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Title: Assessment of the cutaneous immune response during *Arthroderma benhamiae* and *Arthroderma vanbreuseghemii* infection using an experimental mouse model

Running head: Mouse model for dermatophyte immune response study

Authors: L. Cambier [1†], A. Weatherspoon [2†], V. Defaweux [2], E.T. Bagut [1, 3], M.P. Heinen [1], N. Antoine [4], B. Mignon [1*]

Affiliations: [1]Veterinary Mycology Laboratory, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium [2]Human Histology Laboratory, Department of Preclinical Science, Faculty of Medicine, CHU Liège, Liège, Belgium [3]Parasitology and Parasitic Diseases Unit, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Cluj-Napoca, Romania [4]Veterinary Histology Laboratory, Department of Morphology and Pathology, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium

[†]Both authors have contributed equally to this work

***Corresponding author**: Bernard Mignon, Veterinary Mycology Laboratory, Department of Infectious and Parasitic Diseases, University of Liège, Liège, Belgium

Phone: +32 4 366 40 99 ; Fax: +32 4 366 40 97; Email: bmignon@ulg.ac.be

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What's already known about this topic?

- Pathophysiology and immunology of dermatophytoses are still poorly understood.
- Most *in vivo* studies have been performed using guinea pig-based models.
- Several limitations, notably the lack of immunological tools for this animal species, render the development of a modern mouse model necessary for progress in pathogenesis understanding.

What does this study add?

- Using two peculiar fungal species isolated from men, *Arthroderma benhamiae* and *Arthroderma vanbreuseghemii*, a new mouse model of dermatophytosis was developed.
- Clinical, histopathological and immunohistological evaluations showed that this model is reproducible, fits with previous experimental infection models using guinea pigs and mimics superficial tinea in humans.

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- For the first time, the cutaneous cytokine response was assessed during a dermatophyte infection, showing that the role of the Th17 pathway should now be considered.

Abstract

Background Dermatophytoses are common but poorly understood skin infections. Most *in vivo* studies have been performed using the guinea pig as the experimental animal model, which has several limitations.

Objectives To develop a mouse model of dermatophytosis suitable for multiple purposes, including the investigation of immunity against dermatophytes.

Methods Two peculiar fungal species, *Arthroderma benhamiae* and *Arthroderma vanbreuseghemii*, isolated from tinea in men having contact with rodents were used for epicutaneous inoculation. During the infection, clinical and histopathological follow-up were performed. The recruitment of immune cells was evaluated by immunofluorescence staining and the levels of cytokines mRNA were quantified by quantitative RT-PCR in skin of infected mice.

Results The skin symptoms and microscopic lesions, including the colonization of keratinized epidermal and follicular structures by both dermatophytes, were highly similar to those observed in guinea pig infection models and in natural infections, mimicking acute superficial tinea in humans. The dermal inflammatory cellular infiltrate consisted of macrophages, dendritic cells, and especially polymorphonuclear neutrophils, which are one of the histologic ‘clues’ to the diagnosis of dermatophytosis. The *in situ* cytokine profile was characterized by the overexpression of TGF-β, IL-1β, and IL-6 mRNAs during infection, suggesting a role of the Th17 pathway in the establishment of immunity.

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Conclusions Our new reproducible and validated mouse model of dermatophytosis is a modern *in vivo* tool that allows a more in-depth understanding of the pathogenesis of human dermatophyte infections.

Introduction

Dermatophytes are pathogenic filamentous fungi responsible for superficial cutaneous infections called dermatophytoses.[1] They specifically invade keratinized structures such as *stratum corneum*, hair, nails and claws. Based on their ecological features, three groups of dermatophyte species are recognized, namely anthropophilic, restricted to humans, zoophilic, found predominantly in animals and geophilic, recovered from the soil without or only sporadically causing disease.[2]

Despite their superficial localization in keratinized structures, dermatophytes can induce an adaptive immune response. The Th1 cellular response, associated with delayed type hypersensitivity (DTH), appears to be correlated with clinical recovery and protection against reinfection.[3-5] Nevertheless, the Th17 pathway could also be involved in the establishment of a protective immunity in dermatophytoses as has been shown for other fungi such as *Candida albicans*[6-8] and *Aspergillus fumigatus*.[7, 9] Little is known about the mechanisms involved in the establishment of a protective immune response during dermatophytosis.[4, 5, 10] Some *in vitro* studies about immunity against dermatophytes have demonstrated that keratinocytes,[11] neutrophils[12] and dendritic cells[13] are able to produce pro-inflammatory cytokines in response to infection. Nevertheless, *in vivo* studies are essential in furthering our understanding of immunity against dermatophytes. Therefore, the establishment of an animal model of dermatophytosis is required. Currently, the most frequently used animal model is the guinea pig[14-17] but the inbred nature, and the genetic and immunologic tools available in the mouse

