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Production of extracellular enzymes by *Microsporum canis* and their role in its virulence

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Microsporum canis is the most prevalent dermatophyte of domestic animals. Several enzymes produced by dermatophytes, particularly keratinases, are considered to play a role in the virulence of this fungus. To investigate the possible relationship between the clinical status of *M. canis* infection and enzymatic activity of isolates, we studied the relationship between keratinase, elastase, lipase and DNase levels produced *in vitro* by different isolates and virulence as expressed in a guinea pig model. Samples isolated from symptomatic dogs and cats showed a statistically significantly ($P < 0{\cdot}05$) higher keratinase activity than samples isolated from asymptomatic animals. Experimental infection of guinea pigs showed that a strain with high *in vitro* keratinase activity induced acute infection, which resolved clinically and mycologically faster than the infection induced by a strain with low keratinase activity. This suggested a strong correlation between high keratinase activity and the development of symptoms. The same correlation was not observed for other enzymes tested.

Keywords dermatophytes, keratinase, *Microsporum canis*, virulence

Introduction

Microsporum canis is the most important dermatophyte of domestic animals [1]. The frequency of *M. canis* infection in dogs and cats varies from 40% to 90% [2–4], with a variable number of dogs (9%) and cats (2·2–80%) becoming otherwise healthy dermatophyte carriers [5–9]. In histology symptomatic cats show acute or subacute perifolliculitis and folliculitis, while the asymptomatic carriers show chronic inflammation characterized by an infiltrate of mast cells at the superficial dermis layer [10].

The pathological reactions observed in dermatophytosis are mediated by substances, produced by dermatophytes, that diffuse through the horny layer during infection [11]. These substances include enzymes such as keratinases [12–14], elastases [15–17], deoxyribonuclease (DNase) [17], collagenases [18–21] and lipases [21]. Among them, keratinases have been speculated to be the most important dermatophyte virulence factors, since they can aid the fungal invasion of host tissues [22]. During *Trichophyton mentagrophytes* infection of the guinea pig, keratinase is produced in the hair follicle, inducing strong cutaneous delayed hypersensitivity and antibody production [23–25]. In *M. canis* infection, keratinases with molecular weights of 45 kDa [26], 33 kDa [14] and 31·5 kDa [27], have been described. However, the role of these enzymes in virulence is not completely understood.

The objectives of this study were to compare elastase, lipase, DNase and keratinase activities, to quantify keratinase levels in samples of *M. canis* isolated from animals with dermatophytosis and asymptomatic animals and to evaluate experimentally the patterns of pathogenicity shown by these isolates in guinea pigs.

Materials and methods

M. canis *isolates*

Thirty strains of *M. canis* were isolated from dogs and cats with dermatophytosis (symptomatic group) and

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another 30 strains were obtained from dogs and cats with no apparent lesions (asymptomatic group). The samples were grown from the inoculation of perilesional hair of the animals and of material obtained through friction of their corporal surface by a piece of sterile carpet [28], in Mycosel agar (Oxoid, Basingstoke, UK) at 25 °C. Identifications were carried out by morphological evaluation [29].

Keratinolytic activity assay

The *M. canis* isolates were cultured in 2 ml of Sabouraud broth (Oxoid) in 16 160 mm sterile plugged tubes at 25 °C for 15 days. The mycelium was then collected, inoculated in 50 ml Erlenmeyer flasks with keratin medium (3 g keratin powder from hooves and horns [ICN, Montréal, Canada], 6 g $MgSO_4$, 1 ml Protovit vitamin mixture [vitamin A 5000 UI; vitamin B1 4 mg; vitamin B2 1 mg; vitamin PP 10 mg; vitamin B6 1 mg; vitamin B5 10 mg; vitamin H 0·1 mg; vitamin C 50 mg; vitamin D 1000 UI; vitamin E 3 mg; Roche, São Paulo, Brazil], 0·111 g $CaCl_2$, in 1000 ml distilled water), and incubated at 25 °C [30]. The culture supernatant was collected on the 7th, 14th, 21st and 28th day of incubation. One millilitre of 1500 *g* centrifuged culture supernatant was then mixed with 2 ml of 200 mM Tris-HCl and 100 mM $CaCl_2$, pH 8·0 plus 10 mg of keratin azure (Sigma, St. Louis, MO, USA), and afterwards incubated at 37 °C for 24 h. Substrate degradation was measured in a spectrophotometer at 595 nm using uninoculated substrate buffer as a negative control. An increase of 0·01 in the absorbance was considered equal to one keratinase unit (KU).

