A modified approach to the histologic diagnosis of onychomycosis

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Background: Histologic examination of nail clippings with periodic acid—Schiff staining is the most sensitive diagnostic test for onychomycosis; however, difficulties in processing nail plates limit its use. In onychomycosis, fungi are most concentrated in the subungual hyperkeratosis rather than in the nail plate. We hypothesized that the diagnosis of onychomycosis could be effectively made from histologic examination of subungual hyperkeratosis alone. Specimens of subungual hyperkeratosis, unlike nail plates, can be processed in the same routine manner as skin specimens, allowing for the diagnosis of onychomycosis to be made more quickly and at lower cost.

Objective: We investigated whether the diagnosis of onychomycosis could be effectively made from histologic examination of subungual hyperkeratosis alone.

Methods: We selected all nail specimens submitted during an 8-month period to the New York University Dermatopathology Section for evaluation of onychomycosis that had subungual hyperkeratosis associated with the nail plate. Nail specimens were divided into two components: a subungual hyperkeratosis component and a nail plate component. The subungual hyperkeratosis was processed separately in a routine fashion and embedded in paraffin and examined. We determined the percentage of cases of onychomycosis in which hyphae were present in the subungual component.

Results: Sixty-six cases of onychomycosis were diagnosed histologically during the study period. Ninety-seven percent of these cases had hyphae in the subungual component. In 3% of cases, hyphae were present in the nail plate component but not in the subungual component.

Limitations: This modified approach to diagnosing onychomycosis can only be utilized when an adequate amount of subungual hyperkeratosis is submitted.

Conclusions: The diagnosis of onychomycosis can be effectively made from histologic examination of subungual hyperkeratosis alone in most cases. This method circumvents the need to process nail plates in the vast majority of cases of onychomycosis (97%), resulting in a more efficient, less costly, and technically easier way of diagnosing onychomycosis. Submitting ample amounts of subungual hyperkeratosis is essential to increasing the diagnostic yield of nail clippings. (J Am Acad Dermatol 2007;57:849-53.)

Potassium hydroxide preparations and fungal cultures have limited sensitivities for the diagnosis of onychomycosis. It has been reported that histologic examination with periodic acid—Schiff (PAS) stain of nail clippings is the most sensitive diagnostic test for onychomycosis.[1] Processing nail plates for histologic examination is technically demanding and potentially very time consuming.

As onychomycosis progresses, subungual hyperkeratosis accumulates and produces onychodystrophy and distal onycholysis (Fig 1). Although subungual hyperkeratosis is often collected for potassium hydroxide preparations and fungal cultures, it is infrequently collected for histologic sections. Indeed, nail clippings target the nail plate instead of subungual hyperkeratosis. Yet experience has shown that hyphae, when present, are most concentrated in the subungual hyperkeratosis rather than the nail plate. In addition, when in the

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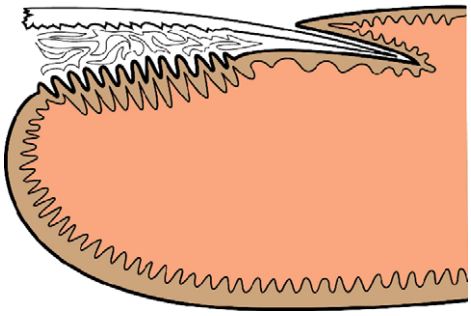

Fig 1. Schematic of nail with accumulation of subungual hyperkeratosis and distal onycholysis.

nail plate, hyphae are predominantly in the deepest ventral portion of the nail plate. We hypothesized that examination of subungual hyperkeratosis alone would be equally or more effective in diagnosing onychomycosis as current methods that examine the nail plate with or without associated subunugual hyperkeratosis. By bypassing the difficulties associated with processing the hard keratin of the nail plate, the diagnosis of onychomycosis could be made more efficiently and at lower cost.

