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Extracellular Protein Profile of *Microsporum canis* Secreted Proteolytic Enzymes (Exoantigens)

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Abstract: *Microsporum canis* is the major zoonotic agent causing dermatophytosis for cats and dogs. The exoenzyme (Keratinolytic proteases) are considered as the main virulence component secreted by *M. canis*. The detailed study of *M. canis* secreted enzymes is so important for understanding the disease pathogenesis and also important in developing a protective vaccines. As they are a potential source of antigen to induce protective immunity thus they are also named "exoantigens". The protein patterns of *M. canis* isolated strains were compared with those obtained from a reference strain using SDS-PAGE on 12.5% polyacrylamide resolving gels revealing about 18 clearly detectable protein bands over a wide range of molecular weight 6.500 Da to 240, 000 Da. Proteins of: 6.5, 10, 15, 20, 24, 28, 31.5, 35, 40, 45, 50, 58, 64, 87, 93, 122, 147, 163, 180, 240 KDa were present but their frequencies varied among the isolates. Protein bands of: 6.5, 10, 15, 20, 25, 31.5, 35, 40, 45, 50, 60, 64, 93, 122, 147, 163, 180, 240 KDa were common among the local isolates and reference isolates and they were considered species-specific.

Key words: Exoenzymes · Dermatophytes · *M. canis* · SDS - PAGE · Keratinases · Fungal Proteinases

INTRODUCTION

Dermatophytosis (Tinea or ringworm) is considered one of the most important fungal skin diseases which infects animals and transmitted from animal to animal, from animal to human and from human to animal [1, 2]. This disease is caused by a group of morphologically and physiologically related moulds called Dermatophytes, which are typical both keratinolytic and keratinophilic, as they can digest keratin *in vitro* in saprophytic state, utilizing it as a substrate and also can invade tissues *in vivo* provoking tineas [3]. Yet, the morphology they show at the parasitic growth phase, *in vivo*, differs from that occur *in vitro* [4]. So we can conclude that dermatophytes are a group of parasitic fungi which have the ability to attack the keratinized structures like nails, hair and *stratum corneum* provoking superficial infections for humans and animals [5]. Dermatophytes are classified into three anamorphic genera, Epidermophyton, Microsporum and Trichophyton. *Microsporum canis* considered the main dermatophyte for domestic animals [6] and is responsible for a frequent zoonosis that has increased in several countries [7, 8]. *M. canis* infection frequency in dogs and cats ranges from 40% to 90% [9] by a variable number of dogs (9%) and in cats (80%) which seems to be a healthy carriers for dermatophyte [10]. Histologically, symptomatic cats reveal acute or sub-acute folliculitis and perifolliculitis, on the other hand asymptomatic carriers reveal chronic inflammation which is characterized by infiltrate of mast cells in the superficial dermis layer [11]. Pathological reactions of dermatophytosis are mediated by substances secreted by these dermatophytes which diffuse through the stratum corneum during the course of infection [12]. So the ability of *M. canis* in: producing enzymes is involved in the skin infection pathogenesis, as it can overcome the host defense mechanisms and fulfill

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its nutritional needs. It has been reported that *Microsporum canis* secretes proteolytic enzymes (Exoantigens), comprise keratinases, elastases, proteinases, peptidases, catalases, aminopeptidases and peroxidases [13]. While keratinases are the most important dermatophyte virulence factors as they can help in invasion of host keratinized tissues [14]. As Immunoprophylaxis can be an important alternative for the current control measures that include usage of topical and systemic antifungals for five weeks at least, detention of infected pet until completely cured and environmental decontamination. All These actions make treatment of dermatophytosis, expensive and time consuming [15]. So, it is so important to get detailed knowledge about different exoantigens in the immune response to understand the pathogenesis of dermatophytosis and also so important in developing a protective vaccines [16]. In the present study we described the protein patterns of secreted extracellular proteins or exoantigens of a reference strain and two local strains of *M. canis* using Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE) as a useful tool in identification of *M. canis* and its classification.

