Dermatophytosis in dogs and cats: molecular identification and epidemiology

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If you can’t fly then run, if you can’t run then walk, if you can’t walk then crawl, but whatever you do you have to keep moving forward.

Martin Luther King, Jr.

DERMATOPHYTOSIS IN CATS AND DOGS: MOLECULAR IDENTIFICATION AND EPIDEMIOLOGY

Abstract

In field of veterinary medicine, dermatology of the small animals represents between 25 and 30 percentages of appointments, being dermatophytosis one of the most frequent reasons (Pinho *et al*., 2013). Dermatophytosis is one of the most common infections in the world and important fungal skin illness of cats and dogs (Frymus *et al*., 2013; Refai *et al*., 2016). Referred as *ringworm* or *tinea*, it defined as infection of cutaneous cornified layers by group of keratinophilic and keratinolytic fungi (HCMI, 2011; Saraiva, 2017). Reactions may range from mild to severe, as a consequence of the host's reactions to the metabolic products of the fungus, the virulence of the strain or species, the anatomic location, and environmental factors (®HCMI, 2011; Lakshmipathy *et al*., 2010). It can lead to acute or chronic disease, with high morbidity but not mortality (Behzadi *et al*., 2014).

There are many zoonotic diseases, among them are the fungal diseases, and dermatophytosis are significant part of them (Frymus *et al*., 2012; Bahri, 2013).

Considering "One Health" concept, immunosuppressive therapy widely used, preventive antibiotic therapy, growing globalization and ubiquity of fungi, increasing number of mycosis (and dermatomycosis as a part) are clearly justified (Anaissie *et al*., 2009; Alves, 2017).

This work has two main goals: at first, optimize an existing identification method for direct detection of the dermatophytes in clinical veterinary samples. The second goal is analysis of statistical data collected over the 16 years (from 2001 to 2016) in Portugal, Lisbon, for epidemiological estimation and identification of risk factors in dermatophytosis of the dogs and cats. Nowadays, similar processed data about the epidemiological situation with dermatophytosis in Portugal, and the validated molecular technique for identifying of dermatophytes in clinical veterinary specimens does not exist.

Keywords: Dermatophytosis, dogs, cats, human, identification, epidemiology

DERMATOPHYTOSIS IN CATS AND DOGS: MOLECULAR IDENTIFICATION AND EPIDEMIOLOGY

Resumo

Em medicina veterinária a dermatologia de animais de companhia representa entre 25 e 30 por cento de todas as consultas, sendo a dermatofitose um dos motivos mais frequentes. Dermatofitose é uma das infeções mais comuns a nível mundial e é uma doença de origem fúngica importante em cães e gatos (Frymus *et al*., 2013; Refai *et al*., 2016). Mencionada como *tinea* ou *ringworm*, definida como infeção cutânea de camada queratinizada por grupo de fungos queratinofílicos e queratitolíticos (HCMI, 2011; Saraiva, 2017). A reação pode variar de leve a grave, em consequência da resposta do hospedeiro aos produtos de metabolismo do fungo, virulência da estirpe ou espécie, localização anatómica da infeção e fatores ambientais (HCMI, 2011; Lakshmipathy *et al*., 2010). Pode resultar em doença aguda ou crónica, de alta morbidade, mas não de mortalidade (Behzadi *et al*., 2014).

Existem muitas doenças zoonóticas, entre elas também se encontram as de origem fúngica, e as dermatofitoses fazem uma parte considerável destas últimas (Frymus *et al*., 2012; Bahri, 2013).

Tendo em consideração o conceito de "Uma Só Saúde", tendências de uso prolongado de imunossupressores e antibioterapia preventiva, globalização crescente e ubiquidade de fungos, o número crescente de micoses em geral, e dermatomicoses em particular, está claramente provado.

Este trabalho tem dois objetivos principais: em primeiro lugar, otimizar um método de identificação existente para a identificação direta de dermatófitos em amostras clínicas veterinárias. O segundo objetivo é a análise epidemiológica de dados estatísticos recolhidos ao longo de 16 anos (2001 - 2016) em zona de Lisboa, Portugal. Atualmente não existem dados processados semelhantes sobre a situação epidemiológica com dermatofitóse em Portugal, como a técnica molecular de identificação de dermatófitos em amostras clínicas veterinárias validada.

Palavras-chave: dermatofitóse, cães, gatos, humano, identificação, epidemiologia.

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List of Abbreviations

AUC – Area Under the Curve

BT – Beta Tubulin

BSA – Bovine Serum Albumin

CECT – Colección Española de Cultivos Tipo

CL – Confidence Limits

CRB – Cooke Rose Bengal

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DTM – Dermatophyte Test Medium

EST – Expressed Sequence Tag

Hfs - Hydrogenase-Fe-S

HIV – Human Immunodeficiency Virus

ITS – Internal Transcribed Spacer

LSU – Large Ribosomal RNA Subunit

mtDNA – Mitochondrial DNA

PacC – pH-responsive Transcription Facto

PCR – Polymerase Chain Reaction

pH –Potential of Hydrogen

rDNA – Ribosomal DNA

RFLP – Restriction Fragment Length Polymorphism

SDA – Sabouraud´s Dextrose Agar

Tef – Translation Elongation Factor

TOP - Topoisomerase

TUB – Partial β-tubulin

UV – Ultraviolet

Chapter I – Introduction

1. General description

Fungi represent one of the three major evolutionary branches of multicellular organisms. They have a status of a kingdom – Fungi or Eumycota (Deacon, 2006).

It is important make a distinction between the true fungi and a range of fungus-like organisms that have traditionally been studied by mycologists, but are fundamentally different from fungi (Deacon, 2006; Perevedenceva, 2009).

All true fungi are eukaryotic, it means that membrane-bound nuclei contained several chromosomes, presence of cytoplasmic organelles – vacuoles, mitochondria and others, DNA containing introns, ribosomes of the 80S type. Typically, haploid nuclei. However, fungal hyphae often have several nuclei in each hyphal compartment. Many budding yeasts are diploid.

Typically, they grow in branching filamentous structure, calls hyphae, giving a rise to a network, designated mycelium. The hypha is essentially a tube with a rigid wall, containing a moving slug of protoplasm, it´s length depending on the species and growth conditions. Hyphae grow only at their tips, where there is a tapered region termed the extension zone. Behind the growing tip, the hypha ages progressively and in the oldest regions it may break down by autolysis or be broken down by the enzymes of other organisms - heterolysis. While the tip is growing, the protoplasm moves continuously from the older regions of the hypha towards the tip. The hyphae of most fungi have cross walls - septa at regular intervals, nevertheless, the functional distinction between septate and aseptate fungi is not as great as might be thought, because septa have pores through which the cytoplasm and even the nuclei can migrate. The growth is apical. However, some fungi grow as single-celled yeasts, which reproduce by bidding. Moreover, some can switch between yeast and hypha phases in response to environmental conditions – they are dimorphic fungi. (Deacon, 2006). The way in which a fungus grows – whether as cylindrical hyphae or as yeasts – is determined by the wall components and the ways in which these are assembled and bonded to one another. In chistosomes and as integral membrane protein is found a chitin synthase, which catalyze the synthesis of chitin chains. This enzyme is one of the principal enzymes involved in fungal wall growth, and as we will see later, has a particular importance for identification techniques. The wall is also the interface between a fungus and its environment:

- protects against osmotic lysis;
- it acts as a molecular sieve regulating the passage of large molecules through the wall pore space;

- if the wall contains pigments such as melanin it can protect the cells against ultraviolet radiation or the lytic enzymes of other organisms.

In addition to these points, the wall can have several physiological roles:

- it can contain binding sites for enzymes, because many disaccharides (e.g. sucrose and cellobiose) and small peptides need to be degraded to monomers before they can pass through the cell membrane, and this is typically achieved by the actions of wall-bound enzymes;
- the wall also can have surface components that mediate the interactions of fungi with other organisms, including plant and animal hosts (Deacon, 2006).

Fungi have a distinctive range of wall components, usually chitin or glucans), however do not have cellulose-rich cell walls. Characteristic range of soluble carbohydrates and storage compounds, which are mannitol and other sugar alcohols, trechalose, glycogen. Devoid of chlorophyll. There are two main wall-synthetic enzymes anchored in fungi plasma membrane, chitin synthase and glucan synthase, are integral membrane proteins. Typically contains ergosterol as the main membrane sterol.

Fungi are heterotrophs, they need organic compounds as source. They release digestive enzymes and absorb simple and soluble nutrients through the wall and cell membrane (Deacon, 2006; Perevedenceva, 2009).

Generally, they are aerobic. Optimal growth temperature +15+30°C, however this condition is variable, because these organisms are very adaptable (Deacon, 2006; Perevedenceva, 2009). They inhabit the land, air and waters of the Earth (HCMI, 2011).

Fungi typically have haploid nuclei – an important difference from almost all other eukaryotes. However, fungal hyphae often have several nuclei within each hyphal compartment, and many budding yeasts are diploid.

Reproduction in fungi occurs in three manners:

- Vegetatively (fragmentation, fission, budding, sclerotia, rhizomorphs);
- Asexual, by spores (exospores – conidia, and endospores);
- Sexual (planogametic copulation, gametangial contact, gametangial copulation, spermatization and somatogamy). (Samiksha, in 01.11.2017)

All fungi require organic nutrients for their energy source and as carbon nutrients for cellular synthesis. But a broad distinction can be made according to how these nutrients are obtained:

- by growing as a parasite (or a pathogen – a disease-causing agent) of another living organism;

- by growing as a symbiont in association with another organism;
- by growing as a saprotroph (saprophyte) on nonliving materials (Deacon, 2006).

They are more highly developed than bacteria and viruses and there are many more species than are found in the microbes (HCMI, 2011). In terms of biodiversity there are estimated to be at least 1,5 million different species of fungi, but only about 75 000 (70 000 to 120 000, from the other sources) have been described to date. Estimates, based on high-throughput sequencing methods, suggest that as many as 5,1 million fungal species exist (Blackwell, 2011). However, one of the authors of the first referred prevision in 1,5 million species, with basis on new data (fungus ratio and environmental sequence studies) conclude, that the commonly cited estimate of 1.5 million species is conservative and that the actual range is properly estimated at 2.2 to 3.8 million species (Hawksworth *et al*., 2017).

All these specific characteristics in physiology and morphology of the fungi still represent a problem for taxonomic and systematic studies, and consequentially, for identification (Perevedenceva, 2009)

In contrast to the many fungal parasites of plants, there are only some 200 fungi that infect warm-blooded animals and humans (Deacon, 2006).

2. Mycosis

Mycosis - any infection or disease caused by fungus. Mycosis can affect many areas of the body, more commonly the skin. Fungi adversely affect human or animal health through three processes – allergy, infection or toxicity (HCMI, 2011). Fungi produce metabolites. During colonization fungi secrete enzyme to digest organic materials into simpler compounds. The simpler compounds are primary and secondary metabolites. The secondary metabolites are called mycotoxins. In nature mycotoxins are elements of defense and provide competitive advantage. But they are toxins, resistant to decomposition by temperature and digestion, and in multicellular organism have an immunological effect, organ-specific toxicity, lead to cancer and in some cases, to death.

Usually, they are opportunistic pathogens. While plants, animals and humans are alive and well, the fungi around are unable to overcome the natural defense mechanisms which higher forms of life possess (HCMI, 2011).

Growing number of mycosis could be explained:

- statement of weakness of animal/human immune system;

- preventive or long-termed antibiotic therapy against bacteria´s;
- strong relations between humans and some worm-blooded animal species;
- growing globalization and circulation of persons and goods;
- etc.

The most common types of fungal infections include athlete's foot, jock itch and ringworm (dermatophytosis). Another common type of fungal infection is a yeast infection, caused by the fungus Candida.

3. Dermatophytosis: overview

Dermatophytosis – is a skin disease, caused by a superficial fungal infection of keratinized skin structures by fungal organisms called dermatophytes, mostly belonging to the genera *Microsporum*, *Trichophyton*, *Epidermophyton* and *Arthroderma* (Varrier *et al*., 2012; Moriello *et al*., 2017). Regarding dogs and cats, normally involving genera are *Microsporum* and *Trichophyton* (Menelaos, 2006; Moriello *et al*., 2017)

Infection is usually cutaneous and restricted to the non-living cornified layers because of an inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts. However, reactions to the dermatophyte infection may range from mild to severe, and it depends on the hosts response to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection, and local environmental factors (Weitzman *et al*., 1995), The disease transmission occurs by direct contact with a source of mycotic infection (a sick or carrier organism) or in contact with environmental objects contaminated with dermatomycetes (Cafarchia *et al*., 2006)

Since the pathogenic process, as a rule, begins and finish at the skin, it is important to know the structure and function of the skin and it´s appendage. It is a one of the critical points in treatment, general diagnosis approach, and has a particular significance in sampling and posterior identification of dermatophytes.

3.1 Skin and it´s appendages

The skin is the largest organ of the body and, depending on the species and age, may represent 12%–24% of an animal's body weight. The skin has many functions, including serving as an enclosing barrier and providing environmental protection, regulating temperature, producing pigment and vitamin D, and sensory perception. Anatomically, the skin consists of the following structures: epidermis, basement membrane zone, dermis, appendage system, and subcutaneous muscles and fat (**Figure 1.1**)

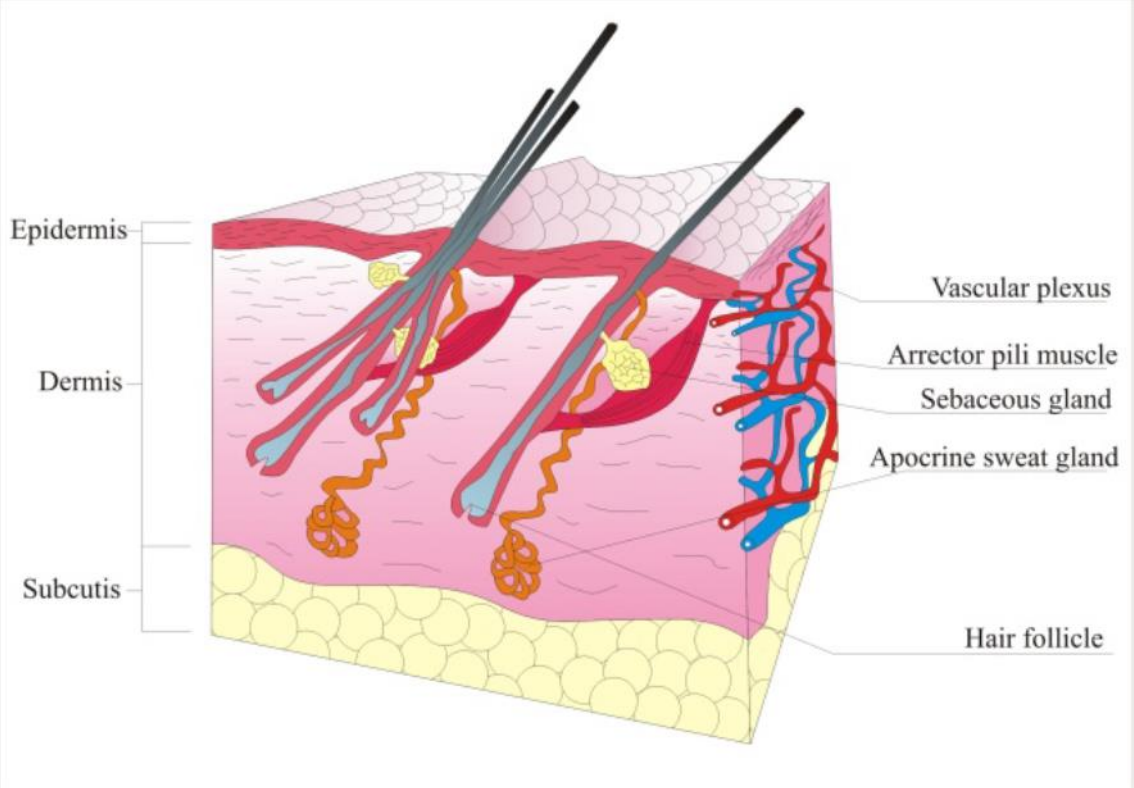


Figure 1.1 - Structure of the skin (Adapted from Bourguignon *et al*., 2013).

- Epidermis.

The epidermis is composed of multiple layers of cells consisting of keratinocytes, melanocytes, Langerhans cells, and Merkel cells.

Keratinocytes function to produce a protective barrier. They are produced from columnar basal cells attached to a basement membrane. The rate of cell mitosis and subsequent keratinization are controlled by a variety of factors, including nutrition, hormones, tissue factors, immune cells in the skin, and genetics. The dermis may also exert significant control over the growth of the epidermis. As keratinocytes migrate upward, they undergo a complex process of programmed cell death or keratinization. Keratin is a protein composed of microfibrils, with low sulfur content, immersed in an amorphous matrix, rich in sulfur. The goal of

this process is to produce a compact layer of dead cells called the stratum corneum, which functions as an impermeable barrier to the loss of fluids, electrolytes, minerals, nutrients, and water, while preventing the penetration of infectious or noxious agents into the skin. The structural arrangement of keratin and the lipid content of the skin are critical to this function. The vitamin D precursor, 7-dehydrocholesterol, is formed in the epidermis. The epidermis is thickest in large animals. The stratum corneum is continuously shed or desquamated.

Melanocytes are located in the basal cell layer, outer root sheath, and ducts of sebaceous and sweat glands. They are responsible for the production of skin and hair pigment (melanin). Production of pigment is under hormonal and genetic control.

Langerhans cells are mononuclear dendritic cells that are intimately involved in regulating the immune system of the skin. They are damaged by excessive UV light exposure and glucocorticoids. Antigenic and allergenic material is processed by these cells and transported to local and nodal T cells to induce hypersensitivity reactions. Epidermal proteins may also conjugate with exogenous haptens, rendering them antigenic.

Merkel cells are specialized sensory cells associated with skin sensory organs, i.g, whiskers and tylotrich pads.

- Basement membrane zone.

This area serves as a site for attachment of basal epidermal cells and as a protective barrier between the epidermis and dermis. A variety of skin diseases, including several autoimmune conditions, can cause damage to this zone. Vesicles are an example of a damaged basement membrane zone

- Dermis.

The dermis is a mesenchymal structure that supports, nourishes, and to some degree, regulates the epidermis and appendages. The dermis consists of ground substance, dermal collagen fibers, and cells (fibroblasts, melanocytes, mast cells, and occasionally eosinophils, neutrophils, lymphocytes, histiocytes, and plasma cells). Blood vessels responsible for thermoregulation, nerve plexuses associated with cutaneous sensation, and both myelinated and unmyelinated nerves are present in the dermis. Motor nerves are primarily adrenergic and innervate blood vessels and arrector pili muscles. Apocrine glands do not appear to be innervated. Sensory nerves are distributed in the dermis, hair follicles, and specialized tactile structures. The skin responds to the sensations of touch, pain, itch, heat, and cold (Moriello, 2016).

- Appendage system.

These structures grow out of (and are continuous with) the epidermis and consist of hair follicles, sebaceous and sweat glands, and specialized structures (eg, claw, hoof). The hair follicles of dogs and cats are compound, i.e. the follicles have a central hair surrounded by 3–15

smaller hairs all exiting from a common pore. Animals with compound hair follicles are born with simple hair follicles that develop into compound hair follicles. For understanding of the anatomy of the hair follicle, it can be divided into 3 regions: the lower segment (bulb and suprabulb), the middle segment (isthmus), and the upper segment. The upper segment extends from the entrance of the sebaceous gland duct to the follicular orifice and is called the infundibulum. The epithelium of the infundibulum is continuous with the epidermis; thus its cells can regenerate the epidermis and replenish it after wounding or injury. This uppermost part of the follicle is the first structure in the developing skin to contain keratohyalin granules and keratin. Although the epithelium is similar in appearance to the epidermis, its cells appear to have a higher proliferative capacity and they can regenerate the after its removal. The lumen of the infundibulum normally contains the hair shaft, keratin material and sebum. The isthmus is the shortened segment of the hair follicle, extending from the attachment of the erector pili muscle (bulge region) into the entrance of the sebaceous gland duct. It is a border zone peculiarly devoid of specific features. The outer root sheath of the isthmus, in contrast to the epithelium of the infundibulum, has no granular layer and its cells have pale cytoplasm, an indication of an increased amount of glycogen. The isthmus has a distinctive corrugated, compact, eosinophilic keratinization called trichilemmal keratinization, a sharp contrast to the basket-weave keratinization of the infundibulum. Trichilemmal keratinization converts the stratified epithelium of the outer root sheath into a nuclear keratin without an intervening keratohyalin layer. The bulge is composed of a biochemically distinct population of keratinocytes that possesses the characteristics of epithelial stem cells. Keratinocytes in the bulge area are relatively undifferentiated structurally. They are normally slow cycling but can be stimulated to proliferate transiently. Moreover, they are multipotent, giving rise to several different cell types including epidermal keratinocytes, sebaceous gland cells, and at least seven different types of epithelial cells in the lower follicle. The suprabulbar area of the follicle is the region below the isthmus and above the bulb. This region is comprised of three layers from outermost to innermost: outer root sheath, inner root sheath, and hair shaft. The inner root sheath typically features three distinct layers of epithelial cells, which are known as Henle's layer, Huxley's layer, and the inner root sheath cuticle (from outermost to innermost). However, in the suprabulbar region, these three layers completely keratinize and become indistinguishable. The cells at the periphery of the outer root sheath are columnar and walled. The outer root sheath cells progressively contain less glycogen in the superior portion (Souza *et al*., 2009).

