

Scanning Electron Microscope Imaging of Onychomycosis

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Although scanning electron microscope technology has been used for more than 60 years in many fields of medical research, no studies have focused on obtaining high-resolution microscopic images of onychomycosis of the toenail caused by *Trichophyton rubrum* in a geriatric population. To provide new insight into the intricate structure and behavior of chronic toenail onychomycosis, we produced three-dimensional images of onychomycosis obtained from two geriatric patients with confirmed growth of *T rubrum*. The photomicrographs illustrate the pervasive integration and penetration of the fungus hyphal elements, underscoring the clinical difficulty of obtaining rapid treatment of fungal infections in the distal and lateral subungual space of the human toenail. Although the scanning electron microscope may not be a practical diagnostic tool for most physicians, it remains invaluable for the researcher to obtain insight into the spatial orientation, behavior, and appearance of onychomycosis. (J Am Podiatr Med Assoc 94(4): 356-362, 2004)

Toenail onychomycosis is the most common condition diagnosed and treated by podiatric physicians in the United States.¹ Onychomycosis is usually diagnosed using several scientific methods, including clinical observation, dermatophyte test medium (DTM) culture, fluorescent potassium hydroxide (KOH) preparation, toenail biopsy with surgical pathology diagnostic testing, periodic acid-Schiff reaction, mycology laboratory fungal culture growth analysis, and light microscope imagery.¹⁻³ Conventional light microscope imaging provides a rapid screening test for the presence or absence of fungi to aid physicians when instituting a treatment regimen for onychomycosis.³ Fungal culture growth by a mycology laboratory of specimens obtained by physicians can be used to identify the specific genus and species of particular fungi causing onychomycosis.

Although current methods of diagnosing onychomycosis remain effective, they are limited in defining and detailing the spatial orientation and pervasiveness of fungi associated with onychomycosis. Although electron microscope technology has been used for more than 60 years in many fields of medical research, no studies have focused on obtaining high-resolution microscopic images of onychomycosis of the toenail caused by *Trichophyton rubrum* in a geriatric population. Rashid et al⁴ grew fungal cultures of *Trichophyton mentagrophytes in vitro*, finding evidence of fungal elements penetrating into the nail plate. Meyer et al⁵ investigated infected nails with *T mentagrophytes* under the scanning electron microscope (SEM) and performed a more detailed examination of the interaction between the fungus and the nail. Our study was undertaken to provide new insight into the intricate structure and behavior of chronic toenail onychomycosis involving *T rubrum*. We obtained samples from the toenails of two geriatric patients suspected of having mycotic infection and studied the specimens using mycology laboratory analysis and SEM imaging.

Scanning electron microscopes use electrons rather than light to form an image. Electrons have a

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much smaller wavelength than light, so the SEM can resolve much smaller structures than the traditional light microscope. The smallest wavelength of visible light is approximately 400 nm (400 millionths of a meter), whereas the wavelength of electrons used in the SEM is approximately 0.005 nm (5 trillionths of a meter). There are many advantages to using the SEM for medical research instead of a light microscope. The SEM can produce three-dimensional (3-D) images with a large depth of field, which allows a large amount of the sample to be in focus at one time. The SEM also produces images of high resolution, which means that closely spaced features can be examined at high magnification (up to $\times 200,000$) and that structures as small as 2.0 nm can be seen.⁶ The ability to produce 3-D medical images with a combination of a larger depth of field, greater resolution, and higher magnification than light microscopes makes the SEM an important diagnostic tool in medical research.

