

Chapter 8

Diagnosis of Sporotrichosis: Current Status and Perspectives

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Abstract Definitive diagnosis of sporotrichosis is based on fungal detection in culture. Microscopic methods for the detection of *Sporothrix* yeast cells in clinical samples have low sensitivity. Although culture methods have high sensitivity, they also have some limitations, such as the time required to conclude the diagnosis, usually from 10 to 15 days, and the difficulty of obtaining an adequate clinical specimen for the test in cases of extracutaneous sporotrichosis. Serological methods are useful tools for a presumptive diagnosis of this infection. The most-used antigenic *Sporothrix* molecules are the peptide-rhamnomannan and secreted exoantigens. The enzyme-linked immunosorbant assay (ELISA) technique using the peptide-rhamnomannan has high efficiency, and it is useful in the serological follow-up of infection. Exoantigens were first used in immunoprecipitation and agglutination tests, but they have been used more recently in immunoenzymatic tests, with high sensitivity and specificity for both human and feline disease. A glycoprotein of 70 kDa was purified from *Sporothrix* exoantigens, presenting high immunogenicity, which allows its use in the development of more sensitive and specific methods for sporotrichosis serodiagnosis. Molecular methods of diagnosis can lower the time for diagnosis conclusion, but described methodologies in this field are scarce. In conclusion, the diagnosis of sporotrichosis is a challenging field, and the development of new serological and molecular diagnostic methods is mandatory.

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8.1 Introduction

Mycoses can be challenging to diagnose, and accurate interpretation of laboratory data is important to ensure appropriate treatment. Although the clinical manifestations of sporotrichosis are well described, the diagnosis of this mycosis cannot be based on clinical information alone because the symptoms of sporotrichosis overlap with those of other diseases.

Sporotrichosis is classically diagnosed by correlating clinical, epidemiological, and laboratory data (Zancope-Oliveira et al. 2011). Typical laboratory analyses include microscopic examination using 10 % potassium hydroxide or 4 % sodium hydroxide to detect parasitic cigar-shaped, budding, yeast-like cells. These fungal cells are small (2–6 μm in diameter), rare, and difficult to detect during direct examination of specimens obtained from human patients (Kwon-Chung and Bennett 1992) or from domestic animals, such as dogs (Schubach et al. 2006). On the other hand, when this test is performed on skin biopsies collected from cats infected with *Sporothrix schenckii*, yeast cells are easily observed because cats have a high fungal burden on their lesions (Schubach et al. 2004). Microscopic methods for yeast detection in clinical samples are of low sensitivity (Barros et al. 2011), and asteroid body observation varies among different works (Quintella et al. 2011). However, definitive diagnosis of sporotrichosis is based on fungal detection in culture (Zhang et al. 2011). Cultivation of clinical specimens in mycological media such as Sabouraud dextrose agar or mycobiotic agar yields white filamentous colonies that become brown to black after a few days. Subculturing these colonies in brain–heart infusion at 35–37 °C results in white to creamy yeast-like colonies (Barros et al. 2011). *S. schenckii* identification is based on the macro- and micro-morphologies of the mycelial (Fig. 8.1) and yeast forms (Zancope-Oliveira et al. 2011). However, these characteristics do not differentiate the newly described species of the *Sporothrix* complex. To physiologically differentiate the species within this complex, other tests such as carbohydrate assimilation (especially sucrose and raffinose), growth rates at 30 and 37 °C, as well as production of dematiaceous sessile conidia are necessary (Marimon et al. 2007, 2008). However, discrepancies between physiological and molecular methods of identification have been described (Oliveira et al. 2011). Moreover, although positive cultures provide the strongest evidence for sporotrichosis, there are some significant limitations. In particular, in some manifestations of the disease, such as *S. schenckii*-induced arthritis, the collection of material for culture is difficult (Morris-Jones 2002). In addition, sporotrichosis may be mistaken for other infections, such as tuberculosis, leishmaniasis, paracoccidioidomycosis, gummatous syphilis, and chromoblastomycosis (Rippon 1988; Sharma et al. 2005).

