PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
ESCOLA DE CIÊNCIAS DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

KUNG DARH CHI

ESPOROTRICOSE FELINA: UMA ABORDAGEM DE SAÚDE COLETIVA

(FELINE SPOROTRICHOSIS: A PUBLIC HEALTH APROACH)

CURITIBA
2019

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ESPOROTRICOSE FELINA: UMA ABORDAGEM DE SAÚDE COLETIVA

(FELINE SPOROTRICHOSIS: A PUBLIC HEALTH APROACH)

Tese apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Saúde, Tecnologia e Produção Animal, da Escola de Ciências da Vida da Pontifícia Universidade Católica do Paraná, para obtenção do título de Doutor em Saúde, Tecnologia e Produção Animal Integrada.

Orientador: Prof. Dr. Marconi Rodrigues de Farias

CURITIBA

2019

TERMO DE APROVAÇÃO

Pontifícia Universidade Católica do Paraná
Programa de Pós-Graduação em Ciência Animal
Câmpus Curitiba

PUCPR
GRUPO MARISTA

ATA Nº 0013 E PARECER FINAL DA DEFESA DE TESE DE DOUTORADO EM CIÊNCIA ANIMAL DA ALUNA KUNG DARH CHI

Aos dezenove dias do mês de novembro do ano de dois mil e dezenove, às 8horas, realizou-se na sala de Pós 08, 2º andar, Bloco Amarelo, da Pontifícia Universidade Católica do Paraná, localizada no Campus de Curitiba, Rua Imaculada Conceição, nº 1155, Prado Velho – Curitiba – PR, a sessão pública de defesa da tese da doutoranda Kung Darh Chi, intitulada: **"ESPOROTRICOSE FELINA: UMA ABORDAGEM DE SAÚDE ÚNICA"**. A doutoranda concluiu os créditos exigidos para obtenção do título de Doutor em Ciência Animal, segundo os registros constantes na secretaria do Programa. Os trabalhos foram conduzidos pelo Professor orientador e Presidente da banca, Dr. Marconi Rodrigues de Farias (PUCPR), auxiliado pelos Professores Doutores Vivien Midori Morikawa (UFPR), Marisol Dominguez Muro (UFPR), Sandra de Moraes Gimenes Bosco (UNESP) e Hélio Langoni (UNESP). Procedeu-se à exposição da tese, seguida de sua argüição pública e defesa. Encerrada a fase, os examinadores expediram o parecer final sobre a tese, que foi considerada Aprovada.

MEMBROS	ASSINATURA
Prof Dr Marconi Rodrigues de Farias - Orientador	
Profa Dra Vivien Midori Morikawa (UFPR)	
Profa Dra Marisol Dominguez Muro (UFPR)	
Profa Dra Sandra de Moraes Gimenes Bosco (UNESP)	
Prof Dr Hélio Langoni (UNESP)	

Proclamado o resultado, a Presidente da Banca Examinadora encerrou os trabalhos, e para que tudo conste, eu Caroline Nocera Bertton, confiro e assino a presente ata juntamente com os membros da Banca Examinadora.

Curitiba, 19 de novembro de 2019.

Caroline Nocera Bertton
Caroline Nocera Bertton
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SUMÁRIO

Página

Dedicatória

Aos meus pais

AGRADECIMENTOS

A Pontifícia Universidade Católica do Paraná através da Coordencão do Curso de Medicina Veterinária pelo apoio e pela oportunidade de executar um projeto de tamanha importância para saúde pública.

Aos meus alunos que sempre foram minha motivação para fazer melhor.

À minha família que tão graciosamente aceitou as minhas ausências em tantas importantes ocasiões

Ao meu eterno “orientador” Prof. Dr. Helio Langoni por me mostrar que escolhi a profissão certa e como exercê-la com um propósito maior.

Ao meu amigo, colega e orietador Prof. Dr. Marconi Rodrigues de Farias pelo exemplo de profissional sem nunca esquecer o lado humano e pela confiança em me deixar fazer parte de um projeto tão importante.

Aos queridos amigos e maravilhosos colegas pelo apoio e estímulo nos momentos difíceis e pelas risadas largadas nos momentos mais difíceis ainda.

Às minhas amigas e colegas de doutorado Ana Paula Sarraff e Fabiana Souza Monti por compatilhar das alegrias e dos revezes dessa emocionante jornada.

Às médicas veterinárias Ana Paula Mafra, Cristiane Plahinnsce e Tatianna Hartin da Unidade de Vigilância em Zoonoses da Secretaria Municipal da Saúde, Curitiba-PR, pelos trabalhos de triagem dos animais com as quais fizeram com esmero e profissionalismo.

À equipe de médicos veterinários “residentes”, de colaboradores e estagiários da CVE meus agradecimentos pelo apoio e dedicação nos cuidados dos “esporotriquentos”.

À equipe do Projeto de Esporotricose Felina, num belo exemplo de abnegação e de empatia, abrindo mão de finais de semana, feirados e horas de descanso e lazer para contribuir nos cuidados dos felinos acometidos por uma doença tão devastadora, a minha eterna admiração e respeito: Amanda Araújo, André França, Ariane Cruz, Camila Villatore, Clara Werneck, Clara Rossoni, Daiana Sicuro, Franciny Kerecs, Gabriela Giustins, Gessica Slompo, Giovana Gonçalves, Giovana Proença, Giulia Ferracin, Grazielle Sepúlveda, Karina Francini, Larissa Condas, Larissa Rüncos, Laura Stammer, Leticia Cruz, Lorena de Oliveira, Louise Bacher, Louise Sales, Luana Ballardin, Madya Krinski, Marcia Dal Magro, Mariana Catoia, Mariana Costa, Mariana Mateus, Mariah Stange, Michel Soares, Renata Ferreira, Suelen Ribeiro, Vitoria Jakimiu.

RESUMO GERAL

O presente estudo avaliou os aspectos epidemiológicos da esporotricose felina causada pelo *Sporothrix brasiliensis* em Curitiba, Brasil. A análise dos dados demográficos e clínicos de 295 felinos com esporotricose diagnosticados e acompanhados pelos médicos veterinários da CVE/PUCPR entre maio de 2016 a junho de 2019 resultaram em: a maioria dos animais eram domiciliados em área urbana, sem raça definida, machos, adultos, não castrados, com acesso à rua. Os animais eram, na sua maioria, portadores da forma cutânea disseminada e mais da metade destes com sintomas respiratórios associados. Os casos de esporotricose zoonótica foram de lesões cutânea fixa causadas por arranhões e contato com exudato de gatos doentes. Pelo escopo dos casos há indicação que em Curitiba esteja ocorrendo o segundo maior surto de esporotricose no Brasil. Derivado do primeiro estudo, o segundo trabalho foi de avaliar a susceptibilidade à itraconazol de 67 isolados de *S. brasiliensis* isolados de felinos e comparar com o desfecho clínico. Os testes de susceptibilidade antifúngica foram executados após a conversão dos fungos da forma filamentosa para levedura de acordo com os protocolos da CLSI. Dos 67 casos, somente 37 (54,4%) converteram da forma filamentosa para leveduriforme para a execução dos testes de susceptibilidade à itraconazol (**ITZ**). Dos 37 isolados convertidos para levedura, somente 17 (25,4%) foram computados os dados do desfecho clínico, destes 13 (76,5%) casos foram da forma cutânea disseminada e associado a sintomas respiratórias, 12 (70,5%) eram Wild Type (**WT**) e cinco (29,5%) Non-Wild Type (**NWT**). Dos 12 casos de WT, 10 (83%) chegaram a cura, onde 8 (80%) foram curados com terapia com **ITZ** associado com iodeto de potássio (**KI**). Dos cinco isolados de **NWT**, três (60%) foram curados com terapia

associada de **ITZ+KI** e outros dois casos foram a óbito. Em conclusão, os casos de esporotricose feline causada por S*. brasiliensis* **ITZ WT**, a monoterapia foi ineficiente e a maioria dos casos atingiram a cura com a terapia associada do **ITZ+KI**. Os casos causados por *S. brasiliensis* **ITZ NWT** estão mais associados a ter prognóstico desfavorável.

Palavras-chave: micose, gato, *Sporothrix brasiliensis*, zoonose, itraconazol

ABSTRACT

The present study evaluated epidemiological aspects of feline sporotrichosis caused by *Sporothrix brasiliensis* in Curitiba, Brazil. Analysis of demographic and clinical data of 295 feline sporotrichosis diagnosed and accompanied by the veterinarian staff at the CVE/PUCPR from May 2016 to June 2019 resulted in: majority housed in urban area, mix breed, adult, intact male felines with outdoor access. The felines bearer predominantly disseminated cutaneous form, over half associated to respiratory symptoms. The zoonotic cases were fixed cutaneous form caused by cats' scratch and exudate contact evolving to. The scope of the cases indicates that Curitiba is undergoing the second largest feline sporotrichosis outbreak in Brazil. Derived from the main study group, the second study group was related to 67 *S. brasiliensis* feline-derived isolates itraconazole ITZ antifungal susceptibility result and their clinical outcome. Susceptibility tests were preceded by fungus filamentous/yeast conversion according to CLSI protocols. Out of these 67 cases, 37 (54.4%) isolates converted from filamentous to yeast form to perform itraconazole (ITZ) susceptibility tests. From the 37 yeast converted samples, only 17 (25.4%) isolates with complete clinical outcome data were computed as clinical result and these, 13 (76.5%) cases were disseminated cutaneous form and only associated with respiratory symptoms, 12 (70.5%) were Wild Type (**WT**) and five (29.5%) Non-Wild Type (**NWT**). Of the 12 WT, 10 (83%) cured cases, whereas 8 (80%) were cured by **ITZ** associated to potassium iodide (**KI**) therapy, and two (16.7%) deceased cases by euthanasia. Of the five NWT, three (60%) were cured by **ITZ** and **KI** therapy, and two deceased cases deaths by disease. In conclusion, **ITZ WT** *S. brasiliensis* causing feline sporotrichosis, however, the monotherapy was ineffective, and most of the cases

reached cure with **ITZ** and **KI** therapy. **ITZ NWT** *S. brasiliensis* strains were most often associated to unfavorable prognosis.