DNase activity assay

The samples were inoculated in Petri dishes containing DNase agar test (Oxoid) and incubated at 25 °C for 15 days. The test was considered positive when a degradation halo around the colony, formed by the addition of 5 N HCl, could be visualized [17].

Lipase activity assay

The samples were inoculated at the centre of Petri dishes containing lipase medium (10 g peptone, 5 g NaCl, 0·1 g $CaCl_2$, 20 g agar, 10 ml Tween 20 in 1000 ml distilled water) [21]. The plates were incubated at 25 °C for 15 days. The assay was considered positive when a precipitation halo around the colony could be seen.

Elastase activity assay

The samples were inoculated at the centre of Petri dishes containing elastin medium (30 g trypticase soy broth [Oxoid], 3 g elastin powder [Sigma], 20 g bacteriological agar, in 1000 ml of distilled water) [18]. The test was considered positive when a degradation halo around the colony could be visualized.

Experimental infection of guinea pigs

Two *M. canis* isolates, one with high keratinase activity and another with low keratinase activity, were used for experimental infection of Dunkin–Hartley guinea pigs. Macroconidial suspensions were prepared as previously described [31], with some modifications. The samples were cultivated in Borelli's lactrimel agar [32] at 25 °C for 20 days. The cultures were covered with saline solution containing 0·001% Tween 80 and scraped with a sterile bacteriological loop. The material was centrifuged at 1500 *g* for 5 min and washed twice with phosphate buffered saline containing 0·1% cycloheximide and 0·01% chloramphenicol. The viability of fungal cells were evaluated by fluorescence [33], and 80% of macroconidia was observed to be viable in both samples. The inoculum was adjusted to 5 10^5 viable cells ml^{-1}. Two groups of six dermatophyte-free guinea pigs, three males and three females, were used for inoculation of the samples. They were shaved on a 2-cm diameter area to be inoculated with the samples and, after scarification of the area, 0·1 ml of macroconidial suspension was applied. The animals were evaluated daily for 3 months for any of the following factors, which were graded on a 0–4 plus scale: erythema, alopecia, scaling, regrowth of hair and *M. canis* isolation. Using these scales, 0 is apparently healthy, 1+ is discrete presence of the symptoms on a small area, 2+ is discrete presence of symptoms on a large area, 3+ is intense symptoms on a small area and 4+ is intense symptoms on a large area.

Statistical analysis

Student's *t* test and Fisher's exact test were utilized for the statistical analysis of the results.

Results

Keratinase activity of isolates

Keratinase levels produced by the two groups of isolates tested are shown in Table 1. In most samples (87%), the peak of enzyme activity occurred on day 14. Samples isolated from the symptomatic group had higher keratinase activity ($P < 0.05$) than samples from the asymptomatic group. For experimental infection studies, we selected isolates 29 and 251, with high (7·75 KU) and low keratinase activity (0·14 KU), respectively.

DNase, lipase and elastase activities of isolates

The results of DNase activity evaluation are shown in Table 2. In the symptomatic group, 50% of isolates gave positive results, while in the asymptomatic group, 43% were positive, a difference that was not statistically significant. The results of lipase activity are shown in Table 2. Most isolates (90%) in both groups were lipase secretors. The difference between groups was insignificant. With regard to elastase (Table 2), seven of 30 isolates from symptomatic animals were positive in contrast to two of 30 from asymptomatic animals (not significant).

Experimental infection of guinea pigs

Guinea pigs inoculated with the *M. canis* isolate with low keratinase activity (strain 251) rapidly developed erythema by day 7 after inoculation. By day 14, an intense alopecia and whitish crusts in the inoculated area could be observed. By day 21, the females, but not the males, displayed the crusts. By day 28, hair growth could be observed in the centre of the lesion. By week 4, the female guinea pigs still showed a small number of crusts. Regrowth of hair was completed between days 77 and 80.

