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Keratinolytic testing for diagnosing dermatophytosis in small domestic animals

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Abstract

Background and Aim. Keratin is used as a structural element by numerous animal groups. Because of the strength and stability of keratin, few organisms can break it down and use it as a food source. Only a few insects, bacteria, actinomycetes, and fungi can use keratin as their sole carbon and nitrogen source. The enzymatic ability of fungi to degrade keratin has long been considered a key innovation in the evolution of animal dermatology.

The aim of the work is to develop keratinolytic tests for use and to identify their pathogenicity in the diagnosis of dermatomycetes of small domestic animals.

Materials and Methods. There were investigated the strains of the genera *Trichophyton, Microsporum,* and animal hair. The diagnosis for the detection of keratinolytic properties was carried out on three tests: hair perforation, nutrient media with grind animal hair and keratin hydrolysate.

Results. The authors of this article present studies for diagnosing dermatophytosis in small domestic animals for pathogenicity using three keratinolytic tests: hair perforation, the addition of ground animal hair, and keratin hydrolysate to the culture medium. The three tests demonstrated high keratinolytic activity, which manifested as the formation of pegs on hair and the appearance of a clearing zone in the culture media under the influence of keratinase. It was also established that in media containing hair and keratin hydrolysate, the growth of cultures was faster, and the cultures did not exhibit pleomorphism, which is a positive aspect in terms of the accuracy and speed of diagnosis.

Conclusion. Keratinolytic test allows to determine the pathogenicity of small domestic animal dermaticosis pathogens. The test showed that cultures do not form pleomorphisms, which contributes to the accurate identification of pathogens.

Key words: dermatophytosis; hair perforation; keratin; keratin hydrolysate; *Microsporum canis; Trichophyton benhamiae.*

Introduction

Dermatophytes are keratinophilic fungi belonging to the family Arthrodermataceae (Onygenales, Ascomycota), which includes dozens of related species that are primarily distinguished by their anamorph or asexual forms and are grouped into three classical genera: *Trichophyton, Microsporum,* and *Epidermophyton* [1-3]. Diseases caused by dermatophytes, such as dermatophytosis, are widespread across the globe, and the number of infections is increasing annually, not only in animals but also in humans. Of particular importance is the spread of dermatophytosis among small domestic animals, such as cats and dogs, which are human companions [4].

Until recently, the diagnosis of dermatophytosis was based on the analysis of clinical signs of the disease, which is unreliable because of the variable nature of dermatological lesions and their similarity to other skin conditions that mimic the symptoms of dermatophytosis [5]. Keratinolytic fungi are considered natural colonizers of keratinized substrates and play key roles in the natural hydrolysis of keratinized tissues [6]. Therefore, it was initially believed that dermatophytes obtain the nutrients necessary for their vital functions from easily hydrolyzed proteins, which make up only about 5% of the total composition of affected tissues [7]. It has been shown that keratinases perform not only nutritional functions, but are also the most important factors in the virulence of dermatophytes at the first stage of infection [8, 9]. *Aspergillus, Paecilomyces, Doratomyces, Trichoderma, Fusarium, Acremonium, Onygena, Cladosporium, Microsporum, Lichtheimia, Chrysosporium, Aphanoascus, Trichophyton,* and *Scopulariopsis* are among the recorded keratinolytic fungi [10-17]. Among fungal strains, keratinolytic activity has been widely described in dermatophytes. The keratinolytic properties of this group may reflect pathogenic tendencies and contribute to skin mycoses in humans and animals [18].

The complexity of keratin biodegradation by dermatophytes begins with sulfitolysis, which involves the intracellular generation of sulfite from cysteine catabolism via the enzyme cysteine dioxygenase (Cdo1). The keratinolytic activity and pathogenicity of this medically important group of fungi are interrelated, as during infection, dermatophytes secrete a variety of proteases that break down keratinized structures into oligopeptides as well as free amino acids, which are then used by the fungi as nutrient sources [19]. Keratin waste of agro-industrial origin presents an ecological problem, with feathers and hair from the poultry and leather industries at the forefront as they strive to meet the demands of a growing population. The authors of this article propose using bacteria and fungi that produce keratinase instead of chemical hydrolysis [20, 21].