The growth of hair is controlled by several factors, including nutrition, hormones, and photoperiod. The hairs have three distinct regions, the cuticle, the cortex and the medulla. The cuticle is a monolayer of keratinized and anucleated cells that are interdigitalized with the cuticle of the inner root sheath. The cortex is formed by several layers of cells fusiform and keratinized ones that contain hard keratin. The medulla is formed by rows of cuboid cells or flattened cells. Size, shape, and length of hair is controlled by genetic factors but may be influenced by disease, exogenous drugs, nutritional deficiencies, and environment. Hormones have a significant effect on hair growth. The primary functions of the hair coat are to provide a

mechanical barrier, to protect the host from actinic damage, and to provide thermoregulation. In most species, trapping dead air space between secondary hairs conserves heat. This requires that the hairs be dry and waterproof; the cold-weather coat of many animals is often longer and finer to facilitate heat conservation. The hair coat can also help cool the skin. The warm-weather coat of animals, particularly large animals, consists of shorter thicker hairs and fewer secondary hairs. This anatomic change allows air to move easily through the coat, which facilitates cooling (Souza *et al*., 2009).

Sebaceous glands are simple or branched alveolar, holocrine glands that secrete sebum into the hair follicles and onto the epidermal surface. They are present in large numbers near the mucocutaneous junction, interdigital spaces, dorsal neck area, rump, chin, and tail area; in some species, they are part of the scent-marking system. Cats mark territories by rubbing their face on objects and depositing a layer of sebum laced with feline facial pheromones. Sebum is a complex lipid material containing cholesterol, cholesterol esters, triglycerides, diester waxes, and fatty acids. Sebum is important to keep the skin soft and pliable and to maintain proper hydration; it gives the hair coat sheen and has antimicrobial properties.

Sweat glands (epitrichial, formerly apocrine, and atrichial, formerly eccrine) are part of the thermoregulatory system. There is some clinical evidence to suggest that limited sweating and it´s evaporation occurs in dogs and cats, and that it may have a minor role in cooling the body. Dogs and cats thermoregulate primarily by panting, drooling, and spreading saliva on their coats (cats). Cats also sweat through their paws, especially when excited; this is most commonly seen as wet paw prints on surfaces, eg, examination tables.

The subcutaneous fat (panniculus adiposus) serves many functions, including insulation; reservoir for fluids, electrolytes, and energy; and shock absorber (Moriello, 2016).

3.2 Pathogenesis

The possible route of entry for the dermatophytes into the host body is injured skin, scars and burns. However, unlike other fungi, dermatophytes are able to cause superficial infections in healthy, immune-competent individuals as well as in those with immune dysfunction. Infection is caused by arthrospores or conidia. Resting hairs lack he essential nutrient required for the growth of the organism. Hence these hairs are not invaded during the process of infection. The pathogen invades the uppermost, non-living, keratinized layer of the skin namely the stratum corneum, produces exoenzyme keratinase and induces inflammatory reaction at the site of infection. The customary signs of inflammatory reactions such as redness (ruber), swelling (induration), heat and alopecia (loss of hair) are seen at the infection site. Inflammation causes the pathogen to move away from the site of infection and take residence at a new site. This movement of the organism away from the infection site produces the classical ringed lesion. Host immune response to the invading pathogen is responsible for the clinical manifestations. The fungal pathogens induce both, immediate hypersensitivity as well as cell

mediated or delayed type hypersensitivity. The fungal growth is restricted by the inflammatory reactions, produced as a result of infection with dermatophytes. The ability of dermatophytes to adhere to these substrates and adapt to the host environment is essential for the establishment of infection. Several fungal enzymes and proteins participate in this adaptive response to the environment and to keratin degradation. The expression of fungal adhesins on the cell surface allows for rapid attachment to host tissue and the extracellular matrix, preventing elimination of the pathogen by the host defense mechanisms. Transcription factors such as *PacC* and *Hfs* 1, as well as heat shock proteins, are involved in sensing and adapting to the acidic pH of the skin in the early stages of fungal–host interaction. Once enclosed in the host tissue, the pathogen must scavenge nutrients to survive while evading innate immune cells and molecules. Fungi secrete a broad spectrum of enzymes to degrade host cells, such as proteases including collagenolytic and elastolytic enzymes, lipases, nucleosidases, and mucolytic enzymes. During dermatophyte growth, with keratin as the sole carbon source, the extracellular pH shifts from acidic to alkaline. This creates an environment in which most of the known keratinolytic proteases exhibit optimal activity. These events culminate in the establishment and maintenance of the infection (**Figure 1.2**), which can be chronic or acute depending on the dermatophyte species (Lakshmipathy *et al*., 2010; Martinez-Rossi *et al*., 2016).

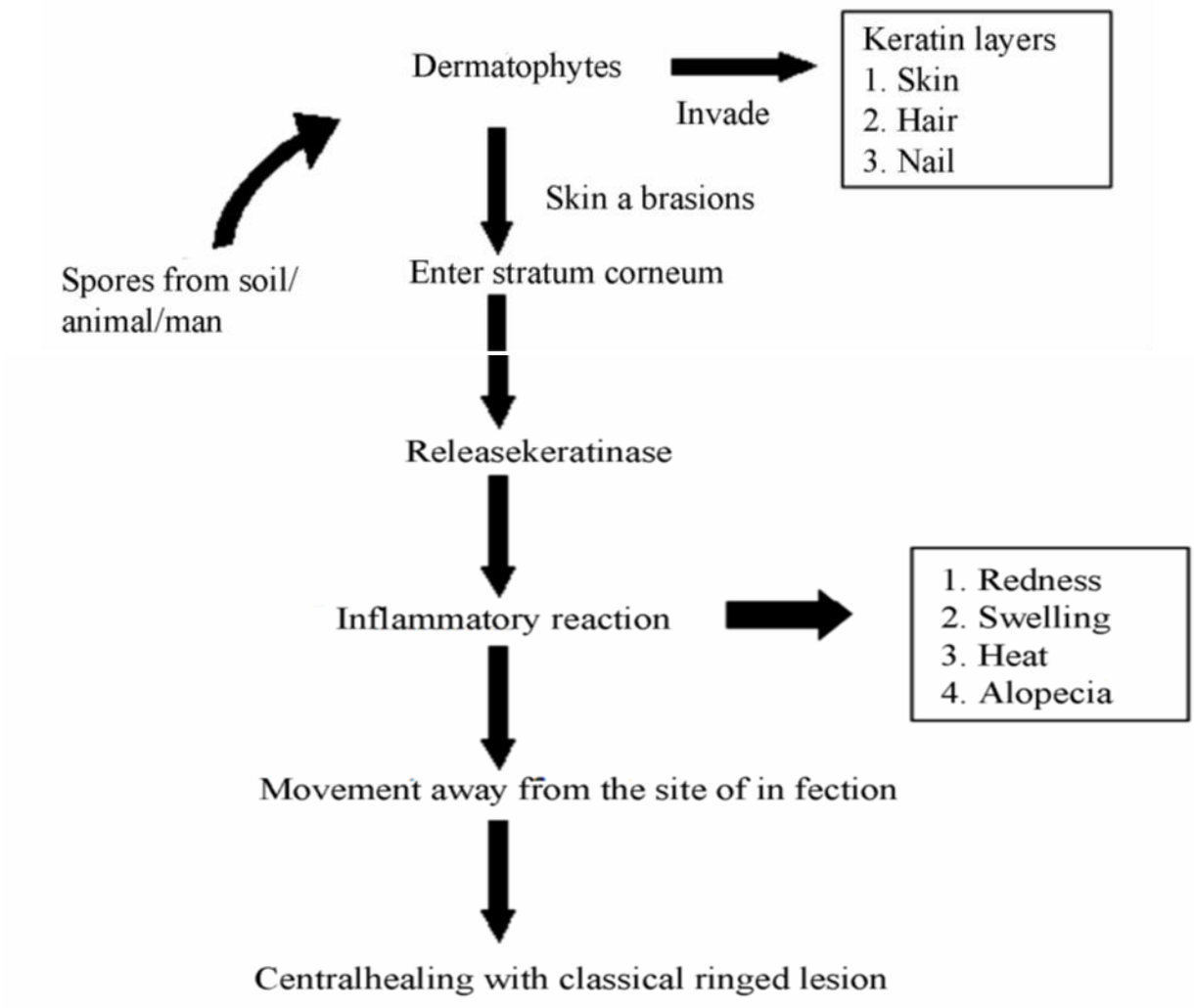


Figure 1.2 - The schematic route of entry of dermatophytes into the host system and onset of immune response in the host in response to the pathogen entry.

(Adapted from Lakshmipathy *et al*., 2010).

Biofilm formation by dermatophytes can explain dermatophytomas (chronic dermatophytosis local lesion of skin), there are confirmed *in vitro* formation of biofilm by two of the most prevalent species worldwide: *Trichophyton rubrum* and *T*. *mentagrophytes* (Costa-Orlandi *et al*., 2014).

3.3 Clinical presentation

The clinical signs of dermatophytosis are extremely variable, and the owners are many times unaware that their pet is infected until they themselves contract the infection (Bernardo *et al*., 2005).

The general presentation of ringworm in animals is a regular and circular alopecia, with erythematous margin and a thin desquamation. Pruritus is generally absent although described in a noticeable proportion of animals in some surveys. Lesions can be single or multiple and are localized on any part of the animal although the anterior part of the body and the head seem more frequently involved. Usually, there is a centrifugal spread of lesions. Multiple lesions may coalesce, while a spontaneous healing at the center with regrowth of hairs is generally observed.

Kerion (nodular dermatophytosis) is a round and nodular edematous reaction to dermatophyte infection, with a patch of erythematous scaling alopecia, characterized by a granulomatous dermatitis to a deep pyogranulomatous inflammation. Mycetoma-like (chronic subcutaneous infection) lesions have been reported, mostly in Persian cats, in which the dermatophyte develops in the dermis and the subcutis (Mattei *et al*., 2014).

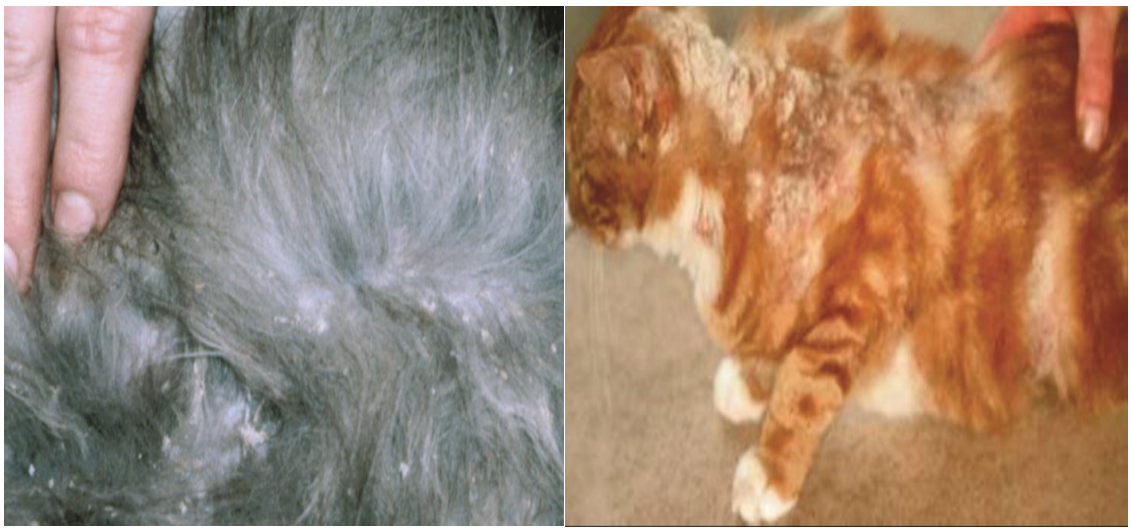
Feline and canine dermatophytosis causes so many different clinical pictures that they could reasonably be included in the differential diagnosis of all skin diseases in cats and dogs. Signs and symptoms vary greatly with the host-fungus interaction.

Canine dermatophytosis is characterized by typical round alopecic lesions and brittle hairs. The scaly crusted lesions may be single or multi- focal and are rarely symmetrical. Local or widespread folliculitis may be observed, with or without furunculosis. Other clinical signs include dry seborrhea, focal or multi-focal crusted dermatitis with a well-defined erythematous margin, kerion. In dogs *M. canis*-related dermatophytosis usually presents with more marked inflammation than in cats. Scaly or papule-pustular and crusted facial lesions could be present. *M. gypseum* or *T. mentagrophytes* often cause a deep, infiltrated inflammatory swelling, with a damp, ulcerated pus-exuding surface and is often associated with secondary bacterial infection

(**Figure 1.3**). Lesions of the nail is very rare in dogs and usually caused by *M. gypseum* or *T. mentagrophytes* (Moretti *et al*., 2013).

Figure 1.3 - Superficial lesions of a dog with dermatophytosis (Adapted from Bahri, 2013).

Clinical pictures in cats are very polymorphous (**Figure 1.4**). Alopecic and inflamed lesions are uncommon and healthy carriers are often found. Typical lesions observed in kittens are non-inflammatory alopecic areas, with central desquamation, which are surrounded by brittle or easy to extract hair. Other forms are characterized by small, crusted scaly, sometimes itchy, lesions. Other aspects are multifocal distribution of skin lesions with no identifiable pattern and ring-shaped lesions with inflammation or papules on the periphery and hair regrowth in the center. Deep and exudative infection is rare (Moretti *et al*., 2013).



A B

Figure 1.4 – Superficial lesions of a cat with dermatophytosis

A) Immunocompetent adult cat (Adopted from Frymus *et al*., 2017).
B) Immunocompromised adult cat (Adopted from Feline Advisory Bureau.uk)

3.4 Epidemiology and ecology

At the world level this is one of the most frequent fungal infections with zoonotic character, affecting humans and many species of animals: dogs, cats, horses, cattles, rabbits and wild animals (Moretti *et al*., 2013). In Portugal particularly, speaking in cats and dogs, dermatophytosis are a common infectious skin diseases, especially during Spring and Autumn (Bernardo *et al*., 2005).

Massive genome-sequencing project provided an analysis of gene content and conservation across different dermatophyte species. Comparative genome analyses revealed few differences in genome organization and content among the species analyzed, suggesting that differences in gene regulation and post-transcriptional mechanisms might be responsible for the niche-specific adaptation of the species and even strains (Achterman *et al*., 2011; Martinez *et al*., 2012).

In according to one of classifications the dermatophytes divided into next categories - zoophilic, geophilic and anthropophilic, depending on their major reservoir in nature – animals, soil or humans, respectively (**Table 1.1**) (Varrier *et al*., 2012; Behzadi, *et al*., 2014; Moriello *et al*., 2017).

Table 1.1 - Examples of dermatophyte species classified in three types: anthropophilic, zoophilic and geophilic (Adapted from Aneja, K.L. *et al*., 2012).

Anthropophilic	Zoophilic	Geophilic
Epidermophyton floccosum	*Microsporum spp.*	*Microsporum spp.*
Microsporum spp.	*M.canis*	*M.gypseum complex*
M.audouinii	*M.galinae*	*M.praecox*
M.ferrugineum	*M.nanum*	*M.racemosum*
Trichophyton spp.	*M.persicolor*	*M.vanbreuseghemii*
T.concentricum	*Trichophyton spp.*	*Trichophyton vanbreuseghemii*
T.megninii	*T.equinum*	
T.mentagrophytes complex (velvety and cottony isol.)	*T.erinacei*	
	T.mentagrophytes comlex (granular isol.)	
T.rubrum	*T.simii*	
T.schoenlenii	*T.verrucosum*	
T.soudanense		
T.tonsurans		
T.violaceum		

The most commonly pathogens of dermatophytosis in cat´s and dog´s belongs to genera *Microsporum* and *Trichophyton* (Mattei *et al*., 2014; Moriello *et al*., 2017). Within these genera was identified the species of dermatophytes that isolate with the highest frequency from animal samples (**Table 1.2**).

Anthropophilic dermatophytes are primarily associated with humans and rarely infect other animals. Zoophilic dermatophytes usually infect animals or are associated with animals but occasionally infect humans. Geophilic dermatophytes are primarily associated with keratinous materials such as hair, feathers, hooves, and horns after these materials have been dissociated from living animals and are in the process of decomposition. These species may cause human and animal infection. Geophilic species are thought to have been ancestral to the pathogenic dermatophytes, preadapted to cutaneous pathogenesis by their ability to decompose keratin and their consequent close association with animals living in hair and feather-lined nests in contact with soil (Weitzman *et al*., 1995).

Table 1.2 - The most frequent zoophilic and geophilic dermatophyte species in animals.

Adapted from Mattei *et al*., 2014

Dermatophyt species	**Main source**	**Others**
Microsporum canis	Cat, dog, horse	All mammals
Microsporum gallinae	poultry	Dog, cat
Microsporum gypseum	Soil	All mammals
Microsporum nanum	Soil	Pig
Microsporum persicolor	Microtid rodents	Dog, cat
Trichophyton equinum	Horse	Cat, dog (rare)
Trichophyton erinacei	Hedgehog	Dog
Trichophyton mentagroph.	Rodents	All mammals
Trichophyton simii	Primate	Fowl, dog, cat
Trichophyton verrucosum	Cattle, other ruminants	All mammals

3.5 Zoonotic reflections

Pet-associated illnesses can occur in any individual, but people at the extremes of age (<5 years of age; ≥65 year), pregnant women or people with immunocompromised conditions are at greater risk. Immunocompromised individuals include, but are not limited to: congenital immunodeficiency, transplant recipients (bone marrow and solid organs), infectious diseases (e.g. HIV), metabolic diseases (e.g. diabetes mellitus, chronic kidney failure), splenectomy, cancers, and treatments with immunosuppressive drugs or chemotherapeutics (Hayette *et al*., 2015; Seyedmousavi *et al*., 2015). Small animal dermatophytosis is a pet-associated zoonosis. The disease is primarily transmitted from contact with the hair coat or skin lesions of an infected animal. Contact with accumulated scales and hair in the environment are possible sources (Cafarchia *et al*., 2006; Moriello *et al*., 2017; Saraiva, 2017).

Understanding the clinical pictures in animals as well as the complex epidemiological and management issues (as a distribution, prevalence, incidence of dermatophytosis in animal species; analysis of the animal's habitat for making a predictive risk assessment of transmission to the receptive host) will provide the dermatologist with in-depth information for treating and managing the infection in humans (Moretti *et al*., 2013).

None of the patients died from the dermatophyte infection and the disease was treatable with the most common complication being prolonged treatment (Moriello *et al*., 2017).

Changes in a lifestyle inevitably lead to the changes in the spectrum of fungal biota, responsible for skin infections. Thus, it is possible to assume that, due to the improvement of hygiene, generalization of animal's quarantine and modern lifestyle, these organisms will change from zoophilic group (*M. canis* and *T. mentagrophytes*) to anthropophilic group of species (*T. rubrum, T. tonsurans* and *T. violaceum*), which transmit in a latent way and cause mild inflammation instead of severe.

Interdisciplinary cooperation is needed to control this kind of disease and achieve maximum results, including efforts from public health organization, accurate diagnosis from clinical works, governmental intervention and social surveillance (Skerlev *et al*., 2010; Zhan *et al*., 2016).

3.6 Current state of diagnosis

The definitive diagnosis of clinical dermatophytosis should be performed before the start of antifungal medication, since the prescribed drugs, besides having high costs, have undesirable side effects (Calvacante Bin, *et al*., 2010).

3.6.1 Routine diagnostic techniques

Dermatophytosis diagnosis is based on the history of the patient, physical examination, microscopic examination of scraping and hairs from the lesions in conjunction with fungal cultures, histology of the tissues and other techniques like a Wood´s lamp examination.

The application of molecular techniques for routine diagnosis of dermatophytosis is still limited, particularly in veterinary medicine. Therefore, they are used mostly for scientific proposes.

Among the routine diagnosis techniques, the **direct microscopic examination** is a simple and rapid method to detect dermatophytes on hairs and scales. Used to confirm the presence of a dermatophyte infection it involves the examination for hyphae and/or fungal spores, allow the rapid confirmation of infection. Hairs and scales are mounted in solutions (DMSO, potassium hydroxide, mineral oil of varying concentrations) to aid in visualization of fungal elements by microscopic examination (Moriello *et al*., 2017). This technique originates false-positive result, especially in presence of saprophytic fungi or due to the wrong interpretation of structural elements. Rendering sensitivity relatively poor (Frymus *et al*., 2013).

Dermoscopy (epiluminescence microscopy) is a noninvasive method, performed using an illuminated camera, allowing the *in vivo* evaluation the colors and microstructures of the epidermis, dermo-epidermal junction and papillary dermis not visible to the naked eye. It is based on the identification of specific diagnostic patterns, such as comma-like hairs in infected cats. Is a clinical tool, being a frequently used with or without concurrent use of Wood´s lamp, to identify hairs for culture and/or direct examination (Moriello *et al*., 2017; Dong *et al*., 2016).