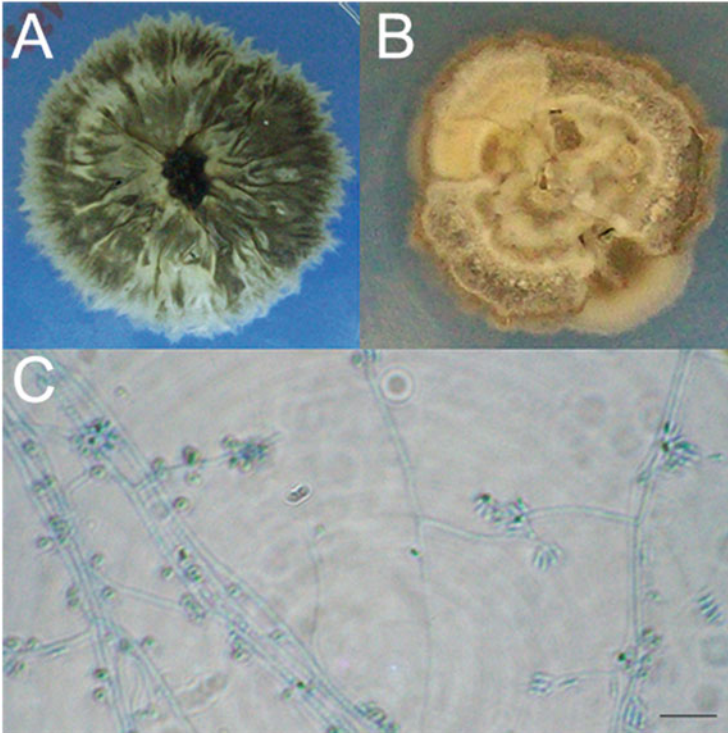


Fig. 8.1 Morphologic characteristics of the mycelial form of the *Sporothrix* complex. (a) *Sporothrix schenckii* sensu stricto colony on potato dextrose agar, incubated at 30 °C for 21 days. (b) Colony of a *S. brasiliensis* strain, showing a similar morphology to the *S. schenckii* strain. (c) Slide culture of the *S. schenckii* strain, showing hyaline conidia on sympodial conidiophores and dematiaceous conidia on denticles arising from the hyphae. Bar 10 μ m

Non-culture methods have been developed to improve the rate and speed of diagnosis. Additional diagnostic tools are currently available for diagnosis of sporotrichosis to supplement culture and microscopic examination. These laboratory tests have a rapid turnaround time and reasonable specificity and sensitivity. For instance, serological techniques involving antibody detection have been developed using different methodologies. Molecular methods to detect *Sporothrix* species complex DNA in clinical specimens, including tissue fragments, are also being studied in several laboratories to facilitate rapid diagnosis of infection (Ruiz-Baca et al. 2013; Oliveira et al. 2014). The results from the described tests can provide a presumptive diagnosis of sporotrichosis and require clinical correlation for the correct evaluation and determination of the final diagnosis.

Serological techniques are usually simpler than culture and very useful in the diagnosis and follow-up of patients with sporotrichosis. The following serologic tests have been applied in the diagnosis of Diagnostic methods: agar gel immunodiffusion (ID), slide (SLA), tube (TA) and latex agglutination (LA), complement

fixation reaction (CF), immunofluorescence (IF) (Blumer et al. 1973), immunoelectrophoresis (IEP) (Albornoz et al 1984), and immunoenzyme assays in several formats, such as ELISAs, and immunoblots. These techniques have advanced considerably in recent decades because of the development of innovative detection schemes in the identification of relevant *Sporothrix* spp. antigens. However, the literature concerning the serodiagnosis of sporotrichosis is neither extensive nor diverse.

A range of antigenic preparations, derived both from whole yeast cells and from culture filtrate in their crude and/or purified states, have been applied in serological tests, which has resulted in high cross-reactivity, one of the most persistent problems found in the serodiagnosis of sporotrichosis. Moreover, these antigenic preparations are highly variable, making it very difficult to standardize diagnostic techniques in different laboratories.

The next sections focus on the main antigens and serologic methods applied for the presumptive diagnosis of sporotrichosis, as well as the application of some antigenic preparations in skin tests and molecular diagnosis.

8.2 Antigen Detection

The antigenic composition of the members of the *Sporothrix* complex is poorly understood. The most studied molecules with immunological reactivity of these fungi are the peptide-rhamnomannan, the exoantigens, and the newly described gp-70.

