Joint doctoral research thesis held by the University of Turin (UNITO) (Italy) and the Universidade Paulista (UNIP) (Brasil)

Contribution to the knowledge of the pathogenesis and epidemiology of infections by *Microsporum canis*

CHAPTER 1

Phagocytosis of *Microsporum canis* by murine raw-264.7 macrophages - an *in vitro* study

CHAPTER 2

Molecular epidemiology of *Microsporum canis* isolates from diseased animals and environment

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BACKGROUND

Taxonomy and biological features

Microsporum canis belongs to the dermatophyte fungi, a group of closely related organisms that can invade the stratum corneum of the epidermis and keratinized tissues derived from it, such as skin, nail, and hair of humans and animals (Weitzman and Summerbell, 1995). They produce an infection named dermatophytosis, commonly referred to as ringworm or *tinea*. Dermatophytes are divided into anthropophilic, zoophilic and geophilic species based on their primary habitat associations. Anthropophilic species are primarily associated with humans and rarely infect animals. Zoophilic dermatophytes usually infect animals or are associated with animals but occasionally infect humans. Geophilic dermatophytes are primarily associated with keratinous materials spread in the environment from living animals. They have, with few exceptions, little or no pathogen value (Chermette et al., 2008).

M. canis is the most common dermatophyte in cats and dogs, with cats considered to be the most important reservoir hosts. This organism is also found regularly in horses and rabbits (Pasquetti et al., 2013)(Cafarchia et al., 2012)(Chermette et al., 2008)(Sharma et al., 2007) and it has been occasionally reported in several other domestic and wild animals (e.g. cattle, sheep, goats, ferrets, camelids, marmots, eastern cottontails, foxes) (Pignon and Mayer, 2011)(Chermette et al., 2008)(Gallo et al., 2005a, 2005b)(Chermette et al., 2008). *M. canis* is distributed worldwide and plays an important zoonotic role. In some countries, it tends to surpass anthropophilic dermatophytes as a cause of human ringworm episodes (Chermette et al., 2008). It is a widespread agent of *tinea capitis* in Europe, the eastern Mediterranean, and South America. It can also cause inflammatory lesions on glabrous skin (*tinea corporis* and *tinea faciei*) (Ginter-Hanselmayer et al., 2007; Skerlev and Miklić, 2010; Weitzman and Summerbell, 1995) (fig. 1).

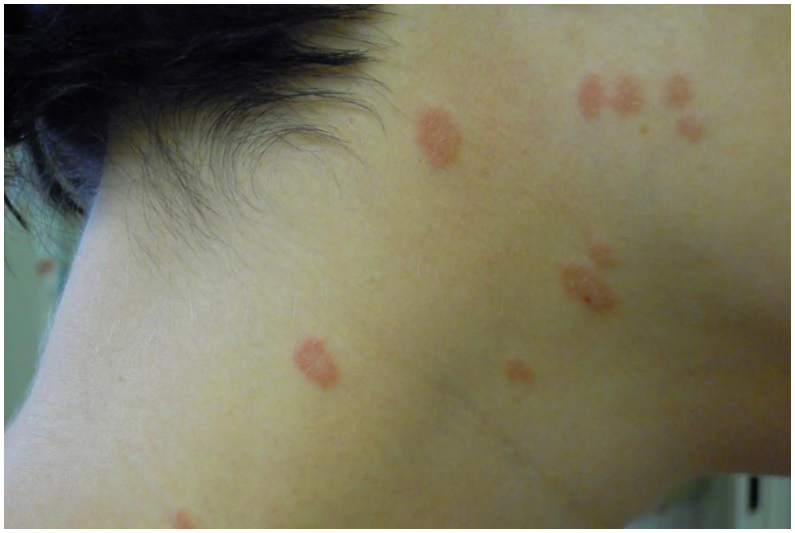

Fig. 1. *Tinea faciei* due to *M. canis* in a cat owner

M. canis is known to reproduce mainly asexually through a mitotic process, although it is also capable of mating with tester strains producing the sexual state. The fungus in this state was classically named *Arthroderma otae*. Following the indications for the new nomenclature of dermatophytes (de Hoog et al., 2017) and the so-called "one fungus-one name" concept (Taylor, 2011), this double nomenclature should not be used any more. One should now refer to this fungus only as *M. canis*.

It is widely that sexual reproduction occurs under laboratory conditions, while it is infrequent in nature (Hironaga et al., 1980). The propagules originating from asexual reproduction (conidia) differ according to the context. During invasion of hair and stratum corneum, hyphae are fragmented to produce masses of small (2-3 µm size) arthroconidia in a mosaic pattern on the surface of the hair. (Miller WH, Griffin CE, Campbell KL, 2013)(DeBoer DJ, Moriello KA, 2006) They represent the infective portions of the fungus. In culture plates, fungal reproduction yields macro- and microconidia. They are formed starting from the terminal part of a hypha, that undergoes a gradual swelling, with the appearance of transversal partitions. At the end of the process, mature conidia are released from the hyphae. Macro-conidia of *M. canis* are spindle-shaped with thick echinulate walls. The echinulations (spines) are more pronounced at the terminal end, which often forms a knob. The macroconidia are composed of six or more cells. One-celled microconidia may be seen. Importantly, *M. canis*, like all dermatophytes, do not form macroconidia in tissues (DeBoer

DJ, Moriello KA, 2006; Miller WH, Griffin CE, Campbell KL, 2013). Generally, the colonies must grow for 7 to 10 days before macroconidia are produced. Conidia are sometimes not or poorly produced on Sabouraud dextrose agar, which is the medium most commonly employed for first isolation of the fungus from clinical samples (Miller WH, Griffin CE, Campbell KL, 2013). On Sabouraud dextrose agar, *M. canis* becomes apparent after 2-3 days of incubation at 25-30°C in the form of tiny, evanescent radiating colonies. With age they become cottony to woolly to more powdery, they have a central depressed area and may show radial folds. The pigment, particularly appreciable on the undersurface of the colony, is yellow-orange, becoming dull orange-brown. However, the intensity of pigmentation is variable, with colonies sometimes appearing pale yellow or white. Thus, considerable variability of colony morphology can be found from strain to strain. This variability is also reflected at the microscopic level since macroconidia occasionally do not present with the "classical" shape described above (Dhieb et al., 2014). According to several studies, the phenotypic variations could depend on the conditions of culture (medium composition, incubation time and temperature) (Brilhante et al., 2003; Faggi et al., 2001; Makimura et al., 1999). Some examples of the macro- and microscopical aspect of the fungus are presented in figures 2-4.

M. canis is closely related to two anthropophilic species, *Microsporum audouinii* and *Microsporum ferrugineum* (Sharma et al., 2007).

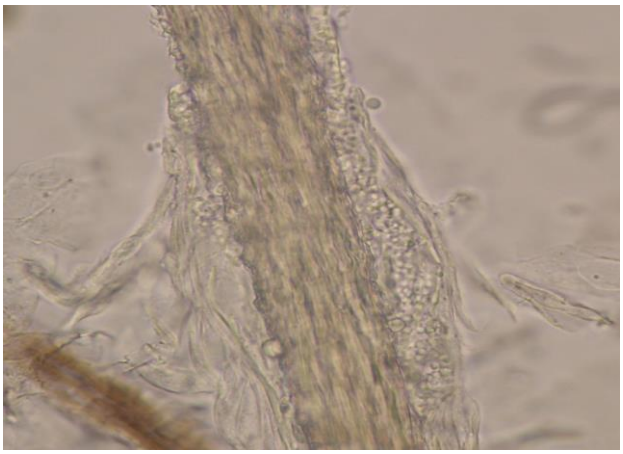

Figure 2. Hyphae and arthroconidia invading a hair shaft

Figure 3. *M. canis* colonies in a culture plate

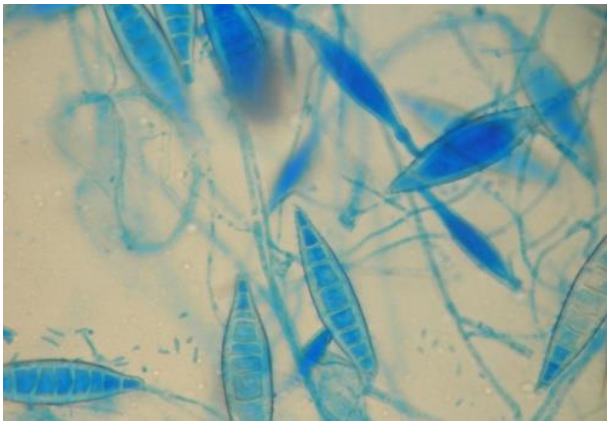

Figure 4. Macroconidia of *M. canis*

Transmission and risk factors

M.canis is transmitted by contact with infected hair and scales or fungal elements on animals, in the environment, or on fomites. All circumstances that favour contacts act thus as predisposing factors, which explains the higher occurrence of infection when numerous animals are kept in confined environments. This is particularly true as regards cats, with enzootic situations frequently occurring in shelters, catteries and multiple-cat households (Chermette et al., 2008; DeBoer DJ, Moriello KA, 2006; Miller WH, Griffin CE, Campbell KL, 2013). In these contexts, the infection is complicated to eradicate and creates a significant health hazard for people in contact with animals. Most recommendations for the control of infection in catteries are based on the concept of a total treatment program, which includes

both topical and systemic treatment of all the cats, strong environmental decontamination procedures, interruption of exchanges of animals, and isolation of new cats (European Scientific Counsel Companion Animal Parasites (ESCCAP), 2011). The difficulty of applying such a demanding approach usually accounts for lack of compliance with treatment. This ultimately leads to the persistence of the problem in time. The occurrence of infection is also high in wandering cats (Chermette et al., 2008). Dogs in kennels, dogs living outdoors, stray or hunting dogs may be at greater risk of acquiring different dermatophytes, including *M. canis* (Chermette et al., 2008; Miller WH, Griffin CE, Campbell KL, 2013). Also, the environment plays a key role as a source of infection and reinfection, since arthroconidia may persist for months or years, particularly when embedded in hair or skin scales, on floors, walls and objects associated with grooming, transportation, and housing of animals (cages, combs, brushes, clippers, blankets). (Chermette et al., 2008; Miller WH, Griffin CE, Campbell KL, 2013)(Peano A, Gallo MG, Molinar Min AR, Mancianti F, 2002). *M. canis* has been also isolated from air samples, which indicates a great potential for environmental dispersion (Peano A, Gallo MG, Molinar Min AR, Mancianti F, 2002). Even when a regular disinfection schedule is employed, the environment may become severely contaminated. Therefore, many cats in multi-cat households and catteries typically test positive on cultures repeated over time because they are constantly being contaminated from their environment (Chermette et al., 2008; Miller WH, Griffin CE, Campbell KL, 2013).

As regards human transmission, all activities and situations which involve contacts with dogs and, above all, cats, cause an increased risk of infection. Humans can become infected also by contact with contaminated dust, brushes, or clothing. The disease is frequently found in veterinarians, veterinary technicians, veterinary students, personnel of catteries and pet shops (Chermette et al., 2008; Miller WH, Griffin CE, Campbell KL, 2013). Besides, many cases occur in familiar contexts. Often the source of infection proves to be a cat (very often a kitten) recently adopted from the road or a shelter. The pathogen can also be conveyed by an animal already present in the household which has outdoor access and has thus the possibility of entering to contact with infected stray animals or contaminated environments. The infection may also be acquired participating in animal shows and competitions, in the course of veterinary visits, or at the groomer. It is noteworthy that infection is not uncommonly caused by animals that would be expected to be more controlled, namely

purebred cats (e.g. Persians, Maine Coons) purchased from breeders or pet shops. These episodes are facilitated by the fact that cats, especially the long-haired ones, present often with poor or null evidence of clinical signs (DeBoer DJ, Moriello KA, 2006; Miller WH, Griffin CE, Campbell KL, 2013). Also, an infected dog may be the source of infection, but, overall, dogs appear to be involved to a lesser extent (Sonck, 1970)(Lunder, 1992) (Weitzman and Summerbell, 1995). In dogs, asymptomatic infections occur more rarely than in cats (Lefebvre et al., 2006). A human-to-human transfer is rare but possible. This possibility is supported by the reports of epidemic episodes in situations without an animal presence (e.g. in schools and hospitals) (Weitzman and Summerbell, 1995). There have been also reports of human infections due to contacts with animals other than cats and dogs such as cheetahs, (Pasquetti et al., 2013); tigers (Sykes and Ramsay, 2007); farm rabbits (Cafarchia et al., 2012), and goats (Pal and Dave, 2005). In disagreement with most literature, a few authors appear to lessen the role of animals as a source of human infection. For example, (Patel et al., 2005) reported a relatively low rate of isolation in asymptomatic cats in England, and on this basis, they hypothesized that infection in humans arises rather from the environment or by contact between people. Likewise, (Dawson and Noddle, 1968) attributed importance to environmental contamination rather than to the actual presence of infected animals.

Notable epidemic episodes due to dermatophytes are considered to be rare in human medicine, especially compared with other pathogens, such as bacteria and viruses. However, there are in the literature articles describing outbreaks due to *M. canis*. For example, an important outbreak occurred in in the port city of Eliat (Israel), with a total of 78 cases diagnosed between 1975 and 1976. Cases came from different families living in the same neighbourhoods. The numerous stray cats and dogs living in the area were blamed as the source of the epidemic (Dvoretzky I, Semah D, Sommer B, 1978). In another case, which occurred between 1955 and 1956 in Belfast (UK), an intensive outbreak involved humans, dogs and cats from different households who had received infected Persian kittens from a breeder (LAWSON and McLEOD, 1957). The degree of exposure to the infection source appeared as a critical factor in determining the transmission of the fungus. Indeed, the high infectivity rate of exposed children (93%) compared with that of adults (30%) was attributed to the fact that the former had stricter and more prolonged contacts with the infected kittens.

Other interesting episodes reported in the literature are nosocomial epidemics, infections in schools, and an outbreak in a nursing home for elderly people (Drusin et al., 2000; Grills et al., 2007; Gürtler et al., 2005; Hillary and Suys, 2014; Kopel et al., 2012; Shah et al., 1988; Snider et al., 1993; Subelj et al., 2014; Yu et al., 2004). In all these contexts the infection spread without an animal intervention, which shows that human-to-human transfer of *M. canis*, although considered rare and self-limiting, can occasionally be very efficient. Different factors likely concurred to facilitate the inter-human passage, e.g. the repeated contacts between nurses and neonates and the immature immune system of these latter; the proximity of children in the course of school activities; the old age of hosts of the nursing home for older people; massive environmental contamination by fungal spores.

Other reports of outbreaks by *M. canis* regard "mini-epidemics" in familiar contexts caused by infected pets adopted from the road or purchased from a breeder or a shop (Alteras and Feuerman, 1979; Hermoso de Mendoza et al., 2010; LAWSON and McLEOD, 1957; Pasquetti et al., 2013; PREISER, 1991) . Similar episodes are likely to occur frequently worldwide, although they are hardly reported in the official literature, probably also because dermatophytosis, both in humans and animals, is not a notifiable disease in most countries.

In some episodes, the involvement of a single strain of the fungus was demonstrated by molecular typing methods (Pasquetti et al., 2013; Yu et al., 2004).

There have also been sporadic reports of outbreaks in uncommon hosts, such as laboratory mice (Di Fonzo et al. 1986) (most frequently affected by *T. mentagrophytes*) and pigs (Gonzalez Cabo et al. 1995).

Pathogenesis

Arthroconidia adhere strongly to keratin and germinate within 6 hours after contact with the skin (Vermout et al., 2008). Fungal hyphae invade the stratum corneum and the ostium of hair follicles and proliferate downward to the hair bulb. Mechanical disruption of the stratum corneum consequent to microtrauma of different origin, e.g. due to ectoparasites in case of cats and dogs (European Scientific Counsel Companion Animal Parasites (ESCCAP), 2011), appears to be important in facilitating penetration and invasion of hair follicles (Miller WH, Griffin CE, Campbell KL, 2013). Clipping the haircoat of infected cats sometimes leads to a worsening of clinical signs. This illustrates the importance of skin trauma in the spread of

infection. Hyphae penetrate the hair cuticle and grow within the hair shaft until the keratogenous zone is reached. At this point, the fungus establishes a sort of equilibrium between its downward growth and the production of keratin. Spontaneous resolution occurs when infected hairs enter the telogen phase or if an inflammatory reaction is incited. When a hair enters telogen, the fungal growth slows and stops due to the lack of newly formed keratinized hair. Infectious arthroconidia may remain on the hair, but reinfection of that particular hair follicle does not occur until it reenters anagen (Miller WH, Griffin CE, Campbell KL, 2013). In experimental models of *M. canis* infection the incubation period between inoculation and development of lesions was 7 to 14 days and 10 to 12 days for cats (DeBoer and Moriello, 1994)(Sparkes et al., 1996) and dogs (Khosla R, Rai P, 1989) respectively. Because the infection is almost always follicular in animals, the most consistent clinical sign is single or multifocal circular patches of alopecia with variable scaling, which may be self-limiting within a few weeks to a few months (DeBoer DJ, Moriello KA, 2006; Miller WH, Griffin CE, Campbell KL, 2013). In practice the degree of susceptibility, the clinical signs and the length of infection are highly variable and depend on several factors – many of which not yet fully elucidated – related to the fungus, the host and their interaction. *M. canis* has been shown to possess multiple enzymatic properties (keratinase, elastase and collagenase), and a role for these proteases has been proposed in the initiation and progression of infection (Papini and Mancianti, 1995)(Brouta et al., 2001)(Brouta et al., 2002)(Descamps et al., 2002). A keratinolytic subtilisin-like protease (Sub3) has been recently shown to be necessary for the adherence of *M. canis* arthroconidia to skin explants from humans, dogs, horses, rabbits, guinea pigs, mice and cats (Băguţ et al., 2012). Enzymes secreted, or present in the fungal cell wall have been shown to act also as antigens in the host immune response(DeBoer DJ, Moriello KA, 2006). The number and quantity of enzymes produced vary from strain to strain and may, in part, explain the variability in clinical presentations (Papini and Mancianti, 1995). In support of this, a correlation between keratinase activity found for some strains *in vitro* and severity of lesions in cats, dogs and a guinea-pig model has been observed (Viani et al., 2007)(Viani et al., 2001). However, keratinase production was not associated with any clinical picture in a previous study (Mignon et al., 1998). Other enzymes that were evaluated (lipase, elastase and DNase) play no role in the virulence of *M. canis* infection (Viani et al., 2001).

Regardless of the possibility of more virulent strains, many factors related to the host play a critical role in determining the type of clinical lesions produced and terminating the infection. The overall immunological status influence the development of infection, which is more easily established in very young, very old or immunocompromised animals and people (DeBoer DJ, Moriello KA, 2006). The increased susceptibility of young animals may also reflect differences in the biochemical properties of the skin and skin secretions (especially sebum), growth and replacement of hair, and the physiologic status of the host as related to age (Miller WH, Griffin CE, Campbell KL, 2013).

As regards the host immune response to *M. canis* infection, most information in companion animals comes from studies performed in cats. It is widely accepted that cellular immune response (CMI), rather than humoral immunity, is more critical for the resolution of dermatophytosis. The majority of human patients with dermatophytosis develop positive intradermal test reactions to dermatophyte antigens. Development of a delayed (48 to 72 hours)-type hypersensitivity (DTH) represents CMI and correlates with a positive lymphocyte blastogenesis test result and at least partial immunity to reinfection (Achterman and White, 2012). As a general rule, human patients who recover from acute dermatophyte infections develop DTH reactions and usually are relatively immune to further infection.

