

ORIGINAL ARTICLE

Clinical study of Dermatophyte Test Strip, an immunochromatographic method, to detect tinea unguium dermatophytes

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ABSTRACT

The Dermatophyte Test Strip visualizes mycotic antigens by immunochromatography. It allows easy and fast detection of dermatophytes. A multicenter, single-arm, comparative clinical study was designed to evaluate the capacity of Dermatophyte Test Strip to detect dermatophytes in suspected tinea unguium specimens in comparison with direct microscopy and polymerase chain reaction (PCR). Signed consent was obtained from 222 subjects and all subjects completed the study. With the Dermatophyte Test Strip, dermatophytes were detected in 201 of 222 (90.5%) specimens but not in 21 of 222 (9.5%) specimens. With direct microscopy, dermatophytes were detected in 170 of 222 (76.6%) specimens but not in 52 of 222 (23.4%). Of the 45 specimens that showed inconsistent results between the two methods, PCR gave further results for 40 specimens, of which 37 (92.5%) specimens were positive and three (7.5%) were negative for dermatophytes. The positive concordance rate, negative concordance rate and overall concordance rate between the Dermatophyte Test Strip and direct microscopy were 81.1%, 66.7% and 79.7%, respectively. When inconsistent results were corrected using the results of PCR, these rates were 97.5%, 71.4% and 95.0%, respectively. When five specimens that could not be tested by PCR because no piece for the PCR test was left were excluded from analysis, these rates were 99.0%, 78.9% and 97.2%, respectively. The present results indicate good detection capacity of the Dermatophyte Test Strip. The Dermatophyte Test Strip provides a reliable, convenient and quick method to test for tinea unguium.

Key words: dermatophytes, immunochromatography, immunological diagnosis, tinea unguium, *Trichophyton*.

INTRODUCTION

Tinea unguium is a nail disease caused by dermatophyte infection of the nail plate.^{1,2} Its prevalence in advanced countries is considered as at least 10% of the population,² and in Japan it is estimated at approximately 10%.¹ The incidence increases with aging in all countries and it is therefore predicted to increase in number in association with the aging of society in the future.² Patients with tinea unguium pose a risk of becoming the source of infection of other people.³ Tinea unguium is particularly considered as a predictor of diabetic foot syndrome.³ Thus, tinea unguium does not only cause problems with nail appearance, but it also seriously damages patients' quality of life. It should therefore be treated actively as far as possible.⁴

Because many other diseases have similar symptoms as tinea unguium,⁵ differentiation is not easy, and a definitive diagnosis by mycological examination is necessary before treatment is started. Dermatophytes are classified into three

genera, namely, *Trichophyton* (*T.*), *Microsporum* (*M.*) and *Epidermophyton* (*E.*).^{6,7} *T. rubrum* accounts for the primary cause of tinea unguium, followed by *T. mentagrophytes*.^{7,8}

The diagnostic tests for tinea unguium include direct microscopy with potassium hydroxide (KOH), fungal culture, periodic acid-Schiff staining (PAS), fluorescence staining, confocal microscopy and molecular biological methods such as polymerase chain reaction (PCR).⁹ Although direct microscopy with KOH and fungal culture have been the gold standard for the diagnosis of tinea unguium,^{7,9} these tests sometimes are a burden in the clinical setting, because direct microscopy requires experience to identify the fungal elements, and dissolution of nails is time-consuming. It takes 2–3 weeks to obtain results using fungal culture, and the detection rate is lower than with direct microscopy.¹⁰ Therefore, the development of an easy and quick test to diagnose tinea unguium accurately is in high demand.

