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Imprint and spreading techniques for the isolation and identification of subungueal fungi in claws of healthy cats

Técnicas de imprint e espalhamento para isolamento e identificação de fungos subungueais em garras de gatos saudáveis

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ABSTRACT: The aim of this study was to compare imprint and spreading techniques for the isolation and identification of colonies of pathogenic and non-pathogenic fungus in the claws of semidomiciliated cats. For that propose, 150 cats were evaluated, subdivided into three groups of 50 animals. In the first and second groups, the cats were submitted to the imprint technique in Petri dishes containing Selective Mycobiotic Agar: In the first group, the cats were subjected underwent antisepsis with 70% ethanol of the claws of the thoracic limbs and in the second group the animals were subjected underwent antisepsis with 70% ethanol of the claws of only one of the thoracic limbs. The third group was submitted to the spreading technique, whose material was collected by rubbing a sterile swab moistened with brain-heart infusion broth, in the claws of the forelimbs, where an aliquot of the material was transferred to Petri dishes containing Selective Mycobiotic Agar. The material was stored at 25°C for 30 days. The readings were performed on days 5, 7, 15, and 30 post incubation. Using the imprint technique performed under the conditions of this experiment, we were not able to isolate and identify the colonies because since day 5, they were overlapped. From the spreading technique, *Mucor* sp. (54,34%), *Rhodotorula* sp. (28,26%), *Fusarium* sp. (21,73%), *Aspergillus* sp. (21,73%), *Trichoderma* sp. (19,56%), *Penicillium* sp. (19,56%), *Cladosporium* sp. (10,86%), *Rhizopus* sp. (8,68%), *Acremonium* sp. (6,5%), *Exophialia* sp. (6,5%), *Paecilomyces* sp. (4,34%), *Trichosporon* sp. (4,34%), and G*eotrichum* sp. (2,17%) were isolated. It was concluded that the spreading technique proved to be useful in isolating fungal colonies from feline claws, and the animals do not present symptoms, which signals the importance of them as possible sources of exposure for tutors. The cats were negative for *Sporothrix* sp. by the imprint and spreading techniques.

KEYWORDS: claws; *Felis catus;* fungal identification; microbiology

RESUMO: O objetivo deste estudo foi comparar as técnicas de imprint e espalhamento para o isolamento e identificação de colônias de fungos patogênicos e não patogênicos em garras de gatos semidomiciliados. Para tanto, foram avaliados 150 gatos, subdivididos em três grupos de 50 animais. No primeiro e segundo grupos, os gatos foram submetidos à técnica de imprint em placas de Petri contendo Agar Micobiótico Seletivo. No primeiro grupo, os gatos foram submetidos à antissepsia com etanol 70% das garras dos membros torácicos e no segundo grupo os animais foram submetidos à antissepsia com etanol 70% das garras de apenas um dos membros torácicos. O terceiro grupo foi submetido à técnica de espalhamento, cujo material foi coletado esfregando-se um swab estéril umedecido em caldo infusão cérebro-coração nas garras dos membros anteriores, a partir do qual uma alíquota do material foi transferida para placas de Petri contendo Ágar Micobiótico Seletivo. O material foi armazenado a 25 ° C por 30 dias. As leituras foram realizadas nos dias 5, 7, 15 e 30 após a incubação. Utilizando a técnica de imprint realizada nas condições deste experimento, não fomos capazes de isolar e identificar as colônias, uma vez que desde o dia 5 elas estavam sobrepostas. A partir da técnica de espalhamento, *Mucor* sp. (54,34%), *Rhodotorula* sp. (28,26%), *Fusarium* sp. (21,73%), *Aspergillus* sp. (21,73%), *Trichoderma* sp. (19,56%), *Penicillium* sp. (19,56%), *Cladosporium* sp. (10,86%), *Rhizopus* sp. (8,68%), *Acremonium* sp. (6,5%), *Exophialia* sp. (6,5%), *Paecilomyces* sp. (4,34%), *Trichosporon* sp. (4,34%) e *Geotrichum* sp. (2,17%) foram isolados. Concluiu-se que a técnica de espalhamento mostrou-se útil no isolamento de colônias de fungos em garras felinas, e os animais não apresentam sintomas, o que sinaliza a importância deles como possíveis fontes de exposição para os tutores. Os gatos foram negativos para *Sporothrix* sp. pelas técnicas de imprint e espalhamento.

PALAVRAS-CHAVE: *Felis catus*; garras; identificação de fungos; microbiologia.

