Laboratory Mill for Pulverizing and Homogenizing Nail Specimens as an Aid to Microscopy and Culture Confirmation of Onychomycosis

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A small mill has been developed for reducing nail clippings to a convenient size for microscopy and inoculation onto isolation media. The milling process acts to pulverize and homogenize the specimen. The use of a homogeneous sample in microscopy increases the opportunity for the discovery of fungal hyphae in a specimen. The use of a pulverized specimen increases the possibility of culture isolation by permitting greater numbers of potentially viable fungal cells to come into direct contact with the isolation medium.

The diagnosis of onychomycosis should be based upon microscopic confirmation of fungal elements present in the suspected nail specimen and isolation and identification of the pathogenic organism (2, 4, 5, 7). Preparing a nail specimen for microscopic examination can be frustrating, particularly if one is not able to take the specimen oneself. We have received for laboratory diagnosis nails of all shapes and sizes, from particles and small clippings to excised nails complete with fingernail polish. The nail is tough and resilient, and defies sub-division with tenacity. We were pleasantly surprised one day to receive a specimen that contained nail particles of a discrete and uniform size. On inquiring how this particular specimen had been taken, we learned that a burr was used to grind down the nail. These particles, when treated with 10% KOH and gently warmed, spread out rapidly and evenly beneath a cover slip and preserved cellular morphology and fungal filaments. With the knowledge of the size nail particle we desired, a small mill was designed and constructed.

MATERIALS AND METHODS

Construction of mill. The construction of the nail mill is relatively simple and can be accomplished by most machine shops. The two holders are cut from a piece of 2-inch (5-cm) stainless steel or aluminum round stock and are turned, trued, and knurled on a lathe. The two milling heads are cut from a piece of ½-inch (1.6-cm) stainless-steel round stock and are trued on a lathe and then set up on a miller with a ½-inch (0.08-cm) end mill used to construct the cutting surface on the milling heads (Fig. 1). The weight of the assembled mill made with aluminum holders averages 230 g, and with stainless-steel holders, approximately 520 g.

When assembling the mill, one cutting head is inserted into the large holder and recessed approximately ⅛ inch (1.27 cm). It is held in place with two ¼-inch (0.64-cm)—20 NC by ¼-inch (0.95-cm) cup point socket set screws (Fig. 2). The second cutting head is then inserted into the small holder so as to protrude approximately ⅛ inch below the holder (Fig. 3). The head (on the smaller holder) is then inserted into the milling pit of the large holder and is spaced with a piece of shim stock (approximately 0.004 inch (0.1 mm)) to set the distance between the cutting heads. In the laboratory, a piece of white bond paper (approximately 0.004 inch) may be substituted for the shim stock in adjusting the distance between cutting surfaces.

Preparation of specimen. Nail specimens are placed in the mill cavity. A single large nail fragment or several small ones may be used, but care should be taken not to overload the mill cutting surfaces. The mill halves are firmly pressed together and rotated in opposite directions, about half a turn, several times. Progress of the disintegration is visually monitored by removing the small holder. The major particle size is determined by the spacing between the teeth of the milling surface.

The particles are concentrated on one edge of the milling pit by gently tapping the mill half on the table at a slight angle to the horizontal. A large number of nail particles may be rapidly obtained in this manner (Fig. 4). It is convenient to transfer these small particles by using a glass rod or dissecting needle. A drop of 10% KOH is placed on a
microscope slide, and the transfer needle is touched to this drop so as to moisten the tip. The transfer needle is then touched to the pile of nail fragments, a number of which readily adhere. The transfer needle and nail particles are then returned to the KOH drop on the microscope slide, and the nail particles are deposited.

Gently warm the slide containing KOH and nail particles without a cover slip and set the slide aside for 10 to 15 min to allow the particles to soften. The nail particles may be further reduced in size by macerating with the end of a small glass rod which has not been fire-polished. This step is optional, as it is possible but not practical to reduce the nail particles to their component cells. A drop of water or KOH may be added to reconstitute the preparation prior to depositing the cover slip. A cover slip is touched to this mass of cell material at an angle to reduce trapping of air bubbles in the final preparation. The cover slip may be gently pressed down on this mass with the eraser end of a pencil to flatten and spread out the softened nail particles. Ideally, a unicellular layer would be best for viewing, but is rarely attained throughout a preparation. We prefer to make four to six double preparation slides from each specimen, as a negative microscope finding based on the examination of one slide is not very meaningful.

