Title: Sub3 is involved in adherence of *Microsporum canis* to human and animal epidermis

Authors: Elena Tatiana Băguţ, Aline Baldo, Anne Mathy, Ludivine Cambier, Nadine Antoine, Vasile Cozma, Bernard Mignon

PII: S0378-1135(12)00363-X
DOI: doi:10.1016/j.vetmic.2012.06.011
Reference: VETMIC 5805

To appear in: *VETMIC*

Received date: 28-3-2012
Revised date: 6-6-2012
Accepted date: 8-6-2012

Please cite this article as: Băguţ, E.T., Baldo, A., Mathy, A., Cambier, L., Antoine, N., Cozma, V., Mignon, B., Sub3 is involved in adherence of *Microsporum canis* to human and animal epidermis, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2012.06.011

Sub3 is involved in adherence of *Microsporum canis* to human and animal epidermis

Elena Tatiana Băguț [a, b], Aline Baldo [b], Anne Mathy [b], Ludivine Cambier [b], Nadine Antoine [c], Vasile Cozma [a], Bernard Mignon [b]*

[a] Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, 400372 Cluj-Napoca, Romania

[b] Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20 B-43a, 4000 Liège, Belgium

[c] Laboratory of Animal Histology, Department of Morphology and Pathology, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20 B-43a, 4000 Liège, Belgium

* Corresponding author: Bernard Mignon, Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20 B-43a, 4000 Liège, Belgium. Tel.: +32 4 366 40 99; Fax: +32 4 366 40 97; e-mail: bmignon@ulg.ac.be

Abstract

The aim of this study was to assess the role of the secreted keratinolytic subtilisin-like protease Sub3 in adherence of *Microsporum canis* to epidermis from various susceptible species, in addition to cat for which this role was recently demonstrated. Firstly, we showed by immunostaining that Sub3 is not expressed in arthroconidia from an *M. canis SUB3* RNA-silenced strain but is present on the surface of arthroconidia from a *SUB3* non-silenced parental strain. Secondly, comparative adherence assays using arthroconidia from both *M. canis* strains and skin explants from humans, dogs, horses, rabbits, guinea pigs, mice and cats revealed that only 8 to 16% of arthroconidia from the *SUB3* silenced strain adhered to different types of epidermis when compared to the control strain. Attempts to restore fungal adherence by addition of recombinant Sub3 failed in the tested conditions. Overall results show for the first time that Sub3 is necessary for the adherence of *M. canis* arthroconidia to epidermis from humans and other animal species than cat, supporting the idea that Sub3 plays a central role in colonization of keratinized host structures by *M. canis*, whatever the host.

Key words: *Microsporum canis*, adherence, secreted proteases, subtilisin Sub3

Introduction

Microsporum canis is a pathogenic filamentous fungus responsible for a superficial cutaneous infection called dermatophytosis in both humans and animals. This zoophilic dermatophyte is most commonly isolated from infected or carrier cats, its natural host (Sparkes et al., 1994; Scott et al., 2001), but it can also infect rabbits, rodents, other mammals, including wildlife (Chermette et al., 2008; Kraemer et al., 2011) and humans, in which it causes the most frequently reported dermatozoonosis (Ginter-Hanselmayer et al., 2007).

Although little is known about the physiopathology of the infection, secreted proteases from dermatophytes are considered to be key factors involved in adherence and invasion of superficial cornified skin layers and keratinized epidermal appendages (Monod, 2008). Currently, an increasing number of dermatophyte secreted proteases is being identified at either the protein or gene level (Monod, 2008; Grumbt et al., 2011) but their role during infection remains quite elusive. In *M. canis*, a major fungalysin (Mep3) (Brouta et al., 2002; Jousson et al., 2004), and a major subtilisin (Sub3) (Descamps et al., 2002) which are both endoproteases, as well as two dipeptidyl peptidases (Vermout et al., 2008) have been characterized. Among Sub, the protease Sub3 is the most studied. Its secretion is strongly induced during *in vitro* growth on feline keratin and the native Sub3 shows a strong activity on keratin as a substrate (Mignon et al., 1998). Additionally, although some dermatophyte genes encoding proteases other than Sub3 have been shown to be over-expressed *in vivo* (Grumbt et al., 2011), Sub3 from *M. canis* is the unique dermatophyte secreted protease for which *in vivo* production during skin infection has been clearly demonstrated (Mignon et al., 1998; 1999). To date, no other dermatophyte protease has been detected by immunohistochemistry, the suitable method for protein detection, in infected epidermal or hair structures from either humans or animals. In addition, Sub3 was found to be necessary for the adherence of *M. canis* to feline epidermis (Baldo et al., 2010). It was the first time that a role in pathogenicity was attributed to a dermatophyte secreted protease. Because the potential involvement of Sub3 in adherence to skin from species other than cats remains unknown, the aim of this study was to assess, using a specific *M. canis SUB3* RNA-silenced strain together with *ex vivo* adherence models, whether Sub3 from *M. canis* is involved in fungal adherence to epidermis from both humans and various receptive animal species.