Keywords: mycosis, cat, *Sporothrix brasiliensis*, zoonosis, itraconazole

CAPITULO 1

Esta tese é composta por:

Artigo 1 intitulado: **“FELINE SPOROTRICHOSIS OUTBREAK AND ZOONOTIC TRANSMISSION IN CURITIBA, BRAZIL”** a ser enviado para revista: MYCOSIS: 3.065

Artigo 2 intitulado: “**ANTIFUNGAL SUSCEPTIBILITY OF *Sporothrix brasiliensis* FROM THE FELINE SPOROTRICHOSIS”** a ser enviado para revista: MEDICAL MYCOLOGY: 2.851

CAPITULO 2

“FELINE SPOROTRICHOSIS OUTBREAK IN CURITIBA- BRAZIL” a ser enviado para revista: MYCOSIS

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ACKNOWLEDGMENTS

Veterinary Clinic School - CVE, Veterinary Medicine (Head Veterinary Medicine), School of Life Sciences, Pontifícia Universidade Católica do Paraná.

Prof Dr Vania Aparecida Vicente, LabMicro, Pathology Department, Biological Science Sector, Paraná Federal University, Curitiba, Paraná, Brazil.

Prof Dr. Anderson Messias Rodrigues, Medical Mycology Laboratory, Microbiology, Immunology and Parasitology Department, São Paulo Federal University, São Paulo, Brazil.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Finance Code 001.

CONFLICT OF INTEREST

None.

Abstract

The present study issues evaluate the outbreak of 295 cases of the feline sporotrichosis caused by *Sporothrix brasiliensis* in Curitiba, southern Brazil, from May 2016 to June 2019. Cytopathological or histopathological and mycological exams were performed for the final diagnosis. The clinical isolates proceeded for molecular identification by calmodulin gene species-specific region. The demography variables analyzed included breed, age, sex, reproductive status, outdoors access, soil/decay vegetation contact, presence of cohabitating or contacting outdoors animals. Clinical data included evolution time, lesions classification and topography. Zoonotic data were analyzed by type of transmission, topography and classification of lesions. Demographic data yield 275 (93%) felines were housed in urban area, 283 (96%) mix breed, adult (average 44-months-old), 203 (69%) males, 160 (58%) not castrated, 223 (77%) with outdoor access, and 231 (84%) in contact to soil/decay vegetation. The clinical evolution of the 275 cats was over 14 weeks before definitive diagnosis; the majority 290 (71%) cats carried disseminated cutaneous form, and 87 (79%) of these cases were associated to respiratory symptoms. Head and forelimbs were affected by more lesions. Sixty-nine owners that carried zoonotic sporotrichosis were caused by their cats' scratch and exudate contact affecting mainly their upper limbs, evolving to fixed cutaneous form. In conclusion, male intact adult cats represent the most exposed group to *S. brasiliensis* developing disseminated cutaneous lesions consisting the most important host and propagator of sporotrichosis in urban environment within low-income areas.

Keywords: sporotrichosis, cat, *Sporothrix brasiliensis*, zoonosis, Curitiba, Brazil

INTRODUCTION

Sporotrichosis is an implantation mycosis, of subacute or chronic evolution, caused by dimorphic fungi of complex *Sporothrix* spp., that can involve skin, subcutaneous tissue and adjacent lymphatic tissues or even worsens to systemic dissemination in immunocompromised patients (QUEIROZ-TELLES *et al*., 2011, DELLA-TERRA *et. al*., 2017). The classical sporotrichosis is caused by traumatic implantation of soil or organic matter contaminated with *S. schenckii*, and associated to agriculture/floriculture occupational activities. The zoonotic sporotrichosis is caused by *S. brasiliensis*, the main etiological agent of feline sporotrichosis and the most virulent species of the *Sporothrix* complex, is transmitted through infected cat's exudate, bite and scratch (RODRIGUES *et al*., 2016; GREMIÃO *et al.*, 2017)

The cat-transmitted sporotrichosis has been described in different countries over different continents, such as Australia, Malaysia, Japan, India, Spain, Germany, USA, Mexico, Panama, and Argentina as isolated cases or in small outbreaks (SINGER *et al.*, 1952; MACKAY *et al*., 1986; GONZALEZ CABO *et al.*, 1989; HIRANO *et al*., 2006; BOVE-SEVILLA *et al.*, 2008; YEGNESWARAN *et al.*, 2009; TANG *et al.*, 2012; FERNÁNDEZ *et al*., 2015; SCHEUFEN *et al*., 2015; RIOS *et al.*, 2018).

In the past 20 years in Brazil, cat-transmitted sporotrichosis has evolved from a sporadic family cat infection (MARQUES *et al.*, 1993), to an emergent expanding outbreak that might affect thousands of people (QUEIROZ-TELLES *et al*., 2017). The largest ongoing zoonotic outbreak in Rio de Janeiro state (Brazil), human cases are over 4000 (SILVA *et al.*, 2012) as so 4703 feline cases (GREMIÃO *et al*., 2017). Other Brazilian states are also reporting significant feline sporotrichosis and zoonotic cases, such as São Paulo metropolitan area reported 163 feline sporotrichosis cases

over 3 years' period (MONTENEGRO *et al*., 2014), and Rio Grande do Sul state reported 372 felines and 34 canine sporotrichosis cases and 83 human cases from 2010-2016 (POESTER *et al*., 2018).

In Curitiba, south of Brazil, since 2014 a number of feline sporotrichosis has been registered with zoonotic transmission; however, no epidemiological survey describing the feline sporotrichosis demographics nor the *Sporothrix* spp. identification was ever done. These data are fundamental for the pathogenesis understanding, also to prescribe control and prevention protocols and establish public health policies, therefore the aim of this study is to identify and evaluate the extension of feline sporotrichosis outbreak in Curitiba and metropolitan area, Paraná state.

MATERIALS AND METHODS

The present study was submitted and approved by Committee for Ethical Animal Use of the Pontifíca Universidade Católica do Paraná (CEUA/PUCPR) by protocol no. 01197.

This study was conducted in cats with sporothricosis diagnosed and accompanied by the veterinarian staff at the Veterinary School Clinic/PUCPR from May 2016 to June 2019. The animals were sent by veterinarians from the Zoonosis Surveillance Unit of the Curitiba, as part of the partnership surveillance.

Anamnesis & Clinical Exam

Demographic information such as breed, age, sex, reproductive status, housing area, outdoor access, co-habitation with other animals, contact with outdoors animals, household location (district) and owner's zoonotic status were collected during anamnesis.

Each sporotrichosis suspected cat undergone complete physical and dermatological exams, anamnesis, and full record on standard form of the cutaneous lesions, its topography and the clinical signs.

Clinical forms of sporotrichosis were classified as cutaneous and extra-cutaneous (BARROS *et al*., 2011). The cutaneous lesions were reclassified as: 1) fixed cutaneous (**FC**) for single lesion, usually restrain to the inoculation site; 2) lymph cutaneous (**LYC**), for presence of lymphangitis and lesions adjacent to lymphatic route and local lymphadenitis and 3) disseminated cutaneous (**DC**) for multifocal or generalized ulcers, plaques and nodules. The extra-cutaneous (**EC**) lesions were classified for any mucosa site as respiratory system (**R**) or ophthalmic (**O**). The **R** form was established by upper respiratory exam identifying presence of

nodules and tumors in the nasal cavity and planum, as respiratory symptoms of sneeze, shrill, rhinorrhea and epiphora. The **O** form by presence of granulomatous blefaroconjuntivitis and epiphora.

Systemic symptoms or signs (**SyS**) such as anorexia/hiporexia, emaciation, apathy, vomit, and diarrhea were also described.

Cytopathology & Histopathology Exam

After the clinical exam, each animal undergone for sample collection for cytopathology and mycological exam. The cytopathology sample collected on glass slide by imprinting the lesion exudate or by fine-needle aspiration. After collection, each slide was stained by hematologic fast stain (Panóptico®, Laborclin, Brazil). For negative cytopathology results, histopathological sample was collected by skin punch of 5 mm, fixed in buffered formalin 10%, embedded in paraffin, sliced by microtome into 2-5 μm films and stained by hematoxylin-eosin (HE) and periodic acid of Schiff (PAS). Cytopathology or histopathology exam considered positive for at least one yeast cell identified with pyogranulomatous exudate background (FARIAS *et al.*, 2004; PEREIRA *et al*., 2011).

Mycological Exam

Clinical samples for mycological examination (gold-standard) (RIPPON, 1988) was inoculated in Sabouraud-dextrose agar with chloramphenicol (0.05 g/L) and agar Mycosel, incubated at 25 ^{o}C and 37 ^{o}C for 3 weeks, with daily observations after the 5th day. Duct tape method stained with cotton blue was performed on the filamentous colony samples for microscopic examination. Positivity was confirmed at 400x microscopy by hyaline, septate thin branching hyphae with conidia along

conidiophore, and in younger cultures conidia might form flowerlike petals consistent to *Sporothrix* spp.