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INTRODUCTION

Given the increasing occurrence of zoonosis, associated with the fact that dogs and cats coexist closely with humans, investigative studies on this interaction and the risk of disease transmission have been arousing the interest of researchers (FARIAS et al., 2011). In this context, it is noteworthy that cats play an important role in spreading zoonosis: fungal diseases such as sporotrichosis, either through scratching or direct contact (LARSSON, 2011).

Dermatological disorders of fungal etiology are common in the veterinary medical routine (SCOTT; MILLER, 2011), including zoonosis (ANDRADE, 2002), and not all animals carrying the disease show clinical signs (LARSSON, 2011). Thus, the diseases can often be misdiagnosed, increasing the risk of transmission to humans, which reveals the importance of identifying the most frequently observed fungi in feline claws.

Of the fungi present in the coat of cats, several of them are saprophytes and are part of the microbiota, like *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp., *Mucor* sp. e *Rhodotorula* sp. (CABAÑES, 2000; GAMBALE et al., 1993; MORIELLO; DEBOER, 1991; PAIXÃO et al., 2001; SCOTT; MILLER, 2011). However, some of these may promote the development of clinically important, opportunistic systemic mycoses in the human population, especially in patients with immune impairment (CAREY et al., 2009).

The diagnostic technique considered as gold standard for the identification of fungal colonies consists of culture and subsequent identification of the agent (CARTER, 1988). For this, the material for cultivation can be obtained through impressions of the claws on plates containing suitable culture media for fungal growth, the most suitable technique, or even fragments of the claws (BORGES et al., 2013). The treatment is based on the use of antifungal drugs, such as ketoconazole, itraconazole, fluconazole, miconazole, among others. The dosage of these drugs varies according to the chosen drug (NOBRE et al., 2002).

Optimal temperature and humidity conditions are known to favor fungal proliferation (TANG et al., 2015) and consequently, the development of mycoses. In this context, although the Ilhéus-Itabuna microregion is not endemic for sporotrichosis: one of the most important mycotic zoonoses in clinical routine, it has a favorable climate for the development of fungal agents. Thus, the objective of this work was to compare the imprint and spreading techniques for the isolation and identification of the colonies of pathogenic and non-pathogenic fungi present in the claws of semidomiciliated felines in the Ilhéus and Itabuna microregion of the state of Bahia, and to use these techniques to determine the fungal population present in the claws of cats.

MATERIAL AND METHODS

This study was approved by the Animal Use Ethics Commission (CEUA), Santa Cruz State University (UESC), protocol number 020/16.

The work was developed in the Ilhéus-Itabuna microregion, located in the southern region of Bahia, with a humid tropical climate. Ilhéus: Latitude, 14º 47 '20 "S; Longitude, 39º 02' 58" W, and 78% humidity and Itabuna: Latitude, 14º 47 '08 "S; Longitude: 39º 16' 49" W, and 83% humidity.

For the sample size calculation, the prevalence of sporotrichosis in healthy feline cat nails in the State of Rio de Janeiro (29%) was used (SOUZA et al., 2006), due to a lack of the epidemiology data of this disease in the state of Bahia (GUTIERREZ-GALHARDO et al., 2015). Data of Brazilian Institute of Geography and Statistics (IBGE) 2010, available in <https://biblioteca.ibge.gov.br/visualizacao/livros/liv94074.pdf>, showed that in the Northeast, 23.6% of households had cats. IBGE also estimated that each household had three people. Thus, the average population of Ilhéus and Itabuna according to the 2010 census was 194,451 people, with 64,817 households, with an estimated 15,296 felines. The Sample Size Program was used, available on the platform <http://sampsize.sourceforge.net/iface/index.html>, with an accuracy of 8%, an estimated cat population of 15,296, a prevalence of 29%, and a confidence level of 95%, giving a sample of 123 cats.

Thus, 150 semidomiciliated cats living in the above-mentioned municipalities participated in this study. These animals came from visits to the Veterinary Hospital of UESC, units of zoonosis control, and household visits. Samples were collected for convenience from May 2017 to November 2018.

After the owner's` authorization, a brief anamnesis was done and the cats were submitted to clinical and dermatological evaluation, followed by the collection of materials from the claws of the anterior and thoracic limbs. The choice of thoracic limbs was made based on cat´s habit of burying their excrement with the thoracic limbs, which determines a higher probability of finding the pathogenic fungi. To collect the materials, the cats were physically contained, wrapped in individual towels to prevent sudden movements or contained in a bag of synthetic material specially made for cats' immobilization, according to the methodology of Souza (2003).