The recently developed Dermatophyte Test Strip detects dermatophytes easily and rapidly by immunochromatography

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using monoclonal antibodies that react specifically with the polysaccharide present in the cell wall of dermatophytes.^{11–14} This antibody was found to react specifically with seven dermatophytes (*T. rubrum*, *T. mentagrophytes*, *T. violaceum*, *T. tonsurans*, *M. gypseum*, *M. canis* and *E. floccosum*).^{12,13} There have been a few preliminary reports on screening results of dermatomycoses using a Dermatophyte Test Strip.^{15–17} Higashi and his colleagues have collected 88 specimens from skin and 72 from nails, examined them using direct microscopy and Dermatophyte Test Strip, and compared the results.¹⁵

In this study, tinea unguium-suspected specimens were subjected both to testing with the Dermatophyte Test Strip as well as the gold standard of direct microscopy with KOH to detect dermatophytes. Specimens that showed inconsistent results between the two methods were subjected to PCR to further confirm the presence or absence of dermatophytes. The detection capacity of the Dermatophyte Test Strip was then investigated for all specimens by comparing the results of medical experts' comprehensive evaluations based on the results of direct microscopy and PCR.

METHODS

A multicenter, single-arm, comparative clinical study was conducted to evaluate the efficacy of the Dermatophyte Test Strip (JNC Corporation, Tokyo, Japan) to detect dermatophytes in tinea unguium-suspected specimens in comparison with direct microscopic examination. The study was conducted at 11 study sites (Table S1) from June to August 2014.

The detection of dermatophytes using the Dermatophyte Test Strip was performed at Yokohama Research Center of JNC Corporation. Direct microscopic examination was performed by Professor Shinichi Watanabe of Teikyo University School of Medicine. PCR was performed by Professor Takashi Sugita of Meiji Pharmaceutical University. This study was performed in compliance with Good Clinical Practice based on the Declaration of Helsinki and other applicable regulations. The institutional review board of each study site reviewed and approved the study protocol. Prior to the start of screening procedures, signed informed consent was obtained from each subject. The present study was monitored and audited by EPS Associates Co., Ltd.

Study design

Within 4 weeks after obtaining signed consent, the investigator or subinvestigator confirmed the eligibility of the subject, collected the baseline characteristic information (date of birth, age and sex) and examined and interviewed the subject (main reason for visit, disease or major complaint, medical history and complications). A nail specimen was collected from each subject within 1 week after confirming the eligibility of a subject, and the specimen was sent to the central laboratory where all specimens were subjected to dermatophyte detection tests with the Dermatophyte Test Strip and direct microscopy.¹⁸ Specimens that showed inconsistent results between the two methods were subjected to PCR to judge the presence or absence of tinea unguium.^{19–22}

This trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (no. UMIN 000016760).

Subjects

Male and female subjects aged 20 years or more who had a nail lesion suspected of tinea unguium, and who provided signed informed consent were included in the study. Subjects were excluded from the study if any of the following applied: (i) history of oral antifungal agent use within 6 months or of topical antifungal agent use within 1 month; (ii) participation in another clinical trial or other clinical studies; and (iii) participation determined to be inappropriate by the study investigator or subinvestigator.

Primary end-point

The primary end-points were the concordance rate (positive concordance rate, negative concordance rate and overall concordance rate) and the inconsistent rate of detecting dermatophytes in the suspected specimens between the Dermatophyte Test Strip and direct microscopy or the adjusted results based on PCR and final determination by medical experts' comprehensive evaluations based on the results of direct microscopy, PCR, the clinical picture, underlying disease information and specimen collection site.

Test specimen collection

Specimens were collected by the dermatologists listed in Table S1. Dermatologists at the 11 study sites selected patients with suspected tinea unguium by visual examination and collected specimens from those patients. A specimen of approximately 20 mg was collected from a suspected tinea unguium nail lesion in accordance with the guideline.²³ The lesions were recorded by taking pictures and sketches.

The collected nail was sandwiched between autoclaved aluminum plates and crushed into small pieces by using a hammer. The pieces were transferred to a test tube, further cut and crushed using a pair of dissecting scissors and divided into three portions after mixing for direct microscopy, the Dermatophyte Test Strip and PCR, respectively. Because nine specimens were extremely small, these specimens were only cut into two pieces, forfeiting the piece for PCR.

Dermatophyte Test Strip

Monoclonal antibodies that react specifically with the polysaccharide present in the cell wall of dermatophytes^{12,13} are linearly immobilized on the test strip. The polysaccharides in the suspected specimens are extracted using the extraction solution.^{14,24} In short, a nail specimen was cut into smaller pieces with a nail clipper or nipper. These pieces were then added to 0.25 mL of extraction solution, and the mixture was stirred and lightly ground with a plastic rod, and then left at rest for 1 min. The test strip was then added. A positive judgment was made if a dark purple line appeared on the test strip after 5–30 min and a negative judgment if it was not visible within this time limit (Fig. 1).

