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Original Article

Sensitivities of periodic acid-Schiff staining, Grocott's silver staining and calcofluor white staining in the diagnosis of human sporotrichosis

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Abstract: Objective: This study aimed to investigate the sensitivity of periodic acid-Schiff (PAS) staining, Grocott's silver staining (GSS) and calcofluor white (CFW) staining in the diagnosis of sporotrichosis. Methods: Paraffin embedded tissues (n = 100) which were diagnosed with sporotrichosis by fungal culture were subjected to PAS, GSS, and CFW staining, and the detection rate of sporotrichosis was determined. Results: The sensitivity of PAS, GSS, and CFW staining was 31%, 40% and 74%, respectively, in the diagnosis of sporotrichosis. Conclusion: CFW staining has a high sensitivity in the diagnosis of sporotrichosis, and sections are easily observed and can be repeatedly stained after CFW staining. For patients suspected to have sporotrichosis, CFW staining may be employed for early diagnosis before a fungal culture.

Keywords: Sporotrichosis, special histopathological staining, calcofluor white

Introduction

Sporotrichosis is a subacute or chronic infection caused by thermodimorphic fungi of the genus *Sporothrix* [1], and in recent years, the prevalence of sporotrichosis is increasing worldwide [2]. Humans usually acquire the infection through traumatic inoculation of the fungus during outdoor activities (such as farming, gardening, animal husbandry) and similar occupations [3]. However, zoonotic sporotrichosis seems to be occupation-independent and any contact with an infected animal can predispose to the infection [2]. Some parts of China, especially Northeast areas (including Jilin, Heilongjiang and Liaoning), have a relatively high prevalence of sporotrichosis [4]. Jilin is an important agricultural province in northeast China and Sporothrix isolates have been noted cornstalks, dead branches, rotten wood, sludge, soil, and tree bark in this region with a rate from 8.6% to 15% [5, 6]. This may be one of the reasons for the high prevalence of sporotrichosis in Jilin.

Based on its clinical manifestations, sporotrichosis can be classified into fixed cutaneous, lymphocutaneous, disseminated cutaneous, and extracutaneous forms [7]. Recently, a new classification was proposed as new clinical presentations were identified, to better describe the clinical features of sporotrichosis [8]. The lesions of sporotrichosis may be plaque-like, nodular, verrucous, or ulcerated papules. Special clinical presentations were also observed in our department, such as acne-like, verruca-like, and cutaneous tuberculosis-like lesions [4]. The diagnosis of atypical sporotrichosis is still challenging because of its similarities to many other dermatologic diseases in clinical presentations [9].

The primary screening of sporotrichosis is based on clinical experience. Histopathological examination and fungal culture are usually employed once sporotrichosis is suspected. The most important use that is given to the tissue obtained from a biopsy is for a fungal culture because it is rarely identified in the histopathology study with haematoxylin and eosin (H&E) [10]. The definitive diagnosis of sporotrichosis is still dependent on the fungal culture. However, fungal culture will take up to four we-

eks, which may delay the antifungal treatment in some cases [11]. The PAS and GSS staining are mandatory due to the difficult observation of fungal structures in the tissues [12]. Special histochemical stains, such as GSS, has been proposed for the diagnosis of human and animal sporotrichosis and are usually employed to enhance the visualization of yeast-like cells in tissues [13, 14].

Calcofluor white (CFW) that can bind to chitin and cellulose and fluorescein that can emit fluorescence after exposure to UV can be used for the detection of fungal components [15]. CFW staining has been used in the auxiliary diagnosis of candida, onychomycosis, and fungal pathogen of corneal ulcer [16-18]. In China, CFW was also reported to be used in the diagnosis of sporotrichosis with favorable performance. In our previous study, results showed it was easier to observe yeast-like forms by CFW.

Convenient tools that can enable rapid and reliable results are highly desirable for the diagnosis of sporotrichosis, which is crucial for early treatment [19]. The present study investigated the sensitivity of PAS, GSS, and CFW staining in the diagnosis of sporotrichosis.

Materials and methods

Samples

100 paraffin embedded tissues that were diagnosed with sporotrichosis by fungal culture were employed for independent PAS, GSS and CFW staining in three sections from the same tissue, respectively.

PAS

After routine deparaffinization, sections were washed with distilled water for 1-2 min, and then treated with periodic acid solution for 10 min. After washing in distilled water, sections were subjected to Schiff staining for 10-15 min, followed by washing in flowing water. Nuclear staining with hematoxylin was done for 2-5 min, followed by washing in flowing water. After dehydration and transparentization, mounting was done with neutral gum. The mold, glycogen and neutral mucus material are red and the nucleus is blue on PAS staining. Materials were purchased from Zhuhai Beisuo Biotech Co., Ltd.

