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ORIGINAL RESEARCH ARTICLE

Molecular Diagnostic Techniques for Onychomycosis: Validity and Potential Application

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Abstract
Background Diagnosis of onychomycosis requires positive findings by direct microscopy and fungal culture. Fungal culture is slow and difficult, with low yields. We compared two dermatophyte identification methods, one using a real-time polymerase chain reaction (PCR) method, and the other using fungal culture to validate the molecular method.
Methods Nail specimens were collected from 149 patients with distal and lateral subungual onychomycosis who were positive for fungal elements by direct microscopy using potassium hydroxide. Each specimen was subjected to the modified real-time PCR assay of Miyajima et al. and fungal culture.
Results Of 149 specimens, 142 (95.3%) were positive for *Trichophyton rubrum* or *Trichophyton mentagrophytes* including *Trichophyton interdigitale* by PCR, while only 104 (69.8%) were positive by fungal culture performed simultaneously. No specimen was negative by PCR, but positive by culture. All specimens positive for *T. rubrum* or *T. mentagrophytes* by culture were also positive by PCR, showing complete concordance for *Trichophyton* species. The culture of 17 specimens yielded fungi other than *T. rubrum* or *T. mentagrophytes*, whereas PCR identified *T. rubrum* in 11 of these specimens. Among 28 culture-negative specimens, 23 showed *T. rubrum* and four showed *T. mentagrophytes* by PCR. PCR allowed more rapid identification of causative fungi (≤2 days vs. ≤28 days).
Conclusion Real-time PCR achieved a higher dermatophyte identification rate and showed complete concordance with conventional culture for two *Trichophyton* species. Specimens never yielded both *T. mentagrophytes* and *T. rubrum* simultaneously, suggesting that mixed infection is uncommon.

Key Point

Real time polymerase chain reaction is a simple, rapid, and sensitive method for detecting/identifying dermatophytes causing onychomycosis.

1 Introduction

Tinea unguium accounts for more than 90% of all cases of onychomycosis [1]. Clinical features of tinea unguium include thickening and yellow-brown discoloration of the nail, and partial detachment of the nail plate. Most cases of tinea unguium are due to *Trichophyton rubrum* or *Trichophyton mentagrophytes var. interdigitale*, with the former being the leading cause of this form of trichophytosis [1–5].

1.1 Pathology of Tinea Unguium

Onychomycosis has several pathological types [6] and can usefully be classified into superficial white onychomycosis (SWO) and hyperkeratotic subungual onychomycosis for

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the purpose of collecting culture specimens. SWO is rare in the outpatients in departments of dermatology in Japan and Serbia [7], although there are many SWO patients in elderly care facilities in Japan, and results from fungal invasion through a nail surface injury. For diagnosis of SWO, it is sufficient to collect a specimen from the surface of the discolored nail [8]. In contrast, distal and lateral subungual onychomycosis (DLSO), which is the most common form of onychomycosis, results from fungal invasion through the distal or lateral end of the nail.

Thus, tinea unguium (which accounts for most onychomycosis) has its pathogenic basis in tinea pedis, because the majority of patients with DLSO attending dermatology departments during the summer months are associated with tinea pedis, and have a history of having tinea pedis. Therefore, DLSO results from the invasion of dermatophytes into the nail bed from the horny cell layer of the tinea pedis lesion. Nails are rich in keratin, but have a low water content. However, the nail bed near the dermis has a higher water content than the nail plate and is therefore more vulnerable to dermatophyte invasion and growth (Fig. 1). As a nail grows, fungi that have invaded the nail bed are moved forward and/or upward. Fungal invasion makes the subungual tissue weaker and more powdery, causing subungual hyperkeratosis. Physical removal of the hyperkeratotic subungual tissue results in nail plate detachment and drying of the tissue by exposure to air, which often leads to a degeneration of dermatophytes and/or inhibition of growth. Therefore, fungal culture of specimens collected from hyperkeratotic subungual lesions is often unsuccessful, and even direct microscopy may fail to identify typical morphologic characteristics of the causative fungus. This commonly leads to misdiagnosis.