The *Sporothrix* peptide-rhamnomannan is a fraction of the fungal cell wall that presents an affinity to concanavalin A(Con-A) and that reacts with antibodies present in sera from patients with sporotrichosis (Lloyd and Bitoon 1971). Through a western blot analysis, this Con-A binding fraction of the *Sporothrix* cell wall was characterized as a mixture of three antigenic fractions of 84, 70, and 58 kDa. Moreover, through a β -elimination procedure, it was verified that the 70-kDa molecule is a deglycosylated form of the 84-kDa antigen (Lopes-Bezerra and Lima 1997). This antigen is stable, and its immunological reactivity does not significantly change if different *Sporothrix* strains are used in the extract preparation (Bernardes-Engemann et al. 2009). The potential application of this antigen to the serodiagnosis of sporotrichosis was initially suggested by the observation that cross-reactions with paracoccidioidomycosis, cryptococcosis, aspergillosis, candidiasis, and histoplasmosis were absent in the Con-A binding fraction of the cell wall peptide-rhamnomannan (Loureiro Y Penha and Lopes-Bezerra 2000). Later, an ELISA to detect immunoglobulin (Ig)-G in serum from patients with several clinical forms of sporotrichosis was described using this specific antigenic fraction, with a sensitivity of 90 % and specificity of 80 % (Bernardes-Engemann et al. 2005). The Con-A binding fraction of the *Sporothrix* cell wall peptide-rhamnomannan can also be used to detect antibodies in the synovial fluid (Costa

et al. 2008) and to monitor therapeutic response to antifungal treatment (Orofino-Costa et al. 2009).

The exoantigens from *Sporothrix* spp. were first applied in several immunological assays, such as IEP, LA, and double ID (Karlin and Nielsen 1970; Blumer et al. 1973; Casserone et al. 1983; Albornoz et al. 1984). However, there were no standard procedures for the production of the antigenic extracts used. In fact, changes in sugar composition of the exoantigens occur during *Sporothrix* growth, indicating a need for standardization (Takata and Ishizaki 1983). Differences in exoantigen composition and immunological reactivity were also related to the morphological state of the fungus (Albornoz et al. 1984), to the culture medium employed (Mendoza et al. 2002; Fernandes et al. 2009), and to the geographical origin of strains (Fernandes et al. 2009). Differences in exoantigen composition among the species of the *Sporothrix* complex are poorly studied, but it does not appear to significantly impact the antigenic composition of secreted molecules. A recent study showed heterogeneous protein profiles of exoantigens obtained from the mycelial form of *S. brasiliensis*, *S. globosa*, and *S. schenckii sensu stricto* in Sabouraud dextrose medium. The proteins of 60 and 46 kDa were observed in all extracts, regardless of species. Moreover, it was not possible to characterize specific secreted molecules for these species (Fernandes et al. 2013). Similar observations were obtained with the secreted antigens from the yeast form of *S. brasiliensis* and *S. schenckii s. str.* A molecule of 85 kDa was detected in both species, and no significant differences in antigenic composition or immunological reactivity were observed among the species (Almeida-Paes et al. 2012).

In a study of the yeast-phase *Sporothrix* exoantigens, it was verified that a 70-kDa antigenic fraction was always reactive against serum antibodies present in experimentally infected mice after 14 days of infection (Nascimento and Almeida 2005). Further studies against this antigen showed that a monoclonal antibody with affinity to this protein was able to enhance phagocytosis by macrophages. Moreover, this monoclonal antibody reduced the fungal burden and inhibited the *Sporothrix* interaction with the extracellular matrix (Nascimento et al. 2008). The role of this antigen as an adhesin was elucidated in subsequent studies (Ruiz-Baca et al. 2009; Teixeira et al. 2009). It is interesting to note that an immunological reactivity against the *Sporothrix* gp-70 was consistently observed in these studies, which encourages its use in the serodiagnosis of sporotrichosis. Furthermore, this antigen is produced by the three major species of the *Sporothrix* complex (*S. brasiliensis*, *S. globosa*, and *S. schenckii*), thus allowing the sporotrichosis diagnosis regardless of the infective species (Ruiz-Baca et al. 2014).