Guinea pigs inoculated with the high keratinase activity isolate showed intense erythema by day 7. After 14 days, the erythema persisted and a great number of crusts and areas of alopecia could be observed. After 21 days there was a decrease in erythema with the resolution of the lesions and the hair began to grow by day 35. By day 56, hair completely covered the inoculated area (Table 3). The guinea pigs were mycologically examined for an additional 15 days until they were considered dermatophyte-free. The guinea

Table 1 *In vitro* keratinolytic activity of *Microsporum canis* strains isolated from symptomatic and asymptomatic dogs and cats

Symptomatic group				Asymptomatic group			
Sample	Host	Day 7	Day 14	Sample	Host	Day 7	Day 14
1	Cat	0·43*	1·48	70	Cat	–†	1·4
2	Dog	0·24	3·23	75	Cat	0·53	2·18
3	Dog	–	0·1	83	Cat	–	0·88
4	Cat	1·14	2·53	99	Cat	0·07	1
5	Cat	0·9	2·13	101	Cat	0·27	5
6	Cat	–	0·55	112	Cat	–	1·33
7	Cat	0·59	1·75	184	Cat	0·57	3·55
8	Dog	0·27	3·55	186	Cat	0·26	2·78
9	Cat	0·54	1·14	187	Cat	0·14	1·98
11	Cat	0·74	5·77	188	Cat	0	1·68
12	Dog	1·83	2·03	189	Cat	0·5	3·35
14	Dog	1·83	5·9	191	Cat	0	3·16
16	Dog	0·52	2·95	192	Cat	0·49	2·83
17	Cat	1·29	3·45	194	Cat	–	0·48
18	Dog	0·58	0·5	195	Cat	0·08	1·85
22	Cat	0·47	4·87	197	Cat	0·56	1·9
23	Dog	0·61	0·70	198	Cat	0·54	1·9
27	Dog	1·29	4·86	199	Cat	0·30	0·8
29	Cat	0·51	7·75	200	Cat	0·57	2·7
30	Cat	0·07	1·79	202	Cat	0·18	1·25
31	Cat	0·89	1·37	204	Cat	0·58	1·47
32	Cat	0·05	1·50	205	Cat	0·26	1·19
33	Cat	0·20	1·55	206	Cat	–	0·93
34	Dog	0·48	2·65	207	Cat	0·95	1·37
38	Cat	0·63	1·0	212	Cat	0·06	2·75
39	Cat	–	1·1	220	Cat	0·11	0·61
40	Cat	0·55	5·4	230	Cat	0·22	1·58
41	Dog	0·01	1·1	251	Dog	0·25	0·14
44	Cat	0·57	2·49	255	Dog	0·07	–
45	Cat	0·16	0·33	256	Dog	0·27	1·85
Mean		0·58	2·52‡	Mean		0·26	1·79‡

*, Keratinase units;
†, null data;
‡, statistically significant difference ($P < 0·05$) between strains of symptomatic and asymptomatic group.

Table 2 DNase, lipase and elastase activities of *M. canis* isolated from symptomatic and asymptomatic animals

	Symptomatic group ($n = 30$)		Asymptomatic group ($n = 30$)	
	Positive	Negative	Positive	Negative
DNase	15 (50·0%)	15 (50·0%)	13 (43·3%)	17 (56·7%)
Lipase	26 (86·7%)	4 (13·3%)	28 (93·3%)	2 (6·7%)
Elastase	7 (23·3%)	23 (76·3%)	2 (6·7%)	28 (93·3%)

pigs inoculated with high-keratinase strain 29 were positive in mycological evaluation for up to 7 days while the animals inoculated with low-keratinase strain 251 were positive for up to 90 days after the inoculation (Table 4).

Discussion

According to some authors [17], DNase activity has been associated with clinical characteristics of dermatophytosis, since dermatophyte samples isolated from chronic dermatophytosis lesions express high DNase activity, while samples isolated from acute dermatophytosis lesions express low DNase activity. However, we did not find any difference between the production of DNase in samples isolated from symptomatic animals and those isolated from animals with frank dermatophytosis. In our opinion, this suggests that this enzyme does not play any clinical role in *M. canis* infections of cats and dogs.

Elastase production by dermatophytes has been associated with acute lesions, as observed with *T. mentagrophytes*, although most *M. canis* strains have

Table 3 Intensity of symptoms in guinea pigs experimentally infected with *M. canis* isolates producing low (strain 251) and high (strain 29) levels of keratinase

