Successive Mycological Nail Tests for Onychomycosis: A Strategy to Improve Diagnosis Efficiency

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Onychomycosis is a fungal infection of nails caused by dermatophytes, yeasts and moulds, accounting for about 50% of onychopathies. A high frequency of onychomycosis caused by *Candida* species has been reported during the last few years in northeast Brazil, as well as in other regions of the world. A clinical diagnosis of onychomycosis needs to be confirmed through laboratory exams. We evaluated the importance of serial repetition of direct microscopic exams and fungal culture for the diagnosis of onychomycosis in the city of Fortaleza, Ceará, in northeast Brazil. We first made a retrospective study of 127 patients with onychomycosis, identifying the fungi that had been isolated from fingernails and toenails. We then made a prospective study of 120 patients, who were submitted to three successive mycological examinations. Ungual residues were scraped off and directly examined with a microscope and fungal cultures were made. In the retrospective study, in which only one sample was analyzed, the incidence of onychomycosis was 25.0%. In our prospective study, in which we had data from successive mycological examinations, 37.8% had onychomycosis. The most commonly isolated fungi in both studies were yeasts from the genera *Candida*, especially *C albicans, C. parapsilosis* and *C. tropicalis*. We found a high proportion of onychomycosis caused by *Candida* species. We also concluded that serial repetition of direct microscopic examination and fungal culture, with intervals of 2-5 days improved the diagnosis of onychomycosis. We suggest that this laboratorial strategy is necessary for accurate diagnosis of this type of mycosis, especially when the standard procedures fail to diagnose fungal infection, despite strong clinical suspicion.

Key-Words: Diagnosis, onychomycosis, *Candida*, moulds and dermatophytes.

Onychomycosis is a chronic fungal infection of fingernails and/or toenails, caused by dermatophytes, yeasts and moulds, leading to gradual destruction of the nail plate [1-3]. It is not self healing and may be a source of more widespread fungal lesions of the skin [1]. *Trichophyton* is the most frequent dermatophyte genus found affecting nails. *Trichophyton rubrum* is the cause of most onychomycosis cases, followed by *T. mentagrophytes* and *T. tonsurans* [1,4-6]. Some yeasts, such as *Candida* [1,4,5,7-9], *Trichosporon* [10] and *Malassezia* [11] species, are also able to cause ungual infection, invading the ungual folds and the ungual bed, as well as the ungual sheet. *Candida albicans* predominates in most yeast-caused onychomycosis cases [1,8]. However, other *Candida* species, including *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondi*, *C. krusei* and *C. famata*, have also been isolated in infected nails [1,7-9,12,13]. There have been reports of a high frequency of onychomycosis caused by *Candida* species in northeast Brazil [8,9]. These moulds are saprophytic fungi, normally found in the soil, air and some plants; they are considered opportunistic fungi. Their pathogenic role in onychomycosis is still controversial; however, their isolation in nails is becoming more and more frequent [4,14,15]. *Scopulariopsis* spp., *Fusarium* spp., and *Aspergillus* spp., for example, have been identified in nails as primary pathogens of onychomycosis [1,4,16-18].

Received on 12 April 2008; revised 15 July 2008.

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The Brazilian Journal of Infectious Diseases 2008;12(4):333-337.

Diagnosis of onychomycosis is made by direct microscopic examination and fungal culture [1-3,19,20]. However, when only one sample is analyzed, the frequency of false-negative results is very high. Other laboratory methods, such as ungual biopsy, PCR, flow cytometry and immunohistochemical techniques, have been used to improve onychomycosis diagnosis; however, these methods are not usually available in common dermatology centers [1,21-23]. As a viable alternative in such labs, we investigated whether serial repetition of routine direct microscopy examination and fungal culture improves diagnosis efficacy.