Melentyev A.I. et al. proposed a method for diagnosing dermatomycosis by culturing samples of biological material on solid selective Sabouraud agar with the addition of an antibiotic, such as chloramphenicol and keratin hydrolysate, derived from chicken feathers. The cultivation conditions were 22 °C–24 °C for 4–8 days, followed by identification of fungal colonies at the species level. The method was effective against not only *Microsporum canis* but also *Trichophyton verrucosum* and *Trichophyton mentagrophytes*. The culture method allows isolation of pathogens and determination of its species affiliations [22].

Materials and Methods

The materials used in this study were

- strains of dermatophytes from the Trichophyton genus (No. 19 and No. 20) and Microsporum genus (No. 35 and No. 68) (isolated from cats and dogs). The strains were selected with an erased form of manifestation of the pathogen when making a diagnosis; four samples were selected from 198 samples.;
- Sabouraud glucose agar (40 g glucose, 10 g peptone, 20 g microbiological agar, and 1 L distilled water) for hydrolysis with 10% NaOH;
- pet hair (cat hair) obtained from veterinary clinics;
- equipment: Esco Class II Biological Safety Cabinet, 2010; BD-53 Thermostat, Germany, 2017; and Retsch MM 200 Vibratory Mill.

Research Methods:

Hair perforation test. To perform this test, several sterilized hairs (not chemically treated) are placed in a Petri dish containing sterile water and a few drops of 10% yeast extract are added. Then an inoculum in the form of several fragments of the isolate being tested is added and the dish is incubated for two weeks at 25 °C. A positive result is considered to be the appearance of cone-shaped perforations in the hair or erosion on the hair surface.

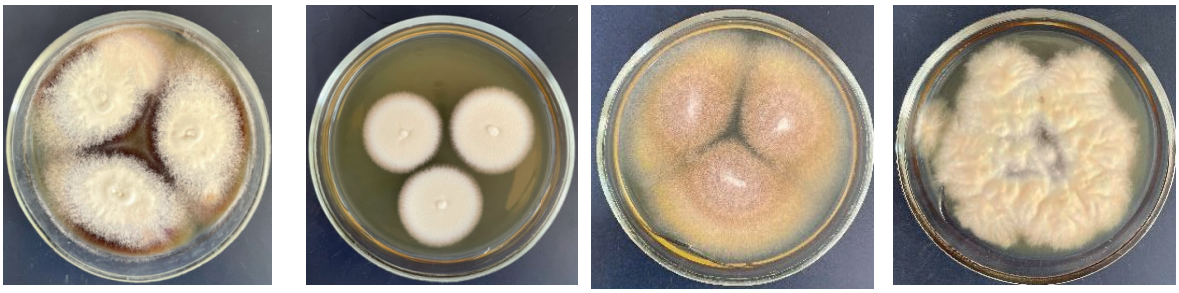
Animal hair provided by veterinary clinics was finely ground using a vibratory mill. The Sabouraud medium was supplemented with 2 g of ground hair per 100 mL of medium and autoclaved at 0.5 atm for 15 min. The prepared hair-containing medium was then poured into Petri dishes.

Keratin hydrolysate was obtained by mixing animal hair with 10% NaOH until the hair was fully dissolved. The resulting mixture was added to the Sabouraud medium and autoclaved at 0.5 atm for 15 min. The prepared keratin hydrolysate-containing medium was then poured into Petri dishes.

Inoculation was performed under sterile conditions in a biological safety cabinet. The cultures were incubated in a thermostat at 27 °C until colonies were formed.

Results

The studies were conducted with isolates that had previously been isolated from small domestic animals, with an erased etiology, to confirm their pathogenicity, a keratinolytic test was performed with chopped wool, keratin hydrolysate and hair perforation. In this study, strains of *Trichophyton benhamiae* and *M. canis* were used. (Figure 1).



