Extracellular proteolytic activity and molecular analysis of *Microsporum canis* strains isolated from symptomatic and asymptomatic cats

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Summary

Microsporum canis is the main zoophylic dermatophyte in dogs and cats, and it is also an important zoonotic agent. The literature showed that cats are asymptomatic carriers of *M. canis*. This is apperently due to host resistance and/or the presence of strains with lower virulence. This study was aimed to evaluate the keratinolytic, elastinolytic and collagenolytic activities of *M. canis* strains and their relationship with symptomatic and asymptomatic cats. In addition, these strains were analysed by RFLP. The strains isolated from cats with clinical dermatophytosis had higher keratinase and elastase activity than those isolated from asymptomatic animals ($p < 0.05$). There were not differences in RFLP patterns based on *Hind* III digestion.

Key words Dermatophyte, Dermatophytosis, *Microsporum*, Enzymes, RFLP

Actividad proteolítica extracelular y análisis molecular de cepas de *Microsporum canis* aisladas de gatos con y sin sintomatología

Resumen

Microsporum canis es el principal dermatofito en perros y gatos, siendo agente importante de zoonosis. La literatura informa la existencia de gatos que son portadores asintomáticos de *M. canis*, debido a la resistencia del portador y a la presencia de cepas con menor virulencia.
Este estudio fue realizado para evaluar la actividad queratinolítica, elastinolítica y colagenolítica de *M. canis* y su relación con gatos (con y sin síntomas) y para analizar molecularmente las cepas por el método de RFLP.
Las cepas aisladas de gatos con dermatofitosis tenían actividad queratinasa y elastasa mayores que las cepas aisladas de los gatos asintomáticos ($p < 0.05$). No hay diferencias en el análisis de RFLP basado en el patrón de digestión con *Hind* III.

Palabras clave Dermatofito, Dermatofitosis, *Microsporum*, Enzima, RFLP

Microsporum canis is one of the main etiological agents of dermatophytosis in animals and one of the most prevalent agent in dogs and cats [4,9,21,35] and recently detected in asymptomatic cats [10,12,38,40]. The wide-scale incidence of asymptomatic cats could be related to immunity acquired during a primary infection [8] or to the existence of less-virulent strains able to produce less-apparent tissue damage [38].

The dermatophytes produce enzymes with the ability to degrade various substrates, such as keratinase [2,18,28,30,41-43], elastase [2,17,30,32,37,42], lipase [41], DNase [22,42] and collagenase [15,23,31,26]. Keratinase bears a direct relation with the clinical profile of experimental infections in guinea pigs and with the occurrence or nonoccurrence of lesions in naturally infected cats [42]. The elastases play an important role in the pathogenesis of other fungi such as *Aspergillus fumigatus* [37] and probably for the dermatophytes as well. In humans, the elastase-producing strains of dermatophytes give rise to acute lesions, while the non-elastase-producing strains result in chronic lesions [22,34].

This study was aimed to demonstrate the quantitative keratinase, elastase and collagenase activity of *M. canis*, isolated from symptomatic and asymptomatic cats, to compare the degree of enzyme production with the clinical profile of the original host; and to determine differences in RFLP (Restriction Fragment Length Polymorphisms) analysis by *Hind III* digestion.

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Material and methods

Strains, cultivation and identification. The hairs from cats with dermatophytosis were collected from the infected region with tweezers, wheras in the asymptomatic cats the material was collected by the sterile carpet method [26]. The material collected was smeared into selective agar for pathogenic fungi (Merck, Frankfurter, Germany). The isolated fungi were cultivated in Sabouraud dextrose agar (Oxoid, Basingstoke, UK) and identified by macroscopic and microscopic morphology [18,27,33].

Determination of the strains enzymatic profil. The strains of *M. canis* isolated were cultivated in 2 ml of Sabouraud dextrose broth (Oxoid) at 25 °C for 15 days.

Keratinase: the mycelium was collected and smeared in 50 ml of medium with Keratin Powder from Hooves and Horn (ICN, Montreal, Canada) [36] and incubated at 25 °C for 14 days without light. After 14 days [42], the supernatant was evaluated for keratinolytic activity using keratin azure (Sigma, St Louis, MO, USA); the material was incubated for 24 h in a 37 °C incubator and degradation of keratin was quantified by spectrophotometer at a wavelength of 595 nm. One unit of keratinase (UK) was defined as an increment of 0.1 in the absorbency reading in relation to the control (uninoculated substrate buffer) [3].