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species, support the use of a model in the study of immune response against dermatophytes.[18] Only few mouse models of dermatophytoses are available. A former model using *Trichophyton quinckeanum* was successfully used for both histopathologic [19, 20] and immunologic precursory studies.[21, 22] However, this model is questionable because *T. quinckeanum* is a rare agent of dermatophytosis in humans, moreover responsible for a particular clinical entity named mouse favus.[23] Two other models using dermatophytes in mice were recently described but they are not representative of a superficial natural infection.[24, 25]

In this study, we focused on two zoophilic dermatophyte species that are frequently isolated from superficial tinea in men: *Arthroderma benhamiae*, which primarily affects guinea pigs and the closely related *Arthroderma vanbreuseghemii* which affects other animal species, notably cats and dogs but very rarely rodents including mice.[26] Both species belong to the *Trichophyton mentagrophytes* complex[26-28] and are readily manipulable at the genetic level.[29-31] These features urged us to test both *A. benhamiae* and *A. vanbreuseghemii* species by using two isolates originating from humans in contact with either the guinea pig or mouse, respectively. Although some mycologists still use the nomenclature *T. mentagrophytes* complex, the sexual names *A. benhamiae* and *A. vanbreuseghemii* should always be used according to the rules proposed by the Amsterdam declaration on Fungal Nomenclature.[32]

The aim of this work was to develop a reproducible experimental mouse model of dermatophytosis using *A. benhamiae* or *A. vanbreuseghemii*, and subsequently to assess the cutaneous immune response generated during infection. To this purpose, the immune cells recruited to the site of infection were defined and the cytokine response induced in skin of infected mice was assessed.

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Materials and Methods

Fungal strains

Arthroderma benhamiae IHEM 20163 (Institute of Hygiene and Epidemiology-Mycology [IHEM], Brussels, Belgium) was originally isolated from a patient suffering from inflammatory tinea faciei and who had had previous contact with a guinea pig.[33] This *A. benhamiae* strain has been characterized at the molecular level[27] and was successfully used to develop a guinea pig infection model for this species.[29] *Arthroderma vanbreuseghemii* IHEM 22740 was chosen because of its peculiar origin, namely a patient in contact with mouse and having highly inflammatory tinea manum.[27] This strain is closely related to *A. vanbreuseghemii* isolated from dogs and cats and causing various inflammatory tinea in humans.[27] Both strains were grown for 15 days at 27 °C on Sabouraud's plates (Sab; 2% glucose-1% peptone), and used for the experimental infection.

Animals and experimental infection

Eighteen specific-pathogen-free 2-month-old female mice of C57BL/6 strain (Charles River, Wilmington, MA, USA) were used and housed separately during the entire study period. Mice were divided into 3 groups. The *A. benhamiae* group (n=6) was inoculated with 4.2×10^{10} colony forming units (CFU), the *A. vanbreuseghemii* group (n=6) received 2×10^{11} CFU while the mice from the control group (n=6) remained uninfected. Experimental infection was performed under general anaesthesia (medetomidine [1 mg kg^{-1}] and ketamine [40 mg kg^{-1}]) administered by intraperitoneal injection. Backs of the animals were shaved and lightly abraded with a 25G needle. The inoculum (300 µl) consisting of mycelia and spores collected from cultures on Sab plates was suspended in 5% (w/w) poloxamer 407 and gently rubbed onto the skin with a sterile pipette tip. Mice from the control group received 300 µl of poloxamer only. For histology, immunoflorescence staining and quantification of

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cytokines, 3 infected mice were randomly selected from each group and skin biopsies samples were collected. Animal experiments were approved by the local ethics committee (University of Liège, ethics protocol no. 943).

Clinical follow-up

Infected mice were monitored twice a week for 5 weeks by two independent examiners using clinical criteria i.e., alopecia and squamosis-crusting. Both investigators who remained the same throughout the experiment were blinded as to the status of mice (infected or not). For each clinical criterion, a score of 0, 1, 2 or 3 (absence, mild, moderate or severe skin lesion) was attributed. A clinical score was then calculated for each mouse by adding scores for both criteria. Finally, a median clinical score was calculated for each group of mice.

