Development of an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of ringworm infection in cattle

Elena Tatiana Băguţă,¹,² Ludivine Cambier,³ Marie-Pierre Heinen,³ Vasile Cozma,⁴ Michel Monod,⁵ Bernard Mignon⁶#

Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Cluj-Napoca, Romania; Veterinary Mycology, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium; Dermatology and Venereology Department, Laboratory of Mycology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

Correspondent footnote

#Address correspondence to Bernard Mignon: bmignon@ulg.ac.be
ABSTRACT
The aim of this study was to develop an in-house ELISA for the serological diagnosis of ringworm infection in cattle. We used available recombinant forms of *Trichophyton rubrum* dipeptidyl peptidase V (TruDppV) and leucin aminopeptidase 2 (TruLap2), which are 98% identical to *Trichophyton verrucosum* orthologues. Field serum samples from 135 cattle with ringworm infection as confirmed by direct microscopy, fluorescence microscopy and PCR, and from 55 cattle without any apparent skin lesions or history of ringworm that served as negative controls were used. Sensitivity, specificity, and positive and negative predictive values were determined to evaluate the diagnostic value. Overall, the ELISAs based on recombinant TruDppV and TruLap2 discriminated well between infected animals and healthy controls. A highly significant difference (*P* < 0.0001, Mann-Whitney U test) was noted between optical density values obtained when testing sera from infected versus control cattle. The ELISA developed for the detection of specific antibodies against DppV gave 89.6% sensitivity, 92.7% specificity, 96.8% positive predictive value and 78.4% negative predictive value. The recombinant TruLap2-based ELISA displayed 88.1% sensitivity, 90.9% specificity, 95.9% positive predictive value and 75.7% negative predictive value. To the best of our knowledge this is the first ELISA based on recombinant antigens assessing the immune response to ringworm in cattle, being particularly suitable for epidemiological studies and also for the evaluation of vaccines and/or vaccination procedures.

INTRODUCTION
The zoophilic dermatophyte *Trichophyton verrucosum* is the most common agent of dermatophytosis (commonly known as ringworm) in cattle, which represents its natural reservoir (1). Although *T. verrucosum* has been reported to be one of the most important morbidity factors...
in calves, there have also been reports of infections in sheep, goats and horses (2). Ringworm in cattle has received particular attention due not only to its contagiousness among animal communities, but also to its zoonotic transmission to humans (3).

At present, there are few studies devoted to assessing the immune response in ringworm of cattle. Both antibody- and cell-mediated immune responses have been found in cattle after experimental infection (4) or vaccination (5, 6). Although one study in experimentally infected calves indicated that a combination of cell-mediated and humoral immune responses is associated with *T. verrucosum* immunity and clearance of the infection (4), the anti-dermatophyte antibody response is commonly considered not to be protective (7–9). In another study, vaccinated cows developed immunity which was not transferred to their progeny (10). The production of specific antibodies has also been detected in domestic carnivores (11–14), guinea pigs (15), rabbits (16) and humans (17, 18), and their potential use for monitoring dermatophyte infections has been demonstrated. The development of an antibody response as a consequence of dermatophyte infection offers the possibility of using serological diagnosis as a screening method for detection of the infection. To our knowledge, several enzyme-linked immunosorbent assays (ELISA) have been developed for the evaluation of antibody responses in animal dermatophytosis, but only a few focused on detecting specific antibodies in cattle infected with *T. verrucosum*. For these reasons, the aim of this study was to develop an in-house ELISA based on two available *Trichophyton rubrum* (Tru) recombinant antigens consisting of secreted exopeptidases, dipeptidyl peptidase V (DppV) and leucin aminopeptidase 2 (Lap2), for serological diagnosis of ringworm infection in cattle.

**MATERIALS AND METHODS**

**Animals and sera**
Between January and April 2010, 135 beef and dairy cattle with suspected ringworm from intensive breeding system in Romania, ranging in age from 3 months to 3 years, underwent clinical examination. To confirm infection, samples consisting of scales and hairs were collected by skin scraping. A part of each sample was analyzed using lactophenol followed by direct microscopic examination, as well as with 20% KOH associated with Calcofluor White (Fluorescent Brightener 28, F3543; Sigma Aldrich, St Louis, MO, USA) followed by fluorescence microscopy (19). The remainder was tested in parallel by PCR using primers reported in the literature as specific for fungi belonging to the genus *Trichophyton* (20), i.e. Trich302for 5’ TTG CTA AAC GCT CAG ACT GAC AGC 3’ and Trich302rev 5’ CGG AAG GAT CAT TAA CGC GCA GGC C 3’ (Invitrogen Life Technologies, Carlsbad, CA, USA). Evidence of fungal infection was found in all 135 samples according to all the methods employed.

