Archives of Veterinary Science **ISSN 1517-784X**
v.26, n.3, p. 72-81, 2021 ww.ser.ufpr.br/veterinary

SPECIES-SPECIFIC PCR FOR THE DIAGNOSIS OF SPOROTRICHOSIS CAUSED BY *Sporothrix brasiliensis*

(PCR espécie-específico para o diagnóstico de esporotricose causada por *Sporothrix brasiliensis*)

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Editora: Julia Arantes Galvão

ABSTRACT - Despite thousands of sporotrichosis cases related to the zoonotic transmission of *Sporothrix brasiliensis* have been described, the diagnostic gold standard is still the classical culturing methods. The mycological culture results are available after seven to 30 days of incubation. Since an early diagnosis contributes to improving the treatment and to the spread control of this mycosis, studies evaluating faster diagnostic methods are needed. Therefore, we aimed to evaluate the species-specific PCR for the sporotrichosis diagnosis caused by *S. brasiliensis*, using samples collected with a non-invasive technique. Seventy-four swabs from feline (n=64), canine (n=5), and human (n=5) suspect sporotrichosis cases were included. All samples were submitted to classical methods for diagnosis and to *S. brasiliensis*-specific PCR. Using mycological culture as the gold standard, the diagnosis of *S. brasiliensis*-caused infection was confirmed in 69% (51/74) of the cases. PCR was positive in 30 out of these 51 cases, showing 59% sensitivity, 100% specificity, 72% accuracy, 100% positive predictive value, and 52% negative predictive value. Consequently, more studies are needed to elucidate the interference factors that culminated with the high rate of false-negative results and then optimize this molecular test for the accurate diagnosis of infections caused by *S. brasiliensis*.

Key words: endemic mycoses; feline sporotrichosis; molecular diagnosis; subcutaneous mycoses; zoonosis.

RESUMO - Apesar de milhares de casos de esporotricose relacionados a transmissão zoonótica por *Sporothrix brasiliensis* venham sendo descritos, o diagnóstico padrão-ouro ainda é a partir do método clássico de cultura. O resultado da cultura micológica demora de sete a trinta dias para ficar disponível. Considerando que o diagnóstico precoce contribuiria para um tratamento prematuro e com isso, com o controle na disseminação desta micose, estudos avaliando um diagnóstico precoce são necessários. Portanto, o objetivo do presente estudo foi avaliar o PCR espécie-específico para o diagnóstico da esporotricose causada por *S. brasiliensis,* utilizando amostras coletas por uma técnica não invasiva. Foram incluídas setenta e quatro amostras de casos suspeitos de esporotricose (swabs) oriundos de felinos (n=64), caninos (n=5) e humanos (n=5). Todas as amostras foram submetidas ao método clássico de diagnóstico e ao PCR específico para *S. brasiliensis*. Utilizando a cultura micológica como padrão-ouro, o

Received in 09/12/2020
Approved in 07/13/2021

diagnóstico da infecção causada por *S. brasiliensis* foi confirmado em 69% (51/74) dos casos. O PCR foi positivo em 30 dos 51 casos, demonstrando 59% de sensibilidade, 100% de especificidade, 72% de acurácia, 100% de valor preditivo positivo e 52% de valor preditivo negativo. Consequentemente, mais estudos são necessários para elucidar os fatores de transferência que culminaram com a alta taxa de resultados falsos-negativos e então otimizar este teste molecular para a acurácia diagnóstica para infecções causadas por *S. brasiliensis*.

Palavras-chave - diagnóstico molecular; esporotricose felina; micoses endêmicas; micose subcutânea; zoonose.

