Dokkyo Journal of Medical Sciences
48(3) : 143 ～ 149, 2021

Original

PCR for Undiagnosed Sporotrichosis using DNA from Paraffin-embedded Skin Specimens : A Case Study Report from a Single Center

Tomoko Kaminaga, Shujiro Hayashi, Masami Koike, Ken Igawa

Department of Dermatology, Dokkyo Medical University

SUMMARY

Background : The gold standard for diagnosing cutaneous sporotrichosis involves microbiological culture. However, occasionally, culture returns false negatives. In other patients, when mycosis is not suspected, culture specimens are not collected. For example, there are cases in which this disease is not suspected initially because it is difficult to distinguish these lesions from similar manifestations that result from other infectious agents and cutaneous neoplastic lesions. We present a representative case, which imitated squamous epithelial carcinoma clinically and histopathologically. Recently, our group performed nested PCR of DNA extracted from formalin-fixed and paraffin-embedded tissues using *Sporothrix*-specific primers.

Objectives : To assess the clinical utility of this method among cases in which deep cutaneous sporotrichosis was not suspected before skin biopsy, so specimens were not collected, and in cases with clinically suspected but culture-negative sporotrichosis.

Materials & Methods : Biopsy specimens were collected from three groups : suspected cutaneous sporotrichosis cases that were histology-positive, but not previously sent for culture ; clinically suspected cases, histology-positive and culture-negative ; and clinically suspected cases, histology-negative and culture-negative. PCR was performed on these formalin-fixed and paraffin-embedded (FFPE) biopsy samples.

Results : Among the cases for which initial cultures were not submitted, 81.3% were positive for sporotrichosis. In the groups with clinically suspected but culture-negative sporotrichosis, there was also some sporotrichosis detected.

Discussions : For patients with negative-culture results, or in situations in which specimens have not been submitted for culture, this method might be useful for diagnosis. Because this was a single-center study, further research on this method at other facilities appears warranted.

INTRODUCTION

Cutaneous sporotrichosis is a deep dermatomycosis caused by organisms of the *Sporothrix schenkii* complex. The gold standard for diagnosis involves the isolation of the fungus by culture[1]. Although tissue from a fresh sample obtained under local anesthesia for biopsy is used for culture, the culture might return a false-negative result[1]. Moreover, there are cases in which this disease is not suspected, and culture is therefore not performed at the time of skin biopsy, because the clinical form of cutaneous sporotrichosis is not disease-specific and it is difficult to distinguish these lesions from similar manifestations that result from other infectious agents and cutaneous neoplastic

Received June 9, 2021 ; accepted August 3, 2021
Reprint requests to : Shujiro Hayashi
Department of Dermatology, Dokkyo Medical University, Mibu, 321-0293, Japan.