Under the national program for surveillance, control and eradication of animal diseases in Romania, blood was collected by jugular vein puncture. The serum was separated by centrifugation and stored at –20°C until assayed. The 135 sera from cattle with confirmed ringworm were further referred to as “positive sera” (group A, \( n = 135 \)). The control “negative sera” (group B, \( n = 55 \)) consisted of 55 sera collected from beef and dairy cattle from intensive breeding system in Belgium with ages ranging from 3 months to 3 years, without any apparent skin lesions or history of ringworm. These animals were examined by a veterinarian and considered clinically healthy.

All activities have been performed according to European Welfare Legislation (Directive 2010/63/EU).

**Antigens**
Although several secreted proteases from dermatophytes have been obtained as recombinant proteins, currently no *T. verrucosum* secreted proteases are available in recombinant form. In spite of their different ecologies, the genome of *T. rubrum* (21) proved to be more closely related to those of *T. verrucosum* and *Arthroderma benhamiae*, two phylogenetically closely related dermatophytes inducing highly inflammatory cutaneous infections in humans (22, 23). In addition, most *Trichophyton* species reveal similar secreted protein profiles (24). For these reasons, recombinant TruDppV and TruLap2, which have already been characterized (25), were selected for use as coating antigens in this assay.

The *T. rubrum* antigens were produced as recombinant proteins using the *Pichia pastoris* expression system with the GS115 strain (25). Briefly, the culture supernatant was separated from the cells by centrifugation (10 min at 1500 × g), concentrated by ultrafiltration using an Amicon cell (Millipore, Billerica, MA, USA) and a filtration membrane with a size threshold of 30 kDa, and then dialyzed against PBS. All procedures were carried out at 4°C. The protein concentrations were determined using the Bradford assay (26). The clear supernatants were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and the presence of each recombinant protein was confirmed by visualization as a single individual protein band. The *P. pastoris* supernatants were stored at −20°C until use.

The non-transformed *P. pastoris* strain GS115, which did not secrete any DppV and Lap2 activities into the culture medium, was grown under identical conditions and the culture supernatant, prepared as described above, was used as a control.

**ELISA assays**

Initially, checkboard titrations were performed to optimize the concentrations of the coating antigens, primary antibody and conjugate required (data not shown). Polystyrene microtiter 96-
well plates (Microlon 600, Greiner Bio-one, Kremsmuenster, Austria) were coated with 100 μl per well of 10 μg/ml antigen solution in phosphate buffered saline (PBS, pH 7.2) as a coating buffer and incubated for 1 h at 37°C. Odd rows were sensitized with the antigen while even ones were coated with *P. pastoris* GS115 supernatant as control wells. After washing with PBS, unoccupied protein-binding sites were blocked by the addition of 200 μl per well of a 3.6% solution of casein hydrolysate (Merck, Whitehouse Station, NJ, USA) in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at 37°C. According to the linear range of dilution curves of ELISA, triplicate serum samples were diluted 1:500 in PBS-T and 50 μl of each were added for 1 h at 37°C to both the antigen-coated and control wells. After washing with PBS-T, 50 μl of peroxidase-conjugated rabbit anti-cow immunoglobulins G (IgG) (Polyclonal Anti-Cow Immunoglobulins/HRP, Dako, Glostrup, Denmark), diluted 1:1000, were added to each well. After a further 1-h incubation at 37°C and 3 subsequent washes with PBS-T, peroxidase activity was revealed by addition of 100 μl of a solution containing tetramethylbenzidine and hydrogen peroxide. The reaction was stopped after 5 min by adding 100 μl of 1 N phosphoric acid and the absorbance at 450 nm was measured directly with a Multiscan RC spectrophotometer (Thermo Labsystems, Vantaa, Finland). On each ELISA plate, a positive reference consisting of serum from one 6-month-old calf with extensive ringworm lesions, two negative references consisting of serum from one 6-month-old calf without evidence or history of ringworm and fetal calf serum (Gibco, Life Technologies, Carlsbad, CA, USA) diluted 1:500, and wells without added sera as reaction blanks were processed alongside the samples in triplicate.

**Assay evaluation**

Optical density (OD) was defined as the difference between the mean absorbance for each triplicate serum tested and the control wells. The cutoff was obtained as mean OD + 2× SD
(standard deviation) of sera from group B. All sera (from groups A and B) were classified as
TP = true positive (sera from group A that presented OD values above the cutoff in the
serological test); FP = false positive (sera from group B that presented OD values above the
cutoff in the serological test); TN = true negative (sera from group B that presented OD values
below the cutoff in the serological test) and FN = false negative (sera from group A that
presented OD values below the cutoff in the serological test). Sensitivity, specificity, positive and
negative predictive values were obtained using the following formulas: sensitivity = TP/(TP +
FN); specificity = TN/(TN + FP); positive predictive value = TP/(TP + FP); negative predictive
value = TN/(TN + FN).