Molecular identification - GESSICA

Isolates confirmed at mycological exam were submitted to DNA extraction based on protocol developed by Vicente *et al.* (2008). Further molecular identification was performed based on the protocol of Rodrigues *et al.* (2015), in which the calmodulin gene species-specific region determined cryptic species of *Sporothrix schenckii* complex.

Briefly, DNA extraction was performed in micro tubes containing 300μL CTBA (cetyltrimethylammonium) buffer in a mixture with NaCl (1,4 M), Tris-HCL (100 mM), EDTA (20 mM), b-mercapto-ethanol (0.2%), and silica (80 mg). Subsequent washing steps were performed with chloroform, ethanol, from which final pellet was re-suspended with TE/ultrapure water, incubated at 37°C and stored at -20°C (VICENTE *et al.*, 2018).

PCR reaction was performed with a total volume of 25 µl, consistent of buffer, Taq Polymerase, dNTPs, water, specific primers (amplifying 18-25 calmodulin gene nucleotides), and 2 µl of targeted DNA. The reaction was performed in an Eppendorf Mastercyle Pro machine (Eppendorf, Hamburg, Germany), using a touchdown method in order to use all designed primers at the same time. PCR conditions included a 10 minutes denaturing step at 95°C, followed by 35 cycles of 1 min at 95°C, 1 minute at 48°C annealing temperature, and 1 minute extension at 72°C. Subsequent annealing temperatures decreased 1°C every 2 cycles for the next 20 cycles in the touchdown protocol. Finally, PCR amplicons were observed with agarose gel electrophoresis, and stained bands visualized at UV light with L-Pix

Touch imaging system (Loccus Biotectonologia, Sao Paulo, Brazil) (RODRIGUES *et al.*, 2015).

Zoonotic Aspects

Information of zoonotic transmission (scratches, bites or exudate contact) were obtained during anamnesis. The owners were also referred to the infectologist for evaluation and classification of the lesions.

Statistical Analysis

Demographic data from the animals, as well as their clinical data, were presented as percentages or means with standard deviation.

The variables included were breed, age, sex, reproductive status, housing district, outdoors access, soil/decay vegetation contact, presence of cohabitating or contacting outdoors animals. Clinical data were evaluated by the time elapsed from the signs before the first admittance to examination, presence of systemic symptoms, lesion classification, lesion topography, and respiratory symptoms. Zoonotic transmission was analyzed by the number of cases, type of transmission, topography and classification of lesions in affected owners.

Proportions of affected animals were, according to demographic variables, compared using a one-sample proportion test, with confidence interval and α set as 95% and <0,05, respectively (MOORE *et al.*, 2012). Analyses were performed in STATA, version 14 (College Station, Texas).

RESULTS

Demography

Of the 355 feline sporotrichosis original cases, in 295 clinical and epidemiological signs were complete. Of these cats, 283 (96%) were mix breed ($p<0.05$), over 44 months old, 203 (69%) male ($p<0.05$), 160 (58%) intact animals ($p<0.05$), 223 (77%) with outdoor access ($p<0.05$), 231 (84%) in contact soil/decay vegetation ($p<0.001$), and 275 (98%) housed in urban area ($p<0.05$) (TABLE 1).

According to this study, only 4 (2%) cats live in rural area and 3.7 cats per household (TABLE 1). Despite statistically not significant 145 (49%) felines have outdoors contacts with cats bearing skin lesions (TABLE 1). Geographically, the present study indicates that the outbreak occurred in Curitiba's west and southwest districts.

TABLE 1. Distribution of epidemiological variables of 295 sporotrichotic cats diagnosed and treated at CVE/PUCPR, Curitiba, Brazil 2016-2019.

Demographic variable	**Distribution n (%)**
Breed	295 (100%)
Mixed breed	283 (96%) *
Siamese	9 (3%)
Persian	1 (0.3%)
Age	266 cats **
Mean (sd)	44. 2 mo (sd 32.6)
Min- max	7 – 156 mo
Sex (n=295)	295(100%)
Male	203 (69%) *
Female	92 (31%)
Reproductive status	274 (100%)
Intact	160 (58%) *
Neutered/Spayed	114 (41%)
Not reported	21
Housing	289 (100%)
Outdoor access	223 (77.1%) *
Indoors	66 (23%)
Not reported	6

Soil/vegetation contact	274 (100%)
Yes	231 (84%) *
No	43 (16%)
Not reported	21
Co-habitant animals	222 (100%)
Sick	112 (50%)
Healthy	101 (45%)
Do not know	9 (4%)
Mean of feline co-habitants (sd; max-min)	Mean 3.7 (6.6; 1 – 47)
Mean of canine co-habitants (sd; max-min)	Mean 0.2 (0.5; 0 – 3)
Not reported	73
Outdoors contacts	240 (100%)
Skin lesions	145 (49%)
No skin lesions	95 (32%)
Not reported	55
Housing area	295 (100%)
Urban– Curitiba and metropolitan area	275 (98%) **
Rural	4 (2%)
Not reported	16

n number of the cases; **mo**: months-old; **sd**: standard deviation; max: maximum; min: minimum; ***** statistically significant ($p<0.05$); ****** another 29 cats defined as adults, however no information available about the precise age

Molecular results

Molecular identification based on calmodulin gene detected a 469bp fragment for all 91 isolates. This specific fragment size characterized all isolates obtained from Curitiba feline sporotrichosis outbreak as *S. brasiliensis.*

Clinical Aspects

Clinical evolution prior the first consultation over 14 weeks was found in this study, 141 (47.7%) cats were under antifungal treatment over 3 months prior the first appointment and around 145 (49%) untreated sick cats had accessing outdoors access and potential infectious source for other animals and humans.

From the 295 feline sporotrichosis confirmed cases, 69 (23%) were **FC** form 209 (71%) **DC** form, 154 (52.5%) **R** symptoms, and 111 (37.6%) **SyS** (TABLE 2).

The present study also registered 87 (79%) **DC**+**R** associated, also 86 (77.4%) **SyS**+**DC** associated (TABLE 2).

TABLE 2. Distribution of clinical forms of sporotrichosis diagnosed from the 295 sporotrichotic cats' first consultation.

Clinical form	**Distribution n (%)**
Fixed Cutaneous (**FC)**	69 (23%)
FC + Extracutaneous form	32
FC + Respiratory	30 (94%)
FC + Ophthalmic (**O**)	2 (06%)
FC + Respiratory + Ophthalmic	1 (3%)
Disseminated Cutaneous **(DC)**	209 (71%) ***
DC + Extra-cutaneous form	111
DC + Respiratory	87 (79%)
DC + Ophthalmic	8 (07%)
DC + Respiratory + Ophthalmic	16 (14%)
Extra-Cutaneous (**EC)**	
No other clinical forms associated	17 (6%)
Systemic symptoms (**SYS)**	111
SYS + FC	23 (20.7%)
SYS + DC	86 (77.4%)
SYS + R	62 (55.8%)
SYS + EC	66 (59.4%)

n number of the cases**; DC** Disseminated Cutaneous form; **FC** Fixed Cutaneous form; **EC** Extra-cutaneous form; **R** Respiratory symptoms; **O** Ophthalmic form; **SYS** Systemic Symptoms

The cutaneous lesions classification and its topography are summarized on TABLE 3 and TABLE 4

TABLE 3. Distribution of lesions classification from the 295 sporotrichotic cats' first consultation.

Lesions	**Distribution n (%)**
Ulcer/Exudative-ulcer	254 (98%)
Purulent blood exudation	189 (64 %)
Crust	171 (58%)
Nodule	116 (39%)
Swelling	74 (16%)
Papule	24 (8%)
Chancre	7 (2%)
Other lesions (plaque/erythema/erosion/fistula/bone exposition/paronychia)	37 (12%)

n number of the cases

TABLE 4. Distribution of lesions topography from the 295 sporotrichotic cats' first consultation.

Topography	**Distribution n=292 (100%)**
Head	240 (82%)
Frontal	76 (32%)
Temporal	76 (32%)
Pina	98 (41%)
Nasal	139 (58%)
Periocular	56 (23%)
Malar	71 (31%)
Lips	27 (11%)
Limbs	167 (57%)
Right Forelimb	80 (48%)
Left Forelimb	71 (42%)
Right Hind limb	55 (33%)
Left Hind limb	54 (32%)
Torso	111 (38%)
Cervical	62 (56%)
Thoracic	46 (41%)
Abdominal	15 (13%)
Lumbosacral	17 (15%)
Flank	7 (06%)
Penial	2 (02%)
Perianal	4 (04%)
Testicular	5 (05%)
Tail	34 (12%)
Not reported	3

n number of the cases

Besides the **DC**+**R** and **SyS+DC** associations, it was also observed within **SyS** group, 75 (67.5%) cats with emaciation and 55 (49.5%) anorexia/hiporexia, also within **R** group, 125 (81%) felines with sneeze and 99 (64%) with shrill (TABLE 5).