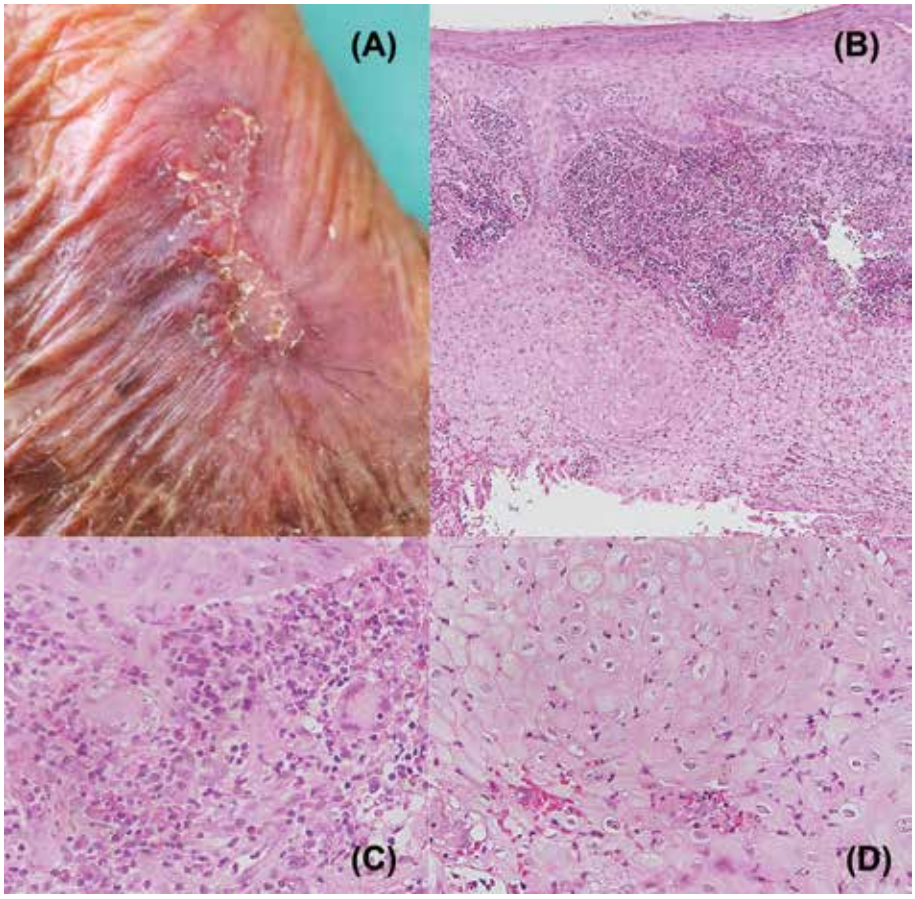

Figure 1

Clinical presentation of a 85-year-old man whose differential diagnosis ofcutaneous squamous cell carcinoma (**A**). Histopathologic study of the first skin biopsy (HE) : Acanthosis, pseudocancerous proliferation of the epidermis, and inflammatory cells (lymphocytes, histiocytes, neutrophils, and giant cells) revealed in the dermis ; ×100 (**B**), ×400 (**C**). Nuclear division was observed in a portion of the proliferating epidermal cells ; ×400 (**D**).

lesions[2]. Furthermore, microscopic examinations after applying potassium hydroxide to purulent exudate from a lesion or histopathological studies of tissue specimens are insufficient to diagnose sporotrichosis[1]. The classic cigar-shaped fungal cells or asteroid bodies characteristic of sporotrichosis are detected in only a small number of cases[3].

We have previously attempted to detect *Sporothrix* by extracting DNA from formalin-fixed and paraffin-embedded (FFPE) tissues using nested PCR[4]. This diagnostic method makes it possible to prove *Sporothrix* existence in FFPE tissue.

Here, we present a patient who was suspected cutaneous neoplastic lesions prior to operation and was not submitted to a culture test but in whom sporotrichosis was detected by PCR. Moreover, to verify whether this method is actually useful in clinical practice, we also investigated patients who were suspected histopathologically to have sporotrichosis without submitting for culture as well as those being clinically suspected but culture-negative.

CASE PRESENTATION

An 85-year-old man was referred to our department because of an irregularly raised, crusted mass on the back of his right hand (Figure 1A). The lesion had increased in size gradually for approximately 2 months and measured 3.5 cm. Histopathological study of the first skin biopsy revealed acanthosis, pseudocancerous proliferation of the epidermis, and inflam-

