

Rapid Detection of Sporotrichosis by ABC-ELISA

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Abstract

Background: Avidin-biotin-horseradish peroxidase enzyme complex, enzyme-linked immunosorbent assay (ABC-ELISA) is widely used in detecting the soluble bacterial, viral antigen and antibody, but the reports of detecting mycotic antigens were few. We used the method to detect *Sporothrix schenckii* pathogen of sporotrichosis in smear.

Aim: To provide a rapid diagnostic method for sporotrichosis.

Methods: 20 cases of sporotrichosis-in doubt we scraped pus or tissue fluid from the skin lesion and perform ABC-ELISA by reagent vectastatin ABC kit, rabbit anti *Sporothrix schenckii* serum.

Results: 18 of 20 cases were diagnosed as sporotrichosis. 14 of 18 cases were positive (77.8%) by ABC-ELISA stain, 5 of 18 cases were positive (27.7%) by Gram stain.

Conclusion: ABC ELISA is new fashioned biological amplification technique due to its sensitivity of this method is 77.8% in smear, the specificity is 100%. Only three hours is needed, so this method can be used to detect *Sporothrix schenckii* rapidly.

Keywords: *Sporothrix schenckii*; Sporotrichosis; ABC-ELISA

Introduction

Sporotrichosis is a subcutaneous mycosis caused by *Sporothrix schenckii* that is widely distributed worldwide particularly common in the tropics, where moisture and temperature promotes fungal growth, and can be found in soil, plants and other Humus. There are three types in clinical findings: a fixed type, lymphocutaneous type and disseminated cutaneous form. It was reported that sporotrichosis was endemic [1-4]. In China sporotrichosis is more frequent in Jilin Province mainly affecting farmers who are infected due to skin injuries and by fungal spores. A definite diagnosis is based on the culture method, biopsy to find spores in histochemical stains, such like haematoxylin and eosin (HE), periodic acid Schiff, (PAS) and Gomori's methenamine silver (GMS) methods. The culture method requires at least one week but can take up to four weeks for final results. A rapid sensitive detection method is required for early detection of Sporotrichosis. Here we report an immunohistochemical method, Avidin-biotin complex (ABC)-ELISA to detect *Sporothrix schenckii* in tissue fluid or pus to improve sensitivity in diagnosis of sporotrichosis. This method takes three hours for diagnosis.

Materials

Reagent: Vectastain ABC Kit (Ig G of rabbit) (Vector - USA).

Preparation of rabbit anti sporotrichosis serum (to independently manufacture)

A total of 20 suspected patients with sporotrichosis were enrolled. All of them are farmers who came from epidemic area of sporotrichosis in Jilin Province, northeast of China. There are 12 males and 8 females. Male to female ratio is 1 to 0.67. The minimum age is 7 years old, the maximum age is 56 years old, with an average age of 36 years old. The shortest course is one month, the longest course is 12 months, and Mean duration was 3 months.

Methods

Preparation of rabbit anti sporotrichosis serum

Yeast form of *Sporothrix schenckii* was grown on 5% rabbit blood infusion agar. Cells were collected from the plate and suspended in 10 mL normal saline. Formaldehyde 5% mL was added to this suspension and incubated for 24 hours to inactivate the cells. Cells were washed three times using distilled water and finally re-suspended in 1: 100v/v in normal saline. 1 mL of this inactivated cells were injected in the vein of the rabbit ears for first day, the rabbit was 2.5 kg weight, 6 months old, Chinese white rabbit. We increased 1 mL of this inactivated cells everyday next three days, total four times for injection. After seven days, rabbit was bled by puncturing carotid, blood was collected 50 mL and serum was collected by spinning at 10,000 rpm for 10 min and stored at -20 degrees Celsius.

Clinical group

To do punch biopsy, used half of specimen to culture onto Sabouraud dextrose agar (SDA), incubated at 25 degrees Celsius for 4 weeks and remaining half specimen for histopathology with HE stain. Smear was prepared from pathological tissue fluids or pus from 20 suspected patients.

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Control group: Smear or Micro culture slide of *S. schenckii*, and common pathogenic fungi: *Trichophyton rubrum*, *Trichophyton gypseum*, *Microsporium lanosum*, and *Candida albicans*.