INTRODUCTION

In the last decades, *Sporothrix brasiliensis* acquired great importance in worldwide public health. This species is associated with zoonotic transmission to humans by infected felines. Nowadays, sporotrichosis caused by *S. brasiliensis* has been expanding geographically with thousands of cases described in different Brazilian regions (Montenegro et al., 2014; Gremião et al., 2017; Orofino-Costa et al., 2017; Almeida & Giordano, 2018; Poester et al., 2018; Lacerda Filho et al., 2019). Since domestic cats are predisposed to severe forms of the disease, showing a great number of infective yeasts in their lesions, they are potential *S. brasiliensis* vectors. In addition, feline life habits, such as frequent fights and disputes over territory, females, or food, allow the fast disease spread by intra- and inter-species fungal transmission when scratching or bitting (Barros et al., 2011; Sanchotene et al., 2015; Gremião et al., 2017).

The diagnostic gold standard for sporotrichosis is mycological culture from samples, such as skin biopsy or secretions from lesions. However, a period of seven to thirty days is required for the fungal growth and identification, negatively impacting both the treatment and the clinical recovery (Barros et al., 2011). Early diagnosis and appropriate treatment, associated with vigilance and educational programs are essentials for disease control and containment (Barros et al., 2011; Rodrigues et al., 2015; Gremião et al., 2017; Zhang et al., 2019). In view of this, alternative and direct diagnostic methods to identify the fungus directly from the clinical sample are needed, aiming to provide faster results (Barros et al., 2011; Sanchotene et al., 2015).

Molecular techniques are potential tools for this purpose, showing high sensitivity and specificity rates in the sporotrichosis diagnosis, however, these results were using experimental samples or clinical skin biopsy (Rodrigues et al., 2015; Zhang et al., 2019). Taking into account that swabs from ulcerative lesions are a noninvasive and easy sample collection method, which allows the sporotrichosis diagnosis in the majority of the cases (Sanchotene et al., 2015; Brandolt et al., 2018; Zhang et al., 2019), a molecular diagnostic method using this clinical sample would have important applicability. Therefore, our study

aimed to evaluate the species-specific polymerase chain reaction (PCR) in the diagnosis of the *S. brasiliensis*-caused sporotrichosis from samples collected by swabs, using culture as the gold standard.

MATERIALS AND METHODS

Study design and period

A retrospective cross-sectional study was carried out to evaluate the accuracy of species-specific PCR as a diagnostic method, using mycological culture as the gold standard. For this, all secretion samples collected with a swab (in Stuart transport medium) from human patients and animals with suspected sporotrichosis sent to the Mycology Laboratory of the School of Medicine of the Federal University of Rio Grande (FAMED-FURG) from January to December 2018 were included.

Mycological diagnosis and exclusion criteria

All samples received in the laboratory were processed on the same day within a maximum period of 72 h after collection. The mycological cultures were performed in duplicate on Sabouraud agar with and without chloramphenicol (15mg/L) and/or cycloheximide (Neogen®, Lansing, United States of America; Biolog®, São Paulo, Brazil), and incubated at 25°C for up to 30 days, with a daily fungal growth assessment. Samples were considered negative in cases without fungal growth; positive in cases when it was possible to isolate fungal colonies with macro and micromorphological characteristics compatible with *Sporothrix* spp.; or contaminated in cases with abundant bacterial or anemophilous fungal growth. All contaminated samples were excluded from the study due to the impossibility to classify them as positive or negative, according to the gold standard method (culture). Positive samples were categorized as cases with high fungal load (>100 Colony Formation Units - CFUs of *Sporothrix* spp.) or low fungal load (1-100 CFUs).

Subsequently, direct mycological examination (DME) of clinical samples (swabs) was performed, by slide smear stained by the Gomori-Grocott method (Swisher & Chandler, 1982). Samples showing small oval to elongated blastoconidia under a microscopical examination were considered positives.

After these procedures, all swabs were kept at 6-10°C, until used for DNA extraction and species-specific PCR.