Clinical Studies

Onychomycosis is a clinical term used to describe a fungal infection characterized by thickening, splitting, roughening, and discoloration of the toenail.⁷ People infected with onychomycosis suffer mainly from cosmetic difficulties; however, left untreated, the fungal infection may spread to other toenails and make everyday activities painful.¹ Studies show that fungal infections affect nearly every person who wears shoes at one time or another; darkness, heat, and moisture associated with hosiery and shoes make conditions inviting for the development of onychomycosis.⁷ The primary fungi associated with onychomycosis are *T rubrum*, *T mentagrophytes*, and *Candida* spp. The former two fungi are classified as dermatophytes—a group of taxonomically related fungi with affinity for cornified epidermis, hair, horns, nails, and feathers.⁸ Dermatophytes are known to infect the ventral nail plate by penetrating keratin layers through enzymatic or mechanical processes.⁹ *Trichophyton rubrum* is a common dermatophytic fungus in southern Florida and throughout the United States. Approximately 90% of the published cases of onychomycosis are caused by dermatophytes, with 90% of these cases caused by *T rubrum*.¹⁰ Fungal infections are often spread through contact with soils, locker rooms, and bathrooms. *Trichophyton rubrum* is an anthropophilic organism commonly isolated in the laboratory from podiatric medical specimens that are usually present as distal subungual infections (tinea unguium).¹¹ Treatment of onychomycosis can include debridement, topical antifungal medications, and a regimen of oral antifungal

medications that have an 80% to 90% success rate.^{12, 13} Owing to a lack of published SEM studies of *T rubrum* and other mycoses of onychomycosis, a clinical sampling of two geriatric patients suspected of having fungal infection was conducted for this study.

Materials and Methods

Two geriatric patients with clinical signs and symptoms of distal and lateral subungual onychomycosis were chosen at random from a nursing home setting in Fort Lauderdale, Florida. Patient 1 is an 88-year-old woman and patient 2 is an 88-year-old man. Neither patient had diabetes mellitus or was using any topical or oral antifungal medication before a specimen was obtained. Clinical photographs were taken of each patient's most affected hallux toenail before specimen collection, and the hallux that appeared to have the most clinically infected toenail was chosen for this study. Individual specimens were obtained by first cleansing the affected toenail and surrounding skin with a sterile alcohol pad. The infected toenail and subungual debris were aggressively debrided as proximally as possible without causing excessive discomfort to the patient. Each specimen was obtained from the proximal end of the growing edge of the suspected infection and placed in a small sealed bag (to prevent contamination) labeled with the patient's name.

Using sterile technique, both specimens were divided into equal halves, with both halves representing the same amount of clinically infected toenail and subungual debris. Half of each specimen was left in the plastic bag and sent to the Mycology Division of Quest Diagnostics in Baltimore, Maryland, for fluorescent KOH preparation and microscopic fungal culture examination. The other halves were placed into small jars containing a 2.5% buffered glutaraldehyde solution. The jars were labeled with the patient's name, stored in a refrigerated container, and transported to the University of Miami Dauer Electron Microscope Laboratory in Coral Gables, Florida.

To preserve the structure of living tissue with no alteration from the living state, the two toenail samples collected for SEM imaging were fixed in a 2.5% glutaraldehyde solution with Millonig's phosphate buffer (pH = 7.3) for 2 hours at room temperature.⁶ After cooling at 4°C overnight, the samples were washed three times at 5-min intervals with the same Millonig's buffer. The two samples were cross-sectioned using a microscopy-grade razor blade to obtain images of the lateral nail morphology. To add density and contrast to the biologic tissue by reacting with lipid moieties, postfixation was conducted using

a 1% osmium tetroxide solution diluted in buffer for 1.5 hours at room temperature.⁶ After postfixation, the specimens were again washed three times at 5-min intervals with Millonig's phosphate buffer. In accordance with an unpublished procedure for SEM fungal specimen preparation, the samples were dehydrated at room temperature in a graded series of 10% to 90% ethanol two times at 5-min intervals for each step followed by 100% ethanol three times at 10-min intervals (Dennis Kunkel, PhD, personal communication, 2003). After the samples were run through the dehydration series and contained in absolute ethanol, acetone was slowly added to the samples in a 2:1, 1:1, 2:1, and 100% grade at 10-min intervals. The acetone was gradually replaced with hexamethyldisilazane in an analogous manner according to the dehydration protocol to complete critical-point drying of the specimens. The dried samples were mounted ventral side up on aluminum stub mounts using 12-mm carbon adhesive tabs coated with carbon-conducting glue and sputter coated with 6 nm of palladium using a Hummer 6.2 sputtering system (Anatach, Ltd, Union City, California). Images were obtained of the two patient samples using a Jeol JSM-5600LV SEM (Jeol-USA, Inc, Peabody, Massachusetts) in high-vacuum mode with accelerating voltages at or around 2.0 kV.