1.2 Diagnosis of Onychomycosis

Diagnosis of onychomycosis requires proof that a pathogenic fungus is present in the lesion based on direct microscopy and culture. For both methods, collection of a suitable specimen is the key to the diagnosis. Direct microscopy can detect pathogenic fungal elements in a specimen even if the fungal cells are dead, although some skill is needed to interpret the microscopic findings. In contrast, successful fungal culture requires the specimen to contain a sufficient number of viable fungal cells, which means that fungal culture of nail specimens from patients with onychomycosis has a low success rate [2–5]. In addition, the presence of organisms in the nail such as saprophytes, or contamination of the culture plate, can make it difficult to determine whether the fungus identified by culture is actually the causative agent. To overcome the disadvantages associated with fungal culture, diagnostic methods based on molecular biological techniques have been developed. These include conventional polymerase chain reaction (PCR), real-time PCR, and post-PCR

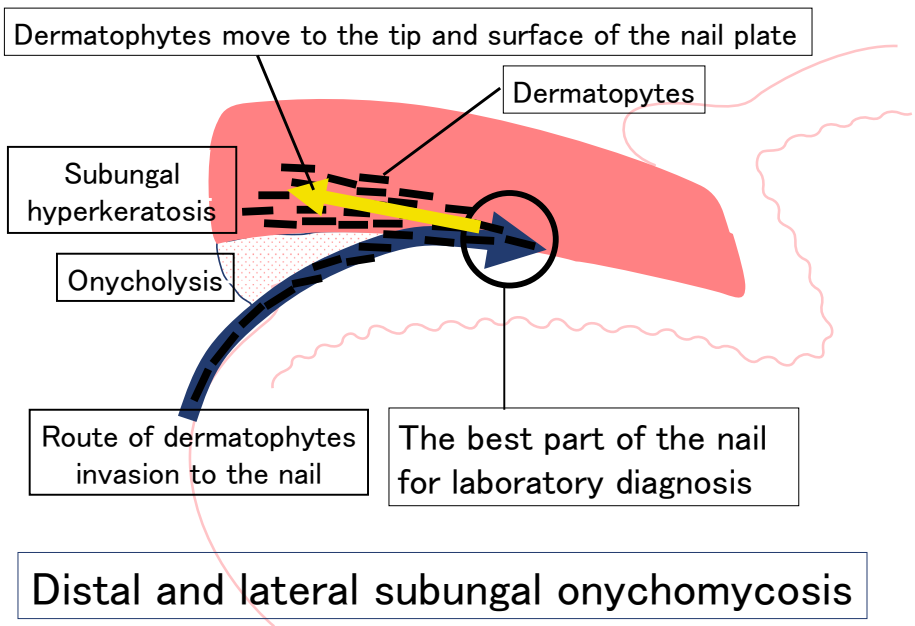

Fig. 1 Pathogenesis of distal and lateral subungal onychomycosis. Distal and lateral subungual onychomycosis results from the invasion of dermatophytes into the nail bed from the horny cell layer of the tinea pedis lesion. As a nail grows, fungi that have invaded the nail bed are moved forward and/or upward. Fungal invasion makes the subungual tissue weaker and more powdery, causing subungual hyperkeratosis. Physical removal of the hyperkeratotic subungual tissue results in onycholysis. The best part of the nail for laboratory diagnosis is the nail bed beneath the affected part of the nail as close as possible to the healthy part of the nail plate

techniques [6]. Conventional PCR methods are usually simple and inexpensive. Real-time PCR methods expand the possibility for simultaneous recognition of multiple species and limit the risk of contamination, whereas post-PCR methods have a longer turnaround time and may be associated with an increased risk of contamination [9]. In 2013, Miyajima et al. [10] established a real-time PCR method with specific fluorescent probes for *T. rubrum* and *T. mentagrophytes* including *Trichophyton interdigitale* to allow rapid and sensitive identification of dermatophytes in specimens from patients with tinea unguium or tinea pedis. We subsequently modified this method to improve its operability. In the present study, the modified Miyajima method was compared with conventional fungal culture for identification of fungi in specimens from 149 patients with onychomycosis diagnosed by direct microscopy to validate the PCR method.

2 Materials and Methods

2.1 Nail Specimens

In each patient, the detached part of the nail plate was removed with clippers and a specimen was collected from the nail bed beneath the affected part of the nail, as close as possible to the healthy part of the nail plate, with this site being selected for the above reasons (Fig. 1).

2.2 Test Specimens

Between February 21 and July 6, 2011, having obtained written informed consent, 300 nail specimens from patients with suspected onychomycosis were collected from the patients attending Samoncho Clinic (Tokyo, Japan) as part of routine medical management. Nail specimens collected from 149 patients were positive by direct microscopy using potassium hydroxide and were employed in this study. The 149 specimens were transported at 17.5 ± 2.5 °C to BLM Inc. (Tokyo, Japan), and each was divided into two parts. One part was maintained at 27 ± 1 °C and subjected to culture within 48 h, while the other part was stored at −85 ± 5 °C until assay by real-time PCR. Both fungal culture and real-time PCR were conducted at BLM Inc., which also interpreted the results.