GSS

After routine deparaffinization, sections were treated with periodic acid solution for 15 min, followed by washing in flowing water. Then, sections were incubated with hexamethylamine silver at 62°C for 30-60 min until black was present on the yellow-brown background. Following washing in flowing water, sections were treated with sodium thiosulfate for 3 min, followed by washing in flowing water. Counterstaining was performed with eosin for 30-60 s, followed by routine dehydration and transparentization. Mounting was done with neutral gum. Mold, elastic fibers and some reticular fibers are black on GSS. Materials were purchased from Zhuhai Beisuo Biotech Co., Ltd.

Fluorescence staining

After deparaffinization and hydration, sections were stained for 2 min. Following mounting, sections were observed under a fluorescence microscope. Positive result was defined once identifiable strong or weak green fluorescence was observed; very weak fluorescence or no fluorescence was considered negative; repeated examination was needed for suspected positive tissues. Fungal fluorescence staining solution was purchased from Jiangsu Laifu Shidai Technology Co., Ltd.

Statistical analysis

Sections were assessed by an experienced pathologist and a skin pathologist. The presence of yeast-like forms consistent with Sporothrix schenkii was considered positive. Statistical analysis was performed with SPSS version 21.0, and quantitative data were compared by Chi square test. A value of $P < 0.05$ was considered significant.

Results

100 tissues were diagnosed with sporotrichosis by fungal culture before this study. Among these tissues, the detection rate of sporotrichosis was 31% (n = 31) for PAS, 40% (n = 40) for GSS and 74% (n = 74) for CFW. Statistical analysis showed the detection rate of CFW was significantly higher than that of PAS and GSS, but there was no marked difference between PAS and GSS (**Table 1**). In 100 tissues, positivity was confirmed in 3 tissues on PAS and 1 tis-

Table 1. Detection rate of CFW, PAS, and GSS in the diagnosis of Sporotrichosis

Method	Negative	Positive	Detection rate
PAS	69	31	31%[a,c]
GSS	60	40	40%[b]
CFW	26	74	74%

Notes: [a]P < 0.05 vs CFW; [b]P < 0.05 vs CFW; [c]P > 0.05 vs GSS.

Table 2. Detection rate of PAS+GSS+CFW and CFW alone

Methods	Detection rate
CFW	74%[a]
PAS+GSS+CFW	77%

Notes: [a]P > 0.05 vs PAS+GSS+CFW.

sue on silver staining, but negative result was found on fluorescence staining. Moreover, the detection of PAS+GSS+CFW was only 77%, which was comparable to that of CFW (P > 0.05) (**Table 2**). On PAS, round or oval spores could be observed, the sporular wall was red, and the nucleus was blue (**Figures 1A** and **2A**). On GSS, black round spores were observed (**Figures 1B** and **2B**). On CFW, round spores with strong or weak green fluorescence were noted (**Figures 1C** and **2C**). As shown in Figures, the spores were observable by the three stains when the amount of spores was relatively large; when the amount of spores was small, spores with strong or weak fluorescence were observable on CFW (**Figure 3**).

Discussion

Unlike tumors with relatively high homogeneity, fungal infections possess heterogenicity, and therefore the diagnosis of fungal infection is dependent on the sections used.

In the diagnosis of cat sporotrichosis, Silva et al found the sensitivity of GSS was as high as 91.3% [20]. Miranda investigated the histopathology of cat sporotrichosis and they found the sensitivity was 94% for GSS in the diagnosis of Cat sporotrichosis, but the sensitivity of PAS in the diagnosis of cat sporotrichosis is still unclear. In addition, they also proposed that the skin lesions had a large amount of spores in cats with sporotrichosis, and more a precise method is needed when the amount of fungi is small [13, 21]. In the diagnosis of dog sporotrichosis, Miranda found the sensitivity of PAS and GSS was 19.5% and 43.7%, respectively [22]. In the present study, the sensitivity of PAS was 31% and that of GSS was 40% in the diagnosis of human sporotrichosis. In 100 tissues, a large amount of spores were observed in 10 tissues (> 10 spores per section), and no more than 5 spores were observable in the