In general, serological tests as an aid for diagnosis do not use purified or recombinant antigens, because described immune reactive proteins are scarce, especially for the newly described *Sporothrix* species such as *S. brasiliensis*. An immunoblot assay allied with computer-based analysis was used to identify putative antigenic molecules in cell-free extracts of both morphological phases of this fungus, and to delineate antigenic polymorphism among seven *S. brasiliensis* isolates and one *S. schenckii* Brazilian strain. The mycelial and yeast phase of the fungus originated 14 and 23 reactive bands, respectively, which varied in intensity.

An 85-kDa antigen, verified in the yeast phase of the fungus, was observed in all strains used, and the immunodominant protein was identified. However, this protein cross-reacted with sera samples from patients infected with other pathogens (Almeida-Paes et al. 2012). It was also demonstrated that the use of different strains or even the morphological form of *Sporothrix* isolates could have an effect on the antigenic reactivity of a *Sporothrix* extract. Therefore, an adequate standardization of antigens must be produced before their general use in the serodiagnosis of sporotrichosis.

8.3 Antibody Detection

8.3.1 Immunoprecipitation and Agglutination Techniques

Immunoprecipitation and agglutination methodologies were first used in the diagnosis of sporotrichosis in the period 1970–1980 (Albornoz et al. 1984; Blumer et al. 1973; Casserone et al. 1983; Karlin and Nielsen 1970). The first TA and CF tests for sporotrichosis were reported in 1910 (Widal et al. 1910), and a diagnostic precipitin test that employed a polysaccharide antigen was subsequently described by González-Ochoa and Figueroa (1947). The ID test for sporotrichosis usually does not cross-react with sera from patients with chromoblastomycosis or leishmaniasis, infectious diseases with similar clinical manifestations (Albornoz et al. 1984). IEP has also been used, and in all positive cases, an anodic arc, called an S arc, is observed (Albornoz et al. 1984). Both methodologies that use an antigenic complex from fungal culture filtrate are highly sensitive. TA and LA both have high sensitivity and specificity and have been used for sporotrichosis serodiagnosis since the 1970s (Blumer et al. 1973; Casserone et al. 1983; Karlin and Nielsen 1970). However, these tests lack sensitivity in cases of cutaneous sporotrichosis (Albornoz et al. 1984; Rippon 1988) and do not enable the determination of the immunoglobulin isotype involved. The lack of standardization of reagents and methodologies applied in these techniques means they are not routinely used in the diagnosis of sporotrichosis in clinical laboratories.

8.3.2 Immunoenzymatic Assays

The serodiagnosis of this mycosis has increasingly used immunoassays. The first immunoblot assay used for diagnosis of sporotrichosis dates back to 1989, when exoantigen preparations from the *S. schenckii* yeast form showed 100 % sensitivity and 95 % specificity for the detection of antibodies (Scott and Muchmore 1989). Later, another immunoassay (ELISA) was developed, using the Con-A binding peptide-rhamnomannan from the *S. schenckii* yeast cell wall, and antibodies were

detected in 35 serum samples from patients with culture-proven sporotrichosis, resulting in 100 % sensitivity. However, the specificity was lower than previous tests because there was cross-reactivity with sera from patients with cutaneous leishmaniasis (Penha and Lopes-Bezerra 2000). The same group reported on an ELISA test using the same antigenic preparation against sera from 92 patients with different clinical forms of sporotrichosis in Rio de Janeiro and reported 90 % sensitivity, 80 % specificity, and a global efficiency of 86 % (Bernardes-Engemann et al. 2005). Other studies showed that the use of different strains during the preparation of the antigen might result in different sensitivity and specificity, despite the purification of the antigen involved in this methodology. This difference is due to the *O*-glycan residues linked to the molecules (Bernardes-Engemann et al. 2009).

An ELISA to detect IgG antibodies reactive to the mycelial-phase *Sporothrix* exoantigens produced after 21 days of growth in Sabouraud dextrose medium at 28 °C showed 97 % sensitivity and 89 % specificity when performed on 90 sera from patients with different clinical forms of sporotrichosis, 72 sera from patients with other infectious diseases, and 76 healthy controls. The major antigenic components present in this preparation were proteins of 90, 70, 63, 51, and 42 kDa (Almeida-Paes et al. 2007a). This assay was further improved to detect IgG, IgM, and IgA antibodies, which improves the global efficiency for diagnosis and therapeutic follow-up of sporotrichosis (Almeida-Paes et al. 2007b). Mendoza et al. (2002) previously described this exoantigen, and the lack of cross-reactivity of this preparation with sera from patients with other mycoses was remarkable. The same antigen was used previously in ID and IEP techniques without cross-reactivity with sera from patients with leishmaniasis or chromoblastomycosis (Albornoz et al. 1984).

