, isolates of both colony phenotypes were found in the animal facility, sometimes even isolated from the same animal.

In skin sections of naturally infected GP, hyphae and numerous conidia were found concentrated in and around hair follicles;
the infection seemed to spread from these postulated portals of entry. The expression of the in vivo trichophytosis marker Sub6 was confirmed using the described customised anti-peptide antibody (Figure 2D; Sub3 and MCPA were also demonstrated; data not shown).

Sections of infected GPSE were analysed immunohistochemically for the abundance of the virulence factors Sub3, Sub6 and MCPA (Figure 3; non-infected GPSE were stained as negative controls, no signal was observed; data not shown). IF signals for Sub6 were found dispersed throughout the whole mycelium grown on and in GPSE (distinct progression proximal to distal from point of inoculation; Figure 3A). Strongest signals were seen mainly around keratinised structures (stratum corneum, hair follicles) and in conidia and hyphae, respectively (Figure 3B,C). Sub3 and MCPA were found at cell peripheries in hyphae and conidia (Figure 3D-F). Furthermore, Sub3 was most abundant in conidia...
and concentrated on one side of the spore: it could be described as a ‘cap’ (Figure 3E).

Semiquantitative and statistical analysis of the acquired IF images (Figure 4) revealed a partly significant increase in the expression of all three virulence factors by human- and GP-derived isolates towards the end of culture. Sub6 expression was found significantly higher in human-derived isolates on d3 of culture; also in human-derived isolates, Sub3 expression was significantly higher on d7 compared with T. benhamiae isolates derived from GP. MCPA was expressed by all dermatophyte isolates throughout the culture without a significant difference between the compared groups (data not shown).

Selected isolates with white and yellow tally—all being confirmed members of the yellow growth type—were depicted in Figure S1, and a phylogenetic tree comprising all applied isolates and outgroups is shown in Figure S2.

4 | DISCUSSION

We used an ex vivo skin infection model that previously proved suitable to study early stages of dermatophytoses and analysed its ability to support the expression of important virulence factors. Therefore, isolates of the zoonotic dermatophyte T. benhamiae derived from two different groups of hosts were applied to the skin model and the infection was compared with skin biopsies of naturally infected GP.

To ensure species identity of all applied isolates, their ITS region was sequenced revealing that only one belongs genetically to the white colony phenotype group although more white and brown tally were observed (see Figure S1). This emphasises the need for species identification through molecular biological methods such as PCR-ELISA, (real time) PCR, sequencing and/or MALDI-TOF-MS. Moreover, even using these sophisticated methods, caution must be exercised when deducing identity from deposited database sequences/spectra since a universal consensus in species description and classification is still not reached.

On top of that, Trichophyton spp. are very closely related. In our study, for example the above-mentioned white phenotype isolate (GP-derived) showed a very high similarity to GenBank sequences deposited for T. verrucosum (per cent identity 98.77%; acc. no. MN295947.1) and T. erinacei (99.15%, acc. no. MF153407.1). Therefore, we strongly recommend to consider all data at hand (direct specimens, sequence data, colony morphology, medical history of the patient, etc) and critically evaluate sequences/spectra and given information to conclude secured results.

The phylogenetic analysis of the T. benhamiae isolates disclosed distinct clusters according to their colony phenotype (y vs w) as it was seen by others as well. The different origin of isolation was not reflected, which is also documented for T. verrucosum isolates.
derived from humans and cattle. The authors ascribe this observation to the fact that human infection mostly originates from contact with infected or asymptomatic animals. Such a zoonotic transmission is also hypothesised for humans with *T. benhamiae* infections especially when contact with GP is reported. 12,16,28,30