Histology

Skin samples collected at days 3, 7, 14 and 21 post-inoculation (PI) were fixed in 10% neutralized buffered formalin and paraffin embedded for routine processing. Sections of 4 µm thick sections were stained with haematoxylin and eosin (H&E) for histopathologic evaluation or with periodic acid-Schiff (counterstained with haematoxylin) to assess the fungal invasion in keratinized skin structures.

Immunofluorescence staining

At day 7 PI, skin samples collected from mice were immersed in tissue-TEK OCT embedding medium (Sakura, Zouterwoude, The Netherlands) and quickly frozen at −80 °C. Sections (8-10 µm) were cut at −20 °C with a microtome (Microtom HM 500 OM, Thermo Fisher Scientific, Waltham, MA, USA), mounted on glass slides coated with poly-L-Lysine (Sigma, St. Louis, MO, USA), fixed in acetone for 10 min at 4 °C, and stored at −20 °C until

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used. After rehydration in PBS, cryosections were incubated for 1 h at room temperature with appropriate primary antibodies diluted 1:100 in PBS. After washing in PBS, sections were incubated for 30 min at room temperature with Alexa Fluor488 streptavidin or species-specific secondary antibody (Molecular Probes, Leiden, The Netherlands). The characteristics of primary and secondary antibodies are listed in Table 1. A fluorescent dye for nuclear staining, the TOPRO-3-iodide (Molecular Probes) was added to cryosections labelled with anti-major histocompatibility complex (MHC) II and anti-CD54 antibodies. To test the general specificity of the antibodies used, samples were incubated with irrelevant antibodies. Negative controls were obtained by incubating samples with secondary antibodies only.

Quantification of cytokine mRNA levels in skin biopsies

At day 0, 7 and 21 PI, skin biopsy specimens were homogenized using the FastPrep® instrument (MP Biomedicals, Santa Ana, CA, USA). Total RNA was isolated using TRIzol reagent following the manufacturer's instructions (Invitrogen, Burlington, ON, Canada). The purified RNA was treated with DNase I (Invitrogen). Template cDNA was synthesized from RNA by reverse transcription, using iScript™ cDNA Synthesis kit (Bio-rad, Hercules, CA, USA). Reverse transcriptase was omitted in control reactions. The sequences of oligonucleotide primers have already been published (12S rRNA, Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL-6 and IL-22)[34-36] or were selected (IL-10 and Transforming Growth Factor (TGF)-β) using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd). The primers for mouse 12S rRNA (internal control) and cytokines were synthesized by Eurogentec (Liège, Belgium) (Table 2). The quantitative PCR (qPCR) reactions were assembled using the iQ™ SYBR® Green Supermix (Bio-rad) and subjected to the following protocol in a MiniOpticon System (Bio-rad): 10 min at 95 °C and 50 cycles of 45 s at 95 °C,

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45 s at 60 °C and 45 s at 72 °C. The melting curve was performed from 45 °C to 95 °C in 1 °C/15 s increments. All assays were performed in duplicate. Results in terms of cycle thresholds were converted in folds 12S rRNA expression using the $2^{-\Delta\Delta Ct}$ method. The level of cytokine mRNA in skin biopsies at day 7 or 21 PI was expressed relative to that in skin biopsies at day 0.

Statistical analysis

The two-way analysis of variance (ANOVA) test was used for the statistical comparison of median clinical scores between the infected and control mice. This test was performed with the Graph pad Prism 5.0 statistical software (GraphPad Software, San Diego, CA, USA). The levels of cytokine mRNA in skin biopsies, 7 and 21 days PI were compared with those determined in skin biopsies at day 0 using a general linear model (GLM procedure of SAS; SAS Institute Inc., Cary, NC, USA). $P<0.05$ was considered as statistically significant.

Results

Mice infected with *A. benhamiae* and *A. vanbreuseghemii* develop clinical lesions of dermatophytosis

All animals infected with *A. vanbreuseghemii* and 80% of mice infected with *A. benhamiae* showed consistent dermatophytosis lesions. This contrasts with previous experiments suggesting that the C57BL/6 strain mice are resistant to dermatophytosis.[21] Clinical signs of dermatophytosis were obvious 4 days after infection and were maximal at day 19 PI for both dermatophyte species (Fig. 1). The lesions healed progressively until day 35 PI. The main clinical signs consisted of severe squamosis-crusting followed by transient alopecia. From day 11 PI until day 24 and 28 PI for *A. benhamiae* and *A. vanbreuseghemii* respectively, median clinical scores were significantly higher in both infected groups in comparison with

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the controls (Fig. 2). The application of poloxamer 407 without fungus did not produce any lesions in the control group.

