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Development of an Enzyme-Linked Immunosorbent Assay for Serodiagnosis of Ringworm Infection in Cattle

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The aim of this study was to develop an in-house enzyme-linked immunosorbent assay (ELISA) for the serological diagnosis of ringworm infection in cattle. We used available recombinant forms of *Trichophyton rubrum* dipeptidyl peptidase V (TruDppV) and *T. rubrum* 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 infection that served as negative controls were used. Sensitivities, specificities, and positive and negative predictive values were determined to evaluate the diagnostic value of our ELISA. Overall, the ELISAs based on recombinant TruDppV and TruLap2 discriminated well between infected animals and healthy controls. Highly significant differences ($P < 0.0001$, Mann-Whitney U test) were noted between optical density values obtained when sera from infected versus control cattle were tested. The ELISA developed for the detection of specific antibodies against DppV gave 89.6% sensitivity, 92.7% specificity, a 96.8% positive predictive value, and a 78.4% negative predictive value. The recombinant TruLap2-based ELISA displayed 88.1% sensitivity, 90.9% specificity, a 95.9% positive predictive value, and a 75.7% negative predictive value. To the best of our knowledge, this is the first ELISA based on recombinant antigens for assessing immune responses to ringworm infection in cattle; it is particularly suitable for epidemiological studies and also for the evaluation of vaccines and/or vaccination procedures.

The zoophilic dermatophyte *Trichophyton verrucosum* is the most common agent of dermatophytosis (commonly known as ringworm infection) in cattle, which represent 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 infection in cattle has received particular attention due to not only its contagiousness among animal communities but also its zoonotic transmission to humans (3).

To date, there have been few studies devoted to assessing the immune responses to ringworm infection in 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 antidermatophyte 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 (ELISAs) 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 infection, from intensive breeding systems in Romania and ranging in age from 3 months to 3 years, underwent clinical examination. To confirm the 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 and with 20% KOH associated with calcofluor white (fluorescent brightener 28, F3543; Sigma-Aldrich, St. Louis, MO) followed by fluorescence microscopy (19). The remainder of the sample 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). Evidence of fungal infection was found in all 135 samples according to the methods employed.

Under the national program for surveillance, control, and eradication of animal diseases in Romania, blood samples were collected by jugular vein puncture. The serum samples were separated by centrifugation and stored at −20°C until they were assayed. The 135 serum samples from cattle with confirmed ringworm infection were referred to as "positive

Received 16 April 2013 Returned for modification 20 May 2013
Accepted 28 May 2013
Published ahead of print 5 June 2013
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doi:10.1128/CVI.00243-13

sera" (group A, $n = 135$). The control "negative sera" (group B, $n = 55$) consisted of 55 serum samples collected from beef and dairy cattle from an intensive breeding system in Belgium, with ages ranging from 3 months to 3 years, without any apparent skin lesions or history of ringworm infection. These animals were examined by a veterinarian and considered clinically healthy.

All activities were performed according to European Welfare Legislation (directive 2010/63/EU).

Antigens. Although several secreted proteases from dermatophytes have been obtained as recombinant proteins, no *T. verrucosum*-secreted proteases are currently available in recombinant form. In spite of their different ecologies, the genome of *T. rubrum* (21) proved to be most closely related to the genomes of *T. verrucosum* and *Arthroderma benhamiae*, two phylogenetically closely related dermatophytes that induce highly inflammatory cutaneous infections in humans (22, 23). In addition, most *Trichophyton* species revealed 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 1,500 × *g*), concentrated by ultrafiltration using an Amicon cell (Millipore, Billerica, MA) and a filtration membrane with a size threshold of 30 kDa, and then dialyzed against phosphate-buffered saline (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 nontransformed *P. pastoris* strain GS115, which did not secrete any DppV or Lap2 activities into the culture medium, was grown under the same conditions described above, and the culture supernatant, also prepared as described above, was used as a control.