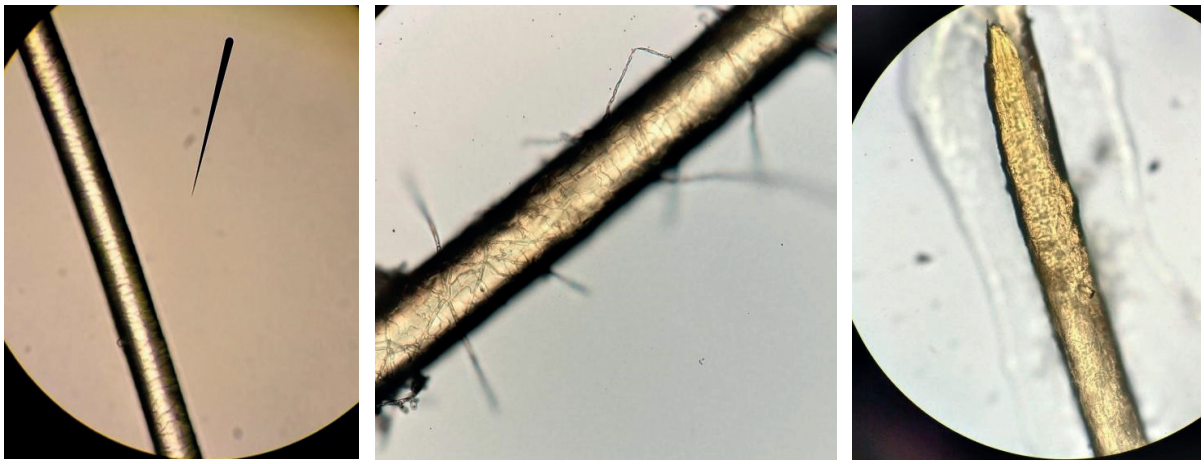
a b c d

Figure 1 – Pure cultures of *Trichophyton benhamiae* (a) No. 19 and (b) No. 20 and *Microsporum canis* (c) No. 35 and (d) No. 68

Figure 1 shows the colonies of pure cultures of *T. benhamiae* No. 19 and No. 20 and *M. canis* No. 35 and No. 68 in classical Sabouraud medium. Colony formation on this medium occurs in 18–30 days for *T. benhamiae* and *M. canis*.

To evaluate the pathogenicity of the strains, we conducted three tests for keratinolytic activity: a hair perforation test, addition of ground hair to the medium, and medium supplemented with keratin hydrolysate. The experimental results are summarized as follows.

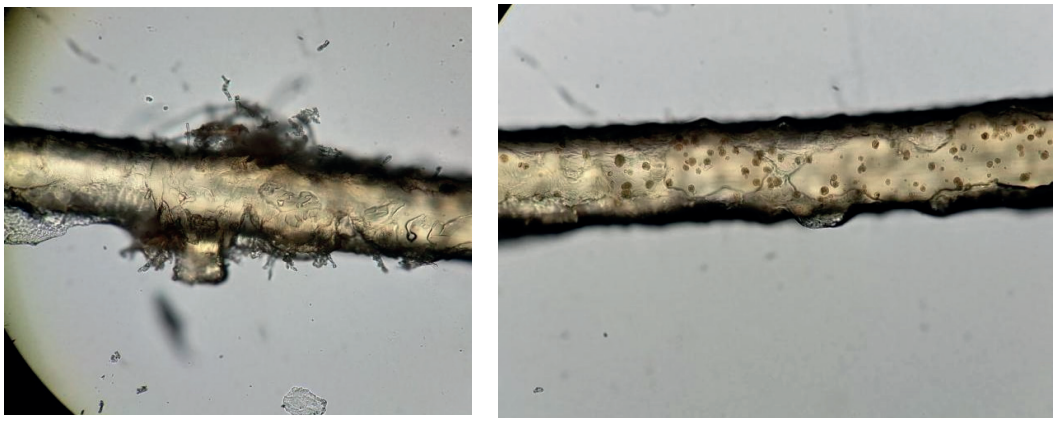
Both strains of *T. benhamiae* exhibited pronounced keratinolytic properties during the classic hair perforation test, as evidenced by abundant growth on the surface of the hair and the appearance of noticeable “pegs” or erosion on the surface of the hair (Figure 2).