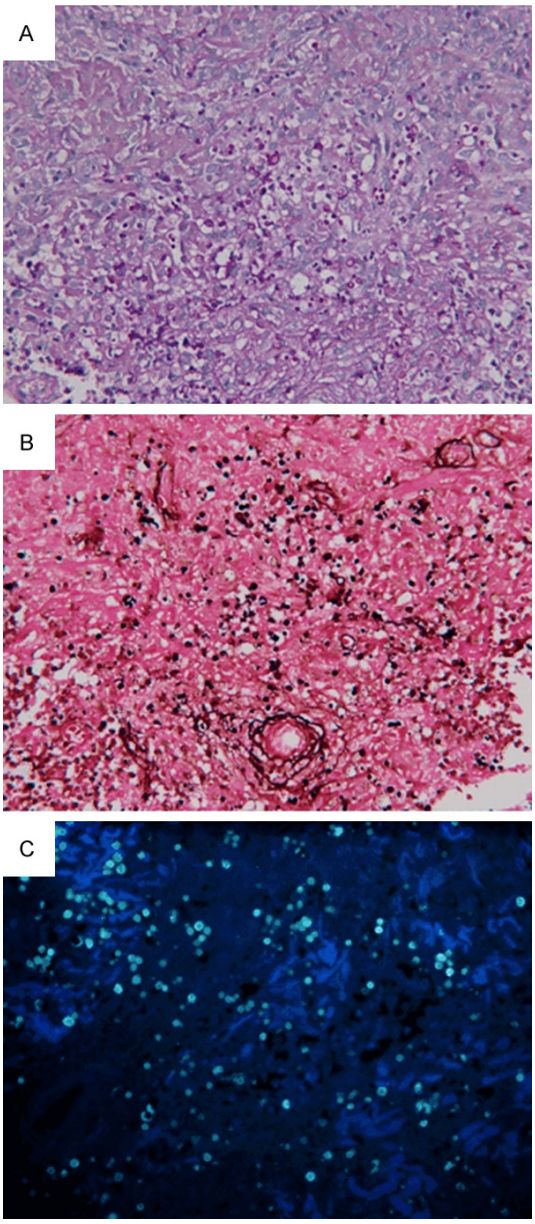

Figure 1. PAS, GSS, and CFW staining of the same tissue (No 50). A: 400×; on PAS, the sporular wall was red and the nucleus in addition to the wall was red in round or oval spores; B: 400×; on GSS, round or oval blackbrown spores were observed. C: 200×; spores with strong or weak green fluorescence were observed.

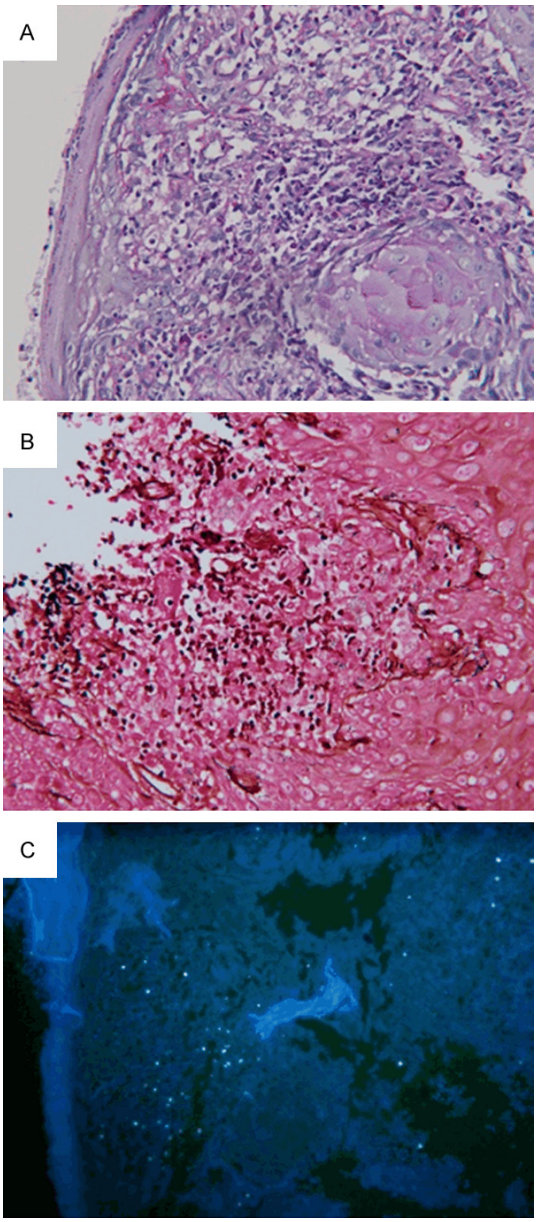

Figure 2. PAS, GSS, and CFW staining of the same tissue (No 52). A: 400×; on PAS, the sporular wall was red and the nucleus in addition to the wall was red in round or oval spores; B: 400×; on GSS, round or oval blackbrown spores were observed. C: 200×; spores with strong or weak green fluorescence were observed.

majority of tissues. This indicates that the amount of spores in human sporotrichosis lesions is small.