MATERIALS AND METHODS

Strains: In this study we used a reference strain CBS 136538 *Microsporum canis* obtained from Westerdijk Fungal Biodiversity Institute (Lately named CBS-KNAW), Utrecht, the Netherlands. Beside, two local isolates of *M. canis* were isolated from infected cats showing ringworm lesions.

Sampling (Hair Brush Technique): The entire body of the cat was examined thoroughly for any evidence of scaling, crusting, hair loss and erythema. Samples of hair and crusts were collected from skin lesions showing clinical signs combined with dermatophytosis. Skin samples were collected by brushing the lesions with a sterile brush after cleaning the lesions with gauze moistened with 70% alcohol. Samples were collected in a clean paper envelop [4]. Each sample was divided into two parts. The ?rst was examined microscopically under a light microscope at 40 X magni?cation power after treating with 20% potassium hydroxide (KOH) and lactophenol cotton blue. The second was cultured on Sabouraud's dextrose agar (SDA) with (0.05% chloramphenicol and 0.5% cycloheximide). The slopes were incubated at 25°C for 2 weeks and examined for any growth at 3 days intervals. Isolates were identified on the basis of phenotypic characteristics of the colonies on SDA (Texture, growth rate, pigmentation, surface and reverse color) and microscopic examination of lactophenol cotton blue wet mounts [3].

Proteinases Production in Broth Cultures: The strains of *M. canis* were cultivated on Sabouraud's Dextrose Broth (Oxoid) and on mineral media containing defatted human hair. Chloramphenicol and cyclohexamide were added on both media. Incubated at 30 °C for 21 days. All media were autoclaved at 121°C for 15 minutes. The extracellular fluid was then filtrated using Whatman's no.1 filter paper.

Estimating Protein Concentration: The protein content of each sample was determined by the Bradford's method [17].

Protein Precipitation: Protein precipitation was done by using tricholoro acetic acid 72% (w/v) according to Mignon [18].

SDS –PAGE: The protein extracts were dissolved in an equal volume of double strength loading buffer (0.125 mol Tris-HCl containing 2% SDS, 2% 2ME, 10% glycerol, 2% bromophenol blue). Volumes of 10-20 microliter, containing 3-10 micrograms protein were electrophoresed in 10% polyacrylamide gel. using a stacking gel buffer of 0.125 mol Tris- HCl, pH 6.8, a separating gel buffer of 0.375 mol Tris-HCl, pH 8.8 and tank buffer of 0.025 mol Tris-HCl, 0.192 mol glycine pH 8.3. The resulted gel was run at 5 mA through the stacking gel and 7 mA through the separating gel in Electrophoresis Unit, until the tracking dye had reached the bottom of the gel. A wide range marker (Sigma marker) molecular weight ranges from 6, 500 to 200, 000 Da was electrophoresed in parallel. The gel was removed and stained for protein with Coomassie Brilliant Blue R-250 (Sigma Chemical, USA) and distained. Protein patterns were analyzed using a scanner, printed and their molecular weights were calculated by comparing them with those of the standards [19, 20].

RESULTS AND DISCUSSION

Many studies have described the large quantity of enzymes produced by dermatophytes, as well as the lysing activity of these enzymes on various substrates [21]. These enzymes especially Keratinase, have been considered as virulence factor [4] thus they are correlated with the clinical form of dermatophytosis. There are evidence that some enzymes produced by dermatophyte