Elastase: the strains were cultivated in mediums with elastin (Sigma) for 14 days. Two ml supernatant was added to 2 ml of 10 mM, pH 7.0 phosphate buffer along with 20 mg of Elastin Congo-Red (Sigma), and the resulting mixture was incubated at 37 °C for 2 h. The degradation of elastin was measured in a spectrophotometer at a wavelength of 495 nm. One unit of elastase (UE) was defined as an increment of 0.1 in the absorbency reading in relation to the control (4 ml of the same phosphate buffer and 20 mg of substrate) [17,35].

Collagenase: the strains were cultivated in medium with type 1 collagen (Sigma) for 14 days. To quantify the collagenolytic action of the resulting supernatant a suspension was made containing 0.1 ml of a solution containing 1.0 mmol/l of a synthetic peptide [PZ-Pro-Leu-Gly-Pro-d-Arg] (Sigma) prepared with 0.1 mol/l of Tris-HCl, pH 7.2 buffer, 0.1 ml of the supernatant of the cultures and 0.8 ml of Tris-HCl buffer, and incubated at 37 °C for 1 h. The degradation of the peptide, forming PZ-Pro-Leu, was measured in a spectrophotometer at a wavelength of 320 nm. One unit of collagenase (UC) was defined as an increment of 0.1 in the absorbency reading in relation to the control (0.2 ml of the same buffer and 0.1 ml of the substrate) [6,38].

Genomic DNA isolation [20,21]. *M. canis* isolates were cultured in Sabouraud agar at 25 °C for 20 days. The mycelial samples were collected and homogenized in liquid nitrogen. The samples were lysed with 1.5 mg of lisozyme (Amersham-Pharmacia, Piscataway, USA) per ml in lysis buffer containing 0.1 mM EDTA, 10 mM Tris hydrochloride (pH 8.0) at 37 °C for 1 h. Then 25 µl of a 10 mg RNase (Amersham-Pharmacia) per ml was aded and incubeted at 37 °C for 30 min. Then was incubated at 37 °C for 1 h with 300 µl of 10% sodium dodecyl sulphate (SDS) and 50 µl of 10 mg/ml of Proteinase K (Amersham-Pharmacia). It was mixed with 1.7 ml of 3.0 M sodium acetate, kept at -20 °C for 10 min, and then centrifuged at 3000 *g*. The supernatant was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and was subsequently extract once with chloroform. The DNA was precipted with a equal volume of isopropanol at -20 °C for 10 min, washed with ethanol, dried and suspended in 50 µl of ultra pure water (Milli-Q Synthesis A10; Millipore).

RFLP analysis. The total DNA isolated was digested with *Eco*RI, *Hinf* I, *Msp* I and *Hind* III restriction enzymes according manufacture's instruction (Fermentas, Hanover, Germany) and compared with Lambda DNA/HindIII Marker (Amersham-Pharmacia). Electrophoresis was performed with 1.4% agarose gel and it was run in TAE [Tris (Amersham-Pharmacia), acetate (Merck), EDTA (Merck)] at 35 V for 15 h. Analysis of the patterns was done with computer assistance, using Molecular Analyst TM (1.4.1, Bio-Rad) and Photo Capt MW program.

Statistical analysis. The results were analyzed statistically using umpaired T-test.

Results

A total of 30 strains of *M. canis* were isolated from asymptomatic cats in the city of São Paulo, whereas the other 30 strains analysed in this study were isolated from cats with proven dermatophytosis and treated at the University of São Paulo veterinary hospital.

Keratinolytic activity. The average keratinolytic activity of the strains isolated from symptomatic animals was 2.82 +/- 1.32 UK, with strain #23 presenting the