2.3 Fungal Culture

Part of each specimen was inoculated onto Sabouraud dextrose CG agar containing chloramphenicol and gentamicin (Becton Dickinson Japan, Tokyo, Japan) and incubated at 27 °C for 4 weeks. If culture yielded a morphologically distinguishable organism, it was judged to be 'positive' and identification was performed. If culture did not yield any fungal growth within the specified time, it was judged to be 'negative'. If there was simultaneous growth of multiple organisms, isolation culture was performed subsequently. For isolation culture, an organism with a filamentous appearance was re-inoculated onto Sabouraud dextrose CG agar or potato agar (Einscience, Tokyo, Japan) and incubated at 27 °C together with slide culture. Identification of molds was based on a combined assessment of growth rate, colony morphology, and other macroscopic (e.g., color) and microscopic features, as well as findings from the slide culture. If morphological identification of the species was difficult, the colony was identified at the genus level. If a yeast-like fungus grew, it was only identified at the genus level.

2.4 Real-Time PCR

Dermatophyte DNA was extracted from part of each specimen and real-time PCR was performed using the method developed by Miyajima et al. [10], with some modifications. In brief, dermatophyte DNA was extracted from nail specimens by the method developed by Boom using the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) and was assayed using a real-time PCR system StepOnePlus™ (Foster City, CA, USA) with fluorescent TaqMan® MGB (Applied Biosystems) probes. If exponential PCR amplification was observed with the specific fluorescent probe for *T. rubrum* or *T. mentagrophytes* including *Trichophyton interdigitale*, the result was assessed as 'positive' for that species, while failure to detect exponential amplification was assessed as 'negative'. If the initial amplification cycles were not exponential and only yielded moderate fluorescence compared with the positive control, the result was also defined as 'negative'. In addition, if PCR efficiency was definitely lower than the control, the result was also defined as 'negative'. If PCR amplification only occurred with a general fungal probe, the result was recorded as 'fungi' and was assessed as 'negative'.

The following reagents and instruments were used for PCR. Detection of fungal DNA in nail specimens was performed with a QIAamp® DNA Micro Kit, and the amplification reagent was Premix EX Taq™ (Perfect Real Time; Takara Bio, Shiga, Japan).

The following PCR probes from the TaqMan® MGB Probe series were employed: vanb-ITS1F: 5′-[FAM]-CTCTCTTTAGTGGCTAAAC-[NFQ-MGB]-3′, rubrum-ITS1V: 5′-[VIC]-CGCGCTCCCCCTGC-[NFQ-MGB]-3′, and fungi-ITS1N: 5′-[NED-TTYAACAAYGGATCTCT-[NFQ-MGB]-3′.

The following PCR primers were tailor made by Greiner Japan (Tokyo, Japan): derma-F: 5′-TAACAAGGTTTCCGT

AGGTGAACCT, and derma-R: 5′-TCGCTGCGTTCTTCATCGA.

The positive controls for PCR were three *Escherichia coli* clones that possessed one of the following plasmids containing the target DNA sequence and the plasmid DNAs isolated and purified from these clones: (1) strain No. TIMM/LMB 217-449 (species: *T. mentagrophytes* 7 derma-FR/PCR2.1/217 *E. coli* DH5α), (2) strain No. TIMM/LMB 217-452 (species: *T. rubrum* 99 derma-FR/PCR2.1/217 *E. coli* DH5α), and (3) strain No. TIMM/LMB 217-458 (species: *Candida albicans* TIMM1768 derma-FR/PCR2.1/217 *E. coli* DH5α). The plasmid DNAs isolated and purified from these clones were TIMM/LMB 217-449P, TIMM/LMB 217-452P, and TIMM/LMB 217-458P, respectively. PCR commenced with initialization at 95 °C for 30 s, followed by 50 cycles of 95 °C for 10 s and 60 °C for 30 s.

2.5 Statistical Analysis

Data were analyzed by SAS 9.3 (SAS Institute Japan Ltd, Tokyo, Japan). Positive rates and detected *Trichophyton* species of fungal culture and real-time PCR were summarized descriptively. The sensitivity of real-time PCR was also calculated. Concordance between fungal culture and real-time PCR was determined using kappa statistics. McNemar's test was performed to compare the positive rates of the two examinations. A p value <0.05 was considered statistically significant.