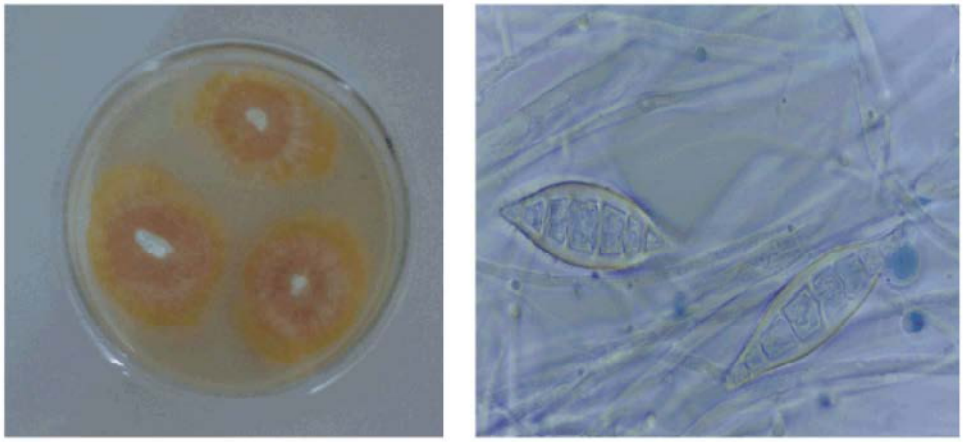

Fig. 1: Showing the macroscopic and microscopic appearance of *M. canis* isolates

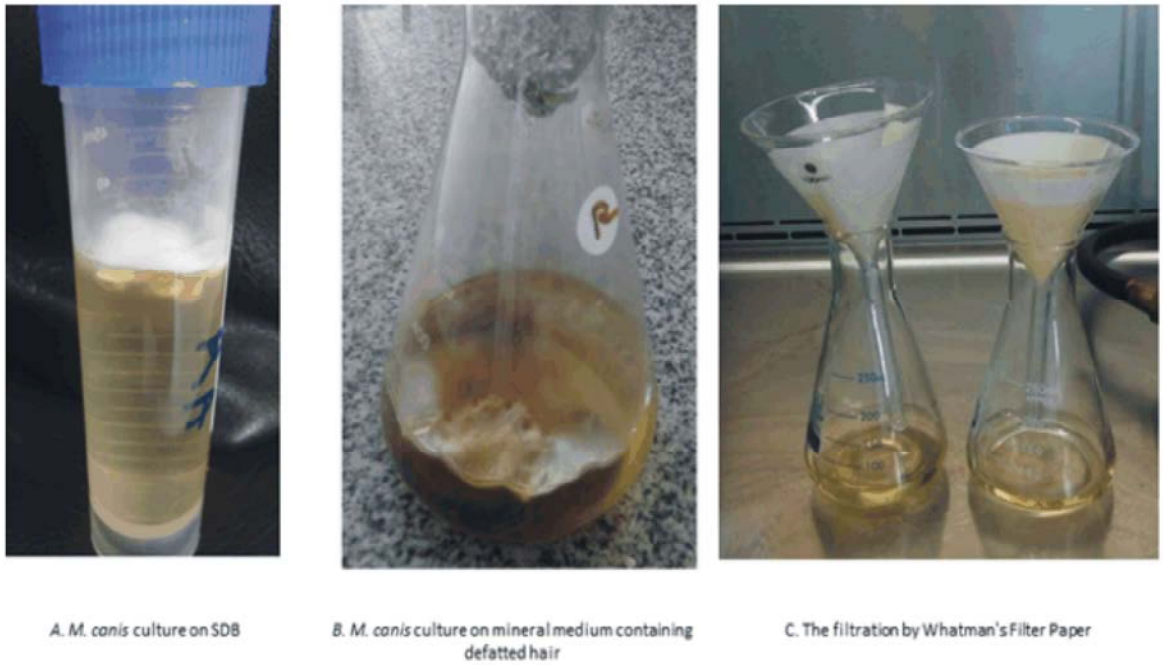

Fig. 2: a-*M. canis* culture on SD broth, b-*M. canis* culture on mineral media, c-Filtration using Whatman's filter paper

give rise to hydrolytic activity against keratin substance [15, 20]. Wearly *et al.* [22] reported that *M. canis* is capable of exerting a keratinolytic effect on Ethylene oxide sterilized wool obtained from sheep.

In current study, *M. canis* strain was isolated from collected samples and confirmatory test was applied on the reference strain (Fig. 1).

The media containing secreted proteolytic Enzymes was filtrated as show in Figure (2).

Exoantigen was separated by SDS-PAGE from the culture filtrates of *M. canis* isolates grown on Sabouraud's broth as shown in Fig. (3) and that was separated from the culture filtrates of *M. canis* strains grown on mineral medium as shown in Fig. (4).