The results show this technique may be suspended by several factors, namely patient cooperation, focus only on certain small area and dependence on experience and skill of the examiner

Fungal cultures are often stated as the gold standard method for dermatophyte diagnosis. This method is very sensitive and can determine the species (Frymus *et al*., 2013). Some of the culture media that can be used: Sabouraud dextrose agar (SDA), Dermatophyte Test Medium (DTM), Mycosel, Cooke Rose Bengal Agar (CRB agar). They are selective media for growth and isolation of fungi, usually incubated for 2 – 3 weeks at +25º to +30ºC, allowing colony development and subsequent identification, based on macro and microscopic examination.

However, false-positive and false-negative result can also occur; due to sampling techniques, storage and incubation of cultures and result interpretation (Moriello *et al*., 2017).

Wood´s examination is performing by using a Wood´s ultraviolet lamp to detect the presence of dermatophyte fungi by emission of fluorescence. Fluorescence occurs when light of

shorter wavelengths initially emitted by the lamp are absorbed, and radiation with longer wavelengths is emitted (longer than 400 nm in this case). Many microorganisms produce phosphors as result of their growth on skin and/or on hairs, and this characteristic can aid in detection or confirmation of infection. Fluorescence develops as early as day 5 – 7, and usually by day 10 – 14 post-infection. Apart from *Trichophyton schoenleinii*, dermatophytes that produce fluorescence are members of the *Microsporum* genus. The characteristic green fluorescence observed on *M*. *canis*-infected hair shafts is due to the production of a water-soluble chemical metabolite (pteridine) located within the cortex or medulla of the hair.

Nevertheless, there are some strains of *M*. *canis* that show negative florescence. The sensitivity of the exam depends on the distance between lamp and skin, that must be close, not more than 10-12 cm. Also, some drugs can destroy fluorescence (Moriello *et al*., 2017).

There is a study, that compare results of testing (sensitivity) of above-mentioned identification techniques (**Table 1.3**).

Table 1.3 - Results of direct microscopic examination, fungal culture and fluorescence in light of Wood´s lamp, in relation to gender for 71 dogs. Adapted from Calvacante *et al*., 2010.

	Direct microscopy			**Culture**			**Fluorescence**		
Gender	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total
Male	28(39%)	11(15%)	39(55%)	7(10%)	32(45%)	39(55%)	11(15%)	28(39%)	39(55%)
Female	23(32%)	9(13%)	32(45%)	6(8%)	26(37%)	32(45%)	14(20%)	18(25%)	32(45%)
Total	51(72%)	20(28%)	71(100)	13(18%)	58(82%)	71(100)	25(35%)	46(55%)	71(100%)

3.6.2 Molecular identification based on PCR

There are many published reports on the identification of *Microsporum* and *Trichophyton* via PCR, but studies on the use of PCR on clinical specimens from veterinary patients are scarce (Garg *et al*., 2009; Bergman *et al*., 2013; Moriello *et al*., 2017).

One-step PCR is conferred to be highly accurate (AUC>90) for the testing of samples from dogs, but only moderately accurate (AUC=78,6) for cats (Cafarchia *et al*., 2013).

Nested-PCR is described as accurate (AUC=93,6) for dermatophyte identification from samples from cats, and achieved high specificity for dog's sample (AUC=94,1 – 94,4). Sensitivity vary between 94,9 and 100% for detection in samples from cats and dogs

respectively. Technique non-already applied to the differentiation of *M*icrosporum *canis* from *Trichophyton interdigitale*, and geophilic dermatophytes (Cafarchia *et al*., 2013).

Real-time PCR presents sensitivity (100%, with confidence interval 95%) and specificity (88,5%, with confidence interval 95%) for diagnosis of *M*. *canis* in cats. In a study performed by Jacobson at al. at 2017 no false-negative results were observed, but false-positive results were relatively common (Jacobson *et al*., 2017).

Identification by **Real-time multiplex PCR** is commonly applied to clinical specimens from humans for the accurate identification of casual and common agents. In comparison with the conventional 'gold standard' procedures for dermatophyte diagnostics, positive predictive value of the multiplex assay is 95,7%, while the negative predictive value is it 100% (Arabatzis *et al*., 2007).

A **Real-time LightCycler PCR** protocol followed by **RFLP** is also available for clinical specimens from humans. Using seven primer sets specific for fungal DNA. Two subsequent LightCycler PCR reactions and one RFLP reaction allow the differentiation of dermatophytes and non-dermatophyte molds and the sub classification of yeasts. However, melting point detection is much more sensitive than detection of bands on agarose gels after restriction digestion (Gutzmer *et al*., 2004).

Even having a sensitivity, specificity and accuracy far above the ones from conventional diagnostic methods for dermatophytosis, PCR techniques may vary between themselves, and results depend on some critical points, including the presence of the other skin lesions at the sampling site, already established antifungal treatment, presence of several fungal species in the sample, amount of sample available for PCR assay, presence of dead fungal material in the sample and the stage of the infectious process from which the sample was obtained.

4. Dermatophytes

Dermatophytes – highly specialized pathogenic fungi that are the most common agents of superficial mycoses. Dermatophytes as keratinophilic fungi able to infect keratinous tissues of skin (the stratum corneum layer), hair, and nail in humans via their keratinase enzymes. They also degrade claws, feathers, hooves, horns, wools in animals (Behzadi *et al*., 2014).

In nature these pathogenic fungi are saprophytic microorganisms (Dabas *et al*., 2017)

4.1 Classification

Based on conidia morphology, three genera of dermatophytes were recognized: *Trichophyton*, *Microsporum*, and *Epidermophyton* (Weitzman *et al*., 1995).

However, classification depending on reproduction type of fungi include four genera, i.e., *Microsporum*, *Trichophyton*, and *Epidermophyton* are anamorphic (asexual or imperfect) and *Arthroderma* (their telomorph or perfect, in sexual reproductive stage) (Weitzman *et al*., 1995; Aneja *et al*., 2012).

Fungal identification has been based traditionally on subjective morphological and phenotypic characteristics, often leading to multiple names for a single species or, conversely, a single name for distinct species, resulting in erroneous species identifications. Traditional methods based on morphological or biochemical characteristics enable, in most cases, genus- and species-level identifications, but they are slow and can be inaccurate. For molecular species identification, an increasingly popular concept of utilizing short DNA sequences, called DNA barcodes, was recommended. A DNA barcode constitutes of short conserved (500- to 800-bp) regions containing species-specific genome diversity. Currently, there is consensus among the mycology community to use the internal transcribed spacer (ITS) sequences of rDNA as the primary barcode for fungi. However, for many taxa, additional barcodes are necessary. These secondary and even tertiary barcodes, usually based on sequences of housekeeping genes, are needed for accurate species identification. The Broad Institute databases have exhaustive sequence repositories for fungal data, with specific links to dermatophytes, dimorphic fungal pathogens, and medically important yeasts. The Dermatophyte Comparative, hosted at the Broad Institute, utilizes an expressed sequence tag (EST) approach and contains genome assemblies and annotations for dermatophytes of the genera *Trichophyton* and *Microsporum* (45). This database is exceptionally useful for the zoophilic, geophilic, and anthropophilic dermatophytes, viz. *Trichophyton rubrum, Trichophyton tonsurans, Trichophyton equinum, Microsporum canis*, and *Microsporum gypseum*, and has features for comparative genome studies which are specific to this group, including gain or loss of gene functions and mating competencies. This database is supplemented by the T. rubrum Expression Database (TrED) for specialized analysis of sequence data sets for the aforementioned superficial fungi (Prakash *et al*., 2017).

A recent multilocus phylogenetic study has reviewed the taxonomy of the dermatophytes**.** A phylogenetic tree was constructed for all genera referred right below (**Figure 1**.**5**) *Arthroderma* now contains 21 species, *Ctenomyces* one species, *Epidermophyton* one species, *Lophophyton* one species, *Microsporum* three species, *Nannizzia* nine species and *Trichophyton* 16 species. In addition, two new genera have been introduced: *Guarromyces* containing one species and *Paraphyton* three species. Although the number of genera has increased, the species that are relevant to routine diagnostics now belong to smaller groups, which should enhance their identification (De Hoog *et al*., 2017).

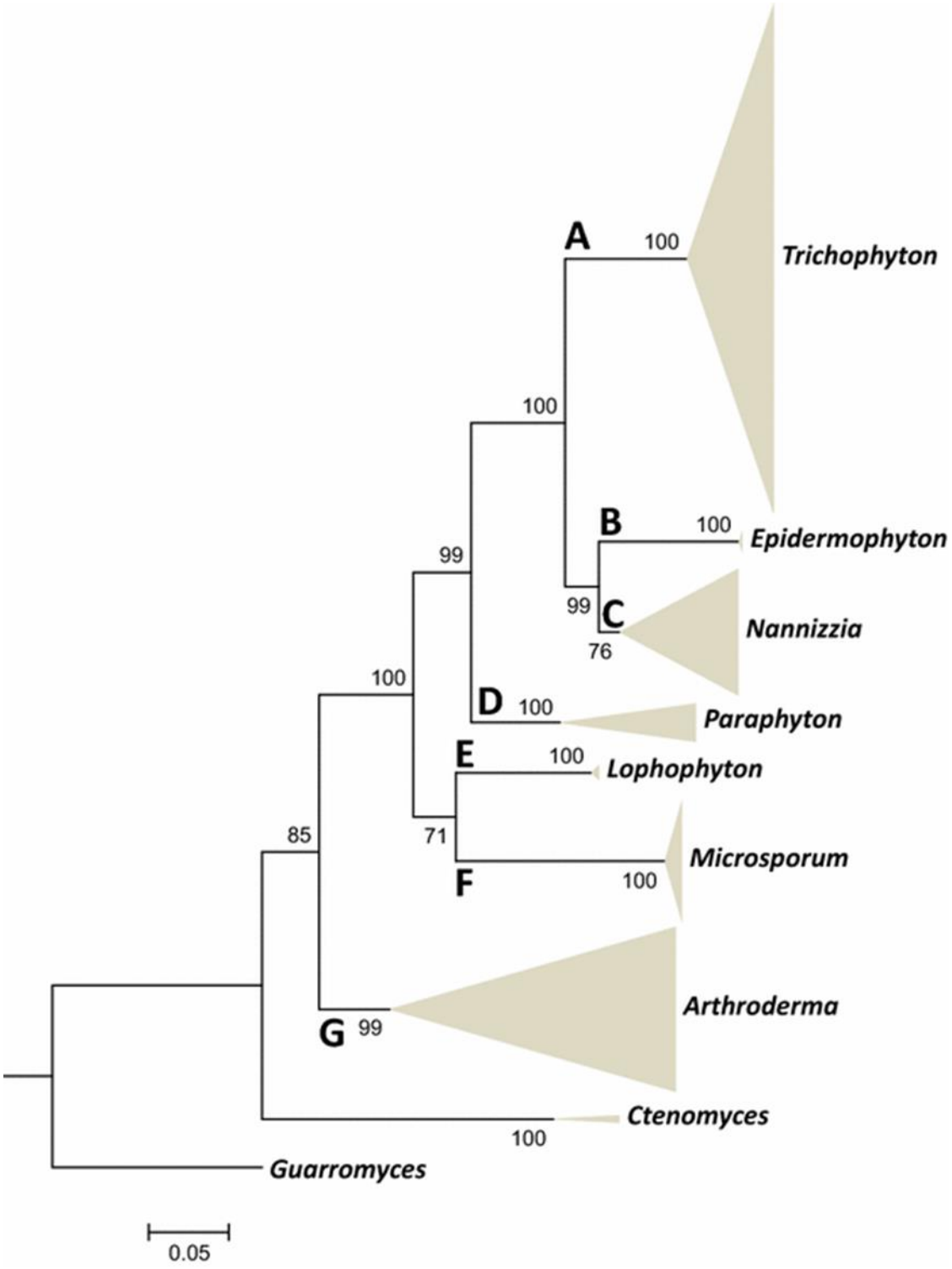


Figure 1.5 - Phylogenetic tree based on ITS and partial LSU, *TUB* and 60S L10 sequences of *Arthrodermataceae* species. *Guarromyces ceretanicus* was selected as outgroup (Adapted from de Hoog *et al*., 2017).

The most frequent etiologic agents of the dermatophytoses in dogs and cats are two genera: *Microsporum* and *Trichophyton* (**Figure 1.6**) (Weitzman *et al*., 1995; Moretti *et al*., 2013; Moriello *et al*., 2017).

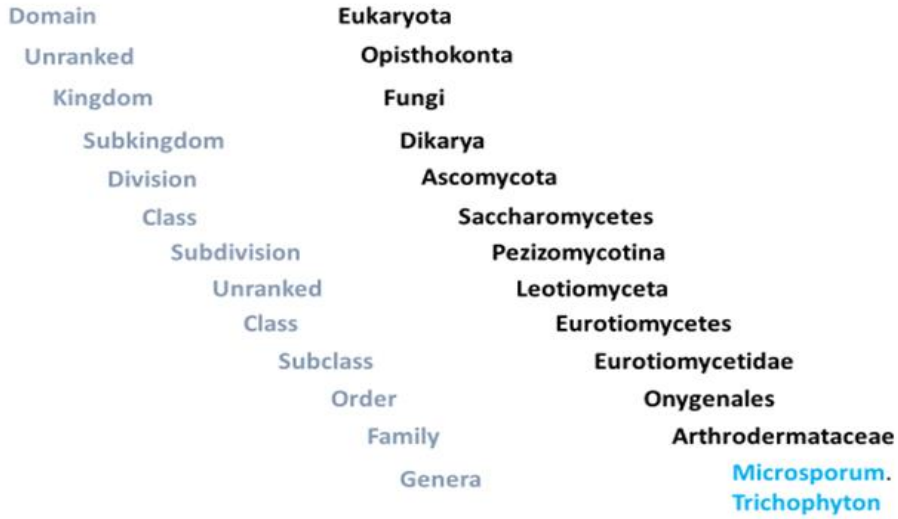


Figure 1.6 - Genera *Trichophyton* and *Microsporum* taxonomy

(Adapted from Encyclopedia of Life, classified by NCBI Taxonomy).

4.2 Genus *Trichophyton*: general description

Sixteen species are now recognized as belonging to the genus *Trichophyton* (The University of Adelaide, Mycology online).

These are found on humans and animals, and in soil and river sediments (Aneja *et al*., 2012).

The genus is characterized morphologically by the development of both smooth-walled macro- and microconidia (Kidd *et al*., 2016). Cultures are whitish to yellowish.

The type species is *Trichophyton mentagrophytes* var. *Mentagrophytes.* The colony aspects are plane, white to cream color, powdery to granular surface and reverse yellowish brown to reddish-brown in Sabouraud dextrose agar at 25°C (Mattei *et al*., 2014).

Macroconidia, if present, is elongated and cigar or pencil-shaped, 8-50 µm × 4-8 µm, with thin and smooth walls. Clavate to fusiform, born laterally, directly from hyphae. Mostly sessile macroconidia differentiate it from *Microsporum* (Kidd *et al*., 2016). Some species rarely produce macroconidia. Microconidia are usually numerous or develop singly along the hyphae or in grape-like clusters, spherical 2.5 to 4 µm or clavate 2-3×3-4 µm (**Figure 1.7**) (Quinn *et al*.,1994; Aneja *et al*., 2012).

In practice, two groups may be recognized microscopically:

- Species, that usually produce microconidia (macroconidia may or may not be present): *T.rubrum, T. interdigitale, T.mentagrophytes, T.equinum, T.eriotephon, T.tonsurans*, lesser extend *T.verrucosum*;
- Species, that usually do not produce conidia (other hyphae stuctures may be present, but microscopy generally non-diagnostic): *T.verrucosum, T.violaceum, T.concentricum, T.schoenleinii, T. soudanense* (Kidd *et al*., 2016).

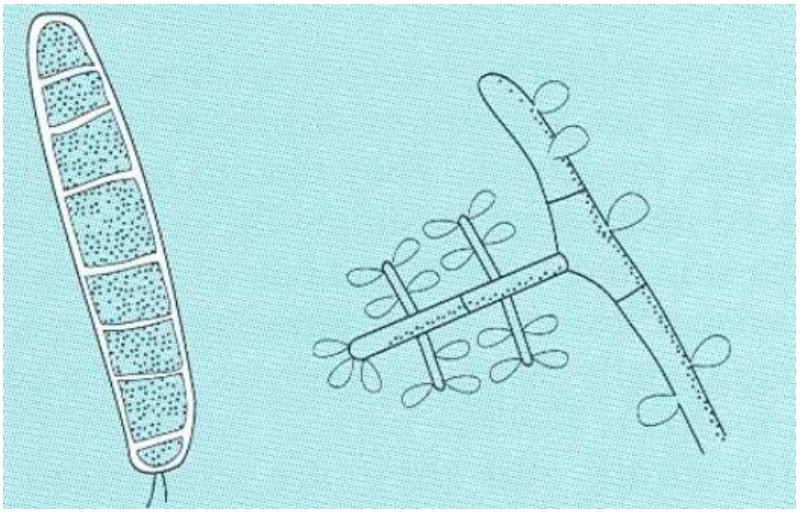


Figure 1.7 - *Trichophyton spp*., macro and microconidia (Adapted from Quinn *et al*., 1994).

4.3 Genus *Microsporum*: general description

The genus now includes only three species: *M. audoiniii, M. canis* and *M. ferrugineum.* (The University of Adelaide, Mycology online).

Cultures are mostly granular to cottony, yellowish to brownish, with a cream-colored or brown colony reverse (Kidd *et al*., 2016).

The type species is *Microsporum canis*. The colony aspects are plane, velvety or cottony surface which may show some radial grooves, white or yellowish color and brown or golden-yellow reverse in Sabouraud dextrose agar at 25°C, but non-pigmented strains may also occur (Mattei *et al*., 2014; Kidd *et al*., 2016).

Microsporum macroconidia are large thick-walled and divided into many cells by transverse septa, presenting a spindle or boat-shaped form. The outer surface of the wall is pitted, a sporulate or spiny. The macroconidia are 35 - 125 µm (sometimes up to 160 µm) × 7-20 µm with 4 to 15 septa (**Figure 1**.**8**) (Aneja *et al*., 2012).

Microconidia are relatively few or absent. If present, they are tear-shaped and born singly on the hyphae, 4-7µm × 2.5 µm (Figure 1.8) (Quinn *et al*., 1994; Aneja *et al*., 2012).

The essential distinguishing feature of this genus is the echinulations on the macroconidial wall (Aneja *et al*, 2012).

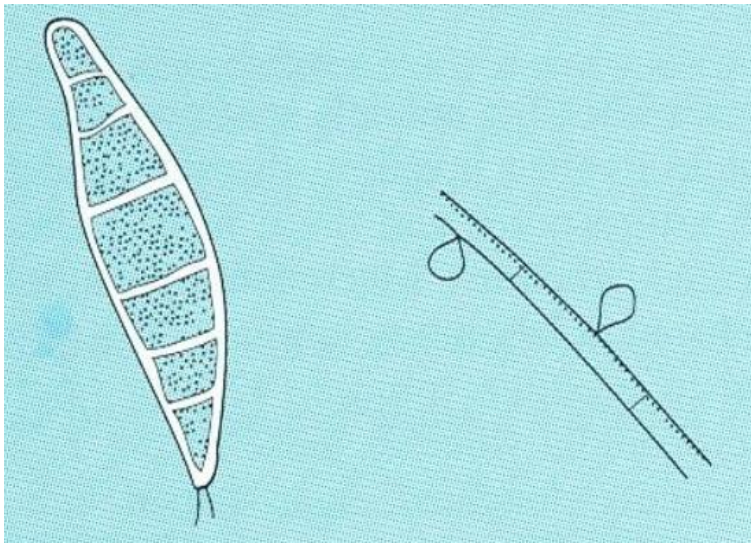


Figure 1.8 - *Microsporum spp.,* macro and microconidia (Adapted from Quinn *et al*., 1994).

Chapter II – Molecular identification

1. Introduction

The most commonly used diagnostic methods of dermatophytosis at the present days are fungal culture, evaluations of symptoms and using of the Wood´s ultraviolet lamp. They all have some restrictions, as time consuming, lack sensitivity and depends strongly on experience of the operator.

Even having a sensitivity and specificity far above the ones from conventional diagnostic techniques, methods of molecular biology based on Polymerase Chain Reaction may vary between themselves, and results depend on many factors too.

PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications (Joshi *et al*., 2011). These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases (Patel *et al*., 2015).

PCR was developed in 1984 by the American biochemist, Kary Mullis. It consists in amplifying of single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. There are three major steps involved in the PCR technique:

- denaturation, the DNA is denatured at high temperatures, from 90 - 97 degrees Celsius);
- annealing, primers anneal to the DNA template strands to prime extension, at 50-60 degrees Celsius;
- extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA, at temperature approx. 72 ° C.

All stages have a specific temperature and duration, optimal for the reaction and interaction between enzymes and other components. These steps are grouped in cycles. At the end of 25 - 35 cycles the final product is analyzed (Joshi *et al*., 2011). The wide range of applications of PCR has led to an ever-growing list of variants of the technique. While some are optimizations to suit specific requirements and are very similar to basic PCR, others completely turn the technique on its head to formulate novel creative applications in various fields. Some of the variations have been created for very specific purposes (Patel *et al*., 2015).