In contrast, some patients develop chronic, unrelenting infections that persist for months or years. These patients, frequently infected by anthropophilic dermatophytes, develop strong immediate hypersensitivity (IH) reactions but often fail to exhibit delayed reactions even after prolonged disease (Vermout et al., 2008). In cats infected by *M. canis*, also, the CMI response is probably associated with clinical recovery (DeBoer and Moriello, 1993). Indeed, DTH responses to *M. canis* antigens were shown to be stronger in cats recovered from infection than in unexposed cats and cats with an active infection (Moriello et al., 2003). It is quite clear that in the cat, the response to *M. canis* infection tends – more often than in dogs - to resemble that described in human patients with chronic infection by anthropophilic dermatophytes. This is illustrated by the high number of cats which, although being truly infected, develop minimal and persisting lesions, just due to a "tolerant" immune response. This is evidence of the steady adaptation of the fungus to the feline host.

As happens in humans, also in cats at least partial immunity to reinfection is present. Cats that had recovered from previous *M. canis* infections experienced either no clinical lesions

or lesions that were more inflammatory and transient than those in *M. canis*–naive cats (Sparkes et al., 1996). This partial immunity may be the reason for the relatively higher susceptibility to *M. canis* infection of younger subjects, which could be related to the development of o a stronger immunity in older animals due to the multiplicity of contacts with the fungus rather than to an intrinsic role of age.

Some studies have recently begun to consider the interaction between *M. canis* and the host at a molecular level. For example, disruption of *dnr1* gene, which has sequence similarity to nitrogen regulatory genes of other filamentous fungi, caused a reduction in the ability of the fungus to grow on medium containing keratin as the sole nitrogen source (Yamada et al., 2006).

Susceptibility to infection also depends on the general health status of the hosts. The concomitance of diseases such as hyperadrenocorticism, or the use of some treatments, mainly corticotherapy, may favour the development of fungal lesions through impairment of immunity. A recent study has found elevated levels of cortisol in the hair of infected cats compared with healthy subjects (Galuppi et al., 2013). In cats, the association between retroviral (FIV or FeLV) infection and dermatophytosis is still a matter of controversy. In one study (Mancianti et al., 1992), dermatophytosis was three times more common in FIV-infected cats; whereas in another (Sierra et al., 2000), no association between FIV or FeLV and dermatophyte infection was apparent. Also, the stress of pregnancy and lactation may increase susceptibility to fungal infection (DeBoer DJ, Moriello KA, 2006). Genetic influences probably play an essential role in the transmission and clinical course of the disease. For example, it is known that Persian cats are predisposed to *M. canis* infection (DeBoer DJ, Moriello KA, 2006; Miller WH, Griffin CE, Campbell KL, 2013; Sparkes et al., 1993) and to the development of the more aggressive forms (such as pseudomycetoma) of the disease (Bond et al., 2001)(Zimmerman et al., 2003). However, apart from a possible genetic breed predisposition, this may also reflect less efficient grooming of the haircoat. Coat length has been reported as being an important factor in the carriage of *M canis* spores (Sparkes et al., 1993). A genetic predisposition to develop a generalized form of *M. canis* infection seems to exist also in Yorkshire Terrier dogs (Sparkes et al., 1993). However, it has been shown that the cause may be, at least in some instances, an underlying disease (such as leishmaniosis, ehrlichiosis or diabetes mellitus) able to cause an immune dysregulation (Cerundolo, 2004).

Clinical signs

Clinical features are highly variable and depend on the degree of inflammation and hair shaft destruction. In cats, *M. canis* infection is not generally a localized disease, despite appearances to the contrary (DeBoer DJ, Moriello KA, 2006). Clinical lesions often appear as one or more irregular or annular areas of alopecia with or without scales, but spores or areas of inapparent infection, or both, will be present throughout the haircoat (DeBoer DJ, Moriello KA, 2006). Hairs in lesions often appear broken and frayed (Miller WH, Griffin CE, Campbell KL, 2013). More apparent cases tend to be seen in kittens, with the early lesions often found on the face, ears and paws. Depending on the overall health of the kitten, lesions may also be generalized. In addition to focal alopecia and scales, affected areas may develop a thin, greyish white crust or a thick, moist scab. They are sometimes pruritic. Extensive lesions are sometimes associated with chronic ringworm in a debilitated animal, or with inappropriate corticotherapy. In these cases, atypical large alopecic areas, erythema, pruritus, exudation and crusts can be observed (Chermette et al. 2008). Other presentations that have been reported in cats include miliary dermatitis and recurrent chin acne. (Miller WH, Griffin CE, Campbell KL, 2013). The infection sometimes presents with unusual clinical features. For example, three Devon Rex cats were presented with multiple erythematous papules, occasionally associated with crusting and hyperpigmentation (Colombo et al. 2012). In another case, lesions of *M. canis* infection in a hypotrichotic Rex cat were characterized by annular areas where hairs were darker and longer than usual (Reedy, 1995). *M. canis* is uncommonly a cause of recurrent otitis externa (DeBoer DJ, Moriello KA, 2006). Onychomycosis is rare and may present as an asymmetric (one digit, or multiple digits on one paw) paronychia or onychodystrophy (Miller WH, Griffin CE, Campbell KL, 2013). Many infected cats have few or no lesions. Long-haired adults, in particular, can be subclinical carriers or have only minimal signs, such as patchy areas of short stubble, alopecia, scales or erythematous plaques, visible only on close inspection. Sometimes lesions become evident after shaving of hair. Also, short-haired cats can be healthy carriers of the fungus. Isolation of *M. canis* from the haircoat in the absence of apparent lesions indicates either infection or fomite carriage from exposure to a contaminated environment. Distinguishing between these two possibilities is often impossible. Wood's lamp can help to detect minimal lesions invisible at naked eye. A mechanical carriage will only be revealed through fungal cultures.

The epidemiological consequences and control measures differ significantly according to these two different carrier states. The mechanical carriage is usually associated with a limited number of fungal colonies in culture, while infection leads to massive production of arthroconidia (Chermette et al., 2008).

Occasionally, as described in man, infection may appear as one or more firm, subcutaneous nodules with chronic evolution known as pseudomycetoma (PM). This condition has been reported mostly in Persian cats (Chermette et al. 2008). An exceptional intra-abdominal localization has been also reported (Black et al., 2001). On examination, single or multiple cutaneous nodules, firm and not painful at palpation, usually situated on the back and the neck are visible. Draining tracts are sometimes present. These cats may have more typical superficial lesions on other areas of the body, or they may be clinically healthy except for the nodules (Miller WH, Griffin CE, Campbell KL, 2013). A complete cure is frequently not achieved despite a systemic antifungal therapy of long duration. Incision of the nodule reveals an orange honey-like and finely granulous content that shows fungal hyaline vesiculous elements and short septate hyphae at microscopy. A pyogranulomatous inflammation characterises histological lesions, with giant cells, macrophages, neutrophils and lymphocytes surrounding the fungal elements, which are embedded in an eosinophilic amorphous material. *M. canis* is isolated in most cases, but colonies frequently have an atypical aspect on primary cultures and macroconidia are often lacking (Chermette et al. 2008). This, together with the conclusions of a study which described different strains of *M. canis* isolated from the granulomatous lesions and the surface infection of the same cat (Morganti et al., 1992), may indicate that PM is caused by specific strains of the fungus with particular virulence factors. Against this possibility, a recent study (Pasquetti et al., 2012) that characterized several episodes of PM by genetic methods (multilocus microsatellite typing), concluded that the same genotypes cause of PM cases could be found in the course of typical surface infections.

Dogs more often exhibit the classic annular areas of peripherally expanding alopecia, scale, crust, and follicular papules and pustules, with sometimes a central area of hyperpigmentation. However, other manifestations may occur. The dermatophytic kerion is a boggy, exudative, variably well-circumscribed, nodular type of furunculosis that may develop multiple draining tracts (this lesion is less frequent in cats). More generalized,

widespread lesions should prompt a search for a potential underlying systemic cause predisposing to the infection (e.g., cancer, hypercortisolism). Severe, disseminated forms have been reported in Yorkshire terriers (DeBoer DJ, Moriello KA, 2006; Miller WH, Griffin CE, Campbell KL, 2013). Asymptomatic infections are more rare than in cats. Figures 4-11 provide some examples of clinical presentations in cats and dogs.

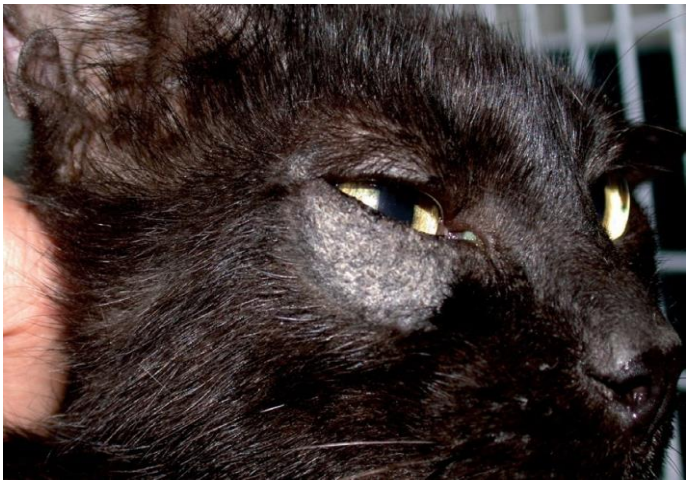

Figure 4. Alopecic lesion in a cat with infection by *M. canis*

Figure 5 – Infection by *M. canis* in a kitten

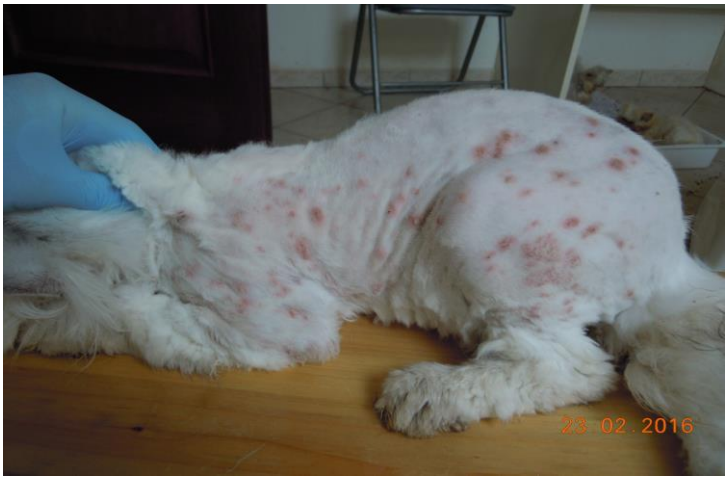

Figure 6. Lesions due to *M. canis* in a Persian cat. The lesions became visible after shaving of the hair.

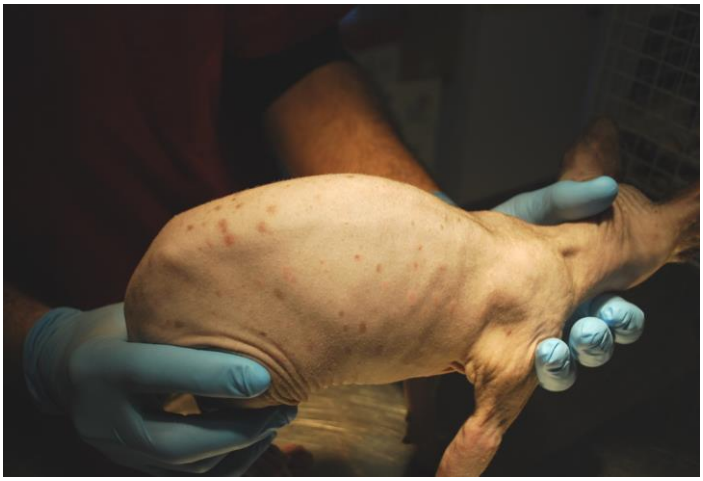

Figure 7. Pigmented lesions due to *M. canis* infection in a Sphynx cat.

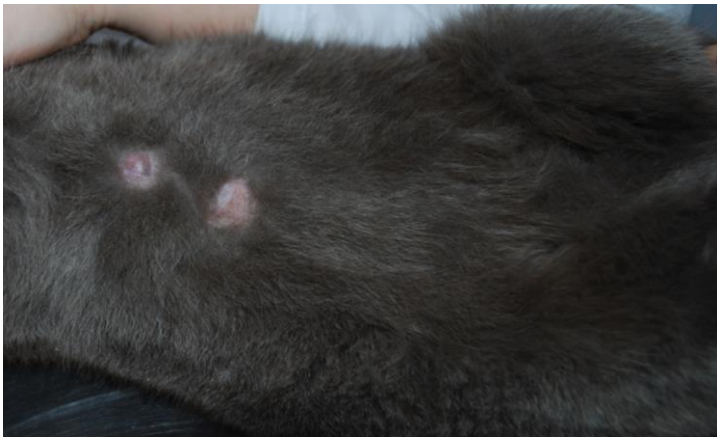

Figure 8. Subcutaneous nodular lesions due to *M. canis* (pseudomycetoma) in a cat

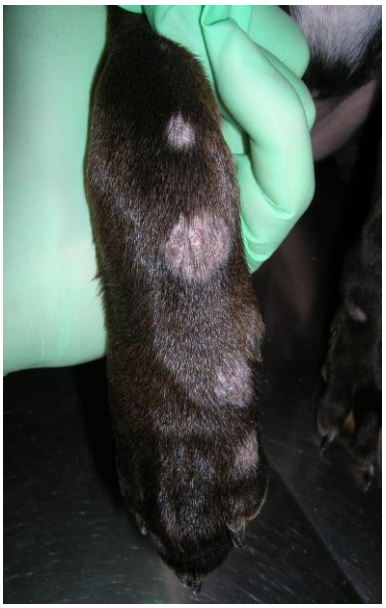

Figure 9. Circular alopecic lesions on the paw of a dog with *M. canis* infection

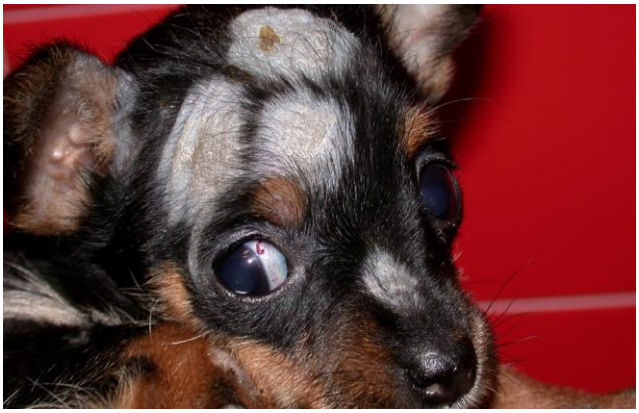

Figure 10. Circular alopecic lesions on the face of a dog with *M. canis* infection

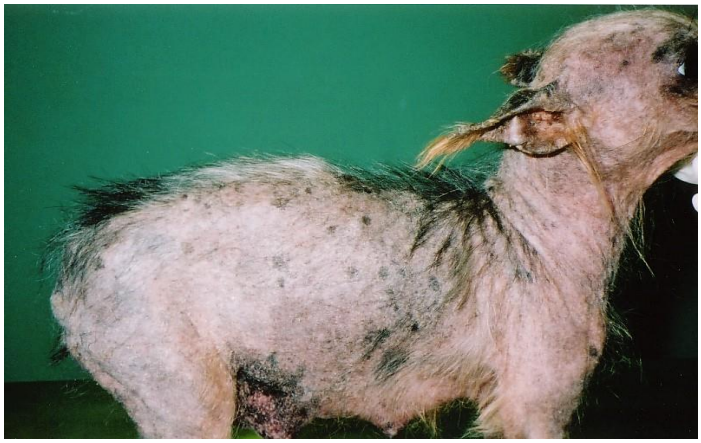

Figure 11. Extensive alopecia in a Yorkshire Terrier dog with infection by *M. canis*

The differential diagnosis for dermatophytosis is extensive, both in cats and dogs, because of the variable clinical appearance of the disease (Miller WH, Griffin CE, Campbell KL, 2013). Diagnosis of *M. canis* infection must thus rely on results of different mycological tests.

Examination with Wood's lamp allows – in many cases – visualizing infected hairs which appear with an "apple green" fluorescence (Moriello et al., 2017). Direct microscopic examination of hair and scales is a quick inexpensive method for detecting the arthroconidia of the fungus. However, even in experienced hands, this test may be diagnostic in a very variable proportion of cases (Moriello et al., 2017). Definitive diagnosis is made by culture of hair samples or using the so-called toothbrush technique in case of animals without evidence of dermatological lesions (DeBoer DJ, Moriello KA, 2006). The culture is generally performed on Sabouraud Dextrose medium supplemented with antibacterial agents and cycloheximide, which aids in preventing the growth of contaminating moulds. Several variants of this medium are commercially available. A formulation that includes a pH indicator and that should aid in tracking the growth of dermatophytes thanks to colour change from yellow to red (the so-called dermatophyte test medium, or DTM) is widely used in veterinary laboratories (Moriello, 2001).

PCR-based methods are now also used to detect the DNA of *M. canis*, as well as that of other pathogenic dermatophytes, directly in biological samples (hair, crusts, toothbrushes) (Moriello et al., 2017).

Distribution in the world: an overview

Available data show that *M. canis* is the most common cause of dermatophytosis in cats (>90% of cases) worldwide. It is generally also the most prevalent dermatophyte isolated from dogs, but with more considerable variation. In Brazil, during the 1998–2003 period, dermatophytosis was diagnosed in 27.8% of cats presented with dermatological problems at several veterinary clinics, with *M. canis* responsible for 100% of cases. The prevalence of infection was 9.8% in dogs, with *M. canis* isolated less frequently (68.5% of all cases of dermatophytosis) (Copetti, 2006). In the USA, a study concerning animals with cutaneous lesions over ten years reported the isolation of dermatophytes from 61 out of 408 cats (14.9%) and 70 out of 1824 dogs (3.8%). *M. canis* accounted for 43% and 92% of canine and feline cases, respectively (LEWIS et al., 1991). In Croatia, dermatophytes were isolated from 748 (40.7%) out of 1,838 cats with dermatological lesions, and *M. canis* represented 98.7% of the isolates (Pinter LJ, Jurak Z, Ukalovic MM, Susic V, 1999). In Italy, during fifteen years,

dermatophytosis was diagnosed in 1,890 (24.7%) out of 7,650 cats and 566 (18.7%) out of 3,028 dogs with skin disorders, with *M. canis* representing 97% and 83% of the isolates, respectively (Mancianti et al., 2002). Whatever the animal host, climatic conditions appear to play a significant role in the distribution of the pathogen, with prevalence higher in hot humid climates and lower in cold, dry climates. For example, *M. canis* was isolated from 4% of cats in various animal shelters in the southeastern United States (Florida), but in none of the shelter cats sampled in the northern United States (Wisconsin) (MORIELLO et al., 1994). In the centre and southern Italy, the fungus was isolated from 47% of asymptomatic stray cats (Romano et al., 1997) and respectively 15 % and 23% of dogs and cats with cutaneous lesions (Cafarchia et al., 2004). A recent survey in stray cats from Northern Italy reported a much lower prevalence (5%) (Proverbio et al., 2014). The influence of climatic factors is emphasized by the results of studies on the microflora of apparently healthy dogs. One study did not isolate *M. canis* in a group of 102 dogs visiting hospitalized people in Ontario (Canada). (Lefebvre et al., 2006), which reinforces the common opinion that the condition of unapparent carrier is more typical of cats. However, two other studies performed in warmer regions, such as Southern Italy and Greece, reported instead of the prevalence of healthy *M. canis*-carrier dogs around 15% (Bourdzi 1997) and 6% (Cafarchia et al., 2006). The prevalence was as high as 36.4% in case of dogs cohabiting with owners diagnosed with *M. canis* infection (Cafarchia et al., 2006). Seasonal differences in the incidence of infection have been found in some countries. For example, in New Zealand, the frequency of isolation of *M. canis* was significantly higher in the winter months ((Simpanya and Baxter, 1996). With similar climates, prevalence varies in relation to other factors, firstly the lifestyle of animals taken into consideration. In the UK, show cats were reported to have a carrier rate of 12.5 % among 241 cats sampled (Quaife and Wormar 1982), while in household cats the isolation rate was 2.2 % (Sparks et al. 1994). In a report from Belgium in 1997, 2.1 % of pet cats were found to be asymptomatic carriers of *M. canis* (Mignon and Losson 1997). The same study found that clinically healthy cats in shelters had a higher prevalence of infection (16%) than those kept as pets. (Mignon and Losson, 1997). Moreover, in catteries, the isolation of *M. canis* depends significantly on whether the cattery has a history of dermatophytosis. In one study of catteries from temperate regions of the USA, cats had negative culture results, while in *M. canis*- affected catteries, virtually all cats had positive culture results (Moriello and Deboer, 1991). These findings were identical to those of another study from a warmer

region of the USA that also failed to isolate the fungus from the haircoat of cattery cats from facilities that were free of the disease (Thomas MLE, Scheidt VJ, Walker RL, 1989). Environmental factors play a role not only in terms of climatic conditions. For example, in the study mentioned above performed in Greece (Bourdzi HE, 1997), *M. canis* was isolated only from the hair coat of healthy dogs living in urban areas, whereas other dermatophytes, such as *M. gypseum*, *T. mentagrophytes*, and *T. terrestre*, were most commonly isolated from dogs living in rural areas.