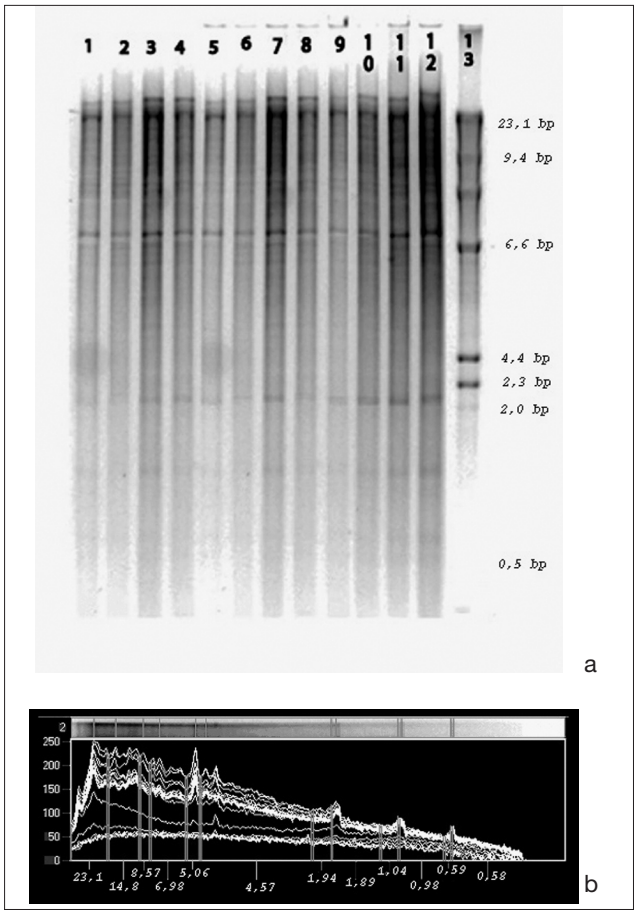

Figure 1. a) RFLP analysis by *Hind III* digestion obtained for 12 of 60 *M. canis* strains from symptomatic and assymptomatic cats showed 12 distinct fragments (23.1; 14.8; 8.57; 6.98; 5.06; 4.57; 1.94; 1.89; 1.04; 0.98; 0.59 and 0.58 kilobases). Columns 01-06: strains from symptomatic cats, columns 07-12 strains from asymptotic cats, column 13, Lambda DNA/*Hind*III marker. b) Analysis of the patterns by Photo Capt MW program.

Table 1. In vitro keratinase, elastase and collagenase activity of *M. canis* isolated from symptomatic and asymptomatic cats.

Symptomatic group				Asymptomatic group			
Strains	UK*	UE*	UC*	Strains	UK*	UE*	UC*
1-MC_ICBUSP	0.94	1.10	0.10	1001-MC_ICBUSP	0.23	1.49	0.40
2-MC_ICBUSP	2.00	0.73	1.05	1002-MC_ICBUSP	1.64	0.73	1.40
3-MC_ICBUSP	1.90	3.70	2.16	1003-MC_ICBUSP	1.23	0.26	1.80
4-MC_ICBUSP	3.10	–	2.14	1004-MC_ICBUSP	0.87	0.73	1.20
5-MC_ICBUSP	4.70	–	1.00	1005-MC_ICBUSP	0.63	0.13	1.80
6-MC_ICBUSP	3.54	0.18	0.76	1006-MC_ICBUSP	1.58	0.73	3.80
7-MC_ICBUSP	4.51	–	0.53	1007-MC_ICBUSP	1.68	0.10	1.80
8-MC_ICBUSP	2.84	0.47	0.75	1008-MC_ICBUSP	1.54	0.10	3.40
9-MC_ICBUSP	4.51	0.08	1.20	1009-MC_ICBUSP	1.40	–	–
10-MC_ICBUSP	5.05	4.51	0.45	1010-MC_ICBUSP	0.96	0.62	1.40
11-MC_ICBUSP	2.68	1.53	1.03	1011-MC_ICBUSP	0.13	0.36	1.80
12-MC_ICBUSP	2.78	4.99	0.73	1012-MC_ICBUSP	0.73	0.12	0.32
13-MC_ICBUSP	1.84	1.21	0.85	1013-MC_ICBUSP	0.68	0.13	2.00
14-MC_ICBUSP	2.80	0.92	1.01	1014-MC_ICBUSP	0.32	0.39	1.80
15-MC_ICBUSP	1.81	1.31	0.25	1015-MC_ICBUSP	0.80	4.19	1.00
16-MC_ICBUSP	1.81	1.06	5.03	1016-MC_ICBUSP	0.85	1.32	0.80
17-MC_ICBUSP	1.89	1.29	–	1017-MC_ICBUSP	1.26	0.29	2.60
18-MC_ICBUSP	3.04	0.71	0.56	1018-MC_ICBUSP	1.77	–	0.80
19-MC_ICBUSP	3.08	0.83	0.89	1019-MC_ICBUSP	2.52	1.06	3.40
20-MC_ICBUSP	2.60	2.25	1.15	1020-MC_ICBUSP	2.35	0.33	1.40
21-MC_ICBUSP	2.91	1.12	0.94	1021-MC_ICBUSP	1.14	0.25	1.40
22-MC_ICBUSP	4.04	0.80	1.30	1022-MC_ICBUSP	0.93	–	0.40
23-MC_ICBUSP	5.75	0.31	1.08	1023-MC_ICBUSP	0.76	0.47	1.00
24-MC_ICBUSP	2.06	0.61	2.58	1024-MC_ICBUSP	0.89	0.36	0.40
25-MC_ICBUSP	2.69	1.32	1.01	1025-MC_ICBUSP	1.80	0.03	1.40
26-MC_ICBUSP	4.35	1.30	0.40	1026-MC_ICBUSP	1.47	–	0.40
27-MC_ICBUSP	2.80	1.24	1.01	1027-MC_ICBUSP	0.92	0.71	0.40
28-MC_ICBUSP	2.60	0.67	–	1028-MC_ICBUSP	1.08	–	0.08
29-MC_ICBUSP	1.25	1.96	0.17	1029-MC_ICBUSP	2.27	0.50	–
30-MC_ICBUSP	2.12	1.08	0.18	1030-MC_ICBUSP	1.14	0.36	–
Average	2.93**	1.24**	1.01***	Average	1.19**	0.53**	1.28***