Control Strain No.19 Strain No. 20

Figure 2 – Hair destruction under the action of *T. benhamiae* enzymes

Comparable results were obtained for hair infected with *M. canis.* The strains of *M. canis* exhibited pronounced keratinolytic properties during the hair perforation test, with more evident damage to the hair compared with that in the experiment with *T. benhamiae* (Figure 3).

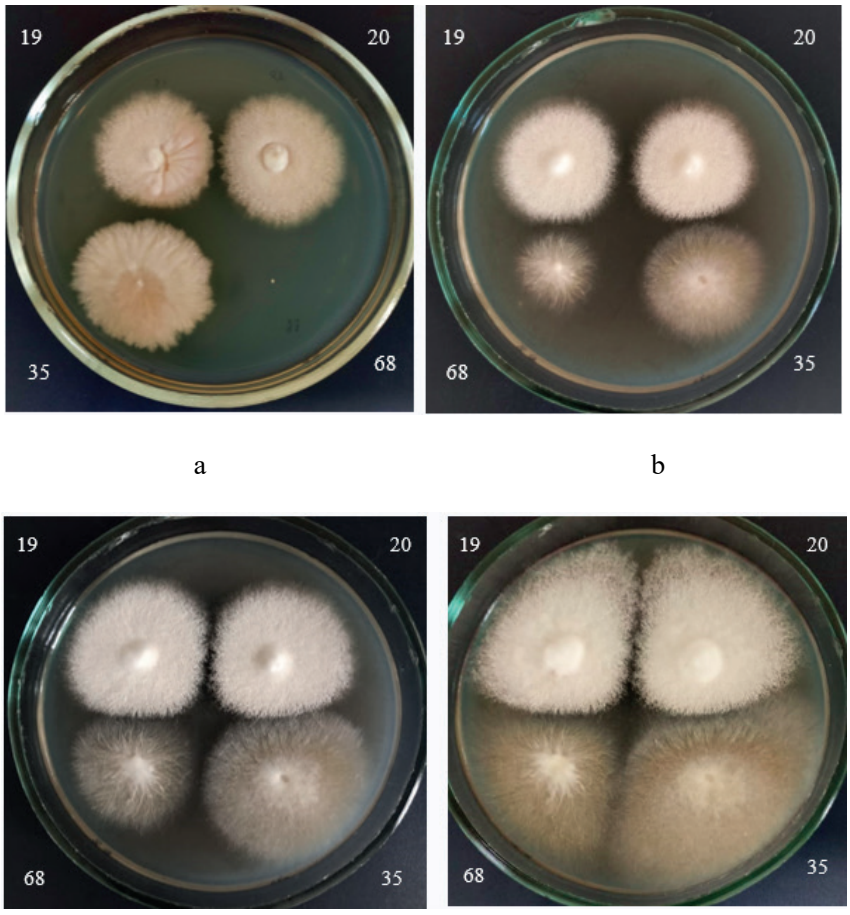


Strain No. 35 Control

Figure 3 – Hair damage under the action of keratinolytic enzymes of *M. canis*

To further identify the keratinolytic properties of dermatophyte cultures, we prepared Sabouraud media with the addition of keratin hydrolysate from cat hair and ground cat hair.

We observed more pronounced species differences in the manifestation of cultural–morphological characteristics in fungal strains grown on media with keratin. The surface of the colonies on control medium was more delicately structured and velvety, whereas that on medium supplemented with keratin was more granular and dense (Figure 4).