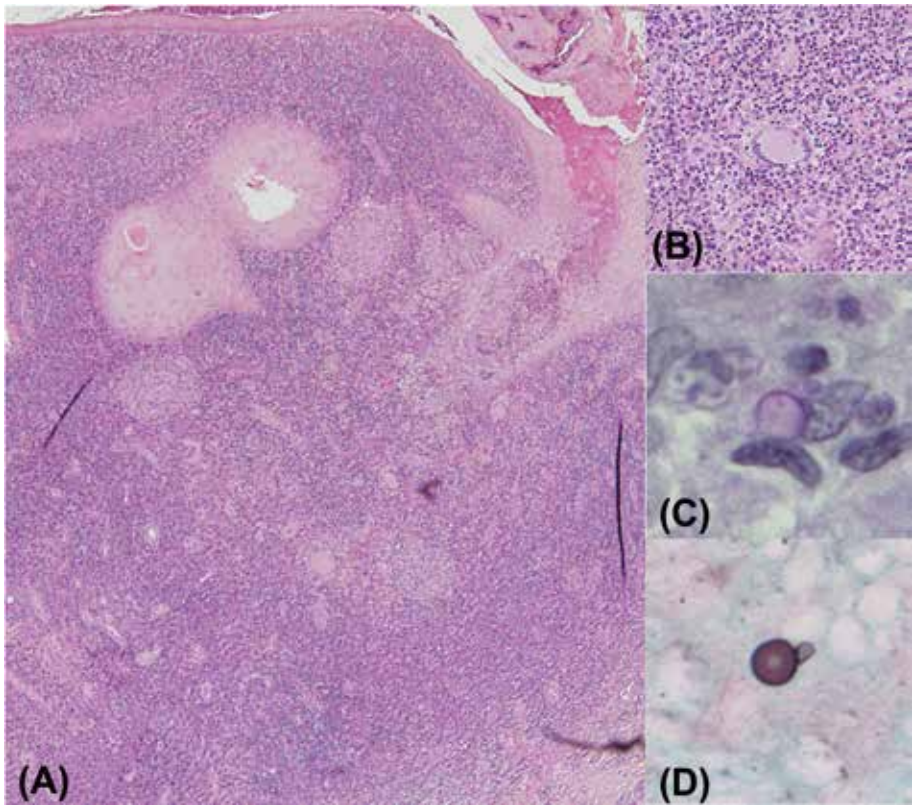

Figure 2

Histopathological findingsin whole tissue showed inflammatory granulation tissue and giant cells suspicious for fungal infection (HE, ×100) (**A**). Histopathological findings at higher magnification (HE, ×400) (**B**). Fungal elements (PAS, ×400) (**C**), (Grocott, ×400) (**D**).

matory cells (lymphocytes, histiocytes, neutrophils, and giant cells) in the dermis (Figure 1B, C). Nuclear division was observed in a portion of the proliferating epidermal cells (Figure 1D). The clinical and initial pathological findings suggested it as highly differentiated squamous cell carcinoma. All the lesions were excised, and the skin was surgically grafted. A specimen was not sent for culture. However, histopathological examination in the whole tissue samples showed inflammatory granulation tissue and giant cells in Hematoxylin-Eosin (HE) staining, suspicious of findings for fungal infection (Figure 2A, B). There was no evidence based on diagnosing of squamous cell carcinoma in whole removed tissue sample. Special staining, both Periodic acid-Schiff stain (PAS) and Grocott's methenamine silver staining (Grocott), showed fungal elements (Figure 2C, D). Therefore, PCR by our established method was performed from the FFPE sample and a positive result was obtained. We explained to the patient that the skin lesions most likely developed due to fungal infections such as *Sporothrix*, and was not neoplastic lesions, and that fungal culture would be performed if the lesions recurred.

MATERIALS AND METHODS

Patients and histopathological examination

Over a 40-year period (1979-2019), we collected biopsy specimens from patients who were thought to have cutaneous sporotrichosis and were subject to histopathological evaluation but were not submitted for cultures. We also collected biopsy specimens from patients with clinically suspected cutaneous sporotrichosis, but whose cultures were negative. In this study, we investigated whether the lesion tissues (FFPE samples) from patients who failed to obtain positive results from culture tests and had no definitive diagnosis contained a DNA fragment specific to *Sporothrix*. All patients underwent histopathological examination using PAS, Grocott, and HE for FFPE tissues.

Patients were divided into three groups : histopathological-positive without submitting for culture, including the aforementioned case (N=16, group 1), clinically suspected cutaneous sporotrichosis, histopathological-positive and culture-negative (N=5, group 2), and clinically suspected cutaneous sporotrichosis with no any other histopathological diagnosis (the cases that were finally histologically diagnosed with other diseases were excluded), in addition to negative fungal elements and culture-negative (N=31, group 3).

Nested PCR

Primers SS1, SS3, and SS4 were used in the nested PCR, as reported previously[4]. The design of oligonucleotides used in this study was based on the comparison of the sequence of the 18S rRNA gene from *S. schenckii* (accession no. M85032) with the corresponding sequences from other fungi in the GenBank database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA). Two sets of primers binding to the 18S rRNA gene sequence of *Sporothrix*, but not to the corresponding sequences from humans and other common human pathogens, were selected. These primers have been verified previously for the specific detection of *Sporothrix* DNA[5]. The outer primer set was SS1 and SS4, which are sequences complementary to those at positions 1007-1026 and 1297-1275 in the GenBank sequence, respectively. The inner primer set consisted of SS3 and SS4. SS3 is complementary to positions 1146-1168 in the deposited sequence ; SS3 and SS4 were expected to generate a 152-nucleotide amplicon.

