Re-discovery of Trichophyton bullosum in North Africa as a cause of severe dermatophytosis in donkeys

Re-discovery of *Trichophyton bullosum* in North Africa as a cause of severe dermatophytosis in donkeys

Andrea Peano[1] (ORCID: 0000-0002-6783-4742)**, Sara Arnoldi**[1]**, Adéla Čmoková**[2,3] (ORCID: 0000-0002-6783-4742), **Vit Hubka**[2,3] (ORCID: 0000-0003-4583-6496)

[1]Department of Veterinary Sciences, , University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco (Turin), Italy

[2]Department of Botany, Faculty of Science, Charles University, Benátská 2, 128 01 Prague 2, Czech Republic

[3]Laboratory of Fungal Genetics and Metabolism, Institute of Microbiology of the Academy of Sciences of the Czech Republic, v. v. i., Vídeňská 1083, 142 20 Prague 4, Czech Republic

ABSTRACT

This article reports the first verified cases of infection by *Trichophyton bullosum* in Africa since the description of the fungus, isolated in 1933 from the coat of horses in Tunisia and Mali. We re-discovered the fungus in cutaneous samples obtained from donkeys suffering for severe dermatitis with areas of alopecia and scaling in the surroundings of Cairo (Egypt). Fungal elements (arthroconidia and hyphae) were seen at the microscopy of material collected by skin scraping and digested in NaOH. Fungal colonies grown on various culture media were identified through PCR and sequencing of the ITS rDNA region. Since the original report in Africa and the Middle East, only a few cases have been reported thus far in humans in France and two cases in horses in the Czech Republic and Japan. *Trichophyton bullosum* seems thus an infrequent cause of dermatophytosis. However, the actual prevalence of this pathogen may be underestimated due to the similarity with *T. verrucosum*, a diffuse cause of infection in cattle

and humans, occasionally found on horses and donkeys too. Indeed, the two fungi can be distinguished only via molecular methods, which are poorly employed in epidemiological studies on equine and bovine dermatophytosis. The results of the present study add to our knowledge on the ecology of this poorly explored dermatophyte, reinforcing the idea that equines are the main hosts of *T. bullosum*, and confirming the presence of this pathogen in Africa.

Keywords

Equidae, molecular identification, animal skin diseases, *Trichophyton benhamiae* complex, zoophilic dermatophytes, zoonotic mycoses

INTRODUCTION

Trichophyton bullosum is a poorly known zoophilic dermatophyte that was originally described from the coat of horses in Africa (Tunisia and Mali) and the Middle East (Syria) (Lebasque 1933). The species was almost forgotten for decades, but recently, the use of the species name was justified using the sequence data and phylogeny (Heidemann et al. 2010). The morphology of this slow-growing pathogen is very similar to that of *Trichophyton verrucosum,* and reliable identification can only be achieved through molecular methods (Sitterle et al. 2012; Lysková et al. 2015).

Horses and donkeys are supposed to be the main hosts of *T. bullosum*, in contrast to *T. verrucosum,* with cattle as the primary host. However, infections in horses and donkeys can be caused by both mentioned species (Lysková et al. 2015). The distribution of *T. bullosum* is almost unknown due to the meagre number of reported cases in both animals and humans and the lack of comprehensive studies on the epidemiology of dermatophytosis in cattle, horses, and donkeys using molecular methods.

Eighty years after discovering *T. bullosum* in Africa and the Middle East, the first cases were described in Europe and Asia. Two cases of human dermatophytosis were reported in France (Sitterle et al. 2012; Sabou et al. 2018), but no sample was collected from the source animals. The only two cases of verified animal dermatophytosis were reported in horses from the Czech Republic (Lysková et al. 2015) and Japan (Watanabe et al. 2021).

This article reports some cases of severe dermatophytosis caused by *T. bullosum* in donkeys from Egypt. These cases are the first verified infections in Africa since the description of *T. bullosum* in 1933. They add to our knowledge of this poorly explored dermatophyte ecology and confirm its continuing occurrence in Northern Africa.

MATERIAL AND METHODS

Animals and sampling site

The findings presented in this article take origin from experience made by one of the authors (SA) within a training program on the medicine of donkeys under the guidance of local Veterinarians operating for "The Donkey Sanctuary" (www.thedonkeysanctuary.org.uk).