Important details in the development and optimization of the PCR protocol as a diagnostic tool for dermatophytosis:

- Molecular biological surveys of fungal phylogeny by different methods such as the GC content of chromosomal DNA, total DNA homology, random amplification of polymorphic DNA, restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA), and determination of nucleotide sequences have shown low genetic diversity in dermatophytes, indicating a homogeneous group of species contrasting the high phenotypic heterogeneity. Nevertheless, a few gene targets including large ribosomal RNA subunit (LSU rRNA – 28S rRNA), nuclear ribosomal internal transcribed spacers (ITS), *CHS1*, *TOP-II*, and recently *BT2* and *Tef- 1α* have been used as genetic markers for dermatophyte species (Ninet *et al*., 2003; Cafarchia *et al*., 2009; Bergman *et al*., 2013; Ahmadi *et al*., 2014);
- During the last 15 years the internal transcribed spacer (ITS) of nuclear DNA has been used as a target for analyzing fungal diversity in environmental samples and has recently been selected as the standard marker for fungal DNA barcoding. Nevertheless, some of the ITS primers, such as *ITS1-F*, were hampered with a high proportion of mismatches relative to the target sequences, and most of them appeared to introduce taxonomic biases during PCR. It was concluded that ITS primers have to be selected carefully. Different primer combinations or different parts of the ITS region should be analyzed in parallel, or that alternative ITS primers should be searched for (Bellemain *et al*., 2010);
- Sometimes closely related dermatophyte species could not be distinguished even using molecular approach. It happens because of using of common fragment patterns, high level of similarity of genome characteristics (genome size - mostly between 22 – 25 Mb), very low rate of predicted single nucleotide variation (that may represent background noise), small number of inversions and a high percent identity of the amino acids, similarity in gene contents (for instance, core set of 6,168 orthologous groups common in seven prioritized dermatophyte species genomes) (Martinez *et al*., 2012);
- One of the genes most frequently used as the main targets for dermatophyte PCR, and used by us in this work too, is the Chitin Synthase 1 (*CHS1*) gene (Garg *et al*, 2009; Nasrin *et al*., 2017). Chitin is a linear polysaccharide, and it is a key cell wall component of fungi, providing structure and protection for cells. The general chitin synthesis pathway can be divided into 3 sequential reactions; the final reaction is catalyzed by chitin synthase (CHS), which is specifically associated with chitin biosynthesis. Fungal CHS gene family is diversified at least at 7 classes, contraction of the CHS gene family is morphology-specific. (Liu *et al*., 2017). There are also studies that reveal a chitin size-dependent immune reactivity of the host.mutants lacking class *CHS1* genes are invariably viable with mild phenotypes under non-stressed conditions (Lenardon *et al*., 2010). The *CHS1* nucleotide sequences of *Microsporum equinum* e *Trichophyton equinum*. from horses, showed more than 80% similarity to those of *Arthroderma*

benhamiae, A. fulvum, A. grubyi, A. gypseum, A. incruvatum, A. otae, A. simii, A. vanbreuseghemii, Epidermophyton floccosum, T. mentagrophytes var. *interdigitale* (*T. interdigitale*), *T. rubrum* and *T. violaceum*. Especially high degree of nucleotide sequence similarity of more than 99% was noted between the *CHS1* gene fragments of *M. equinum* and *A. otae*, and those of *T. equinum, T. interdigitale* and *A. vanbreuseghemii*, respectively (Kano *et al*., 2001). The CHSI gene of some dermatophytes showed 75-85% amino acid sequence similarity with non-dermatophyte fungi*, Exophiala dermatitidis, Penicillium chrysogenum, Coccidioides immiti.r* and *Aspergillus nidulans* (Kano *et al*., 1997)

The PCR-protocol chosen for work was developed and described by Dabrowska I. and colleagues in 2014. They based on the work by Brillowska-Dabrowska A. and colleagues from 2007.

1.1 Primary methodology

A Brillowska-Dabrowska A. and colleagues, at their work, titled “Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum*” described and evaluated a general technique for detection of human-pathogenic dermatophytes. Work also allowed the specific detection of *T*. *rubrum* specifically, from pure cultures and clinically diseased nails. Generally, it is a different approach of DNA extraction from dermatophyte cultures and from nail samples, then used for Pan-dermatophyte PCR, using primers specific for a DNA fragment encoding chitin synthase, panDerm1 and panDerm2, established based on comparison of nucleotide sequences of different dermatophytes in NCBI nucleotide-database. DNA was also evaluated using a T. rubrum-specific PCR. Sequences used for primers design, based on alignment of ITS2 (universal and T. rubrum-specific), and a Multiplex-PCR, performed using two specific sets of primers: panDerm1 + panDerm2, universal and T. rubrum-specific primers. In conclusion, this five-hours diagnostic test allows to increase not only the speed of results availability, but also the sensitivity of dermatophyte detection directly from nails (Brillowska-Dabrowska *et al*., 2007).

This method was adapted by Dabrowska and cowokers, in their work entitled “The use of one-step PCR method for the identification of *Microsporum canis* and *Trichophyton mentagrophytes* infection of pets” for the detection of veterinary ringworm-causing species. It involves a fungal DNA extraction step followed by the Pan-dermatophyte PCR and electrophoresis, and it allows to confirm the presence of dermatophyte-specific DNA in specimens derived from cultured clinical samples.

DNA preparation from dermatophyte cultures was performed using the method proposed by Brillowska-Dabrowska and coworkers in 2007 - briefly, fungal colonies were picked and incubated for 10-min in 100 µl of extraction buffer A (60 mM sodium bicarbonate (NaHCO3), 250 mM potassium chloride (KCl)] and 50 mM Tris, pH 9.5) at 95°C. Subsequently,

a 100 µl of 2% bovine serum albumin, (BSA) was added and mixed thoroughly. The DNA-containing solution was used for PCR assay.

Pan-dermatophyte PCR was realized under the next conditions: set of primers detecting a DNA fragment encoding chitin synthase1 of dermatophytes, panDerm_for (5'GAAGAAGATTGTCG TTTGCATCGTCTC3') and panDerm_rev (5'CTCGAGGTCAAAAGCACGCCAGAG3'), was used for PCR assay. Two different PCR-mixture was compared, commercial and self-composed. Both contained 10 µl of PCR Mix, 0.1 µl of each primer (panDerm_for and panDerm_rev) and 2 µl of DNA in a total volume of 20 µl. PCR was performed in an Eppendorf Mastercycler thermocycler. The time-temperature profile for PCR was; initial denaturation for 3 min at 95°C followed by 45 s at 94°C, 45 s at 54°C or 56°C or 58°C and finally 45 s at 72°C for a total of 35 cycles. The presence of a specific PCR product of approximately 366 bp was determined by electrophoresis on a 2% agarose gel containing ethidium bromide. Using the pan-dermatophyte PCR primers and cycling conditions described by Bsrillowska-Dabrowska and coworkers (2007), both specific and unspecific products were obtained at annealing temperature under 58°C in the presence of commercial PCR mix. A specific product (approx. 366 bp) was visible only at the annealing temperature of 58°C. Although the one-step procedure described here cannot distinguish individual fungal species, the results indicate that the PCR approach may allow detection of dermatophyte specific DNA from *M. canis* and *T. mentagrophytes*.

The aim of this part of the study was to adjust one-step PCR protocol to find an ideal condition for direct detection of dermatophytes in clinical specimens from dogs and cats. The selection of the technique with one-step PCR was based on its sensitivity, specificity, being rapid and easy to perform. It has advantages over conventional identification methods, and at the same time is one of the simplest protocols in "PCR family".

2. Materials and methods

Materials used for our research: reference dermatophyte strains, Sabouraud Dextrose Agar (normal and modified), commercial kit for fungi DNA isolation, PCR ingredients, electrophoresis ingredients.

Equipment: Thermocycler BioRad MyCycler, gel electrophoresis apparatus – VWR PowerSource.

2.1 Fungal cultures

Reference dermatophyte strains:

- *T. mentagrophytes,* CECT 2795. Collection CECT. Strain designation CBS 572.75 (ATCC 32457). Source – skin of man. (Universitat de Valéncia, Lista de hongos)
- *M. canis,* CECT 20910. Collection CECT. Strain designation HCUV-391522. Source – scaly areas and hair of patient with Tinea capitis. (Universitat de Valéncia, Lista de hongos).

Conditions for recovery of cryopreserved cultures:

- *T*. *mentagrophytes*. Temperature +24º C, aerobic conditions, media 87 or 72;
- *M*. *canis*. Temperature +28º C, aerobic conditions, media 72 or 87.

Culture media CECT 87 (1:10 SDA +) Salt)*:* Glucose 2,0 g., Mycopeptone 1,0 g., $MgSO_4$ 1,0 g., KH_2PO_4 1,0 g., Agar powder (only for solid media) 15,0 g., Distilled water 1 L.

Culture media CECT 72 (Emmons modification of SDA): Glucose 20 g., Peptone 10 g., Agar powder (only for solid media) 15 g., Distilled water 1 L.

All fungal cultures, included cryopreserved after being recovered, was placed on medium SDA, at 26ºC

2.2 Dermatophyte DNA isolation

Performed using NZY Plant/Fungi gDNA Isolation kit:

1 - Sample preparation. Wash 50-200 mg mycelium or fruiting body (from liquid culture or scraping surface) of macro fungi in ethanol. Cover sample completely with ethanol (400µl). Remove the ethanol by pipetting and squeezing the mycelium;

2 – Cell lysis. Sample placed into a 1,5 mL microcentrifuge tube, add 150 µg of sea sand and 200 µg of Buffer PNL1. Homogenization (using micro pistil and vortex). Add additional 100 µg of Buffer PNL1 and homogenize the sample again. Incubate for 10 min at 65ºC. Add 100 µl chloroform, vortex for 10 seconds and separate phases by centrifugation for 15 min at 20 000 x*g*

3 - Pipette the top aqueous layer into a NZYSpin Homogenization column (violet ring) placed in a new 2 mL collection tube. Centrifuge for 2 min at 11 000 x*g*, collect the clear flow-through and discard the NZYSpin Homogenization column;

4 – Adjust DNA binding conditions. Add. 450 µl of Buffer PN and mix thoroughly by pipetting up and down for 5 times;

5 – DNA binding. Place the NZYSpin Plant column into a new collection tube and load 700 µl of the sample. Centrifuge for 1 min at > 11 000 x*g* and discard flow-through.

6 – Wash silica membrane. Add 400 µl of Buffer PNW 1 to the NZYSpin Plant column. Centrifuge for 1 min at > 11 000 x*g*. Discard flow-through and place the column back into the collection tube. Add 700 µl of Buffer PNW 2 to the NZY Spin Plant column and centrifuge for 1 min at > 11 000 x*g*, discard flow-through. Add another 200µl of Buffer PNW2 to the NZYSpin Plant column and centrifuge for 2 min at > 11 000 x*g* in order to remove wash buffer and dry the silica membrane completely;

7 – Elute DNA. Place the NZYSpin Plant column into a clean microcentrifuge tube and add 50 µl of Buffer PNE directly in the membrane column (preheating of elution buffer). Incubate 1 min at room temperature and centrifuge at > 11 000 x*g* for 2 min to elute DNA. Repeat the step with another 50 µl and elute at the same tube. Genomic DNA was stored at 4ºC and -20ºC.

2.3 PCR Pan-Dermatophyte

PCR Pan-Dermatophyte protocol parameters:

A set of primers detecting a DNA fragment encoding chitin synthase1 of dermatophytes, panDerm_for (5'GAAGAAGATTGTCGTTTGCATCGTCTC3') and panDerm_rev (5'CTCGAGGTCAAAAGCACGCCAGAG3').

PCR mixture composition: 13,2 µl of PCR water, 0,3 µl of each primer at 50 µM, 0,5 µl of genomic/analyzed DNA and 10 µl of Master Mix (Supreme NZY Taq II 2x Green Master Mix).

Time-temperature profile: initial denaturation for 3 min at 95°C followed by 45 sec at 94°C, 45 sec at 58°C and finally 45 sec at 72°C for a total of 35 cycles. Final elongation step for 10 min at 72ºC.

2.4 Electrophoresis

Electrophoresis parameters:

Detection and analysis of a specific PCR product, amplicon of approximately size 366 bp, was accomplished by electrophoresis on a 1,5% gel agarose (0,75 g Canvax AgarPure Agarose LE and 50 ml of TBE water). Conditions: 5 µl of the PCR product + 0,5µl of GreenSafe, for 30 minutes, 90 V, 500 A. For procedure was used NZYDNA Ladder VII as molecular-weight size marker (**Figure S1**).

2.5 Research phases

- Verification of the effectiveness of the protocols and ingredients;
- Examination of the specificity;
- Examination of the sensitivity.

2.5.1 Effectiveness of the protocol and ingredients

Cryopreserved dermatophyte reference strains were recovered on respective mediums, after that same cultures plated on SDA medium. Genomic DNA isolation and PCR Pan-Dermatophyte in accordance to protocols described above. Electrophoresis confirm the presence of the genomic DNA of the dermatophytes (**Figure 2.1**)

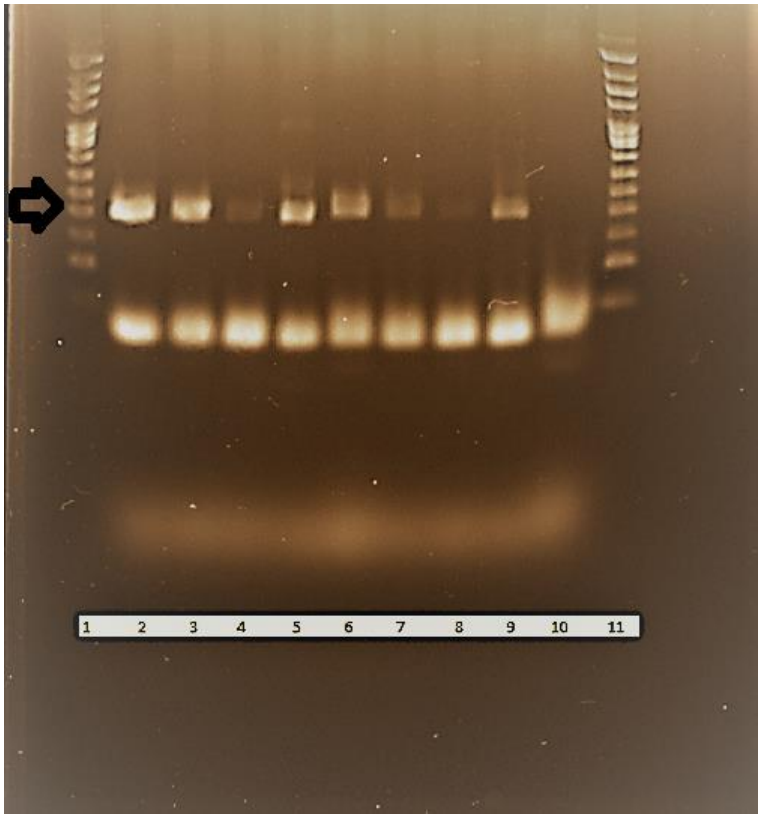


Figure 2.1 - Testing the protocol and DNA extraction kits (NZYTech and Canvax), image of agarose gel electrophoresis: 1 and 11 – Ladder; 2 – *Trichophyton mentagrophytes* NZYTech (DNA 1:1); 3 – *T. mentagrophytes* Canvax (DNA 1:1); 4 – *Microsporum canis* NZYTech (DNA 1:1); 5 – *M. canis* Canvax (DNA 1:1); 6 - *T. mentagrophytes* NZYTech (DNA 10^{-1}); 7 – *T. mentagrophytes* Canvax (DNA 1:10); 8 – *M. canis* NZYTech (DNA 10^{-1}); 9 – *M. canis* Canvax (DNA 10^{-1}); 10 – Negative control.

2.5.2 Specificity of the PCR reaction

For this step was isolated DNA of the reference dermatophyte strains: *T. mentagrophytes* and *M. canis*. For confirmation of the specificity also was isolated DNA from fungi, that frequently can be found in the same type of the samples (Alves, 2017). They are pathogens too and may cause similar clinical signs or be present in the same sample as a fungal biota. It was extracted DNA from the next fungi cultures: *Fusarium sp., Rhodotorula sp., Penicillium sp., Candida sp., Alternaria sp., Chrysosporium sp., Aspergillus niger* and *Aspergillus flavus*.

After performing PCR Pan-Dermatophyte protocol, the presence of dermatophyte DNA was confirmed and distinguished from the DNA of the other fungi (**Figure 2.2**). By this way was confirmed specificity of the PAN-Dermatophyte PCR.

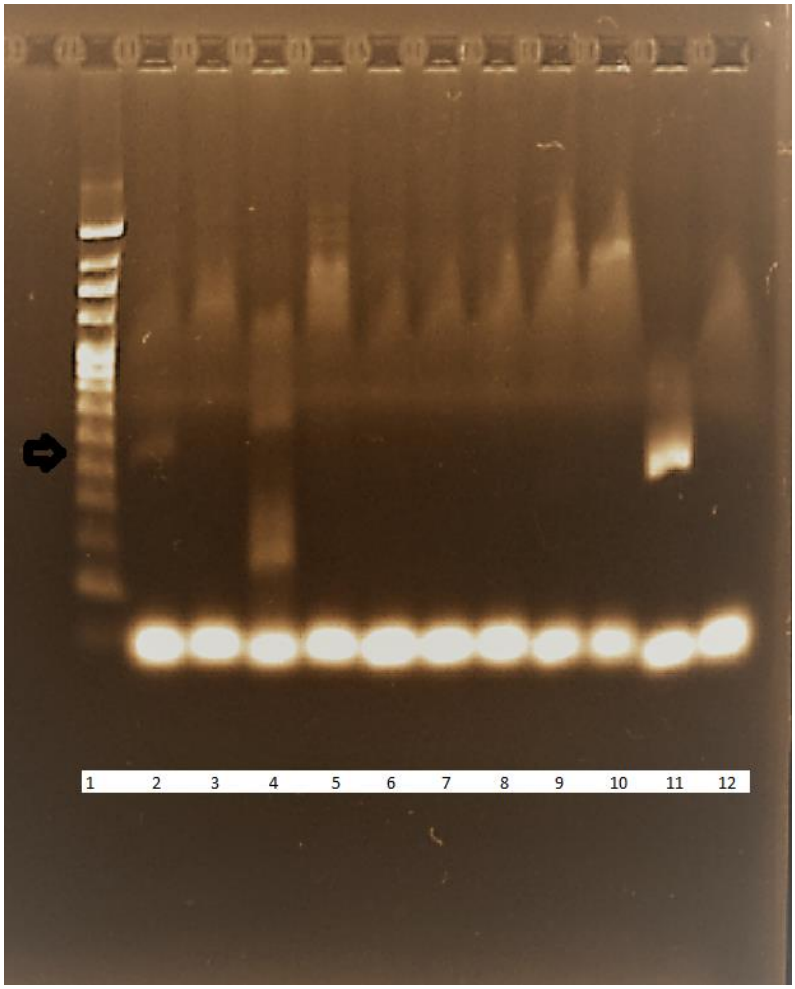


Figure 2.2 - Testing specificity of the protocol, image of agarose gel electrophoresis 1 – Ladder; 2 – *M. canis*; 3 – *Fusarium sp.*; 4 – *Rhodotorula sp.*; 5 – *Penicillium sp.*; 6 – *Candida sp.*; 7 - *Alternaria sp.*; 8 – *Aspergillus niger*; 9 – *Aspergillus flavus*; 10 – *Chrysosporium sp.*; 11 – *T. mentagrophytes*; 12 – Negative control

2.5.3 Sensitivity of the protocol

After unsuccessful attempt, with use of Iodine for sterilization of the hair before inoculation and posterior DNA extraction, was developed next protocol:

- was brushed a hair from four different dogs and four different cats. Then hair for every species was mixed under sterile conditions;
- inoculate from cryopreserved reference strains, 10 µL on Petri dish with SDA medium, in lawn, using sterile spreaders. For 5 days at 26ºC;
- after, scrape with a sterile scalpel a fungal culture in order to try to obtain vegetative and reproductive mycelium. Suspend in 5 ml of 0.9% saline until a concentration of 0.5 on the MacFarland scale;
- carry out 8 dilutions of base 10 (9 ml of 0.9% NaCl to 1 ml of inoculum) from the original suspension;
- for each dilution, inoculate 50 µL to the surface of 4 plates with SDA (in lawn, using sterile spreaders, 8 dilutions and original) and incubate for 5 days at 25ºC;
- place an approximate amount of hair in a test tube (eg 20) and add 100 µl of each dilution. In separate tubes. 1 for each species and each dilution and incubate for 24 hours at 25ºC
- count the number of colonies per plate, average the 4 plates and multiply by 20 to obtain CFU / ml for each dilution;
- perform DNA extraction protocol as described above, but with some changes (for step of the sample preparation at first remove dilutions by pipetting, add 200 µl of ethanol to resting hair and incubate for 24 hours at 25ºC. After 24 hours pipetting liquid for 4-5 times and then use it as a sample). After that follow previously, described protocol.

