

## Diagnostic Value of ELISA Tests for the Detection of Specific Antibodies in Cats and Rabbits with Dermatophytosis

Petra Zrimšek<sup>1\*</sup> and Marinka Drobnič-Košorok<sup>2</sup>

<sup>1</sup>Clinic for Reproduction and Horses, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia

<sup>2</sup>Institute of Physiology, Pharmacology and Toxicology, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia

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### Summary

Two indirect ELISA tests developed for the detection of specific IgG in cats and rabbits, infected with *M. canis* and *T. mentagrophytes*, respectively, were evaluated and compared. The levels of specific antibodies were determined in sera of 20 cats and 25 rabbits naturally infected with *M. canis* and *T. mentagrophytes*, respectively. Infection was confirmed by the results of fungal culture. Blood samples from 12 cats and 17 rabbits, previously unexposed to dermatophytes, served as negative controls. A significant increase in the level of specific antibodies in groups of infected animals was demonstrated. Sensitivity, specificity and predictive values of a positive and a negative test were determined to evaluate the diagnostic potential. ELISA for the detection of specific antibodies in cats infected with *M. canis* (ELISA-cats test) exhibited 75.0 % of sensitivity at 91.7 % of specificity, whereas the test for the detection of specific antibodies in rabbits, infected with *T. mentagrophytes* (ELISA-rabbits test) is highly sensitive (96.0 %) and highly specific (94.1 %), confirming its encouraging diagnostic potential. The cross-reactivity of fungal antigens was tested by performing the assays with antigens *M. canis*, *T. mentagrophytes*, *M. pachydermatis* and *A. fumigatus*. There were no significant indications of cross-reactions in the test *T. mentagrophytes*-rabbits, whereas strong cross-reaction between dermatophyte antigens was observed in the test *M. canis*-cats.

*Key words:* ELISA, diagnostic evaluation, rabbit, cat, dermatophytes

### Introduction

Dermatophytosis is an infection of superficial keratinised tissues by a species of fungus, named dermatophytes. Infections in animals are due to the genera *Microsporum* and *Trichophyton* (1–3). In cats, *M. canis* accounts for nearly all occurrences of dermatophyte infection (4). Recently the incidence of *Microsporum canis* infection has been increasing steeply in Slovenia (5,6).

Dermatophytosis in domestic rabbits is usually attributable to infection with *Trichophyton mentagrophytes*. It is a zoonotic hazard and a costly problem, especially in large commercial rabbit hutches (7).

Studies of dermatophytosis in cats and rabbits have demonstrated the development of both cellular and humoral immune responses to the organism (4,8–11).

\* Corresponding author; Phone: +386 14 779 271; Fax: +386 12 832 243; E-mail: petra.zrimsek@vf.uni-lj.si

Although cell-mediated immunity is crucial for recovery from dermatophytosis, antibodies may, nevertheless, have some role in eliminating infection. Some fungal antigens have been regarded as important in the pathogenesis of dermatophytosis. Opsonization and activation of complement, due to specific antibodies induced to the antigens mentioned above, may contribute to inactivation and elimination of the fungal elements (12). Investigation of the immunological response of the host to dermatophyte infection has led to the development of effective vaccines to eradicate bovine dermatophytosis from several countries and may produce a similar benefit in feline and rabbit dermatophytosis (4,13).

Dermatophytosis is usually diagnosed on the basis of its clinical characteristics and on microscopic and cell-culture examination. However, a reliable serological test could contribute to earlier and more convenient diagnosis. To our knowledge, there have been few studies of the diagnostic evaluation of serological tests for dermatophytosis. ELISA tests for detecting specific antibodies in cats infected with *M. canis* and in rabbits infected with *T. mentagrophytes* have been developed (11,14). The aim of this study was to assess the contribution of ELISA tests in order to improve diagnosis of dermatophytosis. To evaluate the diagnostic potential of ELISA tests, their sensitivity, specificity and predictive values were determined using a 2x2 method and ROC (receiver operating characteristic) analysis (15). Sensitivity and specificity of the tests provide measures of their validity and give an indication of the ability of the tests to correctly identify ill and healthy animals, respectively. Predictive values of tests give an indication of the usefulness of the tests in relation to the fungal culture test regarded as a definitive diagnostic test for dermatophytosis (16).