TABLE 5. Distribution of clinical symptoms and reactive lymph-nodes of 295 sporotrichotic cats' first consultation**.**

Clinical signs	**Distribution n (%)**
Systemic symptoms (**SYS)**	111
Emaciation	75 (67.5%)
Anorexia/Hiporexia	55 (49.5%)
Apathy	30 (27%)
Digestive disorders	30 (27%)
Fever/Jaundice/Pregnancy	5 (04.5%)

Respiratory symptoms (**R**)	154
Sneeze	125 (81%)
Shrill	99 (64%)
Cough	16 (07%)
Nasal secretion	79 (51%)
Polyp	1 (01%)
Nodule	7 (04%)
Nasal deformity	122 (79%)
Respiratory distress	4 (02%)
Reactive Lymph nodes	247
Not reported	48
Submandibular	103 (42%)
Pre-scapular	23 (10%)
Popliteal	67 (27%)
Inguinal	3 (01%)
three reactive	7 (03%)
all reactive	1 (0.4%)
not reactive	110 (44%)

n number of the cases

Zoonotic Aspects

Personal clinical information was provided by 69 owners during their animal consultation. In 42 cases the information was limited to be infected by their cats. In 27 (39%) cases the owners were infected by their own sick cats, 12 (44,5%) was infected by scratch and 11 (40%) by exudate contact infecting. No bite lesions were reported by the owners. The most common clinical presentation of human sporotrichosis lesions were **FC** (n=17, 63%), **LYC** (n=10, 37%) and **O** (n=2, 7%). No **DC** form nor **SyS** were reported by the owners. The topography of the lesions indicated that the upper limbs were affected in 13 (68%), followed by thoracic area in 3 (16%), face in 2 (10%), and 1 (5%) lesion on the legs.

DISCUSSION

The demographic profile presented in this study that 275 (98%) of the cats live in metropolitan area, indicating that the pathogen is circulating among urban cats, as already proposed by Schubach *et al*. (2004), Montenegro *et al*. (2014), Gremião *et al*. (2015), Poester *et al*. (2018).

Feline population is usually larger in urban areas and high cat population density is an important component for feline sporotrichosis occurrence (RODRIGUES *et al*., 2016). It is a fact that intact adult males with outdoor access represent the significant population affected by sporotrichosis. Intact male at its prime, reinforces their territorial domain and matting behavior by physical confrontation, therefore they are more exposed to cutaneous lesions as result of fighting, endorsing their higher incidence (BARROS *et al*., 2008a). Close encounters with intact felines, usually results in scratches and bites lesions amplifying the odds for fungal infection, sustaining animal-animal and animal-human transmission (GREMIÃO *et al.*, 2017).

It is important to state that *S. brasiliensis* is the most virulent species of the *S. schenkii* complex and it has the domestic cat as the most sensitive mammal species natural host (RODRIGUES *et al.,* 2013; RODRIGUES *et al.,* 2016). As *S. brasiliensis* is highly adapted to mammal tissues, it is hardly isolated from the soil, and as parasitic form, yeasts were found in high cell numbers or high burdens disseminating from scratches, bites or exudate contact from sick cat. Therefore, semi-housed or feral adult intact male cat is the pivotal character on the origin and perpetuation of urban zoonotic and animal sporotrichosis (GREMIÃO *et al.*, 2017).

Barros *et al.*, (2008b) reported that up to 78.9% of sick cats would roam freely around their neighborhood and 66.9% in contact with other animals. Cats with outdoor access are the most vulnerable hosts for *Sporothrix* spp. infection, considering feline high susceptibility for *S. brasiliensis*.

It is being postulated that zoonotic sporotrichosis is related to low family income, poor sanitation, irregular housing and little or no access to health services (COSTA *et al*., 2019).

Curitiba's Human Development Index (HDI), 0.823 is the 10th in national ranking according IBGE (PNUD, 2014), but underprivileged areas remain a challenge for health authorities (COSTA, *et al*., 2019) and socioeconomic aspects for sporotrichosis outbreak occurrence demands further studies. Urban areas with high feline density are seemly to be propelling the *S. brasiliensis* epizootic and the absence of feline population control policies should lead to the dissemination of this antropozoonosis, already stated years ago as a recurrence problem neglected by the public health authorities in Brazil (SILVA *et al*., 2012).

Though the felines are the most affected mammals, other species might host the fungus. Under this scenario, urban sporotrichosis demands more studies in order to clarify the roles of other mammals (rodents for instance) as well insects as an alternative transmission route for cats, since the cats are raised in the low-income households for pest control (BARROS *et al.* 2010; RODRIGUES *et al.*, 2016). Also, low-income populations usually have limited access to veterinary services as spay/neuter or treatments for sick animals, which fuels the rat-cat-human alternative cycle (RODRIGUES *et al.*, 2016), and outdoor access for owned and feral sick cats allowed the pathogen migrate from the epidemic area to surrounding areas, setting new epizootic sites and increasing the disease dispersion in urban areas.

Over 14 weeks of clinical evolution prior the cat's first consultation was found in this study. This extension of delay can be related to owners' difficulties to access veterinarian services due low income. Also, this lag time for proper diagnosis and treatment aggravates clinical symptoms. Therefore, represents a long period for susceptible humans and animals be exposed to a highly virulent parasitic yeast form with no standard recommendations of safe handling, animal indoors confinement and treatment for the sick animal taking place. Thus, favoring the infection of 23.3% of reported owners.

The 23% **FC** form rates (TABLE 2) was lower than in other studies as 27% by Miranda *et al.* (2018); 33.3% by Madrid *et al.* (2010), 43.8% by Macêdo-Sales *et al.* (2018), and 72.4% by Rossi, *et al.* (2013). **DC** form was observed in 209 (71%) cases and was the most common clinical presentation in feline sporotrichosis (TABLE 2) and also higher rates than other studies such as 39.5% by Schubach *et al.* (2005), 55% by Madrid *et al.*, 2012, and 56.1% by Poester *et al.* (2018).

Compared to other studies, the **FC** low rates and **DC** high rates found in the present study, at least two possibilities emerges: 1) long time clinical evolution (14 weeks) prior to diagnosis favors the fungus dissemination, and 2) the occurrence of highly pathogenic clone of *S. brasiliensis* with high dissemination ability, a virulence feature already characterized in murine model (ARRILLAGA-MONCRIEFF *et al.*, 2009; ALMEIDA-PAES *et al.*, 2015; DELLA-TERRA *et al.*, 2017), but yet to be verified in feline system. Both possibilities should be accessed in further studies for possible establishment of early diagnosis and treatment protocols avoiding **DC** form sets in and the all consequences that comes along. Regarding majority of sick cats accessing outdoor environment it should be taken in consideration for their

vulnerable dietary status and parasite infestation, factors that might affect their overall health and favoring the **DC** form.

The **DC** form can be due to multiple inoculation sites by scratches and bites during cat fights or by infection expansion from subcutaneous sites: 1) fungal dissemination by haemato-lymphatic via colonizing new subcutaneous sites; 2) cat self-inflicted inoculation by licking open wounds and later grooming themselves other sites (ROSSI *et al.*, 2013), and 3) feline contaminating its claws by scratching the wounds and later scratch new cutaneous sites.

In some severe **DC** forms, muscle and bone exposition are results of large extensions of necrotic cutaneous lesions. Multiple necrotic-ulcers covered by hemorrhagic crusts can easily be found in feline sporotrichosis, and are usually associated to local bacterial infection, emaciation and poor body score. As the infection progresses, like **DC** form, fungi exude from the original site and disperse by circulation to systemic sites and organs such as liver, spleen, kidney, brain, testis, epididymis, articulations and bone tissue (DELLA-TERRA *et. al*., 2017; QUEIROZ-TELLES *et a*l., 2011).

Though most felines cutaneous lesions appear to be restricted to the skin, respiratory sings as sneeze, nasal secretion and deformities indicate mucosa, sinus and cartilage/bone lesions might follow or precede cutaneous form.

Maybe in Curitiba, the 14 weeks' delay for proper diagnosis and treatment explains the 52.2% rates of **R** symptoms when compared to 8 weeks' clinical evolution and 27.7% rates of respiratory signs reported by Miranda *et al.* (2018). Three routes can take place to establish **R** form. 1) fungus primary infects nasal mucosa by inhalation or by self-inoculation when leaking the highly yeast burden

skin lesions, exposing nasal planus and nostrils evolving to respiratory symptoms; 2) fungi migration from contiguous cutaneous lesions, since cats bearing respiratory symptoms also usually presents cutaneous disseminated form; 3) via haemato-lymphatic, as the fungi spread from a nearby subcutaneous site through circulation reaching respiratory mucosa, developing the respiratory symptoms. In any of the cases, the infected nasal mucosa leads to sneezing/discharging secretion with parasitic yeasts, and the latter respiratory impairment that aggravates the clinical status are the two most important features for sporotrichosis respiratory manifestation, also extending the therapy period and favoring recurrence.

In the present study, **DC+R** and **SyS+DC** associated forms were comparable to the findings of Schubach *et al.* (2005). Usually these types of associations represent the most severe clinical manifestations of feline sporotrichosis. Severe respiratory symptoms combined with liver/kidney impairment eventually leads to severe systemic symptoms form demanding also support therapy in addition to antifungal treatment. The partial nasal airways obstruction by sporotrichosis nodules or by lesions in the nasal mucosa, also cartilage and bone damage (GREMIÃO et al., 2015) can lead to nasal deformity resulting in smell impairment, appetite loss and difficulties for water consumption and respiratory distress. Also, the extension of the lesions in the nasal planum and the high burden of parasitic yeasts, that is a common feature for *S. brasiliensis* in feline infections, might facilitate the dispersion via haemato-lymphatic system affecting internal organs.