***A. benhamiae* and *A. vanbreuseghemii* colonize keratinized skin structures**

To assess the presence and the localization of dermatophytes in keratinized skin structures of infected mice, skin biopsy sections were stained with PAS. In mice infected with both *A. vanbreuseghemii* and *A. benhamiae*, fungal elements consisting of both hyphae and arthroconidia were detectable from day 3 until day 14 PI in the *stratum corneum* and the keratinized part of the infundibular-isthmal hair follicles including the hair shafts. Subjectively, the most severe fungal colonization was observed at day 7 PI for both dermatophyte species (Fig. 3). As expected, no fungus elements were found in skin of control mice.

***A. benhamiae* and *A. vanbreuseghemii* induce severe microscopic inflammatory skin lesions**

To assess histologic lesions in infected mice, skin biopsy sections were stained with H&E. All infected animals showed extensive inflammatory lesions. At day 3 PI, both dermatophytes induced a severe increase of epidermis thickness essentially due to both acanthosis (Figs. 4a, c) and hyperkeratosis, mainly orthokeratotic (Fig. 4a). These lesions were accompanied by moderate to severe multifocal to diffuse spongiosis (Fig. 4c) and by perivascular to diffuse dermal infiltration by immune cells (Fig. 4e). At 7 days PI, subcorneal pustules (Fig. 4d) were also observed. By day 14 PI, lesions had disappeared in mice infected with *A. vanbreuseghemii*, while acanthosis, spongiosis and dermal cellular infiltration persisted beyond day 21 PI after infection with *A. benhamiae*. No lesions were observed in control mice (Fig. 4b).

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Immune cells are recruited early after *A. benhamiae* and *A. vanbreuseghemii* infection

To identify which immune cells were recruited to the infection site at day 7 PI, immunofluorescence staining was performed on frozen skin biopsy sections (Figure 5). Large numbers of polymorphonuclear neutrophils (PMNs), MHC II expressing cells and to a lesser extent, dendritic cells (DCs) (CD11C+) and macrophages (CD54+) were recruited in the dermis of mice infected with both *A. benhamiae* and *A. vanbreuseghemii*. These cells were scarce in the skin of control mice.

Cytokine mRNA expression in infected skin

Cytokine mRNA levels were determined at days 7 and 21 PI and compared with those at day 0 (Fig. 6). Infection with both dermatophytes induced a statistically significant increase in the levels of TGF-β, IL-1β and IL-6 mRNA at day 7 PI. The level of IL-22 mRNA was also significantly increased in mice infected with *A. benhamiae*. At day 21 PI, the levels of TGF-β, IL-1β and IL-6 mRNA were significantly higher in mice infected with *A. benhamiae*, while infection with *A. vanbreuseghemii* induced a statistically significant increase in IL-1β mRNA level only. The levels of cytokine mRNA in control non-infected mice were not significantly different at both days 7 and 21 PI in comparison to day 0.

Discussion

We have developed a new and useful experimental mouse model of dermatophytosis using either *A. benhamiae* or *A. vanbreuseghemii*. Both species were isolated from naturally infected humans in contact with rodents. For both dermatophytes, clinical signs were identical from one animal to another, typical for a primary natural infection and disappeared spontaneously, indicating that this model is reproducible and appears to be suitable for *in vivo* studies on acute dermatophytoses.

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The symptoms and microscopic skin lesions, including the colonization of keratinized epidermal and follicular structures by both dermatophytes, were highly comparable to those described in a recent guinea pig infection model for *A. benhamiae*,[29] which was used to assess dermatophyte virulence factors in vivo.[30] A high amount of fungus was observed at day 7 PI in keratinized structures of mice infected with both dermatophytes before the peak of skin lesions (at day 19 PI). This observation is consistent with the recruitment of immune cells at day 7 PI that are responsible for elimination of the fungus by inducing an inflammatory response.