The cytoplasmic extracts of *Microsporum canis* was revealed by SDS-PAGE. About 18 clearly detectable protein bands over a wide range of molecular weight 6.500 Da to 240, 000 Da were recognized. The best results

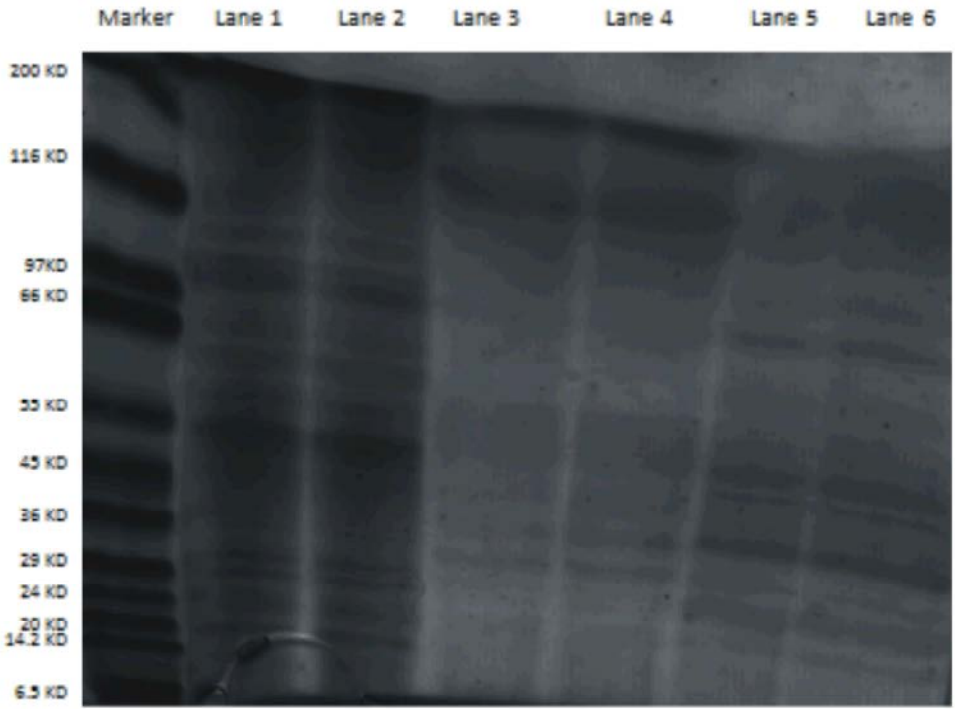

Fig. 3: SDS-PAGE protein profiles of *M. canis* strains grown on SD broth medium
Marker = molecular weight marker, lane 1= local strain a, lane 2 and 3 = reference strain and lane 4 and 5= local strain b