3 Results

Of the 149 nail specimens with microscopic evidence of fungal elements, real-time PCR was positive for *T. rubrum* in 122 specimens (85.9%) and *T. mentagrophytes* in 20 specimens (14.1%), resulting in a detection rate of 95.3% (142/149) for dermatophyte infection. The seven specimens (4.7%) assessed as negative by real-time PCR did not react with either of the specific *Trichophyton* probes, but did react with the general fungal probe, and were therefore considered to contain fungi other than these *Trichophyton* species. Fungal culture of these seven specimens did not yield growth of either *T. rubrum* or *T. mentagrophytes*, with the result being reported as 'non-sporulating unidentifiable fungus' ($n = 2$), *Trichophyton* sp. ($n = 1$), *Fusarium* sp. ($n = 2$), *Exophiala* sp. ($n = 1$), and negative culture ($n = 1$).

Fungal culture detected *Trichophyton* species in 104/149 specimens (69.8%), with *T. rubrum* being identified in 86 specimens (84.6%) and *T. mentagrophytes* in 16 specimens (15.4%). Of the 45 specimens (30.2%) that were negative for both *T. rubrum* and *T. mentagrophytes* by culture, 17 exhibited growth of other fungi, including *Candida* sp. ($n = 3$), *Exophiala* sp. with Candida sp. ($n = 2$), *Exophiala* sp. ($n = 3$), *Fusarium* sp. ($n = 3$), *Penicillium* sp. ($n = 1$), *Trichophyton* sp. ($n = 1$), and non-sporulating unidentifiable fungi ($n = 4$). Real-time PCR detected *T. rubrum* in 11 of these 17 specimens. The remaining six specimens were not positive for either *Trichophyton* species, but a reaction was obtained with the general fungal probe, indicating the presence of other fungi. Of the 28 specimens in which no fungi were detected by culture, 23 were positive for *T. rubrum* and four were positive for *T. mentagrophytes* by real-time PCR, with only one specimen not being positive for either species.

Overall, 104 specimens were positive by both real-time PCR and culture, 38 specimens were positive by PCR but negative by culture, and seven specimens were negative by both PCR and culture. No specimen was negative by real-time PCR, but positive by culture, thus real-time PCR detected fungi in all of the culture-positive specimens (Table 1).

The sensitivity of real-time PCR was 100.0%. The positive rate in real-time PCR was statistically higher than that in culture ($p < 0.0001$, McNemar's test). Identification of *T. rubrum* in 88 specimens and *T. mentagrophytes* in 16 specimens by real-time PCR was in complete correspondence with the results of the culture, and there was no discordance between the two diagnostic methods (Table 2, kappa statistics 1.000). No specimen was positive for both *T. rubrum* and *T. mentagrophytes* by either real-time PCR or culture.

4 Discussion

In the present study, fungal culture had a detection rate of nearly 70% for *T. rubrum* and *T. mentagrophytes*, which was considerably higher than that reported previously in patients with onychomycosis [2–5]. This might have been because specimens were collected from the nail bed beneath the most active site of infection (Fig. 1), adjacent to the healthy nail that contained numerous living fungal cells. However, culture of 30% of the specimens either

Table 1 Comparison of the results of fungal culture and real-time PCR

	Real-time PCR		Total
	+	−	
Fungal culture			
+	104	0	104
−	38	7	45
Total	142	7	149

PCR polymerase chain reaction

Table 2 Comparison of the detection of *Trichophyton rubrum* and *Trichophyton mentagrophytes* by fungal culture or real-time PCR

	Real-time PCR		Total
	T. rubrum	*T. mentagrophytes*	
Fungal culture			
T. rubrum	88	0	88
T. mentagrophytes	0	16	16
Total	88	16	104

PCR polymerase chain reaction

yielded no fungal growth or fungi other than these two *Trichophyton* species. This suggests that even a skilled technician cannot completely avoid the influence of other fungi adherent to the nails or contamination by environmental fungi. Fungal culture is also likely to have a high false-negative rate because it can only be successful if the specimen contains enough viable fungal cells.

In contrast, real-time PCR allows the detection of a small amount of fungal DNA in a specimen, and it achieved a positive rate of 95% in the present study. However, PCR is highly sensitive, which may increase the risk of detecting fungi adherent to the nails or contaminating environmental fungi. In this study, therefore, PCR was defined as positive if *T. rubrum* or *T. mentagrophytes* was detected, while detection of other fungi was considered negative. PCR rarely gives false-negative results and none of the specimens were negative by PCR and positive by culture in the present study. In addition, the relative frequency of detecting *T. rubrum* and *T. mentagrophytes* by real-time PCR (85.9:14.1) was very similar to that shown in fungal culture (84.6:15.4), and was also consistent with that found in Japanese patients with tinea unguium, according to the culture-based epidemiological survey of dermatomycoses performed by the Japanese Society for Medical Mycology [3–5]. These findings confirmed that the PCR method could identify the causative agent of onychomycosis with a similar precision to fungal culture.