The Mann-Whitney nonparametric U test was used for statistical comparison of OD results
obtained from sera from infected animals versus the control group, with significance defined as
$P < 0.0001$.

**RESULTS**

In this study we developed an in-house ELISA for serodiagnosis of ringworm infection in cattle
based on the available recombinant peptidases TruDppV and TruLap2 produced using the
*P. pastoris* expression system, because BLAST analysis revealed that both TruDppV and
TruLap2 are 98% identical and 99% similar to *T. verrucosum* orthologues (27). DppV was used
because *Aspergillus fumigatus* DppV has been identified as a major antigen with great potential
in the serodiagnosis of aspergillosis (28, 29). Lap2 was also tested because it is the major
peptidase secreted by various dermatophyte species during *in vitro* growth in the presence of
keratin (25), the principal component of host structures preferentially colonized by
dermatophytes, i.e. *stratum corneum*, nails, claws or hair. The level of specific antibodies was
measured in field serum samples from cattle with confirmed ringworm (group A, $n=135$) and
healthy control animals (group B, \( n = 55 \)) (Fig. 1). A highly significant difference (\( P < 0.0001 \), Mann-Whitney U test) was noted between the group A and group B OD values.

In total, 190 sera samples were first assessed by recombinant TruDppV-based ELISA employing the protocol described above. The sera from group B assessed using recombinant TruDppV gave OD values ranging from 0.023 to 0.413. The cutoff value, determined as mean OD + 2\( \times \) SD of these sera was fixed at 0.353. The mean OD of sera from group B was 0.173 \( \pm \) 0.089. Of the sera from group B, 51/55 (92.7\%) had OD values below the cutoff value (Fig. 1), indicating the absence of specific antibodies in the vast majority of the healthy animals. Among cattle from group A, 121/135 (89.6\%) had OD values above the cutoff point (Fig. 1), indicating detection of specific antibodies against DppV in individuals in which ringworm infection had been confirmed.

The mean OD of sera from group A was 0.791 \( \pm \) 0.32. A cutoff value of 0.353 gave 89.6\% sensitivity, 92.7\% specificity, 96.8\% positive predictive value and 78.4\% negative predictive value. It is well known that higher cutoff values increase the specificity of the test but reduce its diagnostic sensitivity and might lead to false negative results. As a compromise, a cutoff value of 0.442, determined as mean OD + 3\( \times \) SD, for the recombinant TruDppV-based ELISA displayed 86.6\% sensitivity, 100\% specificity, 100\% positive predictive value and 75.3\% negative predictive value. Thus, the sensitivity and negative predictive values of the test were reduced while the specificity and positive predictive values were maximized.

In parallel, the sera from both group A and B were assessed by the recombinant TruLap2-based ELISA. The sera from group B showed OD values ranging from 0.08 to 0.433. The mean OD + 2\( \times \) SD for these sera was 0.327, which represented the cutoff value. Among the cattle from group B, 50/55 (90.9\%) had OD values below the cutoff point (Fig. 1) indicating the absence of specific antibodies in most non-infected animals. The mean OD of these sera was 0.157 \( \pm \) 0.085.

Of the naturally infected group A cattle, 119/135 (88.1\%) had OD values above the cutoff point
(Fig. 1), confirming the presence of specific antibodies against Lap2 in the sera of most cattle naturally infected with ringworm. The mean OD value was 0.994 ± 0.511. Both the 14 FN and 4 FP sera obtained when using recombinant TruDppV were among the 16 FN and 5 FP sera identified when using recombinant TruLap2. A cutoff value of 0.327 displayed 88.1% sensitivity, 90.9% specificity, 95.9% positive predictive value and 75.7% negative predictive value. By increasing the cutoff value to 0.412 (as the mean OD + 3× SD of sera from group B) the specificity and positive predictive value of the test were improved to 96.3% and 98.3%, respectively, and the sensitivity was slightly reduced to 87.4%, while the negative predictive value remained unchanged.

To rule out laboratory errors, sera classified as either FP or FN were retested under the same conditions and confirmed to yield the same results.