We describe a modified approach to the histologic examination of onychomycosis based on examination of subungual hyperkeratosis rather than nail plates with or without subungual hyperkeratosis. Although prior studies have examined the efficacy of histologic examination of nail clippings in diagnosing onychomycosis,[1,2] the histologic examination of subungual hyperkeratosis alone has not been studied.

METHODS

We reviewed all nail specimens submitted between May 1, 2002 and December 31, 2002 that were sent for histologic evaluation for onychomycosis. All specimens consisted of nail clippings. We identified 87 cases in which subungual hyperkeratosis was available for study with or without a nail plate. When attached to the hard keratin of the nail plate, the subungual hyperkeratosis was manually removed from the nail plate and processed separately (Fig 2). This method was used only when an infectious etiology was considered by the clinician. The subungual hyperkeratotic material was removed with routine grossing instruments either as a thin shave separating the nail plate from the underlying hyperkeratosis, or as crumbly dyscohesive fragments. The subungual hyperkeratosis component (either a thin shave or fragments) was wrapped in 2-inch square lens paper before tissue processing. This method allowed for retention of even tiny fragments of keratotic material.

Thus, specimens were divided into two components: a subungual component (consisting of subungual hyperkeratosis that was removed from the nail plate) and a nail plate component (consisting of the nail plate and any residual subungual hyperkeratosis still attached). All specimens underwent routine formalin fixation. Initially, only the subungual hyperkeratosis components were routinely processed and embedded in paraffin. Nail plate components were processed in those cases in which examination of subungual hyperkeratosis was negative for hyphae. Nail plates were embedded in plastic medium (2-hydroxyethyl methacrylate); charged slides were used for the plastic-embedded sections.

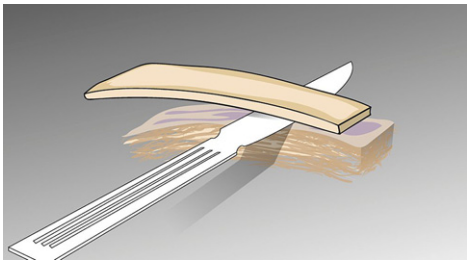

Fig 2. Method of separating subungual hyperkeratosis from nail plate.

For histologic examination, specimens were stained with both hematoxylin-eosin and either PAS with diastase for paraffin-embedded specimens, or PAS without diastase for plastic embedded specimens. The diagnostic criteria for onychomycosis consisted of finding hyphae within either the nail plate or subungual hyperkeratosis. Budding yeast and pseudohyphae did not qualify for the diagnosis of onychomycosis.

RESULTS

Eighty-seven nail specimens containing subungual hyperkeratosis were submitted for histologic evaluation of onychomycosis between May 2002 and December 2002. Sixty-six cases were diagnosed as onychomycosis and 21 cases were diagnosed as dystrophic nail. In cases of onychomycosis, hyphae were generally numerous and most prominent in the subungual hyperkeratosis followed by the deepest portion of the nail plate (Fig 3). Of the 66 cases of onychomycosis, 64 cases (97%) demonstrated hyphae in the subungual component (Fig 4). There were 7 cases of onychomycosis in which both the nail plate and the corresponding subungual component were processed simultaneously. In all 5 cases in which the subungual component demonstrated hyphae, the nail plate component also demonstrated hyphae.

In 2 cases the subungual component lacked hyphae, but the nail plate component was positive

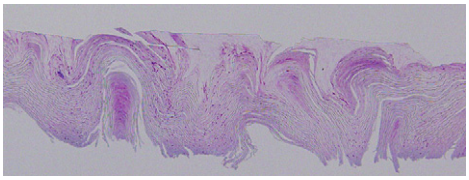

Fig 3. Nail plate with attached subungual hyperkeratosis. Numerous PAS-positive hyphae are present and located mainly in the subungual hyperkeratosis. Few hyphae are present in lowermost ventral portion of the nail plate. (Original magnification: ×100.)

