A Suggested Pathogenic Role for *Trichosporon montevidense* in a Case of Onychomycosis in a Japanese Monkey

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ABSTRACT. Onychomycosis is a fungal infection of fingernails or toenails caused by several species of fungi and yeasts. A Japanese monkey, *Macaca fuscata*, displayed severe onychomycosis in his 4 limbs. Diagnosis and etiological agent identification were performed by conventional and DNA-mediated methods. The accumulated findings of this case revealed *Trichosporon montevidense*, which has long been considered to be a nonpathogenic yeast. Here, we present the first report of an involvement of *T. montevidense* in an onychomycosis case.

KEY WORDS: Japanese monkey, Onychomycosis, *Trichosporon montevidense*.

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Onychomycosis is a mycotic superficial invasion of keratinized tissue of the nail plate. Although it is commonly considered to be caused by the dermatophytic fungi, a variety of other organisms have been implicated as etiologic agents in the disease [7].

A 4-year-old male Japanese monkey, *Macaca fuscata*, showed severe onychomycosis in his four limbs. Clinically, some digits showed onychomycosis of the distal subungual type. However, most of the digits showed inflammation of both the nail and nail bed. In addition, some digits showed a purulent exudate and slight perilesional erythema (Fig. 1). This monkey had been used for neurological research, and it had not been possible to provide adequate veterinary care.

Trichosporon species are yeast-like organisms that belong to Basidiomycota. Several members of this genus are known to be pathogens [8, 10, 15]. The panel of diseases caused by *Trichosporon* spp. has been extended to include nail infection [15]. Several *Trichosporon* spp. have previously been reported to be causative agents of onychomycosis [9]. On the other hand, *Trichosporon montevidense* has long been considered to be a nonpathogenic yeast that naturally inhabits soil, water and human feces and skin [4]. Here, we describe for the first time an involvement of *T. montevidense* in a case of onychomycosis in a monkey.

Nail clippings were obtained and sent to our laboratory under aseptic conditions. A potassium hydroxide preparation (KOH) was made by treating the nail scrapings with drops of 10% KOH-glycerol overnight at room temperature and then examined by optical microscopy.

Nail scrapings were cultured on Potato Dextrose Agar (PDA) supplemented with 50 µg/ml chloramphenicol and on Sabouraud Dextrose Agar (SDA) for up to 3 weeks at

28°C. The cultures were macroscopically examined during the incubation period.

Total DNA was extracted from nail clippings by grinding them using a multibead shocker (MB524UAL; Yasui Kikai, Osaka, Japan) with 3 g of sterilized metal corn at 1800 rpm for 20 sec. Ground nail clippings were soaked in lysis buffer (200 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% w/v SDS and 250 mM NaCl₂) and centrifuged at 10,000 × g for 5 min. The supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and subsequently extracted with chloroform. Finally, DNA was precipitated with an equal volume of 2-propanol at –20°C for 10 min, washed with 150 µl of 70% ethanol, dried and suspended in Ultrapure water.

The grown colonies and the total DNA extracted from nail were subjected to DNA-mediated identification target-

Fig. 1. Onychomycosis in the fingernails of the diseased Japanese monkey.

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ing the 28S region of ribosomal RNA gene (rDNA) with a pair of primers, 28SF1 (5'-AAGCATATCAATAAGCG-GAGG-3') and 635 (5'-GGTCCGTGTTTCAAGACGG-3') [13]. Colony number 5, grown on SDA, was identified by a set of bacterial universal primers, F3L (5'-GTCCCGCAAC-GAGCGCAAC-3') and 1492R (5'-CGGTTACCTTGT-TACGACTT-3') [6, 12] since we could not generate PCR products by the above-described fungal primers. Sequence similarity was searched using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the generated sequences [1].

Physiological and morphological identification of colony number 2 were performed [5]. Its ability to grow on a sole carbon source was checked by an auxanographic method in which 20×10^4 /ml of freshly prepared yeast suspended in yeast nitrogen base (DIFCO, U.S.A.) and incorporated with agar cooled to 45°C. A quantity of 5 mg of 30 carbon sources was spotted on the surface of the previous yeast

incorporated medium. Cultures were incubated at 25°C, and results were read after 2–4 days. Moreover, this isolate was cultivated on SDA medium under different incubation temperatures, 28°C, 35°C and 37°C. A slide culture was prepared on 2% malt extract agar and incubated for 2 days at 28°C.