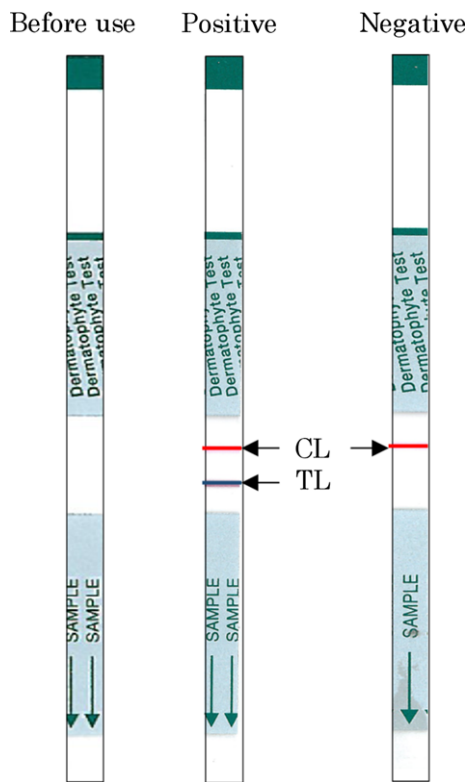


Figure 1. Appearance of the Dermatophyte Test Strip. The control line (CL) should always appear after the assay. If it does not appear, the test is invalid. A positive judgment is made if the test line (TL) is visible in 5–30 min after the assay, and negative if it is not visible.

PCR and sequencing

Nail specimen was weighed and subjected to DNA extraction. The nail specimen and crusher were placed in a 2-mL test tube and liquid nitrogen was added. The nail specimen was crushed using the Automill (TK-AM5-S; Tokken, Chiba, Japan) at 1220 rpm for 30 s, and heated for 15 min at 100°C in the presence of lysing solution (100 mmol/L Tris-HCl [pH 7.0], 1 mmol/L ethylenediaminetetraacetic acid [EDTA] [pH 7.0], 0.1% sodium dodecylsulfate). DNA was extracted by the standard method, dried and reconstituted in 50 μ L Tris-EDTA buffer.

Fungal DNA was amplified using the following primer set that amplified the internal transcribed spacer regions: primer IntF (5'-AACTTGGTCAATTTAGAGGAA-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'),^{19,20} followed by another round of PCR using the IntF primer and a nested primer ITS4R (5'-TCCTCCGCTTATTGATATGC-3').²¹ Sequences of PCR products were confirmed by the direct sequencing using the IntF and ITS4R primers.

Statistical analyses

The planned number of subjects enrolled in this study was 200. The safety analysis set included subjects who provided

signed consent and test specimens. Subjects whose specimens were subjected both to the Dermatophyte Test Strip detection and direct microscopy were included in the efficacy analysis set (intention to treat [ITT]).

To evaluate the endpoints, the concordance rate (positive concordance rate, negative concordance rate and overall concordance rate) and the inconsistent rate of the detection of dermatophytes in the suspected specimens between the Dermatophyte Test Strip test and direct microscopy or adjusted results based on PCR (ITT) were obtained. The results of the medical experts' comprehensive evaluations based on the results of direct microscopy, PCR, the clinical picture, underlying disease information and specimen collection site were utilized as the final determination (per protocol [PP]). The simple κ -coefficient was used to measure the level of agreement between the two methods.²⁵

Any adverse events that occurred after obtaining the signed consent until the day when the test specimens were collected were recorded by the investigators or subinvestigators. Adverse events were coded using the Medical Dictionary for Regulatory Activities, version 17.0.

All statistical analyses and data management were conducted by L Data Science (Tokyo, Japan).

RESULTS

Signed consent was obtained from 222 subjects, and all subjects completed the study. Subjects' age (mean \pm standard deviation) was 64.0 ± 16.1 . This study included 96 males (43.2%) and 126 females (56.8%). Specimens collected from all the subjects were tested for the presence of dermatophytes using the Dermatophyte Test Strip and direct microscopy, and all subjects were therefore included in the efficacy and safety analyses (ITT, $n = 222$).