In general, studies of the fungal flora of asymptomatic cats and dogs highlight a critical point. Although many animals have been found to act as healthy carrier of *M. canis*, this fungus should not be considered part of the *normal* fungal flora of cats (or dogs) (DeBoer DJ, Moriello KA, 2006). If it was, it would have been isolated routinely from healthy animals regardless of geographical region, lifestyle (indoor or outdoor), or status (pet or stray) (DeBoer DJ, Moriello KA, 2006).

Methods for strain typing and population studies

The success in strain typing of dermatophytes, including *M. canis*, according to phenotype criteria such as colony morphology, microscopy or biochemical reaction has been limited. Indeed, the same strain may present with different phenotypic characteristics depending on different factors, firstly the conditions of culture (Faggi et al., 2001)(Brilhante et al., 2003)(Makimura et al., 1999)(Dhieb et al., 2014). On the other hand, strains with similar colonies may belong to different genetic types. More recently, molecular-based approaches using different DNA markers have begun to reveal the intra-specific genetic diversity of *M. canis*. The degree of genetic variability appears profoundly different from study to study, depending on the resolving power of the methods employed. Sequencing of the ITS (internally transcribed spacer region of rRNA genes) region has been claimed as a tool for cluster analysis and estimation of source of infections by *M. canis,* on the ground that identical ITS1 sequences were found in two fungal strains sampled from a cat and its owner, while differences were noted in sequences of five unrelated strains. (Kaneko et al., 2011). In another study, different molecular markers – ITS sequencing, amplification of subrepeat element in the ribosomal DNA nontranscribed spacer (NTS), and random primer amplification polymorphic DNA (RAPD) method– were applied to strains sampled during an outbreak of *tinea capitis* in a school (Yu et al., 2004). The pattern of bands was identical for

all strains sampled from patients and the environment, which suggested that a single clone of the fungus was involved. Another method proposed for strain typing is the inter-single-sequence-repeat (ISSR)-PCR, which was applied to strains coming from some infected patients and their pets (Cano et al., 2005). Out of a total of 24 strains analyzed, 21 different genotypes were found, which may indicate a good discriminatory power of the method employed. More recently, two studies in North-Tunisia and Poland based respectively on a PCR-RFLP (*restriction fragment length polymorphism*) method targeting the ITS region (Dhieb et al., 2014) and on RAPD analysis using (GACA)4 and (ACA)5 primers (Dobrowolska et al., 2011) reported a single genotype shared by all the tested strains. Therefore the areas sampled were apparently dominated by a single clone, although it cannot be excluded that the reason for the lack of variability was instead a low discriminatory power of the markers employed. This latter possibility is supported by findings of other studies which reported a much higher degree of genetic variability in strains of *M. canis*, even from very close locations (in some cases the same city), using microsatellite (MS) markers (da Costa et al., 2013; Pasquetti et al., 2012; Sharma et al., 2007). MS are tandem-repeating DNA sequences comprised of 1-6 bp per repeating unit, that are polymorphic in populations due to their propensity for insertion/deletion mutation of multiples of the repeating unit during replication (Ellegren, 2000). Multiple loci are generally used, so that a multilocus genotype is obtained (multi-locus microsatellite typing – MLMT)(Anderson et al., 1999). Two MS were originally developed by Sharma et al. (2007) (Sharma et al., 2007), while more recently an extended panel of 8 MS has been standardized (Pasquetti et al., 2013). This panel proved to have a very high intra-specific discriminating power, and may thus be a useful tool to track the source and pathway of infections by *M. canis* in humans and animals (Pasquetti et al., 2013).

CHAPTER 1

Phagocytosis of *Microsporum canis* by murine raw-264.7 macrophages - an *in vitro* study

1.1 Introduction

Traditionally dermatophytes were classified into three genera, *Microsporum*, *Trichophyton,* and *Epidermophyton* (CHERMETTE *et al*., 2008; de HOOG *et al*., 2005). However, in 2017, phylogenetic studies using multilocus sequences revealed significant changes in the taxonomy of these fungi. According to the new proposal, these fungi are now classified into seven genera, namely *Trichophyton*, *Epidermophyton*, *Nannizzia*, *Paraphyton*, *Lophophyton*, *Microsporum*, and *Arthroderma* (de HOOG *et al.*, 2017).

Although different dermatophytes can cause infections in humans and animals, some species recognize as reservoirs certain animal species (CHERMETTE *et al*., 2008; VERMOUT *et al*., 2008). Studies in dogs and cats demonstrated the predominance of *M. canis*, followed by *M. gypseum* and *T. mentagrophytes*. (BERALDO *et al*., 2011; COPETTI *et al*., 2006; SILVA *et al*., 2011). *Trichophyton equinum* is more common in horses, *T. verrucosum* in cattle, *M. nanum* in swine and *T. mentagrophytes* in rodents (CHERMETTE *et al*., 2008; COELHO *et al*., 2008a; PEREIRA *et al*., 2006 VIGUIE-VALLANET; PAUGAM, 2009).

Dermatophytes are fungi with keratinolytic properties producing keratinases able to degrade keratin-rich structures such as skin, hair, nails, hooves, horns, and beaks (CHERMETTE *et al*., 2008; de HOOG *et al*., 2005; PIN, 2017; VERMOUT *et al*., 2008).

Dermatophytes can be zoophilic, anthropophilic and geophilic (de HOOG *et al*., 2017; GRÄSER *et al.*, 2018). Human dermatophytosis by zoophilic species is acquired by direct contact with animals or with contaminated fomites (CHERMETTE *et al*., 2008). The presence of diseased hosts or asymptomatic carriers within the home environment increases the chances of other animals and humans to acquire the infection (MANCIANTI *et al*., 2003; NEVES *et al.*, 2018).

Dermatophytosis can affect otherwise healthy humans and animals. However, young and elderly, or humans and animals that have an altered immune system (as observed in

immunosuppressive diseases and also with patients using corticosteroids and undergoing chemotherapeutic treatment) have a higher risk of acquiring the infection and developing more severe and enduring clinical forms (CHERMETTE *et al*., 2008; de HOOG *et al.*, 2005; PERES *et al*., 2010; SILVA, *et al*., 2014; VERMOUT *et al*., 2008).

The diagnosis of dermatophytosis is based on clinical signs, direct examination of hair and skin scales, fluorescence emission under Wood's lamp, culture, histopathology and the identification of species performed by phenotypic and molecular procedures (BOND, 2010; BRILHANTE *et al*., 2006; CHERMETTE *et al*., 2008; GRÄSER *et al.*, 2018; PIHET; Le GOVIC, 2017).

The phenotypic characteristics are not always sufficient to identify the species. Molecular and genetic techniques have been used as tools for diagnosis and taxonomy and polymorphic differences between the species have been verified by these techniques (GRÄSER, *et al*., 2007; PASQUETTI *et al*., 2013; SHEHATA *et al*., 2008; VERRIER; MONOD, 2017; ZHAN; LIU, 2017). The molecular methods minimize false-negative results and accelerate the diagnosis that can be carried out after isolation or can be performed directly in clinical samples, such as nails, hair and skin scales (GRÄSER *et al.*, 2018; HAYETTE; SACHELI, 2015; VERRIER; MONOD, 2017).

The treatment is often performed through the oral route, topical or using a combination of both, mainly with antifungal azoles, griseofulvin, and terbinafine (BOND, 2010; CHERMETTE et al., 2008; COELHO et al., 2008b; PERES et al., 2010; SHARMA et al., 2015; VIGUIE-VALLANET; PAUGAM, 2009).

Dermatophytosis occurs as a result of the interaction between the fungus and host defensive mechanisms, leading to resolution of the infection or its progress (BLANCO; GARCIA, 2008; MARTINEZ-ROSSI *et al.*, 2017). The initial contact between arthroconidia and the stratum corneum is a crucial step that allows dermatophytes to colonize the host skin surfaces, multiply and cause lesions (BRASCH, 2010). In general, the skin acts as a barrier with its outer keratin layer and the phagocytes prevent the occurrence of infections (GINHOUX; JUNG, 2014; MALISSEN *et al*., 2014; YOSHIKAWA; ALMEIDA, 2017).

Dermatophytes induce innate and acquired immune responses in the host (ALMEIDA, 2008; YOSHIKAWA; ALMEIDA, 2017). Innate immunity participates by giving phagocytic cells their

ability to ingest and inhibit fungal growth (BLANCO; GARCIA, 2008; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). Fungal antigens are captured by resident skin macrophages (Langerhans cells), carried to the regional lymph nodes, where they are presented to CD4+ T lymphocytes via class II MHC molecule (BLANCO; GARCIA, 2008; BRASCH, 2010; BROWN, 2011; CRIADO *et al.*, 2011; YOSHIKAWA; ALMEIDA, 2017). These lymphocytes differentiate into Th1, Th2, and Th17 and actively participate in host responses to dermatophytes (BURNSTEIN *et al*., 2018; HEINEN *et al.*, 2017).

The interaction between host cells and the pathogen promotes phagocytes to produce a series of chemotactic and cytokine factors, which act as signal for cell recruitment to the site of infection and stimulate the processes of phagocytosis and death (BLANCO; GARCIA, 2008; BRASCH, 2010; BROWN, 2011; CRIADO *et al.*, 2011).

The chemotactic factors are activated by molecular structures shared by a large number of pathogens, called PAMPs (pathogen-associated molecular patterns) (BRASCH, 2010; BROWN, 2011). PAMPs are recognized by proteins called PRRs (pattern recognition receptors), present in different cells of the body, mainly monocytes, macrophages, dendritic cells, endothelial cells, T and B lymphocytes (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). PRRs include Toll-like receptors (TLRs), which confer recognition of PAMPs (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). These receptors act as signals for the production and release of pro-inflammatory factors and the expression of costimulatory molecules to promote activation of adaptive immunity. Simultaneous activation of multiple PRRs by a fungal pathogen provides the immune system with a wide variety of possibilities for an effective and specific immune response (BRASCH, 2010; BROWN, 2011; HEINEN *et al.*, 2017; YOSHIKAWA; ALMEIDA, 2017). Phagocytosis is perceived as an initiator and effector. This is because on the one hand it encounters and digests the pathogen, while on the other, it helps in antigen presentation, stimulates cytokine production, and initiates the adaptive response (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). Cytokine release favours the recruitment of CD4 + and CD8 + macrophages and lymphocytes, in particular CD4 + T helper cells, increasing their antimicrobial action (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017).

The type of immune response is a determining factor in resistance or susceptibility to infection. In general, Th1-type cellular immunity is required for the control of

dermatophytosis, while Th2 usually increases the susceptibility to infection. Th1 cells predominantly produce cytokines such as IFN-ɣ and promote cellular immunity and activation of macrophages. In contrast, Th2 produces cytokines such as IL-3 and IL-4 (BROWN, 2011; CRIADO *et al*., 2011; SOUSA *et al*., 2015; YOSHIKAWA; ALMEIDA, 2017). In recent years, it has been found that the Th17 subtype-mediated adaptive response can control and resolve infections caused by dermatophytes (BURNSTEIN *et al*., 2018; STADHOUDERS *et al.*, 2018; YOSHIKAWA; ALMEIDA, 2017).

Cell-mediated immunity is considered to be an essential factor in controlling dermatophytosis. It is associated with the development of inflammation and a delayed hypersensitivity reaction to restrict infection (FADLELMULA; MACKENZIE, 2002; SOUSA *et al*., 2015; YOSHIKAWA; ALMEIDA, 2017). Antibodies appear ineffective in protecting against these infections (YOSHIKAWA; ALMEIDA, 2017). However, humoral immunity is considered necessary because the activation of the complement system increases the recruitment and the capacity of destruction by phagocytic cells (YOSHIKAWA; ALMEIDA, 2017). Dermatophytes secrete substances that increase chemotaxis and consequently, cell recruitment and inflammation (CRIADO *et al.*, 2011; TRAYNOR; HUFFNAGLE, 2001; YOSHIKAWA; ALMEIDA, 2017).

Cytokines are proteins produced and released by the phagocytic cells during the phagocytosis, which induce monocyte differentiation into macrophages, promote phagocytosis and antigen presentation, and help in the acquired immune response (YOSHIKAWA; ALMEIDA, 2017). Against dermatophytes, pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12, IL-17, TNF-α, and IFN-γ) and anti-inflammatory cytokines (IL-4 and IL-10) are produced (BRASCH, 2010; BROWN, 2011; CAMPOS *et al*., 2006; CRIADO *et al*., 2011; YOSHIKAWA; ALMEIDA, 2017). Patients with disseminated chronic dermatophytosis caused by *T. rubrum* have reduced levels of pro-inflammatory cytokines and increased IL-10 (anti-inflammatory) which, together, affect the production of an effective cellular immune response (SOUSA *et al*., 2015). Also, cytokines play a central role in the type of response that will be produced by lymphocytes, Th1, Th2 or Th17 (HEINEN *et al*., 2017).

The importance of oxidative products in killing microorganisms is well known. In particular, reactions between hydrogen peroxide, myeloperoxidase, and a halogen are considered central to antimicrobial defence mechanisms (BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017).

Phagocytosis promotes metabolic changes that include increased reactive oxygen intermediates through the production of hydrogen peroxide, superoxide, free oxygen, and hydroxyl radicals (BROWN, 2011). These products are highly toxic to microorganisms in a process called “respiratory burst” (BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). The production of nitric oxide (NO) promotes a toxic reaction against various fungi (FADLELMULA; MACKENZIE, 2002; BROWN, 2011). Neutrophils and macrophages were found to release lower amounts of NO and peroxidase in a group of individuals with disseminated chronic dermatophytosis (REGEV-SHOSHANI *et al*., 2013; SOUSA *et al*., 2015).

Although dermatophytosis is the most common fungal infection worldwide, the effector mechanisms of the immune response that restrict fungal growth in the epidermis are not fully understood. However, the recognition that phagocytosis is a central event in dermatophytosis enhances the interest in exploring these processes in more detail. There are published reports on the process of phagocytosis of *T. rubrum* (CAMPOS *et al.*, 2006; SOUSA *et al*., 2015; YOSHIKAWA *et al*., 2017) and *T. mentagrophytes* (GREGUREK-NOVAC, 2004; RICHARDSON; BELL, 1995), that are common agents in human dermatophytosis. However, there is a lack of research regarding the phagocytosis of *M. canis*, the main species involved in dermatophytosis in dogs and cats.

This study was aimed at contributing to the knowledge on the response of macrophages against *M. canis*, by the standardization of a technique for obtaining *M. canis* microconidia and evaluating the phagocytic, microbicidal and immunomodulatory capacity of macrophages, with and without LPS stimulation, challenged with *M. canis*.

1.2 MATERIAL AND METHODS

1.2.1 Preparation of the microconidia suspension

The standard strain of *Microsporum canis* (ATCC-36299 - Manassas, USA) was grown and kept in a potato agar (Microbiology™ Merck, Darmstadt, Germany) Petri dish at 25 °C for ten days. After this period, five loads of fungal culture were deposited in a sterile grail containing 3 mL of RPMI-1640 (Gibco, New York, USA), supplemented with 10% fetal bovine serum (Gibco, New York, USA) and 20 µg/mL gentamicin (Hipolabor, Belo Horizonte, Brasil) (R-10-medium). The fungus was macerated with the pistil. The content was aspirated and expelled

initially with the P1000 pipette and then with a 1 mL syringe several times; finally it was filtered in cell strainer (40 µm), separating hyphae and macroconidia to obtain individualized microconidia. The individualized microconidia were used for counting, and their viability was checked with Trypan blue (10 µL suspension + 10 µL dye). The viable strains with the viability index greater than 95% were taken for the study. Of the fungal suspension, 10 µL was placed in a Neubauer's chamber and counted at 400x magnification under a bright-field microscope to adjust the ratios to be employed in the study.

1.2.2 Murine macrophages RAW-264.7

Murine macrophage RAW 264.7 cell line, stored at –80 °C were thawed seven days before infection experiments. The cells were cultured in R-10 medium and incubated at 37 °C with 5% CO_2 until the day of the experiment.

1.2.3 Standardization of phagocytosis assay

Pilot test was performed initially with M. canis cultures at different ratios: 1:4, 1:2, 1:1,

1:0.25, and 1:0.125 (macrophages: conidia) and readings were taken at 30 min, 1 h, 2 h, 3 h,

4 h, 6 h, 8 h, and 24 h. The preparation for suspension of microconidia, cell cultures and challenge of macrophages with M. canis for the pilot tests are the same described in items 1.2.1, 1.2.2 and 1.2.4, respectively.

1.2.4 Macrophages challenged with *M. canis*

Macrophages were plated at a concentration of 3×10^5 cells on pre-sterilized 13 mm circular glass coverslips (Knittel, Braunschweig, Germany) in 300 µL of R-10 in 24 well plates at 37 °C with 5% CO_2 until they reached 95% confluence.

The supernatant was then discarded, and the wells were washed with PBS to remove unbound cells. Subsequently, *M. canis* microconidia were added to the wells in 300 µL of R-10 medium at a concentration of 1:0.25 (macrophages: conidia). After 60 min of incubation, the supernatant was collected for further testing, and the adhered cells were washed twice with PBS for removing non-phagocytized conidia. The adhered cells received 1000 µL of R-10 medium and were incubated under the described conditions. The readings were performed at 30 min, one h, three h, and six h. Each test was performed in triplicate and mono-cultures of macrophages were used as negative controls. After the incubation intervals, the coverslips

were removed, washed with PBS, fixed and stained with Giemsa dye for microscopic readings and phagocytosis evaluation. To determine the phagocyte index, 200 macrophages were counted in several microscopic fields in each of the coverslips at 1000x magnification and calculating the percentage of macrophages that internalized at least one microconidium (CAMPOS *et al.*, 2006). Supernatants were collected and stored at –20 °C and –80 °C for further detection of nitric oxide and cytokine levels, respectively. After reaching 95% confluence, the macrophages of four other plates were washed with PBS to remove non-adherent cells and 1 µg/mL LPS (Sigma-Aldrich®, St. Louis, USA) was added into it. The plates were incubated for 24 h in 5% CO_2 at 37 °C for macrophage stimulation. The following procedures were the same as those already described for the evaluation of phagocytosis at different reading intervals at a ratio of 1:0.25 (macrophages: conidia).