Other feline sporotrichosis manifestation was **EC** ophthalmic (**O**) form. It is usually associated to **R** form and nasal area. It occurs usually after the latter form sets in, probably by lymphatic drainage, nasolacrimal duct, or parasitic yeast cells from migrates the cutaneous lesions to conjunctiva and sets new colonization site.

The visual impairment due conjunctiva/eyelid nodule and ulcer growth and high loads of infectious yeasts exuding from the affected eye is just adds up to the already aggravated **DC**+**R** situation.

A total of 30 cases of **FC**+**R** associated forms were found (TABLE 2), that in these cases, two possibilities rise: 1) **FC** form sets contiguous to nasal area or on the nasal planum evolving to nodule/ulcer leading to sneeze, shrill, rhinorrhea, and respiratory impairment, or 2) a single mucosal nodule, obstructing partially nasal airways, leading to sneeze, shrill, rhinorrhea, respiratory impairment, and sinusitis (secondary infection).

Feline lymphocutaneous (**LYC**) form resembles human form developing multiple subcutaneous nodules or linear setting ulcers. Lymphangitis and regional lymphadenitis is not usual in feline clinical presentation and was observed in 19.3% feline cases (SCHUBACH *et al.,* 2005) and only in 10% cases of **DC** form feline sporotrichosis in the present study (TABLE 3), indicating this form in felines usually is associated to **FC** form.

Head lesions registered the highest rate, as nasal and pina areas, limb lesions as cervical and thoracic areas were the most affected (TABLE 4) in agreement to felines physical confrontation behavior.

Personal clinical information provided by 69 owners during animal consultation can evidence how easy feline sporotrichosis can be transmitted to human by scratch and by exudate contact infecting. Owners lesions topography found in the present study are in agreement to Brandolt *et al*., (2018), that reported the enrolment of 73% upper limbs, 14.8% face and 12.1% legs. The occurrence on the anatomical sites in human sporotrichosis is consistent to higher exposure of

hands/arms due cat handling or petting, face and neck are related to cuddling or playing with the animal (BARROS *et.al.*, 2008b, SCHUBACH *et.al.*, 2008), and legs related to cats' scratch and/or face rubbing.

CONCLUSIONS

The feline sporotrichosis in Curitiba is caused by *Sporothrix brasiliensis* and limited to urban area with low income districts. This is affecting adult male intact cats, semi-housed or outdoors exclusive, that are carrying lesions in the head and forelimbs. The lack of early diagnosis is favoring the dissemination, infecting susceptible cats and an endangering dermatozoonotic transmission.

Permanent epidemiological surveillance of feline sporotrichosis is a must for rising awareness among veterinarians, medical workers and health authorities for developing mitigating measures over zoonotic transmission.

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CAPITULO 3

“ANTIFUNGAL SUSCEPTIBILITY OF *Sporothrix brasiliensis* FROM THE FELINE SPOROTRICHOSIS” a ser enviado para revista: MEDICAL MYCOLOGY

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Keywords: itraconazole susceptibility, cat, *Sporotrix brasiliensis*, clinical outcome

Abstract

Itraconazole (**ITZ**) is the drug of choice for treatment of human and feline sporotrichosis, though for cat treatment inconsistecy therapy results remain among the different authors. This study aimed to evaluate **ITZ** susceptibility of *Sporothrix brasiliensis* isolated from sporotricotic cats and relate it to their clinical evolution. Cytopathological or histopathological and mycological exams were performed for the final diagnosis. The clinical isolates proceeded for molecular identification by calmodulin gene species-specific region. Susceptibility tests were preceded by fungus filamentous/yeast conversion according to CLSI protocols. The *S. brasiliensis* isolated from 67 cases of feline sporotrichosis were included in this study. Out of these 67 cases, 37 (54.4%) isolates converted from filamentous to yeast form to perform ITZ susceptibility tests. From the 37 yeast converted samples, 17 (25.4%) isolates with complete clinical outcome data were computed as clinical result and these, 13 (76.5%) cases were disseminated cutaneous form and associated with respiratory symptoms, 12 (70.5%) were Wild Type (**WT**) e five (29.5%) Non-Wild Type (**NWT**). Of the 12 WT, 10 (83%) cured cases, whereas 8 (80%) were cured by **ITZ** associated to potassium iodide (**KI**) therapy, and two (16.7%) deceased cases by euthanasia. Of the five NWT, three (60%) were cured by **ITZ** and **KI** therapy, and two deceased cases deaths by disease. In conclusion, **ITZ WT** *S. brasiliensis* causing feline sporotrichosis, however, the monotherapy was ineffective, and most of the cases reached cure with **ITZ** and **KI** therapy. **ITZ NWT** *S. brasiliensis* strains were most often associated to unfavorable prognosis.

INTRODUCTION

Sporotrichosis is the most prevalent and widespread implantation mycosis (QUEIROZ-TELLES *et al.*, 2017), granulomatous, subacute or chronic infection, that affects animals and humans, caused by dimorphic fungus of the *Sporothrix schenckii* complex. The infection occasionally spreads via lymphatic and hematic systems to other organs and sites (SCHUBACH *et al.,* 2012; MILLER *et al.*, 2013; RODRIGUES *et al.,* 2013).

The *Sporothrix schenckii* is found on organic matter and soil and is usually related to occupational/recreational infection for its accidental cutaneous implantation. On the other hand, zoonotic sporotrichosis is caused mainly by *S. brasiliensis,* the most virulent species of the *S. schenckii* complex (RODRIGUES *et al.,* 2016), and it is transmitted to humans and other animals through infected cat's lesions exudate, bite and scratch.

Zoonotic sporotrichosis has being registered in some Brazilian major metropolitan areas, as an emergent ongoing infectious mycosis in Rio de Janeiro recording 4188 human cases from 1997 to 2011 and 4703 feline cases from 1998 to 2005 (GREMIÃO *et al.,* 2017), São Paulo city with 13 human cases and 114 feline cases from 2011 to 2013 (SILVA *et al.,* 2015) and 83 human cases and 372 feline cases over a 7 years' period in Rio Grande do Sul (POESTER *et al.,* 2018), and in Curitiba (Paraná state) from 2016 to 2019 over 350 feline cases (personal data).

Itraconazole (**ITZ**) is the first-choice treatment for human sporotrichosis for its efficiency and safety due low toxicity and tolerance (FERREIRA *et al.,* 2019). The same is recommended for feline sporotrichosis treatment using azoles, specially **ITZ** alone or in combination to potassium iodide (REIS *et al.*, 2016).

It is noteworthy that the antifungal treatment for feline sporotrichosis remains a hazy area as different studies bringing different outcomes of therapeutic success, failure and recrudescence for same compound (PEREIRA *et al.*, 2010; REIS *et al.*, 2016; SOUZA` *et al.*, 2017). There are no studies to determine whether eventual features might interfere **ITZ** the absorption, distribution and therapeutic cutaneous concentrations in sporotricotic cats, therefore affecting clinical outcome.

Some aspects for therapeutic outcome should be taken account as, 1) owners commitment and compliance to the treatment; 2) as felines as natural hosts to *S. brasiliensis* (RODRIGUES *et al.*, 2016), their immune defense status plays an important part on the treatment outcome, 3) treatment pricing is unfeasible for most owners; 4) systemic symptoms such as dehydration, anemia, hypoalbuminemia are common features in cutaneous disseminated form of feline sporotrichosis and they play a hindrance for **ITZ** absorption and tissue distribution, and 5) emergence of resistance to antifungal drugs.

Intrinsic factors that might favor *S. brasiliensis* drug resistance, increasing its virulence capacity, can be related biofilm formation associated to decreased antifungal susceptibility (BRILHANTE *et al.*, 2018), also *S. brasiliensis* cell wall fibrillary layer forming cell clusters, a structural feature that prevents the yeast cell been phagocyted by human macrophages that might affect drug penetration (LOPES-BEZERRA *et al.*, 2018).

Considering the importance of **ITZ** on feline sporotrichosis treatment, the aim of this study was to evaluate the susceptibility to **ITZ** by *S. brasiliensis* isolated from cats with sporothricosis and relate it to clinical evolution.

MATERIALS AND METHODS

Study group

The present study was submitted and approved by Committee for Ethical Animal Use of the Pontifícia Universidade Católica do Paraná (CEUA/PUCPR protocol No. 01197).

Non-randomized, not controlled and systematized cross-sectional study of 68 feline sporotrichosis cases diagnosed and accompanied by the veterinarian staff at the Veterinary School Clinic/PUCPR. The animals were send by veterinarians from the Zoonosis Surveillance Unit of the Curitiba, as part of the partnership surveillance.

The sporotrichosis diagnosed has been established after complete physical and dermatological exams, cytopathological/histopathological positive exams and fungal isolation and identification. Inclusion parameter based on the obtaining of colony purity (uncontaminated one single filamentous colony) for molecular characterization and antifungal susceptibility tests.

For clinical/epidemiological evaluations of information as breed, age, sex, reproductive status, housing area, outdoor access, co-habitation with other animals, contact with outdoors animals and household location (district) were registered during anamnesis on standard form. Also, evaluation and description of systemic symptoms, previous use of antifungals when first admitted to the clinic, relapse, drug administration failure, therapy failure, cure and deceased were made.