Material and Methods

Patients

We made a two-stage study; the first was a retrospective study of patients with suspected onychomycosis in Fortaleza, Ceará, in northeast Brazil, from January 2004 to August 2006, through analysis of their medical records. The second was a prospective study of 120 patients, who were clinically suspected of having onychomycosis and sought help at a dermatology service. Male and female patients of all ages were included in this study; they all signed an informed consent form. Patients who had undergone treatment with topical antifungals during the previous four months and those who had undergone treatment with systemic antifungals during the previous nine months were excluded from the study. All patients in the prospective study were submitted to anamnesis and had their nails clinically examined. Scrapings were collected from the nails that were most affected, and the samples were separated according to whether they were from fingernails or from toenails. The patient's nails were submitted to three successive mycological analyses. The interval

between each sample collection from the same nail varied from two to five days. We maintained a short interval to ensure isolation of the same fungus in the affected nails. During the sample collection interval the patients could not use any onychomycosis therapy, including alternative medicine. In addition, they were advised that the affected nails could not be scraped or clipped during that period.

Laboratory Methods

Each clinical specimen was processed with 40% KOH for a direct microscope examination; a part of the original sample was cultured on Sabouraud dextrose agar, Sabouraud dextrose agar with chloramphenicol, Sabouraud dextrose agar with chloramphenicol and cycloheximide and Dixon's medium. The cultures on Sabouraud agar were incubated at 25-28°C and at 37°C on Dixon's medium. These cultures were observed once a week for up to three weeks. Identification of dermatophytes was made by observing the macro and micro characteristics of the colonies, microculture in potato agar and biochemical tests. Microculture in potato agar was also used in the identification of the moulds. The following procedures were used to identify the yeasts: microculture in corn meal agar with Tween 80, germination tube tests, as well as zymograms and auxanograms [9].

Diagnosis Criteria

The criteria employed for the diagnosis of onychomycosis in both stages of the study were culture identification of dermatophytes, even with no identification from direct microscopy examination, and positive microscopy examination associated with cultures of yeasts and moulds [17,20].

Statistical Analysis

The data were processed with the software SPSS 10.0. The χ^2 test and the Fisher – Freemann – Halton exact test were used for comparisons, with a significance level of 5%.

Results

Among the 507 mycological records analyzed in the retrospective study, 127 (25.0%) diagnoses of onychomycosis were obtained in accordance with the diagnosis criteria. Among these, 107 (84.3%) were infected by *Candida* spp., 72 being in fingernails, caused mainly ($p < 0.05$) by *C. albicans* (Table 1).

Among the 156 mycological nail examinations in the prospective study, there were only 31 (19.9%) positive diagnoses of onychomycosis in the mycological exams, 25 (80.6%) by yeasts and only one (3.2%) by dermatophytes. In the second analysis (n=125), 13 nail tests that were negative in the first analyses were positive in the second nail evaluation. These new positive diagnoses in the second mycological nail examination were by yeasts (5; 38.5%), moulds (5; 38.5%) and dermatophytes (3; 23.0%). Additionally, 15 mycological nail tests that were negative in the first and second tests were positive in the third mycological analysis (n=112), including two (13.3%), five (33.3%) and eight (53.4%) by dermatophytes, moulds and yeasts, respectively. Fifty-nine diagnoses of onychomycosis were obtained with the three successive mycological nail tests, including 38 (64.4%, $p < 0.05$), 15 (25.4%) and six (10.2%) by yeasts, moulds and dermatophytes, respectively (Table 2).

Among the fungi isolated in the prospective study, *C. albicans* (15, 35.7%) and *C. parapsilosis* (4, 23.5%) were the most frequent yeasts in fingernails and in toenails, respectively. Among the moulds, *Fusarium* sp. (11, 18.6%) was the most commonly isolated fungus, especially in toenails. Dermatophytes were less frequently found, comprised of one case with *T. tonsurans*, two *T. mentagrophytes* and three *T. rubrum* (Table 3).

The incidence of onychomycosis in the retrospective study was 25%; however, the frequency increased with the repetition of the mycological nail analysis in the prospective stage. In the first mycological nail test, an incidence of 19.9% onychomycosis was obtained; in the second mycological nail test, considering the diagnoses in the first test and 13 diagnoses in the second mycological evaluation, the incidence observed was 28.2%. With the third repetition, considering all the diagnoses obtained in the three mycological analyses, the incidence obtained was 37.8% ($p<0.05$) (Table 4).