When the ELISAs probed with different antigenic preparations are compared, the crude exoantigens (Almeida-Paes et al. 2007a) gave slightly higher sensitivity and specificity than those using the con-A binding fraction of the *S. schenckii* yeast cell wall (Bernardes-Engemann et al. 2005). A similar observation was found when using this con-A binding fraction and crude exoantigens for the serodiagnosis of feline sporotrichosis. The use of crude exoantigens showed slightly better results than the purified peptide-rhamnomannan in terms of sensitivity (96 and 90 %) and specificity (98 and 96 %, respectively), suggesting that the *Sporothrix* secreted proteins are highly immunogenic and specific (Fernandes et al. 2011).

More recently, an immunoblot assay using cell-free exoantigens of the yeast form of *S. brasiliensis* was described to detect IgG antibodies in serum samples from human patients. In this assay, a sensitivity of 100 % was achieved, but a low specificity (50 %) was observed. However, if the authors verified a positive serum sample only if at least two immunological bands appeared in the immunoblots, the specificity increased to 80 % with 93 % sensitivity (Almeida-Paes et al. 2012).

8.3.3 *Sporotrichin Skin Test*

The skin test (ST), with mycelial extracts or yeast cells called sporotrichin, has been widely used throughout the world, especially for epidemiological studies of sporotrichosis. It has also been applied as support for diagnosis in Latin America (Lopes-Bezerra et al. 2006; Barros et al. 2011; Bonifaz and Vázquez-González 2013) and in atypical forms of the disease, such as the case of a recent bulbar conjunctival sporotrichosis (Kashima et al. 2010). However, sporotrichin is not available commercially in many countries (Dominguez-Soto and Hojyo-Tomoka 1983; Bonifaz and Vázquez-González 2013), but it is accessible in many institutions dedicated to biomedical research. Reports are contradictory concerning its usefulness as a diagnostic tool due to false-positive results without signs or symptoms of the disease, but this condition suggests a previous immunosensitizing contact (exposure) with *S. schenckii*. Other reports mention almost 100 % positive sporotrichin ST in lymphocutaneous and fixed cutaneous forms of sporotrichosis (Barros et al. 2011; Bonifaz and Vázquez-González 2010).

The use of sporotrichin ST in epidemiological surveys has been extensively developed from the first surveys in Mexico (González-Ochoa and Ricoy 1970), Guatemala (Mayorga et al. 1978), and Brazil (Rocha-Posada 1968), among others. This practical test has been used until the present (Bonifaz et al. 2013), demonstrating the usefulness of these antigens to identify endemic regions of sporotrichosis worldwide, an option to gain insight in disease emergence scenarios such as the recent cat zoonosis in Brazil (Barros et al. 2004; Oliveira et al. 2011; Freitas et al. 2010; Silva et al. 2012).

Concerning the term sporotrichin, there are reports of different type of antigens. Culture filtrate extracts from the mycelial (28 °C) or yeast (37 °C) forms, commonly called metabolic antigens, were first described by González-Ochoa and Figueroa (1947) and consisted of glycopeptide antigens. Another described and used antigen is a 1:1000, 1:2000, or 1:4000 dilution of heat-killed yeast cells. In addition, their biological properties depend on culture conditions (Takata and Ishizaki 1983; Arenas and Toriello 1986). A commonly used antigen for epidemiological surveys is a culture filtrate of the mycelial form standardized to 10 µg protein/0.1 ml of intradermal ST (Toriello et al. 1991; Bonifaz et al. 2013). The ST may be applied on the back or forearm and readings made at 24 and 48 h. A reaction of 8 mm of induration after 24 and 48 h constitutes a positive test.