Donkeys in Egypt are a common means of transport for goods and people. Though these animals represent a fundamental resource for many families, they frequently receive poor or null veterinary care due to the lack of financial resources and availability of veterinary services. To address this criticism, since 2002, the Donkey Sanctuary serves through mobile clinics in villages, offering free veterinary counselling and interventions to donkey owners. SA noticed many animals with skin lesions during her practical activities and collected biological samples to assess a possible fungal aetiology. The animals covered in this study were visited in August 2015 and April 2016 during different working sessions in villages in the surroundings of Cairo.

Microbiological methods

Hair and scales from the donkeys presenting dermatological lesions were collected using sterile scalpel blades through superficial skin scrapings. The samples were digested in 10% NaOH for 3 hours at room temperature. The material was then transferred to slides and observed under the microscope for the presence of fungal elements. Primary cultures were performed on a commercial medium selective for dermatophytes (Mycobios Selective Agar, Biolife, Milan, Italy), on Sabouraud Dextrose agar enriched with thiamine and inositol and on a medium with 4% casein and 0.5% yeast extract (Kane and Smitka 1978). All the media included antibacterial agents and cycloheximide to reduce the growth of contaminant organisms.

Subcultures were grown on Sabourad´s dextrose agar (SGA, Himedia, Milano, Italy), malt extract agar (MEA; malt extract from Oxoid, Basingstoke, UK) and potato dextrose agar

(PDA; Roth, Karlsruhe, Germany) at 25 °C in the dark; growth at 30 °C and 37 °C was tested on SGA. Lactic acid with cotton blue was used as a mounting medium for observation of the micromorphology. Preparations for light microscopy were made from 14–21 days old cultures grown on MEA. Lactic acid (60 %) was used as a mounting medium. Microphotographs were taken by Olympus BX51 microscope with an Olympus DP72 camera. Colony details were observed and photographed on Olympus SZX2-ILLT (Tokyo, Japan) equipped with microscope digital camera Olympus DP27 (Tokyo, Japan). The isolates were deposited into the Culture Collection of Fungi (CCF) housed at the Department of Botany, Charles University, Czech Republic.

Molecular studies

Genomic DNA was extracted from 14-day-old colonies grown on MEA (Oxoid Ltd., Basingstoke, UK) using the ArchivePure DNA yeast and Gram2 + kit (5PRIME Inc., Gaithersburg, MD, USA) according to the updated manufacturer´s instructions (Hubka et al. 2018b). The ITS rDNA region was amplified with the primers ITS1 (5´-TCCGTAGGTGAACCTGCGG) and ITS4 (5´-TCCTCCGCTTATTGATATGC) (White et al. 1990). The reaction mixture and PCR protocol were described previously (Hubka et al. 2018a). The PCR product purification followed the protocol described by Sklenář et al. (Sklenář et al. 2021); sequencing was performed in Seqlab Sequencing Service (Charles University, Czech Republic) using both terminal primers. The obtained sequences were deposited into the ENA (European Nucleotide Archive) database.

The primer pair MF3 and MF6 was used for the detection of the MAT1-1-1 idiomorph of the mating type gene (MAT) encoding protein with an alpha-domain motif, and primers MF1 and MF5 were used for the detection of the MAT1-2-1 idiomorph encoding a regulatory protein

with a HMG (high-mobility group) DNA-binding motif (Kano et al. 2012; Symoens et al. 2013).

Alignments of the ITS was performed using the FFT-NS-i option implemented in the MAFFT online (Katoh et al. 2019). The final dataset contained 33 taxa and a total of 667 characters of which 90 were variable and 51 parsimony-informative. Suitable partitioning schemes and substitution models (Bayesian information criterion) were selected using PartitionFinder 2 (Lanfear et al. 2017) with settings allowing ITS1, 5.8S and ITS2 regions to be independent datasets. Suitable partitioning scheme and substitution models were as follows: the TrNef model was proposed for the ITS1 and ITS2 regions; and K80 model for the 5.8S region. The maximum likelihood tree was constructed with IQ-TREE version 1.4.4 (Nguyen et al. 2015) with nodal support determined by nonparametric bootstrapping with 1000 replicates.

RESULTS

Animals

Out of 187 animals examined, 35 (18,7%) presented dermatological problems.