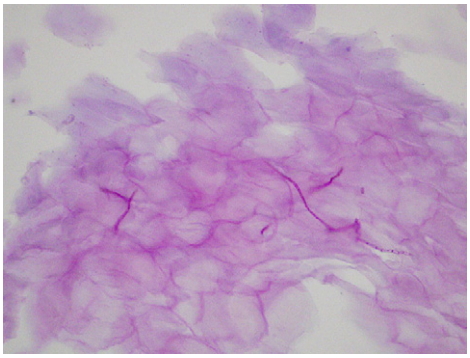

Fig 4. Fragments of detached subungual hyperkeratoses harboring numerous PAS-positive hyphae. (Original magnification: ×200.)

for fungi. In one case, the size of the subungual hyperkeratosis component was very small. Interestingly, the corresponding nail plate component had a significant amount of subungual hyperkeratosis that was still attached to the nail plate and which contained hyphae; however, hyphae were not present in the nail plate itself. In the second case, the size of the subungual component was adequate. The corresponding nail plate had only a small focus of hyphae.

Twenty-one biopsy specimens were diagnosed as dystrophic nail. In these cases, both the subungual and the nail plate components were negative for hyphae.

DISCUSSION

The sensitivity of fungal culture for detecting dermatophytes has been reported to be from 25%[1] to 80%,[2] and the false-negative rate for cultures and KOH mounts is estimated to be 30%.[1] In addition to having poor sensitivities, both fungal cultures and KOH mounts are time consuming. Nail biopsies (including nail clippings) have been shown to be a valuable adjunctive tool for diagnosing onychomycosis and have been shown to detect dermatophytes missed by microscopic examination of KOH mounts and nail cultures.[1-3] Nail clipping examination with PAS stain has been reported to have a sensitivity of 85%.[1] Our prior experience at the New York University Dermatopathology Section has shown that nail plate processing in plastic-embedded medium is very efficacious for the histologic diagnosis of onychomycosis. However, it is technically difficult, costly, and time consuming.

Processing nail specimens using the same method as skin specimens frequently results in inadequate sections for histologic examination.[4] It is often too difficult to cut the hard keratin of the thickened nail plate in paraffin by a microtome.[5,6] This hardness may cause the nail plate to be dislodged from the paraffin block during cutting and lost.[4] In our experience, extra fixation of nails in 10% neutral buffered formalin has no effect on softening nails during paraffin sectioning. Softening nails requires momentary or permanent breakage of the disulfide linkages present in nails, which a cross-linking fixative like formaldehyde cannot do.

Because of these difficulties, numerous softening agents have been suggested. These include combinations of KOH followed by a solution of detergent and ammonia,[5] 5% trichloroacetic acid in 10% formalin, 5% trichloroacetic acid with a modification of the water soluble Carbowax embedding method, chitin-softening agent,[2] cedar oil,[5] and 10% Tween-40.[7] The application of permanent-wave solutions or a depilatory preparation to regularly fixed and embedded nails in paraffin blocks every 2 to 3 slices of microtome has also been described.[6] These methods do work when the nail plate is thin ($\leq$1 mm) to produce a limited number of sections. However, soaking agents do not work well when the nail plate is thick ($\geq$2 mm). In addition, unlike skin, nail plates adhere poorly to glass slides.[5,6] Gelatin-coated slides have been reported to enhance adherence of nails.[2,6] Our experience with the use of casein (Elmer's Glue) is that the adhesive produces higher backgrounds during staining, and it does not prevent the plate from curling away from the glass slide during drying. Plastic embedding has also been described.[8]