Etiological agent identification as *S. brasiliensis*

After fungal growth in the culture medium, colonies phenotypically identified as *Sporothrix* spp. from all positive samples included in the study were subcultured on Sabouraud (Neogen®, Lansing, United States of America) agar at 25°C for 7 to 10 days to obtain pure, young and viable colonies. Then, DNA extraction was performed as described by Woods et al. (1993) and the identification of the isolates at the species level was performed by species-specific PCR, using the primer pair SbraF (5' - CCC CCG TTT GAC GCT TGG) and SbraR (5' - CCC GGA TAA CCG TGT GTC ATA AT), according to Rodrigues et al. (2015). The reaction mix was performed on 25µL, containing 19.25 µL ultrapure water, 2.5 µL of 10x buffer, 0.75 µL de $MgCl_2$ (50mM), 0.5 µL of each primer, 0.5 µL of dNTP (Ludwig Biotecnologia®, Rio Grande do Sul, Brazil) (10mM) and 0.125 µL of Platinum Taq polymerase Invitrogen® (Thermo Fisher Scientific®, California, United States of America), plus 1 µL of extracted DNA. The DNA was quantified by spectrophotometry, using a NanoVue™ (Harvard Bioscience, Massachusetts, United States of America), and DNA integrity was confirmed in 0.8% agarose gel. Amplification was performed in a Mastercycler (Eppendorf®, Hamburg, Germany). PCR products were analyzed by 1.5% agarose gel electrophoresis in the presence of GelRed® (Biotium, California, United States of America) and samples with 469 bp amplicons were considered positive for *S. brasiliensis*, as described (Rodrigues et al., 2015).

Molecular diagnosis

To perform the molecular diagnosis, swabs were subjected to DNA extraction using an in-house method, adapted from the protocol by Breacker et al. (2016). Briefly, swabs were initially arranged in 1.5 mL microtubes with 300µL of DNA extraction buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, pH 8.0, 200 mM NaCl, and 0.2% SDS) with the addition of 10 µL of proteinase K (10mg/mL; Ludwig Biotecnologia®, Rio Grande do Sul, Brazil). Then, this solution was heated at 55°C for 4 h, cooled, centrifuged for one minute at 13.400 x g, and the swab was removed from the microtube. Then, 100 µL of the protein precipitation solution (5 mM potassium acetate and 11.5% acetic acid) was added, the sample mixed in a vortex, and centrifuged for 5 min at 13.400 x g. The supernatant was transferred to another microtube with 400 µL isopropanol, incubated at 25°C for 5 min, and centrifuged for an additional 5 min at 13.400 x g. Afterward, the supernatant was discarded, and the pellet was suspended in 500 µL 70% ethanol, centrifuged for 5 min at 13.400 x g, the supernatant was discarded, repeating this process twice. Finally, the pellet was dried for 25 min and suspended in 50 µL of TE buffer (10

mM Tris-HCl, pH 7.4, and 1 mM EDTA, pH 8). The DNA was quantified by spectrophotometry, using a NanoVue™ (Harvard Bioscience, Massachusetts, United States of America), and DNA integrity was confirmed in 0.8% agarose gel. All PCR reactions were performed with the same DNA amount (80 ng) with a 260/280 nm ratio of over 1.8.

After DNA extraction, samples were submitted to species-specific PCR to *S. brasiliensis* as described by Rodrigues et al. (2015) using the primers SbraF (5′ - CCC CCG TTT GAC GCT TGG) and SbraR (5′ - CCC GGA TAA CCG TGT GTC ATA AT), as described in the previous section.

In addition, DNA extracted from an *S. brasiliensis* colony was used as a positive control in all reactions. PCR products were analyzed by 1.5% agarose gel electrophoresis in the presence of GelRed® (Biotium, California, United States of America) and samples with 469 bp amplicons were considered positive for *S. brasiliensis* (Rodrigues et al., 2015).