RESULT

Representative patient who met the exclusion criteria and analysed patients in group 3

The entry patients whose pathology diagnosed other skin diseases and not cutaneous sporotrichosis were excluded. For example, an 84-year-woman had skin lesions on her face with clinically suspected cutaneous sporotrichosis (Figure 3A) ; however, her culture test returned negative and pathology diagnosis was consistent that of basal cell carcinoma (Figure 3B). Such patients were excluded and nested PCR assay was not analyzed.

We analyzed the patients in group 3. A 6-year-girl had skin lesions on her face with clinically suspected cutaneous sporotrichosis (Figure 3C) ; however, the sample for culture and pathological test were quite small because she was too young to stay still during the skin biopsy under the local anesthesia. Resulting, culture returned negative and pathological test showed no specific finding, which was only inflammatory cells invasion in the dermis (Figure 3D). However, the nested PCR assay demonstrated positive result. Another patient in group 3 was a 72-year-woman with skin lesions on her right knee with clinically suspected cutaneous sporotrichosis (Figure 3E) ; however, culture test returned negative and histopathologic showed only a large number of inflammatory cells with no negative fungal elements and no diagnostic specific findings in order for diagnosis (Figure 3F). The nested PCR assay also demonstrated negative result.

Results of each analyzed group

Results of the PCR testing are shown in Table 1. In group 1, 81.3% (13/16) of patients had *Sporothrix* detectable by PCR (Figure 4A). In groups 2 and 3, 80.0% (4/5) and 12.9% (4/31, Figure 4B), respectively, of clinically suspected but culture-negative patients had positive PCR results.

DISCUSSION

In this study, we demonstrated that the same tissue specimens contained a DNA fragment of *Sporothrix* with clinically and histologically suspected sporotrichosis, even if the culture tests were negative or unsubmitted.

Sporotrichosis is a fungal infection caused by *Sporothrix* species, which have distinct geographical distributions and virulence profiles. Although culturing for cutaneous sporotrichosis using skin tissue provides the definitive diagnosis, some patients test negative because of insufficient sampling of the skin tissue or poor culture technique for the fungus. In addition, because sporotrichosis is a deep-skin mycosis and the removal of skin tissue for the culture test is performed under local anesthesia (an invasive procedure), generally, the sampling for the culture test is performed at the same time as the skin biopsy[4].