The 45 specimens, for which the Dermatophyte Test Strip and direct microscopy results differed, had to be subjected to PCR to further confirm the presence or absence of dermatophytes. However, 40 specimens were subjected to the PCR test because no piece for the PCR test was left in five specimens.

Detection of dermatophytes

Using the Dermatophyte Test Strip, dermatophytes were detected in 201 of 222 (90.5%) specimens but were absent in 21 of 222 (9.5%) specimens (Table 1). Dermatophytes were detected in 170 of 222 (76.6%) specimens but were absent in 52 of 222 (23.4%) by direct microscopy. Of the

Table 1. Results of the Dermatophyte Test Strip and direct microscopy ($n = 222$)

Dermatophyte detection	Dermatophyte Test Strip		Direct microscopy	
	<i>n</i>	Ratio (%)	<i>n</i>	Ratio (%)
Positive	201	90.5	170	76.6
Negative	21	9.5	52	23.4

170 specimens, 163 were positive and seven were negative with the Dermatophyte Test Strip. Of the 222 specimens, 177 showed matched test results between the test strip and direct microscopy, and 45 specimens showed inconsistent results. Of the 177 specimens, 163 were positive with the Dermatophyte Test Strip and direct microscopy, and 14 were negative with the Dermatophyte Test Strip and direct microscopy. Of the 45 specimens that showed inconsistent results between the Dermatophyte Test Strip and direct microscopy, PCR could further clarify the results of 40 specimens (Tables 2, 3). Of the 40 specimens, 35 were positive with the Dermatophyte Test Strip and negative with direct microscopy, and five were negative with the Dermatophyte Test Strip and positive with direct microscopy. Of the 35 specimens that were positive with the Dermatophyte Test Strip, 33 were positive with PCR. Of the five specimens that were negative with the Dermatophyte Test Strip, four were positive with PCR (Table 2). Of the 37 positive specimens, 23 specimens were positive for *T. rubrum* and 14 specimens for *T. mentagrophytes* complex. Of the 40 specimens subjected to PCR, 34 (85.0%) and six (15.0%) specimens showed the same results as the Dermatophyte Test Strip and direct microscopy, respectively (Table 2). Concerning the five specimens that were not left to be subjected to PCR, the results of direct microscopy were used to analyze the efficacy endpoint in the ITT population. In the PP population, the five specimens that were not subjected to PCR were excluded from analysis as undeterminable due to protocol deviations, and the concordance and inconsistent rates between the results of the final determination and the Dermatophyte Test Strip were analyzed ($n = 217$).

Table 2. Results of PCR

Inconsistent (Dermatophyte Test Strip/direct microscopy)	PCR		
	PCR	<i>n</i>	Ratio (%)
Positive/negative	Positive	33	82.5
	Negative	2	5.0
Negative/positive	Positive	4	10.0
	Negative	1	2.5
Total		40	100.0

PCR, polymerase chain reaction.

Table 3. Comparison of the results of the Dermatophyte Test Strip with those of direct microscopy, direct microscopy adjusted by PCR, and final determination

	Direct microscopy			Direct microscopy/PCR			Final determination [†]		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Population (<i>n</i>)	ITT (222)			ITT (222)			PP (217)		
Dermatophyte Test Strip									
Positive	163	38	201	196	5	201	196	2	198
Negative	7	14	21	6	15	21	4	15	19
Total	170	52	222	202	20	222	200	17	217
κ -coefficient	0.29			0.70			0.82		

[†]Determination based on results of direct microscopy, PCR and evaluation by specialists. ITT, intention to treat; PP, per protocol; PCR, polymerase chain reaction.