In the process of working on this phase we faced some complications (for ex. using Iodine for remove bacteria's, even after washing step we don't remove all particles of Iodine, and they interacted lately to PCR reagents). Since the result of the counting of the colonies cannot be accepted as definitive, it will not be shown in this work.

The gel, obtained after performing electrophoresis with the products of this PCR (**Figure 2.3** and **2.4**), shows a lot of conflicting and unnecessary information (because of that realizing of typical calculations is unreasonable), however practically it proves, that with proper protocol optimization we could obtain a valid diagnostic technique.

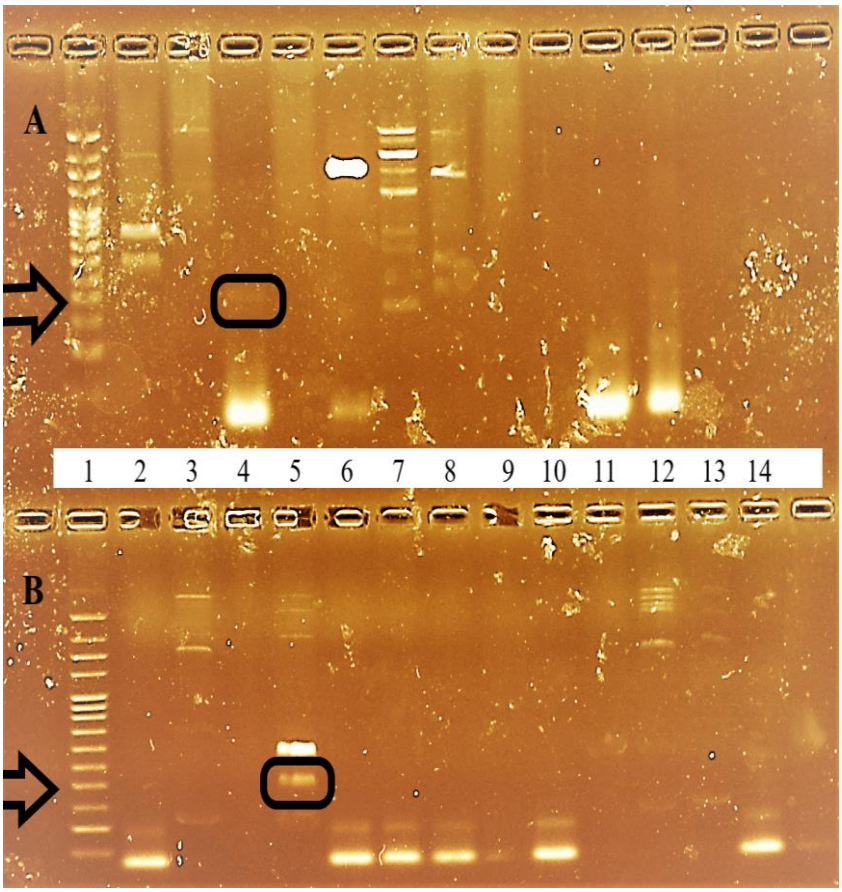


Figure 2.3 - Testing sensitivity for direct detection in cats' hair sample, image of agarose gel electrophoresis

A - (*T.mentagrophytes*) 1 – Ladder; 2 - Reference strain; 3 – Original suspension (Cats´ hair incubated with reference strain suspended in normal saline to 0,5 McFarland scale); 4 - 1st suspension (10^{-1}); 5 – 2nd suspension (10^{-2}); 6 – 3rd suspension (10^{-3}); 7 – 4th suspension (10^{-4}); 8 – 5th suspension (10^{-5}); 9 – 6th suspension (10^{-6}); 10 – 7th suspension (10^{-8}); 11 – 8th suspension (10^{-8}); 12 – Negative control.

B - (*M.canis*) 1 – Ladder; 2 - Reference strain; 3 – Original suspension (Cats´ hair incubated with reference strain suspended in normal saline to 0,5 McFarland scale); 4 – unoccupied; 5 - 1st suspension (10^{-1}); 6 – 2nd suspension (10^{-2}); 7 – 3rd suspension (10^{-3}); 8 – 4th suspension (10^{-4}); 9 – unoccupied; 10 - 5th suspension (10^{-5}); 11 – 6th suspension (10^{-6}); 12 – 7th suspension (10^{-7}); 13 – 8th suspension (10^{-8}); 14 – Negative control.

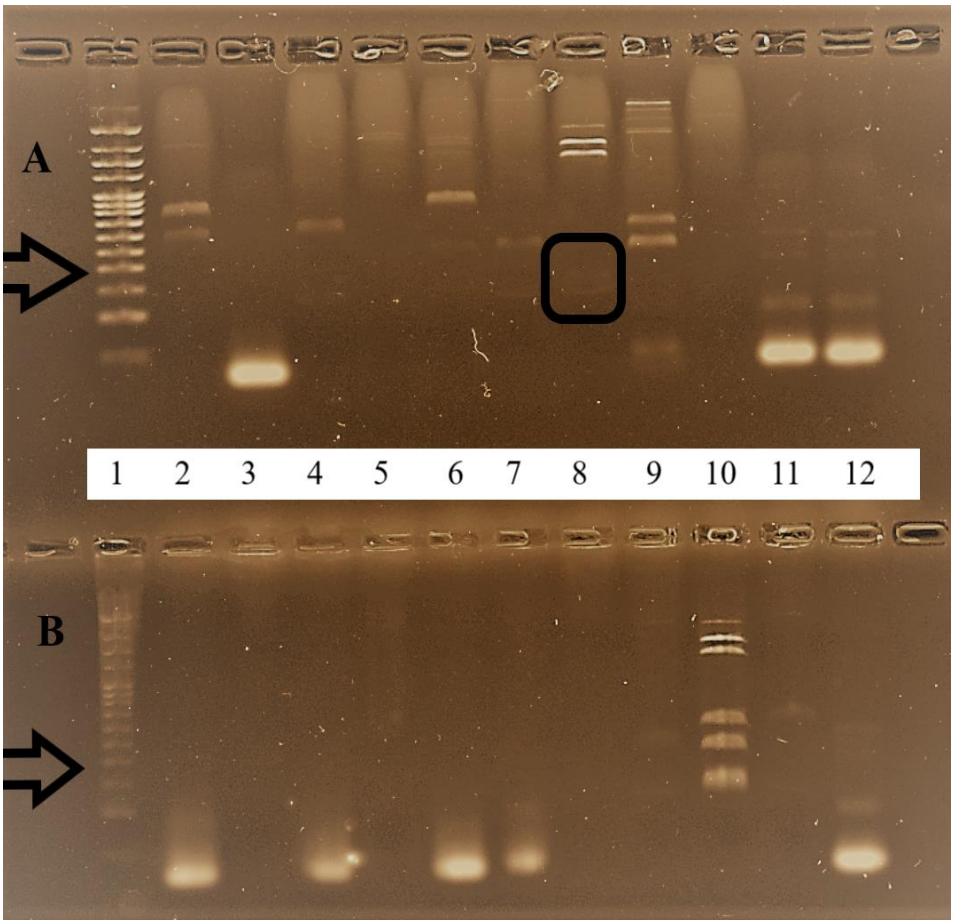


Figure 2.4 - Testing sensitivity for direct detection in dogs' hair sample, image of agarose gel electrophoresis

A - (*T.mentagrophytes*) 1 – Ladder; 2 - Reference strain; 3 – Original suspension (Dogs´ hair incubated with reference strain suspended in normal saline to 0,5 McFarland scale); 4 – 1st suspension (10^{-1}); 5 – 2nd suspension (10^{-2}); 6 – 3rd suspension (10^{-3}); 7 – 4th suspension (10^{-4}); 8 – 5th suspension (10^{-5}); 9 – 6th suspension (10^{-6}); 10 – 7th suspension (10^{-7}); 11 – 8th suspension (10^{-8}); 12 – Negative control.

B - (*M.canis*) 1 – Ladder; 2 - Reference strain; 3 – Original suspension (Dogs´ hair incubated with reference strain suspended in normal saline to 0,5 McFarland scale); 4 – 1st suspension (10^{-1}); 5 – 2nd suspension (10^{-2}); 6 – 3rd suspension (10^{-3}); 7 – 4th suspension (10^{-4}); 8 – 5th suspension (10^{-5}); 9 – 6th suspension (10^{-6}); 10 – 7th suspension (10^{-7}); 11 – 8th suspension (10^{-8}); 12 – Negative control.

3. Discussion

At first was tested the protocol in general and effectiveness of all reagents. Was determined parameters for our search – reagents concentrations, temperature conditions, and order of procedures.

Secondary we tested specificity. In spite of obtained information we can conclude that there is a high level of specificity – two specific PCR products from dermatophytes was distinguished from eight other fungi (without false-positive and false-negative results). Specificity was tested against fungi frequently identified in the same clinical veterinary samples (Cafarchia *et al*., 2009; Alves, 2017). However, by this way we cannot distinguish between dermatophyte species, usually for this propose, and not as clinical routine diagnosis, uses more complex protocols of PCR-based techniques (Gutzmer *et al*., 2004; Shehata *et al*., 2008)

On the next phase of this work we tested sensibility of the method. Analyzing electrophoresis gel, we can see some bands corresponds to specific PCR product in cat´s hair: **Figure 2.3**, gel A, line 4 and gel B, line 5, which are Trichophyton and Microsporum of the 1st dilutions (10^{-1}). In gel from dog´s hair the is one slight band – **Figure 2.4**, gel A, line 8, correspond to the 5th dilution (10^{-5}).

These results are the first part of study with main goal to optimize and validate diagnostic technique for direct identification of the dermatophytes in clinical veterinary samples.

All results are promising even at this stage and shows that is possible to detect the specific DNA of dermatophytes. During subsequent work, among the other details, it is necessary to pay attention to the next factors:

- The hair must be processed before DNA extraction (as we can see at **Figure 2.3** and **2.4** there are many biological materials presents in the sample, and it affects a result);
- Iodine cannot be used for the preparation of the sample because of its interactions with PCR reagents and following obstacle of the reaction;
- Possibility of cross-contamination reaction. Selection of primers is based on existing knowledge about the genome of the microorganisms being diagnosed and similar to it. Theoretically, there is the possibility of the presence of the same fragment in other microorganisms, the genome of which is not currently deciphered, and which have not been tested for the possibility of a cross reaction. The presence of such microorganisms in the sample can lead to a false positive result of the analysis (Maurer, 2011);
- The accuracy, specificity and sensitivity of the methods must be determined according to ISO / FDIS 16140: 2000 (E) and its revised versions - "Microbiology of food and animal feeding stuffs - protocol for the validation of alternative method". The confidence interval (CI) for each of calculations should be also determined, and the degree of similarity between the methods. This standard must consider the results obtained by the reference method and the alternative method;

- Detailed analysis of the PCR properly (stringency, G-C content etc.) to attain better results (Lorenz, 2012)

Chapter III – Epidemiology

1. Introduction

The challenges that animal and public health have in common that they require identification, quantification and intensive examination of multiple, directly or indirectly causal, and often interacting, disease determinants. The science of veterinary epidemiology deals with the investigation of these determinants of disease distribution in animal populations.

The basis for most epidemiological investigations is the assumption that disease does not occur in a random fashion. If this was not the case, it would not be necessary to identify causal relationships between potential risk factors and disease. Disease is assumed to be influenced by multiple, potentially interacting risk factors or determinants (Pfeiffer, 2009).

Descriptive epidemiology is concerned with both the frequency and distribution of a health outcome (or health-related exposure). This is the first stage of an epidemiological investigation. It focuses on describing disease distribution by characteristics relating to time, place and potentially susceptible organism.

Analytical epidemiology (also known as etiological epidemiology) considers the role of individual risk factors in the development of disease; investigating which factors are responsible for increasing or decreasing the risk of an outcome and quantifying their effect. The main objective is to determine whether an exposure just happens to be associated with the outcome of interest, or whether it is causing the outcome (i.e. the association is causal) (Bray *et al*., 2016). Statistical hypothesis testing involves determining whether an observed difference is statistically significant on the basis of testing whether a particular hypothesis can be rejected or not (Pfeiffer, 2009). Hypothesis testing is a statistician's way of trying to confirm or deny a claim about a population using data from a sample (Rumsay, 2010). Usually it is about a null hypothesis indicating that there is no association between two factors in the population from which the sample used in this analysis was selected. This is quite important, since while there may be a difference in the sample, the important question is whether there is one in the target or source population. It is important to recognize, that statistical hypothesis testing does not assess whether the null hypothesis or any hypothesis is correct or false. Instead, it tests if the null hypothesis can be rejected, which in turn leads to the interpretation that a result (usually an association between two factors) is statistically significant. If it cannot be rejected, the result of the analysis is not statistically significant. The decision on whether the null hypothesis can be rejected or not is based on the p-value, which quantifies the probability that the observed

association is the result of random variation, and this value is often set to a level of 0.05 (Pfeiffer, 2009).

Every hypothesis test contains two hypotheses. The first hypothesis is called the **null hypothesis**, (the current state). The null hypothesis always states that the population parameter is equal to the claimed value. Out to test this claim – **research/alternative hypothesis**. **P-value** (probability value) – calculated/estimated probability of rejecting the null hypothesis in a given statistical model (strength of the evidence) (Botelho *et al*., 2008).

The *p*-value is a number between 0 and 1 and interpreted in the following way:

- A small *p*-value (typically ≤ 0.05) indicates strong evidence against the null hypothesis, so you reject the null hypothesis;

- A large *p*-value (> 0.05) indicates weak evidence against the null hypothesis, so you fail to reject the null hypothesis;

- *p*-values very close to the cutoff (0.05) are considered to be marginal (could go either way) (Botelho *et al*., 2008; Rumsay, 2010).

Degrees of freedom - number of values in final calculating is free to vary. Broadly defined as the number of "observations" (pieces of information) in the data that are free to vary when estimating statistical parameters.

A hypothesis test is a statistical procedure that's designed to test a claim. Typically, the claim is being made about a population parameter (one number that characterizes the entire population) (Rumsay, 2010). As an example, a chi-squared test could be used to test the difference in incidence risk between animals exposed and not exposed to a risk factor for statistical significance (Pfeiffer, 2009). A chi-square test for independence compares two variables in a contingency table to see if they are related. In a more general sense, it tests to see whether distributions of categorical variables differ from each another.

2. Materials and methods

In this study, 1988 dogs and 805 cats with clinically suggestive ringworm lesions were investigated for the presence of dermatophytes by fungal culture, the gold standard diagnosis technique for the present time. Samples were collected during a 16-year period (2001-2016) and included hairs, hair and scales plucked from the lesion's periphery. All samples were inoculated in Sabouraud dextrose agar supplemented with cycloheximide and chloramphenicol, incubated for 21 days at 28ºC and observed daily for the growth of dermatophytes. Identification of the dermatophyte species was performed by micro and macroscopic examination of colonies and smears.

For calculations of analytical epidemiology was used an R-Studio software, R version 3.4.3 (2017-11-30), to estimate a chi-test values. For calculating the confidence limits (CL) for the proportions we used site EpiTools – epidemiological calculators, by the Wilson method, confidence level 0,95. Evaluated factors: age, breed, gender and season of the year.

2.1 Descriptive epidemiology

Out of the 1988 dogs included in this study, 1795 animals (90,29%) were negative, and only 193 animals (9,71%) were proved to be positive for dermatophytes. As expected, species identified belonged to the genera *Microsporum* (n=139 / 72,02%) and *Trichophyton* (n=54 / 27,98%). The most frequently identified dermatophyte species are *Microsporum canis* (n=101 / 52,33%), *Trichophyton mentagrophytes* (n=27 / 13,99%) and *Microsporum gypseum* (n=14 / 7,25%) (Table 3.1).

Regarding cats, 638 animals (79,25%) are negative, the percentage of positive animals was higher - 167 animals (20,75%) positive for dermatophytes. In resemblance to the cats, dermatophyte species identified belongs to the genera *Microsporum* (n=142 / 85,03%) and *Trichophyton* (n=25 / 14,97%). In these cases, *M. canis* (n=123 / 73,65%), *T. mentagrophytes* (n=18 / 10,78%) and *Microsporum nanum* (n=7, 4,19%) were the most frequently identified species (**Table 3.1**).

Table 3.1 – *Microsporum* and *Trichophyton* species identified in clinical specimens from cats and dogs with dermatophytosis, by fungal culture, in period from 2001 to 2016, quantity and percentage.

	MICROSPORUM							***TRICHOPHYTON***					**TOTAL**	
	canis	*gypseum*	*nanum*	*persicolor*	*coockei*	*gallinae*	*spp.*	*mentagrophytes*	*terrestre*	*allejoi*	*schoenleinii*	*spp.*		
	N/%	N/%	N/%	N/%	N/%	N/%	N/%	N/%	N/%	N/%	N/%	N/%	N	%
DOGS	101/52,3	14/7,2	7/3,63	2/1,04	1/0,52	-----	14/7,2	27/13,9	3/1,55	2/1,04	1/0,52	19/9,84	193	100
CATS	123/73,6	5/2,99	7/4,19	-----	1/0,60	2/1,20	4/2,40	18/10,7	1/0,60	-----	-----	6/3,59	167	100

The percentage of the dogs and cats, positive for dermatophytes, in general terms, corresponds to published studies on the subject. Prevalence of *M*. *canis* as a most frequently identified pathogenic agent of canine and feline dermatophytosis also coincides with the data presented in previous studies (Bernardo *et al.*, 2006; Frymus *et al*., 2013; Moretti *et al*., 2013).

2.2 Analytical epidemiology

From these calculations data about group of animals with a sample equal or less than three were excluded. In this part of the chapter all the results represented in the form of tables and graphs. For all tables N means negative, P – positive, DF – degree of freedom, confidence level for the sample proportion is 0,95. The results are divided into species, with a subdivision into risk factors

2.2.1 Dogs

2.2.1.1 Age

The age was dividing on the next groups, based on physiological development: dogs before 2 months of age, from 2 to 6 month, from 6 to 12 month, from 1 year to 5 years, from 5 to 9 years, and over 9 years old.