In contrast with guinea pig-based models,[37] one of the major advantages of our mouse model lies in the homogeneity of skin symptoms and lesions between individuals, thanks to the inbred nature of animals. Additional benefits include lower operating costs and availability of many modern immunological tools, which render relevant mouse models of dermatophytoses desirable for furthering a comprehensive understanding of the pathogenesis of these diseases.[18]

In addition to comparable clinical signs and microscopic inflammatory lesions, the nature of the immune cells recruited after the infection of mice with both *A. benhamiae* and *A. vanbreuseghemii* was consistent with what most authors have observed using other models or during natural infections. The inflammatory response observed in skin of mice infected with both dermatophytes was characterized by an inflow of immune cells such as PMNs, macrophages and DCs. Similarly, the infiltration of skin by PMNs has been shown in experimentally infected mouse[20] and is commonly described as one of the histologic ‘clues’ to the diagnosis of dermatophytosis in humans.[38] Human PMNs and macrophages can exert cytotoxic activity against *Trichophyton* species including the most common dermatophyte

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Trichophyton rubrum, via phagocytosis and production of superoxide anions[39-41] and also via the formation of neutrophil extracellular traps (NETs).[13]

In addition to PMN and macrophages, our model also revealed infiltrating DCs, consistent with the role that these professional antigen presenting cells (APCs) are believed to play in the establishment of a protective immune response during dermatophytosis. Such a role is supported by *in vitro* experiments showing that both human DCs[13] and several pattern recognition receptors on mouse DCs[42, 43] can bind fungal components from both *T. rubrum* and *Microsporum andouinii*, and modulate immunity to dermatophytes. Additionally, cellular mediated immunity, which is known to be protective in dermatophytoses[4, 5] is induced by the recognition of the MHC II at the APCs surface and the CD4+ T-cell receptor (TCR).[44] In this work, the considerable infiltration of MHC II expressing cells in the skin of infected mice suggests that these cells could be involved in the immunity against *A. benhamiae* and *A. vanbreuseghemii.* DCs and macrophages, both expressing MHC II on their surface[45] were recruited in the skin of mice infected with both dermatophytes, which suggests that these cells could be important for the initiation of immunity against dermatophytosis.

In order to investigate the cutaneous immune response in our validated dermatophytosis mouse model, parallel to the characterization of the cells recruited to the site of infection, we assessed the cytokine response *in situ*, precisely because the set-up of a protective immune response is also correlated with the establishment of an appropriate cytokine response.[46] The cytokine profile generated by *A. benhamiae* and *A. vanbreuseghemii* infection was a pro-inflammatory profile characterized by TGF-β, IL-1β and IL-6 production. These cytokines are involved in the establishment of the Th17 pathway[47-49]. In mice infected with *A. benhamiae*, an increase in IL-22 mRNA level was also observed. This cytokine, which is also produced

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by Th17 cells,[50] has a significant role in the establishment of skin and mucosa immunity.[51] Altogether, these results indicate that the Th17 pathway could be involved in the establishment of immunity against *A. benhamiae* and *A. vanbreuseghemii*. This hypothesis is strengthened by the weak IL-10 mRNA level produced in the skin of mice infected with both dermatophytes. This cytokine is known to reduce the inflammatory response by activating the Treg pathway.[52] The potential role of the Th17 pathway in the immunity against *A. benhamiae* and *A. vanbreuseghemii* infection is also supported by the recruitment of PMNs to the site of infection, as cross-talk exists between Th17 cells and PMNs.[53-56]

In conclusion, we have developed and validated a new model of dermatophytosis in mice, which is consistent with former *in vivo* models, most of which are based on the guinea pig, a commonly used animal species that is no very suitable for studying this disease. Thanks to the inter-individual homogeneity of clinical signs and skin lesions, our model will be particularly useful for the evaluation of antifungals and vaccines, and for identifying dermatophyte virulence factors. Additionally, our model is consistent with the recent insights gained *in vitro* into the innate and specific immune response against dermatophytes. It appears to be, therefore, a promising modern animal model suitable to study, in depth, the host immune response in dermatophytosis. In the near future, the role of Th17 and the possible involvement of the Th1 or Th2 pathways in the initiation of a protective immunity against dermatophytes will be assessed by evaluating other pro-Th1, pro-Th2 and pro-Th17 cytokines in mice infected with *A. benhamiae* and *A. vanbreuseghemii*, as by using mice deficient for specific cytokines.

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Figure Legends

Figure 1: Comparative clinical follow-up of skin lesions in mice inoculated with *Arthroderma benhamiae, Arthroderma vanbreuseghemii*, and the excipient (control).

Both dermatophyte species induced comparable symptoms, which were obvious since day 4 post-infection (PI) and had spontaneously disappeared by day 32 PI. Macroscopic inflammatory skin lesions consisted of scaling and crusting followed by transient but severe inflammatory alopecia. Control mice developed no skin lesions.