Comparison of the results between the Dermatophyte Test Strip and direct microscopy

Of the 201 specimens that showed a positive result with the Dermatophyte Test Strip, 163 specimens were also positive with direct microscopy. The concordance rate was 81.1% (95% confidence interval [CI], 75.4–86.8) (Tables 3, 4). Of the 21 specimens that showed a negative result with the Dermatophyte Test Strip, 14 specimens also showed a negative result with direct microscopy. The concordance rate was 66.7% (95% CI, 44.1–89.2). Overall, the number of specimens with consistent results between the Dermatophyte Test Strip and direct microscopy, either positive or negative, was 177 of 222 specimens. The concordance rate was 79.7% (95% CI, 74.2–85.2; κ -coefficient, 0.29). The number of inconsistent specimens between the two methods was 45 of 222 specimens (20.3%; 95% CI, 14.8–25.8).

Comparison of the results between the Dermatophyte Test Strip, direct microscopy adjusted by PCR, and final determination

Of the 201 specimens that showed a positive result with the Dermatophyte Test Strip, 196 specimens also showed a positive result for the combined results of direct microscopy and PCR that resulted in a concordance rate of 97.5% (95% CI, 95.1–99.9) in the ITT population (Tables 3, 4). Of the 21 specimens that showed a negative result with the Dermatophyte Test Strip, 15 also showed a negative result with the combined results of direct microscopy and PCR that resulted in a concordance rate of 71.4% (95% CI, 49.7–93.1). Overall, the number of specimens that showed consistent results between the Dermatophyte Test Strip and direct microscopy adjusted by PCR, either positive or negative, was 211 of 222 specimens, which resulted in a concordance rate of 95.0% (95% CI, 92.0–98.1; κ -coefficient, 0.70). The number of inconsistent specimens between the two methods was 11 of 222 specimens (5.0%; 95% CI, 1.9–8.0).

Of the 198 specimens that showed a positive result with the Dermatophyte Test Strip, 196 specimens also showed a positive result with the final determination, which resulted in a concordance rate of 99.0% (95% CI, 97.3–100.0) in the PP population (Tables 3, 4). Of the 19 specimens that showed a negative result with the Dermatophyte Test Strip, 15 specimens also showed a negative result with the final determination,

Table 4. Concordance and inconsistent rates in comparison with the detection results between the Dermatophyte Test Strip and direct microscopy, direct microscopy adjusted by PCR, and final determination

	Concordance rate	(95% CI)	Fisher's exact test (95% CI)
Comparison of Dermatophyte Test Strip with direct microscopy			
Positive concordance	81.1% (163/201)	(75.4–86.8)	(75.0–86.3)
Negative concordance	66.7% (14/21)	(44.1–89.2)	(43.0–85.4)
Overall concordance	79.7% (177/222)	(74.2–85.2)	(73.8–84.8)
Inconsistent	20.3% (45/222)	(14.8–25.8)	(15.2–26.2)
Comparison of Dermatophyte Test Strip with direct microscopy adjusted by PCR			
Positive concordance	97.5% (196/201)	(95.1–99.9)	(94.3–99.2)
Negative concordance	71.4% (15/21)	(49.7–93.1)	(47.8–88.7)
Overall concordance	95.0% (211/222)	(92.0–98.1)	(91.3–97.5)
Inconsistent	5.0% (11/222)	(1.9–8.0)	(2.5–8.7)
Comparison of Dermatophyte Test Strip with final determination			
Positive concordance	99.0% (196/198)	(97.3–100.0)	(96.4–99.9)
Negative concordance	78.9% (15/19)	(58.0–99.9)	(54.4–94.0)
Overall concordance	97.2% (211/217)	(94.8–99.7)	(94.1–99.0)
Inconsistent	2.8% (6/217)	(0.4–5.2)	(1.0–5.9)

CI, confidence interval; PCR, polymerase chain reaction.

which resulted in a concordance rate of 78.9% (95% CI, 58.0–99.9). Overall, the number of specimens for which the results of the Dermatophyte Test Strip and the final determination were consistent, either positive or negative, was 211 of 217 specimens, which resulted in a concordance rate of 97.2% (95% CI, 94.8–99.7; κ -coefficient, 0.82). The number of inconsistent specimens between the two methods was six of 217 specimens (2.8%; 95% CI, 0.4–5.2).

No adverse events were observed in any of the 222 subjects throughout the study.