Results

A complete medical evaluation revealed that patients 1 and 2 demonstrated clinical signs and symptoms of distal and lateral subungual onychomycosis (Figs. 1 and 2). Preliminary reports from Quest Diagnostics

revealed that hyphal elements were seen during the direct fluorescent KOH microscopic examination for both patient samples, indicating the presence of dermatophytes. In the laboratory, the traditional fluorescent KOH preparation method was enhanced by adding the fluorescent dye Calcofluor white, and the specimen was observed under a fluorescent microscope.⁵ After growth on two different types of media—Sabouraud dextrose agar plus chloramphenicol and Sabouraud dextrose agar plus chloramphenicol and cycloheximide—for approximately 3 weeks, the laboratory microscopically examined the colonies for genus and species identification. The laboratory reported the presence of *T rubrum* in both samples.

On initial SEM observation of the morphology of the subungual ventral nail plate, we detected fungus hyphal elements in the toenails of the two patients. The thin layers of semihard keratins of the true cuticle, the bed horny layer, and the thicker solehorn provide an environment allowing fungal elements to thrive in patient 1 (Fig. 3).³ The SEM observation of the cross-sectioned area of the distal toenail in patient 1 illustrates fungal hyphae penetrating the matrix between the keratin folds (Fig. 4). Figure 4 further exemplifies the pervasive nature of *T rubrum* and the difficulty of treatment because of deep penetration into the nail plate. Several unique formations of hyphae on the toenail plates were also observed; Figure 5 portrays a characteristic heart-shaped design from patient 1.

The SEM observations of the sample obtained from patient 2 further illustrate the presence of hyphae on the distal part of the ventral side of the toe-



Figure 1. Distal and lateral subungual onychomycosis due to *Trichophyton rubrum* in patient 1.



Figure 2. Distal and lateral subungual onychomycosis due to *Trichophyton rubrum* in patient 2.

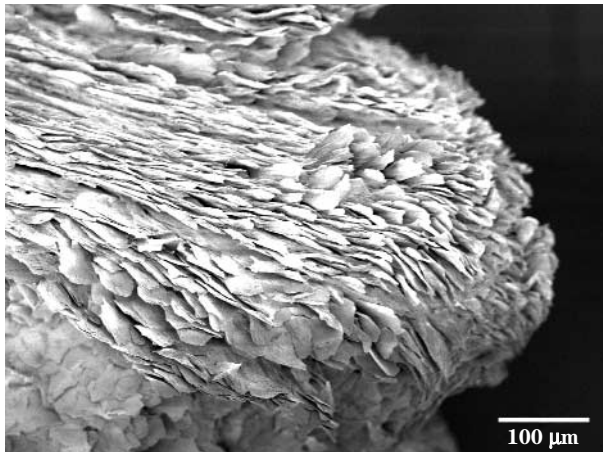


Figure 3. Distal view of the ventral part of the toenail exhibiting its unique structure and morphology ($\times 190$).

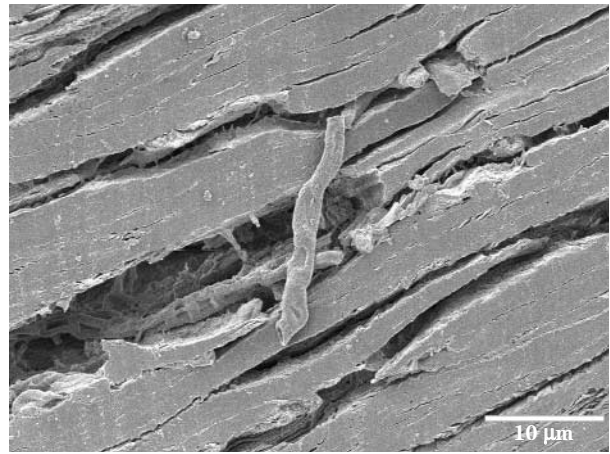


Figure 4. Lateral view of the cross-sectioned nail shows fungal hyphae integrating between nail plates ($\times 2,500$).



Figure 5. Heart-shaped fungal hypha present on the ventral toenail ($\times 600$).