For the claw imprint technique described previously (BORGES et al., 2013), the claws of the thoracic limbs of 100 cats were included. They were divided into 2 experimental groups: group 1 (G1), in which 50 cats underwent antisepsis of both thoracic claws with 70% ethanol and group 2 (G2), in which 50 cats were subjected to 70% ethanol antisepsis in only one of the thoracic claws. Collection was similar for both groups (G1 ad G2). For claw exposure, the thoracic limb pads were cranially pressed to the metacarpal. After exposure and antisepsis of the claws as previously mentioned for each group, the digits were sticked in Petri dishes containing Selective Mycobiotic Agar, using a plate for each thoracic limb.

After sample collection, mycological culture was performed, during which Petri dishes containing Selective Mycobiotic Agar were inoculated at 25°C for 30 days, for mycological analysis. Plaques were observed at 5, 7, 15, and 30 days after inoculation to evaluate colony growth for subsequent identification, based on macroscopic and microscopic characteristics. For G2,

we also observed whether antisepsis would reduce the burden of fungal colonies present on the plaques.

For the cultivation of fungi through the spreading technique, after exposure of the claws of the thoracic limbs, samples of 50 cats were collected for mycological culture, using a sterile swab for each front paw. The collection was performed separately in each paw of each animal. This swab was moistened with brain-heart infusion broth (BHI): previously prepared in glass test tubes, rubbed three times on each side of the nail (cranial, dorsal, right, and left lateral), and then returned to the tube, which contained 2 ml of the broth BHI for further spreading in the Petri dishes with the culture medium.

Soon after the collections, the swabs were removed from the BHI solution and 0.1 mL of the BHI broth was spread with Drigalski spatula on a plate containing Selective Mycobiotic Agar. One plate per paw of each cat was used, totaling 100 plates. After this step, the tubes containing the swab and BHI broth were left at a temperature of 25°C. After the tubes remained at 25°C for 24 hours, the swabs were removed from the BHI solution and again, 0.1 mL of the BHI broth was spread with Drigalski spatula on a plate containing Selective Mycobiotic Agar.

After this spread, 0.25 mL of the solution was transferred to another tube containing 2.25 mL of physiological solution. This solution was homogenized and 0.1 mL was spread on a plate containing Selective Mycobiotic Agar, 1 plate per claw, totaling 100 plates. Thus, by adding the number of plates of the two solutions, four plates per animal were used, totaling 200 plates.

The plates containing Selective Mycobiotic Agar were used for the isolation of the fungal colonies and subsequent identification of the fungi that showed higher rates of macroscopic growth and the colonies that presented characteristics similar to those described for *Sporothrix* spp., during 30 days.

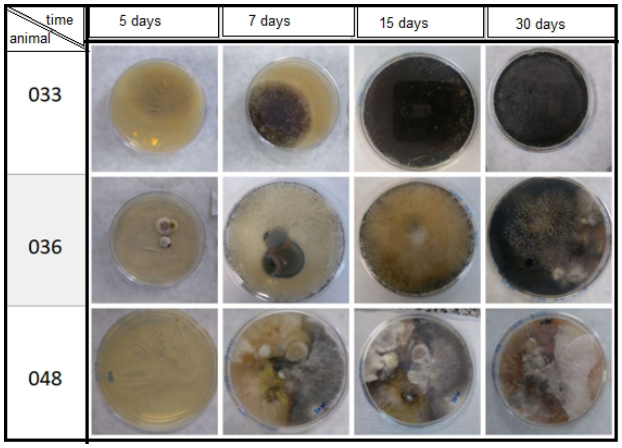
The filamentous colonies were submitted to the micro culture technique to identify the fungi. This technique was performed as follows: fragments of filamentous colonies were transferred to a square of BDA medium on a slide, covered with coverslips and placed in a petri dish with cotton soaked with water, forming a humid chamber. After 5 days at room temperature, the coverslip was removed and placed on a new slide with blue cotton dye (Lactophenol) for later observation under a light microscope for fungal identification.

The colonies that presented macroscopic characteristics similar to those described for the fungi of the genus *Sporothrix* spp., were reinoculated into a sterile environment, containing the BHI infusion agar at 37°C, to confirm the characteristic dimorphism of the fungus (SOUZA et al., 2006).