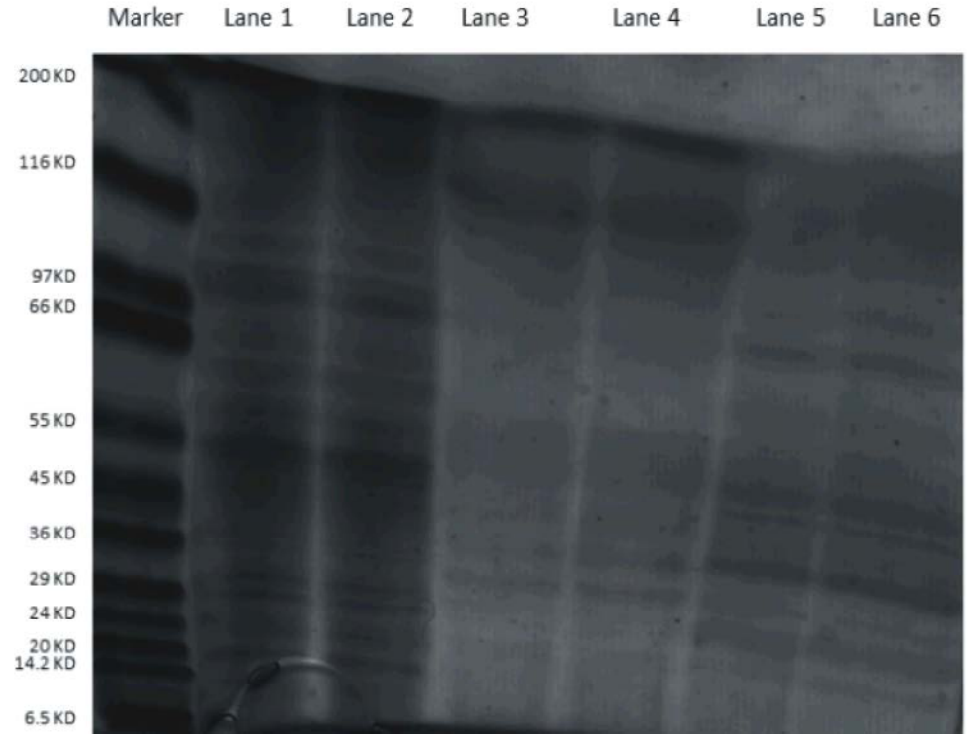

Fig. 4: SDS-PAGE protein profiles of *M. canis* strains grown on mineral medium
Marker = molecular weight marker, lane 1and 2= reference strain , lane 3 and 4 = local strain a and lane 5 and 6= local strain b

were obtained with freshly prepared extracts. Proteins of: 6.5, 10, 15, 20, 25, 31.5, 35, 40, 45, 50, 60, 64, 93, 122, 147, 163, 180, 240 KDa were present but their frequencies varied among the isolates. Protein bands of 6.5, 10, 15, 20, 31.5, 35, 40, 45, 50, 64, 122, 147KDa were common among the local isolates and reference strain and they were considered species-specific. This result was similar to those revealed by Simpanya and Baxter [23] as there were six proteinases at different molecular weights; where bands of molecular weight of122 KDa, 64 KDa, 62 KDa, 45 KDa, 31 KDa and 25 KDa were revealed to be specific to *M. canis*. Similar conclusions also were confirmed for the proteins, 31.5 KDa, 34 and 48 fragments, under the name of Ekase (Keratinase) [24]. Besides, the presence of elastase, 64 and 62 KDa in *M. canis* is of significant clinical value, as it grows on *stratum corneum*, hair and nails utizing them as sole sources of nitrogen and carbon during infection [25]. Also the 20 KDa of collagenase as it was the first collagenase of fungal origin from *Trichophyton schoenleinii* reported by Rippon [26]. Also it is said that the 40 KDa protein band is for fungal lipase as stated by Sumathy *et al*. [27]. Also our results agreed with the results discussed by Takiuchi *et al*. [28] who isolated and purified proteinase enzyme from the culture filtrate of *T. rubrum* collected from human hair samples. Also, a 45 KD extracellular proteinase was detected from *M. canis* filtrate as well [29]. The majority of low molecular weight proteinases, in a range from 18.5 to 35 KDa and around 25 KDa are serine proteinases, while larger enzymes also reported by North [30]. These serine proteinases play a very important role in the disease pathogenesis as they are small size so can easily diffuse into the lower epidermis and dermis causing inflammation. Minocha *et al*. [31] showed dermo-epidermal separation and spongiosis as they injected the fungal extracts intradermally. These changes were suggested to be as a result of proteolytic enzymes. Simpanya [32] used starch gels to demonstrate the cleavage of peptides into amino acids by peptidases and aminopeptidases. Daniels [33] cultured *M. canis* on human hair and reported also the accumulation of amino acids. No difference was observed due to the variation of the media used to grow *M. canis* strains.

The conventional classification of the dermatophytes depends on gross and microscopic morphology with minor emphasis on physiology and nutrition. As colonies of dermatophyte have forms and produce pigmentation by which identification of dermatophyte species takes place [34]. However, identification of dermatophyte isolates has been tricky due to their overlapping characteristics, phenotypic variability and pleomorphic.

This study will be preliminary for further studies aim to evaluate the immunogenicity and protective efficacy of these secreted enzymes.

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