Figure 2: Kinetics of median clinical scores (± SD; n=6) reflecting the severity of dermatophytic lesions in mice inoculated with *Arthroderma benhamiae* (a), *Arthroderma vanbreuseghemii* (b), and the excipient (control). The median clinical scores were calculated using the clinical scores attributed blindly to each mouse in a given group. For each mouse, a clinical score was determined by adding the score for alopecia (0, 1, 2 or 3) and the score for squmosis-crusting (0, 1, 2 or 3). Median clinical scores were significantly different between the control group and the *A. benhamiae* group from day 11 until day 24 post-infection (PI). Significant differences were also observed between the control group and the *A. vanbreuseghemii* group from day 11 until day 28 PI. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Figure 3: Keratinized skin and follicular structures of infected mice are colonized after experimental inoculation with *Arthroderma benhamiae* and *Arthroderma vanbreuseghemii.* The fungal colonization in mice infected with *A. vanbreuseghemii* 7 days post-infection is shown. The *stratum corneum* (a) and hair follicles (b) were colonized by dermatophyte hyphae and arthroconidia (black arrows). Sections were stained with periodic acid-Schiff and counterstained with hematoxylin. Scale bars = 20 µm.

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Figure 4: Mice infected with *Arthroderma benhamiae* (c) and *Arthroderma vanbreuseghemii* (a, d, e) develop comparable inflammatory epidermal and dermal lesions. Micrographs illustrate the main skin lesions consisting of severe acanthosis (a, c) and hyperkeratosis (a), accompanied by spongiosis (c), scattered subcorneal pustules (d), and perivascular-to-diffuse cellular infiltration in the dermis (e) at day 7 (a, b, d, e) and 21 post-infection (c). Control mice inoculated with the excipient (b) developed no skin lesions. Sections were stained with hematoxylin and eosin. †: hyperkeratosis, ‡: acanthosis, ↑: spongiosis. Scale bars = 20 µm.

Figure 5: Immune cells are recruited in the dermis of mice infected with both *Arthroderma benhamiae* and *Arthroderma vanbreuseghemii*. Micrographs show the connective tissue around the infundibular and isthmal hair follicles of infected mice at day 7 post-infection or uninfected controls. Immunofluorescence staining revealed that polymorphonuclear neutrophils (PMNs) (neutrophil+), class II major histocompatibility complex-expressing cells (MHC II+) and, to a lesser extent, dendritic cells (DCs) (CD11c+) and macrophages (CD54+) were recruited mainly around hair follicles. A fluorescent dye for nuclear staining, the TOPRO-3-iodide, was added to cryosections immunostained with anti-MHCII and anti-CD54 antibodies. Scale bars = 75 µm.

Figure 6: Cytokine mRNA profile in the skin of mice at days 7 (a) and 21 (b) post-infection (PI) with *Arthroderma benhamiae* and *Arthroderma vanbreuseghemii*. In each group of mice, both uninfected control and infected, three animals were used to compare the levels of cytokine mRNAs before inoculation (day 0) and at days 7 and 21 PI. A statistically significant increase in TGF-β, IL-1β, IL-6, and IL-22 mRNA levels was observed in mice

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infected with *A. benhamiae* at day 7 PI. Similar results were observed in mice infected with *A. vanbreuseghemii*, except for levels of IL-22 mRNA, which were not significantly higher than at day 0. At day 21 PI, a statistically significant increase in TGF-β, IL-1β, and IL-6 mRNA was observed in mice infected with *A. benhamiae.* In mice infected with *A. vanbreuseghemii*, the increase in the levels of these cytokine mRNAs was statistically significant only for IL-1β. In uninfected control mice, the levels of cytokine mRNAs did not increase significantly through the study period. Data are representative of three independent experiments (mean ± SD). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Tables

Table 1. Antibodies used for immunofluorescence staining

Primary antibody	Target	Secondary antibody	Source
Rat anti-mouse MHC II FITC	MHC II	-	Chemicon International (Temecula, CA, USA)
Rat anti-mouse CD54 (ICAM-I) Biotin	Macrophages	Streptavidin Alexa 488	eBioscience (San Diego, CA, USA)
Hamster Anti-mouse CD11c Biotin	DCs	Streptavidin Alexa 488	eBioscience (San Diego, CA, USA)
Rat anti-mouse neutrophil	PMNs	Goat Anti-rat Alexa 594	Abcam (Cambridge, UK)