Direct examination and primary cultures

Samples from three donkeys were positive for fungal elements at microscopy. The animals were adults (Table 1) belonging to different owners and visited on different days in Abu Ghaleb, close to Cairo. The lesions were represented by multiple areas of alopecia localized in various body sites (Table 1, Fig. 1, Supplementary Fig. S1).

The fungal structures observed were chains/groups of arthroconidia inside or surrounding the hair shafts (Fig. 2). In the cultures of three donkeys (Nos. 1, 2, and 3) it was possible to observe pale, waxy, slow-growing colonies, which were subcultured for identification. As regards donkey No. 4, despite numerous attempts, the fungus seen at direct examination failed to grow, with the culture plates rapidly invaded by contaminant moulds.

Mycological studies

In total, six isolates from three donkeys were further characterized. One isolate from each culture-positive animal was deposited into the CCF collection with accession codes CCF 5730 (donkey No.1), CCF 5731 (donkey No. 2) and CCF 5678 (donkey No. 3). The description of subcultures cultivated at 25 °C in the dark is reported below and shown in Fig. 3.

Colonies on MEA attained 8–12 mm diam after 7 days (10–13 mm after 14 days and 15–17 mm after 21 days), white to pale yellow (#F3E5AB) or vivid orange-yellow (#F6A600), waxy, slightly elevated, margins entire, reverse light yellow (#F8DE7E). Colonies on SGA attained 11–12 mm diam after 7 days (20–21 mm after 14 days and 28–33 mm after 21 days), white to pale yellowish pink (#ECD5C5) or pale orange-yellow (#FAD6A5), submerged, filamentous, reverse light yellow (#F8DE7E). Colonies on PDA attained 7–9 mm diam after 7 days (14–16 mm after 14 days and 25–29 mm after 21 days), white to pale yellowish pink (#ECD5C5) or pale orange yellow (#FAD6A5), waxy, lanuginous in some areas, elevated in the centre, margins entire to delicately filamentous, reverse light yellow (#F8DE7E). Colonies on SGA with cycloheximide and chloramphenicol (Trios, Prague, Czech Republic) were submerged with dendritic growth. Colonies on SGA after 7 d at 30 °C attained 11–14 mm diam (⌀ = 13 mm); at 37 °C 8–9 mm diam (⌀ = 8 mm).

Vegetative hyphae were smooth, septate, frequently inflated, hyaline, 1.5–4 µm diam (Mean ± sd; 2.7 ± 0.7). Chlamydospores abundantly present, spherical, ovate or irregularly shaped, 4–9(–20) µm diam, frequently forming chains. Microconidia, macroconidia and spiral hyphae were not observed.

Molecular studies

The MAT1-1-1 idiomorph of the mating type gene was amplified in all six *T. bullosum* strains with primers MF3 and MF6, while the idiomorph MAT1-2-1 was not detected. The ITS

rDNA sequences obtained from all strains were identical. Three sequences representing isolates preserved in the CCF collection are available under accession numbers LR595961–LR595963 (Table 1). Using the BLAST similarity search, these sequences were identical to those derived from strains of *T. bullosum* isolated from human tinea infections in France (strains IHEM 24321 and 0805m150877; GenBank accession numbers: MK298921 and KY885205), horse in the Czech Republic (isolate CCF 4831; LR794144), ex-type strain of *T. bullosum* (CBS 363.35; FM992675), and strain CBS 557.50 (FM992675) from an unknown source. The strain NUBS 20002 from a horse in Japan (LC592175) showed one substitution in the ITS2 region compared to our strains.

Some sequences deposited in GenBank under the name *T. bullosum* represent in fact a recently described species, namely *T. africanum*, especially some generated in the study of Baert et al. (2020). In the phylogeny based on the ITS rDNA sequences (Fig. 4), this species is resolved as a sister species to *T. bullosum*. Although the majority of known *T. africanum* records originate from human dermatophytoses in Mozambique (IHEM 4032), South Africa (IHEM 4033) or Belgium (IHEM 19628), it is probably a zoophilic dermatophytes as evidenced by strains IHEM 19638 and IHEM 17561 isolated from horse in South Africa and guinea pig in France, respectively.