1.2.5 Cell Viability Assay

For cytometry assays, 1 million cells were used. Cell viability was determined by the Cell Viability kit (BD™, San Jose, EUA). For this purpose, 12 well plates with 1×10^6 macrophages in each well were incubated with R-10 medium overnight in 5% CO_2 at 37 °C. The wells were washed with PBS to remove non-adherent cells, adding 1:0.25 (macrophages: conidia) to the wells in R-10 medium, and incubating the plates for 1 h at 37 °C for phagocytosis to occur. After incubation, the wells were rewashed with PBS to remove non-phagocytized conidia. The plates were incubated with R-10 medium and at each of the different reading intervals (30 min, 1 h, 3 h, and 6 h) cells were detached with a cell scraper, resuspended in PBS, and labelled by the kit. A similar procedure was performed with LPS stimulated macrophages.

1.2.6 Microbicidal Activity

To determine the microbicidal activity, the cell suspensions (50 µL) from the cell viability test were seeded on Sabouraud dextrose agar (Difco™BD, Sparks, EUA) plates. The plates were incubated at 25 °C for two weeks. The growth and phenotypic characteristics of *M. canis* were observed.

1.2.7 Characterization of macrophages

Macrophages were cultivated as described in items 3.2 and 3.4, then 500 µL of cold PBS was added per well, incubated on ice for 15 min and then released with a cell scraper and placed in microcentrifuge tubes. Then the material was centrifuged for 10 min at 1,200 rpm, and

the supernatant was discarded. Of the anti-CD 16/32 antibody, 20 µL was added and incubated for 15 min on ice. After incubation, 100 µL of MACS buffer was added, centrifuged for 10 min at 1,200 rpm, and the supernatant was discarded. Subsequently, 20 µL of each CD80/86/MHC II in Fit (1:100), CD206 Alexa Fluor 647 (1:200), CD40 PECy5 (1:50), and F4/80 (1:100) PE antibodies (BD™, San Jose, EUA) were added. It was incubated for 30 min on ice, protected from light. Further, 200 µL of MACS buffer was added, centrifuged for 10 min at 1,200 rpm, and the supernatant was discarded. Finally, 150 µL of MACS buffer was added, and the reading was taken by flow cytometry on the BD AccuriTMC6 (BD Biosciences, Mountain View, USA).

1.2.8 Dosage of nitric oxide production

Nitric oxide production was quantified by the Griess method, considering the nitrite accumulation in the supernatant of the cultures. Since nitric oxide is a molecule that has a half-life of a few seconds, it was indirectly quantified by detecting its products, such as nitrates and nitrites (DUSSE *et al*., 2003). In this way, 100 µL of the supernatant was transferred to a 96-well plate in duplicates, with the same volume of GRIESS reagent (NEED 0.1% in 60% acetic acid and 1% sulfanylamide in 30% acetic acid). After 15 min at room temperature in the dark, absorbance was determined in a spectrophotometer with 540-nm wavelength filter (Thermo Plate, Belo Horizonte, Brazil). Nitrite concentrations were calculated from a standard curve of sodium nitrite ($NaNO_2$), 0.78-100 µM and the results were expressed in micromoles of nitrite for $3x10^5$ macrophages (ZHANG et al., 2011).

1.2.9 Determination of cytokine production

The assay was performed according to the CBA Mouse Th1/Th2/Th17 Cytokine Kit's (BD Biosciences, Mountain View, USA) instructions for the detection of pro-and anti-inflammatory cytokines: IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α. For this test, 20 µL of each sample (included a standard curve) was incubated for 2 h at room temperature in the dark with 20 µL of a mixture of capture beads and a secondary antibody conjugated to the fluorochrome PE. After washing, centrifuged and resuspended the samples in the same buffer, the data collection was performed using a two-colour BD AccuriC6TM flow cytometer (BD Biosciences, Mountain View, USA). The results were analyzed in Fcap Array software version 3.0 and presented graphically.

1.2.10 Transmission Electron Microscopy

For transmission electron microscopy (TEM), suspensions of 1×10^6 macrophages were infected with *M. canis* in a ratio of 1:0.25 (macrophage: conidia). The samples were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4° C for 10 h and post-fixed in 1% OsO_4 buffered at 1% for 2 h. Subsequently, the samples were dehydrated with different ethanol concentrations and embedded in Epon Resine Blocks. The blocks were cut in semi-thin samples, stained with toluidine blue, and photographed with light microscopy. Ultrathin cuts were stained twice with uranyl acetate and lead citrate and observed in TEM at LEO EM 906E, 80 kV (Zeiss, Oberkochen, Germany).

1.2.11 Statistical analysis

Statistical analysis was performed using a one-way ANOVA test with Tukey's post-test and T-test with GraphPad Prism Software (version 5). All values were reported as mean ± standard deviation, with a significance of 95% considering $p< 0.05$.

1.3. RESULTS

In the pilot experiment, macrophages showed an inability to phagocytize *M. canis* macroconidia (Fig. 1A). When we employed different microconidium concentrations at different time intervals, the better readings were obtained at 1:0.25 concentration of macrophages: microconidia at 30 min, 1 h, 3 h, and 6 h. At 8 h and 24 h, there was an increased amount of dead cells and filamentation, making it difficult to read them (Fig. 1B).

The percentage of non-stimulated LPS macrophages that phagocytized microconidia was 28.0%, 33.5%, 62.5%, and 71.5%, respectively at 30 min, 1 h, 3 h, and 6 h. In LPS stimulated macrophages, this percentage was 34.5%, 47.5%, 67.0%, and 74.0%, respectively at 30 min, 1 h, 3 h, and 6 h. (Fig. 2). The percentage of phagocytosis was increased over time, peaking at 6 h, in macrophages stimulated with or without LPS. The results were statistically significant at 3 h and 6 h compared to that found at 30 min and 1 h intervals.

RAW 264.7 macrophages were found to have a high phagocytic capacity, with more than 25% of them presenting phagocytized conidia with 30 min of contact, an index that exceeded 70% at 6 h (Fig. 2).

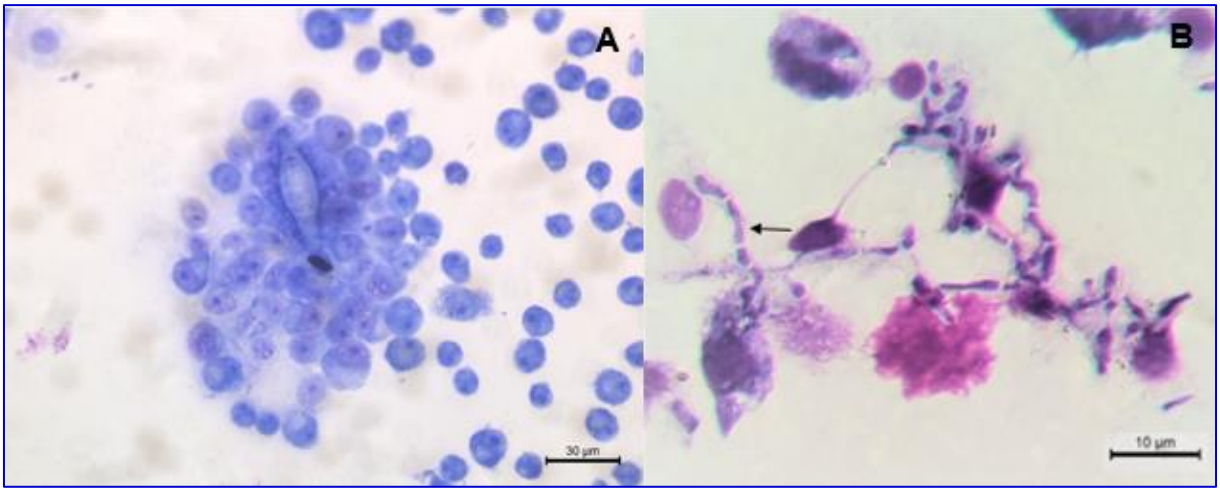

Figure 1 – *M. canis* phagocytosis by RAW-264.7 macrophages. A - large amount of macrophages involving *M. canis* macroconidium; B – *M. canis* phagocytosis at 1:1 ratio (macrophages: conidia) within 24 h, macrophages death and growth of hyphae with arthroconidia formation (arrow) (Giemsa 1,000x).

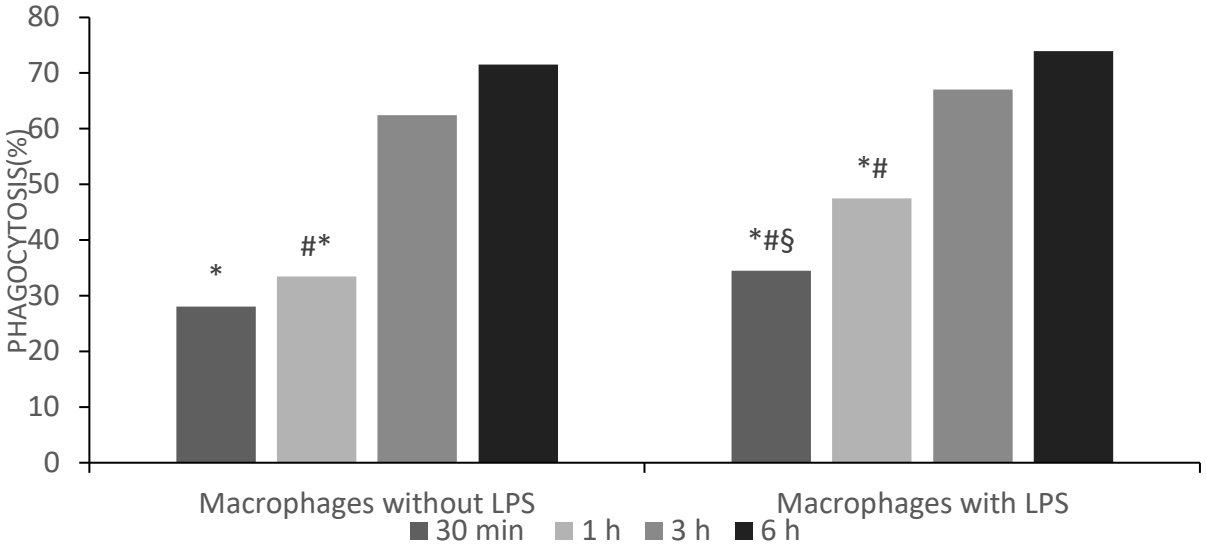

Figure 2 – Evaluation of phagocytic activity of RAW - 264.7 macrophages, stimulated with or without LPS, challenged with *M. canis* at different time intervals in the ratio 1:0.25 (macrophages: conidia). A one-way ANOVA test with Tukey's post-test revealed in not-LPS-stimulated macrophages * p <0.0001 at 30 min for 3 h and 6 h, and 1 h for 6 h; # p <0.001 at 1 h for 3 h. In the group with LPS stimulation * p <0.0001 at 30 min for 6 h, 1 h for 3 h; # p <0.001 at 30 min for 3 h, and 1 h for 6 h. § p <0.05 at 30n min for 1 h.

In both experiments, with and without stimulation by LPS, the presence of conidia phagocytized by macrophages and conidia emitting germ tube and multiplying in the interior were noted (Fig. 3 A e B). On transmission electron microscopy, macrophages were observed in the process of phagocytosis, with the cytoplasmic membrane surrounding the conidium (Fig. 4 A) and phagocytized conidium forming a germ tube and disrupting the macrophage cell wall (Fig. 4 B).

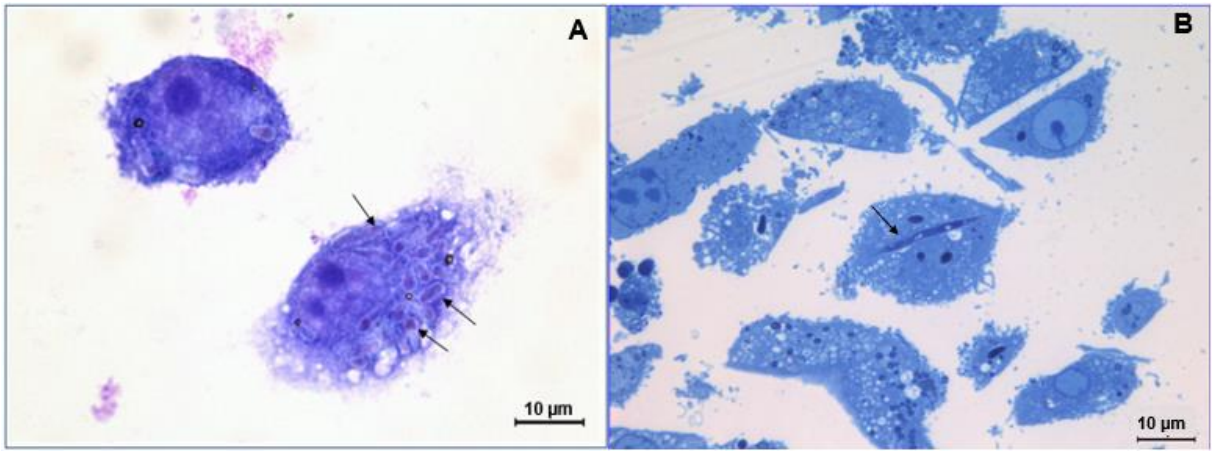

Figure 3 – *Microsporum canis* phagocytized by RAW-264.7 macrophages without LPS at 3 h. A - Presence of a large amount of phagocytized conidia (arrows) (Giemsa 1,000x). B - Hyphae inside macrophage (arrow) (toluidine blue 1,000x).

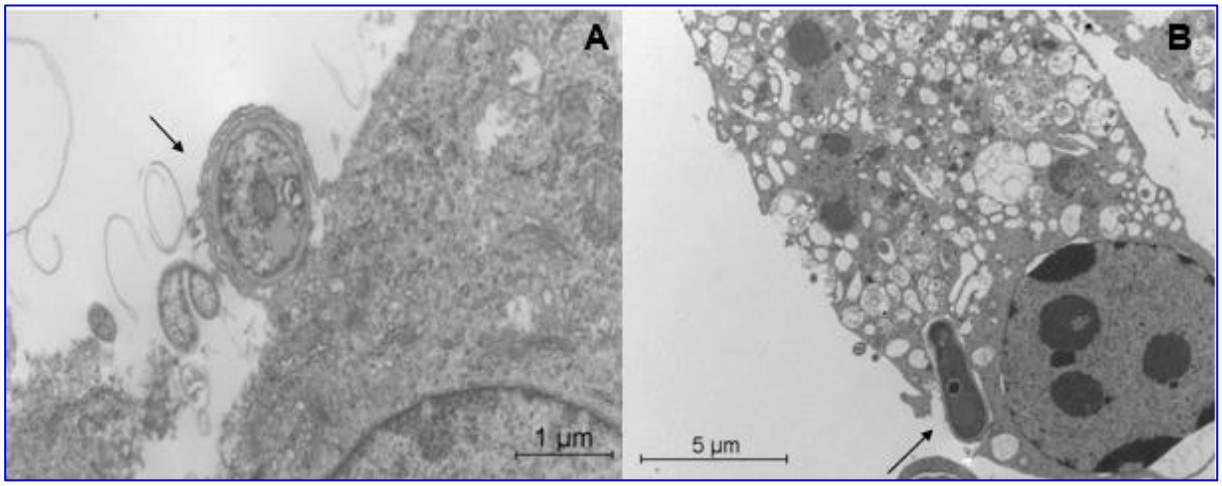

Figure 4 – Transmission electron microscopy. *M. canis* phagocytized by RAW-264.7 macrophages without LPS stimulation at 3 h. A - cytoplasmic membrane surrounding the conidium. B - phagocytized conidium forming a germ tube and disrupting the macrophage cell wall (arrow).

The cell viability declined over time and was maximum within 6 h in the unstimulated LPS challenged macrophages with *M. canis* ($p < 0.05$), with about 40% macrophages being killed (Fig. 5). The viability was higher in LPS-stimulated macrophages, even when challenged with *M. canis*, with about 25% macrophages being killed at 6 h (Fig. 6).

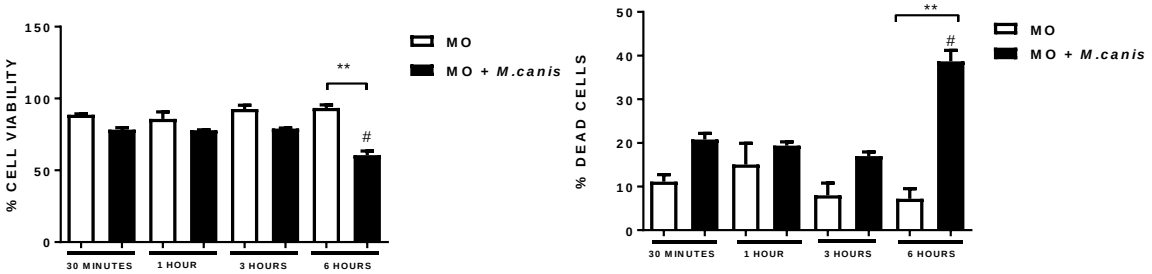

Figure 5 – Cell viability of not-LPS-stimulated RAW-264.7 macrophages (MO) and *M. canis* challenged macrophages (MO+*M. canis*) at 1:0.25 ratio (macrophages: conidia) at 30 min, 1 h, 3 h, and 6 h. A one-way ANOVA test with Tukey's post-test revealed p * <0.05, ** p <0.01.

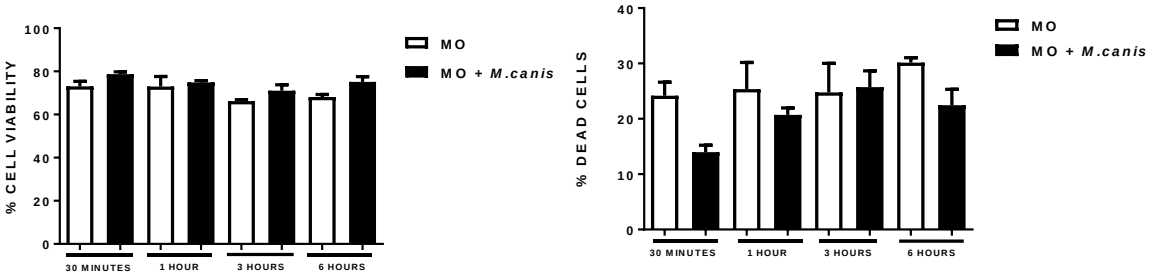

Figure 6 – Cell viability of LPS-stimulated RAW-264.7 macrophages (MO) and *M. canis* challenged macrophages (MO+*M. canis*) at 1:0.25 ratio (macrophages:conidia) at 30 min, 1 h, 3 h, and 6 h.

Microbicidal activity assay showed that even after phagocytosis, *M. canis* remained viable and showed growth when seeded on Sabouraud dextrose agar: white and cottony colony, delicate aerial mycelium and yellowish reverse (Fig.7), phenotypic characteristics observed on the agar plate. This was observed throughout the study period, regardless of macrophage treatments.

The characterization of macrophages in M1 and M2 profile showed that in the group stimulated with LPS at 6 h infected macrophages were less activated compared to the control group. It exhibited reduced CD 80/86 and CD 40 (M1) expression and enhanced CD 206 (M2) expression (Figs. 8, 9, 10, and 11).