RESULTS

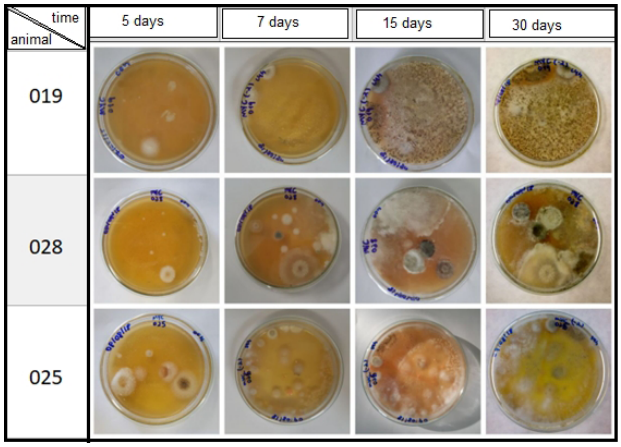
The imprint technique performed under the conditions of this experiment was not viable for the isolation and identification of the colonies, since they overlapped. It was observed from the imprint technique that there is a large fungal load housed in the claws of felines and this colonization promotes the development of fast and slow growing fungi, ascending and overlapping, despite the performed asepsis, making it impossible to identify the fungi. The plates were photographed after 5, 7, 15, and 30 days of incubation each, using the antisepsis technique of only one thoracic limb (Figure 1).

From the spreading technique, in 46 of the 50 cats (92%), the colonies' growth were dispersed on the plate (Figures 2 and 3), and it was possible to isolate and identify the following colonies: *Mucor* sp., 25 (54.34%); *Rhodotorula* sp. 13 (28.26%); *Fusarium* sp., 10 (21.73%); *Aspergillus* sp., 10 (21.73%); *Trichoderma* sp., 9 (19.56%); *Penicillium* sp., 9 (19.56%); *Cladosporium* sp., 5 (10.86%); *Rhizopus* sp., 4



Animal plates 033, 036, and 048, incubated for 5, 7, 15, and 30 days. From the 7th day onwards, fungal isolation was compromised due to exacerbated fungal growth.
Source: personal archive, 2019.

Figure 1. Imprint technique on Selective Mycobiotic Agar.



Animal plates 019, 028, and 025, incubated for 5, 7, 15, and 30 days each. With the spreading technique, where it was observed that the colonies were more dispersed, enabling the isolation and subsequent identification of the colonies.
Source: personal archive, 2019.

Figure 2. Selective Mycobiotic Agar spreading technique.

(8.68%); *Acremonium* sp., 3 (6.5%); *Exophialia* sp., 3 (6.5%); *Paecilomyces* sp., 2 (4.34%); *Trichosporon* sp., 2 (4.34%); and *Geotrichum* sp., 1 (2.17%).

No colonies of the genus *Sporothrix* spp. was isolated, although one of the analyzed colonies had microscopy compatible with *Sporothrix* spp. (Figure 4). However, since thermal dimorphism was not seen, it was not possible to infer that this was the genus. In four cats evaluated (8%), it was not possible to isolate and identify the colonies present in the Petri dishes, due to the exacerbated growth and overlap of the fungal colonies. Importantly, more than one fungal colony per cat was identified.

DISCUSSION

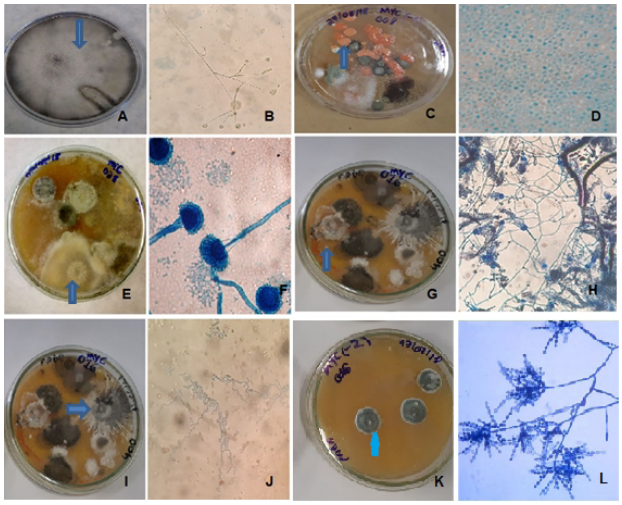
From the results obtained, the imprint technique performed under the conditions of this study was not effective for the isolation and identification of moderately growing fungi. In the present study, it was observed that through this technique, even with the use of specific Mycobiotic Agar culture medium, specific to inhibit fast growing fungi, there was exacerbated and accelerated fungal growth, so that their isolation and identification was impossible. This finding disagrees with what was observed by other researchers (BORGES et al., 2013), who in their study with domestic and wild or exotic felines were able to identify using this technique, the fungi: *Sporothrix schenckii, Microsporum canis, Malassezia pachydermatis, Penicillium* sp., *Aspergillus* sp., *Rhodotorula* sp., *Candida* sp., *Trichoderma* sp. and *Acremonium* sp.