a b

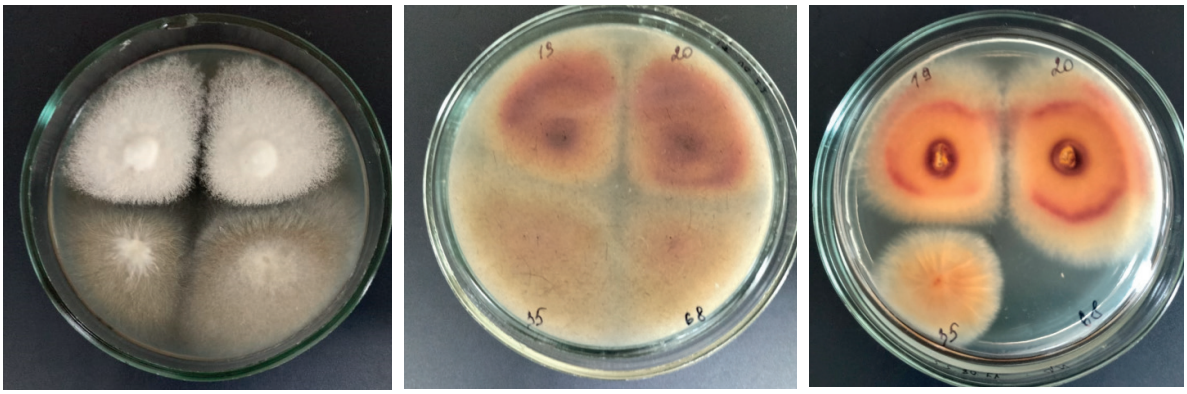
c d

a – control, 8th day; b – colonies, 8th day; c – colonies, 10th day; d – colonies, 14th day.

Figure 4 – Growth of dermatophyte colonies on Sabouraud medium with added keratin hydrolysate

As seen in Figure 4, on media with cat keratin hydrolysate (b–d), the fungal strains accumulated biomass more quickly, formed aerial and substrate mycelium and spores more actively, and exhibited different colony colors and structures compared with those on the control medium without keratin hydrolysate (a), with this effect being particularly pronounced in *T. benhamiae.*

The growth of dermatophyte cultures on Sabouraud media using the method with ground cat hair also indicated that the addition of hair affected the manifestation of cultural properties in fungal colonies (Figure 5).



a b c

a) front; b) back; c) control

Figure 5 – Characteristics of growth of *T. benhamiae* and *M. canis* strains on modified media with added ground hair

As seen in Figure 5, on the underside of colonies grown on media with chopped hair (b), there is more pronounced pigmentation on the reverse side, with zonal pigment accumulation and higher pigment intensity, especially in *T. benhamiae* strains.

Additionally, upon growth and colony formation, a more distinct zone of transparency around each strain was observed, which manifested as a more intense background color in the medium. This phenomenon is believed to be related to the thinning and destruction of hair keratin within the substrate by the keratinolytic enzymes of the fungi, which alters the direction of light dispersion. The presence of hair particles reduces the transparency of the nutrient medium, resulting in light refraction. The absence or thinning of hair particles allows light to partially pass through the boundary of the medium, thereby changing the direction of light dispersion. If the medium is transparent, the image is visually reflected in a more intense shade of the background.

Thus, we identified the keratinolytic activity of the *T. benhamiae* and *M. canis* fungal strains in relation to domestic animal hair. The pronounced keratinolytic properties of these strains provide evidence of their etiological roles in the development of skin pathology and its derivatives in domestic cats and dogs.

Discussion and Conclusion

The method of diagnosing dermatophytes by inoculating clinical material on the Sabouraud medium with keratin hydrolysate and finely chopped hair allows for a reduction in the time required to isolate and identify dermatophyte fungal cultures. Moreover, pleomorphic changes in cultures were not observed when using the proposed media.

In studying the keratinolytic properties of dermatophytes, characteristic hair damage was identified, confirmed by the research of Čmoková, A. (2020), who demonstrated that *T. benhamiae* exhibits pronounced enzymatic activity in the breakdown of hair keratin, indicating that it belongs to the *T. mentagrophytes* complex. The *M.canis* strains exhibited strong keratinolytic properties, with hair damage being more pronounced in the perforation test compared with the experiment with *T. benhamiae.* These results complement the findings of Čmoková A. (2020) and Ajello L. (1967), who described hair perforation and peg formation under the action of keratinase in *T. mentagrophytes* and *M. canis* [23, 24].

According to Li Q. (2021), complete keratin biodegradation requires an appropriate microorganism strain, properly optimized cultivation conditions, and a keratinase degradation system that includes a mixture of enzymes. Considering the influence of easily accessible carbon and energy sources, such as glucose and xylose, on the overall keratinolytic process, including proteolysis, sulfitolysis, and keratinolytic attack, the addition of glucose primarily stimulated protease and keratinase activities in the cultures of the three strains of *Trichophyton ajelloi* [25].