Direct microscopic examination of nail clippings uncovered pyriform to spherical elongated conidia (Fig. 2). Budding cells were also present. Macroscopic examination after 5–7 days of incubation revealed three types of colonies on PDA, 1) pink smooth moist mucoid colonies, 2) cream-colored moist soft colonies that changed into colonies with a deeply fissured marginal zone after a long period of incubation and 3) white-dry colonies, while it revealed two types of colonies on SDA, 4) creamy-colored and 5) white-dry colonies. Molecular analysis of these colonies and BLAST sequence similarity search identified the above-described colonies as 1) *Rhodotorula mucilaginosa*, 2) *Trichosporon montevidense*, 3) *Torulaspora delbrueckii*, 4) *Trichosporon montevidense* and 5) *Leuconostoc mesenteroides*, respectively (Table 1). On the other hand, the total DNA from the nails resulted in two fungal species, *T. montevidense* and *Trichosporon caseorum*, based on the 28S region (Table 1).

The ability of our *T. montevidense* isolate to assimilate variant carbon sources is illustrated in Table 2. Furthermore, it was able to grow at 28°C and 35°C but not at 37°C. Microscopically, the slide culture examination showed hyphae disarticulated into arthroconidia and budding cells (Fig. 3).

The conventional method for diagnosis of an onychomycosis has to be performed by demonstrating the presence of causative agents in clinical materials using microscopic examination, which helps to rule out the non-fungal causes of nail disease, and by culture mediated identification to determine the exact pathogen that is responsible for the onychomycosis case [3]. In the current case, direct microscopy of clinical materials provided evidence of fungal invasion of

Fig. 2. Direct microscopic examination of nail clippings prepared using KOH-glycerol overnight at room temperature. Different fungal conidia are indicated by arrows.

Table 1. The accession numbers and maximum identities of the species identified from nail clippings of the diseased monkey

No.	Source	Accession No. ^{h)}	Maximum identity	Species name
1 ^{a)}	PDA	EU807914.1	100 %	<i>Rhodotorula mucilaginosa</i>
2 ^{b)}	PDA	AF105397.1	100 %	<i>Trichosporon montevidense</i>
3 ^{c)}	PDA	EF063125.1	100 %	<i>Torulaspora delbrueckii</i>
4 ^{d)}	SDA	AF105397.1	100 %	<i>Trichosporon montevidense</i>
5 ^{e)}	SDA	EU074837.1	98 %	<i>Leuconostoc mesenteroides</i>
6 ^{f)}	Nail ⁱ⁾	AF105397.1	100 %	<i>Trichosporon montevidense</i>
7 ^{g)}	Nail ⁱ⁾	AJ319757.1	99 %	<i>Trichosporon caseorum</i>

a) Pink smooth moist mucoid colonies, b) cream-colored moist soft colonies and c) white-dry colonies were all isolated on PDA. d) Creamy-colored colonies and e) white-dry colonies were isolated from SDA. f)–g) Fungal species identified directly from nail clippings by DNA extraction. h) The accession numbers of the identified fungal species from colonies and from the DNA of nail clippings based on BLAST sequence similarity search. i) Refers to the total DNA extracted directly from the nails.

Table 2. Results of carbon assimilation tests for identification of our *T. montevidense* isolate

Tested substances	Results	Tested substances	Results
Glucose	+	D-Arabinose	–
Galactose	+	D-Ribose	+
L-Sorbose	–	L-Rhamnose	–
Sucrose	+	N ^o -Acetyl-D-glucosamine	+
Maltose	+	Methanol	–
Cellobiose	+	Ethanol	–
Trehalose	–	Glycerol	–
Lactose	+	Erythritol	–
Melibiose	–	Ribitol	–
Raffinose	–	Galactitol	+
Melezitose	+	D-Mannitol	+
Inulin	–	D-Glucitol	+
Soluble starch	+	a-Methyl-D-glucoside	–
D-Xylose	+	Salicin	–
L-Arabinose	+	Inositol	–
		DL-Lactate	+

Substances in bold are the identical materials for *T. montevidense* identification. Plus (+) and minus (–) correspond to ability (or disability) of the isolated *T. montevidense* to use carbon sources.