Materials and methods

Fungal strains

Two previously constructed *M. canis* strains were used for all the experiments: a *SUB3* RNA-silenced strain IHEM 22957 (Institute of Hygiene and Epidemiology-Mycology, Brussels, Belgium) obtained by RNA interference and a control strain (IHEM 22958) consisting of the parental *M. canis* wild-type strain IHEM 21239 transformed with the plasmid pSilent-1 without hairpin inserts (Vermout et al., 2007). Arthroconidia were produced as described by Tabart et al. (2007). Briefly, both strains were grown on Sabouraud's agar medium plates (2% glucose-1% peptone) containing 100 µg/ml hygromycin at 27 °C for 15 days. The strains were then transferred onto 2% yeast extract-1% peptone agar containing 100 µg/ml hygromycin in a humidified atmosphere with 12% CO_2 at 30 °C for 21 days. Surface mycelium and conidia were collected by scraping, transferred to PBS, filtered through Miracloth layers (22-25 µm; Calbiochem, La Jolla, CA, USA) and stored at 4 °C until use. Arthroconidia concentration was determined by serial dilutions on Sabouraud's agar medium. In all the experiments, arthroconidia were used within two weeks.

Production and purification of *Microsporum canis* recombinant Sub3

The *M. canis* Sub3 protease was produced in recombinant form by the methylotrophic yeast *Pichia pastoris* expression system and purified as previously described (Descamps et al., 2003). Briefly, culture supernatant containing recombinant Sub3 (rSub3) was concentrated by ultrafiltration on an Amicon cell (Millipore, Billerica, MA, USA) using a filtration membrane with a size threshold of 10 kDa, dialysed against bicine buffer (50 mM bicine, pH 8.2) and applied onto a carboxymethyl-agarose column previously equilibrated in the same buffer. The bound protein was eluted with a linear gradient of salt (1 M NaCl, pH 8.2) and dialysed against PBS. All procedures were carried out at 4 °C. Protein concentrations were determined by the method of Bradford (Bradford, 1976). Subtilisin-like activity was measured using

1 mM *N*-Succinyl-Ala-Ala-Pro-Phe-*p*Nitroanilide (Suc-AAPF-*p*Na; Sigma Aldrich, St Louis, MO, USA) as substrate in Tris buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, pH=7.5).

Production of polyclonal antibody against rSub3

A pre-immune serum and a polyclonal antibody specific for rSub3 were prepared in rabbit following standard procedures and with the agreement of the local ethics committee (University of Liège, ethics protocol no. 946), and were purified. Briefly, one rabbit was subcutaneously immunized four times (day 1, 14, 28 and 56) with purified rSub3 (75 µg) adjuvanted with saponin (20 µg; Sigma). Before and 70 days after the first immunization blood samples were collected, immunoglobulins G (IgG) were purified using the Protein A Antibody Purification kit (Sigma) and their concentrations were adjusted to 450 µg/ml. The specificity of polyclonal antibody against rSub3 and the absence of cross-reactivity with other *M. canis* proteases from a crude exo-antigen (Mignon et al., 1999) were confirmed by ELISA and Western Blot, respectively (data not shown).

Detection of Sub3 in *Microsporum canis* arthroconidia by immunostaining

Arthroconidia (5×10^4 in a volume of 100 µl PBS) from both the *M. canis SUB3* silenced strain and the control strain were filed by cytocentrifugation (2 min at 1000 *g*) on poly-L-lysine slides (Sigma). Cytopreps were air-dried and fixed for 10 min in pure acetone at 4 °C. After rehydration, non-specific antigenic sites were saturated with a blocking agent (Protein block™, Dako, Glostrup, Denmark). Purified polyclonal antibody against rSub3 (100-fold dilution in PBS) was added on the slides for 1 h at room temperature. Slides were then rinsed three times in PBS and incubated with an anti-rabbit immunoglobulin peroxidase-labelled polymer (Amplification EnVision Kit, Dako) for 30 min at room temperature. Peroxydase

activity was revealed with 9-ethyl-3-aminocarbazol (AEC Zymed, San Francisco, USA) and hydrogen peroxide as substrate.

Controls consisted of omission of the primary antibody and use of the purified IgG from pre-immune serum. The specimens were counterstained with Mayer's haematoxylin and observed under an optical microscope.

Adherence assays

Microsporum canis arthroconidia adherence assays were performed using skin explants from humans, cats, dogs, horses, rabbits, guinea pigs and mice. Animal skin explants were harvested from individuals without any apparent skin lesions immediately after euthanasia performed for reasons independent of our experiment. For human epidermis, samples consisted of abdominal skin explants collected from dead patients who had bequeathed their bodies to science at the Faculty of Medicine, University of Liège, Belgium. The *ex vivo* experiments were performed using 4 cm^2-sheared skin explants conditioned and placed on gauze immersed in sterile skin graft fluid (Duek et al., 2004). Each skin explant was inoculated with 5×10^4 arthroconidia in a total volume of 20 µl PBS and incubated for 4 h at 37 °C in humidified atmosphere containing 5% CO_2. To remove non adherent arthroconidia, skin explants were washed for 10 min in PBS-Tween 20 0.1%. Each skin explant was scraped using a sterile scalpel blade and harvested material was spread onto Sabouraud's plates containing 0.05% chloramphenicol and 0.05% cycloheximide. After three days of incubation at 27 °C, colony forming units (CFUs) were counted under the microscope. For each experiment, four pieces of skin were inoculated with the control strain and four other pieces were inoculated with the *SUB3* silenced strain. For each tested skin type, assays were performed in triplicate using skin samples from three unrelated individuals.

Additional adherence assays were conducted to assess whether the addition of rSub3 could restore the adherence of the *M. canis SUB3* silenced strain, previously shown to be defective for adherence to feline epidermis. Three independent assays using skin explants from three unrelated cats were performed as described above, except that before inoculation of skin explants with deficient or control strain arthroconidia, the latter were preincubated with rSub3 (2.5 µg/ml, 1 µg/ml or 0.5 µg/ml reaction mixture) for 1 h at 37 °C in humidified atmosphere containing 5% CO_2. Recombinant Sub3 was substituted with PBS as a control.