Discussion

Various studies of the incidence of onychomycosis, in which only one mycological nail examination of each patient was performed, report incidences of from 6.5% to 20% [6,24,25]. In our retrospective study, we found a slightly higher incidence of 25% onychomycosis. In the prospective study, we analyzed three successive mycological examinations over a short time period (2-5 day intervals between sample collections). Some cases that were negative in the first mycological examination were positive in the second or third analysis, increasing the final incidence of onychomycosis to 37.8%. Possibly, this is because multiple sampling gives better access to nail-bed debris, because it becomes easier to collect an adequate specimen after the first or second nail scraping, favoring isolation of the fungus.

Although a positive microscope exam associated with negative cultures has been observed in certain situations, we did not address this possibility, because the criteria employed for the diagnosis of onychomycosis were culture of dermatophytes, along with positive direct microscopy examination associated with positive cultures for yeasts and moulds [17,20].

Many dermatologists prefer to initiate treatment of onychomycosis based only on symptoms, because laboratory data frequently do not confirm the clinical diagnosis when only one mycological analysis is carried out. This medical conduct can lead to therapeutic errors and relapses [20]. Mycological confirmation of onychomycosis is recommended in dermatological medical practice, to avoid submitting the patient to unnecessary or inefficient treatment

Table 1. Diagnosis of onychomycosis in our retrospective study.

Fungi	Fingernail (n)	Toenail (n)	Total	
			N	%
Yeasts				
C. albicans	*30[a]	12	42	33.1
C. tropicalis	20	10	30	23.6
C. parapsilosis	15	10	25	19.7
Candida sp.	7	3	10	7.9
Subtotal	*72[b]	35	*107[c]	84.2
Moulds				
Fusarium sp.	1	2	3	2.4
Scytalidium sp.	-	2	2	1.6
Aspergillus sp.	-	1	1	0.8
Subtotal	1	5	6	4.8
Dermatophytes				
T. rubrum	4	5	9	7.1
T. mentagrophytes	-	3	3	2.4
T. tonsurans	1	1	2	1.6
Subtotal	5	9	14	11.0
Total	**78**	**49**	**127**	**100.0**

χ^2=10.5; DF=2; *p=0.005. [a](*C. albicans versus* other *Candida species*); [b](*Candida species* in fingernails *versus Candida species* in toenails) and [c](*Candida species versus* other fungi).

Table 2. Diagnosis of onychomycosis from our prospective study.

Serial repetition/ Groups of isolated fungi	First mycological examination (n=156)		Second mycological examination (n=125)		Third mycological examination (n= 112)		Total	
	N	%	N	%	N	%	N	%
Yeasts	25	80.6	5	38.5	8	53.4	38	64.4
Moulds	5	16.1	5	38.5	5	33.3	15	25.4
Dermatophytes	1	3.2	3	23.0	2	13.3	6	10.2
Total	**31**	**100.0**	**13**	**100.0**	**15**	**100.0**	***59[a]**	**100.0**

Fisher – Freeman – Halton exact test: *p= 0.04. [a](first + second + third mycological examination *vs.* first mycological examination).

[20,26,27]. We found that the accuracy of onychomycosis diagnosis was increased with the repetition of direct microscopy examination and fungal culture. Therefore, we recommend this strategy to improve the diagnosis and consequent treatment of this type of mycosis.

Onychomycosis is a common fungal infection of the nails, accounting for 50% of nail diseases, and this percentage has been progressively increasing [4,13,28]. Several studies have reported dermatophytes as the main etiological agent of onychomycosis, especially in temperate zones. *Trichophyton rubrum* and *T. mentagrophytes* are the main dermatophytes isolated from toenail infections [4-6]. However, reports of onychomycosis caused by yeasts [7-13] and moulds [14,15,24] have become more and more frequent.

We observed a low percentage of dermatophytes isolated from onychomycosis cases of patients from Fortaleza city, northeast Brazil. This differs from what was found in studies made in Canada [6], USA [25] and Europe [29,30]. Nevertheless, *T. rubrum* was the most commonly isolated dermatophyte, followed by *T. mentagrophytes* in both ours and these other studies.