In our laboratory, plastic embedding of nail plate specimens has been superior to the use of softening agents. Softening agents soften only the surface of the nail plate, and repeated soaks are frequently needed for adequate sections. In addition, the sections produced vary in thickness. Plastic embedding allows for thinner, uniform sections, which also decreases the possibility of dislodging the nail plate. Unlike paraffin, plastic processing covalently binds with the tissue during polymerization and allows thin sections to be produced from hard tissue. Sections with thicknesses of 2 to 2.5 μm can be cut from plastic-embedded specimens in contrast to standard

paraffin sections that are 5 μm in thickness. Plastic sections adhere very well on a variety of coated slides used for immunostains (e.g., silanized slides or Fisher Superfrost). However, plastic embedding takes approximately 2 weeks and is costly,[4] requires specialized labor, expensive reagents, a motorized microtome, and glass knives. There are no automated steps with plastic embedding and all steps must be hand processed. The processing time (and subsequent staining time) is longer for plastic-embedded sections than paraffin-embedded sections. Of note, a few reports have described successful processing of nail specimens without special procedures[1,3]; however, the plethora of different softening agents and techniques for nail processing described in the literature suggests that in most instances, special techniques are needed to produce adequate sections for histologic analysis.

In onychomycosis, there is subungual orthokeratosis and parakeratosis with variable amounts of inflammation. Hyphae are generally numerous and localized to the subungual hyperkeratosis of the nail bed more than the nail plate. Moreover, when in the nail plate, hyphae usually reside in the deepest (ventral) portion of the nail plate.[9] Indeed, cultures from subungual debris are more likely to yield positive results, compared with cultures from the nail plate alone.[10,11] Our experience supports these observations. This suggests that subungual hyperkeratosis may be the ideal material for nail biopsies performed to evaluate for onychomycosis. We postulated that examination of subungual hyperkeratosis alone may be equally effective in diagnosing onychomycosis as examination of the nail plate. In addition, because subungual hyperkeratosis can be processed in the same manner as skin specimens, the time, technical difficulty, and cost would be significantly reduced. Ultimately, sectioning and staining the subungual hyperkeratotic tissue is easier than including the nail plate and is the diagnostically significant area to demonstrate organisms. The in vivo relationship of the organism to the nail plate is clinically irrelevant, so fragments of subungual hyperkeratosis alone can initially be examined to make the diagnosis.

Our study of 87 nail specimens clinically suspected of being onychomycosis showed that the histologic diagnoses of most (66/87) of these cases were onychomycosis. Hyphae were seen in the subungual hyperkeratosis component in 64 of the 66 cases of onychomycosis. In cases in which both the subungual and nail plate components were examined, the presence of hyphae in the subungual hyperkeratosis correlated with the presence of hyphae in the nail plate component. In 2 cases, the subungual components did not demonstrate hyphae that were seen in the nail plate components. In one case, inadequate removal of the subungual hyperkeratosis from the nail plate during processing resulted in the small size of the subungual component, which likely accounts for the false-negative result. The subungual hyperkeratosis that remained attached to the nail plate contained hyphae. If the subungual hyperkeratosis had been well separated from the nail plate, one would expect that the subungual component would have been positive for hyphae.

Because a small number of cases of onychomycosis can be missed by examination of the subungual tissue alone, we recommend processing and examining the nail plate in cases when the subungual component lacks hyphae, particularly when the size of the subungual component is small. Histotechnicians need to be trained to separate subungual hyperkeratosis from nail plates to further increase the sensitivity.

It is likely that in cases of white superficial onychomycosis the diagnosis may be missed by histologic examination of subungual hyperkeratosis alone. In white superficial onychomycosis, hyphae are on the surface of the nail plate. This subtype accounts for a minority of cases of onychomycosis. In these cases, we suggest submitting scrapings from the white affected portion of the nail plate for histologic examination. In proximal subungual onychomycosis, we suggest submitting subungual debris from the proximal nail (the subungual debris could be obtained by paring the proximal nail with a scalpel). In the most common form of onychomycosis, distal and lateral subungual onychomycosis, the use of a small curet to obtain subungual debris after clipping the nail plate as proximal as possible to obtain a sample for diagnosis has been described.[12] However, we prefer the submission of clippings of the nail plate with the attached underlying subungual hyperkeratosis.

