

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 (1). 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 derma-

tophytosis (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

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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 centriprep YM-3 (Amicon). Total protein concentration was determined by Bio-Rad Protein Assay (II) (Bio-Rad, Germany). The protein extract was frozen in aliquots at -20 °C.

Hair and serum samples

Hair and serum samples were collected from 21 rabbits (New Zealand White) naturally infected with dermatophyte *Trichophyton mentagrophytes*. The infection was confirmed by microscopic examination and culture of the hair. Samples from 10 rabbits (New Zealand White) from an uninfected rabbitry, with no evidence of previous clinical history of dermatophytosis, served as controls. Blood samples were clotted and centrifuged at 3 000 rpm; sera were separated and stored at -20 °C until assayed.

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 %. 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).

Diagnostic evaluation

Diagnostic parameters were determined for each band observed on immunoblots on the basis of the 2x2 method (14,15). Sera used for detecting immunogenic proteins correlating with *T. mentagrophytes* infection were divided into positive (D+) and negative (D-) samples according to the disease. Results of immunoblot analysis for each band were divided into positive (T+) and negative (T-) on the basis of their presence or absence on the immunoblots. Immunoblot results for each band were divided into four categories: TP: true positive (D+, T+); TN: true negative (D-, T-); FN: false negative (D+, T-); FP: false positive (D-, T+). Sensitivity and specificity were calculated using the 2x2 table. Sensitivity for each band was calculated as the proportion of true positives identified as positive in immunoblot analysis (TP/TP+FN) and specificity as the proportion of true negatives identified as negative in immunoblot analysis (TN/TN+FP). The predictive value of a positive result was calculated as the proportion of animals with a positive immunoblot result that were really positive (TP/TP+FP) and the predictive value of a negative result as the proportion of animals showing a negative immunoblot result that were really negative (TN/TN+FN).

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.

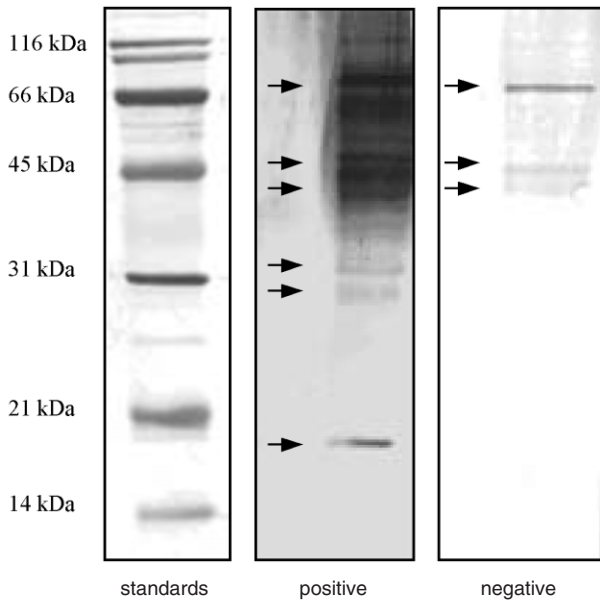


Fig. 1. Molecular weight markers (standards), and the immunoblot of positive and negative samples. Dominant bands are indicated with (→)

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 1. Diagnostic parameters for individual bands on immunoblots

Bands/ kDa	Diagnostic parameters/%				
	sensitivity	specificity	accuracy	positive predictive value	negative predictive value
20	76.19	100.00	88.10	100.00	66.67
26	9.52	100.00	54.76	100.00	34.48
28	23.81	100.00	61.91	100.00	38.46
30	76.19	100.00	88.10	100.00	66.67
33	76.19	100.00	88.10	100.00	66.67
42	85.71	30.00	57.86	72.00	50.00
45	100.00	20.00	60.00	72.41	100.00
75	100.00	10.00	55.00	70.00	100.00

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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Použdanost imunoblot analize za utvrđivanje zaraženosti zečeva s *Trichophyton mentagrophytes*

Sažetak

Svrha je ovoga rada bila istražiti pouzdanost imunoblot analize za utvrđivanje zaraženosti zečeva prirodnim putem sa *T. mentagrophytes*. Pripravljen je proteinski ekstrakt dermatofita *T. mentagrophytes* iz fungalnoga sloja uzgojenog u tekućem mediju u kojem je proizvodnja keratinaze stimulirana dodatkom keratina kao izvora dušika. Proteini dobiveni razgradnjom *T. mentagrophytes* razdvojeni su postupkom SDS-PAGE uz naknadni »Western blotting«. Ispitan je 21 kunić zaražen s *T. mentagrophytes* i uspoređen s 10 nezaraženih kunića. Identificirano je 8 dominantnih vrpca približne molekularne mase od 20 do 75 kDa na imunoblotima od zaraženih kunića. Dijagnostička slika inficiranosti kunića dobivena je pomoću vrpce na osnovi ispitivanja senzitivnosti, specifičnosti, pozitivne i negativne predvidljive vrijednosti i ukupne preciznosti. Vrpce od 20, 30 i 33 kDa utvrđene su sa 100 %-tnom specifičnosti, a senzitivnost je iznosila 76,19 %. Prema tome, mogu se smatrati sigurnim i ispravnim dokazom zaraženosti životinja. Preciznost je ostalih dominantnih vrpca iznosila od 54 do 62 %. Čini se da vrpce sa 42, 45 i 75 kDa, osjetljivosti od 75,71 do 100 %, nisu specifične za utvrđivanje infekcije dermatofitima. Iako su one od 26 i 28 kDa imale specifičnost od 100 %, bile su osrednje precizne jer im je senzitivnost bila samo 9,52 ili 23,81 %.