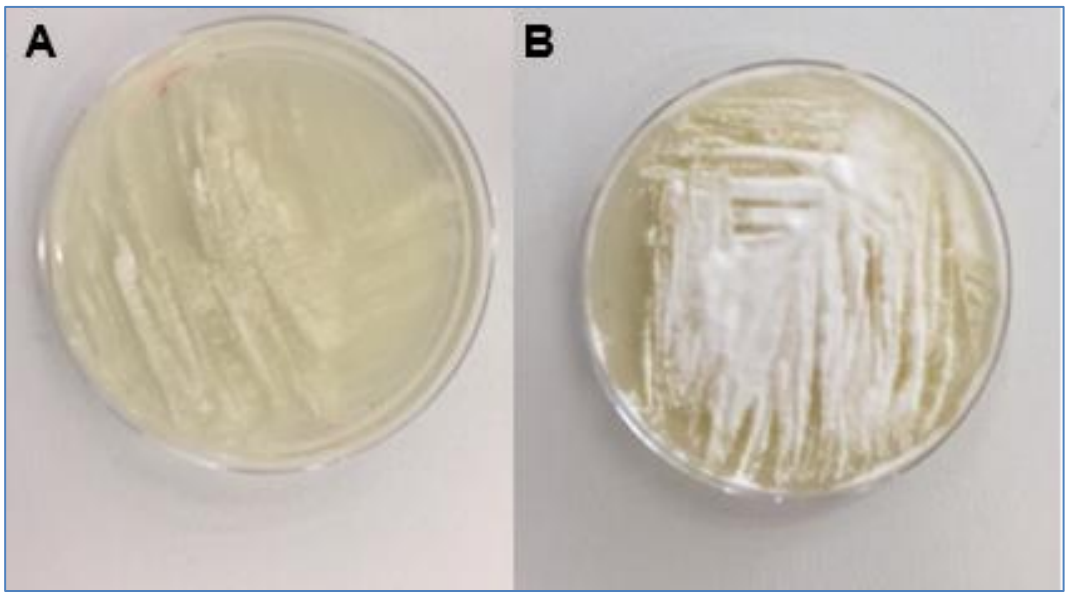

Figure 7 – Microbicidal assay on Sabouraud dextrose agar. A - Colonies of *M. canis* after 48 h growth at 25°C; B - Colonies of *M. canis* after 120 h growth at 25°C.

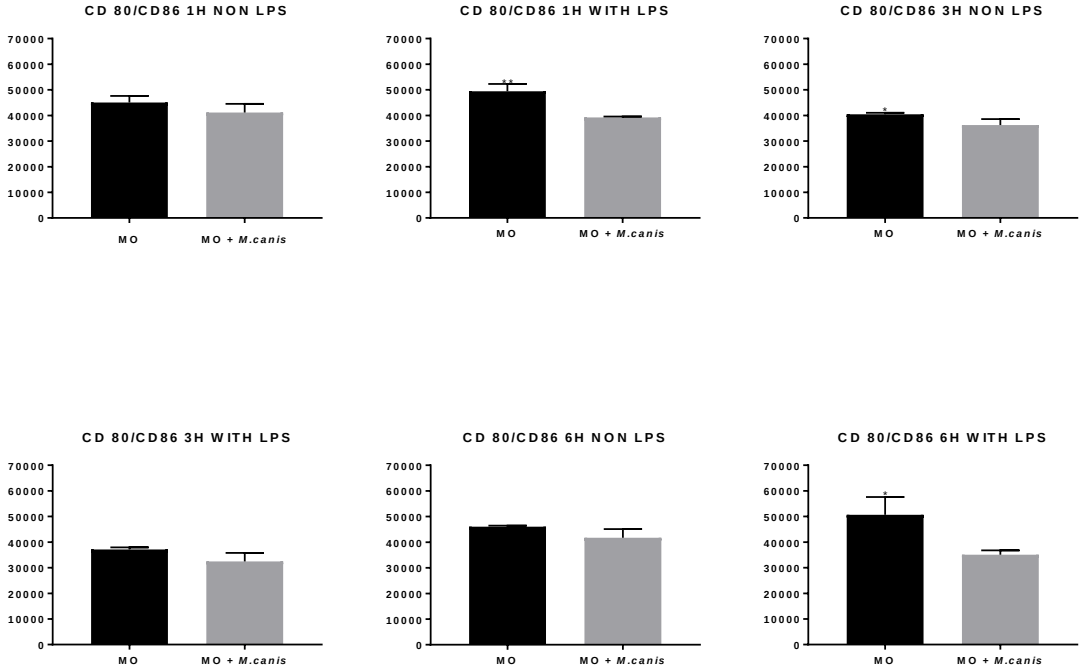

Figure 8 – Characterization of RAW - 264.7 macrophages, stimulated with or without LPS (MO) and *M. canis* challenged macrophages (MO + M. canis) at 1:0.25 ratio (macrophages: conidia), labelled with CD80/86 on time intervals 30 min, 1 h, 3 h, and 6 h.

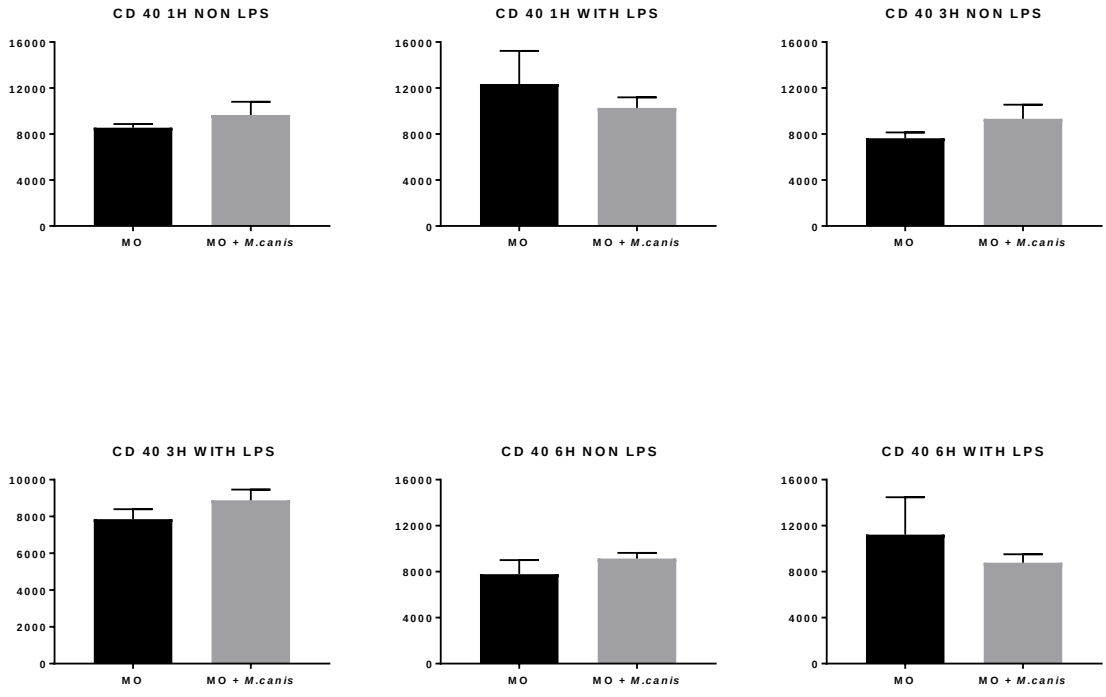

Figure 9 – Characterization of RAW - 264.7 macrophages, stimulated with or without LPS (MO) and *M. canis* challenged macrophages (MO + M. canis) at 1:0.25 ratio (macrophages: conidia), labelled with CD40 on time intervals 30 min, 1 h, 3 h, and 6 h.

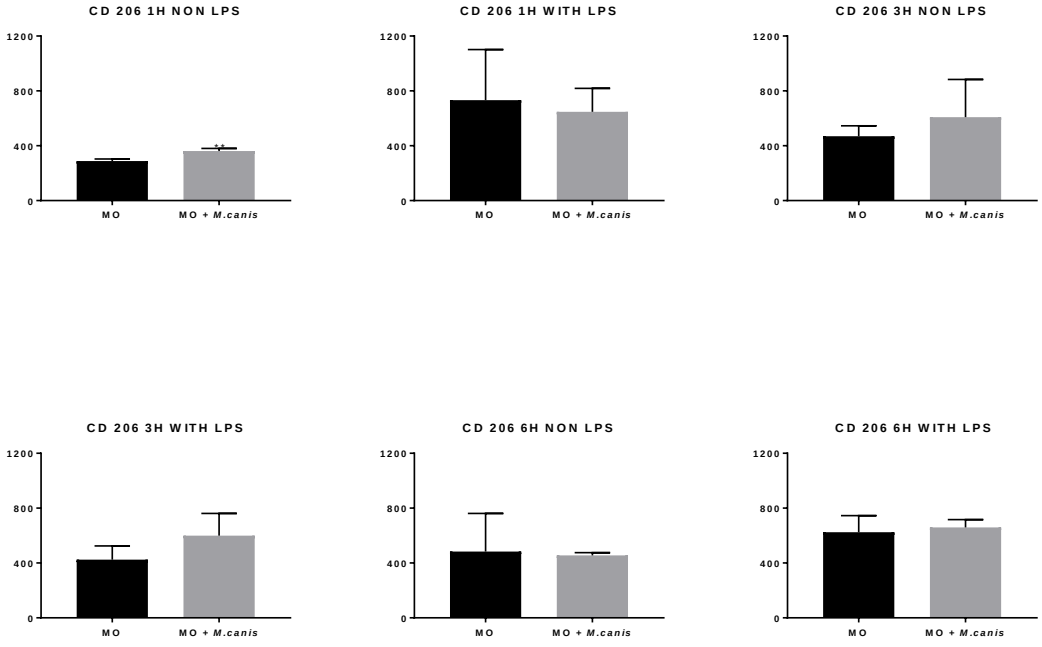

Figure 10 – Characterization of RAW - 264.7 macrophages, stimulated with or without LPS (MO) and *M. canis* challenged macrophages (MO + M. canis) at 1:0.25 ratio (macrophages: conidia), labelled with CD206 on time intervals 30 min, 1 h, 3 h, and 6 h.

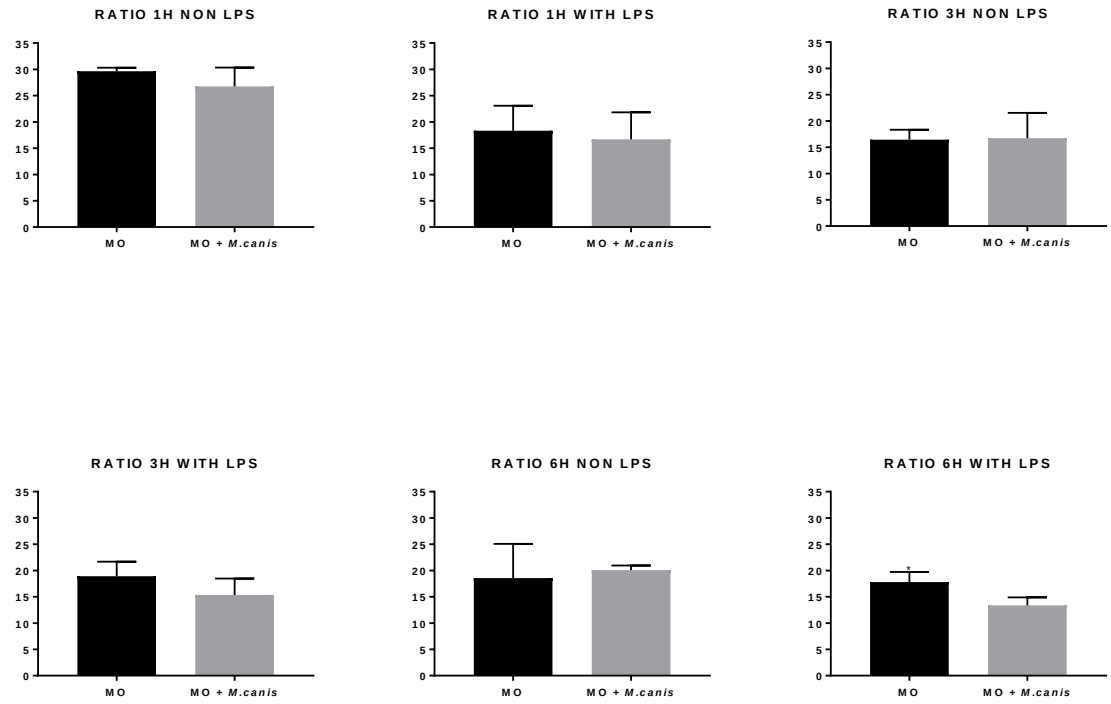

Figure 11 – Characterization of RAW - 264.7 macrophages, stimulated with or without LPS (MO) and *M. canis* challenged macrophages (MO + M. canis) at 1:0.25 ratio (macrophages: conidia), on-time intervals 30 min, 1 h, 3 h, and 6 h.

Although the production of NO by the macrophages challenged with *M. canis*, stimulated with or without LPS showed a statistically significant difference at 1 h interval, in general, NO production was very similar (Fig. 12).

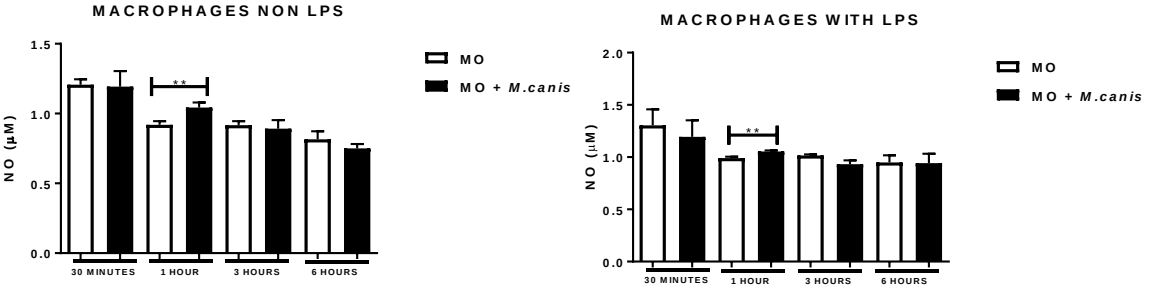

Figure 12 – Production of nitric oxide (NO) by RAW-264.7 macrophages (MO) stimulated with or without LPS, challenged with *M. canis* (MO + *M. canis*) at 1:0.25 ratio (macrophages: conidia), on-time intervals 30 min, 1 h, 3 h, and 6 h. T-test revealed p*<0.05, **p<0.01.

Regarding cytokine production, the concentration of TNF-α increased over time. It was found to be statistically significant in macrophages challenged with *M. canis*, both in LPS stimulated and unstimulated cells (Fig. 13). Macrophages infected with *M. canis* but without stimulation of LPS produced a higher concentration of TNF-α than those stimulated (Fig. 13).

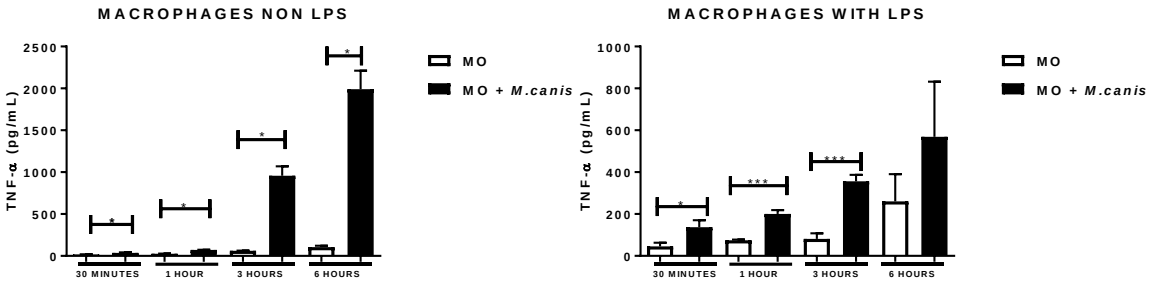

Figure 13 – Production of TNF-α by RAW-264.7 macrophages (MO) stimulated with or without LPS, challenged with *M. canis* (MO + *M. canis*) at 1:0.25 ratio (macrophages: conidia), on-time intervals 30 min, 1 h, 3 h, and 6 h. T-test revealed p*<0.05, **p<0.01, ***p<0.001.

It was also observed that the increase in IL-6 production in macrophages stimulated with LPS and challenged with *M. canis* showed a significant difference in the range of 1 h (Fig. 14). No differences were found in IL-6 production in macrophages without LPS stimulation challenged with *M. canis* (Fig. 14). No significant production of the other cytokines IL-2, IL-4, IL-10, IL-17A, and IFN-γ was detected.

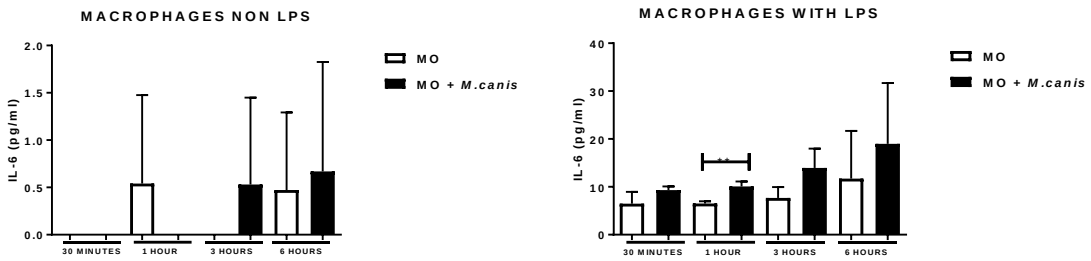

Figure 14 – Production of IL-6 by RAW-264.7 macrophages (MO) stimulated with or without LPS, challenged with *M. canis* (MO + *M. canis*) at 1:0.25 ratio (macrophages: conidia), on-time intervals 30 min, 1 h, 3 h, and 6 h. T-test revealed p*<0.05, **p<0.01.

1.4 DISCUSSION

The macrophages of the RAW 264.7 lineage efficiently phagocytized *M. canis* conidia, with percentages higher than 70% along with the presence of several conidia inside. Other published reports stated similar observation with macrophages from other lineages, neutrophils and dendritic cells against *T. rubrum* (CAMPOS *et al.*, 2006; SANTIAGO *et al*., 2014; SOUSA *et al*., 2015; YOSHIKAWA *et al*., 2017) and *T. mentagrophytes* (GREGUREK-NOVAK, 2004; RICHARDSON; BELL, 1995). In cases of generalized chronic dermatophytosis, it was found that the patient's phagocytes had lower phagocytic capacity than the phagocytes of healthy individuals. As a consequence, there was a decrease in the production of cytokine and reactive oxygen species (SOUZA *et al.*, 2015; WOODFOLK *et al*., 2015).

Phagocytosis is the first step in triggering the host immune response and phagocytes play a central role in resistance to fungal infections; deficiencies in these cells or their effector mechanisms result in susceptibility to infection (BRASCH, 2010; BROWN, 2011). The presence of dermatophyte at the skin causes overregulation of its pathogenic genes, its virulence factors are released, and resident macrophages are mobilized to phagocytize their spores (BRASCH, 2010; EPELMAN *et al.*, 2014; GINHOUX; JUNG, 2014; SZEPES *et al.*, 1993). Besides, dermatophytes have been found to release components that act as chemotactic factors, which increase cell recruitment during inflammation (BROWN, 2011; DAHL; CARPENTER, 1986; DAVIES; ZAINI, 1984; SOUZA, *et al.*, 2015; SUITE *et*. *al.*, 1987; YOSHIKAWA; ALMEIDA, 2017). All major carbohydrates in the fungal cell wall can be

recognized by the immune system (BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). After internalization, the phagosome matures leading to the formation of a phagolysosome, which exerts antimicrobial activity along with pathogen digestion and death. It is essential in the process the presentation of fungal antigens, this, in turn, induces the release of cytokines that promote adaptive immunity (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017).