MHC II: class II major histocompatibility complex

DCs: dendritic cells

PMNs: polymorphonuclear neutrophils

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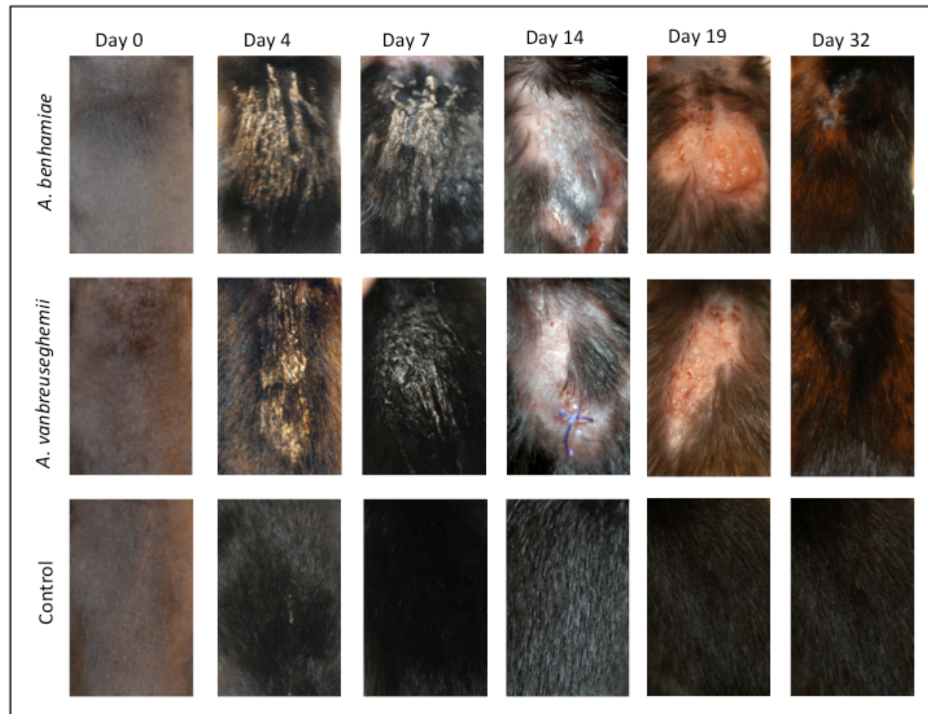
Table 2. Sequences of oligonucleotide primers

Target gene	**Forward sense (5'-> 3')**	**Reverse sense (5'-> 3')**
12s rRNA	GGAAGGCATAGCTGCTGGAGGT	CGATGACATCCTTGGCCTGA
TNFα	TCTCATCAGTTCTATGGCCC	GGGAGTAGACAAGGTACAAC
IL-1β	TTGACGGACCCCAAAAGATG	AGAAGGTGCTCATGTCCTCA
IL-6	GTTCTCTGGGAAATCGTGGA	TGTACTCCAGGTAGCTAT GG
TGFβ	GCTTTCGATTCAGCGCTCACT	TCCAACCCAGGTCCTTCCTAA
IL-10	AGCCGGGAAGACAATAACTG	CATTTCCGATAAGGCTTGG
IL-22	GGCCAGCCTTGCAGATAACA	GCTGATGTGACAGGAGCTGA



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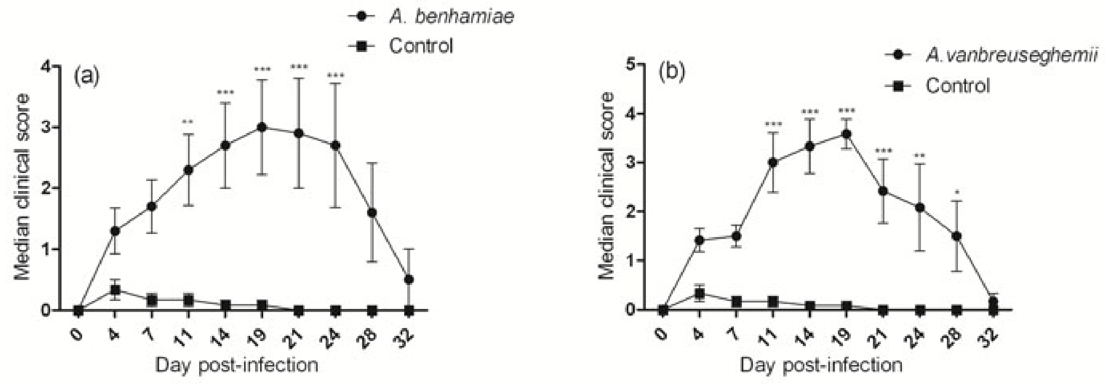