There were 28 specimens with no fungal growth by culture, among which real-time PCR detected *T. rubrum* in 23 specimens and *T. mentagrophytes* in four specimens, while one specimen only reacted with the general fungal probe. Thus, the real-time PCR method was able to identify the causative agent of onychomycosis in 96.4% (27/28) of the specimens for which culture was unsuccessful. This difference in sensitivity arises because culture is likely to give a false-negative result if the specimen does not contain sufficient viable fungal cells.

There were also 17 specimens in which culture detected fungi other than *T. rubrum* or *T. mentagrophytes*. Among these specimens, 11 were positive for *T. rubrum* by real-time PCR. The remaining six specimens reacted with the general fungal probe and were diagnosed as tinea unguium caused by other dermatophytes or non-dermatophyte onychomycosis. Thus, only seven patients (these six and one patient mentioned above) had onychomycosis caused by fungi other than *T. rubrum* or *T. mentagrophytes*, which means that only 4.7% (7/149) of the patients studied had tinea unguium as a result of dermatophytes that could not be detected by the specific probes used in the present real-time PCR or had non-dermatophyte onychomycosis.

Thus, fungal culture was often unsuccessful in identifying a causative agent or else detected saprophytes (including *Candida* sp., *Exophiala* sp., *Fusarium* sp., and *Penicillium* sp.) that might have been adherent to the nails, or may have contaminated the specimens during processing and culture rather than being the cause of onychomycosis. Among the 17 specimens showing the growth of saprophytes or potentially environmental fungi, 11 were positive for *T. rubrum* by real-time PCR. This indicates that fungal culture may fail to correctly identify the causative agent, leading to misdiagnosis of tinea unguium as non-dermatophyte onychomycosis. Therefore, it may be necessary to confirm culture-based mycological diagnoses of non-dermatophyte onychomycosis by using a method such as PCR.

None of the 147 nail specimens tested in this study showed mixed infection with *T. rubrum* and *T. mentagrophytes*, indicating that mixed infection is extremely rare in tinea unguium. Patients sometimes have tinea pedis and tinea unguium caused by different species, and it is not surprising that different fungi may be detected from different tinea unguium lesions. Some patients with DLSO also have concomitant proximal subungual onychomycosis and SWO affecting the same nail, thus fungal culture of specimens collected from various lesions of such patients may well yield multiple species. However, simultaneous detection of *T. rubrum* and *T. mentagrophytes* in specimens obtained from a specific type of lesion seems to be very rare.

To diagnose onychomycosis caused by other fungi (e.g., non-dermatophyte onychomycosis), multiple culture- or PCR-based tests would need to be performed to confirm repeated detection of the same species because the real-time PCR method is susceptible to the risk of contamination and even a trace amount of an environmental fungus contaminating the specimen could be detected by a specific probe for such an organism.

5 Conclusion

The real-time PCR method was as accurate as direct microscopy for diagnosing tinea unguium, suggesting that PCR is able to compensate for the insufficient sensitivity of

fungal culture and decrease the false-negative rate. While PCR can only detect species for which probes are designed, fungal culture can detect any species, but it requires a specimen containing a sufficient number of viable fungal cells. Therefore, fungal culture alone, without any other diagnostic method, often fails to accurately diagnose onychomycosis. In contrast, PCR can detect a causative agent even if the specimen is small or the agent has low viability. Unlike conventional PCR, the real-time PCR technique permits high-throughput analysis, which may make it more attractive as a diagnostic method for onychomycosis, and it does not require specialized skills once the procedures have been established. However, real-time PCR requires relatively expensive instruments and reagents, so its cost is higher than that of direct microscopy and fungal culture. This has prevented the method from becoming popular in the clinical setting. At present, the cost of an investigational PCR test is US$100/sample in Japan. Once it becomes available in clinical practice, the cost will likely be reduced to around $US50/sample.

In addition, PCR cannot be used to monitor a patient's response to treatment because it can yield a positive result by detecting DNA from dead fungal cells. Despite such disadvantages, real-time PCR seems to be an excellent supplement to conventional fungal culture for the diagnosis of onychomycosis.

Compliance with Ethical Standards

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Conflict of interest Kazuya Ishida is an employee of Basic Research Laboratories, Hisamitsu Pharmaceutical Co., Inc. (Ibaraki, Japan). Shinichi Watanabe has no conflicts of interest to declare.

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