Cytokines play an important role in fungal response by signalling and recruiting more phagocytic cells to the site of infection, maintaining epithelial integrity, and producing antimicrobial peptides (BURSNTEIN *et al*., 2018; DAVIES; ZAINI, 1984; SHIRAKI et al., 2006; TRAYNOR; HUFFNAGLE, 2001). The production of cytokines is essential and also occurs by keratinocytes, even before the adaptive response begins (SHIRAKI *et al*., 2006). In this study, TNF-α production was higher in *M. canis*-infected macrophages than controls during all time intervals in LPS-stimulated or non-stimulated macrophages. These findings reinforce the participation of this cytokine in an attempt to resolve the infection. The concentration of TNF-α was also increased in macrophages and neutrophils challenged with *T. rubrum* (CAMPOS *et al.*, 2006; SOUZA *et al*., 2015). This pro-inflammatory cytokine participates in fungal infections and has a role in cell recruitment and in determining the type of adaptive response that T cells will induce. Predominantly, Th1 is induced that is crucial in defending against intracellular microorganisms (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). However, it has been recently observed that superficial infection caused by dermatophytes also generates Th17 adaptive response. Th17 induces IL-17 that helps in combating fungal and bacterial infections (BURNSTEIN et al., 2018; STADHOUDERS *et al*., 2018). A more significant production of TNF-α by not-LPS-stimulated macrophages and challenged with *M. canis* was verified. This increase could be attributed to the higher percentage of unstimulated macrophages killed at 6 h than those stimulated with LPS, as can be seen in the viability test.

Another pro-inflammatory cytokine produced during the experiment, particularly by macrophages treated with LPS and challenged with *M. canis*, was IL-6. IL-6 may induce a Th17 response, which has been implicated in autoimmune and infectious diseases (BURNSTEIN *et al*., 2018; STADHOUDERS *et al.*, 2018). Similar to TNF-α, IL-6 plays a role in recruiting new phagocytic cells to the site of injury. IL-6 production was also found in a study comparing the response to *Arthroderma benhamiae* and *A. vanbreuseghemii* infections in

rats and by keratinocytes in infection caused by *A. benhamiae* (HEINEN, *et*. *al.*, 2017; SHIRAKI, *et*. *al*., 2006).

The production of reactive oxygen intermediates is one of the components of the antifungal mechanism of phagocytes. This mechanism involves the production of superoxide (O_2^-), which is converted to toxic oxygen intermediates such as hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). In this experiment, the production of NO by macrophages challenged with *M. canis* in 1 h was higher ($p < 0.05$) than uninfected macrophages, regardless of LPS stimulation. In the other intervals, the production was similar in macrophages infected or not with *M. canis*. In *T. rubrum*-infected macrophages, no NO production was detected, concluding that other mechanisms might participate in the fungal death (CAMPOS *et al.*, 2006). Although reactive oxygen species play an essential role in the killing of microorganisms, it is also known that the fungal death may be due to other processes, besides the oxidative damage (HERNÁNDEZ-CHAVES et al., 2017).

In this research, the reading intervals were not extended, because after 6 h most of the macrophages were already dead. Concerning dermatophytes, the binding between PAMP and PRR activates transcriptional and non-transcriptional responses, followed by cytokine production, phagocytosis, and death (BRASCH, 2010; BROWN, 2011; YOSHIKAWA; ALMEIDA, 2017). In the experiment described here, although phagocytized, *M. canis* remained viable in the RAW-264.7 macrophages, even inducing its death. This inefficiency in the elimination of the fungus was also observed by other researchers with *T. rubrum*, where it continued its multiplication within macrophages (CAMPOS *et al.*, 2006; SOUZA *et*. *al*., 2015; YOSHIKAWA *et al*., 2017).

Fungal pathogens have a variety of strategies that allow them to survive after phagocytosis. In recent years, several mechanisms of fungal survival in host tissues have been described (HERNÁNDEZ-CHAVES et al., 2017), such as inhibition of phagosome maturation, resistance to environmental degradation in phagolysosome, ability to replicate within phagosome (SMITH et al., 2015; TÖTH et al, 2014), and production of antioxidant enzymes such as catalase and superoxide dismutase (BROWN, 2011; HERNÁNDEZ-CHAVES et al., 2017). In this work, the multiplication of *M. canis* conidia inside macrophages, with the formation of germ tubes and small hyphal fragments, was observed. This is also reported in the infection of

macrophages with *T. rubrum* (CAMPOS, *et al*., 2006). It may be possible that the macrophages were killed by pyroptosis, which is an alternative mode of macrophage killing related to inflammasome activation. It releases pro-inflammatory cytokines in the presence of hyphae. Hyphal morphogenesis is essential for phagocyte membrane lysis in pyroptosis (WELLINGTON et al., 2014).

The characterization of macrophages infected with *M. canis* showed a tendency to anti-inflammatory M2 profile, which favours fungal growth; this tendency has already been observed by researchers in both *Candida albicans* (ZHENG, et al., 2013) and cryptococcal pneumonia (DAVIS, et al., 2013).

Macrophage stimulation with LPS did not influence their phagocytic capacity, but instead increased survival when challenged with *M. canis* and increased IL-6 response. Researchers also suggest that the viability of phagocytic cells when stimulated with PMA and concanavalin increases in dermatophyte infections (CARDERON; HAY, 1987).

On a timeline, 1 h after infection, there was a rapid response with the production of NO by macrophages challenged with *M. canis*, which was not sufficient to overcome infection and the subsequent upward production of pro-inflammatory cytokines during the 6 h observation.

There is a need to understand better the mechanisms involved in the relationship of *M. canis* with host defence cells. The knowledge of the processes by which fungi are recognized or evade from macrophages will contribute to the design of new strategies in the treatment of fungal infections.

1.5 CONCLUSIONS

Although phagocytized, *M. canis* remained viable inside the RAW 264.7 macrophages, inducing cell death.

The low production of NO suggests that this may not be the primary microbicidal process regarding *M. canis* infection. The presence of TNF-α and IL-6, pro-inflammatory cytokines, indicates that their participation is essential in the attempt to resolve the infectious process.

CHAPTER 2

Molecular epidemiology of *Microsporum canis* isolates from diseased animals and environment

2.1 INTRODUCTION

Methods able to characterize a pathogen at a strain level are expected to provide useful information for examining the origins of phenetic diversity within a species, to track the introduction, maintenance and disappearance of strains within a community, to gain insight into the dynamics of disease transmission and to track features of interest (virulence, drug resistance) within populations of the pathogen (Abdel-Rahman, 2008).

The molecular characterization of *M. canis* has been the subject of different studies in recent years. Some of the strategies employed (e.g. sequencing of internally transcribed spacer and non-transcribed spacer regions of rRNA genes, intergenic spacers of nuclear DNA, and mitochondrial DNA genes) were shown to possess a low discriminatory power within the species, being thus useful only for species identification and for a basic understanding of phylogenetic relationships towards other dermatophytes. Other markers, such as microsatellites (MS), have been instead proven to reveal a certain degree of genetic variation within the fungus (Sharma et al., 2007). MS are tandem-repeating DNA sequences comprised of 1-6 bp per repeating unit, that are polymorphic in populations due to their propensity for insertion/deletion mutation of multiples of the repeating unit during replication (Ellegren, 2000). Multiple loci are generally used, so that a multilocus genotype is obtained (multi-locus microsatellite typing – MLMT) (Anderson et al., 1999). In other pathogenic fungi, including other dermatophyte species, such as *Trichophyton rubrum* and *Microsporum persicolor*, the MLMT has proved to be a useful tool for uncovering intraspecific diversity and giving information on population structure and dynamics of transmission (Gong et al., 2016; Graser et al., 2007; Sharma et al., 2008).

As regards *M. canis*, two MS markers were originally developed by Sharma et al. (2007), while more recently an extended panel of 8 MS has been standardized (Pasquetti et al., 2013). This panel has a very high intra-specific discriminating power and represents thus a

useful tool to track the source and pathway of infections by *M. canis* in humans and animals (Pasquetti et al., 2013). Using this panel, in the last years, the team of the Italian supervisor of the present study has characterized hundreds of strains of *M. canis* from different hosts (animals and humans) and geographical origins. Strains analyzed came predominantly from Europe and, to a lesser extent, from Egypt, Turkey, Egypt and Korea.

The present study was aimed at applying the above panel of 8 MS to strains of *M. canis* sampled in animals and environmental sites in Brazil. Another objective was that of preparing a database of MS-Types on a worldwide scale. An easily accessible database including metadata (such as geographic and host provenance) regarding the fungal isolates tested may represent a tool to track the diffusion of different genotypes of *M. canis*, which in turn may provide information useful to clarify the dynamics of transmission of this fungal pathogen. Such a study is expected to augment the knowledge of the biological characteristics of this fungal species by addressing questions that are still unsolved, e.g. whether specific genotypes of the fungus colonize preferentially determined areas/animal groups; whether there are lineages of the fungus more diffuse due to a higher degree of virulence; whether all animal derived strains have the same potential to infect humans.

Moreover, the continuous enrichment of the database with results for strains coming from various geographic locations (Brazil in this case) represents a pivotal step to make the database a more and more powerful reference in the study of the dynamics of transmission of *M. canis*. Indeed, it must be pointed out that studies that report the same strain among all isolates from a suspected outbreak, occurring in a geographic region for which no baseline data on the degree of variation in the population exists, remain uninterpretable.

2.2 MATERIALS AND METHODS

2.2.1 Fungal strains

Strains from Brasil were obtained in the course of a previous study on pets with dermatophytosis (Neves et al. 2018). Fungal strains were collected in different locations, mainly in the city of Sao Paulo and its surroundings. Overall, 71 strains (8 cats, 13 dogs and 47 environmental samples) from 13 distinct infection episodes were included in the genetic

analyses (table 1). Fungal isolates were cultured on Mycobios Selective agar (Biolife Italiana S.r.l., Milan, Italy) and identified following morphological keys.

Figure 1. Sampling area in Brasil

episode	Samples	City
1	Atman 4, cat, animal	São Paulo
1	Atman 4, cat, bed	São Paulo
1	Atman 4, cat, toy	São Paulo
2	Belinha 5, dog, animal	Osasco
2	Belinha 5, dog, pillow	Osasco
2	Belinha 5, dog, carpet	Osasco
3	Charlisie 6, dog, animal	Osasco
3	Charlisie 6, dog, toy	Osasco
3	Charlisie 6, dog, floor balcony	Osasco
3	Charlisie 6, dog, sheet	Osasco
3	Charlisie 6, dog, floor room	Osasco
3	Charlisie 6, dog, blanket	Osasco
3	Charlisie 6, dog, bed	Osasco

4	Bam bam, dog, animal	Osasco
4	Bam bam, dog, floor living room	Osasco
5	Marie 34, cat, animal	Osasco
5	Marie 34, cat, animal, sofa	Osasco
5	Marie 34, cat, floor living room	Osasco
5	Marie 34, cat, carpet bathroom	Osasco
5	Marie 34, cat, carpet balcony	Osasco
5	Marie 34, cat, floor around feeder	Osasco
5	Marie 34, cat, table	Osasco
5	Marie 34, cat, bathroom sink bench	Osasco
5	Marie 34, cat, sink bathroom	Osasco
6	Marie 37, dog, animal	Osasco
6	Marie 37, dog, floor room	Osasco
6	Marie 37, dog, sheet	Osasco
7	Simba 38, cat, animal	Osasco
7	Simba 38, cat, bathroom floor	Osasco
7	Simba 38, cat, lepe (animal contact)	Osasco
7	Simba 38, cat, jade (animal contact)	Osasco
7	Simba 38, cat, kiara (animal contact)	Osasco
7	Simba 38, cat, house animal	Osasco
7	Simba 38, cat, cloth animal	Osasco
7	Simba 38, cat, apron proprietary	Osasco
8	Luna 52, dog, animal	Carapicuíba
8	Luna 52, dog, caos (animal contact)	Carapicuíba
8	Luna 52, dog, gaia (animal contact)	Carapicuíba
8	Luna 52, dog, eros (animal contact)	Carapicuíba
8	Luna 52, dog, baco (animal contact)	Carapicuíba
8	Luna 52, dog, floor living room	Carapicuíba
8	Luna 52, dog, enclousure cubs	Carapicuíba
8	Luna 52, dog, brush animal	Carapicuíba
8	Luna 52, dog, hall floor	Carapicuíba
8	Luna 52, dog, sofa	Carapicuíba
8	Luna 52, dog, cardboard box	Carapicuíba
8	Luna 52, dog, blanket animal	Carapicuíba
9	Pacho 53, dog, animal	Osasco
9	Pacho 53, dog, air	Osasco
9	Pacho 53, dog, sofa	Osasco
9	Pacho 53, dog, bag proprietary	Osasco
9	Pacho 53, dog, feeder	Osasco
9	Pacho 53, dog, blanket animal	Osasco
9	Pacho 53, dog, bed animal	Osasco
9	Pacho 53, dog, slippers	Osasco

9	Pacho 53, dog, living room floor	Osasco
10	Jack, dog, animal	Osasco
10	Jack, dog, cardboard box	Osasco
10	Jack, dog, sofa	Osasco
10	Jack, dog, bed	Osasco
11	Tody, dog, animal	São Paulo
11	Tody, dog, bed	São Paulo
11	Tody, dog, toy	São Paulo
11	Tody, dog, sofa	São Paulo
11	Tody, dog, pillow	São Paulo
11	Tody, dog, air	São Paulo
12	Mel, cat, animal	São Paulo
12	Mel, cat, cristal (contact)	São Paulo
12	Mel, cat, bed mother	São Paulo
12	Mel, cat, bed son	São Paulo
13	Apollo, dog, animal	Osasco

Table 1. List of samples obtained in Brasil

Some strains of *M. audouinii* and *M. ferrugineum*, and of the two main other dermatophyte species found in dogs and cats, namely *Trichophyton mentagrophytes* and *Nannizia gypsea* (formerly *Microsporum gypseum)* (Chermette et al., 2008), were also included in the analyses.

The fungal DNA was extracted using a commercially available kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany).

2.2.2 PCR and Microsatellite Fragment Analysis

The MLMT procedure employed and its validation have been described in depth previously (Pasquetti et al. 2013). Briefly, PCR primers were designed against sequences flanking the MS detected by a BLAST (Basic Local Alignment Search Tool) search using the nucleotide sequence information assembled by the *Microsporum canis* CBS 113480 genome project (http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative/MultiHome.html). Primer sequences for the 8 MS markers recognized as the most polymorphic (Pasquetti et al., 2013) were custom synthesized (Applied Biosystems UK) with a fluorescent label attached to the 5' end of each forward primer. Different dyes (FAM: MS4 and 6; VIC:

MS1 and 7; NED: MS2 and 5; PET: MS3 and 8) were employed to allow loading of the PCR products onto the genetic analyzer in two panels, each including four of the MS markers. This avoided confusion due to possible overlapping of allele ranges. Each PCR mixture contained 200 µM of each dNTP, 0.5 U Hot-Start *Taq* DNA polymerase (Qiagen), and 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl and 1.5 mM $MgCl_2$ (3 for MS1, 3, 4, 5, 6 and 4.5 for MS8), 20 ng template DNA, and 5 pmol of each primer in a final volume of 25 µl. All amplification reactions were performed in a GeneAmp PCR system 2400 thermal cycler (Applied Biosystems , Foster City, CA). After an initial denaturation step of 15 min at 95°C, samples were processed through 35 cycles consisting of 30 s at 95°C, 30 s at the specific temperature for each primer, and 1 min at 72°C, followed by a terminal elongation step of 6 min at 72°C. Microsatellite fragment analysis was performed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, FosterCity, CA) for capillary electrophoresis. Allele calling and analysis were performed using the "microsatellite detector" option and default analysis settings of the GeneMapper version 3.7 software (GeneMapper software version 3.7 User Guide or http://www.appliedbiosystems.com). The software was also used for panel design and allele binning. For each strain we obtained results consisting of 8 peaks, each corresponding to a MS marker. Each peak was assigned a size by aligning it to an internal size standard (fig. 2). The combination of these 8 peaks yielded a multilocus genotype (starting now named microsatellite type [MS-Type]).

Each MS-Type was assigned a number taking into account, for comparison, the MS type profiles obtained with previous analyses on European samples. In other words, in case of a Brazilian strain with an MS Type identical to a profile obtained for a European strain, we assigned to both these strains the same MS-Type number.

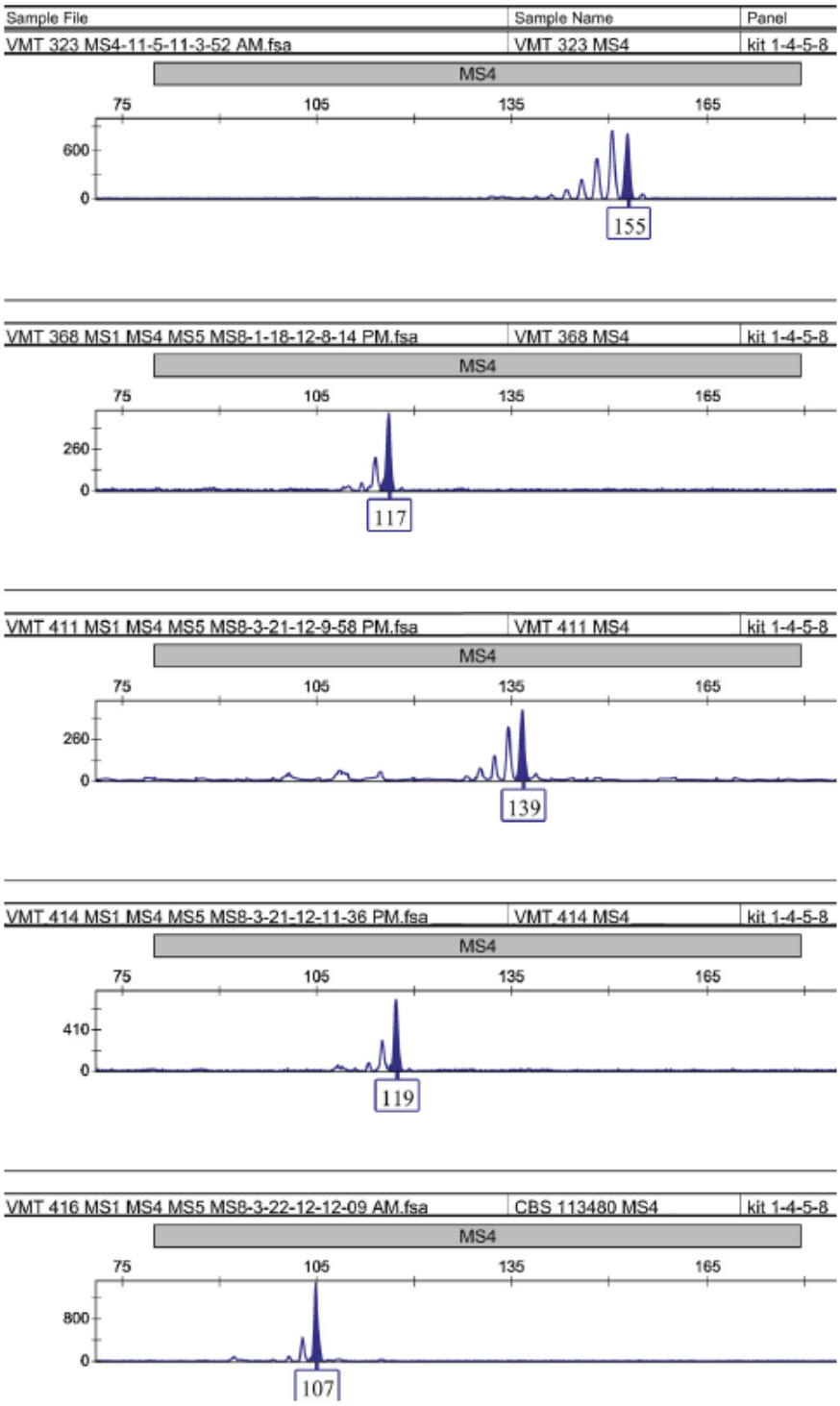

Figure 2. Examples of results of capillary electrophoresis for the marker microsatellite 4 (MS4) regarding five strains of *M. canis*. Below the peaks is reported the fragment size

2.2.3 Preparation of the database

Past results obtained with the panel of 8 MS were retrieved and ordered in an excel file which included the available metadata regarding the fungal isolates. Results regarded

isolates analyzed from 2012 to 2019. The file is provided separately as a supplementary document (S1, the file also includes the Brasilian isolates). Besides, sampling sites were reported on a “google maps” document to allow an overview of the MS-Type distribution and an accession to the data “geographically-based”.