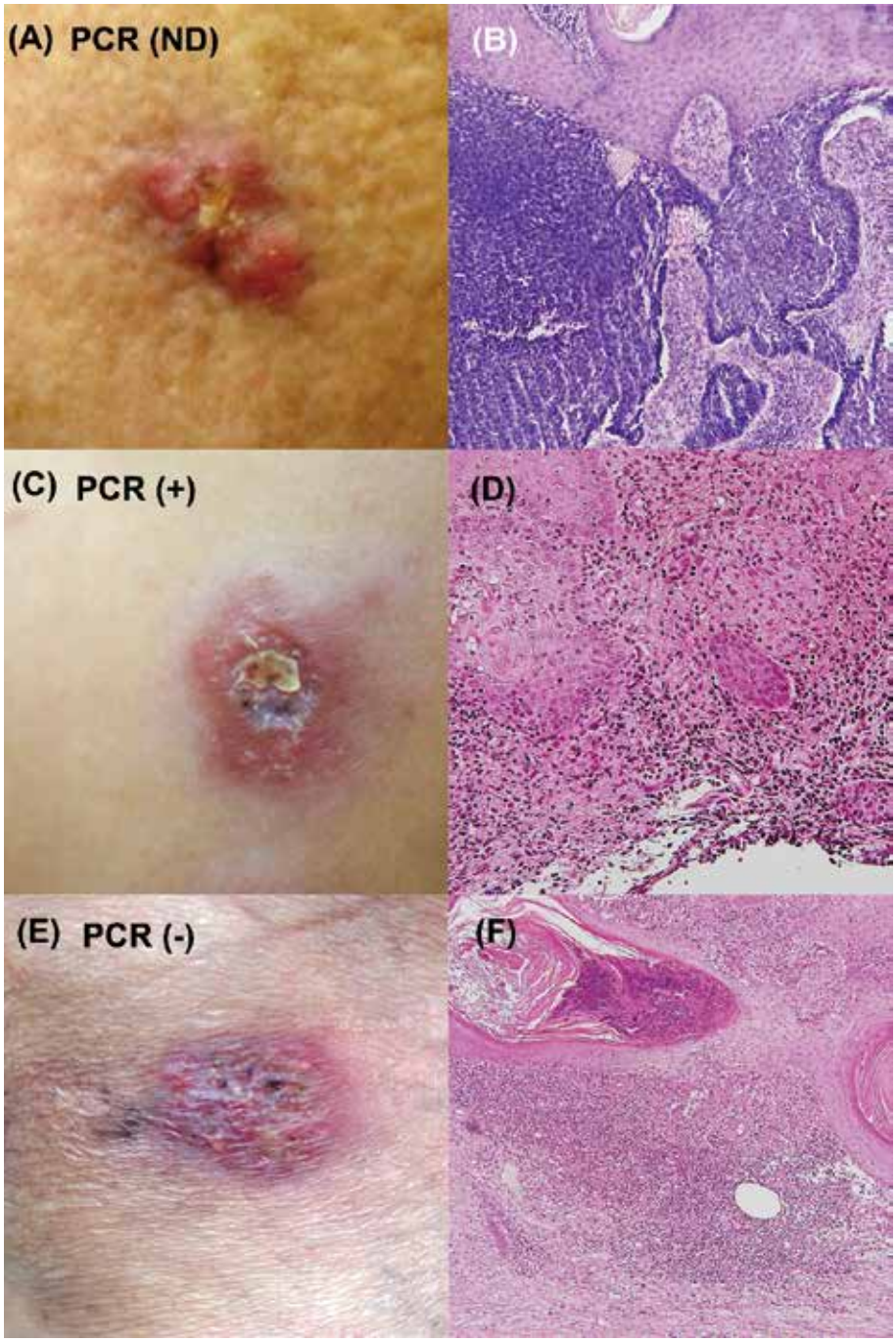

Figure 3

Representative patient who met the exclusion criteria and analysed patients in group 3. The clinical and pathological findings of the representative patient who meet the exclusion criteria and analysed patients in group 3. The skin lesion clinically suspected cutaneous sporotrichosis (**A**) ; however, pathology diagnosis consisted of basal cell carcinoma (**B**). The skin lesion with also clinically suspected cutaneous sporotrichosis (**C** and **E**) ; however, culture returned negative and pathological test showed no specific finding, which was only inflammatory cells invasion in the dermal (**D** and **F**). B, D and F ; hematoxylin & eosin staining, ×400 (**B**, **D**), ×200 (**F**). ND ; no date, PCR ; Polymerase chain reaction.

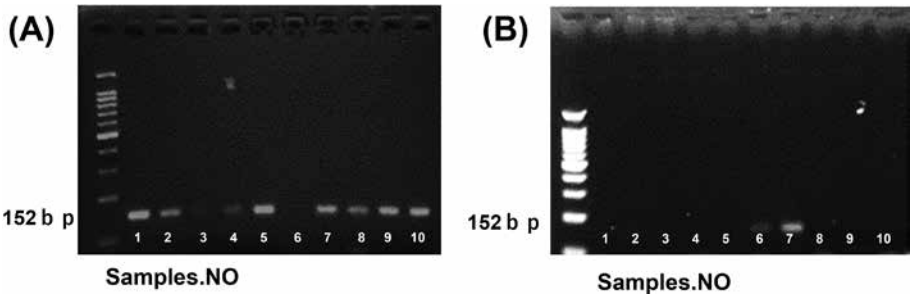

Figure 4

The representative results of 10 cases were showed in Figure. Of the 16 patients (group 1) for whom specimens were not submitted for culture, 13 patients (81.3%) had Sporothrix detectable by the PCR assay (**A**). Of the 36 patients (groups 2 and 3) with clinically suspected but culture-negative cutaneous sporotrichosis, eight patients (22.2%) had positive results by the PCR assay (**B**).

Table 1 Patient characteristics and nested PCR assay results

	Cases	Fungal elements identified on histopathological examination	Sporotrichin skin test result	Culture result	Positive by nested PCR assay
Group1 Positive histopathologic test and not submitted for culture	16	16/16	10/14*	ND	13/16**
Group2*** Positive histopathologic test and negative culture test	5	5/5	0/3	0/5	4/5
group3*** Negative histopathologic and negative culture test	31	0/31	0/3	0/31	4/31

ND ; not done, PCR ; polymerase chain reaction

* Three patients with a negative sporotrichin skin test were positive by the nested PCR assay.