DISCUSSION

Trichophyton bullosum is a member of the *Trichophyton benhamiae* complex along with other ten predominantly zoophilic pathogens; the only exception is an anthropophilic species, *T. concentricum* (Čmoková et al. 2021). *Trichophyton verrucosum*, a dominant causal agent of dermatophytosis in cattle and other ruminants, belongs to the most well-known members of this complex (Chermette et al. 2008). Some other representatives, especially *T. benhamiae*, *T. europaeum*, *T. japonicum* and *T. erinacei*, have also received considerable attention in recent

years due to the increasing incidence of infections in pets and pet owners, most noticeably in the European countries (Nenoff et al. 2014; Abarca et al. 2017; Hubka et al. 2018c; Le Barzic et al. 2021). Compared to these extensively studied pathogens, little is known about the host spectrum of the remaining zoophilic species, which are usually known only from several case reports in humans and animals.

Trichophyton bullosum is phylogenetically relatively distant from all other species in the *T. benhamiae* complex. It is most closely related to *T. africanum (Fig. 4),* which is strikingly different by morphology (Čmoková et al. 2020). It is also phylogenetically distant from *T. verrucosum,* which is superficially and microscopically very similar, and it has similar nutritional requirements and growth pattern *in vitro* (Lysková et al. 2015; Watanabe et al. 2021). Both species show a low level of intraspecific genetic variability, and their populations most likely consist only of strains displaying one mating type gene (MAT) idiomorph. The MAT idiomorph, however, differs between these pathogens. Only *MAT1-1-1* idiomorph has been detected in *T. bullosum* strains, while *MAT1-2-1* was detected in all hitherto examined *T. verrucosum* strains (Kano et al. 2014; Kosanke et al. 2018; Čmoková et al. 2020). These facts suggest that clonal reproduction is dominant or the only mode of dissemination of these species. Because there is no reliable phenotypic character distinguishing these species, molecular methods are necessary to for the identification.

Apart from molecular and phylogenetic differences, the main difference between *T. bullosum* and *T. verrucosum*, therefore, lies in their host spectrum, which can, however, partly overlap. Based on the known data, *T. bullosum* is specific for horses and donkeys, while *T. verrucosum* is mainly reported from ruminants but occasionally from other animals, including horses and donkeys. Lysková et al. (2015) hypothesized that a significant part of dermatophytosis cases in horses and donkeys due to *T. verrucosum* could be caused by *T. bullosum* due to the overall lack of large-scale studies examining agents of dermatophytosis in

these animals using molecular methods. Consequently, this is why we do not have exact data on geographical distribution, host spectrum and prevalence of *T. bullosum*.

Probable endemic areas of *T. bullosum* are Africa and the Middle East, where the species was initially found. Our findings confirm its occurrence at present in Africa.

Lysková et al. (2015) suggested that this species could be introduced to Europe with the animal trade between France and former French colonies and then spread to other countries, including the Czech Republic. In this regard, the case discovered in a horse in Japan could represent a significant turning point in this thinking (Watanabe et al. 2021). This case may indicate the introduction of a pathogen to remote areas along with animal transport and trade. Still, on the other hand, it can also show the worldwide distribution of an underdiagnosed pathogen.

Clinical manifestation of infection due to *T. bullosum* in donkeys has not been described except in the present study. Thus the only well-documented animal cases due to this pathogen come from horses and, in general, manifested as circular erythematous lesions with hair loss in the saddle area, shoulders, chest, withers, and less commonly on the head, neck, buttocks and limbs (Lebasque 1933; Lebasque 1934; Lysková et al. 2015; Watanabe et al. 2021). There are few well-documented cases of dermatophytosis in donkeys due to *T. verrucosum* (Suleiman et al. 2017) or *T. verrucosum*-like isolates (Abdalla et al. 2005), which could in fact represent *T. bullosum*, while the remaining records lack a detailed clinical description. In agreement with the cases described here, these infections predominantly manifested as generalized superficial infections with non-itchy, slightly elevated, well-demarcated lesions accompanied by alopecia. The lesions were mainly distributed on the head, neck, limbs and flank.

Dermatophytosis in equids is usually a self-limiting disease that resolves spontaneously within several months (Rochette et al. 2003; Knottenbelt 2005; Cafarchia et al.