2.2.4 Data analysis

The genotype frequency of each MS marker was calculated using the software MSA version 3.12 (Dieringer D, 2003), while MULTILOCUS 1.3 (Agapow and Burt, 2001) (http://www.agapow.net/software/multilocus/) was employed to analyze the genetic diversity of the sample and the frequency of each MS-Type.

In case of microorganisms, such as *M. canis*, that are known to reproduce clonally via mitosis, the use of clone correction has been proposed as a means of reducing the overrepresentation of individual genotypes derived from local-specific clonality (Xu et al., 1999). Accordingly, in our study, analyses were conducted using a clone-corrected approach, namely considering only one strain in case all the isolates from a related sampling (e.g. in an outbreak episode, in a kennel, in a cattery) showing the same MS Type. A further clone-correction was also applied, considering the host of provenance. For example, in case of an infection episode with more humans involved – provided that the same MS-Type was obtained for the fungal isolates coming from all the patients – only one "human-associated" strain was considered. The same was done for "cat-" and "dog- associated" samples.

The expected clonality was verified, versus possible recombination in the *M. canis* sample, by calculating the index of association (I_A) using the software MULTILOCUS 1.3 (Agapow and Burt, 2001). The I_A is the traditional measure of multilocus linkage disequilibrium (Brown et al. 1980; Maynard Smith et al. 1993; Haubold et al. 1998). In this test, the observed data are compared against the null hypothesis of random mating (random association of alleles from different DNA loci). When the null hypothesis is rejected, a clonal population structure is suggested.

The results were also evaluated to infer a possible population structure. For this purpose, as the interpretation of population structures is not always straightforward in clonal fungi (Graser et al., 2007), we applied two distinct models of analysis. Firstly, microsatellite genotype data were analyzed by calculating genetic distances between individuals based on Dc (Cavalli-Sforza & Edwards, 1967), Dm and Ds (Saitou & Nei, 1987) distances, implemented

in the software package POPULATIONS version 1.2.28 (http://www.pge.cnrs-gif.fr/bioinfo/populations/index.ph-p?lang=en). Neighbour-joining trees were constructed from the distance matrices and were displayed and analyzed using *MEGA* version 4 (http://www.megasoftware.net/mega4/mega.html) (Tamura et al., 2007).

As an alternative, a Bayesian approach was implemented in the program STRUCTURE 2.3 (Pritchard et al., 2000). Various models were used, including the no-admixture model, which can deal with clonal reproduction. One million Markov chain replications and a burn-in period of 100000 were used. The probability of the data, assuming one to ten clusters (K) was estimated in ten runs. The most likely number of clusters was determined using the Evanno 's method (http://taylor0.biology.ucla.edu/structureHarvester/) (Earl and vonHoldt, 2012) and taking into account the results of the distance analysis.

2.3 RESULTS

As regards the Brasilian isolates, five different MS Types were detected. An identical MS Type was shared by related strains, i.e. the strains sampled in each episode (with a lone exception in the episode 8; the presence of two genotypes was probably due to the introduction of the fungus by more than one source) (Table 2)

episode	Samples	City	MS Type
1	Atman 4, cat, animal	São Paulo	10
1	Atman 4, cat, bed	São Paulo	10
1	Atman 4, cat, toy	São Paulo	10
2	Belinha 5, dog, animal	Osasco	39
2	Belinha 5, dog, pillow	Osasco	39
2	Belinha 5, dog, carpet	Osasco	39
3	Charlisie 6, dog, animal	Osasco	39
3	Charlisie 6, dog, toy	Osasco	39
3	Charlisie 6, dog, floor balcony	Osasco	39
3	Charlisie 6, dog, sheet	Osasco	39
3	Charlisie 6, dog, floor room	Osasco	39
3	Charlisie 6, dog, blanket	Osasco	39
3	Charlisie 6, dog, bed	Osasco	39
4	Bam bam, dog, animal	Osasco	39
4	Bam bam, dog, floor living room	Osasco	39
5	Marie 34, cat, animal	Osasco	39

5	Marie 34, cat, animal, sofa	Osasco	39
5	Marie 34, cat, floor living room	Osasco	39
5	Marie 34, cat, carpet bathroom	Osasco	39
5	Marie 34, cat, carpet balcony	Osasco	39
5	Marie 34, cat, floor around feeder	Osasco	39
5	Marie 34, cat, table	Osasco	39
5	Marie 34, cat, bathroom sink bench	Osasco	39
5	Marie 34, cat, sink bathroom	Osasco	39
6	Marie 37, dog, animal	Osasco	39
6	Marie 37, dog, floor room	Osasco	39
6	Marie 37, dog, sheet	Osasco	39
7	Simba 38, cat, animal	Osasco	39
7	Simba 38, cat, bathroom floor	Osasco	39
7	Simba 38, cat, lepe (animal contact)	Osasco	39
7	Simba 38, cat, jade (animal contact)	Osasco	39
7	Simba 38, cat, kiara (animal contact)	Osasco	39
7	Simba 38, cat, house animal	Osasco	39
7	Simba 38, cat, cloth animal	Osasco	39
7	Simba 38, cat, apron proprietary	Osasco	39
8	Luna 52, dog, animal	Carapicuíba	39
8	Luna 52, dog, caos (animal contact)	Carapicuíba	40
8	Luna 52, dog, gaia (animal contact)	Carapicuíba	39
8	Luna 52, dog, eros (animal contact)	Carapicuíba	39
8	Luna 52, dog, baco (animal contact)	Carapicuíba	40
8	Luna 52, dog, floor living room	Carapicuíba	39
8	Luna 52, dog, enclousure cubs	Carapicuíba	39
8	Luna 52, dog, brush animal	Carapicuíba	39
8	Luna 52, dog, hall floor	Carapicuíba	39
8	Luna 52, dog, sofa	Carapicuíba	39
8	Luna 52, dog, cardboard box	Carapicuíba	39
8	Luna 52, dog, blanket animal	Carapicuíba	39
9	Pacho 53, dog, animal	Osasco	44
9	Pacho 53, dog, air	Osasco	44
9	Pacho 53, dog, sofa	Osasco	44
9	Pacho 53, dog, bag proprietary	Osasco	44
9	Pacho 53, dog, feeder	Osasco	44
9	Pacho 53, dog, blanket animal	Osasco	44
9	Pacho 53, dog, bed animal	Osasco	44
9	Pacho 53, dog, slippers	Osasco	44
9	Pacho 53, dog, living room floor	Osasco	44
10	Jack, dog, animal	Osasco	5
10	Jack, dog, cardboard box	Osasco	5

10	Jack, dog, sofa	Osasco	5
10	Jack, dog, bed	Osasco	5
11	Tody, dog, animal	São Paulo	39
11	Tody, dog, bed	São Paulo	39
11	Tody, dog, toy	São Paulo	39
11	Tody, dog, sofa	São Paulo	39
11	Tody, dog, pillow	São Paulo	39
11	Tody, dog, air	São Paulo	39
12	Mel, cat, animal	São Paulo	39
12	Mel, cat, cristal (contact)	São Paulo	39
12	Mel, cat, bed mother	São Paulo	39
12	Mel, cat, bed son	São Paulo	39
13	Apollo, dog, animal	Osasco	40

Table 2 – MS Types found for the Brazilian isolates

Following a clone-corrected approach, one genotype (MS-Type 39) was more prevalent as it was shared by nine unrelated samplings (9/14; 64%). MS-type 40 was shared by two unrelated samplings, while the other MS-types (MS-type 5, 10 and 44) were found only once.

As expected from previous studies (Sharma et al., 2007), PCR products were obtained using *M. audouinii* and *M. ferrugineum*. However, the genetic profiles were distinct from those obtained for *M. canis* strains. For this reason, they could be used as an outgroup in the distance analyses (see below). Instead, testing of *N. gypsea* and *T. mentagrophytes* yielded negative results.

From the consultation of the database produced (document S1, see also below), we could note that none of the genotypes found in Brazil was typical of this country since all of them were found also in Europe.

Considering the whole database produced, it includes to date a total of 424 isolates of *M. canis* coming from 10 countries (Italy, France, Austria, Germany, Belgium, Brazil, Turkey, Egypt, China, South Korea). Isolates were sampled from human patients (n=146), cats (n=151), dogs (n=50), environmental sites (n=73), and other animal species (cheetah n=2; dwarf rabbit n=1; chamois n=1) (document S1). Samplings from Europe – in particular from

Italy and Germany – were the most balanced as regards human and animal provenance, while only isolates from human patients were available as regards Egypt, Turkey, South Korea and China. As mentioned, only isolates of animal provenance were available for Brazil.

The analysis of the combinations of the eight markers allowed detecting a total of 91 MS-Types (document S1). With few exceptions, an identical MS Type was shared by "related" strains, i.e. strains isolated from episodes of infections involving multiple people or animals, kennels, catteries and in a pet shop (document S1). The calculation of the allele and MS Type frequency was thus performed using 264 clone-corrected samples.

The existence of 91 MS-Types within the dataset of 264 clone-corrected samples corresponds to a genotypic diversity of 96%. According to this results and the high resolving power proven for the set of MS markers employed (Pasquetti et al., 2013), we assumed that the isolates found with the same genotype were clonal or at least highly genetically related. Some MS Types were found with higher prevalence, especially MS Type 5 and 90, followed by MS Type 39, 23, 10, 12. Many MS Types were found only once (Table 3)

MS TYPE	NUMBER OF UNRELATED SAMPLINGS	%
5	33	12,5
90	28	10,6
39	15	5,7
23	14	5,3
10	11	4,2
12	9	3,4
86	8	3,0
1	7	2,7
40	7	2,7
16	6	2,3
4	4	1,5
9	4	1,5
22	4	1,5
48	4	1,5
56	4	1,5
6	3	1,1
13	3	1,1
21	3	1,1
38	3	1,1
44	3	1,1
46	3	1,1
87	3	1,1

26	2	0,8
30	2	0,8
35	2	0,8
41	2	0,8
45	2	0,8
49	2	0,8
57	2	0,8
61	2	0,8
69	2	0,8
70	2	0,8
76	2	0,8
81	2	0,8
82	2	0,8
83	2	0,8
84	2	0,8
89	2	0,8
2	1	0,4
3	1	0,4
7	1	0,4
8	1	0,4
11	1	0,4
14	1	0,4
15	1	0,4
17	1	0,4
18	1	0,4
19	1	0,4
20	1	0,4
24	1	0,4
25	1	0,4
27	1	0,4
28	1	0,4
29	1	0,4
31	1	0,4
32	1	0,4
33	1	0,4
34	1	0,4
36	1	0,4
37	1	0,4
42	1	0,4
43	1	0,4
47	1	0,4
50	1	0,4
51	1	0,4
52	1	0,4
53	1	0,4

54	1	0,4
55	1	0,4
58	1	0,4
59	1	0,4
60	1	0,4
62	1	0,4
63	1	0,4
64	1	0,4
65	1	0,4
66	1	0,4
67	1	0,4
68	1	0,4
71	1	0,4
72	1	0,4
73	1	0,4
74	1	0,4
75	1	0,4
77	1	0,4
78	1	0,4
79	1	0,4
80	1	0,4
85	1	0,4
88	1	0,4
91	1	0,4
tot	**264**	

Table 3 – Frequency of the different MS Types found

The different genotypes appear variably distributed in the world. To allow an easier visualization, we prepared a google map that reports the sites of samplings with the corresponding MS Types. Different symbols and colors indicate each MS Type.

The map can be accessed at

https://drive.google.com/open?id=1GIXqRHrnZhORSCo7RW4US2ZlybZe04G_&usp=sharing

In case the link does not work after clicking on it, it will be sufficient to copy and paste it in the browser bar. At the opening of the map, the user will have the possibility of visualizing all the samplings or thicking the different MS Types to see the corresponding samplings.

The map is intended as an user friendly tool that allows moving throughout the areas sampled and giving a graphical overview of the MS Types distribution. Moreover it is possible to thick on the different sampling points to obtain further information (provenance

and number of isolates for that sampling). It is possible to examine the map at low magnification to cover a vast extension of territory, for example, to appreciate the distribution of the two most prevalent MS Types, namely MS Type 5, widespread in Europe but absent in Asia, and MS-Type 90, present in China and South Korea but not in Europe (Figure 2).

Figure 2 – Screenshot taken from the google map showing the distribution of MS Type 5 and MS Type 90

Another example is reported in figure 3, showing the presence of MS Type 39 in Brasil and Europe. Navigating on the map with higher magnification allows instead to evidence the distribution of genotypes in more localized areas, for example, at the city level. By focusing the visualization only on San Paulo city, it is possible to appreciate the above mentioned higher prevalence of the genotype 39 (figure 4).

Figure 3 – Screenshot taken from the google map showing the distribution of MS Type 39

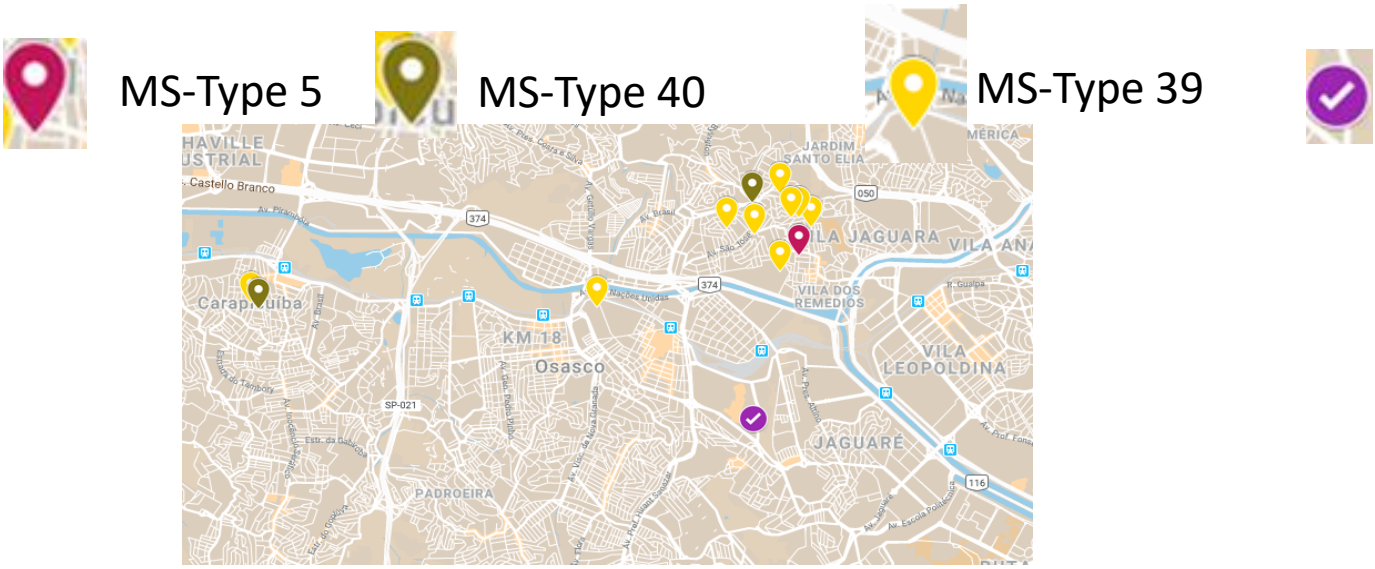

Figure 4 –Screenshot of the map at higher magnification allows showing the distribution of MS Types in San Paulo city and surroundings

The database includes many samples coming from other metropolitan areas, in which instead a lot of different MS Types were found (see examples in the next figures).

Figure 5 –Screenshot of the map showing the distribution of MS Types in the city of Torino (Italy) (for the legend regarding the MS Types consult the google map)

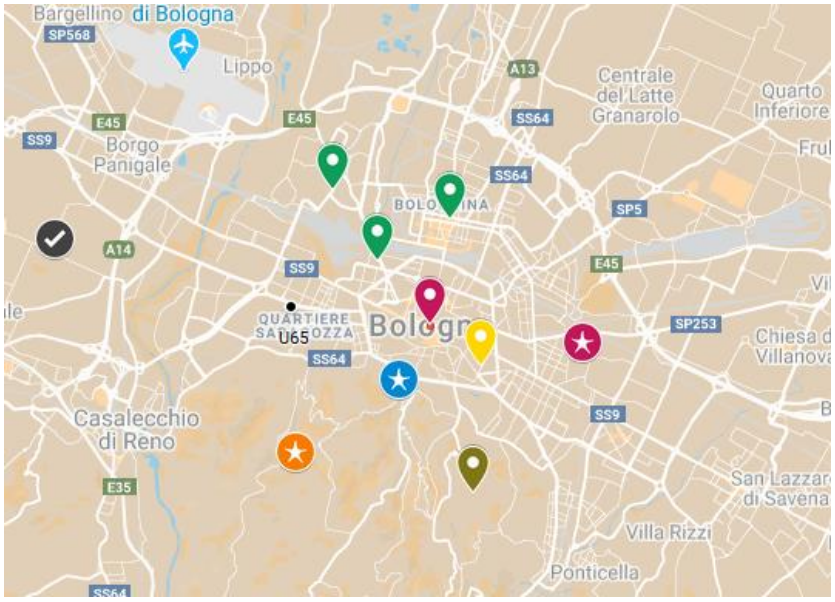

Figure 6 –Screenshot of the map showing the distribution of MS Types in the city of Bologna (Italy) (for the legend regarding the MS Types consult the google map)

Another worth noting finding is that Asia appears dominated by a single genotype (MS Type 90, accounting for 87.5% of the samplings, 28/32). At the same time, in Europe a much higher variability is present (83 different genotypes represented, with the most prevalent MS Type [MS Type 5] accounting for 16.1% of the samples, and many genotypes found only once (see the google map and document S1).

The linkage disequilibrium analysis rejected the null hypothesis of random mating (I_A 0.67, $p < 0.01$).

With the further clone-correction according to the host of provenance, we found 124 "human-associated" samples (with 45 different MS Types), 112 "cat-associated" samples (with 55 different MS Types) and 39 "dog-associated" samples (with 24 different MS-Types) (tables 4-6).

MS TYPE	NUMBER OF SAMPLES	%
90	28	22,6
23	12	9,7
86	8	6,5
1	5	4,0
5	5	4,0
16	5	4,0
10	4	3,2

9	3	2,4
87	3	2,4
4	2	1,6
12	2	1,6
13	2	1,6
21	2	1,6
39	2	1,6
40	2	1,6
49	2	1,6
69	2	1,6
70	2	1,6
76	2	1,6
81	2	1,6
82	2	1,6
83	2	1,6
84	2	1,6
89	2	1,6
22	1	0,8
38	1	0,8
41	1	0,8
42	1	0,8
48	1	0,8
64	1	0,8
66	1	0,8
67	1	0,8
68	1	0,8
71	1	0,8
72	1	0,8
73	1	0,8
74	1	0,8
75	1	0,8
77	1	0,8
78	1	0,8
79	1	0,8
80	1	0,8
85	1	0,8
88	1	0,8
91	1	0,8
TOT **124**		100

Table 4 – Frequency of MS Types for "human-associated samples."