It might also be argued that without seeing actual nail plate invasion by hyphae, the diagnosis of onychomycosis should not be rendered. However, it is not uncommon in onychomycosis to see rare or no hyphae in the nail plate despite seeing numerous hyphae in the subungual hyperkeratosis, especially in early cases. Multiple sections may have to be examined to detect hyphae in the nail plate. Most important, the presence of hyphae in subungual hyperkeratosis denotes pathology. It might also be argued that one cannot differentiate psoriatic nails secondarily colonized by dermatophytes from simple onychomycosis. It is well known that psoriasis can mimic onychomycosis and that the presence of

hyphae can be the only differentiating factor. We agree with Fleckman and Omura[5] that hyphae are seen only in fungal infections. The presence of hyphae in either subungual hyperkeratosis or nail plate indicates a pathologic state, unlike the presence of the budding yeast or pseudohyphae of *Candida*, whose significance is debated.[3,5] Thus the finding of hyphae in subungual hyperkeratosis indicates onychomycosis, but does not rule out other concomitant causes of nail dystrophy. In such cases, clinical correlation would be helpful.

Nail biopsies are particularly valuable in determining whether an organism identified from fungal cultures is pathogenic or merely a contaminant.[6] In these cases, finding hyphae within subungual hyperkeratosis would argue against a contaminant.

In conclusion, our modification to the histologic diagnosis of onychomycosis consists of separating the subungual hyperkeratosis and initially processing and examining only this component. The nail plate would be held and not processed unless examination of the subungual hyperkeratosis was negative for hyphae. With this method, significantly fewer nail plates would be processed, resulting in a more efficient, easier, and less costly method of diagnosing onychomycosis. In our series of 87 nail specimens, only 23 (26%) would require nail plate processing. Because the nail plate would be processed in all cases where the subungual hyperkeratosis component is negative, the sensitivity of histologic diagnosis of nail biopsies would not be compromised. The additional time in processing the subungual hyperkeratosis separately from the nail plate would be only 1 or 2 days. Finally, dermatologists should know that submitting ample amounts of subungual hyperkeratosis is key to increasing the diagnostic yield of nail biopsies for onychomycosis.

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REFERENCES

1. Lawry MA, Haneke E, Strobeck K, Martin S, Zimmer B, Romano PS. Methods for diagnosing onychomycosis. Arch Dermatol 2000;136:1112-6.
2. Suarez SM, Silvers DN, Scher RK, Pearlstein HH, Auerbach R. Histologic evaluation of nail clippings for diagnosing onychomycosis. Arch Dermatol 1991;127:1517-9.
3. Machler BC, Kirsner RS, Elgart GW. Routine histologic examination for the diagnosis of onychomycosis: an evaluation of sensitivity and specificity. Cutis 1998;61:217-9.
4. Mondragon G. Histotechnologist to histopathologist: a method for processing specimens of nails. Dermatopathol Practical Conceptual 1996;2:41-2.
5. Fleckman P, Omura EF. Histopathology of the nail. Adv Dermatol 2001;17:385-406.
6. Omura EF. Histopathology of the nail. Dermatol Clin 1985;3:531-41.
7. Pierard GE, Arrese JE, De Doncker P, Pierard-Franchimont C. Present and potential diagnostic techniques in onychomycosis. J Am Acad Dermatol 1996;34:273-7.
8. Alvarez R, Zaias N. A modified polyethylene glycol-pyroxylin embedding method specially suited for nails. J Invest Dermatol 1967;49:409-10.
9. Scher RK, Ackerman AB. Subtle clues to diagnosis from biopsies of nails. Am J Dermatopathol 1980;2:55-7.
10. Harvey CK, Richardson A. Techniques for obtaining specimens for culture to confirm onychomycosis. J Am Podiatr Med Assoc 2000;90:394-6.
11. Hull PR, Gupta AK, Summerbell RC. Onychomycosis: An evaluation of three sampling methods. J Am Acad Dermatol 1998;39:1015-7.
12. Gupta AK, Ryder JE, Summerbell RC. Onychomycosis: classification and diagnosis. J Drugs Dermatol 2004;3:51-6.