In the present study, no evaluated feline had dermatological lesions compatible with the characteristics of sporotrichosis. The results of the present study corroborate those described by other researchers (BORGES et al., 2013), since in the felines they evaluated, without clinical signs, it was not possible to identify the *Sporothrix* spp.

It is noteworthy that although the present study used the technique of Borges et al. (2013), following the protocol described, an adaptation of this technique was also employed. The adaptation consisted of subjecting only one of the thoracic limbs of a group of animals to antisepsis before collection to compare colony growth between the same animal's paws. However, in both cases there was an accelerated and exacerbated growth, as previously mentioned, negatively influencing the isolation and subsequent fungal identification.

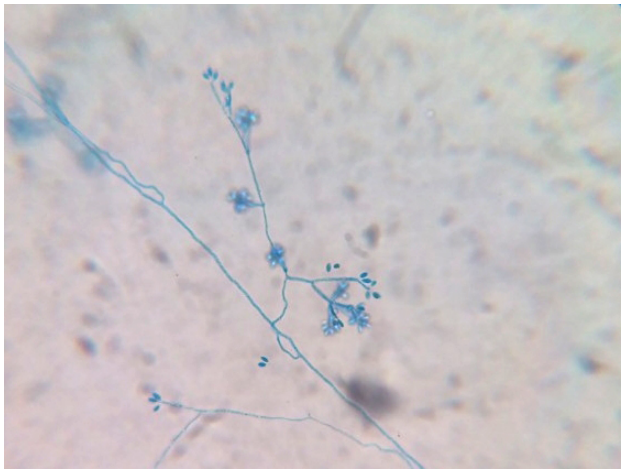
According to Venturelli et al. (2009), 70% alcohol is an intermediate level antiseptic and can be used on surfaces and the skin, penetrating microorganisms without causing cellular dehydration, but promoting protein denaturation, resulting in the reduction of microorganisms. However, a study by other researchers (FERNANDO et al., 2013), even after antisepsis with 70% alcohol, found the persistence of yeast in the mattresses subjected to the research, and this corroborates with what was observed in the present study.

In the current research, unlike the imprint technique, the spreading technique was useful for the isolation of moderately growing fungi (8 to 14 days), which also fit the *Sporothrix* complex species. The fungi identified with the spreading technique were similar to those described by other researchers (CABAÑES, 2000; GAMBALE et al., 1993; MORIELLO; DEBOER, 1991; PAIXÃO et al., 2001; SCOTT; MILLER, 2011), who described these microorganisms as the most



A. Colony macroscopy (blue arrow - one can observe the presence of the "white" cotton colony throughout the petri dish). B. Microscopy of the fungus of the genus *Mucor* sp., shown in A, 40× magnification. C. Colony macroscopy (blue arrow). D. Microscopy of *Rhodotorula* sp., shown in C, 40× magnification. E. Colony macroscopy (blue arrow). F. Microscopy of the fungus of the genus *Aspergillus* sp., shown in E, 40× magnification. G. Colony macroscopy (blue arrow). H. Microscopy of the genus *Penicillium* sp., shown in G, 40× magnification. I. Colony macroscopy (blue arrow). J. Microscopy of the genus *Geotrichum* sp., shown in I, 40× magnification. K. Colony macroscopy (blue arrow). L. Microscopy of genus *Cladosporum* sp., shown in K, 40× magnification. All cultures obtained by the spreading technique.
Source: personal archive, 2019.

Figure 3. Colonies macroscopy and microscopy.



Source: personal archive, 2019.

Figure 4. Colony showing microscopic characteristics compatible with *Sporothrix* spp, but without thermal dimorphism.

commonly isolated fungi of cats, housing the tegument and coat of felines, being saprophytes as most of them that are part of the natural microbiota.