**Isolation.** When an attempt to isolate a culture of the pathogen from a diseased nail is made, we have found that the milled nail particles are ideal for making multiple inoculations. The end of a sterile transfer needle is moistened in the isolation agar and touched to the pile of nail particles in the mill pit. The adhering particles are then embedded and streaked across the isolation agar. It is preferable to make at least a dozen inoculations with visible nail particles deposited at each inoculation site. Generally three inoculations are made per plate, or three slants of each medium may be used.

The choice of isolation media varies with the investigator. Mycosel (BBL) or Mycobiotic Agar (Difco) are commercially available agars useful for this purpose, as are also pH indicator media such as DTM or ink blue agar (1-3). Our personal preference is to use several media for each specimen. We routinely include Sabouraud dextrose agar, Littman oxgall agar, corn meal dextrose agar, and a personal concoction (1111G agar) composed of 0.1% yeast

![Fig. 1. Close-up of protruding cutting head with "waffle iron" type cutting pattern and toenail clippings prior to milling. Scale marker represents 1 inch.](image1)

![Fig. 2. Assembled mill. Lateral view of knurled holders with channels in which socket set screws hold milling (cutting) heads in place. Excess length of cutting head protrudes above holder. Scale marker represents 1 inch.](image2)

![Fig. 3. Mill halves. View of cutting surfaces as they are locked into knurled holders. The left half bears the recessed cutting head and and the right half bears the protruding cutting head. Scale marker represents 1 inch.](image3)

![Fig. 4. Milled nail fragments compared against millimeter scale and dissecting needle used to transfer fragments to microscope slide or agar media.](image4)
extract, 0.1% dextrose, 0.1% soluble starch, 0.1% CaCO₃, 1.5% agar, and 100 μg of the antibiotic gentamicin per ml available as Gentamicin Reagent Solution, Schering Diagnostic Division, Port Reading, N.J. 07064). *Trichophyton rubrum* is the fungal species most frequently isolated from nail specimens in our geographical area, as was also reported for skin, nail, and hair specimens by Rosenthal and Furnari (3). The inclusion of the last three of the above media allows a rapid presumptive identification of *T. rubrum*, the colonies of which appear small, bristly, and yellowish-tinged on Littman's oxgall agar; white, thin, and spreading on corn meal dextrose agar, with a characteristic cherry-red diffusible pigment; and white, thin, and widely spreading on the 1111G agar, with a characteristic maroon diffusible pigment rapidly developing. The latter two media are also good places to search for the typical *Trichophyton* microconidia and mycelial configurations, such as spiral hyphae, raquet hyphae, pectinate bodies, and nodular organs. Other dermatophytic fungal species also develop characteristically on these media.

The nail mill after use should be sterilized by steam or boiling. A clean dissecting needle has been found useful to remove adhering particles from the milling surface teeth, or the mill may be disassembled and cleaned with a small wire brush.

Photomicrographs were taken with a standard GFL Zeiss microscope equipped with phase-contrast optics.

**RESULTS**

The following observations have been made on the use of pulverized, homogenized nail particles. (i) Often, clearly defined hyphae or portions of hyphae are found free from nail cells. It appears that nail cells often separate along planes that are traversed by fungal filaments, the filaments becoming free much as individual pebbles in a weakly consolidated rock conglomerate may be found to be easily dislodged from the matrix (Fig. 5-8). (ii) Most cells of the nail when subjected to milling remain intact, rupture occurring between the cells. Small clusters of a few cells are easily scanned for fungal filaments (Fig. 5). (iii) Small clusters of nail cells are more readily softened by the action of KOH than are large nail fragments, which may require several hours or overnight KOH treatment (Fig. 9 and 10). Fewer bizarre structures are introduced from the KOH digestion by a shorter softening period. (iv) Fungal hyphae appear little damaged by the milling treatment of the nail and are more readily detected in microscope mounts and more easily cultured from the nail particles.