Finally, the stability of rSub3 in the conditions of our adherence assays, i.e., after contact with either feline epidermis or arthroconidia, was also assessed. The rSub3 was either deposited on feline epidermis or mixed with deficient or control strain arthroconidia for 4 h at 37 °C. Samples were then subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and assessed for enzymatic activities using Suc-AAPF-*p*Na, as described above.

Statistical evaluation

Inhibition of *M. canis SUB3* silenced strain adherence was expressed as the residual percentage of adherence to each type of epidermis with regard to the control strain, set up as 100%. Significant differences between sets of data were assessed using the Student's *t*-test, with significance defined as $P < 0.01$.

Results and discussion

Expression of the protease Sub3 in *in vitro* produced *Microsporum canis* arthroconidia

Arthroconidia were used in the *ex vivo* adherence assays because they represent the *in vivo* infectious fungal elements and constitute the primary way of transmitting dermatophyte

infections in humans and animals, by remaining infective for several months after shedding from the host (Sparkes et al., 1994). However, while Sub3 has been shown to be produced *in vivo* by *M. canis* in both naturally infected cats (Mignon et al., 1998) and experimentally infected guinea pigs (Mignon et al., 1999), the expression of Sub3 in *in vitro*-produced arthroconidia had never been demonstrated. Consequently, as a first step in this study, the expression of Sub3 in *M. canis SUB3* silenced and control strain arthroconidia was assessed and compared, with the aim of determining whether *in vitro*-produced arthroconidia were suitable for use in further adherence assays testing the role of Sub3.

The positive labelling obtained after immunostaining of *M. canis* control strain arthroconidia using specific anti-Sub3 antibody highlighted the fact that Sub3 is present on the surface of arthroconidia (Fig. 1a). This is in accordance with previous results showing that *SUB3* is transcribed in *M. canis* arthroconidia produced *in vitro* (Baldo et al., 2008). By contrast, the arthroconidia from the *M. canis SUB3* silenced strain were not stained (Fig. 1c). As a control the expression of Sub3 on the surface of arthroconidia in the control strain, which consisted of an *M. canis* wild-type strain transformed with the plasmid pSilent-1 without hairpin inserts, showed that the production of Sub3 was not altered after transformation. Taken together, these results indicated that both chosen strains were suitable for further comparative adherence assays using *in vitro*-produced arthroconidia. The specificity of the antibody against rSub3 was proved by the absence of any Sub3 positive staining of the arthroconidia incubated with pre-immune antibody (Fig. 1b). Interestingly, to date, *M. canis* Sub3 is the only dermatophyte secreted protease that has been identified as a protein by immunostaining both on the surface of arthroconidia produced *in vitro*, as our results show, and in hyphae invading *in vivo* hair follicles from infected cats and guinea pigs.

Sub3 is involved in *Microsporum canis* adherence to epidermis from humans and other animal species

In addition to not expressing Sub3 on *in vitro*-produced arthroconidia, the *SUB3* RNA-silenced strain had a high rate of inhibition for the *in vitro* production of the Sub3 protease (2% residual Sub3 activity) (Vermout et al., 2007). Much more importantly, the *SUB3* RNA-silenced strain was stable after both 4 *in vitro* and one *in vivo* transfer (Baldo et al., 2010). The use of arthroconidia from this defective strain appeared therefore perfectly suitable to assess the role of Sub3 in fungal adherence using repetitive comparative assays on skin explants. Establishing the number of CFUs revealed that only between 8 and 16% of arthroconidia from the *M. canis SUB3* silenced strain adhered to the different types of epidermis when compared to the control strain set up as 100% (Fig. 2). These results show that Sub3 is necessary for the adherence of *M. canis* arthroconidia not only to feline epidermis, but also to epidermis from humans and all tested animal species, supporting the idea that Sub3 plays a central role in colonization of keratinized host structures by *M. canis*. Our data also confirm the results obtained previously on feline epidermis, where only 7% of arthroconidia from the *M. canis SUB3* silenced strain adhered to corneocytes when compared to the control strain (Baldo et al., 2010).

The gene coding for Sub3 has been found to be transcribed in *M. canis* arthroconidia produced both *in vitro* (Baldo et al., 2008) and *in vivo*, in hairs and scales of *M. canis* experimentally infected guinea pigs (Descamps et al., 2002) and naturally infected cats (Mignon et al., 2004). Although it is possible that each species of dermatophyte has a unique programme for the expression of proteases, an orthologue of *M. canis SUB3* has been found to be expressed in multiple dermatophyte species. Experimentally, the expression of the gene encoding Sub3 has been monitored in *Trichophyton rubrum* during *in vitro* growth in the presence of keratin, elastin, collagen and human skin (Maranhão et al., 2007; Leng et al.,

2009), in soy or keratin-soy medium (Giddey et al., 2007; Zaugg et al., 2009; Chen et al., 2010), and *SUB3* was found to have an increased expression under all tested conditions. The expression of *SUB3* has also been revealed in *Arthroderma benhamiae* in which the gene was upregulated during *in vitro* growth on keratin, but not *in vivo* in experimentally infected guinea pigs (Staib et al., 2010; Sriranganadane et al., 2011). It would be of major interest to assess whether Sub3 has a similar involvement in adherence of other dermatophyte species in which its expression has also been proven.