Clinical Exam and Therapy Evolution

Every feline was subject the clinical and dermatological exams, were the cutaneous lesions registered by classification (BARROS *et al*., 2011): 1) fixed cutaneous (**FC**) for single lesion, usually restrain to the inoculation site; 2) lymph cutaneous (**LC**) for presence of lymphangitis and lesions adjacent to lymphatic route associated to local lymphadenitis, and 3) disseminated cutaneous (**DC**) for multifocal or generalized ulcers or exudative ulcer lesions. Extra-cutaneous lesions (**EC**) for any mucosa site as respiratory (**R**) or ophthalmic (**O**) system. The **R** form can be established by upper respiratory exam identifying presence of nodules and tumors in the nasal cavity, nose planum and nose bridge, as respiratory symptoms of sneeze, rhinorrhea, respiratory stridulum, and epiphora. The **O** form by presence of granulomatous blefaroconjunctivitis. Also, systemic symptoms (**SyS**) as apathy, anorexia, hyporexia, emaciation, vomit and diarrhea would be registered.

Cytopathology and Histopathology Exams

Following the clinical exam, samples were collected form cytopathology and mycological exam. The cytopathology sample collected on glass slide by imprinting the lesion exudate or by fine-needle aspiration. For cytopathology, each slide was stained by hematologic fast stain (Panóptico®, Laborclin, Brazil). If necessary, histological sample would be collected by skin punch of 5 mm, fixed in 10% buffered formalin, blocked in paraffin, sliced by microtome into 2-5 μm films and stained by hematoxylin-eosin (H&E) and periodic acid of Schiff (PAS). Cytopathology exam confirmed positive at 1000x microscopy at least a single yeast cell or multiple

pleomorphic yeasts cells associated to a pyogranulomatous infiltrate would be founded, also for histopathology exam (FARIAS *et al.*, 2004; PEREIRA *et al.*, 2011).

Mycological Exam

Clinical samples undergone mycological examination (gold-standard) (RIPPON, 1988) were inoculated in two sets of Sabouraud-dextrose agar added chloramphenicol (0.05 g/L) and agar Mycosel incubated at 25ºC and 37ºC for up to 3 weeks, with daily observations after the 5th day. For microorphology identification of filamentous colony, duct tape method was applied and stained by cotton blue method. Positivity should be confirmed at 1000X microscopy by presence of hyaline, septate thin branching hyphae with conidia along conidiophore, and flowerlike petals arrange consistent to *Sporothrix* spp. As inclusion criteria, only single pure colony yield from clinical sample was selected for this study.

Molecular identification

After confirmed by mycological exam, the isolates were submitted to DNA extraction based on protocol developed by Vicente *et al.* (2008). Molecular identification was performed based on the protocol of Rodrigues *et al.* (2015), in which the calmodulin gene species-specific region determined cryptic species of *Sporothrix schenckii* complex.

DNA extraction was performed in micro tubes containing 300μL CTBA (cetyltrimethylammonium) buffer in a mixture with NaCl (1,4 M), Tris-HCL (100 mM), EDTA (20 mM), b-mercapto-ethanol (0.2%), and silica (80 mg). Washing steps were

performed with chloroform, ethanol, and the final pellet was re-suspended with TE/ultrapure water, incubated at 37°C and stored at -20°C (VICENTE *et al.*, 2018).

PCR reaction was performed with a total volume of 25 µl, consistent of buffer, Taq Polymerase, dNTPs, water, specific primers (amplifying 18-25 calmodulin gene nucleotides), and 2 µl of targeted DNA. The reaction was performed in an Eppendorf Mastercyle Pro machine (Eppendorf, Hamburg, Germany), using a touchdown method in order to use all designed primers at the same time. PCR conditions included a 10 minutes denaturing step at 95°C, followed by 35 cycles of 1 min at 95°C, 1 minute at 48°C annealing temperature, and 1 minute extension at 72°C. Subsequent annealing temperatures decreased 1°C every 2 cycles for the next 20 cycles in the touchdown protocol. Finally, PCR amplicons were observed with agarose gel electrophoresis, and stained bands visualized at UV light with L-Pix Touch imaging system (Loccus Biotectonologia, Sao Paulo, Brazil) (RODRIGUES *et al.*, 2015).

Fungal Susceptibility Test

Susceptibility test were performed based on M27-S4 document of Clinical and Laboratory Standards Institute (CLSI, 2012) modified by Borba-Santos *et al.,* (2017) for yeast form of dimorphic fungi. Minimum inhibitory concentrations (MIC) of ITZ were determined against yeasts of feline-borne isolates and a reference isolate *S. brasiliensis* CBS 133006.

ITZ (Sigma Chemical, CO., St. Louis) was stored as stock solution of 1600 µg/mL in DMSO (at -20 °C). ITZ was diluted in RPMI 1640 medium supplemented with 2% glucose and buffered to pH 7.2 with 0.165 M 3-(*N*-morpholine) propane

sulfonic acid (MOPS) to obtain final concentrations ranged from 0.03 to 16 µg/ml. Susceptibility tests were performed in 96-well microtiter plates containing 200 µL/well of medium with ITZ and 0.5–1.5 × 10^5 CFU/mL of yeasts. For each strain, MIC was defined as the lowest concentration of ITZ that inhibited 50% of fungal growth relative to untreated controls, after 48 hours of incubation at 35 ºC in the dark in a humid chamber with 5% CO_2. MIC breakpoints or epidemiological cut-off values have not been established by CLSI for both filamentous (CLSI, 2008a) and yeast (CLSI, 2008b) forms of *S. schenckii* complex. For the clinical evaluation purpose, this study considered the cut-off value of MIC $\leq 2\mu$g/mL for ITZ as proposed by Espinel-Ingoff *et al.,* (2017). The MIC was determined by visual inspection in an inverted light microscope and confirmed by spectrophotometric readings at 492 ηm, in a microtiter plate reader (EMax Plus, Molecular Devices). In accordance to CLSI supplement M59 (CLSI, 2018), fungi are to be designated as wild type (**WT**) for those without intrinsic or acquired resistance and non-wild-type (**NWT**) for those harboring intrinsic or acquired resistance mechanisms.

Experiments were made in duplicate. The quality control isolates *Candida parapsilosis* ATCC® 22019™ and *Candida krusei* ATCC® 6258™ were included in susceptibility tests as recommended by the CLSI.

Evaluation of therapeutic response

On the first consultation, the prescription of ITZ were 25mg for felines ≤ 3kg and 100 mg > 3kg body weight, and the owner would fill the prescription by choice, whereas generic, compound or reference ITZ, as for the KI always compound form. At the follow-up examination two weeks later, for the no improvement cases, KI 2.5

to 5.0 mg/kg/SID/orally would be prescribed. At the next consultation, when the owner reportedly no clinical improvement, alternative treatment as intra-lesion amphotericin-B infiltration for **R** cases, or cryosurgery for refractory **FC** cases would be recommended. Clinical discharge or cured cases were considered after complete healed lesions, deceased cases were those deaths by disease or euthanized cases and relapses were those cured cases presenting new lesions.

Statistical Analysis

Statistical analysis was performed on demographic, clinical and therapeutic data from feline patients. Data was presented as percentages and median (age). The variables were breed, age, sex, reproductive status, lesion classification, systemic clinical signs, previous use of antifungals when first admitted to the clinic, relapse, therapy abandonment, drug administration failure, therapy failure, cure and deceased. One sample proportion test was used to evaluate the distributions of affected animals according to demographic variables (MOORE *et al.*, 2012a).

Geometric means, range, maximum and minimum values, 50 and 90% MIC/MFC were described. Statistical differences among MIC geometric means were calculated according to the outcome group (cure, deceased) using the Kruskal Wallis test (MOORE *et al.*, 2012b). The proportions of therapeutic variables, between deceased animals, of animals that reached cure were compared using chi square test, with 95% confidence intervals and α set as 5%, respectively (MOORE *et al.*, 2012a). The statistical analyses were performed in STATA, version 14 (College Station, Texas, USA).

RESULTS

Demography and Clinical Data

Mycological samples from 68 feline sporotrichosis cases diagnosed by clinical and laboratorial results, however only 37 (54.4%) isolates converted from filamentous to yeast form for the MIC determination.

Out of these 37 felines demographic data (TABLE 1), 34 (92%) mix breed (p=0.0001), median of 24 months old, 22 (66.5%) intact (p=0.05), 28 (75%) male (p=0.001), and 30 (83.3%) outdoors access (p=0.0001).

The clinical evolution or interval from onset of clinical signs to the first consultation was 16.3 weeks (standard deviation 21.1 weeks, 96 max - 1 min weeks) before definitive diagnosis, 16 (47%) animals were under previous antifungal treatment for a mean of 3.66 weeks before first consultation. Lesion distribution was 7 (20%) **FC** form, 11 (31.4%) **DC** form, 17 (48.6%) **DC**+**R** association and 16 (52%) cases registered of **DC** form with **SyS** such as apathy, anorexia, hyporexia, emaciation and digestive disorders (TABLE 1).

TABLE 1. Demographic and lesion classification variables distribution of 37 sporotrichotic cats.