The percentage of dermatophytes isolated from nails is usually low. For example, Pontes et al. [8] found that the main fungi involved in onychomycosis in João Pessoa city, northeast Brazil were *Candida* species (82%), followed by dermatophytes (13.4%). Also, Brilhante et al. [9] reported that the etiological agents most frequently found in cases of onychomycosis in Ceará (northeast Brazil) were *Candida* species (74.42%), followed by dermatophytes (12.99%) and *Fusarium* sp. (8.19%).

We found yeasts to be common; there were 107 (84.3%) positive diagnoses in our retrospective study, including 72 ($p<0.05$) and 35 onychomycosis cases from fingernails and toenails, respectively. In our prospective study, 64.4% of the diagnoses by yeasts were obtained from fingernails ($p<0.05$). Other researchers also observed frequent involvement of yeasts in fingernail onychomycosis [7,8,12].

Identification of the *Candida* species was based on phenotypical features, such as macro and micromorphological descriptions, as well as through zymograms and auxanograms [9]. Some micromorphological characteristics, especially round

Table 3. Isolated fungi in our prospective study, based on three successive mycological nail analysis.

Attacked nails/	Fingernails (n)	Toenails (n)	Total	
Fungi			**N**	**%**
Yeasts				
C. albicans	*15[a]	-	15	25.4
C. parapsilosis	8	4	12	20.3
C. tropicalis	8	1	9	15.3
C. glabrata	-	1	1	1.7
Candida sp.	-	1	1	1.7
Subtotal	*31[b]	7	*38[c]	64.4
Moulds				
Fusarium sp.	4	*7[d]	*11	18.6
Aspergillus sp.	1	2	3	5.1
Scytalidium sp.	1	-	1	1.7
Subtotal	6	9	15	25.4
Dermatophytes				
T. rubrum	3 7.1	-	3	5.1
T. mentagrophytes	1 2.4	1	2	3.4
T. tonsurans	1 2.4	-	1	1.7
Subtotal	5 11.9	1	6	10.2
Total	**42 100.0**	**17**	**59**	**100.0**

Fisher – Freemann – Halton exact test: *p= 0.001. [a](*C. albicans versus* other *Candida species*); [b](*Candida species* in fingernail *versus Candida species* in toenail); [c](*Candida species versus* others fungi) and [d](*Fusarium* sp. *vs*. other moulds).

Table 4. Incidence of onychomycosis, considering the positive results obtained in the three mycological examinations.

Serial nail examination/	1st nail test		1st + 2nd nail test		1st+ 2nd+ 3rd nail test	
Results	**N**	**%**	**N**	**%**	**N**	**%**
Positive	31	19.9	44	28.2	*59	37.8
Negative	125	80.1	112	71.8	97	62.2
Total	156	100.0	156	100.0	156	100.0

χ^2=12.3; DF=2; *p=0.002. [a](first + second + third nail test *versus* first nail test).

to oval cells (isolated blastoconidia), were more frequent than the blastoconidia form in association with pseudohyphae. Possibly nail characteristics favor the isolated blastoconidia form.

Candida albicans was the most frequently isolated yeast in both our retrospective and prospective studies. However, *C. parapsilosis* was the most commonly isolated fungus in toenail onychomycosis in our prospective study. Other researchers also reported that *C. parapsilosis* is the most common yeast in toenail infections [12,31]. The much greater proportion of *Candida* species compared to dermatophytes and moulds could be due to *Candida* contamination in the altered nail. However, this possibility appears to be unlikely, as there was no evidence of mixed cultures of *Candida* spp. plus dermatophytes or moulds.

Isolation of moulds from nail infections has been frequently observed, especially from toenails of patients from tropical countries. Some studies have reported onychomycosis by *S. dimidiatum* and *S. brevicaulis* [1]. In our prospective analysis, *Fusarium* sp., was the most frequent mould in toenail onychomycosis. Other research carried out in Brazil came to the same conclusion [15].

In conclusion, we found a high proportion of onychomycosis caused by *Candida* species in the city of Fortaleza, Ceará, northeast Brazil and we found that serial repetition of direct microscopic examination and fungal culture improves onychomycosis diagnosis efficiency. This strategy of repetition would allow for more accurate diagnosis of this mycoses.

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