For dermatophytes, the precise mechanisms by which subtilisins contribute to virulence are not clear and need to be further investigated, notably because their role in pathogenicity is either highly suspected, as for Sub6 from *A. benhamiae* (Staib et al., 2010) or already demonstrated, as for Sub3 in *M. canis* adherence (Baldo et al., 2010). Aside from dermatophytes, the involvement of subtilisins in the adherence process has been extensively studied in other pathogens, including fungi. For instance, *Candida* spp. isolated from linear gingival erythema, secretes serine proteases suspected to be involved in initial colonization events (Portela et al., 2010). In parasites, a surface-associated subtilisin-like serine protease that interferes with adherence to host cells has been identified in the pathogenic protozoan *Trichomonas vaginalis* (Hernández-Romano et al., 2010). Also, it has been recently shown that host cell invasion by *Toxoplasma gondii* is critically dependent upon TgSUB1, a subtilisin-like serine protease required for efficient adhesion of tachyzoites (Lagal et al., 2010).

Recombinant Sub3 failed to restore the adherence of arthroconidia from the *Microsporum canis SUB3* silenced strain to feline epidermis

In order to evaluate whether the addition of rSub3 could restore the adherence of the *SUB3* silenced *M. canis* strain to feline epidermis, feline skin explants were inoculated with both

SUB3 silenced and control strain arthroconidia preincubated with purified exogenous rSub3 protease. Determining the number of CFUs revealed that addition of rSub3 had no influence on the adherence of either silenced or control strain arthroconidia (Fig. 3). The absence of adherence restoration upon addition of rSub3 was not related to degradation of exogenous rSub3 during adherence assays. Indeed, after contact with either feline epidermis (Fig. 4) or with arthroconidia, no degradation was observed. Additionally, rSub3 retained 99% of enzymatic activity towards Suc-AAPF-*p*Na in all tested conditions. One explanation could be that the native Sub3 from *M. canis* is a cell-wall associated protein present on the surface of arthroconidia, interfering in adherence through intimate mechanisms that have not yet been elucidated. This has been notably reported for several pathogenic bacteria, such as *Campylobacter jejuni* (Konkel et al., 1997), some strains of *Escherichia coli* (Spencer et al., 1998) and *Mannheimia haemolytica* (Dagmara and Czuprynski, 2009).

Although the exact cause of the failure of the restoration of *M. canis* adherence using rSub3 remains currently unknown, similar results have been reported for other pathogens. For example, Pracht et al. (2005) reported that a *Streptococcus pneumoniae* strain deficient in the pneumococcal adherence and virulence factor A (PavA) showed substantially reduced adherence to various cell types when compared with a control strain, while complementation of the *pavA* knockout strain with exogenously added PavA recombinant protein did not restore the adherence of the mutant. In some other studies, the use of an exogenous serine protease in restoring the capacity of adherence lost after genetic elimination of the gene encoding the corresponding protease was, however, shown to be successful. For example, EspP, a serine protease of enterohaemorrhagic *E. coli*, plays an important role in intestinal colonization in calves. Dziva et al. (2007) showed that, *in vitro*, the adherence of *E. coli* O157:H7 to intestinal epithelial cells was significantly impaired due to *espP* gene deletion but was restored upon addition of purified exogenous EspP.

In conclusion, this study is, to our knowledge, the first to reveal the expression of the keratinolytic protease Sub3 in *M. canis* arthroconidia produced *in vitro*. Sub3 is detectable on the surface of arthroconidia and is an indispensable factor in fungal adherence not only to feline epidermis, but also to epidermis from humans and various animal species. These data support previous hypotheses concerning the major role of Sub in the earliest events of the infectious process, i.e. adherence to corneocytes. We believe that our findings provide a positive catalyst to prompt further experiments devoted to developing an intimate understanding of cellular and molecular mechanisms involving Sub in dermatophyte pathogenesis.

Conflict of interest statement

None.

Acknowledgments

This study was supported by grant 3.4558.10 from Fonds de la Recherche Scientifique Médicale (FRSM). E.T.B. is the recipient of a research grant provided by the University of Liège. A.M. was and L.C. is the recipient of a studentship from FRIA (Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture, 1000 Brussels, Belgium). The authors thank Dr V. Defaweux for providing human skin explants. They also thank M. Leclercq and J. Collard for their excellent technical assistance.

298 **References**

299 Baldo, A., Tabart, J., Vermout, S., Mathy, A., Collard, A., Losson, B., Mignon, B., 2008.
300 Secreted subtilisins of *Microsporum canis* are involved in adherence of arthroconidia
301 to feline corneocytes. J. Med. Microbiol. 57, 1152-1156.

302 Baldo, A., Mathy, A., Tabart, J., Camponova, P., Vermout, S., Massart, L., Maréchal, F.,
303 Galleni, M., Mignon, B., 2010. Secreted subtilisin Sub3 from *Microsporum canis* is
304 required for adherence to but not for invasion of the epidermis. Br. J. Dermatol. 162,
305 990-997.

306 Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram
307 quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72,
308 248-254.

309 Brouta, F., Descamps, F., Monod, M., Vermout, S., Losson, B., Mignon, B., 2002. Secreted
310 metalloprotease gene family of *Microsporum canis*. Infect. Immun. 70, 5676-5638.

311 Chen, J., Yi, J., Liu, L., Yin, S., Chen, R., Li, M., Ye, C., Zhang, Y., Lai, Z., 2010. Substrate
312 adaptation of *Trichophyton rubrum* secreted endoproteases. Microb. Pathogenesis. 48,
313 57-61.