	Male												Female											
	Animal 1				Animal 2				Animal 3				Animal 4				Animal 5				Animal 6			
Days	E	A	C	R	E	A	C	R	E	A	C	R	E	A	C	R	E	A	C	R	E	A	C	R
Strain 251																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	2	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0
14	2	4	2	0	2	4	2	0	2	4	2	0	2	4	2	0	2	4	2	0	2	4	2	0
21	0	4	0	0	0	4	0	0	0	4	0	0	0	0	2	2	0	0	2	2	0	0	2	2
28	0	0	0	2	0	0	0	2	0	0	0	2	0	0	1	2	0	0	1	2	0	0	1	2
35	0	0	0	2	0	0	0	2	0	3	0	2	0	0	0	2	0	0	0	2	0	0	0	2
42	0	0	0	3	0	0	0	2	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3
49	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3
56	0	0	0	4	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3
63	0	0	0	4	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	4	0	0	0	3
70	0	0	0	4	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3
77	0	0	0	4	0	0	0	3	0	0	0	4	0	0	0	3	0	0	0	4	0	0	0	3
94	0	0	0	4	0	0	0	3	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4
101	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4
Strain 29																								
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0
14	4	1	3	0	4	4	1	0	3	3	3	0	4	1	3	0	3	3	2	0	3	2	3	0
21	2	4	2	0	2	4	2	0	2	4	2	0	2	4	2	0	2	4	2	0	2	4	2	0
28	0	4	1	0	0	4	1	0	0	4	1	0	0	4	1	0	0	4	1	0	0	4	1	0
35	0	0	1	2	0	0	1	2	0	0	1	2	0	0	1	2	0	0	1	2	0	0	1	2
42	0	0	1	2	0	0	1	2	0	0	1	2	0	0	1	2	0	0	1	3	0	0	1	2
49	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3
56	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4	0	0	0	4

Intensity of clinical signs: 0 = absent; 1 = discrete presence of the symptoms on a small area; 2 = discrete presence of symptoms on a large area, 3 = intense symptoms on a small area; 4 = intense symptoms on a large area. E, erythema; A, alopecia; C, scaling; R, hair growth.

Table 4 Duration of *M. canis* infection in guinea pigs experimentally infected with strain 251 (low keratinase producer) and strain 29 (high keratinase producer), as assessed by laboratory isolation

		Duration of infection (days)																
Strain	Animals	1	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105	112
251	1	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−
251	2	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−	−
251	3	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
251	4	+	+	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−
251	5	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
251	6	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−	−	−
29	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	2	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	3	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	4	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	5	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	6	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, Positive for *M. canis*; −, negative for *M. canis*.

been reported as weak secretors of this enzyme [34]. In another study, elastase activity was expressed by *T. mentagrophytes* and *T. tonsurans* isolated from acute human lesions [17]. However, although elastase activity was detected in 80% of *M. canis* isolates tested, these authors could not correlate high elastase levels of this dermatophyte to acute lesions [17].

Recently, elastase activity in *T. mentagrophytes*, *T. verrucosum* and *M. gypseum*, but not in *M. canis*, was also observed, suggesting that the first three species could be more virulent than the last [21]. In our study, 27·6% of the samples of *M. canis* showed elastase activity, but no link between expression of this enzyme and level of symptoms could be shown, suggesting that elastase does not play a significant role in *M. canis* pathogenesis.

Few studies on lipase production by dermatophytes have been reported [17,21,35]. In one of them, 30% of *M. canis* strains displayed lipase activity, which was associated with a chronic dermatophytosis [17]. This finding was not supported by our results which showed no significant difference in lipase activity between strains isolated from acute and chronic infections. DNase, elastase and lipase levels have been studied in relation to human dermatophytosis and high levels appear to correlate with inflammatory intensity [17,21]. The mechanisms underlying this correlation appear not to be significant in dog and cat dermatophytosis.

All *M. canis* strains studied were capable of producing keratinase, but their calculated mean value levels differed greatly in the two groups, showing a clear association between high levels of keratinase activity and symptomatic dermatophytosis. These results differ from those of Mignon *et al.* [36], who found that keratinase production was not associated with any particular clinical picture. However, in this study the authors did not measure the enzyme secretion quantitatively, but associated inflammatory intensity qualitatively with enzyme production. We have confirmed our results by experimental infection.

Since guinea pigs inoculated with a high keratinase isolate had relatively severe lesions, plus an acute course and fast mycological clearance, while guinea pigs inoculated with a low keratinase strain showed chronic type lesions, it appears that the intensity of enzyme production, not its simple presence, is a determinant of the clinical course of dermatophytosis. Keratinase seems, then, to be one of the most important virulence factors for *M.canis*, as previously described for *T. mentagrophytes* [23–25]. Mannans from fungal walls may be another major virulence factor, since they are able to suppress the host's immune response, as has been well shown with *T. rubrum* [37].

The other enzymes that were evaluated (lipase, elastase and DNase) apparently play no role in the virulence of *M. canis* infection. Perhaps quantifying their levels, as it has been performed for keratinase, could demonstrate an association of enzyme production with the clinical picture of *M. canis* infection.

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