The lack of standardization in the above-mentioned antigens would account for the difference in reactivity with sporotrichin ST, such as a 6.25 % positivity with a 1:4000 dilution of a yeast antigen in the southern state of Oaxaca, Mexico with *S. schenckii* isolates recovered from soil (Sánchez-Aleman et al. 2004). This is in contrast with the 14 % positivity observed with a mycelial metabolic antigen at 10 µg protein/0.1 ml in an endemic region of sporotrichosis in Puebla, Mexico, without any fungal isolation (Mendez-Tovar et al. 2003).

Additional epidemiological studies of sporotrichosis and the *S. schenckii* complex are necessary because changes in the interplay of pathogens, hosts, and

environment lead to the formation of novel disease patterns as observed for sporotrichosis.

8.4 Molecular Diagnosis

Molecular methods have been developed based on polymerase chain reaction (PCR) techniques that show good sensitivity, specificity, and speed for early diagnosis of *S. schenckii* (Berbee and Taylor 1992; Kano et al. 2001, 2003; Hu et al. 2003; Xu et al. 2010; Mendoza et al. 2012; Liu et al. 2013; Ruiz-Baca et al. 2013; Oliveira et al. 2014). Sandhu et al. (1995) proposed the pioneering DNA-based methodologies used for the diagnosis of fungal infections, and developed 21 specific nucleic acid probes targeting the large subunit of the ribosomal RNA (*rRNA*) gene from several fungi, including *S. schenckii*. Among the major genes described for the diagnosis of sporotrichosis are the chitin-synthase gene 1 (*CHS1*), 18S rDNA, and mitochondrial DNA (mtDNA) (Berbee and Taylor 1992; Kano et al. 2001, 2003; Hu et al. 2003; Xu et al. 2010). The trials with nested PCR using the 18S rDNA gene and mtDNA as targets showed high sensitivity and specificity, indicating that these tests can provide a rapid diagnosis with sufficient accuracy to be used clinically in patients with sporotrichosis (Hu et al. 2003; Xu et al. 2010; Oliveira et al. 2014). A drawback of these methods is that they are not commercially available and require specialized equipment; therefore, they are not carried out in most clinical laboratories.

8.5 Conclusions and Perspectives

Sporotrichosis is classically diagnosed by correlation among clinical, epidemiological, and laboratory data. The conventional method for definitive diagnosis of sporotrichosis is based on etiological agent isolation in culture and its identification. A disadvantage of the culture methods, is that they are difficult to apply in disseminated and/or systemic sporotrichosis; therefore, detection of an antibody response in patients could provide a faster method for diagnosis. Sporotrichin ST remains an option for epidemiological studies to gain insight in disease emergence scenarios such as the recent cat zoonosis in Brazil. To date, serological techniques (like ELISA, Western blot, immunodiffusion, etc.) are used for sporotrichosis diagnosis and involve antibody detection against soluble antigens and different molecules of the *S. schenckii* cell wall, such as peptide-rhamnomannans, exoantigens, and the newly described molecule gp70. The sensitivity and specificity of these methods differ depending on the antigenic fraction used. Fluorescent antibodies and immunohistochemical techniques are alternative methods that also provide a rapid diagnosis of sporotrichosis in tissue samples. The diagnosis of *S. schenckii* by PCR in clinical samples has also shown a high degree of sensitivity

and specificity. These tests can provide a rapid diagnosis with sufficient precision to be used clinically for patients with sporotrichosis, with PCR studies for the disseminated cases of the disease.

The different methods described in this review are not available in many clinical laboratories, which compromises the ability to diagnose and provide individual treatment for sporotrichosis. The search for new protein biomarkers for the development of an easy, sensitive, and specific methodology could help lower the treatment costs and offer alternatives to current tests for the diagnosis of sporotrichoid infections that can be confused by infections caused by other pathogens. To date, efforts continue to improve or develop new diagnostic tests that are more sensitive and specific for this mycosis. However, these tests require validation prior to general application in routine diagnosis. The development and application of new biomarker molecules for the diagnosis of sporotrichosis remains to be done either with exoantigens or cell wall antigens. The recent release of the *S. schenckii* genome, and the use of certain tools such as genomics, proteomics, immunoproteomics, metabolomics, interactomics, etc., would enable major advances in the search and identification of new biomarker antigens for sporotrichosis, which are expected to be introduced to the clinical laboratory in the short or medium term.

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