314 Chermette, R., Ferreiro, L., Guillot, J., 2008. Dermatophytoses in animals. Mycopathologia.
315 166, 385-405.

316 Dagmara, I.K., Czuprynski, C.J., 2009. Identification of *Mannheimia haemolytica* adhesins
317 involved in binding to bovine bronchial epithelial cells. Infect. Immun. 77, 446-455.

318 Descamps, F., Brouta, F., Monod, M., Zaugg, C., Baar, D., Losson, B., Mignon, B., 2002.
319 Isolation of a *Microsporum canis* gene family encoding three subtilisin-like proteases
320 expressed *in vivo*. J. Invest. Dermatol. 119, 830-835.

321 Descamps, F., Brouta, F., Vermout, S., Monod, M., Losson, B., Mignon, B., 2003.
322 Recombinant expression and antigenic properties of a 31.5-kDa keratinolytic

subtilisin-like serine protease from *Microsporum canis*. FEMS Immunol. Med. Microbiol. 38, 29-34.

Duek, L., Kaufman, G., Ulman, Y., Berdicevsky, I., 2004. The pathogenesis of dermatophyte infections in human skin sections. J. Infect. 48, 175-180.

Dziva, F., Mahajan, A., Cameron, P., Currie, C., McKendrick, I.J., Wallis, T.S., Smith, D.G.E., Stevens, M.P., 2007. EspP, a Type V-secreted serine protease of enterohaemorrhagic *Escherichia coli* O157:H7, influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. FEMS Microbiol. Lett. 271, 258-264.

Giddey, K., Monod M., Barblan, J., Potts, A., Waridel, P., Zaugg, C., Quadroni, M., 2007. Comprehensive analysis of proteins secreted by *Trichophyton rubrum* and *Trichophyton violaceum* under *in vitro* conditions. J. Proteome Res. 6, 3081-3092.

Ginter-Hanselmayer, G., Weger, W., Ilkit, M.J., Smolle J., 2007. Epidemiology of tinea capitis in Europe: current state and changing patterns. Mycoses. 50, 6-13.

Grumbt, M., Monod, M., Staib, P., 2011. Genetic advances in dermatophytes. FEMS Microbiol. Lett. 320, 79-86.

Hernández-Romano, P., Hernández, R., Arroyo, R., Alderete, J., López-Villaseñor, I., 2010. Identification and characterization of a surface-associated subtilisin-like serine protease in *Trichomonas vaginalis*. Parasitology. 137, 1621-1635.

Jousson, O., Lechenne, B., Bontems, O., Capoccia, S., Mignon, B., Barblan, J., Quandroni, M., Monod, M., 2004. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiology. 150, 301-310.

Kraemer, A., Mueller, R.S., Werchenthin, C., Straubinger, R.K., Hein, J., 2011. Dermatophytes in pet guinea pigs and rabbits. Vet. Microb., in press

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson, D.E. Jr., Cieplak, W. Jr., 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol. Microbiol. 24, 953-963.

Lagal, V., Binder, M.E., Huynh, M., Kafsack, B.F.C., Harris, P.K., Diez, R., Chen, D., Cole, R.N., Carruthers, C.V., Kim, K., 2010. *Toxoplasma gondii* protease TgSUB1 is required for cell surface processing of micronemal adhesive complexes and efficient adhesion of tachyzoites. Celull. Microbiol. 12, 1792-1808.

Leng, W., Liu, T., Wang, J., Li, R., Jin, Q., 2009. Expression dynamics of secreted protease genes in *Trichophyton rubrum* induced by key host's proteinaceous components. Med. Mycol. 47, 759–765.

Maranhão, F.C., Paiao, F.G., Martinez-Rossi, N.M., 2007. Isolation of transcripts over-expressed in human pathogen *Trichophyton rubrum* during growth in keratin. Microb. Pathog. 43, 166–172.

Mignon, B., Nikkels, A.F., Pierard, G.E., Losson, B., 1998. The *in vitro* and *in vivo* production of a 31.5-kD keratinolytic subtilase from *Microsporum canis* and the clinical status in naturally infected cats. Dermatology. 196, 438-441.

Mignon, B., Leclipteux, T., Focant, C., Nikkels, A., Pierard, G., Losson, B., 1999. Humoral and cellular immune response to a crude exo-antigen and purified keratinase of *Microsporum canis* in experimentally infected guinea pigs. Med. Mycol. 37, 123-129.

Mignon, B., Vermout, S., Brouta, F., Nikkels, A., Losson, B., Descamps, F., 2004. *In vivo* m-RNA expression analysis of *Microsporum canis* secreted subtilisin-like serine proteases in feline dermatophytosis. Advances in Vet. Derm. 5, 255-260.

Monod, M., 2008. Secreted proteases from dermatophytes. Mycopathologia. 166, 285-294.

Portela, M.B., Souza, I.P., Abreu, C.M., Bertolini, M., Holandino, C., Alviano, C.S., Santos, A.L., Soares, R.M., 2010. Effect of serine-type protease of *Candida* spp. isolated from

linear gingival erythema on HIV-positive children: critical factors in the colonization. J. Oral. Pathol. Med. 39, 753-760.

Pracht, D., Elm, C., Gerber, J., Bergmann, S., Rohde, M., Seiler, M., Kim, K.S., Jenkinson, H.F., Nau, R., 2005. PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. Infect. Immun. 73, 2680-2689.

Scott, D.W., Miller, W.H., Griffin, C.E., 2001. Fungal skin diseases. In: Miller, G.H., Kirk, R.W., (Eds.), Small Animal Dermatology, 6th Edition, Saunders, Philadelphia, pp. 339-361.