## Material and Methods

### Serum samples

Blood and hair samples were collected from 25 rabbits (New Zealand White) naturally infected with *Trichophyton mentagrophytes* and from 20 cats naturally infected with *Microsporum canis*. The infection was confirmed by microscopic examination and fungal culture of the hair. Samples from 17 rabbits and 12 cats with no 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.

### Enzyme linked immunosorbent assay (ELISA)

The indirect ELISAs developed for detecting specific antidermatophyte IgG in sera of rabbits infected with *T. mentagrophytes* (ELISA-rabbits) (11) and in sera of cats infected with *M. canis* (ELISA-cats) (14) were used. Briefly, microtiter plates (Maxisorp, Nunc, Denmark) were coated with 50 µL of 5 µg/mL antigen of *T. mentagrophytes* (ELISA-rabbits) and 10 µg/mL antigen of *M. canis* (ELISA-cats), respectively, in 0.01 M carbonate-bicarbonate buffer, pH=9.6 and incubated overnight at 4 °C. The plates were washed three times with phosphate buffered saline containing 0.05 % Tween<sub>20</sub> (PBST). Unoccupied sites on

the plates were blocked with 100 µL per well of 3 % non-fat dried milk in PBST (ELISA-rabbits) or 2 % Tween<sub>20</sub> in PBST (ELISA-cats), respectively, at 37 °C for 1.5 h. According to the linear range of dilution curves of ELISA, doubling dilutions of sera in PBST with 2 % BSA were prepared from 1:640 to 1:5120 (ELISA-rabbits) and from 1:80 to 1:640 (ELISA-cats), respectively. After washing the plates, 50 µL of diluted serum samples were added to the duplicate. The same parallel dilutions of positive and negative control sera were included as well as the blank in each assay. The plates were further incubated at 37 °C for 1.5 h and washed as described above. 50 µL per well of diluted conjugate was added. Diluted goat anti-rabbit IgG 1:8000, conjugated to horseradish peroxidase (Sigma) (ELISA-rabbits) and diluted goat anti-cat IgG 1:3000, conjugated to horseradish peroxidase (Jackson Immunoresearch) (ELISA-cats), respectively, were used. After further 1.5 hours of incubation at 37 °C the plates were washed and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma) was added at a concentration of 1 mg/mL, with 0.012 % H<sub>2</sub>O<sub>2</sub> in 0.01M citrate phosphate buffer, pH=4.5. After an optimal time of incubation at 37 °C, which was found to be 45 min for ELISA-rabbits test and 30 min for ELISA-cats test, the reaction was stopped by adding 50 µL 0.2M hydrofluoric acid to each well. Absorbance at 405/490 nm was measured using a microplate reader (Behring EL 311, Microplate Reader). The working serum dilution was selected from the linear range of dilution curves.

### Cross reactivity of fungal antigens

The potential cross-reactivity of fungal antigens was examined. ELISA tests were modified by replacing the coating antigen with testing antigens and the serum samples were measured under optimal test conditions. Antigen of *T. mentagrophytes* and antigen of *M. canis*, as related dermatophytes antigens, were used in ELISA for detecting the specific antibodies in cats and rabbits, respectively. Antigens of *Malassezia pachydermatis* and *Aspergillus fumigatus* were used as unrelated fungal antigens in both tests.

### Statistical analysis

The non-parametric Mann-Whitney U test was used for statistical comparison of ELISA results obtained from the groups of infected and control animals. The statistical comparison of the results from testing the cross-reactivity of fungal antigens in ELISA was performed with the same test (17,18).

### Diagnostic evaluation

Diagnostic parameters of ELISA tests were determined on the basis of the 2x2 method and ROC (receiver operating characteristic) analysis (15).