DISCUSSION

The clinical presentation of tinea unguium includes clouding, thickening and deformation of the nail plate, and subungual hyperkeratosis. The diagnosis of tinea unguium is sometimes challenging because it mimics many diseases including psoriasis, lichen planus and onychodystrophy.⁵ Therefore, to distinguish tinea unguium from other diseases, mycological examination is essential.¹¹ Direct microscopy and fungal culture are recommended in the guidelines.^{23,26,27} However, there are disadvantages to direct microscopy; for example, detection sensitivity is likely to be affected by the skill and experience level of the person who performs the test. Fungal culture, on the other hand, takes 2–3 weeks to identify the pathogenic fungi and also has low sensitivity (40–77%).^{10,28} Recent developments in molecular biological methods offer highly sensitive and reliable methods such as PCR, which detects dermatophytes directly in the affected lesions, but these methods are not practical in the clinical setting or on a commercial basis, because dedicated equipment and special skills are required. Therefore, there has been demand for the development of a quick, reliable and practical detection method.

In this study, the detection capacity of the Dermatophyte Test Strip was evaluated by comparing the results of the Dermatophyte Test Strip and direct microscopy using nail

specimens obtained from subjects with suspected tinea unguium. The specimens that showed inconsistent results between the two methods were further subjected to PCR, the results of which were then added to the comparison in the ITT population. Finally, the results of medical experts' comprehensive evaluations based on the results of direct microscopy, PCR, the clinical picture, underlying disease information and specimen collection site were utilized as the final determination, and PP analysis was carried out.

The Dermatophyte Test Strip was developed for use in daily dermatological practice; therefore, in this study, the results of the Dermatophyte Test Strip were compared with those of direct microscopy which is a current standard test in daily dermatological practice. However, because direct microscopy is not as accurate as PCR, PCR was supplementally used to obtain more accurate data to assess the accuracy of the Dermatophyte Test Strip. It would be of interest to compare the results of the Dermatophyte Test Strip and those of PCR in a future study.

The present results of direct microscopy showed that 170 and 52 subjects, respectively, were positive and negative (Table 3). When the results were corrected by the PCR results, the numbers turned to 202 and 20 subjects, respectively. The cause of the false-negative results by direct microscopy is considered to be that the morphological fungal element was not preserved although the DNA was intact because most of the specimens were collected from the tip of the nail. When the results were further analyzed in the PP population, in which five specimens could not be subjected to PCR, the concordance rates increased with κ -coefficient of 0.82 (Table 3). These results indicate that the Dermatophyte Test Strip is practical and useful as a detection method that can be implemented in the actual clinical setting.

In a previous study, the sensitivity, specificity and concordance rates of the same test strip have been evaluated by direct microscopy as the standard.¹¹ In this previous study, Tsunemi and his colleagues have shown that the sensitivity, specificity,

positive concordance rate, negative concordance rate and overall concordance rate were 97.8%, 78.4%, 84.8%, 96.7% and 89.1%, respectively. Because the sensitivity of the test strip and negative concordance rate between the two methods were high, the test strip was considered to be useful in screening for tinea unguium. However, in the present study, the results were somewhat different (Tables 3, 4); the positive and overall concordance rates between the previous study and the present study appear to be comparable, but the negative concordance rate differs by 30%. The difference in the negative concordance rate between the present and previous studies appeared to be increased because the number of subjects without tinea unguium in this study was extremely small. In addition, the cases with extremely small specimens had negative results with the Dermatophyte Test Strip in the present study (data not shown).

Both the previous and present studies indicate that the Dermatophyte Test Strip can quickly provide accurate results. The Dermatophyte Test Strip is also convenient and practical in the actual clinical setting because it is provided as a ready-to-use kit. Although direct microscopy is the gold standard for the diagnosis of tinea unguium, it has disadvantages; for example, it takes time to dissolve the nail specimens and it requires skill to identify the fungal elements. The Dermatophyte Test Strip is a useful method that overcomes these disadvantages. It should however be noted that the selection of the sampling site and method of sampling are critical for any test methods. Specialists with sufficient experience should collect the specimens in accordance with the method recommended in the guideline.²³

In conclusion, the present results showed that the Dermatophyte Test Strip has high detection capacity and provides a reliable, convenient and quick test for the detection of tinea unguium.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Study investigators and institutions.