Table 3.2 - Results of estimations for dermatophytosis for dogs age: A – frequency; B – row percentage; C – proportion/prevalence

A

Age	*N*	*P*
≤ 2 m.	25	8
2-6 m.	95	14
6-12 m.	188	21
1-5 y.	416	27
5-9 y.	234	22
≥ 9 y.	143	12

B

Age	*N*	*P*	*Total*	*Count*
≤ 2 m.	75.8	24.2	100	33
2-6 m.	87.2	12.8	100	109
6-12 m.	90.0	10.0	100	209
1-5 y.	93.9	6.1	100	443
5-9 y.	91.4	8.6	100	256
≥ 9 y.	92.3	7.7	100	155

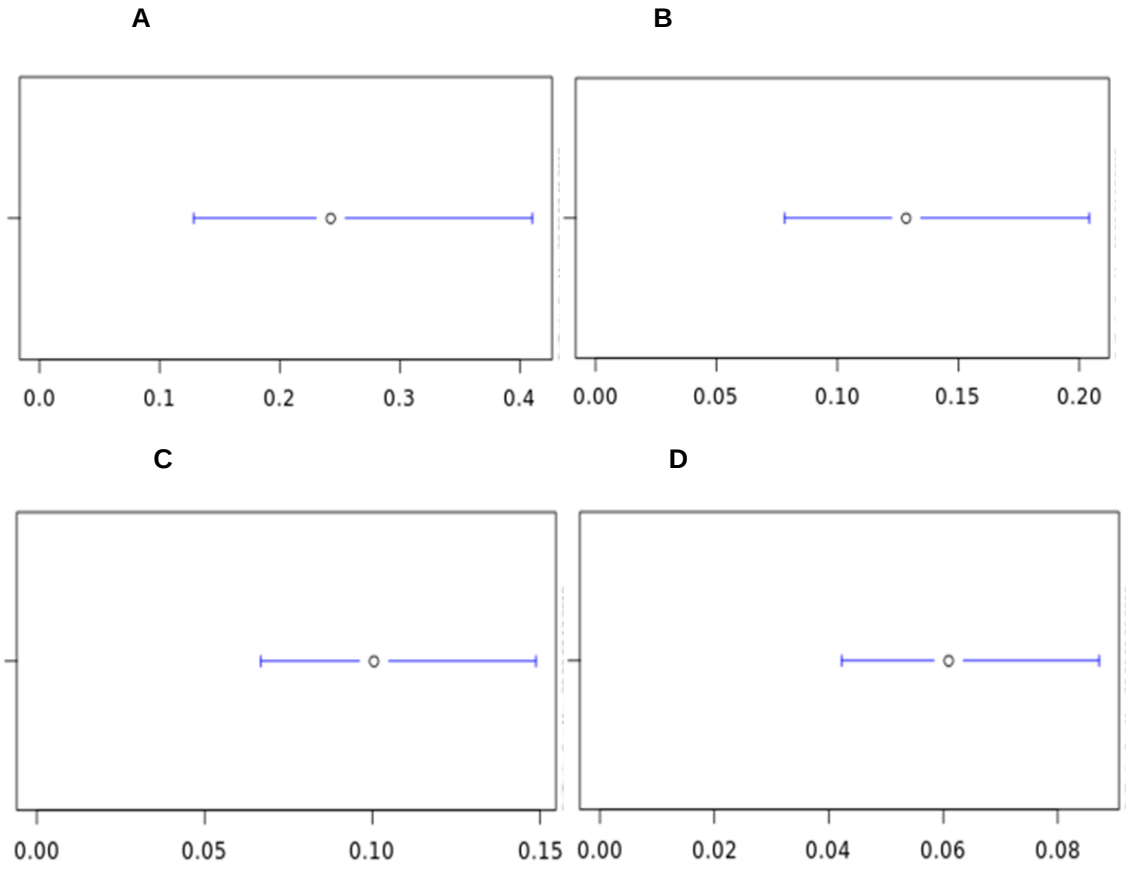
Pearson's Chi-squared test

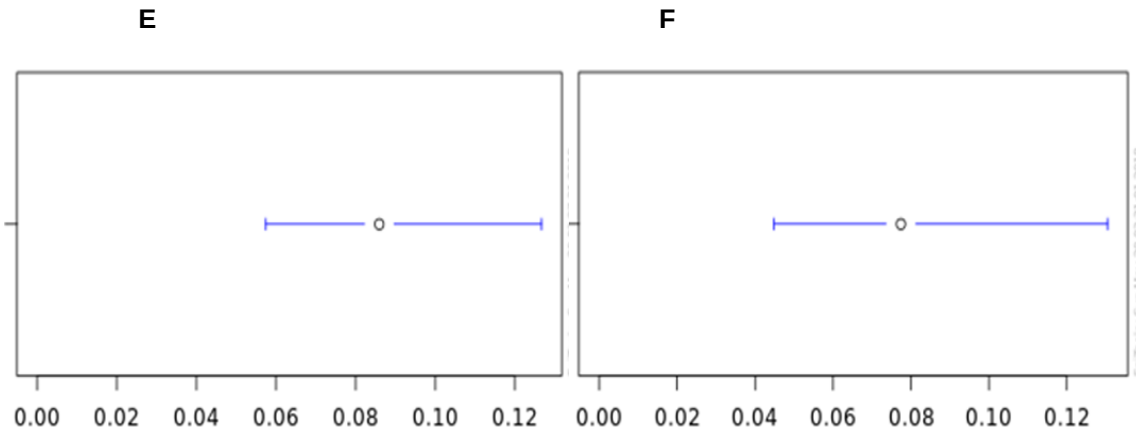
X-squared = 16.954, DF = 5, P-value = 0.004589

C

Age	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
≤ 2 m.	33	8	0,2424	0,1283	0,4102
2-6 m.	109	14	0,1284	0,0781	0,2041
6-12 m.	209	21	0,1005	0,0667	0,1487
1-5 y.	443	27	0,0609	0,0422	0,0872
5-9 y.	256	22	0,0859	0,0574	0,1267
≥ 9 y.	155	12	0,0774	0,0448	0,1304

Figure 3.1 - Set of graphs containing obtained confidence interval for prevalence/proportion for dermatophytosis for dogs age: A - Dogs ≤ 2-month old; B - 2 to 6-month old; C - 6 to 12-month old; D - 1 to 5-years old; E - from 5 to 9-years old; F - over 9-years old





The results of calculations (for this population X-squared 16,954 and P-value = 0.004589) suggest that there is an association between the age of the dog and the possibility of having dermatophytosis. Thus, the age could be considered a risk factor that influences susceptibility of the dogs to dermatophytes. Row Percentage of ill animals in this data set is 24,2% less than 2 months of age, 12,8% for 2 to 6 months, and 10,0% for 6 to 12 months of age. Prevalence / Proportion, respectively, is 0,24; 0,13 and 0,1, with Confidence Level=0,95. Corresponding indicators at the age of more than one year are lower.

2.2.1.2 Breeds

Were chosen the most often mentioned breeds in this pool of data.

T**able 3.3 – Results of estimations for dermatophytosis for dog breeds:** A – frequency; B – row percentage; C – proportion/prevalence

A

Breed	*N*	*P*
Boxer	65	5
French Bulldog	27	3
Poodle	31	6
Husky	19	1
Labrador	94	8
German Shepherd	26	2
Retriever	20	5
Rottweiler	16	2
Yorkshire	20	8

B

Breed	*N*	*P*	*Total*	*Count*
Boxer	92.9	7.1	100	70
French Bulldog	90.0	10.0	100	30
Poodle	83.8	16.2	100	37
Husky	95.0	5.0	100	20
Labrador	92.2	7.8	100	102
German Shepherd	92.9	7.1	100	28
Retriever	80.0	20.0	100	25
Rottweiler	88.9	11.1	100	18
Yorkshire	71.4	28.6	100	28

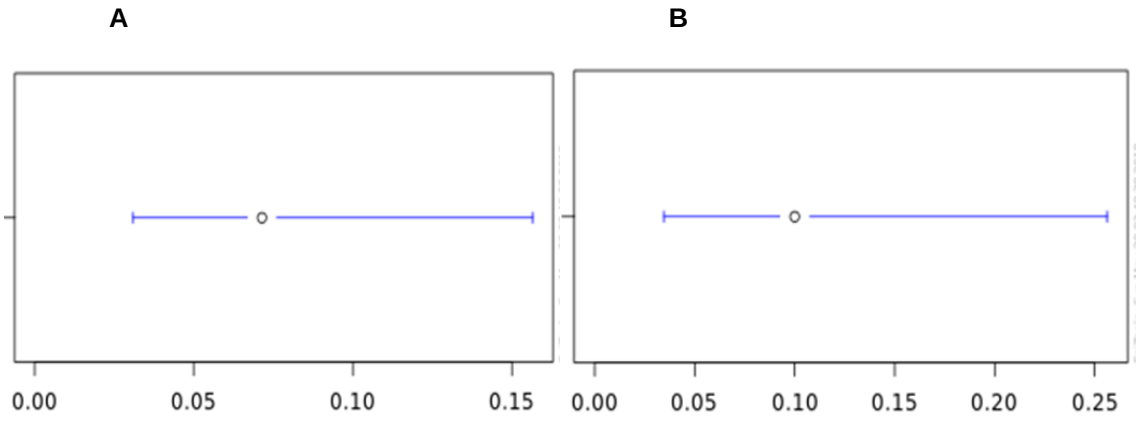
Pearson's Chi-squared test

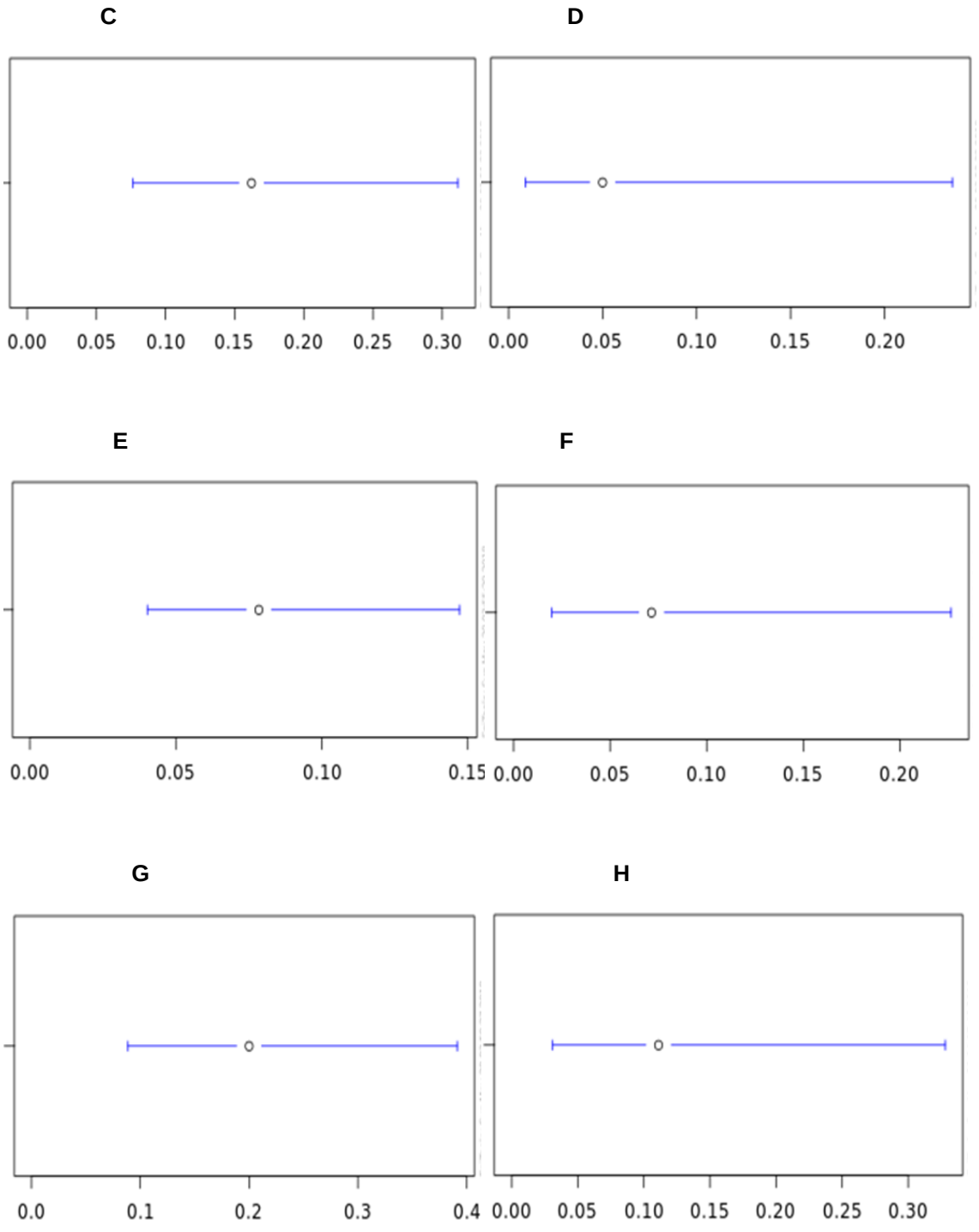
X-squared = 15,004, DF = 8, P-value = 0.05907

C

Breed	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
Boxer	70	5	0,0714	0,0309	0,1566
French Bulldog	30	3	0,1000	0,0346	0,2562
Poodle	37	6	0,1622	0,0765	0,3114
Husky	20	1	0,0500	0,0089	0,2361
Labrador	102	8	0,0784	0,0403	0,1472
German Shepherd	28	2	0,0714	0,0198	0,2265
Retriever	25	5	0,2000	0,0886	0,3913
Rottweiler	18	2	0,1111	0,0310	0,3280
Yorkshire	25	5	0,2000	0,0886	0,3913

Figure 3.2 - Set of the graphs containing obtained confidence interval for prevalence/ proportion for dermatophytosis for dog breeds: A – boxer; B - french bulldog; C – poodle; D – husky; E – Labrador; F - german shepherd; G – retriever; H – rottweiler; I – Yorkshire

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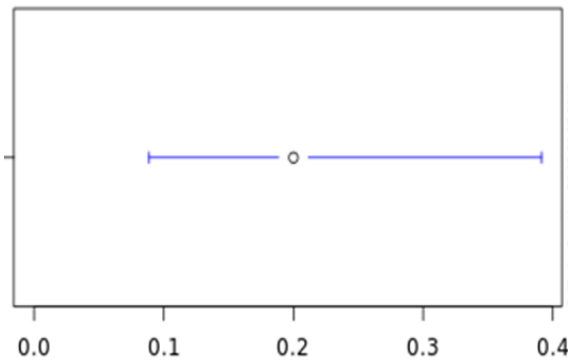
I



The results of calculations (for this population X-squared 15,004 and P-value = 0,05907) are slightly above the boundary value, that suggest that there may exist an association between the breed of the dog and the possibility of having dermatophytosis. Basing on this information, breed couldn´t be considered a risk factor that influences susceptibility of the dogs to dermatophytes, however this possibility not totally excluded.

The Row Percentage of ill animals in this data set is 7,1% for Boxers, 10,0% for French Bulldogs, 16,2% for Poodles, 5,2% for Huskies, 7,8% for Labradors, 7,1% for German Shepherds, 20% for Retrievers, 11,1% for Rottweilers and 28,6% for Yorkshires. Prevalence / Proportion is 0,07; 0,10; 0,16; 0,05; 0,08; 0,07; 0;20; 0,11 and 0,20 respectively, with Confidence Level=0,95.

2.2.1.3 Gender

T**able 3.4 - Results of estimations for dermatophytosis for dog gender:** A – frequency; B – row percentage; C – proportion/prevalence

A

Gender	*N*	*P*
Female	774	80
Male	974	105

B

Gender	*N*	*P*	*Total*	*Count*
Female	90.6	9.4	100	854
M ale	90.3	9.7	100	1079

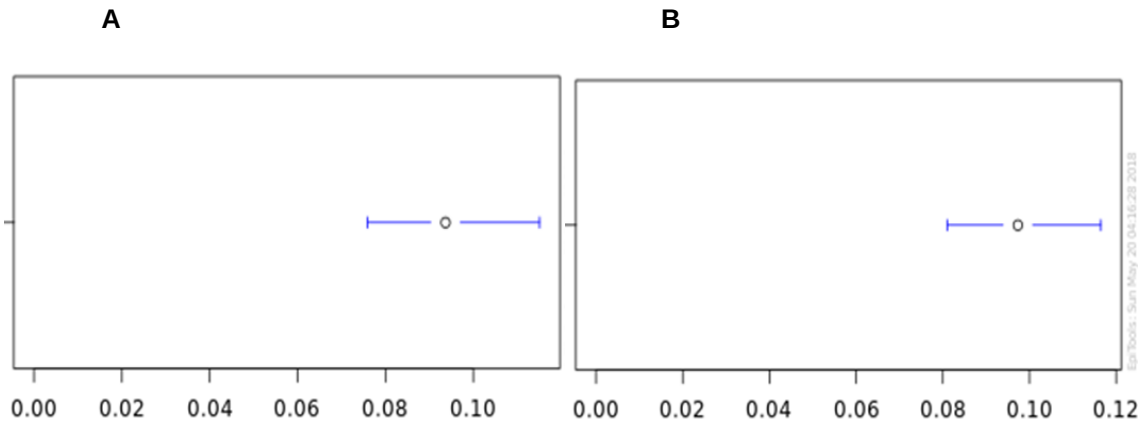
Pearson's Chi-squared test

X-squared = 0.0728, DF = 1, P-value = 0.7873

C

Gender	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
Female	854	80	0,0937	0,0759	0,1151
Male	1079	105	0,0973	0,0810	0,1165

Figure 3.3 - Set of the graphs containing obtained confidence interval of prevalence/ proportion for dermatophytosis for dogs' gender: A – female; B – male



In this case, X-squared = 0.0728, P-value = 0.7873, Row percentage for Female Dogs 9,4 and for Male 9,7 and Prevalence / Proportion for Female 0,09 and for Male 0,1, with Confidence Level 0,95. It suggestы that there are no association between dog gender and susceptibility to dermatophytosis. So, gender in dogs could not be considered a risk factor for this disease.

2.2.1.3 Season of the year

Table 3.5 - Results of estimations for dermatophytosis for season of the year for dogs: A – frequency (per month); B – row percentage (per month); C – proportion/prevalence (per season of the year)

A

Entry date	*N*	*P*
January	146	13
February	155	19
March	151	16
April	132	16
May	136	13
June	161	10
July	113	17
August	144	16
September	165	19
October	197	15
November	161	26
December	132	13

B

Entry date	*N*	*P*	*Total*	*Count*
January	91.8	8.2	100	159
February	89.1	10.9	100	174
March	90.4	9.6	100	167
April	89.2	10.8	100	148
May	91.3	8.7	100	149
June	94.2	5.8	100	171
July	86.9	13.1	100	130
August	90.0	10.0	100	160
September	89.7	10.3	100	184
October	92.9	7.1	100	212
November	86.1	13.9	100	187
December	91.0	9.0	100	145

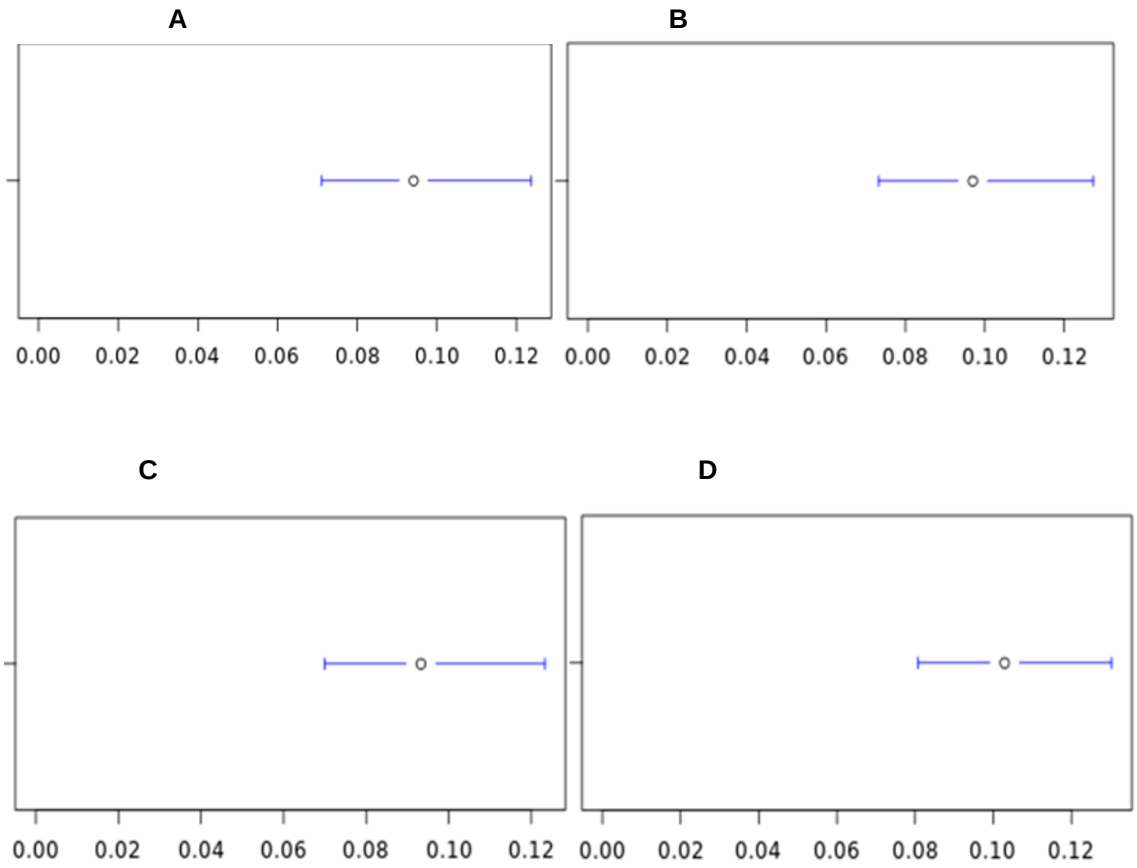
Pearson's Chi-squared test:

X-squared = 11.288, DF = 11, P-value = 0.4195

C

Season	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
Winter	478	45	0,0941	0,0711	0,1237
Spring	464	45	0,0970	0,0733	0,1273
Summer	461	43	0,0933	0,0700	0,1233
Autumn	583	60	0,1029	0,0808	0,1302

Figure 3.4 - Set of the graphs containing obtained confidence interval for prevalence/ proportion for dermatophytosis per season of the year for dogs: A – winter: B – spring; C – summer; D – autumn



In this case, X-squared = 11,288, P-value = 0.4195, Prevalence / Proportion summarily for Winter 0,09, Spring 0.1, Summer 0,09 and Autumn 0,10, with confidence Level 0,95. It suggest that there are no association between season of the year and dogs´ susceptibility to dermatophytosis. So, it could not be considered a risk factor for this disease.