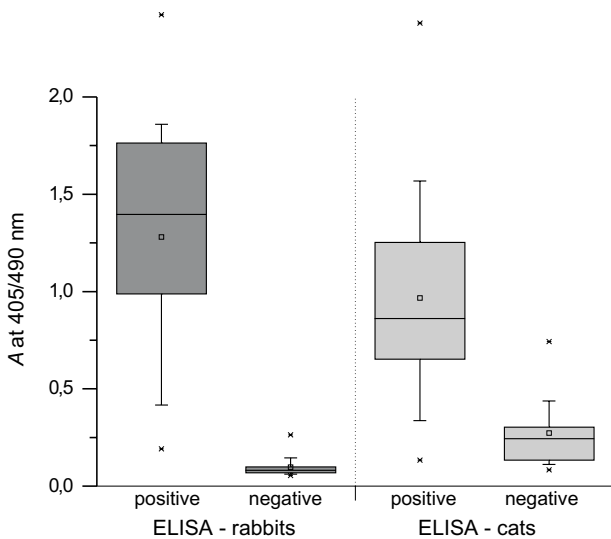
Sensitivity was calculated as the proportion of positive samples according to the disease identified as positive by the test and specificity as the proportion of negative samples according to the disease identified as negative by the test. Diagnostic parameters, mentioned above, were calculated on the basis of the completed 2x2 table, using five different cutoff values. The cutoff val-

ues were calculated as the mean value for the negative group of samples plus 0, 1, 2, 3 and 4 SD, respectively. The relationship between sensitivity and specificity of the test was displayed with ROC curves. The ROC curve plots the true positive rate of sensitivity against the one minus the specificity as a false positive rate of different cut off values. A diagonal line in a plot corresponds to a test that is positive or negative just by chance. Predictive values of a test were determined using optimal sensitivity and specificity, according to the ROC curves. A positive predictive value is the proportion of animals with a positive test result, which are really positive, and negative predictive value is the proportion of animals with negative test result, which are really negative.

**Results**

*Detection of specific antibodies in rabbit and cat sera by ELISA*

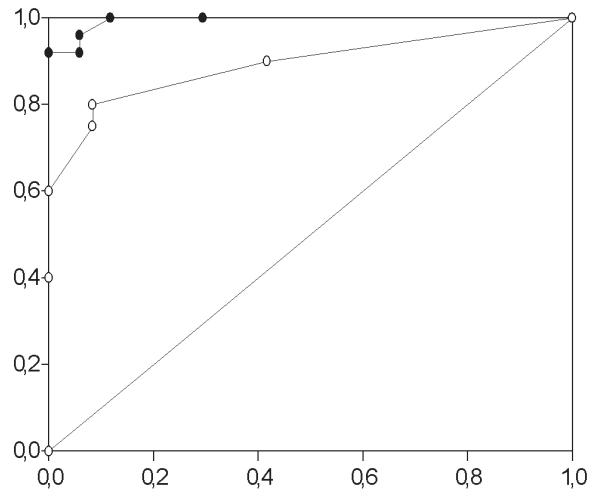
The level of specific antibody was measured in samples from infected and uninfected animals (Fig. 1). Optical density (A at 405/490 nm) represents the level of specific antibodies. The optimal dilution of samples at 1:1280 and 1:320 in ELISA-rabbits and ELISA-cats, respectively, was selected from the linear range of the dilution curves of ELISA. There was a significant difference between the levels of specific IgG in the control group and in infected rabbits ( $P < 0.0001$ ). IgG antibody concentration was also significantly higher in the group of infected cats than in the control group ( $P < 0.0001$ ).



**Fig. 1. Levels of ELISA OD values from infected and control animals.** Box plot shows the median, upper and lower quartiles and the range of values. ELISA-rabbits: blank value =  $0.064 \pm 0.008$ ; positive: *T. mentagrophytes* infected rabbits (N=25); negative: non-infected control rabbits (N=17). ELISA-cats: blank value =  $0.092 \pm 0.004$ ; positive: *M. canis* infected cats (N=20); negative: non-infected control cats (N=12).

*ROC analysis*

The ROC curves were used to compare the diagnostic value of the ELISA tests (Fig. 2). The optimal cutoff values were set as the mean value for the negative group plus two SD for both ELISA tests. At this value the sensitivity was 96.0 % and specificity 94.1% for ELISA-rabbits. Sensitivity and specificity for ELISA-cats were calculated to be 75.0 and 91.7 %, respectively.