Isolation of the pathogenic organism is not always easy (2, 3, 7) and depends upon the condition of the nail specimen and previous therapy which may have impaired the viability of the fungus. Data presented in Table 1 (G. Baker and R. Kishimoto, personal communication) were obtained in a laboratory study in which one of the nail mills described in this paper was used. Although representing a small number of specimens, the results are in favorable agreement with a larger number of specimens run in our laboratory.

A case in our laboratory requiring persistence involved a patient who was clinically diagnosed for onychomyositis and treated with griseofulvin without results. After treatment had been discontinued, nail specimens were submitted for microscopy and culture, but they produced only a rare microscopic positive of degenerative, bizarre-appearing hyphae and were culture-negative except for an occasional saprophyte. Periodically over a 3-year period, we received additional nail specimens which gave similar results. Finally, the patient came to the laboratory concerned about the appearance of her large toe nail which was raised and unattached to the nail bed for 75% of its length. The raised portion of the nail was marked off into an outer, middle, and inner portion and painlessly clipped back to the point of attachment to the nail bed. The three portions of nail clippings were individually homogenized in the mill and cultured. Culture was negative for the outer portion of the nail. From the middle portion of the nail, three unidentified cultures of sterile mycelium, one culture of a *Penicillium* species, and one culture of a *Fusarium* species were obtained. From the inner portion of the nail, 12 cultures were recovered which produced a dark-violet diffusible pigment and produced typical *Fusarium*-type macroconidia. Microscopic examination of nail fragments from this inner portion of the nail revealed a number of bizarre hyphae atypical of the even-diameter hyphae found usually in *T. rubrum* infections but similar to those previously reported for *Fusarium* isolates from nail (4, 5). Failure of the patient to respond to griseofulvin therapy was substantiated by the belated isolation and culture of this organism. Multiple isolations of an organism which commonly occurs as a saprophyte from the area of active invasion provided a confidence factor for reporting this organism, which upon a single culture isolation would have been doubtfully considered as a pathogen.

**DISCUSSION**

The positive microscopic identification of fungal filaments in a nail specimen is conclu-
sive evidence of fungal involvement, but unfortunately a negative microscopic result is not absolutely conclusive. It may mean several things: the diseased condition is not due to a fungus (6); the fungal hyphae were not present in the specimen taken; or the fungal filaments in the preparation have eluded the observation of the microscopist. The problem of microscopically identifying fungal filaments in nail specimens is often complicated by the number of cell layers which must be traversed by the microscopist before a positive identification can be made. Fungal filaments passing between the nail cell layers often require focusing on several planes to differentiate the fungal hyphae from intercellular artifacts. The presence of these multilayers of epidermal cells often obscures the true outline of the fungal hyphae. A single layer of nail cells, if it could be obtained, would be ideal for microscopic examination. Nail specimens which have been reduced to a small particle size in the mill described have made positive identification of

<table>
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<th>Specimen</th>
<th>Without homogenizing</th>
<th>With homogenizing</th>
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<tr>
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<tr>
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<td><em>Candida albicans</em></td>
</tr>
<tr>
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<td>0</td>
<td><em>Alternaria</em> sp.</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
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<tr>
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<td>0</td>
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<tr>
<td>6</td>
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<td>0</td>
</tr>
<tr>
<td>7</td>
<td><em>Curvularia</em> sp.</td>
<td><em>Curvularia</em> sp.</td>
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<tr>
<td>11</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
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fungal filaments rapid and relatively free from false-positive results.

Nail specimens which have been found to contain fungal hyphae by microscopy often fail to yield positive cultures (2, 7). This condition is frustrating and often requires obtaining additional nail specimens from a patient. The use of a pulverized nail specimen increases the opportunity for bringing viable fungal cells into direct contact with the isolation medium and aids in obtaining multiple isolations of the pathogenic fungus.

A schematic diagram suitable for a machinist to reproduce this mill will be included with reprint requests.

LITERATURE CITED