Sparkes, A.H., Werrett, G., Stokes, C.R., Gruffydd-Jones, T.J., 1994. *Microsporum canis*: inapparent carriage by cats and the viability of arthrospores. J. Small. Anim. Pract. 35, 397-401.

Spencer, J., Chart, H., Smith, H.R., Rowe, B., 1998. Expression of membrane-associated proteins by strains of enteroaggregative *Escherichia coli*. FEMS Microbiol. Lett. 161, 325-330.

Sriranganadane, D., Waridel, P., Salamin, K., Feuermann, M., Mignon, B., Staib, P., Neuhaus, J.M., Quadroni, M., Monod, M., 2011. Identification of novel secreted proteases during extracellular proteolysis by dermatophytes at acidic pH. Proteomics 11(22), 4422-4433.

Staib, P., Zaugg, C., Mignon, B., Weber, J., Grumbt, M., Pradervand, S., Harshman, K., Monod, M., 2010. Differential gene expression in the pathogenic dermatophyte *Arthroderma benhamiae in vitro* versus during infection. Microbiology. 156, 884-895.

Tabart, J., Baldo, A., Vermout, S., Nusgens, B., Lapiere, C., Losson, B., Mignon, B., 2007. Reconstructed interfollicular feline epidermis as a model for *Microsporum canis* dermatophytosis. J. Med. Microbiol. 54, 299-308.

Vermout, S., Tabart, J., Baldo, A., Monod, M., Losson, B., Mignon, B., 2007. RNA silencing in the dermatophyte *Microsporum canis*. FEMS Microbiol. Lett. 275, 38-45.

Vermout, S., Baldo, A., Tabart, J., Losson, B., Mignon, B., 2008. Secreted dipeptidyl peptidases as potential virulence factors for Microsporum canis. FEMS Immunol. Med. Microbiol. 54, 299-308.

Zaugg, C., Monod, M., Weber, J., Harshman, K., Pradervand, S., Thomas, J., Bueno, M., Giddey, K., Staib, P., 2009. Gene expression profiling in the human pathogenic dermatophyte *Trichophyton rubrum* during growth on proteins. Eukaryot. Cell. 8, 241-250.

Figures

Fig. 1. Expression of protease Sub3 by *M. canis* in *in vitro*-produced arthroconidia. (a) *M. canis* control strain (IHEM 22958) arthroconidia were intensively stained at their surface after immunostaining with purified rabbit polyclonal antibody raised against Sub3. (b) No staining was observed after immunostaining using the pre-immune rabbit serum as a control. (c) Arthroconidia from the *M. canis SUB3* RNA-silenced strain (IHEM 22957) were also devoid of any positive staining using anti-Sub3 antibody.

Fig. 2. Adherence of arthroconidia from the *M. canis SUB3* RNA-silenced strain to epidermis from humans and various animal species. The figure shows the average number of adherent arthroconidia of control strain IHEM 22958 (□) in comparison with silenced strain IHEM 22957 (◊). For each tested skin type, three independent assays were carried out and are reported in the figure. Dots correspond to means of adherent arthroconidia of four skin explants ± standard deviations. Significant differences between silenced and control strain adherence were obtained for all assays regardless of the skin type tested ($P < 0.01$).

Fig. 3. Adherence of arthroconidia from the *M. canis SUB3* RNA-silenced strain to feline epidermis in the presence of recombinant Sub3. The figure shows the average number of adherent arthroconidia from the silenced strain IHEM 22957 and control strain IHEM 22958 in the presence of rSub3 with regard to the average number of adherent arthroconidia of both strains in the absence of exogenous Sub3. The addition of rSub3 had no effect on adherence of any of the strains. Data correspond to means of adherent arthroconidia from four skin explants ± standard deviations. Three independent assays, using three unrelated cats (cat 1, cat 2, cat 3) were carried out and are reported in the figure.

Fig. 4. Detection of *M. canis* recombinant Sub3 after contact with feline skin by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protease rSub3 (2.5 µg/ml-lane1, 1 µg/ml-lane2 or 0.5 µg/ml-lane3) was deposited on feline skin and incubated for 4 h at 37 °C. The 12% SDS-PAGE was stained with Coomassie brilliant blue. Recombinant Sub3 appeared as a unique band at 31.5 kDa, indicating that the protease is not degraded under conditions of adherence assays. The molecular weights (in kDa) are indicated on the left.

