



## Communication

[Comunicação]

### Direct PCR of lesions suggestive of sporotrichosis in felines

[PCR direta de lesões sugestivas de esporotricose em gatos]

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Sporotrichosis is a zoonotic mycosis present in urban centers in Brazil (Montenegro *et al.*, 2014; Gremião *et al.*, 2017); the agent is often acquired by traumatic inoculation of the skin (Kauffman, 1999). Cases of human sporotrichosis have been reported in 14 of 26 Brazilian states, and the southern and southeast regions of Brazil have the highest incidence of human cases (Rodrigues *et al.*, 2014). According to Pereira *et al.* (2014), sporotrichosis is considered a neglected disease and was a serious public health problem in Rio de Janeiro; from 1997 to 2011, 4118 human cases diagnosed at the INI/FIOCRUZ (Instituto Nacional de Infectologia Evandro Chagas/Fundação Oswaldo Cruz), the main reference center for the treatment of this ringworm in Brazil (Silva *et al.*, 2013; Gremião *et al.*, 2015). In the last 3 years the city of Guarulhos has registered more than 1500 cases in cats, resulting in a status of “compulsory notification”.

The known pathogenic species of *Sporothrix* are *S. schenckii* s. str., *S. brasiliensis*, *S. globosa*, *S. mexicana*, and *S. luei* (Marimon *et al.*, 2007); among these species, *S. brasiliensis* has been described as the most pathogenic (Marimon *et al.*, 2007; Rodrigues *et al.*, 2013) and geographically not only restricted to Brazil, some cases have already been described in Argentina (Córdoba *et al.*, 2018; Etchecopaz *et al.*, 2019). Epidemiological studies have indicated that *S. brasiliensis* is dependent on a feline host for its development in southern and southeastern Brazil (Rodrigues *et al.*, 2014, 2013).

Diagnosis and treatment delays in infected cats can lead to the rapid spread of the disease among animals and community members (Schubach *et al.*, 2005). Therefore, it is fundamental to confirm sporotrichosis in a particular region for adequate public health planning. Direct PCR analysis of lesions in cats could expedite the diagnosis, initiation of treatment and other measures to control the disease; however, this method has not yet been described.

The definitive diagnosis depends on the isolation of the etiologic agent in culture (Silva *et al.*, 2015). Other methodologies proposed for diagnosis are cytopathology (Pereira *et al.*, 2011), histopathology (Miranda *et al.*, 2013), serology (Fernandes *et al.*, 2011), species determination by polymerase chain reaction of fungal isolation (Rodrigues *et al.*, 2015) and cell block cytology (Gonsales *et al.*, 2019). The aim of this study was to detect and identify the species of *Sporothrix* in cats with lesions from an endemic area and for whom sporotrichosis was part of the differential diagnosis (city of Guarulhos) and compare this method with the reference methodology, fungal culture, considering the following variables: type of injury (dry or wet) and treatment status of the cat (being treated or not treated) at the time of sampling.

Samples from skin lesions from 70 cats (28 (40%) female and 42 (60%) male) from Guarulhos city (23°27'49"S 46°32'1"W) were collected for culture and PCR analysis. Regarding the type of injury, there were 9 (12.9%) dry lesions and 61

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(87.1%) wet lesions. Sixteen (22.9%) cats had already started treatment with itraconazole at the time of sampling, one of the cats had 1 week of treatment and the others had months of treatment (ranging from 2 to 9 months). The protocol was approved by the institutional ethics committee (FMVZ/USP n°7029080616). Lesion samples for culture and PCR were collected with sterile swabs. Samples for culture were stored in Stuart's medium, and samples for PCR were stored in dry tubes without medium.

The cultures were seeded on Sabouraud agar with chloramphenicol and incubated at 30°C for 7 days, with growth observed and thermal reversal performed. Subsequently, a slide stained with lactophenol was prepared for microscopic confirmation of the genus; the visualization of septate hyphae with the typical aspect of daisy sporulation confirmed the genus (Hoog *et al.*, 2000).