2.2.2 Cats

2.2.2.1 Age

Table 3.6 - Results of estimations for dermatophytosis for cats age: A – frequency; B – row percentage; C – proportion/prevalence

A

Age	*N*	*P*
≤2 m.	4	15
2-6 m.	20	35
6-12 m.	53	16
1-5 y.	204	33
5-9 y.	83	15
>9 y.	70	7

B

Age	*N*	*P*	*Total*	*Count*
≤2 m.	21.1	78.9	100	19
2-6 m.	36.4	63.6	100	55
6-12 m.	76.8	23.2	100	69
1-5 y.	86.1	13.9	100	237
5-9 y.	84.7	15.3	100	98
>9 y.	90.9	9.1	100	77

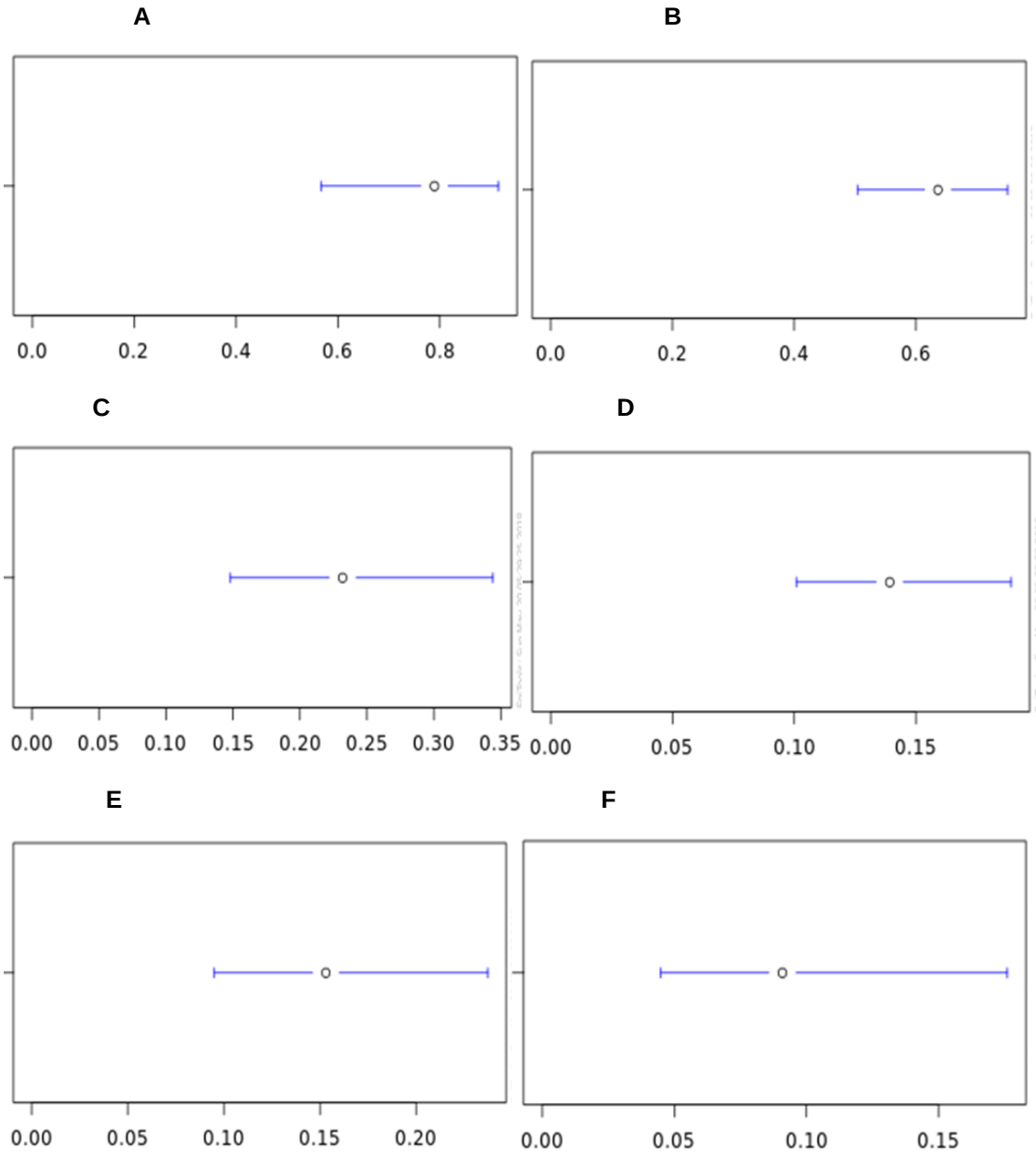
Pearson's Chi-squared test

X-squared = 111.28, DF = 5, P-value < 2.2^{-16}

C

Age	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
≤ 2 m.	19	15	0,7895	0,5667	0,9149
2-6 m.	55	35	0,6364	0,5042	0,7507
6-12 m.	69	16	0,2319	0,1481	0,3440
1-5 y.	237	33	0,1392	0,1009	0,1891
5-9 y.	98	15	0,1531	0,0950	0,2373
>9 y.	77	7	0,0909	0,0447	0,1760

Figure 3.5 - Set of graphs containing obtained confidence interval for prevalence/proportion for dermatophytosis for cats age: A - Cats ≤ 2-month old; B - 2 to 6-month old; C - 6 to 12-month old; D - 1 to 5-years old; E - from 5 to 9-years old; F - over 9-years old



The results of calculations (for this population X-squared 111,28 and P-value $<2,2^{-16}$) strongly suggest that there is an association between the age of the cat and the possibility of having dermatophytosis. Thus, the age could be considered a risk factor that influences susceptibility of the cats to dermatophytes. Row Percentage of ill animals in this data set is 78,9% less than 2 months of age, 63,6% for 2 to 6 months, and 23,2% for 6 to 12 months of age. Prevalence / Proportion, respectively, is 0,79; 0,64 and 0,23, with Confidence Level=0,95. Corresponding indicators at the age of more than one year are lower.

2.2.2.2 Breed

Table 3.7 - Results of estimations for dermatophytosis for cat breeds: A – frequency; B – row percentage; C – proportion/prevalence

A

Breed	*N*	*P*
Norwegian forest	10	3
British	1	3
Europ. Comum	297	65
Ind.	268	69
Persian	44	25
Siamese	14	2

B

Breed	*N*	*P*	*Total*	*Count*
Norwegian forest	76.9	23.1	100	13
British	25.0	75.0	100	4
Europ. Comum	82.0	18.0	100	362
Ind.	79.5	20.5	100	337
Persian	63.8	36.2	100	69
Siamese	87.5	12.5	100	16

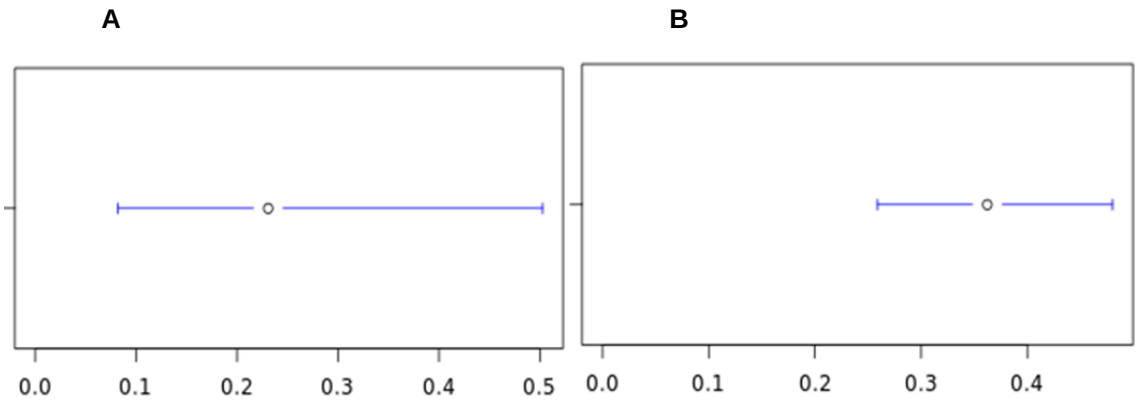
Pearson's Chi-squared test

X-squared = 19.582, DF = 5, P-value = 0.001497

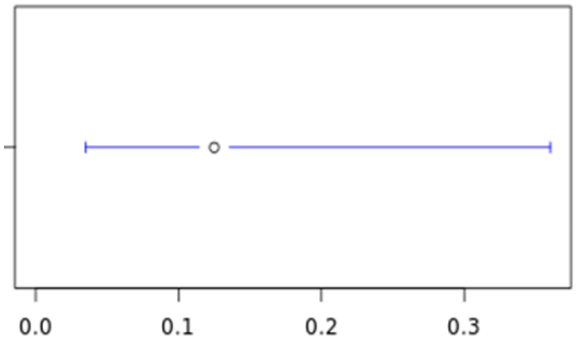
C

Breed	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
Norwegian forest	13	3	0,2308	0,0818	0,5026
Persian	69	25	0,3623	0,2590	0,4802
Siamese	16	2	0,1250	0,0350	0,3602

Figure 3.6 - Set of the graphs containing obtained confidence interval of prevalence/ proportion for dermatophytosis for cat breeds: A – Norwegian forest cat; B - Persian; C – Siamese (Europ. Comum – Europ. Shorthair was excluded)



C



The results of calculations (for this population X-squared 19,582 and P-value = 0.001497) strongly suggest that there is an association between the breed of the cat and the possibility of having dermatophytosis. Thus, breed could be considered a risk factor that influences susceptibility of the cats to dermatophytes. Row Percentage of ill animals in this data set is 23,1 % for Norwegian forest cat, 36,2% for Persian, and 12,5% for Siamese. Prevalence / Proportion, respectively, is 0,23; 0,36 and 0,113, with Confidence Level=0,95.

2.2.2.3 Gender

Table 3.8 - Results of estimations for dermatophytosis for cat gender: A – frequency; B – row percentage; C – proportion/prevalence

A

Gender	*N*	*P*
F	341	71
M	284	92

B

Gender	*N*	*P*	*Total*	*Count*
F	82.8	17.2	100	412
M	75.5	24.5	100	376

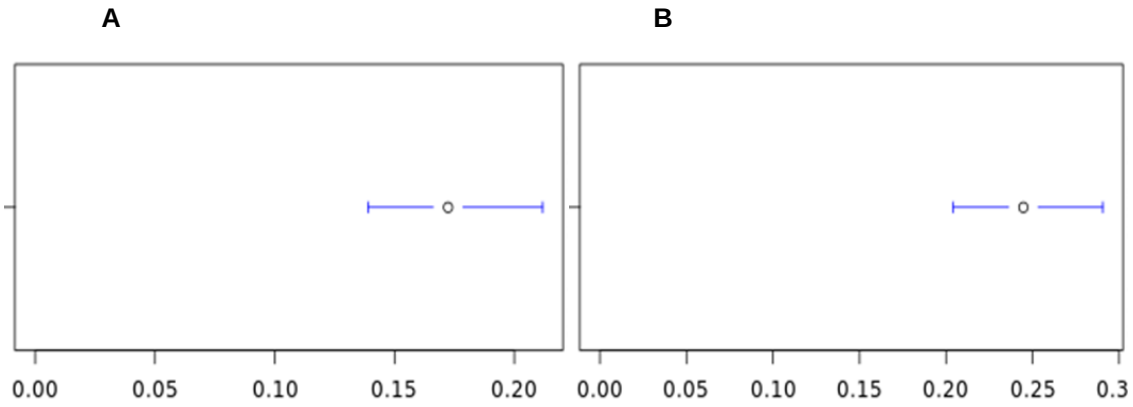
Pearson's Chi-squared test

X-squared = 6.2723, DF = 1, P-value = 0.01226

C

Gender	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
Female	412	71	0,1723	0,1389	0,2118
Male	376	92	0,2447	0,2040	0,2906

Figure 3.7 - Set of the graphs containing obtained confidence interval of prevalence/ proportion for dermatophytosis for cats' gender: A – female; B – male



In this case, X-squared = 6,2723 and P-value = 0.01226, Row percentage for Female Cat´s 17,2% and for Male 24,5 and Prevalence / Proportion for Female 0,17 and for Male 0,2. It suggest that there is possibility to have an association between cat's gender and males are more susceptible to dermatophytosis. So, gender could be considered a risk factor for this disease in cats.

2.2.2.4 Season of the year

Table 3.9 - Results of estimations for dermatophytosis for season of the year for cats: A – frequency (per month); B – row percentage (per month); C – proportion/prevalence (per season of the year)

A

Entry date	*N*	*P*
January	58	23
February	68	4
March	63	9
April	58	10
May	56	18
June	56	19
July	48	8
August	51	10
September	32	20
October	56	26
November	57	12
December	57	10

B

Entry date	*N*	*P*	*Total*	*Count*
January	71.6	28.4	100	81
February	94.4	5.6	100	72
March	87.5	12.5	100	72
April	85.3	14.7	100	68
May	75.7	24.3	100	74
June	74.7	25.3	100	75
July	85.7	14.3	100	56
August	83.6	16.4	100	61
September	61.5	38.5	100	52
October	68.3	31.7	100	82
November	82.6	17.4	100	69
December	85.1	14.9	100	67

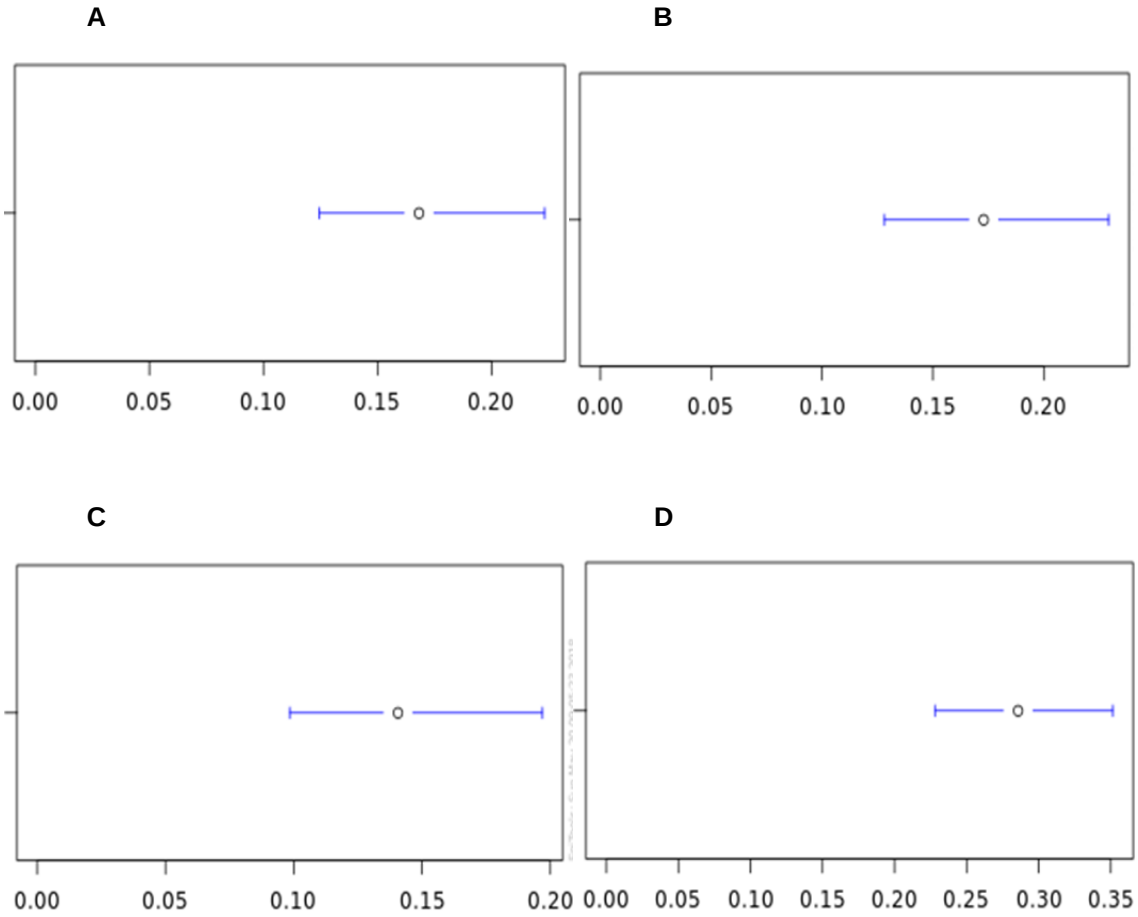
Pearson's Chi-squared test

X-squared = 39.346, DF = 11, P-value = 0.00004625

C

Season	*Sample size*	*Positive*	*Proportion*	*Lower 95% CL*	*Upper 95% CL*
Winter	220	37	0,1682	0,1245	0,2232
Spring	214	37	0,1729	0,1281	0,2292
Summer	192	27	0,1406	0,0985	0,1969
Autumn	203	58	0,2857	0,2280	0,3514

Figure 3.8 - Set of the graphs containing obtained confidence interval for prevalence/ proportion for dermatophytosis per season of the year for cats: A – winter: B – spring; C – summer; D – autumn



In this case, X-squared = 39,346, P-value = 0.00004625. Prevalence / Proportion summarily for Winter 0,17, Spring 0,17, Summer 0,14 and Autumn 0,29, with confidence Level 0,95. It strongly suggest that there is an association between season of the year and cats´ susceptibility to dermatophytosis, with special stand out of the Autumn. So, time of the year should be considered a risk factor in cats for this disease.

2 Discussion

The percentage of the dogs and cats, positive for dermatophytes, in general terms, corresponds to published studies on the subject. Prevalence of *M. canis* as a most frequently identified pathogenic agent of canine and feline dermatophytosis also coincides with the data presented in previous studies (Bernardo *et al.*, 2006; Frymus et *al*., 2013; Moretti et *al*., 2013).

It is necessary to pay attention to the detection of the less frequent species of dermatophytes (*Microsporum persicolor, Microsporum cookie, Microsporum gallinae, Trichophyton ajelloi, Trichophyton scoenleinii, Trichophyton vanbreuseghemii*). Some of dermatophyte cultures were not identified (7,25 % for *Microsporum* and 9,84% for *Trichophyton*) (**Table 3.1**). These data may indicate some changes in actual epidemiology situation comparatively with previously described (Hayette *et al*., 2015), from other view clearly confirm necessity in molecular identification method as highly sensitive and specific.

In spite of analyzing data was concluded, that for dogs as a risk factor can be considered the age (especially for animals under 12 months of age) (**Table 3.2**). It seems that dog´s breed has no predisposition for dermatophytosis, however we cannot exclude it totally as a risk factor, because for some breeds (Boxer, Retriever, Labrador, Poodle) raw percentage of the animals positive for dermatophytosis is higher than for other breeds. Gender and season of the year was not considered as a risk factor. But these results must be interpreted very carefully. Talking about seasons important elements for evaluation is temperature and humidity (because they affect a growth of dermatophytes), they are different in every year, and sometimes have no strong influence on the dog, since it companions specie and frequently live in indoor conditions. In relations of the dog's breeds, they are more or less common in Portugal, and it affects a calculations result.

Regarding cats, results strongly suggest that the animals under 12 months of age are more susceptible to dermatophytosis, so we can conclude that the age is a risk factor for cats for this disease (**Table 3.6**). Cat´s breed was considered a risk factor too, at least for Norwegian forest cat, Persian and Siamese (we exclude a European Shorthair from final discussion because of the strong possibility of incorrect identification of this breed). The gender for cats seems to be a risk factor too, and a male look like a more susceptible to dermatophytosis, (maybe it depends on behavioral characteristics of the males and relatively more freedom than the dogs have). About season of the year, it is also can be considered a risk factor for cats, especially Autumn. Two last factors show them importance, in contrary to dogs.

Repeating about caution in interpretation of these results, we would like to remind that dogs and cats, as very dependent on humans, also depend on them socials and financial conditions. So, it reflects on quality and frequency of preventive and curative procedures, and, consequently, on final result of our study.

Chapter IV – Conclusions

Fungi are ubiquitous microorganisms with a profound influence on agriculture and human and animal life. The health impact of chronic respiratory, mucocutaneous, and allergic fungal diseases is enormous. they are subject to extensive studies, making correct taxonomic identification paramount (Prakash *et al*., 2017).

Dermatophytosis it is a cutaneous infection caused by keratinophylic and keratinolytyc fungi assembled in closely related group – dermatophytes.

Dermatophytes are not life-threatening microbial agents, but they are distributed around the world and cause acute or chronic mycotic infections with high morbidity, but not mortality (Behzadi *et al*., 2014), and secondary bacterial infections too (Dabas *et al*., 2017). Epidemiological, etiological and clinical patterns of fungal infections caused by dermatophytes are changed in many aspects. From one point of view, it leads to changes and the emergence of alternatives in the therapeutic and diagnostic protocols. From another, explains and promotes the search for new methods of identification, processing and periodical updating of the epidemiological information (Skerlev *et al*., 2010; Zhan *et al*, 2016).

The main objective of the present work it was optimization of a molecular diagnostic method based on one-step PCR and apply the protocol for identification of the dermatophytes directly in the veterinary clinical samples. Another important component of the study it was to analyze obtained data about dermatophytosis in dogs and cats and evaluate a risk factors.

To achieve the mentioned goals was created a stock with cryopreserved cultures of *Trichophyton mentagrophytes* and *Microsporum canis*, originated from CECT. Was optimized an identification method based on molecular biology, one-step PCR; included DNA extraction, PCR Pan Dermatophyte and electrophoresis.

Firstly, was tested effectiveness of the method by extraction of dermatophyte DNA from inoculated reference strains, and result was positive.

Secondly, we tested a specificity of the PCR Pan Pan Dermatophyte (for this was extracted DNA from eight fungal species – *Aspergillus niger, Aspergillus flavus, Candida sp., Fusarium sp., Penicillium sp., Chrysosporum sp., Alternaria sp., Rhodotorula sp*, some of them are pathogenic and may cause systemic mycosis or allergenic reactions, others just can be present at the animal skin. Anyway, they are frequently can be find in the same sample). DNA of the dermatophyte reference strains was tested against DNA of the referred fungal species and specificity was total, without false-positive or false-negative results.

Finally, we tested a sensitivity of the protocol. First attempt has no success (for sterilization of the sample before incubation and posterior extraction we used Iodine, and it affects a structure of fungi wall and interacts with reagents). Second attempts give us promising practical result – it was detected some specific PCR products, however it was detected unspecific products too, so for the next steps the part of the sample preparation should be optimized.