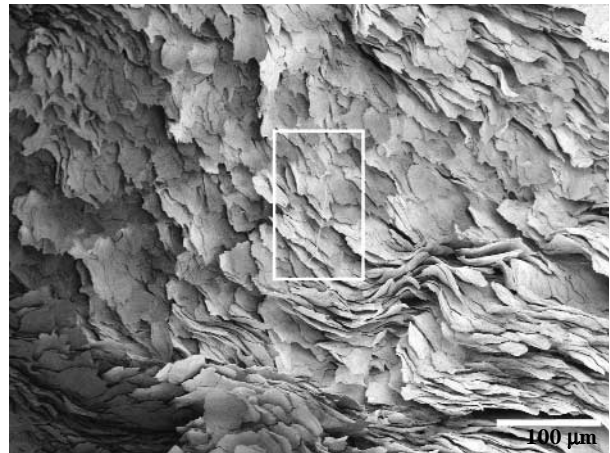


Figure 6. The ridged ventral nail plate with its tilelike appearance provides an ideal environment for fungal growth. The white box indicates the location of *Trichophyton rubrum* hyphae ($\times 220$).

nail. A fungal hypha is clearly shown in Figure 6 running along the keratin plates of the toenail. Further investigation of the element illustrates the unique fungal morphology characteristic of *T rubrum* (Fig. 7). The average size of the fungal hypha is approximately 2 to 5 μm , with macroaleuriospores reaching a size of 10 μm . Many of the fungi present on the ventral side of the toenail showed evidence of penetration into the corneocytes; Figure 8 illustrates a hyphal element protruding from the toenail in patient 2. Figure 9 presents a magnified view of the area in Figure 8 enclosed in a white box. In contrast to the fungal presence in corneocyte layers in Figure 4, this fig-

ure contains a probable site of keratin digestive activity due to the manner in which the fungus is penetrating the nail.¹⁴

Throughout the layers of the toenail in patient 2, arthrospores were seen (Fig. 10); in *T rubrum*, they are classified as spores or segments produced by fragmentation of septate hyphae.⁸ These elements were isolated to the distal part of the ventral side of the patient's toenail and appeared to be penetrating the corneocytes (Fig. 11). *Trichophyton rubrum* also tends to reproduce through branching, in which a hyphal element bifurcates on the outermost layer of the toenail (Fig. 12).

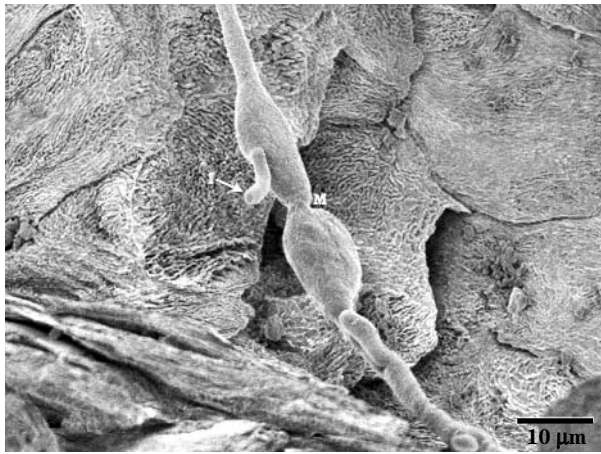


Figure 7. Magnified view of the framed part of Figure 6 illustrating the substructure of fungus. The microaleuriospore (I) projects from one of the two macroaleuriospores (M) ($\times 1,600$).

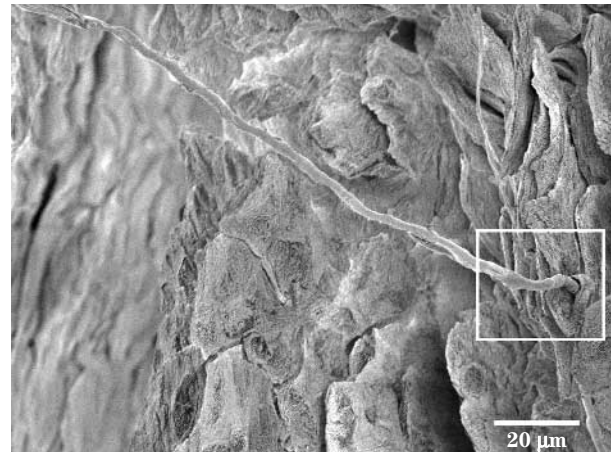


Figure 8. Fungal element suspended above the ventral side of the nail. The white box indicates a hyphal projection from the corneocyte ($\times 900$).