ELISAs. Initially, checkerboard titrations were performed to optimize the required concentrations of the coating antigens, primary antibody, and conjugate (data not shown). Polystyrene microtiter 96-well plates (Microlon 600; Greiner Bio-One, Kremsmuenster, Austria) were coated with 100 μl/well of 10 μg/ml antigen solution in PBS (pH 7.2) as a coating buffer and incubated for 1 h at 37°C. Odd rows of the plates were sensitized with the antigen, while even rows were coated with *P. pastoris* GS115 supernatant as control wells. After being washed with PBS, unoccupied protein-binding sites were blocked by the addition of 200 μl/well of a 3.6% solution of casein hydrolysate (Merck, Whitehouse Station, NJ) in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at 37°C. According to the linear range of the dilution curves of the ELISAs, triplicate serum samples were diluted 1:500 in PBS-T, and 50 μl of each was added over 1 h at 37°C to both the antigen-coated and control wells. After being washed with PBS-T, 50 μl of peroxidase-conjugated rabbit anti-cow immunoglobulin G (IgG) (polyclonal anti-cow immunoglobulins/horseradish peroxidase [HRP]; Dako, Glostrup, Denmark), diluted 1:1,000, was added to each well. After a further 1-h incubation at 37°C and 3 subsequent washes with PBS-T, the peroxidase activity was revealed by the 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 Multiskan 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 infection and fetal calf serum (Gibco Life Technologies, Carlsbad, CA) 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 sample tested and the control wells. The cutoff was obtained as the mean OD + 2 times the standard deviation (SD) of sera from group B. All sera (from groups A and B) were classified as true positive (TP) (sera from group A that presented OD values above the cutoff in the serological test), false positive (FP) (sera from group B that presented OD values above the cutoff in the serological test), true negative (TN) (sera from group B that presented OD values below the cutoff in the serological test), and false negative (FN) (sera from group A that presented OD values below the cutoff in the serological test). Sensitivities, specificities, and 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), and 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 those from the control group, with significance defined as a P value of <0.0001.

RESULTS

In this study, we developed an in-house ELISA for the 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 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, and hair. The levels of specific antibodies were measured in field serum samples from cattle with confirmed ringworm infection (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 in the OD values between group A and group B.

In total, 190 serum samples were first assessed by recombinant TruDppV-based ELISAs 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 the mean OD + 2SD of these sera, was fixed at 0.353. The mean OD of sera from group B was 0.173 ± 0.089. Of the serum samples from group B, 51/55 (92.7%) had ODs 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 ± 0.32. A cutoff value of 0.353 gave 89.6% sensitivity, 92.7% specificity, a 96.8% positive predictive value, and a 78.4% negative predictive value. It is well known that higher cutoff values increase the specificity of a test but reduce its diagnostic sensitivity and might lead to false-negative results. As a compromise, a cutoff value of 0.442, determined as the mean OD + 3SD, for the recombinant TruDppV-based ELISA displayed 86.6% sensitivity, 100% specificity, a 100% positive predictive value, and a 75.3% negative predictive value. Thus, the sensitivities and negative predictive values of the test were

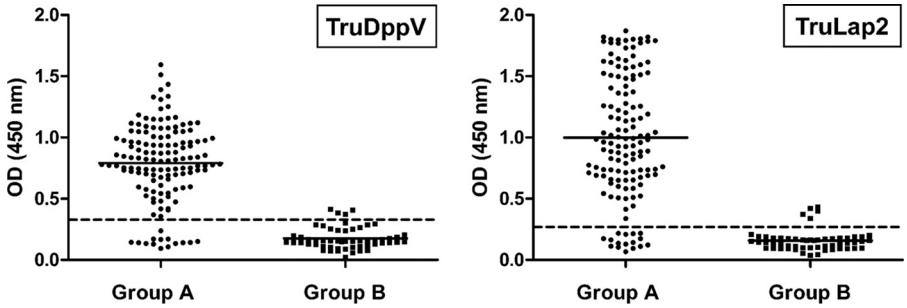

FIG 1 Distribution of optical density (OD) values obtained from the sera of cattle with confirmed ringworm infection (group A) and healthy control cattle (group B) using the recombinant proteases *Trichophyton rubrum* DppV (TruDppV) and Lap2 (TruLap2) as coating antigens. For the TruDppV-based ELISA, the mean ODs of sera from groups A and B were 0.791 ± 0.32 and 0.173 ± 0.089, respectively, and the cutoff value obtained as the mean OD + 2SD of sera from group B was 0.353. For the TruLap2-based ELISA, the mean ODs of sera from groups A and B were 0.994 ± 0.511 and 0.157 ± 0.085, respectively, while the cutoff point was fixed at 0.327 as described above. With both antigens, a highly significant difference ($P < 0.0001$, Mann-Whitney U test) was noted between OD values obtained when sera from group A versus those from group B were tested. Details about sensitivities, specificities, and positive and negative predictive values are given in Results. The horizontal bars represent the mean ODs, and the dashed bars represent the cutoff.