ABC-ELISA staining procedure

Two drops normal sheep serum was added to the smear and incubated under 37 degrees Celsius for 10 minutes in order to block non specific binding; then rabbit serum generated against *S schenckii* diluted 1:100 was added to the smears, followed by incubation at 37 degrees Celsius for 30 minutes., Smear was rinsed with 5 mL TBS (Tris-buffered saline). 100 microliter biotinylated Goat anti rabbit IgG (1: 200 dilution) was added on the smear and incubate at 37 degrees Celsius for 30 minutes, then rinse with TBS add biotin-avidin labeled Ig G of rabbit that diluted 200times, incubate at 37 degrees Celsius for 60 minutes, then rinse them with TBS agent; add DAB (3,3'-Diaminobenzidine) and incubate at 37 degrees Celsius for 20 minutes, then rinse them with water, observe by microscope (Olympus)

Spore with brown coloration is considered positive. The spore with light yellow coloration or without yellow coloration that is considered negative.

Results

Experimental observation

Spores of *S. schenckii* stained dark brown with slight rough fringe with refractivity. *Monilia albicans*, *Trichophyton rubrum*, *Trichophyton gypseum*, *Microsporium lanosum* and *Aspergillus* were not stained. But spores of *Candida albicans* stained light yellow.

Clinical observation

20 suspected patients were included and 18 were diagnosed with sporotrichosis by clinical, endemic, biopsy and culture at last. Out of these 18 diagnosed cases, 14 cases stained positive through the ABC-ELISA method and only 5 showed spores positive by Gram stain. The positive rate to detected spores of *S. schenckii* is 28% by HE and is 78% by ABC-ELISA.

Discussion

Detection of sporotrichosis is done using several methods such as HE stain, PAS stain, silver stain and sporotrichin skin test [5,6]. HE stain is a common and widely used method to diagnose dermatological diseases. Fungal organisms (*S. schenckii* yeast cells, budding spores, cigar shaped bodies and asteroid bodies) are often identified by HE, PAS, and GMS stains. Among these stains PAS is more efficient to visualize fungi, but GMS is better at detecting old and non-viable fungal elements,[7] HE stain is very useful to visualize host response but it is not a specialized stain so it is not useful in staining all fungi. The positive rate is very low to detected spores of *S. schenckii* by HE, PAS and GMS, that is 23%, 23%, and 37%, respectively [8]. All these stains cannot distinguish morphologically similar fungi, so these histochemical methods are not good methods for high accuracy and sensitivity in diagnosing sporotrichosis.

Biopsy

Biopsy was performed, but the histopathological pattern of sporotrichosis was nonspecific which usually represents a combination of granulomatous and pyogenic inflammation [9]. Histopathology is one of the major methods to diagnose fungal infections, due to the advantages of low cost, speed and identify fungus and tissue reactions with different stains.

Culture

Most physicians rely on in vitro culture on Sabouraud's agar to diagnose sporotrichosis, *Sporothrix schenckii* exhibits thermal dimorphism, at 25degrees Celsius it grows as a brown-black mycelial colony producing conidia. Culturing is the gold standard to identify fungus, however culture may takes at least a week, most of time two weeks or longer to yield a result that still might fail because of no growth or contamination.

Serology

Immunological methods such as immunodiffusion and immunoelectrophoresis use antigen complex from fungal culture filtrate. Both the tube and latex agglutination have high sensitivity and specificity. Immunoenzymatic assay are increasingly being used for the serodiagnosis of sporotrichosis. The antigen preparation from *S. schenckii* yeast form provide high sensitivity and specificity [10], ELISA method using concanavalin a binding peptide rhamnomanan from the *S. schenckii* yeast cell wall provide high sensitivity as well.

Immunofluorescence

Both direct and indirect immunofluorescence method has been used to detect *S. schenckii* with high sensitivity and specificity [11,12].