** One patient with a positive sporotrichin skin test was negative by the nested PCR assay.

*** These patients were clinically suspected cutaneous sporotrichosis, but negative culture test.

In 2003, a PCR assay for diagnosing cutaneous sporotrichosis was reported by Hu *et al.*[5] The method uses cryopreserved portions of the fresh skin tissue collected during skin biopsy. When cutaneous sporotrichosis was not suspected clinically at the time of biopsy, but histopathological findings were consistent with cutaneous sporotrichosis, DNA samples from fresh tissues would not have been available.

Generally, FFPE tissue is difficult to amplify by PCR because DNA is fragmented owing to formalin fixation, whereas, in previous reports, PCR can be performed from FFPE by targeting short DNA regions[6]. Moreover, two-step PCR (nested PCR) can target fungal DNA, even small quantities in FFPE[5,6]. We performed PCR using DNA from FFPE with the modified combination of primers in Hu *et al.*[4], targeting short DNA regions. Assaying 52 patients who tested positive by culture as well as 72 patients who were confirmed clinically and histopathologically to have conditions other than sporotrichosis, as a control group, resulted in 100% sensitivity and 98.7% specificity in that study[4]. Considering the sensitivity, many of the cases with negative-PCR and -culture were possibly other infections or bacterial pyoderma (group 3 ; 26/31 ; Figure 3E, F).

PCR-positive findings were obtained in three patients for whom the sporotrichin skin test (SST) was negative, whereas one patient was positive by

SST but negative by PCR (Table 1). The SST is positive for *Sporothrix* infection in 52.2% of patients[1]. However, the SST test cannot make a definitive diagnosis. For example, the SST does not become positive in patients with a low immune response (false-negative). If there is a history of *Sporothrix* infection in the past, positive results can occur even in patients who do not have *Sporothrix* infection at present (false-positive).

A limitation of our method is that it detects not only live fungus but also dead fungus, which might be contaminating the sample. Moreover, this method cannot distinguish among several fungi, such as *Sporothrix schenckii* and/or *Sporothrix Grobosa*. Therefore, the PCR result must be judged comprehensively with clinical/pathological findings and clinical course. Nevertheless, our method could contribute to the provisional diagnosis of cutaneous sporotrichosis in cases in which culture tests are negative or samples have not been submitted for culture that might be clinically helpful.

Acknowledgments

This study was supported by a grant from Dokkyo Medical University, Young Investigator Award (No.2019-10), Tochigi, Japan. We also thank Miki Kanno and Saiko Koike for their technical assistance.

Conflict of interest

None declared.

Author Contributions

Conceptualization : SH and TK ; Data Curation : SH ; Funding Acquisition : MK ; Investigation : SH ; Project Administration : SH ; Supervision : IK ; Writing - Original Draft Preparation : SH and TK.

REFERENCES

1) Bonifaz A, Toriello C, Araiza J, et al : Sporotrichin Skin Test for the Diagnosis of Sporotrichosis. J Fungi **4** : E55, 2018.
2) Rodríguez-Brito S, Camacho E, Mendoza M, et al : Differential identification of Sporothrix spp. and Leishmania spp. by conventional PC and qPCR in multiplex format. Med Mycol **53** : 22-27, 2015.
3) Arenas R, Sánchez-Cardenas CD, Ramirez-Hobak L, et al : Sporotrichosis : From KOH to Molecular Biology. J Fungi **4** : E62, 2018.
4) Hayashi S, Kaminaga T, Baba A et al : Diagnostic value of a nested polymerase chain reaction for diagnosing cutaneous sporotrichosis from paraffin-embedded skin tissue. Mycoses **62** : 1148-1153, 2019.
5) Hu S, Chung WH, Hung S et al : Detection of Sporothrix schenckii in clinical samples by a nested PCR assay. J Clin Microbiol **41** : 1414-1418, 2003.
6) Futatsuya T, Anzawa K, Mochizuki T, et al : Molecular Identification of Fungi in Formalin-Fixed and Paraffin-Embedded Skin Tissue Samples. J Dermatol **46** : 171-172, 2019.