Variable (n)	**Distribution n (%) (*p* value)**
Breed (37)	Mix 34 (92%) (*p=0.0001*) Siamese 3 (8%)
Ages (26) Not reported (7)	Mean 30.8 months, SD 20.4 months Max 84 months - Min 5 months
Sex (35)	Male 28 (75%) (*p=0.001*) Female 9 (25%)

Reproductive status (33) Not reported (4)	Intact 22 (66,5%) (*p=0.05*) Neutered/Spayed 11 (33.5%)
Outdoors access (36) Not reported (1)	Yes 30 (83.3%) *(p=0.0001)* No 3 (16.7%)
Evolution time (28) Not reported (9)	Mean 16.3 weeks, SD 21.2 weeks Max 96 weeks - Min 1 week
Previous treatment (19) No previous treatment (15) Not reported (3)	Antifungal 16 (47%)
Lesion classification (35) Not reported (2)	**DC** 11 (31.4%) **DC**+**R** 17 (48.6%) **FC** 7 (20%)
SYS (16) No symptoms (21) Not reported (6)	**SYS+DC** 13 (81.3%) **SYS+FC** 3 (18.7%)

n number of the cases; **SD** standart deviation, **FC** Fixed Cutaneous form, **DC** Disseminated Cutaneous form, **R** Respiratory symptoms, **SyS** Systemic Symptoms, **LYC** Lymph cutaneous form

Molecular identification and MIC

Molecular identification based on calmodulin gene species-specific primers, specifically Sbra-F and Sbra-R primers, detected a 469bp fragment for all 68 isolates. This specific fragment size characterized all isolates obtained from Curitiba feline sporotrichosis outbreak as *S. brasiliensis.*

ITZ MIC ranged from 0.06-16 μg/Ml. Geometric media (GM) was 1.18, IC 0.6553527-2.12841 (TABLE 2). In the present study, from 37 sample studied, obtained 25 (67.6%) wild-type (**WT**) isolates and 12 (32.4%) non-wild-type (**NWT**).

TABLE 2. In vitro ITZ susceptibility of 37 clinical isolates of *S. brasiliensis*.

	MIC (mg/mL^{-1})				Susceptibility (%) at MIC (mg/mL^{-1}) of						
	GM	**Range**	**50%**	**90%**	**≤0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
Itraconazole ITZ	1.18	0.06-16	1	16	32.4	8.1	13.5	13.5	8.1	8.1	16.2

MIC Minimal Inibitory Concentration

Clinical Outcomes

Of the 37 cases, 18 (48.6%) cases of therapy abandonment or lost contact and 2 (5.4%) *post-mortem* cases were excluded from this item for there were no clinical outcome to be considered. Therefore, for the clinical outcome data from remain **17** feline sporotrichosis cases were applied for the study. Out of the 17 feline sporotrichosis cases, 12 cases were caused by *S. brasiliensis* **WT** isolates and 5 cases by *S. brasiliensis* **NWT** isolates. Lesional aspects, relationship with antifungal resistance and therapeutic evolution these cats are described in TABLE 3.

TABLE 3. Clinical outcomes of 17 feline sporotrichosis cases.

Clinical Form n (%)	**ITZ susceptibility n (%)**	**Clinical Outcome n (%)**
FC 4 (23.5%)	**WT** 4 (100%) **NWT** 0	**Cure** 3 (75%) **Decease** 1† (25%)
DC 13 (76.5%)	**WT** 8 (61.5%) **NWT** 5 (38.5%)	**Cure** 10 (76.9%) **Decease** 2†† (15.4%) 1† (7.7%)
DC*+*R *6 (46.5%)*	**WT** 4 (66.6%) **NWT** 2 (33.3%)	**Cure** 4 (66.6) **Decease** 1† (16.6%) 1†† (16.6%)

n number of the cases, **ITZ** Iitraconazole, **FC** Fixed Cutaneous form, **DC** Disseminated Cutaneous form, **R** Respiratory symptoms, **WT** Wild Type, **NWT** Non-Wild Type, †euthanasia, ††death by disease

Alternative treatments were applied in a mean of 19.3 weeks after clinical treatment has been set, in a total of 7 (41.1%) of the cases. Of these, 4 (57.1%)

amphotericin-B intra-lesion application, 2 (28.6%) cryotherapy, and 1 (14.3%) combined cryotherapy and amphotericin-B.

Of the 17 cases, 13 (76.5%) obtained **cure**, with a mean of 84.5 weeks of treatment for clinical discharge, 3(23.1%) cases undergone alternative treatment and 4 (31%) cases relapsed 72 weeks after clinical discharge.

All four animals of the **decease** group of were under treatment for a mean of 78 weeks before death, also the four cases had treatment failure, three (75%) undergone alternative treatment and two (50%) cases received previous treatment before first consultation. Two deaths (50%) by disease occurred after 38 weeks of treatment and other two (50%) were euthanized after 118 weeks of treatment. A total of three (75%) of **deceased** cases registered **SyS**.

Antifungal Treatment

Within the 12 **WT** isolates cases, ten were **cured**, as eight by ITZ+KI antifungal treatments and two by ITZ monotherapy; and two **deceased** cases, as one by ITZ+KI antifungal treatment and one by FLU monotherapy. Within the 5 **NWT** isolates cases, three were **cured** by ITZ+KI antifungal treatments; and two **deceased** cases were treated by ITZ+KI (TABLE 4). Though not significant ($p \geq 0.05$), higher mortality rates in **NWT** group were found than in **WT** group

TABLE 4. ITZ susceptibility related to potassium iodide (KI) association and clinical outcome

Suscetibility (n)		**Outcome**		**With KI**		**Without KI**	
		n	**%**	**n**	**%**	**n**	**%**
WT (12)	Cure	10	83.3	8	80	2	20
	Decease	2	16.7	1	50	1	50
NWT (5)	Cure	3	60	3	100	-	-
	Decease	2	40	1	50	1	50

n number of the cases, **KI** potassium iodide, **WT** Wild Type, **NWT** Non-Wild Type

DISCUSSION

In the present study, *S. brasiliensis* was confirmed in the 68 feline sporotrichosis cases. *S. brasiliensis* is the most virulent species of the *S. schenkii* complex and has the domestic cat as its natural host (RODRIGUES *et al.*, 2013; RODRIGUES *et al.*, 2016).

The feline sporothricosis demographic profile is in agreement to other studies in Brazil (SCHUBACH et al., 2004; MONTENEGRO *et al.,* 2014; GREMIÃO *et al.,* 2015; REIS *et al.,* 2016; POESTER *et al.,* 2018). It is a common fact that intact adult male with outdoor access are the most exposed population. Domestic felines' territorial and matting behavior expressed by their physical confrontation favors them to get infected with consequent fungal propagation to humans and to other animals by sick cats' scratches, bites and lesions secretions contact. Cats with outdoors access are the most vulnerable hosts for *Sporothrix* spp. infection, considering feline high susceptibility for *S. brasiliensis*.

The most frequent lesion form in feline sporotrichosis is **DC** form occurred in 10 (76.9%) cases in the present study and is a common high rate among various studies (SCHUBACH *et al.,* 2004; REIS *et al.*, 2016; SOUZA *et al.,* 2018; MIRANDA *et al.,* 2018). It can be due multiple inoculation by scratches and bites during cat fights, also the host can self-inoculate by grooming and scratching the open lesions as the parasitic yeasts inoculum evolves from nodules to ulcers. Usually head and forelimbs are the most affected, but other sites are also reported in various studies.

The low rate of conversion (only 37 from original 68 isolates) from filamentous to yeast form for the MIC determination narrows the antifungal susceptibility test as a clinical tool for routine sporotrichosis treatment. In the present study, the low

conversion led to the exclusion of 31 feline sporotrichosis cases for antifungal susceptibility and clinical outcome study. Considering M27-S4 document CLSI (2012) is applied for *Candida* spp., even modified by Borba-Santos *et al.,* (2017), the *Sporothrix* spp. filamentous conversion to yeast form remains a challenge for antifungal susceptibility tests.

Comparing the clinical outcomes of the **WT** and **NWT** groups from the clinical point of view, **WT** *S. brasiliensis* isolates present better cure incidence comparing to **NWT** isolates. The present study evinced that two-thirds of *S. brasiliensis* isolates were **WT**, bearing no intrinsic nor acquired resistance to itraconazole.

The remain one-third of the isolates was **NWT** *S. brasiliensis*. According to Almeida-Paes *et al.* (2017), long term ITZ treatment (42.6 weeks) does not lead to acquisition of resistance in *S. brasiliensis*, and as the mean period of the previous treatment find in this study was of 3.6 weeks, this does not seem to trigger resistance expression from the isolates. The other probable fungal azole resistance development is by environmental exposure to azole group, characterized in *Aspergillus fumigatus* exposition to agricultural azole fungicide (PARENTE-ROCHA *et al*., 2017) within the One Health spectrum.

Besides acquired resistance, intrinsic resistance mechanisms such as *S. brasiliensis* thicker cell wall which interferes on the antifungal input (LOPES-BEZERRA, *et al.*, 2018), is that some isolates express virulence features that overcomes the antifungal action. Biofilm formation related antifungal resistance is a feature verified by Brilhante *et al.,* (2018) in *S. brasiliensis* biofilm formation contributing to antifungal resistance by limiting drug diffusion Cluster formation of yeast cells by *S. brasiliensis* cell wall fibrils might affect antifungal access its action site (LOPES-BEZERRA *et al.*, 2018).

In the present study, ITZ+KI association was used in 11 out of 13 cured cases, 84.6% of **cure** rate, comparable to Miranda *et al.*, (2018) 81.5% cure, and Reis *et al.* (2016) 96% cure rates. Despite more **WT** isolates found here, the majority of the cases did not respond to itraconazole monotherapy and only were cleared by ITZ+KI associated and some after alternative therapy intervention. MIC breakpoints or epidemiological cut-off values have not been established by CLSI for *S. schenckii* complex, and despite the cut-off value of MIC $\leq 2\mu$g/mL for ITZ proposed by of Espinel-Ingoff *et al.,* (2017) multicenter study, there is a chance that this value is been underestimated, hence clinical success only with ITZ+KI association.