**DISCUSSION**

Assessment of dermatophyte antibodies is routinely performed by ELISA, especially when large numbers of samples need to be examined. The *in vitro* production of recombinant antigens is an inexpensive and fast method to obtain proteins of high and constant quality, bypassing the time consuming *in vitro* culture and further purification steps necessary to achieve sufficient native antigens. Using an in-house ELISA based on the *T. rubrum* recombinant exoproteases DppV and Lap2, we confirmed the presence of specific antibodies in the sera of cattle naturally infected with ringworm. The test discriminated well between infected animals and healthy controls, with good intrinsic validity when using both recombinant TruDppV (89.6% sensitivity and 92.7% specificity) and recombinant TruLap2 (88.1% sensitivity and 90.9% specificity). Serological reactivity in the non-infected control group B was observed only in a minority of animals, most seropositive for both TruDppV and TruLap2. This could reflect a previous non-reported
asymptomatic infection with *T. verrucosum* or other dermatophyte species, as there is evidence for inter-species and inter-generic cross-reactions (30). Exposure to environmental non-dermatophyte fungal antigens could also be the source of the cross-reactions, as cattle stables are environments with high levels of exposure to such antigens. Cross-reactions with other mucosal and/or skin fungal commensals such as *Candida albicans* and *Malassezia pachydermatis* could also not be totally excluded, even if these have been reported neither in cattle nor in other animal dermatophytoses (11).

In spite of confirmed infection, several sera from group A had levels of antibodies below the cutoff point. A window exists between the onset of infection and seroconversion during dermatophytosis. Therefore, sampling of cattle with clinical lesions but serologically negative could have occurred, especially because IgM, the first Igs to appear in response to initial fungal exposure, could not be detected with the in-house ELISA developed herein. With regard to the cattle in group A for which the ELISA results were positive, it is not clear if antibodies were produced before or after the onset of clinical lesions. In calves experimentally infected with *T. verrucosum*, specific antibodies appeared between days 33 and 55 post-infection (PI) (4).

During *M. canis* experimental infection, levels of specific IgG were detectable at different intervals after the lesions appeared, either at 14-21 days PI in dogs (11) or at 14 days PI in guinea pigs (30, 15). In *M. canis*-infected cats, specific IgG levels were high in some animals when the first lesions appeared and 4 weeks later in others (31).

At the moment, ELISA assay has not yet been established for the serological diagnosis of ringworm infection in cattle. Conversely, serological assays for the detection of some invasive fungal infections are commercially available. Several ELISA kits have been developed for the detection of both circulating antibodies and antigens for the diagnosis of fungal infections caused by *Candida* and *Aspergillus*, major pathogens in immunocompromised individuals. The tests
developed for the detection of mannan or galactomannan antigens revealed good specificity but variable sensitivity (32). Most data are available for the detection of *Aspergillus* galactomannan antigen using Platelia ELISA format. Although, several studies indicated that the use of this biomarker has good specificity for the diagnosis of invasive aspergillosis, sensitivity is variable ranging from 17% to 100% (33, 34).

The results obtained in the present study constitute the basic support to postulate that the two *T. rubrum* exoproteases used as antigens are exposed to the immune system of the host during ringworm infection. The dermatophyte-secreted exoproteases (Dpps and Laps) interfere with compact keratinized tissue degradation after initial sulphitolysis (35) and endoprotease (subtilisins and fungalysins) digestion (36). So far the expression of genes encoding the dermatophyte secreted exoproteases DppV and Lap2 has only been shown *in vitro* in the presence of keratin (37). The general basis of the pathogenicity of dermatophytes is still far from being elucidated, but it is likely that DppV and Lap2 are secreted by dermatophytes at a late stage of infection during keratin degradation.

To the best of our knowledge this is the first ELISA based on recombinant antigens assessing the humoral immune response in cattle with ringworm. This assay could be valuable in screening large number of samples, making it particularly suitable for epidemiological studies, but also for the evaluation of vaccines and/or vaccination procedures.

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Distribution of optical density (OD) values obtained from the sera of cattle with confirmed ringworm infection (group A) and healthy control cattle (group B) using the recombinant proteases *Trichophyton rubrum* DppV (TruDppV) and Lap2 (TruLap2) as coating antigens. For the TruDppV-based ELISA, the mean OD of sera from groups A and B was 0.791 ± 0.32 and 0.173 ± 0.089, respectively, and the cutoff value obtained as mean OD + 2× SD of sera from group B was 0.353. For the TruLap2-based ELISA, the mean OD of sera from groups A and B was 0.994 ± 0.511 and 0.157 ± 0.085, respectively, while the cutoff point was fixed at 0.327 as mentioned above. Using both antigens, a highly significant difference (*P* < 0.0001, Mann-Whitney U test) was noted between OD values obtained when testing sera from group A versus group B of the cattle. Details about sensitivity, specificity, and positive and negative predictive values are given in the Results section. The horizontal bars represent the mean OD and dashed bars (---) represent the cutoff.