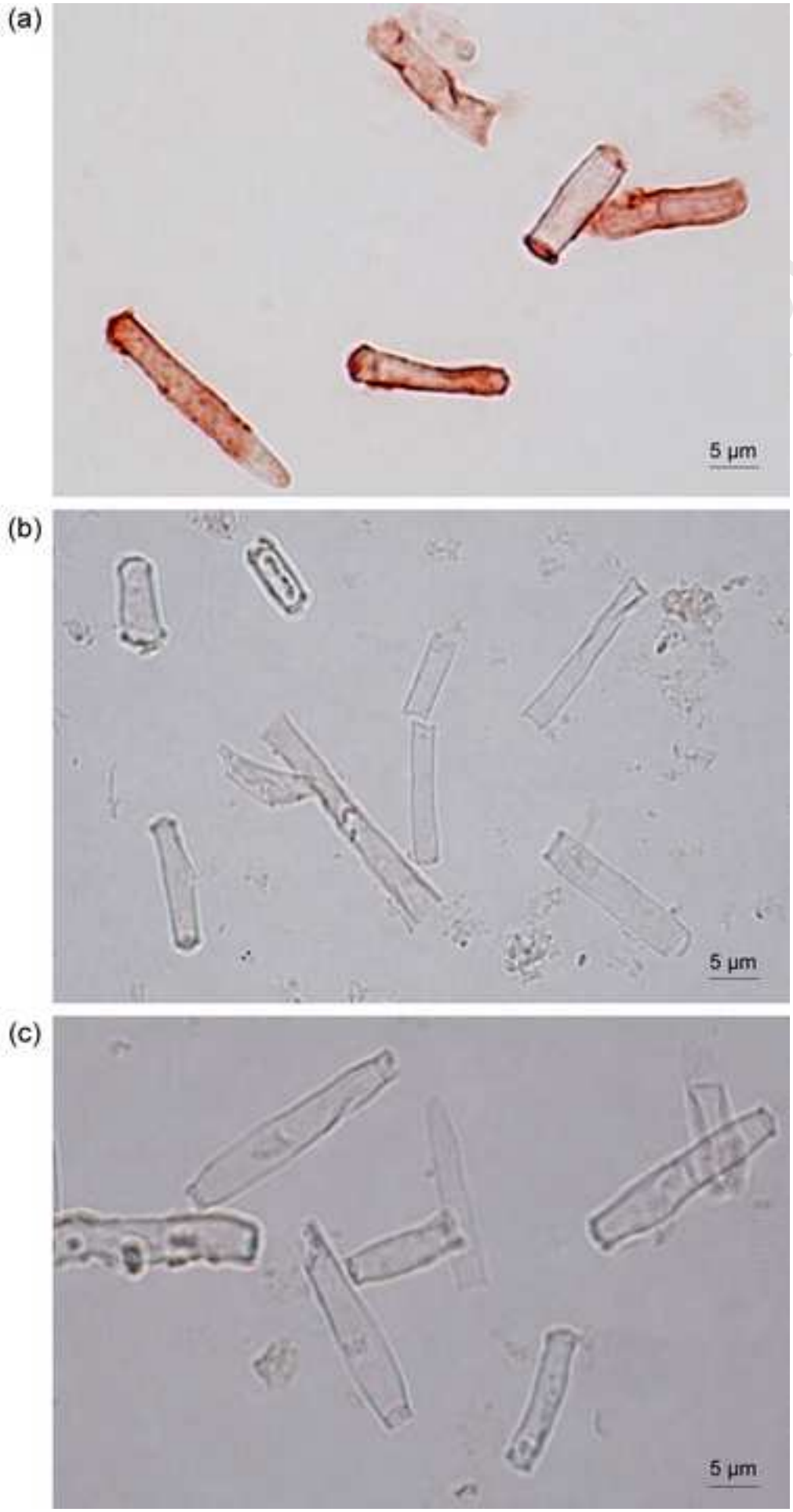


Figure 2

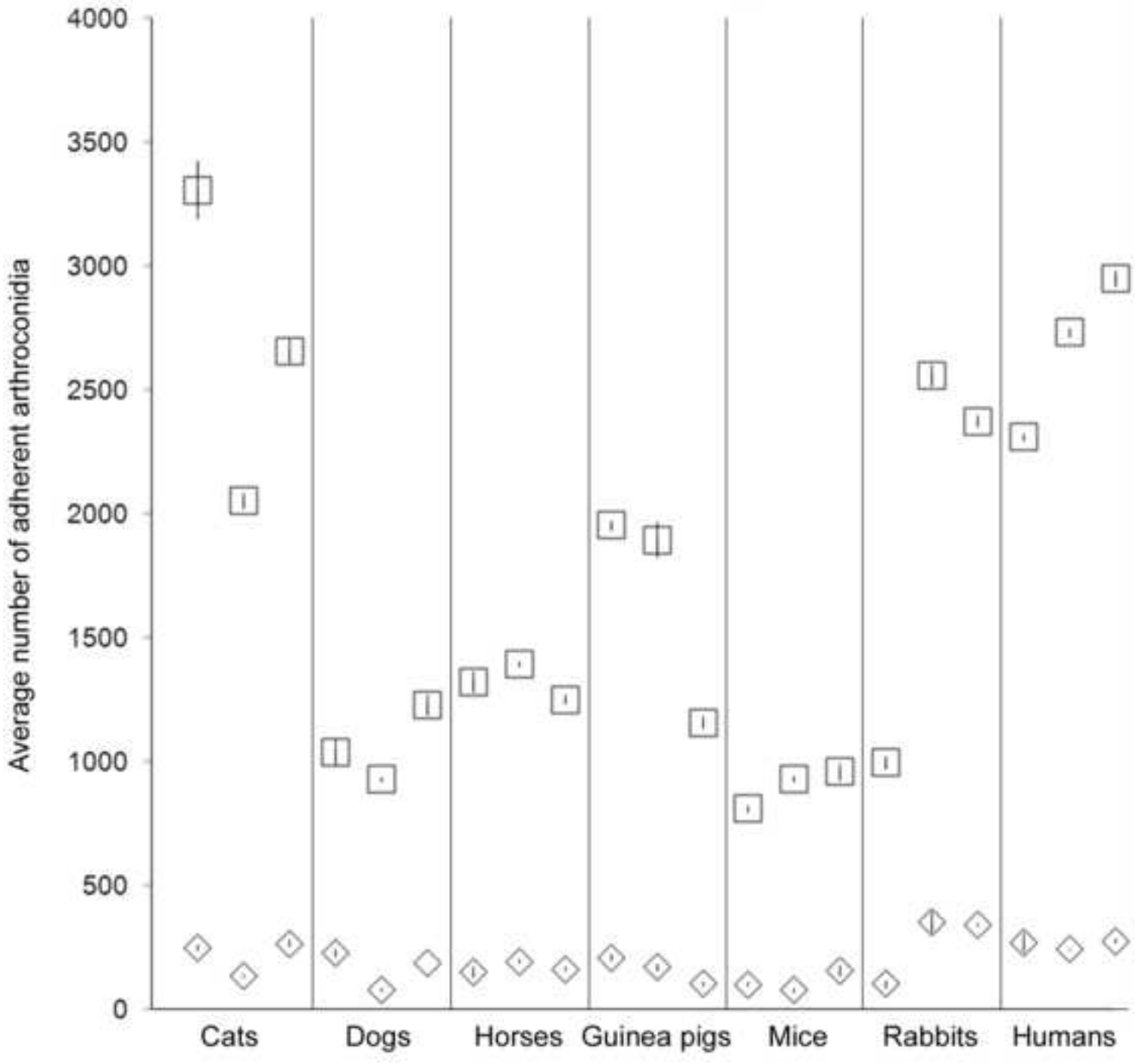
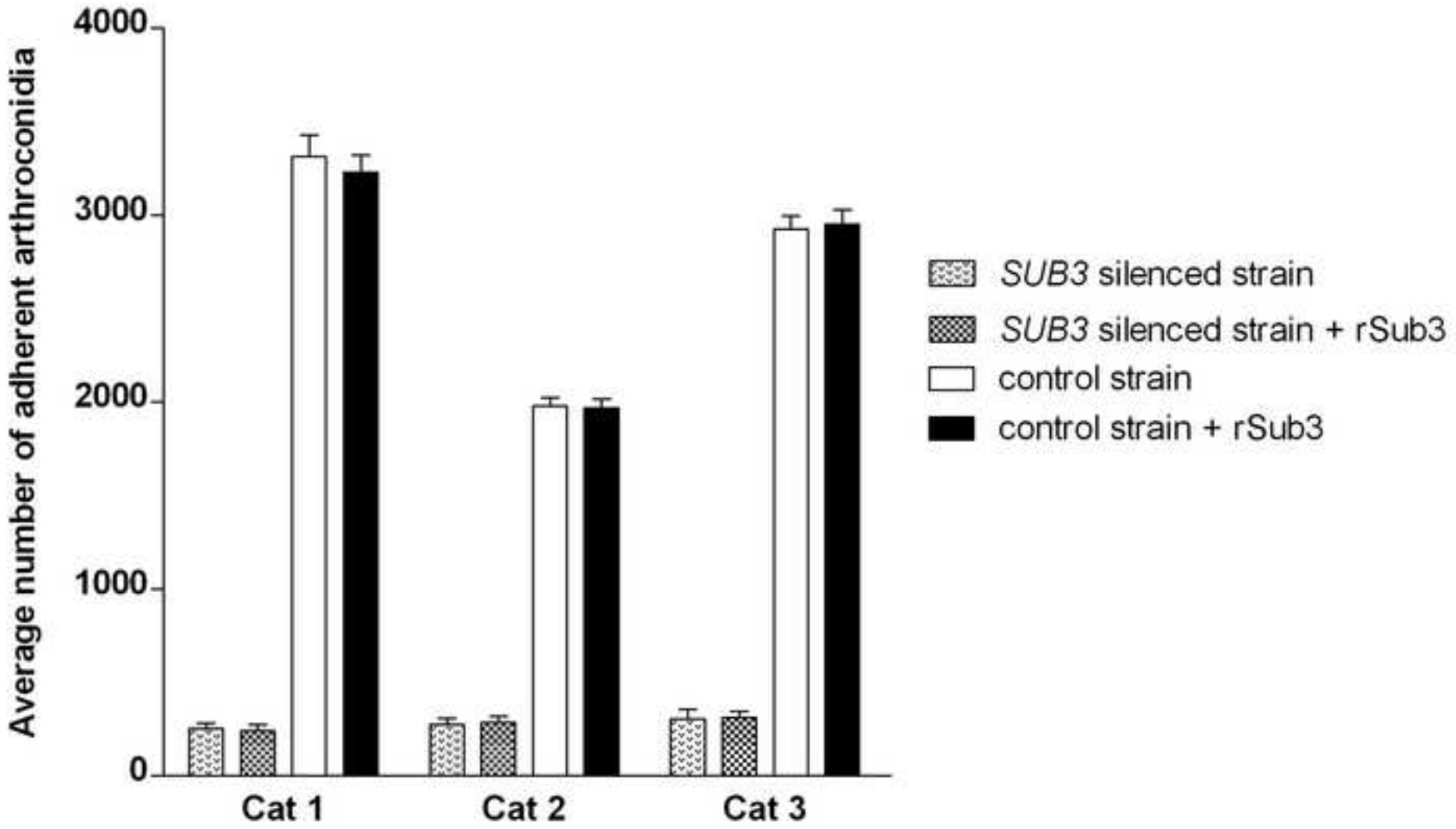

Average number of adherent arthroconidia
4000
3500
3000
2500
2000
1500
1000
500
0
Cats
Dogs
Horses
Guinea pigs
Mice
Rabbits
Humans

Figure 3


Average number of adherent arthroconidia
4000
3000
2000
1000
0
Cat 1
Cat 2
Cat 3
SUB3 silenced strain
SUB3 silenced strain + rSub3
control strain
control strain + rSub3