Statistical methods

PCR sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were calculated using mycological culture as the gold standard. The statistical program SPSS 20.0® was used to evaluate the swab storage period (in days) and the fungal CFU quantity interference in the PCR results, by Kruskal-Wallis and Chi-square tests, respectively, considering $p \leq 0.05$ values as significant. Comparison between PCR and DME was performed by the Kappa index evaluation and interpreted as: no agreement (<0.00), poor agreement (0.00-0.19), fair agreement (0.20-0.39), moderate agreement (0.40-0.59), substantial agreement (0.60-0.79) or almost perfect agreement (0.80-1.00) (Silva et al., 2016).

RESULTS

During the studied period, 93 swabs from human patients and animals with suggestive sporotrichosis lesions were sent to the Mycology Laboratory (FAMED-FURG). Eighty-one of those samples were from felines, seven from dogs, and five from humans. Nineteen out of the 93 samples were excluded from the study due to their contamination in the mycological culture, totalizing 74 samples from suspected sporotrichosis cases in felines (n=64), humans (n=5), and canines (n=5).

Sixty-nine percent (51/74) of the samples were positive in the mycological culture, confirming the sporotrichosis diagnosis in 70% of the felines (45/64), 40% of the dogs (2/5) and 80% of the humans (4/5) samples. In all of them, *S. brasiliensis* was confirmed

as the causative agent. The remaining 31% (23/74) did not show fungal growth on culture and were considered negative.

The molecular detection by PCR identified *S. brasiliensis* DNA in 30 out of the 51 positive samples and was negative in 23 culture-negative cases, resulting in 59% sensitivity, 100% specificity, 72% accuracy, 100% PPV, and 52% NPV. Moreover, the DME was positive in 17 of the 42 samples evaluated for this exam, showing 77% of sensitivity, 100% of specificity, 88% of accuracy, 100% of PPV, and 80% of NPV (Table 1). The Kappa index was 0.54. Table 1 shows the PCR and DME results, which have a moderate agreement.

Table 1 - Results of species-specific PCR and direct mycological examination (DME) in the sporotrichosis diagnosis (feline, canine, and human), using mycological culture as the gold standard.

Origin	CULTURE	PCR		DME		
		POS	NEG	POS	NEG	M
Cats (n=64)	Positives (n=45)	26	19	16	2	27
	Negatives (n=19)	0	19	0	18	1
Dogs (n=5)	Positives (n=2)	0	2	1	1	0
	Negatives (n=3)	0	3	0	1	2
Humans (n=5)	Positives (n=4)	4	0	0	2	2
	Negatives (n=1)	0	1	0	1	0
Total:	74	30	44	17	25	32

PCR: polymerase chain reaction; DME: direct mycological examination; POS: positives; NEG: negatives; M: missing – samples without DME results; n: number of samples.

Swab storage period until molecular diagnostic ranged from 15 to 321 days, with an average of 101 days (standard deviation: 70 days). The median of the period in positive PCR results was 103 days (ranging from 15 to 321) and in the false negatives cases, it was 69 days (ranging from 16 to 165), showing no interference (p=0.284) in the species-specific PCR result. On the other hand, 75% of samples with high fungal load (>100 CFUs) (12/16) were positive in PCR, differing significantly (p=0.006) from the samples with low fungal load (<100 CFUs) within only 12% were positive (01/08).

DISCUSSION

Nowadays, the unrestrained sporotrichosis cases increment in Brazil enhanced the severity of this problem in the public health context (Almeida & Giordano, 2018; Poester et al., 2018). One of the main causes of this increase is the fast evolution of this disease in cats, the most frequent animal vector for humans (Gremião et al., 2017). Therefore, diagnostic techniques that allow an earlier beginning of the treatment are essential to control this epidemiological scenario (Rodrigues et al., 2015; Zhang et al., 2019). Thus, the present study provides unpublished data about the applicability of molecular diagnosis in non-invasive samples from animal and human *S. brasiliensis*-caused sporotrichosis.