Fig. 3. Slide culture of *Trichosporon montevidense* isolated from nail clippings on PDA. The slide shows hyphae disarticulating into arthroconidia. This slide was prepared on 2% malt extract agar after 2 days of incubation at 28°C.

the tissue. However, culture of nail clippings resulted in more than one type of colony. Identifying the resulting colonies by molecular biology was performed using fungal universal primers and BLAST sequence similarity search. Bacterial universal primers had to be employed for colonies that did not respond to the previous primers. Simultaneously, we tried to identify causative agents directly from nails to exclude agents that cultured on media as contaminants, but were not involved in the infection, by direct analysis of DNA extracted from the nails, which revealed two types of fungal species (Table 1).

Although many fungal organisms were identified in culture and/or directly based on the DNA extracted from the nail clippings, *T. montevidense* is the only agent suspected to be responsible for the case of onychomycosis described here. Demonstration of the presence of fungal elements directly in the nails excludes *L. mesenteroides* form being the pathogen in this case. In addition, isolation of *R. mucilaginosus* on PDA was coincidental, as it could not be isolated repeatedly on SDA, and it was not identified in the DNA extracted from the nail clippings. On the other hand, *Rhodotorula* spp. are well-known contaminants of clinical isolates, and have no history of superficial infections [2, 15]. *T. delbrueckii* is a nonpathogenic yeast that is mainly involved in the fermentation processes and wine production, and it could not be identified directly in DNA isolated from the nails or on SDA media [11]. *T. caseorum* has been reported in a few studies as a new species isolated from cheese, but since it could not be isolated on non-selective media (SDA and PDA), the possibility of it having a pathogenic role in this case is poor.

In contrast, *T. montevidense* has previously been described to be associated with summer-type hypersensitivity pneumonitis but not as a real pathogen [14]. The morphologies of the yeasts seen in direct microscopic examination of nail clippings and the yeasts displayed in slide culture of *T. montevidense*, which was isolated from the nails, were nearly symmetrical (Figs. 2, 3). Nevertheless, *T. montevidense* was the only species isolated from all cultures and isolated directly from the DNA of nail clippings, and it had the same accession number in all isolations (Table 1).

The identification of the *T. montevidense* strain was proved not only on molecular biological grounds but also on physiological and morphological bases. Homological search by BLAST of the isolated *T. montevidense* resulted in 100% maximum identity based on the 28S of the rDNA region. In addition, it assimilated galactose, while it could not grow in the presence of L-sorbose, L-rhamnose, melibiose and erythritol, which are identifying to *T. montevidense* [4]. Conversely, it could not assimilate ethanol, glycerol, ribitol, a-Methyl-D-glucoside, salicin and inositol, and this might be attributed to a unique phenotype of this strain. These findings, in addition to the typical microscopic features of *T. montevidense* and the ability to grow at 35°C but not at 37°C, confirm the identification of our strain.

Trichosporonosis is disease caused by *Trichosporon* species. The infection manifests as disseminated or superficial mycoses (white piedra) [2, 15]. Several species of this genus are known to be pathogens of humans and animals [8, 9, 15, 16]. Recently, this genus has undergone taxonomic revision through which the number of species considered as pathogens has been increased from one to seven, which are *T. cutaneum*, *T. asahii*, *T. mucoides*, *T. inkin*, *T. ovoides*, *T. asteroides* and *T. loubieri* [2, 8, 10, 15]. This leaves the door open to reconsider the pathogenicity of the other members of this genus in light of new reports. Considering all our findings, *T. montevidense* has a higher possibility of having

a pathogenic role in this case.

In conclusion, *Trichosporon montevidense* has long been considered to be a nonpathogenic yeast. However, the isolation of *T. montevidense* in this case of onychomycosis in a monkey indicated that this isolate was possibly a pathogenic organism. To our knowledge, this is the first report to implicate of *T. montevidense* in an onychomycosis case.

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