These fungi, although mostly described as saprophytes, may promote in the human population the development of opportunistic systemic mycoses, which are of clinical importance especially in immunocompromised patients (GALIZA et al., 2003). In felines, in turn, they can also promote the development of superficial or deep mycoses, in situations that lead to a drop in immunity, revealing their importance (GALIZA et al., 2014).

In the present study, agents of the genus *Aspergillus* sp. and *Penicillium* sp. were found, which are described in the literature as some of the most abundant microorganisms in nature and with worldwide distribution, often being isolated in soil and organic matter (LACAZ et al., 2002; SIDRIM; ROCHA, 2004; WARD, 2006). This wide distribution and presence on the ground justified its frequent isolation from feline claws. Additionally, fungi such as *Cladosporium* sp. and *Paecilomyces* sp. are often isolated from semidomiciliated feline coats (AMARAL et al., 1998; LEITE, 2001), as is the case with the animals evaluated in the present study, in which five and two animals presented with *Cladosporium* sp. and *Paecilomyces* sp., respectively.

Moreover, there are rare cases of zygomycosis caused by fungi of the genus *Mucor* sp, *Rhizopus* sp., among others (GINN, 2007). In these cases, when the animals are affected, gastrointestinal changes are observed (GROOTERS, 2006). Similarly, there are hardly any reports of phaeohyphomycosis affecting the human and feline population (SEYEDMOUSAVI; GUILLOT; DE HOOG, 2013). This justifies the absence of clinical signs arising from them in animals submitted to the current study.

It is noteworthy that most of the fungi isolated in this research are ubiquitous and present in the environment. These characteristics facilitate the development of diseases by immunocompromised patients, since the fungal population present in feline coat is mostly composed of fungi that inhabit the environment in which humans live (MORIELLO; DEBOER, 1991), such as the *Geotrichum* sp., *Rhodotorula* sp. *Aspergillus* sp., *Penicillium* sp., *Paecilomyces* sp., *Cladosporium* sp., *Trichoderma* sp., *Tricosporon* sp. all of these described in the present study. Thus, according to researchers (GRUMACH, 2001), knowledge about environmental fungi is extremely important, since it can promote advances in technical and scientific development of diseases resulting from infection by these agents.

The non-isolation of *Sporothrix* sp. colonies, even using the spreading technique, which was effective in the isolation and identification of moderately growing fungi, indicates that the prevalence of the causative agent of sporotrichosis is possibly low in the region, due to the low agglomeration of cats in squares or other social environments, as occurs in large centers such as Rio de Janeiro and São Paulo, thus reducing the spread of the disease. Additionally, it can be observed that this case series is reflected by the clinical care at the Veterinary Hospital of UESC, since in the last three years, there was only one diagnosis of fungal injury in a domestic cat, identified as cryptococcosis (GUEDES et al., 2019).

Based on the national health survey conducted in Brazil by the Brazilian Institute of Geography and Statistics in 2013, it is estimated that 21.3% of households in the state of Bahia have at least one cat in their home. The feline population in Brazil is about 22.1 million, demonstrating the importance of felines as potential sources of infection for the human population, as a result of this habitat sharing. In this context, the lack of feline epidemiological studies in the microregion of Ilhéus and Itabuna, Bahia, makes it difficult to estimate these diseases in the region. Thus, investigative studies on the presence of pathogenic fungi in feline claws are important, since many of them as already described, cause important clinical changes in cats, they can also cause zoonoses, such as *Sporothrix* sp. (POESTER et al., 2018).

CONCLUSION

From the results of this study, it was concluded that the spreading technique proved to be useful in isolating some opportunistic pathogenic colonies, revealing its clinical importance in felines, although the evaluated animals were negative for *Sporothrix* sp. by the imprint and spreading techniques.

In addition, it was observed that, using the imprint technique, even after antisepsis, under the conditions of this experiment, there was an exacerbated fungal growth, in an ascending and overlapping manner, making it impossible to identify the fungi, regardless of the antisepsis of the claws with 70% alcohol.

It is also noteworthy that even with their claws colonized by different species of fungi, the evaluated cats did not show any symptoms, signaling the importance of these as possible focus of exposure for tutors, that may promote in the human population the development of opportunistic systemic mycoses, which are of clinical importance especially in immunocompromised patients. In view of these results, it would be relevant for the cat medical clinic to disseminate educational information regarding the importance of personal hygiene for the guardians of these animals, since many dermatological and even systemic problems in humans, transmitted by domestic cats, can be minimized, impacting, even, in the reduction in the number of abandonment of their pets.

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