In addition to classic dermatophyte fungi, saprophytic fungi are used in studies aimed at identifying keratinase enzymes. For example, *S. Timorshina* et al. (2022) evaluated 32 strains of micromycetes belonging to the genera *Aspergillus, Chaetomium, Cladosporium, Paecilomyces, Penicillium,* and *Ulocladium* using three agarized media with various protein substrates: keratin, casein, and gelatin. On the basis of the study results, the following fungi of the genus Aspergillus demonstrated effective keratin hydrolysis: *Aspergillus amstelodami, Aspergillus chevalieri, Aspergillus clavatus, Aspergillus fischeri, Aspergillus ochraceus, Aspergillus sydowii,* and *Cladosporium sphaerospermum* [26].

Among various fungi exhibiting keratinolytic abilities, there also mentioned dermatophytes and related fungi from the Chrysosporium group [27]. In addition to these fungi, some authors [28] report that keratinolytic abilities are also manifested by ubiquitous mold fungi such as *Fusarium sp.* Among dermatophytes, anthropophilic species such as *Trichophyton rubrum,* zoophilic species such as *Trichophyton verrucosum,* and geophilic, including species such as *Trichophyton terrestre, T. georgie, T. ajelloi, Microsporum gypseum* and *M. fulvum* are distinguished [29].

Authors Yu R.J. et al. (1968) conducted an experiment on the isolation and purification of extracellular keratinase of *T. mentagrophytes*. In their research, they used horse hair and guinea pig hair. Cultivation of *T. mentagrophytes* was carried out in a keratin medium consisting of horsehair and a separate medium of guinea pig hair. According to the results of the experiment, keratinase enzyme were obtained using chromatography with a molecular weight of approximately 48,000 from horse hair. An experiment with guinea pig hair gave a negative result [30]

Giudice M.C. et al. (2012) conducted studies to evaluate the activity of extracellular proteolytic enzymes keratinase and elastase in the geophilic fungus *Microsporum gypseum* isolated from various soils in Brazil. According to the results of the experiment, the geophilic fungus *M. gypseum* showed low enzymatic activity, compared with isolates obtained from human and veterinary sources [31].

In addition to fungi, in industry uses microbial hydrolysis. Thus, in the publication R. Bhari et al. (2021), microorganisms of microbial origin were used for keratin hydrolysis. The bioconversion of these dreaded wastes into value-added protein hydrolysate using keratinolytic microbes is an effective way to recycle and manage hard-to-process keratin waste. There is a complete catalog of keratinolytic microbes (*Bacillus* sp. *Amycolatopsis* sp. и *Streptomyces* sp.), known to dissolve feather protein in a short time under mild conditions, and their number is constantly increasing [32].

Currently, pet owners turn to veterinary clinics, who treat the first signs of dermatomycosis pathogens, which subsequently leads to a latent form of the disease. Our research is aimed at allowing veterinarians and mycological laboratory workers to use keratin media to test pathogenicity, which will help in making a diagnosis and prescribing timely treatment.

Thus, we have identified keratinolytic activity in the fungal strains *T. benhamiae*: No. 19 and No. 20; and *M. canis:* No. 35 and No. 68 in relation to the hair of small domestic animals, and also conducted a hair perforation test, where hair destruction was visible, which indicates the pathogenicity of the fungal strains. The use of media containing ground animal hair and keratin hydrolysate allows for determining the pathogenicity of dermatophyte pathogens, and it has been proven that the fungi do not exhibit pleomorphism. This factor enables accurate identification of the pathogen and contributes to the correct diagnosis. The presence of pronounced keratinolytic properties in these fungal strains is evidence of their etiological role in the development of skin pathology and its derivatives, as identified in domestic cats and dogs.

Authors' Contributions

AS and TI: Concept development, design and planning of the study, data collection and analysis, critical review of the article and final approval, research, statistical analysis. TI: Conducted the final revision and proof reading of the manuscript. AS and TI: Conducted a comprehensive literature search

and conducting research. All the authors have read, reviewed and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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