MS TYPE	NUMBER OF SAMPLES	%
5	25	22,3
10	8	7,1
12	8	7,1
39	6	5,4
56	4	3,6
6	3	2,7
48	3	2,7
1	2	1,8
22	2	1,8
23	2	1,8
26	2	1,8
35	2	1,8
44	2	1,8
61	2	1,8
2	1	0,9
4	1	0,9
7	1	0,9
8	1	0,9
9	1	0,9
11	1	0,9
13	1	0,9
14	1	0,9
15	1	0,9
18	1	0,9
19	1	0,9
20	1	0,9
21	1	0,9
24	1	0,9
25	1	0,9
27	1	0,9
29	1	0,9
30	1	0,9
31	1	0,9
32	1	0,9
33	1	0,9
36	1	0,9
38	1	0,9
40	1	0,9
41	1	0,9
42	1	0,9
43	1	0,9
45	1	0,9
46	1	0,9
47	1	0,9

49	1	0,9
50	1	0,9
51	1	0,9
53	1	0,9
54	1	0,9
55	1	0,9
57	1	0,9
62	1	0,9
63	1	0,9
64	1	0,9
65	1	0,9

Table 5 – Frequency of MS Types in “cat- associated samples."

MS TYPE	NUMBER OF SAMPLES	%
39	7	17,9
5	6	15,4
40	4	10,3
46	2	5,1
3	1	2,6
4	1	2,6
10	1	2,6
12	1	2,6
16	1	2,6
17	1	2,6
19	1	2,6
21	1	2,6
22	1	2,6
28	1	2,6
30	1	2,6
34	1	2,6
37	1	2,6
44	1	2,6
45	1	2,6
52	1	2,6
57	1	2,6
58	1	2,6
59	1	2,6
60	1	2,6

Table 6 – Frequency of MS Types for “dog- associated” samples."

As regards the population analyses the most congruent findings between the different approaches employed were obtained when the number of clusters/populations were postulated as five (fig. 7 and 8). The assignment of the samplings to each of the five population is reported in document S1 and summarized in the tables below.

POP	NUMBER OF SAMPLES	%
I	52	19,7
II	27	10,2
III	64	24,2
IV	96	36,4
V	25	9,5
	264	

Table 7. Number and percentage of samples belonging to the different populations

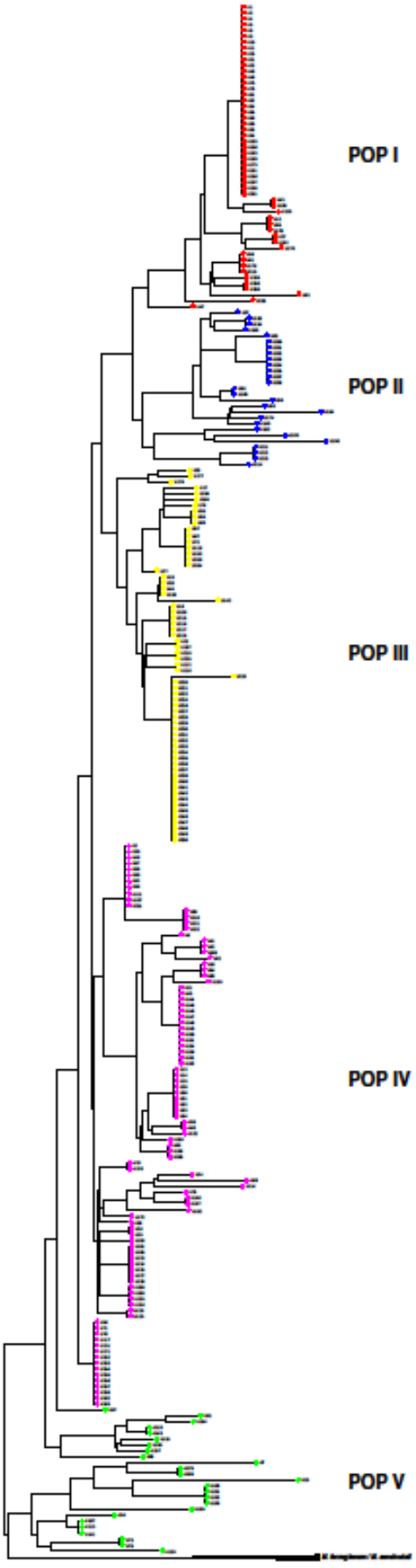

Figure 7 . Neighbour-joining tree constructed from the distance matrices

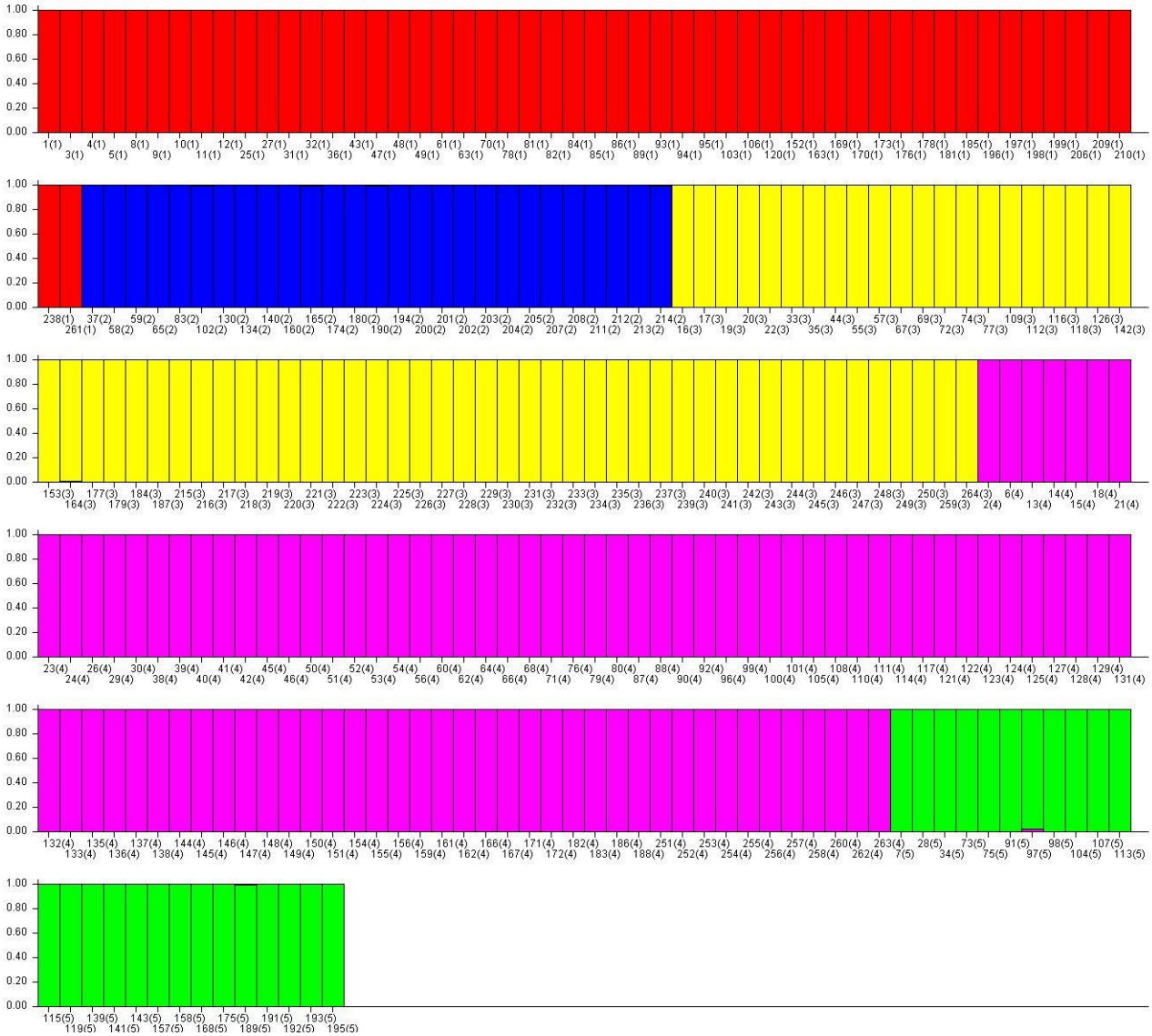

Figure 8. Bar plots for K= 5 using STRUCTURE showing the same five clusters as the distance tree (figure 7). Each individual is represented by a single vertical line broken into K coloured segments, with lengths representing the probability (0-100%) for each fungal isolate to belong to the different inferred clusters (K) (populations in the distance analyses). A different colour represents each genetic cluster.

Europe		
POP	N	%
I	47	23,6
II	15	7,5
III	27	13,6
IV	85	42,7
V	25	12,6
	199	**100**

South Korea/China		
POP	N	%
I	1	3,1
II	0	0,0
III	31	96,9
IV	0	0,0
V	0	0,0
	32	100

Egypt/Turkey		
POP	N	%
I	3	15,8
II	12	63,2
III	4	21,1
IV	0	0,0
V	0	0,0
	19	

Brasil		
POP	N	%
I	1	7,1
II	0	0,0
III	2	14,3
IV	11	78,6
V	0	0,0
	14	

Table 8 – Abundance of samplings belonging to the different populations (I-V) in different geographic areas

	Europe		South Korea/China		Egypt/Turkey		Brasil		
POP	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**tot**
I	47	90,4	1	1,9	3	5,8	1	1,9	**52**
II	15	55,6	0	0,0	12	44,4	0	0,0	**27**
III	27	42,2	31	48,4	4	6,3	2	3,1	**64**
IV	85	88,5	0	0,0	0	0,0	11	11,5	**96**
V	25	100,0	0	0,0	0	0,0	0	0,0	**25**
	199		**32**		**19**		**14**		

Table 9 – Distribution of samplings belonging to the different populations according to geography

Human Associated		
POP	NUMBER OF SAMPLES	%
I	12	9,7
II	17	13,7
III	43	34,7
IV	42	33,9
V	10	8,1
	124	

Cat associated		
POP	NUMBER OF SAMPLES	%
I	37	33,0
II	8	7,1
III	9	8,0
IV	43	38,4
V	15	13,4
	112	

Dog associated		
POP	NUMBER OF SAMPLES	%
I	6	15,4
II	3	7,7
III	11	28,2
IV	17	43,6
V	2	5,1
	39	

Table 10. Abundance of samplings belonging to the different populations (I-V) in different hosts

	"HUMAN ASSOCIATED" SAMPLINGS		"CAT ASSOCIATED" SAMPLINGS		"DOG ASSOCIATED" SAMPLINGS		
POP	N	%	N	**%**	N	**%**	**tot**
I	12	21,8	37	67,3	6	10,9	55
II	17	60,7	8	28,6	3	10,7	28
III	43	68,3	9	14,3	11	17,5	63
IV	42	41,2	43	42,2	17	16,7	102
V	10	37,0	15	55,6	2	7,4	27
TOT	**124**		**112**		**39**		

Table 11 – Distribution of samplings belonging to the different populations according to the host

2.4 DISCUSSION

The initial inspiration for this study was represented by the possibility of enriching the collection of genetic profiles of *M. canis* isolates, available at the Department of Veterinary Sciences of Turin, with data on isolates coming from Brazil. As the study proceeded, it also became an opportunity for establishing a database able to provide, in a user-friendly manner, the results of strain typing – by a multilocus microsatellite technique – of hundred of *M. canis* isolates coming from different countries. This was also achieved by the creation of a google map that permits accessing the data in a "geographic" based manner. Through this map, it is thus possible to have an overview of the spatial distribution of the samplings, with the corresponding MS Types.

Our study showed that the MS Types found in Brazil are present also in Europe, but with different prevalence. Indeed, the most frequent Brazilian MS Type (MS Type 39, 64% of the samples) in Europe accounts only for 2.2% of the samples. Moreover, this MS-Type was not found in other countries. Though these results may suggest a possible correlation between some genotypes and geographical origin, some caution in drawing conclusions is necessary, for a series of reasons. First, only 14 unrelated strains were available for the analyses. Second, the isolates were collected in quite a restricted area - i.e. the metropolitan area of Sao Paulo – which may explain the low genetic variability found and the predominance of genotype 39. On the other hand, data present in the database indicate that not necessarily strains coming from very close locations share the same genotype. For example, a high

degree of genetic variability can be observed in samples obtained in Paris in France or Turin and Bologna in Italy. Thus, the finding of a high percentage of isolates with MS Type 39 in Sao Paulo may reflect an actual higher diffusion of this genotype in Brazil. Of course, a much higher number of samplings from much more locations would be necessary to clarify this issue.

An identical MS Type was shared by the strains sampled in each of the 13 episodes (a lone exception was present in episode 8, in which two of the in-contact animals were shown to harbour a different MS-Type. These animals probably acquired the infection from a different source). The finding of the same MS Types for all the samples (animal and environment) coming from a defined episode confirms the high reproducibility of the MS panel employed (Pasquetti et al., 2013) and reinforces the idea that it represents a useful tool to track the paths of infection due *to M. canis*. For this purpose, other molecular techniques were employed in the past. Kaneko *et al.* (2011) investigated an episode of an animal-derived infection in a human patient by sequencing the ITS1 region. The specific sequence was identical in both the human and animal isolates, while differences were noted in sequences of other unrelated strains. However, the authors failed to comment that ITS is the region of choice for species identification and for a basic understanding of phylogenetic relationships among dermatophyte species, but is not however ideally suited for applications designed to discriminate between different strains of dermatophytes, as only limited sequence variations distinguish related species.

Moreover, within the same species sequences are generally highly conserved, and thus probably shared by hundreds of strains. As confirmation, the unrelated isolates of Kaneko *et al.* (2011) showed very limited sequence variations. Moreover, the study included just one cat with its relative owner and the episode was compared with only five unrelated strains.

Yu *et al.* (2004) [19] identified strain types of isolates coming from 40 patients sampled in a school outbreak in China. Several methods were used, including RAPD (with two primers), NTS amplification, and ITS sequencing. As expected, no differences in the ITS sequence were observed among the isolates. Similarly, both RAPD analyses, along with the NTS typing, returned the same results for all isolates. Cano *et al.* (2005) evaluated 24 unrelated *M. canis* strains in Spain, by inter-single-sequence-repeat (ISSR)-PCR, with some isolates coming from related hosts. Authors identified that two patients from the same family harboured different

M. canis strains, one of which matched the strain isolated from the domestic household cat, while the owner and cat in the second human-pet pair in this study harboured a different *M. canis* strain type. Authors attributed this observed difference to the high endemic rate for *M. canis* in Spain; however, there could have been a relationship between the strains that was missed due to the rapid mutation of the markers employed (Abdel-Rahman, 2008), which were not validated from this point of view.

Going back to our study, the genetic diversity found within the *M. canis* dataset (around 96%) confirms the resolving power of the markers employed (Pasquetti et al., 2013) and shows that *M. canis* possesses a high genetic variability. This supports the fact that the low variability found in previous studies (Kaszubiak et al., 2004; Gräser et al., 2000; Dobrowolska et al., 2011; Brilhante et al., 2005; Faggi et al., 2001) was probably due to an inadequate discriminatory power of the molecular markers used.

Another parameter to take into account is the fact that the reproduction type of *M. canis* should be predominantly mitotic, therefore clonal. This figure, which emerged from the analysis of the index of association (I_A), seems to confirm the assertions made by several works based on a different approach, namely the search for the c.d mating type of the fungus. Dermatophytes during the skin infection process replicate asexually, producing resistance spores, called arthroconidia, considered as the elements responsible for the infection. Sexual reproduction is usually obtained under laboratory-controlled conditions and requires two fungal strains, respectively called mating-type + and –.

The results of several studies lead to believe that one of the two mating types (that +) is to be considered almost extinct in nature, which makes it very improbable the sexual reproduction and confirms, by other means, our findings (Hasegawa & Husui, 1975; Hironaga et al., 1980; Weitzman & Padhye, 1978).

In this study some MS Types were found with higher frequency, which leads to hypothesize the existence of clonal lines of “major success” due to a stronger parasitic aptitude. Besides, some MS-Types appear related to specific geographical contexts (e.g MS Type 5 in Europe, MS Type 90 in Asia; MS Type 12 in Northern Italy; MS Type 86 in Turkey). Notably, while in Europe a MS Type (5) is prevalent, but a lot of other MS Types are also present, in Asia (South Korea and China) the most frequent MS Type (90) is also almost the only present. Perhaps the higher intensity of sampling in Europe allowed discovering more MS Types.

Another hypothesis is that the low intra-specific variability may indicate a more recent introduction of *M. canis* in Asia compared with Europe (Sharma et al., 2008).

The difference between Europe and Asia is reflected also in the results of the population analyses. All the five populations are represented in Europe, while population III accounts for almost the totality of samplings in Asia (about 90%). Interestingly, geographic factors may have been responsible for structuring the populations of *M. canis*. This is shown by the fact that population I, IV and V appears strongly represented in Europe and poorly or not at all (pop V) in other countries. Always with regard to population analyses, strains belonging to pop III are present only in Europe and Brasil, which may indicate an exchange of isolates between continents. The fact that a lot of MS Types were found in Europe and a fewer number in Brasil (though we have already discussed that this should be proved on a higher number of samples) may indicate a direction from Europe towards Brasil. Indeed, previous studies support the conclusion that a high genetic variability within a population is evidence of an ancient introduction of a microorganism in a particular region (Sharma et al., 2008).

As regards the transmission to humans, Sharma et al. (2007), using a panel of two microsatellite markers, reported that a single genotype with pandemic distribution might have a higher potential to infect humans when transmitted from animals. This conclusion was based on the fact that a cluster of genotypes included most human samplings, and that 75% of isolates of human origin shared the same genotype. Our data from South Korea and China may confirm the hypothesis made by Sharma et al, in that we found a single genotype (MS Type 90) mostly dominant. However, there is a very critical issue to consider, namely that animal samplings were not available from those areas. Thus the overrepresentation of an MS type may depend only on geographic reasons. In other words, in Asia MS Type 90 may be overrepresented in animals too.

Moreover, data from Europe, more balanced as regards the hosts of provenance, seem to tell another story. Indeed, it is possible to note that a lot of MS Types were involved in human infections and that the genetic diversity was similar for feline (55 different MS Types out of 112 samples, genetic diversity 93%), human (45 MS Types out of 124 samples, genetic diversity 94%) and canine (27 MS Types out of 39 samples, genetic diversity 94%) populations. Interestingly, it was instead the feline population to show a more prevalent MS-

Type (MS-Type 5) largely diffused from south to north Europe (see the google map), but this MS Type was not the most prevalent in human infection episodes.

In conclusion, this study allowed preparing a well-defined database of MS profiles as regards *M. canis*, and enriching it with data coming from Brasil. A well defined, easily accessible database including metadata (such as geographic and host provenance) regarding the fungal isolates tested, may represent a tool to track the diffusion of different genotypes of *M. canis*, which in turn could provide information useful to clarify the dynamics of transmission of this fungal pathogen.

This database and the related google map represent a useful tool for researchers aiming at work on the genetic variability of *M. canis*. Database and map may indeed allow the comparison of results obtained for isolates in other parts of the world.

The availability of as many MS profiles as possible is essential to interpret better and better future infection episodes, as it must be pointed out that studies that report the same strain among all isolates from a suspected outbreak, occurring in a geographic region for which no baseline data on the degree of variation in the population exists, remain uninterpretable (Abdel-Rahman, 2008).

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