Fungalysin and dipeptidyl-peptidase gene transcription in *Microsporum canis* strains isolated from symptomatic and asymptomatic cats

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Abstract

Microsporum canis is the main pathogenic fungus that causes a superficial cutaneous infection called dermatophytosis in domestic carnivores. In cats, *M. canis* causes symptomatic or asymptomatic infection. Recent conflicting data raise the question of whether the clinical status of the infected cat (symptomatic or asymptomatic) is directly correlated to the proteolytic activity of *M. canis* strains. Here, the transcription of fungalysin and dipeptidyl-peptidase genes (*DPP*) of *M. canis* was compared between four strains isolated from symptomatic and asymptomatic cats during the first steps of the infection process, namely in arthroconidia, during adherence of arthroconidia to corneocytes and during early invasion of the epidermis, using a new *ex vivo* model made of feline epidermis. There was no detectable transcription of the fungalysin genes in arthroconidia or during the first steps of the infection process for any of the tested strains, suggesting that these proteases play a role later in the infection process. Among *DPP*, the *DPP IV* gene was the most frequently transcribed both in arthroconidia and later during infection (adherence and invasion), but no significant differences were observed between *M. canis* strains isolated from symptomatic and asymptomatic cats. This study shows that the clinical aspect of *M. canis* feline dermatophytosis depends upon factors relating to the host rather than to the proteolytic activity of the infective fungal strain.

Keywords: dermatophytes, secreted proteases, strain differentiation, feline ringworm

Introduction

Microsporum canis is the main pathogenic fungus that causes a superficial cutaneous infection called dermatophytosis in domestic carnivores (Scott et al., 2001). In cats, which are the natural hosts for *M. canis*, the fungus can cause both symptomatic and asymptomatic

47 infection (Mignon and Losson, 1997). A recent study showed that strains isolated from cats
48 with symptomatic dermatophytosis have a higher keratinase activity than those isolated from
49 asymptomatic animals (Viani et al., 2007). These results contrast with those obtained by
50 Mignon et al. (1998), who demonstrated that *M. canis* secretes the keratinolytic subtilisin
51 Sub3 (Descamps et al., 2002) in naturally infected cats, both symptomatic and asymptomatic.
52 These conflicting data raise the question of whether the proteolytic activity of *M. canis* strains
53 varies according to the clinical status of the cats from which they are isolated. Besides
54 secreting the subtilisins which are involved in the fungal adherence to skin
55 (Baldo et al., 2008), *M. canis* secretes many other proteases including fungalysins (Mep)
56 (Brouta et al., 2002) and dipeptidyl-peptidases (Dpp) (Vermout et al., 2008a). The role of
57 Mep and Dpp is currently unknown (Monod, 2008). However, Mep and Dpp could be
58 involved in fungal adherence and/or invasion (Vermout et al., 2008b), and their differential
59 expression among *M. canis* strains could reflect the capacity to adhere to and/or invade cat
60 tissues and to induce a symptomatic infection.
61 In this study, we compared the *in vitro* transcription of *MEP* (*MEP1*, *MEP2* and *MEP3*) and
62 *DPP* (*DPP IV* and *DPP V*) genes in *M. canis* strains isolated from two symptomatic and two
63 asymptomatic cats (table 1), both in the infective fungal elements (arthroconidia) and during
64 the first steps of infection, i.e. adherence and invasion, using a new *ex vivo* model of
65 dermatophytosis. The four strains had typical morphological characteristics of *M. canis*.
66
67 Material and Methods
68 Arthroconidia were produced as previously described (Tabart et al., 2007). A concentration of
69 1×10^5 arthroconidia in a 100-µl volume was lysed in a tube containing Lysing Matrix C
70 (Qbiogene, Valencia, CA) and lysis buffer from the Qiagen RNeasy Plant Minikit (Valencia,
71 CA). Samples were homogenized using two 30-s pulses at a speed setting of 6 m/s in the