* Keratinase, Elastase and Collagenase Units
** statistically significant difference ($p < 0.05$) between strains of symptomatic and asymptomatic groups
*** No statistically significant difference ($p < 0.05$)
"-" Null data

highest detected activity. For the strains isolated from asymptomatic animals this average was 1.15 +/- 0.59 UK. The difference between these averages was statistically significant ($p < 0.05$) (Table 1).

Elastinolytic activity. The average elastinolytic activity for the strains isolated from symptomatic animals was 1.24 +/- 1.23 UE, with strain #12 showing the greatest detected activity. For the strains isolated from asymptomatic cats this average was 0.51 com +/- 0.86 UE. The difference between these averages was statistically significant ($p < 0.05$) (Table 1).

Collagenolytic activity. The average collagenolytic activity for the strains isolated from symptomatic animals was 1.01 +/- 1.07 U.C. For the strains isolated from asymptomatic animals this average was 1.35 +/- 1.02 UC. The difference between these averages was not statistically significant ($p < 0.05$) (Table 1).

RFLP analysis. Only *Hind* III digestion showed visible bands. *RFLP* analysis of all *M. canis* total DNA isolated showed 12 distinct fragments (23.1; 14.8; 8.57; 6.98; 5.06; 4.57; 1.94; 1.89; 1.04; 0.98; 0.59 e 0.58 kb) by Photo Capt MW program (Figure 1) and no polymorphism between samples studied was observed. The marker used was Lambda DNA/HindIII Marker.

Discussion

Various studies have described a large quantity of enzymes produced by dermatophytes, as well as the lysing activities of these on various substrates [1,2,7,22,32,37,41-43]. These enzymes, especially keratinase, have been considered as virulence factors [11] and correlated with the clinical form of the dermatophytoses.

The data obtained here on the keratinolytic activity of strains isolated from asymptomatic animals with dermatophytosis are in agreement with those obtained previously [42]. There was a clear quantitative difference in the intensity of keratinase production, where the strains belonging to the group isolated from cats with dermatophytosis produced this enzyme in statistically significant greater amounts than did those strains isolated from asymptomatic cats, thus suggesting a relation between the occurrence of dermatophytosis and the intensity of keratinase production. These results disagree with those obtained by Mignon et al. 1998 [29], who demonstrated the presence of this enzyme in vivo, in asymptomatic cats with dermatophytosis, and in vitro, in strains of *M. canis*, concluding that keratinase production was unrelated to their clinical manifestations. That study did not involve a quan-

titative evaluation and only showed the presence of the enzyme in the animal tissues.

In regard to the production of elastase, the results obtained here show a statistically significant difference between the strains isolated from cats with and without dermatophytosis, suggesting that elastase also influences the tissular reactions in dermatophytosis.

Although reports in the literature have clearly shown that the strains produce an enzyme with collagenolytic activity, and that various strains produce it at different levels, no statistically significant difference has been demonstrated that would show a correlation between collagenase production levels and the presence, or not, of dermatophytosis by *M. canis* in cats.

Among the strains encountered in the two groups of the present study, there was no coincidence between high producers of each of the enzymes, suggesting that there does not exist just one proteinase with keratinolytic, elastinolytic and collagenolytic activity, but rather a specific enzyme for each substrate. Brouta et al. [5] described two proteases that can exert activity on the three substrates studied here, suggesting that these are responsible for the observed keratinase, elastase and collagenase activity.

Due to the fact that this is the first dermatophyte RFLP study on *M. canis*, no RFLP analysis with *Hind* III digestion were available for comparison. This methodology was not capable to biotype the studied strains, demonstrating generated fragments homogeneity and great amount of fragments, what hinders the analysis. When other techniques are applied with the same purpose, like RAPD (Random Amplification of Polymorphic DNA) or internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences, same results were showed. Such techniques only could differentiate the strains in species level [12,14,16,24,44]. Although dermatophytes follow an asexual expansion and rarely reproducing for sexual reproduction, it is evident that these phenotypics differences, related with the virulence, are due specific genotypes not yet detected by the techniques used. For this purpose, other studies should be made for genotypes related to virulence identification.

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