Unfortunately, still remain a big problem of some diagnostic methods, based on molecular biology - the impossibility of distinguishing living organisms from non-living ones without an additional stage of reverse transcription, since the DNA of both living and dead microorganisms is amplified. However, it should be kept hat the molecular methods applied directly on the sample cannot replace microscopic and histopathological examination particularly to assess the involvement of contaminants (Hayette *et al*., 2015). Identification technique should be chosen in accordance to the conditions and objectives.

For epidemiological analysis was evaluated next critical points: the age of the animal, the gender, animal breed and season of the year.

It was concluded, that fog dogs risk factors are the age (more susceptible animals under 12 months of age). The breed can be the risk factor too (even calculation almost exclude this possibility there are some evidence, as a raw percentage, that can be interpreted as predisposition of Boxers, Labradors, Retrievers and Poodles to dermatophytosis). Gender and season of the year was not considered risk factors for this disease.

Regarding cats, all the critical points was considered risk factors for dermatophytosis: the age (animals under 12 months of the age show a strong susceptibility), the breed (especially Norwegian forest cat, Persian and Siamese), results show that male cats are more susceptible then female, and the Autumn was a season of the year with a largest number of cats positive for dermatophytosis.

At the same time, epidemiological calculations must be interpreted careful. Final result depends on collected statistical data, and they in turn not always reflect a real state of the things.

In this study were evaluated data about companion animals, so, they extremally depend on the tutors. Frequently they live in the house and rarely, or even never, walking outside, so, the temperature and humidity of them ambient do not correspond to the nature conditions. They also depend on the social and financial conditions of the owners, and it reflects on the quality and frequency of the preventive and therapeutic procedures for the animals. We also must consider a spreading of the certain breeds in the country/geographical zone.

Unfortunately, our understanding of the epidemiology of fungal infections remains quite far from complete understanding and is hampered by inadequate diagnostic methods and the

lack of mandatory reporting of fungal disease. The epidemiology of fungal infections is in a constant state of flux (Anaissie *et al*., 2009).

This work is the first comprehensive study of the epidemiological situation in dermatophytosis of the dogs and cats in Portugal, and at the same time the first phase of optimization and posterior validation of the molecular identification method based on PCR one-step for direct identification of the dermatophytes in the clinical veterinary samples.

SUPPLEMENTARY DATA

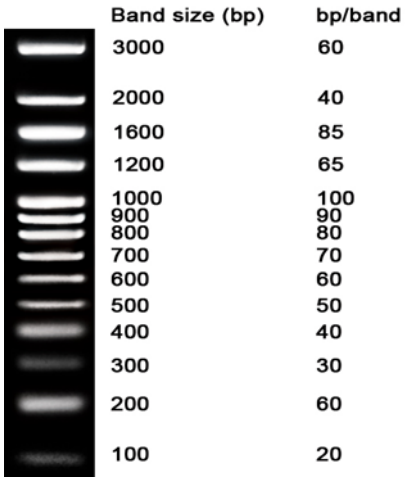


Figure S1 - Patterns produced by NZYDNA Ladder VII. Adapted from (NZY ONLINE).

Supplementary Table S1 – Dermatophytosis data collected from 2001 to 2016

(Summary, samples per year)

	2001	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
DOGS	62	205	234	193	185	144	163	97	65	63	65	118	115	88	74	106
CATS	33	58	52	55	45	63	66	56	33	31	53	51	54	46	65	55

REFERENCES

Achterman, R.R., Smith, A.R., Oliver, B.G., White, T.C., 2011. Sequenced dermatophyte strains: growth rate, conidiation, drug susceptibilities, and virulence in an invertebrate model. *Fungal Genetics and Biology*, 48, (3): 335 – 341

Ahmadi, B., Mirhendi, H., Makimura, K., Sybren de Hoog, G., Shidfar, M.R., Nouripour – Sisakht, S., Jalalizand, N., 2016. Phylogenetic analysis of dermatophyte species using DNA sequence polymorphism in calmodulin gene. *Medical Mycology*, 54, (5): 500 – 514

Alves, P.D.M., 2017. *Rastreio de fungos patogénicos e oportunistas em animais domésticos, selvagens e em humanos: Implicações na saúde pública*. Dissertação de Mestrado em Biologia Clínica Laboratorial. Universidade de Trás-os-Montes e Alto Douro, Portugal, pp. 85

Anaissie, E. J., McGinnis, M. R., Pfaller, M. A., 2009. Clinical Mycology. 2nd Ed., Elsevier, China, pp. 688

Aneja, K.R., Joshi, R., Sharma, C., Surain, P., Aneja, A., 2012. Biodiversity of dermatophytes: an overview. *Review of plant pathology*, 5: 299 – 314

Arabatzis, M., Bruijnesteijn van Coppenraet, L.E.S., Kuijper, E.J., de Hoog, G.S., Lavrijsen, A.P.M., Templeton, K., van der Raaij-Helmer, E.M.H., Velegraki, A., Graser, Y., Summerbell, R.C., 2007. Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme. *British Journal of Dermatology*, 157: 681 – 689

Arthrodermataceae Dermatophytes. [Online]. Available at: < http://eol.org/pages/6106/hierarchy_entries/50982642/overview >, Accessed in 10.04.2018

Bahri, J., 2013. *Dermatofitoses*: *causas e tratamento*. Trabalho de conclusão do curso, Centro universitário CESMAC, 36

Behzadi, P., Behzadi, E., Ranjbar, R., 2014. Dermatophyte fungi: infections, diagnosis and treatment. *SMU Medical Journal*, 1, (2): 50 – 62

Belleman, E., Carlsen, T., Brochmann, C., Colssac, E., Taberlet, P., Kauserud, H., 2010. ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. *BMC Microbiology*, 10, (189). [Online]. Available at < https://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-10-189 >, Accessed in 19.06.2018

Bergman, A., Heiman, D., Kondori, N., Enroth, H., 2013. Fast and specific dermatophyte detection by automated DNA extraction and real-time PCR. *Clinical Mycology and Infection*, 19, (4): E205 – E211

Bernardo, F., Lança, A., Guerra, M.M., Martins, M.M., 2005. Dermatófitos isolados de animais de companhia (cão e gato), em Lisboa, Portugal (2000-2004). *Revista portuguesa de ciências veterinárias*, 100: 85 - 88

Blackwell, M., 2011. The Fungi: 1, 2, 3… 5,1 million species? *American Journal of Botany*, 98, (3): 426 – 438

Bourguignon, E., Guimarâes, L.D., Ferreira, T.S., Favarato, E.S., 2013. *Dermatology in Dogs and Cats***.** Insights from veterinary medicine. pp. 290

Bray, I., Reghmi, K., 2016. *Public Health Intelligence*, Chapter 9, Erratum to: Epidemiology and Public Health Intelligence. Springer International Publishing, Switzerland, pp. 20 – 40

Botelho, F., Silva, C., Cruz, F., 2008. Epidemiologia explicada – O valor de prova (p). Acta Urológica, 25, (3): 55 - 57

Brillowska – Dabrowska, A., Saunte, D.M., Arendrup, M.C., 2007. Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum*. *Journal of Clinical Microbiology*, 45, (4): 1200 – 1204

Calvacante Bin, L.L., Gomes, J., Braz, S.A., Giuffrida, R., 2010. Comparação de métodos diagnósticos para dermatofitose em animais de companhia. *Colloquium Agrariae*, 6, (2): 46 – 51

Cafarchia, C., Gasser, R.B., Figueredo, L.A., Weigl, S., Danesi, P., Capelli, G., Otranto, D., 2013. An improved molecular diagnostic assay for canine and feline dermatophytosis. *Medical Mycology*, 51: 136 – 143

Cafarchia, C., Ortanto, D., Weigi, S., Campbell, B.E., Parisi, A., Cntacessi, C., Mancianti, F., Danesi, P., Gasser, R., 2009. Molecular characterization of selected dermatophytes and their identification by electrophoretic mutation scanning. *Electrophoresis*, 30, (20): 3555 - 3564

Cafarchia, C., Romito, D., Capelli, G., Guillot, J., Otranto, D., 2006. Isolation of the *Microsporum* canis from the hair coat of pet dogs and cats belonging to owners diagnosed with *M*. *canis* tinea corporis. *Veterinary Dermatology*, 17, (5): 327 - 331

Costa-Orlandi, C.B., Sardi, J.C.O., Santos, C.T., Fusco-Almeida, A.M., Mendes-Giannini, M.J.S., 2014. In vitro characterization of Trichophyton rubrum and T. mentagrophytes biofilms. *Biofouling: The Journal of Bioadhesion and Biofilm Research*, 30, (6): 719 – 727

Dabas, Y., Xess, I., Singh, G., Pandey, M., Meena, S., 2017. Molecular identification and antifungal susceptibility patternsof clinical dermatophytes following CLSI and EUCAST guidelines. *Journal of Fungi*, 3, (2), pp. 10

Dabrowska, I., Dworecka – Kaszak, B., Brillowska – Dabrowska, A., 2014. The use of a one-step PCR method for the identification of *Microsporum canis* and *Trichophyton mentagrophytes* infection of pets. *Acta Biochimica Polonica*, 61, (2): 375 - 378

Deacon, J. W., 2006. *Fungal Biology*. Blackwell Publishing. 4th ed. Oxford, UK:210, pp. 371

De Hoog, G.S., Dukik, K., Monod, M.I., Packeu, A., Stubbe, D., Hendrickx, M., Kupsch, C., Stielow, J.B., Freeke, J., Goker, M., Rezaei-Matehkolaei, A., Mirhendi, H., Graser, Y., 2017. Toward a Novel Multilocus Phylogenetic Taxonomy for the Dermatophytes. *Mycopathologia*, 182, (1-2): 5 - 31

Dong, C., Angus, J., Scarampella, F., Neradilek, M., 2016. Evaluation of dermoscopy in the diagnosis of naturally occurring dermatophytosis in cats. *Veterinary Dermatology*, 27: 275 – e65

Frymus, T., Grufydd-Jones, Tl., Pennisi, M.G., Addie, D., Belák, S., Boukraut-Baralon, C., Egberink, H., Hartmann, K., Hosie, M.J., Lloret, A., Lutz, H., Marsilio,F., Mostl, K., Radford, A.D., Thiry, E., Truyen, U., Horzinek, M.C., 2013. Dermatophytosis in cats ABCD guidelines on prevention and management. *Journal of Feline Medicine and Surgery*,15: 598 – 604

Garg, J., Tilak, R., Garg, A., Prakash, P., Gulati, A.K., Nath, G., 2009. Rapid detection of dermatophytes from skin and hair. *BioMed Central Research Notes*, 2, (1): 60

Gutzmer, R., Mommert, S., Kuttler, U., Werfel, T., Kapp, A., 2004. Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. *Journal of Medical Microbiology*, 53: 1207 – 1214

Hayette, M., Sacheli, R., 2015. Dermatophytosis, trends in epidemiology and diagnostic approach. *Current fungal infection reports*, 9, (3): 164 - 179

Hawksworth, D.L., Lucking, R., 2017. Fungal diversity revisited: 2,2 to 3,8 million species. *Microbiology Spectrum*, 5, (4)

HCMI, 2011. Fungus/Mold/Yeast/Mycotoxin/Prion – Complete list and description. [Online]. Available at: <http://www.hcmionline.com/pdfs/funguskit_descriptionlist.pdf> Accessed in 16.04.2018

Jacobson, L.S., McIntyre, L., Mykusz, J., 2017. Comparison of real-time PCR with fungal culture for the diagnosis of *Microsporum canis* dermatophytosis in shelter cats: a field study. *Journal of Feline Medicine and Surgery*, 9: 1 – 5

Joshi, M., Deshpande, J.D., 2010. Polymerase chain reaction: methods, principles and application. *International Journal of Biomedical Research*, 2, (1): 81 - 97

Kano, R., Aihara, S., Nakamura, Y., Watanabe, S., Hasegawa, A., 2001. Chitin synthase 1 (Chs1) gene sequences of *Microsporum equinum* and *Trichophyton equinum. Veterinary Microbiology*, 78, (1): 75 – 90

Kano, R., Nakamura, Y., Watari, T., Watanabe, S., Takahashi, H., Tsujimoto, H., Hasegawa, A., 1997. Phylogenetic analysis of 8 dermatophyte species using chitin synthase 1 gene sequences. *Mycoses*, 40, (11 - 12): 411 - 414

Kidd, S., Halliday, C., Alexiou, H., Ellis, D., 2016. *Descriptions of medical fungi*. Ed. Newstyle Printing, Adelaide, Australia

Lakshmipathy, D.T., Kannabiran, K., 2010. Review on dermatomycosis: pathogenesis and treatment. *Natural Science*, 2, (7): 726-731

Lenardon, M.D., Munro, C.A., Gow, N. AR., 2010. Chitin synthesis and fungal pathogenesis. *Current opinion in microbiology*, 13: 416 – 423

Liu, R., Xu, C., Zhang, Q., Wang, S., Fang, W., 2017. Evolution of the chitin synthase gene family correlates with fungal morphogenesis and adaption to ecological niches. *Scientific Reports*, 7: 44527, [Online], Available at: < https://www.nature.com/articles/srep44527 >.

Lorenz, T.C, 2012. Polymerase Chain Reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*, 63: e3998, [Video article, Online], Available at: < https://www.jove.com/video/3998/polymerase-chain-reaction-basic-protocol-plus-troubleshooting >, Accessed in 19.08.2018

Martinez, D.A., Oliver, B.G., Graser, Y., Goldberg, J.M., Li, W., Martinez-Rossi, N.M., Mohod, M., Shelest, E., Barton, R.C., Birch, E., Brakhage, A.A., Chen, Z., Gurr, S.J., Helman, D., Heltman, J., Kosti, I., Rossi, A., Salf, S., Samalova, M., Saunders, C.W., Shea, T., Sommerbell, R.C., Xu, J., Young, S., Zeng, Q., Birren, B.W., Cuomo, C.A., White, T.C., 2012. Comparative genome analysis of *Trichophyton rubrum* and related dermatophytes reveals candidate genes involved in infection. ®*mBio.asm.org*, 3, (5): e00259-12. [Online]. Available at: < http://mbio.asm.org/content/3/5/e00259-12.full >, Accessed in 13.04.2018

Martinez-Rossi, N.M., Peres, N.T.A., Rossi, A., 2016. Pathogenesis of dermatophytosis: sensing the host tissue. *Mycopathologia*, 182, (1-2): 215 - 227

Mattei, A.S., Beber, M.A., Madrid, I.M., 2014. Dermatophytosis in small animals. *SOJ Microbiology & Infection Diseases*, 2, (3): 1 – 6

Menelaos, L. A., 2006. Dermatophytosis in dog and cat. *Buletin USAMV-CN*, 63: 304 - 308

Moretti, A., Agnetti, F., Mancianti, F., Nardoni, S., Righi, C., Moretta, I., Morganti, G., Papini, M., 2013. Dermatophytosis in animals: epidemiological, clinical and zoonotic aspects. *Giornale Italiano di Dermatologia e Venereologia*, 148, (6): 563 - 572

Moriello, K, A., 2016. *Overview of integumentary system*. MSD veterinary manual, Ed. 11. [Online].Available at: <http://www.msdvetmanual.com/integumentary-system/integumentary-system-introduction/overview-of-integumentary-system#v4657129>, Accessed in 14.02.2018

Moriello, A.K., Coynert, K., Paterson, S., Mignon, B., 2017. Diagnosis and treatment of dermatophytosis in dogs and cats. *Veterinary Dermatology*, 28: 266 - e68

Nasrin, S., Saeed, Z.B., Sina, M., Sahar, N., 2017. Genotyping and Molecular Characterization of dermatophytes isolates collected from clinical samples. *Archives of Pulmonology and Respiratory Care*, 3, (3): 52 – 57

Ninet, B., Jan, I., Bontems, O., Lèchenne, B., Jousson, O., Panizzon, R., Lew, D., Mohod, M., 2003. Identification of dermatophyte species by 28S ribosomal DNAsequencing with a commercial kit. *Journal of Clinical Microbiology*, 41, (2): 826 - 830

Patel, S.V., Bosamia, T.C., Bhalani, H.N., Singh, P., Kumar, A., 2015. Polymerase chain reaction (PCR). *Agrobios Newsletter*, 8, (9): 10 – 12

Perevedenceva L.G., 2009. *Микология*: *Грибы и грибоподобные организмы*, Tutorial. Perm, FR, pp. 76

Pfeiffer, D.U., 2009. *Introduction to veterinary epidemiology*. Unpublished. [Online]. Available at: <https://doi.org/10.13140/rg.2.1.3773.3365 >, Accessed in 25.06.2018

Pinho, R., Monzón, M, F., Simões, J., 2013. Dermatologia veterinária em animais de companhia: a pele e seus aspetos relevantes na prática clínica. *Veterinaria.com.pt*, 5, (1-2): Sec. 2. [Online]. Available at: < http://www.veterinaria.com.pt/133023.html >, Accessed in 27.02.2018

Prakash, P.Y., Irinyi, L., Halliday, C., Chen, S., Robert, V., Meyer, W., 2017. Online databases for taxonomy and identification of pathogenic fungi and proposal for a cloud-basic dynamic data network platform. Journal of Clinical Mycrobiology, 55, (4): 1011 - 1024

Quinn, P.J., Carter, M.E., Markey, B., Carter, G.R., 1994. *Veterinary Clinical Microbiology*. Section 3. Ed. Grafos, S.A. Arte Sobre Papel, Spain, pp. 648

Refai, M, K., El-Yazeed, H, A., Abdel-Haleem, M., Hassan, A., El-Hariri, M., 2016. *Fungal diseases of cats and dogs*. Monograph, a guide for postgraduate students. Cairo University, Cairo, Egypt, pp. 393

Rumsey, **D**., 2010. *Statistics Essentials for dummies*. Wiley Publishing, Indianapoilis, USA, pp. 178

Samiksha, **S**. Reproduction in fungi: vegetative, asexual and sexual methods. [Online]. Available at: <http://www.yourarticlelibrary.com/fungi/reproduction-in-fungi-vegetative-asexual-and-sexual-methods/7296 >, Accessed in 01.11.2017

Saraiva, **S.T**., 2017. *Epidemiologia e diagnóstico de dermatofitose em humanos e animais: uma abordagem "One Health*". Dissertação de Mestrado em Biotecnologia para as Ciências da Saúde. pp. 79

Sergeant, ESG, 2018. Epitools epidemiological calculators. [Online]. Available at: <http://epitools.ausvet.com.au>, Accessed in 28.06.2018

Seyedmousavi, S., Guillot, J., Tolooe, A., Verweij, P.E., de Hoog, G.S., 2015. Neglected fungal zoonoses: hidden treats to man and animals. *Clinical Microbiology and Infection*, 21, (5): 416 – 425

Shehata, A.S., Mukherjee, P.K., Aboulatta, H.N., El Akhras, A.I., Abbadi, S.H., Ghannoum, M.A., 2008. Single-step PCR using (GACA)4 primer: utility for rapid identification of dermatophyte species and strains. Journal of Clinical Mycrobiology, 46, (8): 2641 - 2645

Skerlev, M., Miklic, P., 2010. The changing face of Microsporum spp infections. *Clinics in Dermathology*, 28: 146 – 150

Souza, T.M., Fighera, R.A., Kommers, G.D., Barros, C.S.L., 2009. Aspetos histológicos da pele de cães e gatos como ferramenta para dermatopatologia. *Pesquisa Veterinária Brasileira*, 29, (2): 177-190.

The University of Adelaide, Micology online. [Online]. Available at: <https://mycology.adelaide.edu.au/descriptions/dermatophytes/microsporum/ >, Accessed in 14.11.2017

The University of Adelaide, Micology online. [Online]. Available at: <https://mycology.adelaide.edu.au/descriptions/dermatophytes/trichophyton/ >, Accessed in 14.11.2017

Universitat de València, Colección de cultívos tipo, Lista de hongos. [Online]. Available at: < https://www.uv.es/uvweb/coleccion-espanola-cultivos-tipo/es/cect/catalogo-cepas/medios-cultivo/lista-hongos/m-1285892320209.html >, Accessed in 31.01.2018

Universitat de València, Colección de cultívos tipo, Lista de hongos. [Online]. Available at: < https://www.uv.es/uvweb/coleccion-espanola-cultivos-tipo/es/cect/catalogo-cepas/medios-cultivo/lista-hongos/t-1285892323955.html >, Accessed in 31.01.2018

Verrier, J., Krahenbuhl, L., Bontems, O., Fratti, M., Salamin, K., Mohod, M., 2012. Dermatophyte identification in skin and hair samples using a simple and reliable nested polymerase chain reaction assay, *British Journal of Dermatology* © British Association of Dermatologists 2013, 168: 295 – 301

White, M., Henn, M. Genomic Determinants of Infection Competence in Dermatophyte Fungi. [Online]. Available at < https://www.genome.gov/pages/research/sequencing/seqproposals/dermatophyte_wp_seq.pdf > Accessed in 20.06.2018

Weitzman, I., Summerbell, R.C., 1995. The Dermatophytes. *Clinical Microbiology Reviews*, 8, (2): 240 – 259

Zhan, P., Liu, W., 2016. The Changing Face of Dermatophytic Infections Worldwide. *Mycopathologia*, 182 (1 – 2): 77 - 86