DNA from the obtained samples was extracted and purified using a DNeasy Blood and Tissue kit (Qiagen, Crawley, UK), following the manufacturer's instructions, resulting in DNA eluted in 200µl of buffer at the end of the process. To identify the *Sporothrix* species (*S. schenckii* s.s., *S. brasiliensis*, or *S. globosa*), the calmodulin

locus region was amplified directly from genomic DNA PCR using primers and protocol described by Rodrigues *et al.* (2015). DNA concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, USA); ranging from 5.7 to 156.7ng/µl. The PCR mixture consisted of a 25µL final volume containing 12.5µL of Master Mix buffer for PCR (2X), 1µL of each specific primer (sense and antisense - 10 pmol/µL) per reaction, 1µL of target DNA (100ng/µl) and pure water to the final volume.

The conditions for the PCR were as follows: initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, temperature annealing for 1 minute (touchdown PCR) and 1 minute at 72°C. In the touchdown protocol, the annealing temperature was reduced by 1°C every 2 cycles for 20 cycles; finally, the PCR was completed with an annealing temperature of 60°C for 15 cycles (Rodrigues *et al.*, 2015). The amplification product together with a marker of molecular weight was subjected to agarose gel electrophoresis at 1% containing 0.1µg ethidium bromide per mL and visualized through an ultraviolet image capture system (Figure 1).

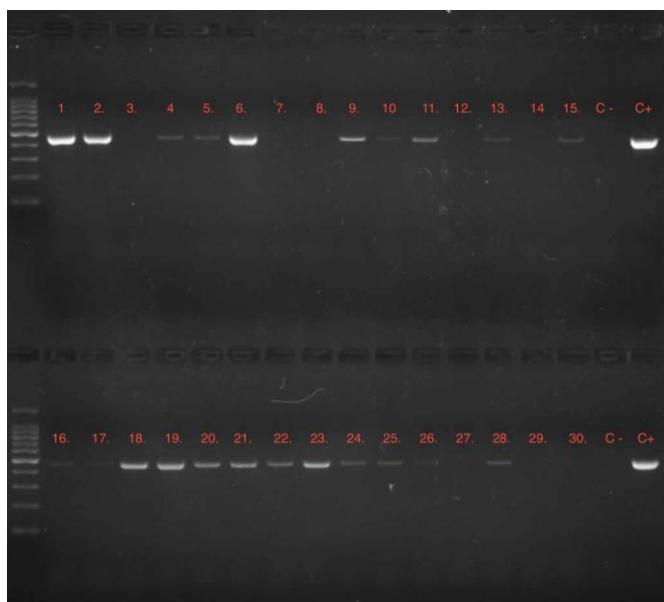


Figure 1. Agarose gel electrophoresis revealing amplicon sizes (469 base pairs) for *S. brasiliensis*. Numbers 1 to 30 refer to clinical samples of cats with suspected sporotrichosis lesion. C+ = positive control; C- = negative control

The results presented in Table 1 show that 61 of 70 cases (which corresponds to 87.1%) were positive for *Sporothrix* by PCR or fungal culture. Also, 57 of 70 cases (which corresponds to 81.4%) were positive for *S. brasiliensis* by PCR. It was also possible to check that 50 of 70 samples (which corresponds to 71.4%) were positive by isolation and 46 of 70 (which corresponds to

65.7%) were positive by both methods. A total of 9 of 70 (which corresponds to 12.9%) cases were negative by both methods; finally, we can highlight that 7 of 9 (which corresponds to 77.8%) samples were taken from dry lesions, and 2 of 9 (which corresponds to 22.2%) samples were taken from wet lesions.

Table 1. Results from PCR and isolation technique and respective kappa agreement values for the samples obtained from dry or wet skin lesions, and in the presence or absence of the initiated treatment with itraconazole (at the time of collection) of cats with suspected sporotrichosis

	PCR+		PCR+		PCR-		PCR-		Kappa (CI)
	Isolation +		Isolation -		Isolation +		Isolation -		
Overall	46	65.7%	11	15.7%	4	5.7%	9	12.9%	0.413 (0.173 to 0.654)
Treated	4	5.7%	7	10%	0	0	5	7.1%	0.263 (-0.014 to 0.540)
Untreated	42	59.9%	4	5.7%	4	5.7%	4	5.7%	0.413 (0.077 to 0.749)
Dry lesion	2	2.9%	1	1.4%	2	2.9%	4	5.7%	0.308 (-0.307 to 0.922)
Wet lesion	44	62.9%	10	14.3%	2	2.9%	5	7.1%	0.353 (0.079 to 0.628)

+ = positive; - = negative; CI confidence interval

Wet lesions had a significantly higher positive rate for *S. brasiliensis* than dry lesions ( $P < 0.01$ ) according to PCR. Sixteen sampled cats had already begun treatment with itraconazole without a previous diagnosis; 15 (93.7%) of them presented wet lesions, and 11/15 (73.3%) samples were confirmed *S. brasiliensis*-positive by PCR. The remaining cat had dry lesions, and the sample was confirmed as *S. brasiliensis*-positive by PCR. There were 65.7% coincident results between both methods (kappa = 0.413), indicating moderate agreement. The treated group presented less agreement than the untreated group, mainly due to false negative culture results.