reduced while the specificities and positive predictive values were maximized.

In parallel, the sera from groups 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 + 2SD 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 noninfected animals. The mean OD of these sera was 0.157 ± 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. The 14 FN and 4 FP serum samples obtained when recombinant TruDppV was used were among the 16 FN and 5 FP sera identified when recombinant TruLap2 was used. A cutoff value of 0.327 resulted in 88.1% sensitivity, 90.9% specificity, a 95.9% positive predictive value, and a 75.7% negative predictive value. By increasing the cutoff value to 0.412 (as the mean OD + 3SD 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 ELISAs, especially when large numbers of samples need to be examined. The *in vitro* production of recombinant antigens is an inexpensive and fast method for obtaining 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 either recombinant TruDppV (89.6% sensitivity and 92.7% specificity) or recombinant TruLap2 (88.1% sensitivity and 90.9% specificity) was used. Serological reactivity in the noninfected control group (group B) was observed in only a minority of animals, with most of them reacting to both TruDppV and TruLap2. This might reflect a previous nonreported asymptomatic infection with *T. verrucosum* or other dermatophyte species, as there is evidence for interspecies and intergeneric cross-reactions (30). Exposure to environmental nondermatophyte fungal antigens might 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 commensal organisms such as *Candida albicans* and *Malassezia pachydermatis* were also not totally excluded, even if they have been reported for neither cattle nor other animal dermatophytoses (11).

In spite of confirmed infections, several serum samples 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 serologically negative cattle with clinical lesions might have occurred, especially because IgM, the first Ig to appear in response to initial fungal exposure, was not detected with the in-house ELISA that we developed. 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 postinfection (p.i.) (4). During experimental *Microsporum canis* infection, levels of specific IgG antibodies were detectable at different intervals after the lesions appeared, either at 14 to 21 days p.i. in dogs (11) or at 14 days p.i. in guinea pigs (15, 30). In *M. canis*-infected cats, specific IgG levels were high in some animals when the first lesions appeared and were high in others 4 weeks later (31).

To date, ELISAs have 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*, which are major pathogens in immunocompromised individuals. The tests developed for the detection of mannan or galactomannan antigens revealed good specificities but variable sensitivities (32). The most data are available for the detection of

Aspergillus galactomannan antigen using the Platelia ELISA format. Although several studies indicated that this biomarker has good specificity for the diagnosis of invasive aspergillosis, the sensitivities have varied, ranging from 17% to 100% (33, 34).

The results obtained in the present study provide basic support for the 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 sulfitolysis (35) and endoprotease (subtilisins and fungalysins) digestion (36). So far, the expression of genes encoding the dermatophyte-secreted exoproteases DppV and Lap2 has been shown only *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 for assessing the humoral immune response in cattle with ringworm infection. This assay may be valuable in screening large numbers of samples, making it particularly suitable not only for epidemiological studies but also for the evaluation of vaccines and/or vaccination procedures.

ACKNOWLEDGMENTS

This study was supported by grant 3.4558.10 from the Fonds de la Recherche Scientifique Médicale (FRSM, Belgium) and grant 594/2012 from the Executive Unit for Higher Education, Research, Development and Innovation Funding (UEFISCDI, Romania). This publication was possible thanks to the agreement that binds Wallonia-Brussels (WBI) and Romania. E.T.B. was the recipient of a research grant provided by the University of Liège. L.C. is the recipient of a studentship from Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA), Brussels, Belgium.

We thank Claude Saegerman and Aline Baldo for useful discussions and Françoise Maréchal for excellent technical assistance.

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