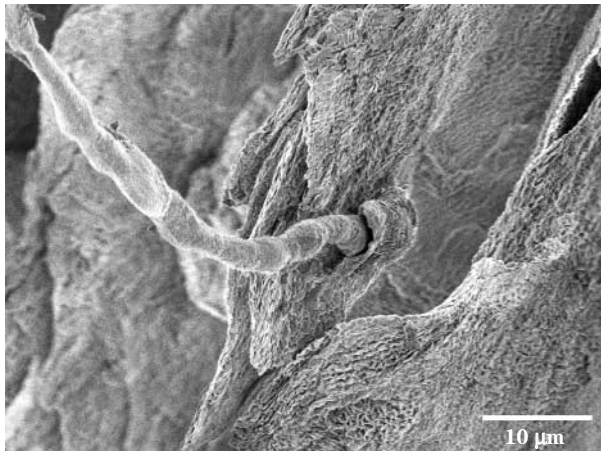


Figure 9. Magnified view of the framed part of Figure 8 illustrating penetration of a fungal element into the corneocyte ($\times 2,300$).

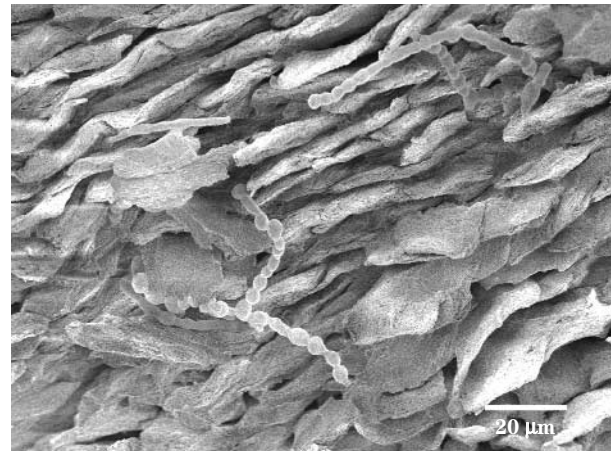


Figure 10. Micrograph of the *Trichophyton rubrum* arthrospore fungal element ($\times 850$).

Discussion

Scanning electron microscopy provides valuable insight into the growth characteristics of the fungi associated with onychomycosis. The SEM allows for far more information about the spatial interaction between dermatophytes and the nail plate than any other medical diagnostic tool. From the study by Meyer et al,⁵ we ascertained the approximate location of the fungi and their probable appearance under the microscope. Although principally investigating *T mentagrophytes*, the study by Meyer et al⁵ concluded that the better resolution, higher magnification, and

enormous focal depth of the SEM give it a great advantage over the light microscope.

Our investigation of the samples taken from two geriatric patients proved complete in providing positive identification of the fungal elements discovered. After laboratory genus and species identification, we proceeded to find hyphae under the SEM. Mycology literature gives insight into the morphologic characteristics of *T rubrum*. According to the fungus identification manual by Funder,¹⁴ the elements present in our samples shared the same characteristics as those listed in his Table 78b. The fluorescent KOH mount illustrating growth on the skin is similar to that of

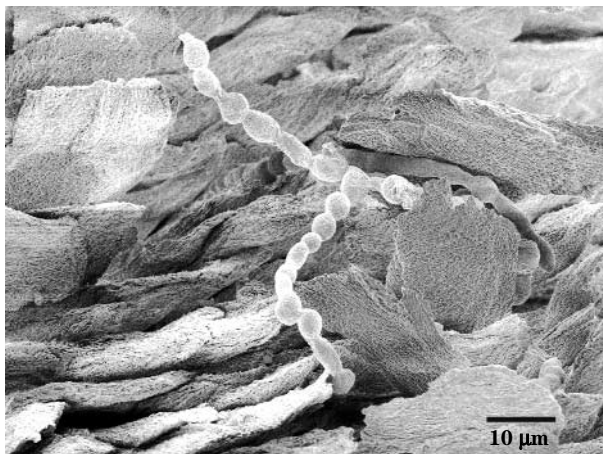


Figure 11. Magnified view of a fungal arthrospore. An arthrospore is a spore or segment produced by fragmentation of septate hyphae ($\times 1,400$).