Regarding the overall frequencies of the results analyzed in this study, an increased frequency of positive results was observed in cats with wet lesions that had not yet begun treatment with itraconazole at the time of sampling. This result is similar to the double positivity results considering both methods (PCR and fungal isolation), which showed an increased frequency (65.7%) in cats with wet lesions that were untreated; these frequencies were much higher than those of the other parameters analyzed ( $P < 0.0001$ ).

Among the cases that were positive only by PCR, 4 were from treated cats and 2 were from dry lesions. Although there was no significant difference between the groups ( $P > 0.05$ ), PCR may be an alternative for diagnosing felines.

Eleven results were PCR positive but with negative fungal isolation, all of these samples were from wet skin lesions. From these, 7 cats were already under the effect of itraconazole. A hypothesis for this result is that the medication has interfered with fungal growth; in the case of the other 4 cats, it could be explained by the higher sensitivity of PCR: it detects a low amount of the agent, without the need for fungal viability. On the other hand, 4 cats were *S. brasiliensis*-negative by PCR with fungal isolation; 2 of dry and 2 of wet skin lesion samples. A conjecture that could justify this result is the limitation of a species-specific primer, like the one adopted in this study, that couldn't detect a different *Sporothrix* species. In this way, we didn't achieve an amplified DNA for sequencing, limiting the diagnosis.

Usually, species determination of *Sporothrix* is achieved after fungal isolation and growth. Direct PCR can increase the speed of the results and avoid these intermediate steps. The majority of evaluated cases were positive by both methods, and 23.9% of positive samples were detected by only PCR, which is a method with high sensitivity. A total of 8.7% samples were positive by only culture, which may suggest that fungal pathogen culture isolation has a lower sensitivity than PCR (Kumar and Mugunthan, 2018). Clinicians report a difficult time in diagnosing sporotrichosis from dry lesions, as it is difficult to obtain culture and cytology results. Although there is no data in the literature on diagnosis rates

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from wet and dry lesions, we tested this hypothesis; we did not observe any significant difference among dry and wet lesions. PCR had a higher frequency of positive results than culture in the studied groups, and studies with a larger sample size than the current one may help verify this possibility.

In conclusion, direct PCR of lesions suggestive of sporotrichosis in cats was a good method to

confirm and identify species of *Sporothrix*, allowing for rapid diagnosis and achieving good agreement with fungal isolation. Parameters such as lesion presentation (wet or dry) and whether or not the treatment had been initiated interfered with the detection of *S. brasiliensis*, as well as the isolation of *Sporothrix* spp

**Keywords:** zoonosis, *Sporothrix brasiliensis*, molecular diagnosis, cats

### RESUMO

A esporotricose é uma doença emergente e a incidência de esporotricose zoonótica tem aumentado, principalmente no Brasil; a maioria dos casos está relacionada à transmissão de gatos infectados. O diagnóstico definitivo da esporotricose felina é feito por cultura fúngica; no entanto, aguardar o longo período de cultura pode atrasar o início do tratamento. O objetivo deste estudo foi detectar e determinar as espécies de *Sporothrix* por PCR realizado diretamente das lesões de gatos, provenientes de área endêmica, nos quais a esporotricose fazia parte do diagnóstico diferencial. Um total de 87,1% dos casos foi positivo por PCR ou cultura fúngica para *Sporothrix*; 81,4% foram confirmados como *S. brasiliensis* por PCR, 71,4% por isolamento e 65,7% pelos dois métodos. Em conclusão, a análise direta por PCR de lesões sugestivas de esporotricose em gatos é um bom método para confirmar a infecção e determinar as espécies de *Sporothrix*, garantindo um diagnóstico rápido; esse método tem uma boa concordância com o isolamento fúngico.

**Palavras-chave:** zoonose, *Sporothrix brasiliensis*, diagnóstico molecular, gatos

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