2013). However, the treatment is mandatory as it shortens disease duration and reduces the possibility of spread to other animals or humans. The decontamination of the environment using hypochlorite bleach and enilconazole sprays can further prevent re-infection and spread of the disease (Rochette et al. 2003). Enilconazole and natamycin are registered in most European countries for the treatment of dermatophytosis in Equids. These drugs are usually employed in topical washes and sprays with satisfactory results (Rochette et al. 2003). Although the data about efficacy are mostly based on the therapy of horse dermatophytosis, enilconazole washes also show good results in treating dermatophytosis in donkeys (White et al. 2019). Dermatophyte vaccine can be used as an alternative treatment option in donkeys, leading to resolution (White et al. 2019).

Available data on treatment of verified *T. bullosum* infections include only three cases, two in horses and one in man. The first horse was successfully treated with topical enilconazole (Lysková et al. 2015) and the second with terbinafine ointment (Watanabe et al. 2021). Tinea corporis infection in a 21-year-old male was successfully treated by a combination of topical ketoconazole and systemic griseofulvin therapy (Sitterle et al. 2012). Regarding the donkeys of the present study, it was not possible to set up a therapeutic protocol. It is essential to remind that the study context was that of a rural area characterized by extreme poverty. Facing the commitment and expense of antifungal treatment in a situation of subsistence rearing is difficult to accept. There are also cultural reasons behind this way of thinking. The role of donkeys in human activities in many African countries, although fundamental, is frequently overshadowed in the collective imagination. At the same time, more attention and resources are dedicated to animals destined for food production. Popular beliefs have often built a distorted vision of donkeys, considered stubborn and able to live and work without any care.

The work context was problematic also concerning the communication with the donkeys' owners, who generally provided only basic data regarding animal history. Unfortunately, this made it impossible to investigate a crucial aspect: whether and to what extent in the area of study the contact with donkeys causes infection by *T. bullosum* in people.

In conclusion, this study allowed demonstrating *T. bullosum* as an agent of dermatomycosis in Northern Africa 90 years after its first report. Further studies will be necessary to ascertain the involvement of animal species other than donkeys and the role of this fungus as a human pathogen.

ACKNOWLEDGEMENTS

The contribution of Vit Hubka was supported by the Charles University Research Centre program no. 204069.

FIGURE LEGENDS

Fig. 1 Clinical manifestation of dermatophytosis due to *Trichophyton bullosum* in donkeys. a-d: donkey No. 1; e-h: donkey No. 2; i-l: donkey No. 3. A detailed description of the lesions is provided in Table 1.

Fig. 2 a-c: hair infected with arthroconidia (microscopic examination after NaOH digestion, 250x magnification). a: donkey No. 1; b: donkey No. 2; c: donkey No. 4; d-e macromorphology of primary cultures; d: donkey No. 1, culture on Sabouraud Dextrose agar with thiamine and inositol (red arrows = contaminant moulds; blue arrows = *T. bullosum*); e: donkey No. 1 (culture on casein medium); f: donkey No. 2 (culture on casein medium); g: donkey No. 3 (culture on Mycobios selective agar).

Fig. 3 Macromorphology and micromorphology of *Trichophyton bullosum*. a. Colonies after 14 days at 25 °C on MEA, SGA and PDA (from left to right). b. detail of colony on MEA. c. detail of colony on SGA. d. detail of colony on PDA. e-f. colony on SGA wih cycloheximide and chloramphenicol (Trios, Prague, Czech Republic) after 3 months of incubation at 25 °C. g-k. thick-walled vegetative hyphae disintegrating into arthroconidia; numerous thick-walled irregular intercalar, terminal or free chlamydospores. Scale bars = 10 µm.

Fig. 4 A best scoring maximum likelihood tree based on ITS rDNA sequences showing relationships of *Trichophyton bullosum* to other members of the the *T. benhamiae* complex. The main primary host(s) of particular species are shown as icons; uncommon occasional hosts are omitted; a question mark means that host spectrum is little known and only based one single or few isolations from animals. Ex-type isolates are designated by a superscript T;

only support values exceeding bootstrap values 70% are shown on branches; *Trichophyton rubrum* CBS 202.88 was used as outgroup.

Supplementary Fig. S1 Donkey No. 4. One large, irregular, alopecic patch with scaling on the head. Another smaller area on the face (close to the nares). Some little alopecic areas on the pinna. Large confluent alopecic areas on the limbs.

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