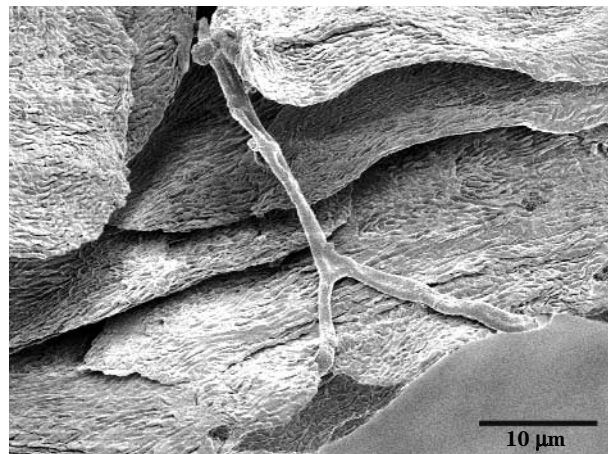


Figure 12. Bifurcating hyphal element present in the layers of the toenail. *Trichophyton rubrum* has the ability to branch off into many different strands of hyphae ($\times 2,500$).

our sample (Figs. 6–9); however, the elements in our samples were mostly isolated fungal hyphae as opposed to structured colonies. The characteristic cigar shape of the macroconidia, as shown in the manual by Funder,¹⁴ closely resembles Figure 7 and supports the laboratory's identification of the genus and species of the dermatophyte infection of the two geriatric patients. Figure 7 illustrates fungal growth characteristics common to *T rubrum*, including the usual fluffy thallus consisting of long strands of hyphae with small, lateral, characteristically tear-shaped or peg-shaped microaleuriospores.⁸

Although many of the fungal hyphae were preserved well in SEM preparation, the elements found in the sample from patient 1 had shrunk considerably and compacted the characteristic fungal shape into one resembling a ribbon (Figs. 4 and 5). The samples from patient 1, however, illustrated the pervasive integration of the dermatophyte into the layers of the patient's nail. As demonstrated in the study by Meyer et al⁵ of the enzymatic destruction of onychomycosis, the SEM allows for visualization of intercellular penetration of fungal hyphae into the nail plate between the corneocytes. Figure 8 in our investigation supports the ability of species in the *Trichophyton* genus to penetrate the nail plate. The fungal element is suspended in the toenail and remains well preserved. On closer examination, we observed evidence supporting direct fungal penetration of the corneocytes (Fig. 9). The hypha, compared with Figure 4, further illustrates the pervasive integration of the species into the nail plate.

The reproductive structures of the fungal infection were also discovered at the distal part of the ventral side of the toenail. On close examination, we concluded that the fungi in Figures 10 and 11 were reproducing actively in these locations. Figure E in *Dermatophytes*, by Rebell and Taplin,⁸ illustrates the microscopic appearance of *T rubrum* arthrospores and supports the presence of these elements in our sample. Dermatophytes, including *T rubrum*, exhibit reduced sporulation in culture and depend on arthrospores produced in scalp hairs and desquamating human epidermis.

Conclusion

The SEM allows for greater insight into the intricate structure and behavior of onychomycosis than any other medical diagnostic tool. By using various techniques, we obtained high-resolution 3-D images of onychomycosis caused by the fungus *T rubrum*. Our photomicrographs illustrate the pervasive integration and penetration of the fungus hyphal elements, underscoring the clinical difficulty of obtaining rapid treatment of fungal infections in the distal and lateral subungual space of the human toenail. The images of *T rubrum* obtained with the SEM show the spatial orientation and pervasiveness of these fungi associated with onychomycosis of the toenail with greater detail than has ever been reported in the medical literature. Although the SEM may not be used by most physicians treating patients infected with onychomycosis, it remains an invaluable tool for the researcher

to obtain new insight into the spatial orientation, behavior, and appearance of onychomycosis caused by *T rubrum*.

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