Diagnostic Potential of Immunoblot Analysis for Identifying Rabbits Infected with *Trichophyton mentagrophytes*

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**Summary**

The aim of this study was to investigate the diagnostic potential of immunoblot analysis for detecting rabbits naturally infected with *Trichophyton mentagrophytes*. A protein extract of the dermatophyte *T. mentagrophytes* was prepared from a fungal mat grown in liquid medium, where the production of keratinases was stimulated by keratin as a nitrogen source. The proteins derived from disrupted *T. mentagrophytes* were separated by SDS-PAGE and visualized by Western blotting. Twenty-one rabbits infected with *T. mentagrophytes* and 10 non-dermatophyte exposed rabbits were studied. Eight dominant bands were identified in immunoblots from dermatophyte-infected rabbits, with apparent molecular weights ranging from 20 to 75 kDa. The diagnostic potential of the bands for identifying infected rabbits was evaluated by determining sensitivity, specificity, positive and negative predictive values, and overall accuracy. Bands at 20, 30 and 33 kDa were observed with 100 % specificity and 76.19 % sensitivity and can thus be considered highly accurate in correct identification of the infected animals. Accuracies from 54 to 62 % were found for the other dominant bands. Bands at 42, 45 and 75 kDa with 85.71 to 100 % sensitivity appeared not to be specific for dermatophyte infection. Although the specificity of bands at 26 and 28 kDa was found to be 100 %, their accuracy was only moderate, due to the low level of sensitivity, 9.52 and 23.81 %, respectively.

**Key words**: immunoblotting, diagnostic evaluation, rabbit, *Trichophyton mentagrophytes*

**Introduction**

Trichophytosis in rabbits is a serious health problem for both animals and humans. The economic consequences of the disease, especially in large commercial rabbitries, are considerable (I). Dermatophytoses are superficial infections, which are accompanied by both cellular and humoral immune responses (2–5). To penetrate the hair and keratinous layers of the skin, dermatophytes release keratinolytic enzymes called keratinases, which are thus important in the pathogenesis of dermatophytosis (6,7). Keratinases may stimulate the production of specific antibodies capable of inactivating the enzymes and eliminating the infecting organism (4,8,9).

Increased levels of specific IgG in rabbits with the naturally occurring disease were observed using ELISA (10). High levels of specific antibodies against keratin-induced *T. mentagrophytes* proteins were also detected in rabbits naturally infected with *T. mentagrophytes* (11). Western immunoblotting has been used to
evaluate the humoral immune response in cats naturally infected with *M. canis* (12), and has also been used for rabbits with naturally occurring trichophytosis (10,11). To our knowledge, a diagnostic evaluation of immunoblot analysis for identifying dermatophyte infection has not previously been described. The purpose of this study was to investigate the diagnostic potential of immunoblot analysis by evaluating the humoral immune response to individual antigens in rabbit trichophytosis.

**Material and Methods**

**Preparation of *T. mentagrophytes* antigen**

A protein extract of *T. mentagrophytes* was prepared from a fungal mat grown in a liquid medium, where the production of keratinases was induced by keratin as a nitrogen source. A pure dermatophyte culture of *T. mentagrophytes*, isolated from rabbit, was incubated at 30 °C with shaking (150 rpm) for 21 days. Mycelia were harvested from the medium (dextrose 12 mg/mL, inositol 0.05 mg/mL, thiamine HCl 0.01 mg/mL, pyridoxine 0.01 mg/mL, gentamicin 0.1 mg/mL, chloramphenicol 0.1 mg/mL, purified keratin from hooves and horn 4 mg/mL) by filtration, washed with distilled water, frozen at –20 °C and, after thawing, disrupted by sonication in phosphate buffer saline (PBS). The supernatant containing the protein extract was separated from the crude mass by centrifugation at 3500 rpm for 20 min at 4 °C, followed by ultracentrifugation at 25 000 rpm for 60 min at 4 °C. This supernatant was concentrated using a centrifprep YM-3 (Amicon). Total protein concentration was determined by Bio-Rad Protein Assay (II) (Bio-Rad, USA). The transfer was carried out in a buffer containing Tween 20, 0.05 % containing Tween 20, 2 %. Twenty-one positive sera from rabbits naturally infected with dermatophyte *Trichophyton mentagrophytes* (positive samples) and 10 control sera (negative samples) were tested for the presence of specific antibodies against proteins from the extract of *T. mentagrophytes*. The membrane was incubated for 1.5 h at room temperature in serum diluted with BSA 3 % in PBST in a ratio 1:600. After incubation the membrane was washed in PBST and further incubated for 1.5 h at room temperature with a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma) in BSA 3 % in PBST. After washing, bound complexes were detected by incubating the membrane in a substrate solution of 2.5 mL of 3-amino-9-ethyl-carbazole (AEC) in dimethylformamide at a concentration of 8 mg/mL, diluted in 47.5 mL of H2O2 0.015 % in 50 mM acetate buffer, pH=5.0. When bands became visible, the strips were washed with distilled water, dried and scanned using a Model GS-700 Imaging Densitometer (Bio-Rad).