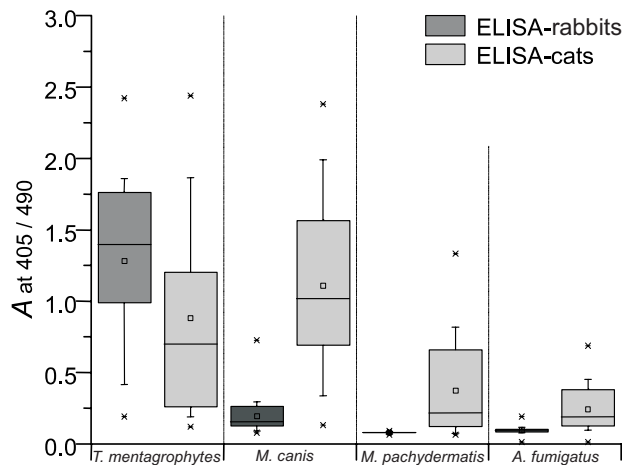


**Fig. 2. Receiver operating characteristic (ROC) curves;** ● – ROC curve for ELISA-rabbits, ○ – ROC curve for ELISA-cats

Predictive values for positive (PV+) and negative (PV-) test were also calculated at optimal cutoff values, and found to be 96.0 and 94.1 % for ELISA-rabbits, respectively and 93.8 and 68.8 % for ELISA-cats, respectively.

*Cross-reactivity of fungal antigens*

The cross-reactivity of fungal antigens in ELISA is shown in Fig. 3. ELISA OD values for rabbit positive



**Fig. 3. Comparison of cross-reactivity between fungal antigens in ELISA-rabbits and ELISA-cats;** Rabbit *T. mentagrophytes* positive sera and cat *M. canis* positive sera were used. Box plot shows the median, upper and lower quartiles and the range of values.

sera, using *T. mentagrophytes* as antigen, differed significantly from the results of the same group tested with any other of the fungal antigens ( $P < 0.0001$ ). A significant difference was also observed between *M. canis* and *M. pachydermatis* ( $P < 0.0001$ ) as well as between *M. canis* and *A. fumigatus* ( $P < 0.0001$ ).

Table 1. Blank values in ELISA tests with different antigen tested

Antigen	Elisa-test	
	rabbits	cats
<i>T. mentagrophytes</i>	0.064 ± 0.008	0.083 ± 0.007
<i>M. canis</i>	0.066 ± 0.004	0.092 ± 0.004
<i>M. pachydermatis</i>	0.069 ± 0.007	0.070 ± 0.005
<i>A. fumigatus</i>	0.071 ± 0.006	0.052 ± 0.008

However, in ELISA-cats no significant difference was observed ( $P = 0.9226$ ) between ELISA OD values for positive cat sera, using antigens from *M. canis* or *T. mentagrophytes*. ELISA results differed significantly between antigens of *M. canis* and *A. fumigatus* ( $P < 0.0001$ ), whereas the difference between *M. canis* and *M. pachydermatis* was slightly lower ( $P = 0.0005$ ).

## Discussion

Several authors have investigated the immune response in cats infected with *M. canis* (4,9,10), but there are only a few reports concerning the immune response against dermatophytes in rabbits. Our previous study was the first to show specific antibodies in rabbits naturally infected with *T. mentagrophytes* (11).

Using ELISA tests we were able to confirm the presence of specific antibodies in the sera of cats naturally infected with *M. canis* and in the sera of rabbits naturally infected with *T. mentagrophytes*. Although these values were significantly higher than values in the control groups, there was a considerable overlap between the two groups in the ELISA-cats test. Similarly, the presence of antibodies was reported in the majority of healthy unexposed human beings (19) and healthy cats (4,9), resulting as a response to antigens common to both dermatophytes and the ubiquitous saprophytic fungi. No such observations were encountered in the ELISA-rabbits test. There are no other studies to which to compare our results. Low antibody values in some individual samples of infected animals may reflect early or localized infections or, in some cases, the positive results from fungal culture could represent a passive carrier status without active infection (9).