In humans, biopsy skin and secretion samples from lesions are standard methods of collection, however, the secretion samples present as a better option due to being a sensitive and non-invasive, and easy-to-collect method (Brandolt et al., 2018). In addition, given the difficulty of handling domestic cats, it is essential to evaluate the usefulness of simple methods during the biological sample collection for the sporotrichosis diagnosis (Santos et al., 2018). In this context, our study included swabs in Stuart medium as samples for the sporotrichosis molecular diagnosis, which are a non-invasive, low-cost, and easy-to-collect method widely used for the diagnosis of both human and animal sporotrichosis (Sanchotene et al., 2015; Brandolt et al., 2018; Santos et al., 2018).

However, in our study conditions, species-specific PCR showed limited accuracy with a great number of false negatives results. Although the "in-house method" for DNA extraction can provide sufficient quantity and quality of DNA, the presence of PCR inhibitors in the clinical samples, such as antibodies, hemoglobin, and others are some limitations that could explain the low rate of sensitivity found in our study (Schrader et al., 2012). Another limitation could be attributed to the sample, since the swab submitted to PCR was the same that was previously submitted to the classical mycological tests, decreasing the fungal load in the sample when reached the stage to perform the molecular technique. In fact, a significant association of PCR results with the CFU amount in culture was shown (p=0.006).

Interestingly, the sample storage period had no interference in the species-specific PCR, possibly due to the long viability of DNA fungal cells (Osmundson et al., 2013). In addition, despite the PCR resulted in 100% specificity, DME also showed this high rate and was also able to provide an early diagnosis of the disease. Therefore, given the low cost and the easy performance of the DME, along with the better sensitivity for this technique (77%) when compared with PCR (59%), this method is still indicated to be used in complement to culture in the early sporotrichosis diagnosis (Barros et al., 2011; Sanchotene et al., 2015).

Promising detection rates of *Sporothrix* spp. DNA on clinical samples, reaching values around 90 to 100% of sensitivity (Rodrigues et al., 2015; Zhang et al., 2019), are described in the literature. Our results are in disagreement with them, however, distinctly from us, authors used tissue samples and commercial DNA extraction kits, which provided higher fungal load and low levels of PCR inhibitors, respectively (Rodrigues et al., 2015; Zhang et al., 2019). Moreover, the most recent study (Zhang et al., 2019), employed a multiplex Real-Time PCR, which present a 93.9% of detection to *Sporothrix* spp. in biopsy samples, indeed this technique allows the detection of smaller amounts of DNA when compared to conventional PCR; and another study (Rodrigues et al., 2015), submitted negative samples to a nested PCR after the conventional species-specific reaction, increasing the detection rate. This same methodology was applied in this article, although our positivity rate did not increase (data not shown).

CONCLUSION

Species-specific PCR and the in-house extraction method were chosen for our study due to their rapid results and low cost, aiming greater accessibility; nevertheless, in view of the high rate of false-negative results, the need to refine this technique is emphasized. The requirement of using a commercial DNA extraction kit or to choose another in-house method to improve DNA concentration and purity is mandatory (Rodrigues et al., 2015; Breacker et al., 2016). Also, in view of the greater sensitivity of the multiplex Real-Time PCR (Zhang et al., 2019), studies of this procedure for the sporotrichosis diagnosis from samples collected by non-invasive methods are instigated. Therefore, the high rate of false-negative results in the species-specific PCR, in our study conditions, showed that this technique could not be used for the exclusion of the sporotrichosis diagnosis in suspected cases.

ACKNOWLEDGMENT

The authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES).

INFORMATIVE NOTES

All ethical international and national guidelines concern for animal welfare were followed to developing the study. The University Ethics Committee (CEP/FURG) approved this study (code number Nº 234/2018), in addition, written consent was obtained from

animal owners. The confidentiality of data from all patients (humans and animals) was ensured during all study.

DECLARATIONS OF INTEREST

None

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