**SDS-PAGE**

Proteins from fungal extract of *T. mentagrophytes* at a concentration of 750 μg/mL were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) on a 12 % separating polyacrylamide gel and 4 % stacking gel. Antigen (fungal extract of *T. mentagrophytes*) was diluted with SDS reducing buffer in a ratio 1:4 and heated in a boiling water bath for 5 min prior to loading on the gel. The mixture of molecular weight markers was treated in a similar way. A modified method of Laemmli (13) was used in the procedure of the SDS-PAGE. Electrophoresis was performed at constant voltage of 200 V for 45 min.

**Western immunoblotting**

After SDS-PAGE, separated proteins from the extract of *T. mentagrophytes* were blotted to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, USA). The transfer was carried out in a buffer containing 25 mM Tris, 192 mM glycine and 20 % volume fraction of methanol, pH=8.3 at a constant voltage setting of 100 V for 45 min in a cell for electrophoretic transfer. After transfer, a strip with molecular weight markers was stained with Coomassie brilliant blue R-250 (Bio-Rad). Residual binding sites on the remaining membrane were blocked by overnight incubation at 4 °C in PBST (PBS supplemented with Tween 20, 0.05 %) containing Tween 20, 2 %. A100 V for 45 min.

**Results**

**Western immunoblotting**

Typical immunoblots for positive and negative samples, where sera were tested for their ability to develop...
an immunoblot of *T. mentagrophytes* proteins, are shown in Fig. 1. Using positive sera, bands between 20 and 75 kDa were exhibited. Immunogenic bands at 20, 30 and 33 kDa occur when sera from *T. mentagrophytes* infected rabbits were tested, but not from the uninfected. Bands at 42, 45 and 75 kDa are also observed with control samples, although with greatly reduced intensity compared to the samples from the infected rabbits.

**Fig. 1.** Molecular weight markers (standards), and the immunoblot of positive and negative samples. Dominant bands are indicated with (+)

### Diagnostic value of immunoblot analysis

Diagnostic parameters for selected dominant bands observed on immunoblots (bands which were commonly observed and stained heavily on blots and were easily identified as individual bands) are presented in Table 1. Wider bands, which could present double or triple band, were not included in the diagnostic evaluation. The highest accuracy, 88.10%, was observed for bands at 20, 30 and 33 kDa. The specificity of 100% was observed for bands at 26 and 28 kDa, but the sensitivity for these bands was only 9.52 and 23.81%, respectively. On the other hand, high sensitivity, but low specificity was calculated for bands at molecular masses of 40 kDa and higher.

### Discussion

The extraction of different antigenic materials from dermatophytes to investigate the humoral immune response after dermatophyte infection in humans (3,16,17), cats (2,4,5,12) and dogs (18) has been described. In the current study we used an antigen prepared from keratin rich media, where the production of keratinolytic enzymes important in the pathogenesis of dermatophytosis was induced (6,7).

The immunogenic proteins of *T. mentagrophytes* were investigated by Garg and Müller (19) using hyperimmune antisera raised in rabbits but samples from naturally infected rabbits were not included. In our previous studies, the humoral immune response in rabbits with naturally occurring dermatophytosis was reported (10,11). In the present work we have evaluated the diagnostic potential of bands on immunoblots for identifying rabbits infected with *T. mentagrophytes*.

Bands at 20, 30 and 33 kDa were observed with 100% specificity and 76.19% sensitivity, and can thus be considered highly accurate (88.10%) in correct identification of the affected animals. Similar accuracy (89%) was reported for ELISA using the same antigen (11). The predictive values of 90% of positive and negative results were obtained, confirming the value of this method for use in detecting specific antibodies in infected rabbits’ sera (11). However, in contrast to ELISA, immunoblotting enables identification of individual epitopes, enabling epitopes important in provoking host immune response to be identified.

Accuracy ranging from 54 to 62% was found for other dominant bands. Bands at 42, 45 and 75 kDa with 85.71 or 100% sensitivity appeared not to be specific for dermatophyte infection. They also occurred in control samples, although at greatly reduced intensity. Sparkes et al. (12), who investigated the humoral immune response in cats naturally infected with *M. canis* using immunoblot analysis, were not able to find a 100% specific marker of the infection. Although the specificity of bands at 26 and 28 kDa is 100% in our study, their accuracy was only moderate, due to the low level of sensitivity, found to be 9.52 and 23.81%, respectively.

<table>
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<th>Bands/kDa</th>
<th>sensitivity</th>
<th>specificity</th>
<th>accuracy</th>
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<th>negative predictive value</th>
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Conclusions

Immunoblot analysis for the detection of rabbits infected with *T. mentagrophytes* has an encouraging diagnostic potential. The identification of immunogenic proteins by immunoblot analysis may contribute to earlier and more convenient diagnosis of dermatophytosis than standard methods. It could also be valuable in identifying epitopes important in the humoral immune response against dermatophyte infection.

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References