Diagnostic evaluation of ELISA tests was performed using completed 2x2 table and ROC curves. The relationship between sensitivity and specificity of ELISA tests was displayed with ROC curves. In both tests the optimal cutoff value was set as the mean value for the negative group plus two SD. 96.0 % sensitivity and 94.1 % specificity of the ELISA-rabbits constitute high validity of the test in relation to fungal culture as the »gold standard« for diagnosis of dermatophytosis. The predic-

tive values of a positive and a negative test were high as well, found to be 96.0 and 94.1 %, respectively. Overlapping in antibody concentration values between the groups of infected and control cats resulted in lower sensitivity and specificity of the ELISA-cats test, calculated as 75.0 and 91.7 %, respectively. The indicated usefulness of the test was therefore lower, resulting in 93.8 % predictive value of a positive test and 68.8 % predictive value of a negative test.

There was evidence for inter-species and inter-genetic cross-reaction among dermatophyte antigens according to Pier *et al.* (10), who investigated experimental immunity to *M. canis* and cross-reaction with some other veterinary important dermatophytes. No significant difference between antigens of *M. canis* and *T. mentagrophytes* was observed in ELISA-cats, whereas the differences between dermatophytes and nondermatophyte antigens were significant, confirming previous studies, revealing as the difference among the patterns of dermatophyte and nondermatophyte antigens (12,20,21). Although there was a relatively high level of antibodies against *M. pachydermatis* in some samples of infected cats, results obtained for the positive group, using *M. canis* as antigen, differed significantly from the results of the same group, tested with antigen of *M. pachydermatis*. The high level of antibodies against *M. pachydermatis* could result from previous infection of the organism. A slightly increased level of antibodies against *M. pachydermatis* was also observed in ELISA as a tool for sero-diagnosis of canine dermatophytosis due to *M. canis* (22). In the ELISA-rabbits no significant cross-reactivity between *T. mentagrophytes* and the other fungal antigens tested was observed.

## Conclusions

An efficient ELISA for detecting specific antibodies in rabbits infected with *T. mentagrophytes* could enable earlier and more convenient diagnosis of dermatophytosis in rabbits as compared to the established diagnostic methods. The high confidence that a positive test result in ELISA for detecting feline anti-dermatophyte antibodies is correct may reflect in a shorter interval from first day of the diagnostic assay to the start of treatment.

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## Dijagnostička važnost ELISA testova pri određivanju specifičnih antitijela u mačkama i kunićima inficiranim dermatofitozom

### Sažetak

Razrađena su dva indirektna ELISA testa za utvrđivanje specifičnih IgG u mačkama i kunićima inficiranim *M. canis* i *T. mentagrophytes*. Testovi su međusobno uspoređeni i procijenjeni. Utvrđena je razina specifičnih antitijela u serumu dvadesetak mačaka i 25 kunića prirodno inficiranih s *M. canis* i *T. mentagrophytes*. Infekcija je potvrđena rezultatima fungalne kulture. Kao negativna kontrola koristili su se uzorci krvi 12 mačaka i 17 kunića koji prethodno nisu bili izloženi dermatofitima. U skupini inficiranih životinja utvrđeno je bitno povećanje razine specifičnih antitijela. Utvrđeni su osjetljivost, specifičnost i očekivane vrijednosti u pozitivnim i negativnim testovima kako bi se procijenio dijagnostički potencijal. ELISA-test (ELISA-cats test) za utvrđivanje specifičnih antitijela u mačaka inficiranih s *M. canis* pokazao je 75,0 % osjetljivosti i 91,7 % specifičnosti, dok je test za utvrđivanje antitijela u kunića inficiranih s *T. mentagrophytes* (ELISA-rabbits test) bio vrlo osjetljiv (96 %) i jako specifičan (94,1 %), potvrđujući time njegov znatan dijagnostički potencijal. Unakrsna reaktivnost fungalnih antigena ispitana je provodeći pokuse s antigenima *M. canis*, *T. mentagrophytes*, *M. pachydermatis* i *A. fumigatus*. U testu *T. mentagrophytes*-rabbits nije opažena značajna unakrsna reakcija dok je u testu *M. canis*-cats došlo do snažne unakrsne reakcije između antigena dermatofita.