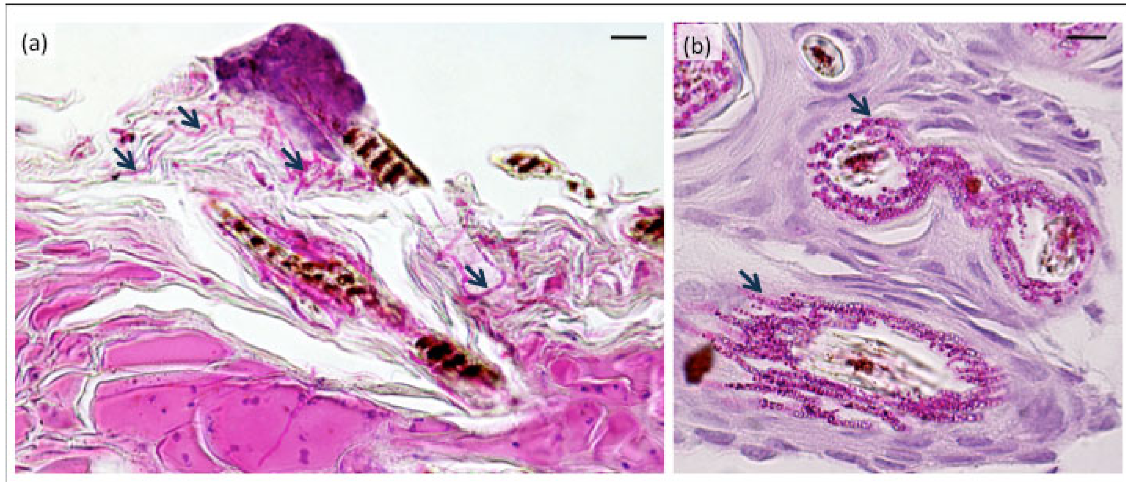

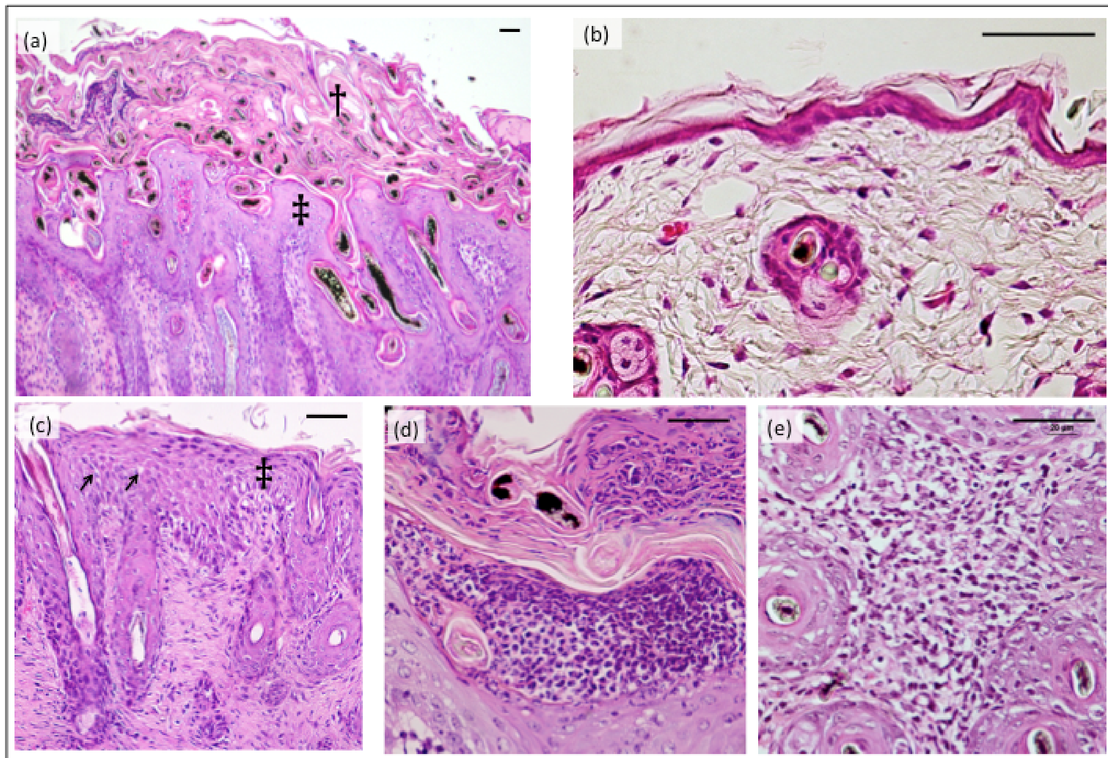

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	A. benhamiae	*A. vanbreuseghemii*	Control
Neutrophil+			
CD54+			
CD11c+			
MHC II+