Sporotrichin test

There are two types of antigens that could be used in sporotrichin tests [5,6]. Poly-saccharidic (P-antigen) is one antigen, which is obtained from cultures of *Sporothrix schenckii* that can precipitate with serum from some patients with sporotrichosis. It is reported that this antigen can elicit positive results with almost 100 percent accuracy [6]. Whether the P-antigen will elicit a positive result on an unaffected person hasn't been reported. These results imply that a sporotrichin test will elicit a positive reaction on persons depends on many factors, including the growing phase of *S. schenckii*, the dilution of the culture filtrates and the person's immune status. As we know tests through skin, like sporotrichin and tuberculin test, usually cause delayed allergic reactions in the skin [5]. People who have been in contact with even little amount of the same organism before and who are carrying the organism may both show positive results. The Sporotrichin made from P-antigen seems to have a relatively higher accuracy than the other, but considering the interference factors; we conclude sporotrichin test may be used as a screening test in diagnosing sporotrichosis. A positive result of sporotrichin can't confirm a definite diagnosis.

Molecular or DNA detection method

PCR based amplification of the fungal gene sequences is a powerful tool for identifying mycoses. First description of PCR for the diagnosis of sporotrichosis was reported by Kano et al. [13,14]. Specific oligonucleotide primers based on the chitin synthase 1 gene were designed, with the primer pair, PCR was able to detect a 10 pg genomic DNA fragment of *S. schenckii*. PCR assay for the detection of *S. schenckii* was evaluated in clinical samples using 18s rRNA gene sequences as the target. PCR detected *S. schenckii* DNA samples from clinical patients with sporotrichosis confirmed by culture or histochemical staining [15]. PCR has high specificity and sensitivity and provides rapid diagnose in suspected patient with sporotrichosis.

Immunocytochemical method

The immunocytochemical method has been proven to be a rapid and specific way in testing *S. schenckii* infection in biopsy sections

[7,16]. It is known that the avidin enhancing the sensitivity of the enzyme staining, because the four high-affinity sites of the avidin can bind with biotin, so we see avidin as a bridge which can make biotin-antibody complex (B-IgG) couple more biotinylated multienzyme complex (B-E). ABC-ELISA is abiomagnification technique, which can greatly improve the sensitivity of antigen-antibody reaction. A common problem we are concerned with regarding this method is the probable cross-reaction with other fungi. The result shows that 18 of the 20 culture-confirmed sporotrichosis patients' exudation and pus sample from skin lesions, 14 of 18 cases are stained brown yellow by ABC-ELISA (Figure 1 and 2) in smear, the sensitivity of 77.8% and specificity of 100%. Other fungi only stained with light yellow or without coloration. Antisera to *S. schenckii* doesn't appear to cross react with *Trichophyton rubrum*, *Trichophyton gypseum*, *Microsporum lanosum* and *Aspergillus*, only *Candida albicans* stained with light yellow. Scott et al. [10] analyzed reactivity of antisera through western immunoblot between the components of sporotrichosis' antigen and the sera from patients with a variety of fungi including *S. schenckii*, and also didn't find cross-reactivity, except that only one disseminated histoplasmosis' serum showed a faint Western blot antibody band to the 40-kDa antigen of *S. schenckii*. On the other hand, Ishizaki [17] reported that a serological cross reactivity of *S. schenckii* with various fungi occurred in half of the experimental rabbits by immunodiffusion technique, but the sample only included four rabbits.

As we know, there are many factors affecting the preparation of antisera specific for *S. schenckii*, including the immunization procedures and the immunological responsiveness of individual experimental animal. In our research, the antisera in the kit (American Vector Company) was made from rabbit too, but in a different immunological procedure which may contribute to the result here. We also consider the light yellow stain to be negative with an antisera dilute of 1:100, thus we get a high specificity of 100% and a sensitivity of 77.8% in smear preparations. As our research shows that is a high positive rate (96.2%) in biopsy sample with a series of sections in 1994 [16].



Figure 1: A brown, slight rough fringe with refractivity in smear X 1000.



Figure 2: A dark brown spore in smear X 400.

The method described herein only needs three hours for completion; therefore we think this immunocytochemical method can be utilized for a rapid screening of sporotrichosis.

Conclusion

Sporotrichosis is diagnosed by culture, biopsy, histochemical methods, immunofluorescence, or polymerase chain reaction (PCR). All of these techniques described for the diagnosis of sporotrichosis, have strengths and weaknesses. We feel that ABC-ELISA method should be used for diagnosis of sporotrichosis with patients who have exudates or pus as this test has high sensitivity and specificity.

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