FastPrep (MP Biomedicals, Solon, OH) system. Isolation of total RNA was performed with the Qiagen RNeasy Plant Minikit. On-column DNase digestion was performed with an RNAse-Free DNAse Set (Qiagen). Total mRNA was transformed into cDNA with the iScript Select cDNA Synthesis Kit (Biorad, Hercules, CA). Reverse transcriptase was omitted in control reactions. The cDNA obtained was used as a template for PCR (47 cycles) using the Go Taq Flexi Polymerase (Promega, Fitchburg, WI) and the primer pairs specific for *M. canis* genes of actin (positive control) (Okeke et al., 2001), Mep (Brouta et al., 2002) and Dpp (Vermout et al., 2008a). The crude reaction product was then used as a template in a second PCR (35 cycles) with internal primers, as previously described (Brouta et al., 2002; Descamps et al., 2003; Vermout et al., 2008a). An additional control consisted of testing all the primer pairs on genomic DNA of the 4 strains. Extraction of the total RNA and PCR experiments from arthroconidia were performed in triplicate. After evaluation of *MEP* and *DPP* gene transcription in arthroconidia, gene expression was assessed during adherence of arthroconidia to feline corneocytes, i.e. 4 h after inoculation (Zurita and Hay, 1987; Baldo et al., in press) and during early invasion of the feline epidermis, i.e. 48 h after inoculation (results not shown), using a new *ex vivo* model made of feline epidermis. Normal feline skin samples were obtained from healthy cats in an animal shelter immediately after euthanasia. Skins were harvested from the flanks and were sheared. The pieces of skin, approximately 4 cm^2, with full epidermal thickness were placed on a stainless steel grid support immersed in cold sterilized skin graft fluid (Duek et al., 2004).

Each piece of skin was inoculated with 1×10^5 arthroconidia suspended in PBS for a total volume of 20 µl and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

For adherence experiments, the skin explants were washed, 4 h post inoculation, with PBS-tween 0.1% for 10 min and were scraped with a sterile scalpel blade. The harvested

material containing adherent arthroconidia was lysed, the total RNA extracted and transformed into cDNA, and PCR experiments were performed as described above.

For invasion experiments, the skin explants were washed, 24 h post inoculation, with PBS-tween 0.1% for 10 min, rinsed in PBS for 10 min to eliminate non adherent arthroconidia and incubated for 24 additional hours. The infected epidermis containing invading hyphae were scraped and treated as described above. The total RNA was extracted and transformed into cDNA, and PCR experiments were performed as described above. For each tested strain, adherence and invasion experiments were performed using three skin explants from three unrelated cats. The results of arthroconidia, adherence and invasion experiments were analyzed by logistical regression with significance defined as $P<0.05$.

Results and Discussion

For arthroconidia experiments, there was no significant effect between experiments and transcribed genes. For adherence and invasion experiments, there was no significant effect of feline explant origin on gene expression, showing that our *ex vivo* model is a reproducible one.

The actin gene was transcribed in the four tested strains in all experiments (arthroconidia, adherence, invasion). There was no detectable transcription of the fungalysin genes in arthroconidia or during adherence or invasion for any of the four tested strains. These results suggest that the proteolytic activity of fungalysins, including the keratinolytic activity of Mep3 (Brouta et al., 2001) is not related to the ability of *M. canis* to adhere to or to invade host tissues. However, we cannot exclude the possibility that fungalysins play a role later in the infection process, because they have been shown to be expressed in the hair of experimentally infected guinea pigs 14 days after inoculation (Brouta et al., 2002).

120 The *DPP IV* gene was transcribed in arthroconidia and later in fungal elements in three out of
121 four *M. canis* strains (table 2). In dermatophytes, the secreted Dpp IV may be a particularly
122 important protease for virulence, as it is necessary for X-Pro degradation at the N-terminal
123 end of peptides (Monod, 2008). In other Ascomycota, such as *Aspergillus fumigatus*, Dpp IV
124 helps the fungus in the first step of infection (e.g. colonization of collagen and elastin)
125 (Beauvais et al., 1997; Rementeria et al., 2005). Dpp IV also plays a significant role in the
126 pathogenicity of some bacteria. In *Porphyromonas gingivalis*, Dpp IV is responsible for the
127 degradation of collagen and interactions between bacteria and extracellular matrix proteins of
128 the host (Kumagai et al., 2000; Yagishita et al., 2001, Kumagai et al., 2003; 2005). In
129 *Streptococcus suis*, Dpp IV is able to link to fibronectin and is involved in the adherence of
130 bacteria to the surface of the host's cell (Ge et al., 2009). In the protozoan *Trypanosoma cruzi*,
131 Dpp IV is also able to degrade collagen types I and IV and is required for invasion of the
132 host's cells (Santana et al., 1997; Grellier et al., 2001; Bastos et al., 2005). All these data
133 support the major interest in studying further the precise role of Dpp IV of *M. canis* in the
134 infection process.
135 Additionally, our results showing that the transcription of *DPP IV* in *M. canis* strains induces
136 symptomatic as well as asymptomatic infections support our previous finding
137 (Mignon et al., 1998) that the proteolytic activity of *M. canis* is not in itself responsible for the
138 clinical aspect of dermatophytosis in cats. This concept is also supported by clinical evidence.
139 Indeed, in *M. canis*-free catteries, the introduction of a single *M. canis* strain, most often
140 through an asymptomatic carrier, induces symptomatic or asymptomatic infections depending
141 on the animals (Mignon, pers. comm.). Additionally, the age of cats has been shown to be
142 determinant for clinical expression of *M. canis* dermatophytosis in stray cats
143 (Romano et al., 1997). Similarly, in humans infected with *Trichophyton tonsurans*, some host
144 factors, such as age (Bergson et al., 2001) but also others (Abdel-Rahman et al., 2006),