In our study, only 3 tissues were positive on PAS and 1 was positive on silver staining among 100 tissues, but they were negative on CFW; in the remaining tissues positive on PAS

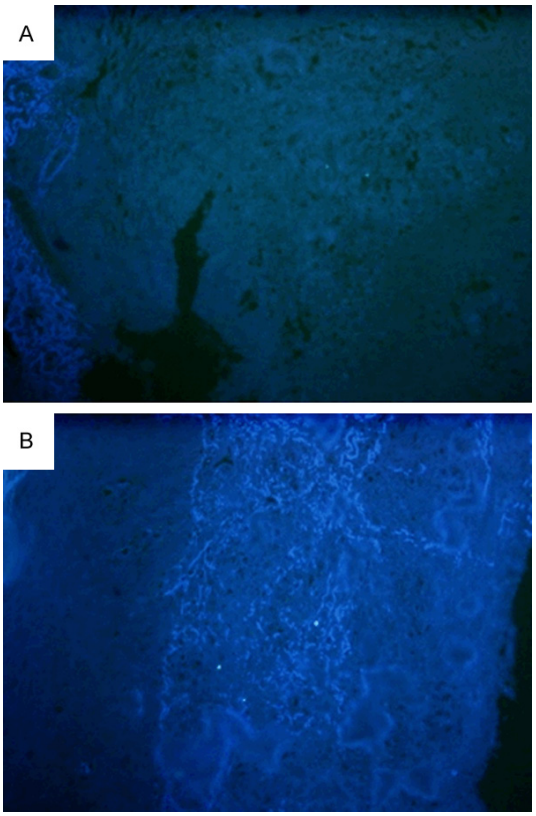

Figure 3. A and B: A small amount of spores was observed on CFW (200×).

and GSS, positivity was seen on CFW. Among the three methods, the detection rate of CFW was significantly higher than that of PAS and GSS, but there was no marked difference between PAS and GSS. Moreover, the detection rate of CFW+PAS+CFW (77%) was comparable to that of CFW alone. This suggests that combined staining may not achieve a better positive rate as compared to CFW alone. In addition, sections can be observed within 2-min CFW fluorescence staining. Observation may be repeated within 10 min and counterstaining is feasible after washing in water, which facilitates the rapid assessment of sections. When the amount of spores is large, the spores show round or oval fluorescence, and strong fluorescence is suggestive of positive staining. When the amount of spores is small, false positive staining is suggested if fluorescence movement is observed on moving the coverslip; true positive staining is indicated if fluorescence location remains stable on moving the coverslip. If the amount of spores is small, it is recommended to repeat examination three

times; true positive staining is confirmed if the fluorescence location remains stable.

Our results showed, when the amount of fungi was large, the spores were easily identified after different stains. In the sections of No 50 and No 52 (**Figures 1A-C**, **2A-C**), a large amount of spores was observed under a microscope, which indicates that an alternative staining may be employed if a stains fails to confirm the presence of fungi.

When the amount of fungi was small, CFW staining achieved a higher detection rate. In CFW, the fluorescein binds to the wall of fungi to form complexes which emit fluorescence at the specific excitation wavelength. Our results showed observation and assessment of sections was relatively convenient and easy after CFW: round or oval fluorescence was suggestive of positive staining; the elastic fibers with blue fluorescence could be discriminated based on the morphology.

Of note, the detection rate was comparable between GSS and PAS. The fungal morphology after PAS is superior to that after GSS [23]. GSS has complex procedures and is time-consuming, and over-staining is common with GSS. In the present study, the findings after PAS and GSS were interfered with by glycogen, neutral mucus, and inflammatory cells, and therefore the assessment of PAS and GSS should be done by an experienced pathologist and/or skin pathologist.

Conclusion

In conclusion, our study indicates the amount of fungi in human sporotrichosis tissues is relatively small; among three staining methods, CFW is easy to perform, observation of sections is simple and convenient, and the sensitivity is higher. We recommend CFW for fungal staining if possible before a fungal culture because CFW is simple, observation is easy and results can be rapidly obtained. Of note, the fluorescence will quench, which makes the long-term storage of sections impossible.

Disclosure of conflict of interest

None.

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