The experimental GPSE infection was compared with skin biopsies obtained from GP naturally infected with *T. benhamiae* and considered very similar concerning the stratum corneum invasion and the concentration of fungal elements in and around hair follicles for both groups of the employed isolates. These localisations were also observed in skin biopsies of human and animal patients, and after experimental GP infection. 5,16,31-34 Co-stainings of sections of infected GPSE with anti-Dsg1 and anti-*Trichophyton* antibodies substantiate the findings of other authors concerning host tissue invasion, which is described simultaneously intra- and intercellularly most likely through concerted mechanical and enzymatic forces.35-38

Proteases are considered the most important dermatophyte virulence factors especially during the establishment of the infection. Consequently, we confirmed Sub3, Sub6 and MCPA immunohistochemically in skin biopsies of naturally infected GP. To the best of our knowledge, this is the first report of a visualisation of virulence factor expression in host tissue. Additionally, this finding proved our customised antibodies valuable tools for further research on, for example, the chronology and secretion levels of these virulence factors during natural infection. Such data are currently not available but urgently needed. 39

We observed most Sub3 signals in conidia, which is in accordance with other groups who found Sub3-mRNA upregulated in...
Microsporum canis arthroconidia and T. rubrum conidia. It is explained with its experimentally proven important role during conidial adhesion to host tissue. During our study, semiquantitative scoring of IF images as a widely used evaluation technique revealed human-derived isolates to express significantly more Sub3 on d7 of culture compared with GP-derived isolates. This observation might be explained with the appearance of conidia as well: 9 of 10 human T. benhamiae isolates sporulated again towards the end of culture.

As outlined before, Sub6 is considered an important marker of in vivo infection with dermatophytes such as T. benhamiae. Some of its biochemical properties, in vitro functionality and immunologic implications are summarised by Monod. However, its specific role during infection is still a matter of research. A study using a Sub6-deletion mutant of T. mentagrophytes for experimental GP infection revealed a delayed onset of symptoms and a milder disease course suggesting an influence on general virulence. In our study, all T. benhamiae isolates expressed Sub6 at a comparatively low level. Why human-derived isolates expressed more Sub6 than GP-derived isolates on culture d3 remains to be elucidated in future experiments. Given the fact that the gene repertoire encoding subtilisins is extended in dermatophytes and by far not homogenously distributed among different species and even isolates, more research on their specific function, regulation and interaction is urgently warranted.

Transcriptome analyses by Tran and colleagues revealed MCPA during in vitro and in vivo experiments; Staib et al. allocated MCPA even a pathogenic relevance in both scenarios. During our protein-based analyses, we found MCPA expressed throughout the culture by all T. benhamiae isolates and it was mainly localised at the cell walls of fungal elements. Zaugg and colleagues report of a secreted T. rubrum carboxypeptidase homologous to human carboxypeptidase A that is secreted to the culture medium but not membrane-bound. However, many proteases are produced as (pre)proproteins inside the cell and further processed until they reach their final destination and full functionality.

Dermatophytoses are an ever-growing global health burden, but the knowledge on specific pathomechanisms remains scarce. The herein employed ex vivo model proved suitable to study early events of dermatophyte infection regarding tissue invasion and virulence factor expression and may be used to answer some of the open questions. Since it can be adapted easily to other dermatophyte species—as proven by the successful application of T. mentagrophytes isolates—and skin donors, other fungal infections of the skin might be studied as well, for example in the context of host specificity. Likewise, the customised antibodies for IF analyses represent
important prerequisites to gain more insight into virulence factor production and functionality during natural and experimentally induced dermatophyte infection.

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CONFLICT OF INTEREST
All authors have nothing to disclose.

AUTHOR CONTRIBUTION
Christina Baumbach: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing-original draft (lead); Writing-review & editing (lead). Jule Michler: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Project administration (equal); Supervision (equal); Writing-original draft (lead); Writing-review & editing (lead). Pietro Nenoff: Resources (supporting); Supervision (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). Silke Uhrläß: Resources (supporting); Validation (equal); Writing-original draft (supporting); Writing-review & editing (supporting). Wieland Schrödl: Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Validation (equal); Writing-original draft (supporting); Writing-review & editing (supporting).

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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