145 enhance the susceptibility of the carrier to clinical diseases. Furthermore, the four strains used
146 in this study were shown to be keratinolytic on keratin azur (Sigma, St. Louis, MO) (data not
147 shown).
148 Considering the results regarding *DPP V* transcription, it would be useful to test a higher
149 number of *M. canis* isolates and to perform additional PCR experiments, especially for this
150 protease even though its role in fungal virulence has not yet been reported.
151 Surprisingly, no *DPP* genes were transcribed in the strain IHEM 22881, while the specificity
152 of primer pairs was proved by the amplification of target genes from genomic DNA.
153 As the cat was treated with itraconazole, it could be hypothesized that this strain had
154 developed antifungal resistance before its isolation (table 1). Indeed, in fluconazole-resistant
155 *Candida albicans* virulent strains cultivated in fluconazole-free medium, an *in vitro* decrease
156 in proteolytic activity has been reported (Angiolella et al., 2008). This hypothesis is however
157 inconsistent, as all the strains used in this study, including IHEM 22881, were sensitive to
158 itraconazole *in vitro* (MIC values between 0.5 and 1 µg ml^{-1}, data not shown). Further
159 investigations are needed to assess the potential effect of antifungal drugs on the transcription
160 of proteolytic enzymes in dermatophytes.
161 In conclusion, we found no differences in the expression of fungalysin and *DPP* genes
162 between *M. canis* strains isolated from symptomatic and asymptomatic cats. This finding
163 supports the fact that the clinical aspect of feline dermatophytosis depends upon factors
164 relating to the host rather than to the fungal strain, including its proteolytic activity. Further
165 studies are needed to determine the role of Dpp in fungal adherence and early invasion of
166 feline epidermis, while others should target the role of Mep later in the infection process.
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168 Conflict of interest statement
169 None.

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Table 1. Origins of *Microsporum canis* strains used in this study.

Fungus reference number	Data of cats				
	Breed	Sex	Age[b]	Clinical status	Treatment
IHEM[a] 21239	Domestic short haired	Male	5	Symptomatic	none
IHEM 22880	Persian	Male	3	Symptomatic	none
IHEM 22627	Birman	Male	4	Asymptomatic	none
IHEM 22881	Persian	Female	6	Asymptomatic	Itraconazole[c]

[a] IHEM: BCCM/IHEM Collection, Scientific Institute of Public Health, Brussels, Belgium.

[b] Age in months.

[c] The treatment (oral itraconazole, 5 mg kg^{-1} every other week for 5 weeks) was completed 4 weeks before the isolation of the fungal strain.

Table 2. Genes of *Microsporum canis* proteases transcribed during the first steps of the infection process.

Infection step	Fungus reference number			
	Symptomatic cats		Asymptomatic cats	
	IHEM[a] 21239	IHEM 22880	IHEM 22627	IHEM 22881
Arthroconidia	*DPP IV*[b]	*DPP IV*	*DPP IV*	none
	DPP V[c]	*DPP V*	*DPP V*	
Adherence	*DPP IV*	*DPP IV*	*DPP IV*	none
			DPP V	
Invasion	none	*DPP IV*	*DPP IV*	none

[a] IHEM: BCCM/IHEM Collection, Scientific Institute of Public Health, Brussels, Belgium.

[b] *DPP IV*: gene of dipeptidyl-peptidase IV.

[c] *DPP V*: gene of dipeptidyl-peptidase V.