In five cases, intra-lesion amphotericin B was applied after 19.3 weeks of oral ITZ+KI association treatment. Most feline ITZ therapy studies refers, after oral dosing, a good drug bioavailability, but only by plasma concentrations. Since sporotrichosis being predominant a cutaneous disease, feline integumentary concentrations should also be aim for future studies. On the other hand, in human studies, it has already been demonstrated ITZ reaching therapeutic concentrations in cutaneous fungal lesions (MORIMOTO *et al.*, 2004; SEISHIMA *et al.*, 2004).

An important feature in feline sporotrichosis **DC** form, besides potential secondary infections form the open wounds, is the loss of plasmatic proteins from lesion exudation, that depending on the extension and number of the ulcers, depletes the albumin, already in critical levels in sporotrichotic cats, and impacts the clinical treatment, hence the on antifungal bioavailability/distribution and ultimately concentration on infected tissues that relies on plasmatic albumin.

Differences among the generic, compound or reference ITZ might interfere its efficiency, therefore the supporting intra-lesion therapy was required. It remains

uncertain the KI action mechanism in antifungal therapy, but apparently, KI modulates inflammatory responses or enhances host's innate immune defense system (STERLING & HEYMANN, 2000; REIS *et al.*, 2012).

On the other hand, alternative therapies such as intra-lesion amphotericin-B application and cryotherapy are the second line of choice for **FC** or **DC+R** forms, indicated low blood supplied sites where is difficult to keep ITZ therapeutic concentrations such as nasal area, pinna, forehead and tail. Low blood supply upper respiratory mucosa also favors fungal propagules formation within concha dorsal and sinus frontal mucosa.

The long treatment period can be related to the majority of the clinical presentation were **DC** form and **DC+R** association. According to Reis *et al.,* (2016), comparing felines with cutaneous presentation and felines with mucosal involvement, the period of treatment until clinical discharge is longer. According to Gremião *et al.,* (2015), the most frequent clinical form in felines is **DC** form associated with respiratory symptoms. **R** form with mucosa lesions, respiratory difficulties, build-up secretion, sinusitis, eventually cartilage and bone degeneration can lead to important respiratory impairment, all affecting olfactory abilities reducing food and water intake.

Some issues were raised by the long-term treatment before clinical discharge. First, delay of 16.3 weeks before definitive diagnosis might impact the treatment extent comparing the period of eight weeks' treatment found by Miranda *et al.,* (2018); therefore, early diagnosis and treatment shortens the treatment length. Second, the clinical severity, primarily **DC** and **R** forms that are related to treatment failure (REIS *et al.,* 2012) might delay the treatment improvement. Third, some more virulent strains down-regulate hosts' innate response causing more disseminated

lesions and long lasting infection (BATISTA-DUHARTE *et al.*, 2018). Also, delay on therapy establishment on ITZ + KI associated, or any alternative therapy, might extend the period for clinical discharge as in the present study of 84.5 weeks, was much longer than Souza *et al.,* (2018) of 16 weeks and Pereira *et al.*, (2010) of 26 weeks.

Relapse occurred after 72 weeks (18 months) in four (30.7%) **WT** cured cases using ITZ+KI in the present study. According to MacDonald *et al.,* (1980), *S. schenckii* can remain viable within healed tissues for at least six months and can be reactivated by any host's immunosuppressive event. Gremião *et al.,* (2015) also stated that recurrence may occur due lesion reactivation, despite the remission from the treatment. The fungal viability within scar tissues in felines is an important topic for further studies, as for relapse occurrence prevention; also, the treatment length might be revised as for relapse prevention as well.

For four **deceased** cases, most were disseminated skin forms. In two cases of antifungal administration failure, besides **SyS** occurrence, that ultimately lead to decease. Anorexia, hiporexia emaciation, digestive disorders, hepatic or renal impairment worsens the overall health condition in any type of disease. Additionally, Della-Terra *et al.,* (2017) using murine model, found that highly virulent strains of *S. brasiliensis* feline-derived showed a highly dissemination power infecting liver, spleen, kidneys, lungs, heart and brain. Also, antifungal treatment can also elicit hepatic and digestive disorders leading to vomit and anorexia, therefore adding up the treatment failure rates.

Other important finding was the deceased rates between **WT** (16.6%) and **NWT** (40%), denoting susceptibility results might be used as prognosis tool, ever overcome the low rate of filamentous/yeast conversion process.

CONCLUSIONS

The present study, the feline sporotrichosis was caused by ITZ **WT** *S. brasiliensis*, however, the monotherapy was ineffective and the most of the cases were cured with ITZ+KI associated therapy and some even alternative treatments. Isolation of **NWT** *S. brasiliensis* strains was associated with an unfavorable prognosis.

ACKNOWLEDGMENTS

Veterinary Clinic School - CVE, Veterinary Medicine (Head Veterinary Medicine), School of Life Sciences, Pontifícia Universidade Católica do Paraná.

Prof Dr Vania Aparecida Vicente, LabMicro, Pathology Department, Biological Science Sector, Paraná Federal University, Curitiba, Paraná, Brazil.

Prof Dr. Anderson Messias Rodrigues, Medical Mycology Laboratory, Microbiology, Immunology and Parasitology Department, São Paulo Federal University, São Paulo, Brazil.

Prof Dr Sonia Rosental, Laboratory of Fungal Cell Biology, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Finance Code 001.

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CAPITULO 4

CONSIDERAÇÕES FINAIS

A esporotricose felina causada pelo *Sporothrix brasiliensis* é uma dermatomicose emergente em importantes regiões metropolitanas do Brasil como Rio de Janeiro, São Paulo e Pelotas. O número de casos felinos em Curitiba e região metropolitana nos últimos três anos, desde 2016 tem aumentado de forma significativa, elevando o município para o segundo lugar no número de novos casos felinos no país, estando apenas atrás do Rio de Janeiro.

Vale ressaltar que a doença é uma antropozoonose, endêmica no Rio de Janeiro a mais de vinte anos que impacta principalmente populações de baixa renda e imunocomprometidos, embora com boas taxas de cura humana, eleva a carga sobre o sistema de saúde público.

Faz-se necessário a adoção de medidas tanto de controle como de prevenção dentro do contexto de Saúde Única, integrando todos os processos e intervenções aplicados em três níveis: **1)** na saúde animal com protocolos de abordagem para médicos veterinários na suspeita, diagnóstico e tratamento da doença, medidas de biossegurança, educação em saúde para os proprietários; **2)** na saúde pública com campanhas de conscientização sobre a doença, importância da posse responsável que inclui castração e restrição de acesso à rua do animal, e acesso ao tratamento humano pele sistema público de saúde; e **3)** na saúde ambiental, avaliando os impactos dos fungicidas utilizados na agricultura sobre fungos de importância clínica.

Entretanto, enquanto o sistema de saúde público não reconhecer a gravidade da doença tornando-a doença de notificação obrigatória, não existirão políticas oficiais para que as medidas de prevenção e controle sejam estabelecidas e executadas.

ANEXO 1 – Parecer de aprovação do CEUA/PUCPR

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação
Comissão de Ética em Pesquisa no Uso de Animais

Curitiba, 30 de novembro de 2017.

PARECER DE PROTOCOLO DE PESQUISA

REGISTRO DO PROJETO: 01197 – 2ª versão

TÍTULO DO PROJETO: *EPIDEMIOLOGIA MOLECULAR DA ESPOROTRICOSE EM GATOS DE CURITIBA PR*

PESQUISADOR RESPONSÁVEL

MARCONI RODRIGUES DE FARIAS

EQUIPE DE PESQUISA

Luana Beatriz Balardin, Larissa Anuska Zeni Condas, Suelen Sicuro Ribeiro, Renata Rodrigues Gomes, Fabiana dos Santos Monti, Karina Franci Braga, Kung Darch Chi

INSTITUIÇÃO

Pontifícia Universidade Católica do Paraná

CURSO

Medicina Veterinária

VIGÊNCIA DO PROJETO	Julho/17 a Julho/2018	**QUANTIDADE DE ANIMAIS**	100
ESPECIE/LINHAGEM	*Felis catus familiaris*	**Nº SISBIO** (Somente animais de vida livre)	Não se aplica
SEXO	Variável	**ATIVIDADES** (Somente animais de vida livre)	Não se aplica
IDADE / PESO	Variável	**ESPECIÉ – GRUPO TAXONÔMICOS** (de vida livre)	Não se aplica
ORIGEM DO ANIMAL	CVE PUCPR - Particulares	**LOCAL (IS)** (Somente animais de vida livre)	Não se aplica

O colegiado da CEUA certifica que este protocolo que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto homem), para fins de pesquisa cientifica, encontra-se de acordo com os preceitos da Lei nº 11.794/2018 e Decreto nº 6.899/2009, e com as normas editadas pelo CONCEA e foi **APROVADO** pela CEUA - PUCPR em reunião de colegiado.

Se houver mudança do protocolo o pesquisador deve enviar um relatório á CEUA descrevendo de forma clara e sucinta, a parte do protocolo a ser modificado e as suas justificativas.

Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciar antes de receber a autorização formal para a sua realização.