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Short note

Biotin-avidin amplified ELISA for detection of antibodies to *Sarcoptes scabiei* in chamois (*Rupicapra* spp.)

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Abstract – Scabies is a major threat to the well being of mountain-dwelling Bovid hosts, *Rupicapra rupicapra* and *Rupicapra pyrenaica*. Severe outbreaks are in progress over a significant part of their distribution area and resource managers demand improved methods to monitor, analyse and possibly forecast the spread and effects of scabies at the population level. An amplified capture enzyme-linked immunosorbent assay was developed to detect antibodies to *Sarcoptes scabiei* in chamois (*Rupicapra* spp.) serum. The method used the biotin-avidin amplification system and was validated on a panel of 144 serum samples, of which 40 were obtained from scabietic and 104 from healthy unexposed individuals originating from a scabies-free area. The antigen, a whole body extract of the various developmental stages of *S. scabiei*, was prepared from mites actively leaving the skin lesions of naturally infested red foxes (*Vulpes vulpes*). The resulting LAB-ELISA was characterised by 93% sensitivity, 97% specificity and a high degree of repeatability. A single seroreactor was found amongst 32 chamois affected with skin pathologies other than scabies, including infestations by other Acarina (*Trombicula* spp. and Ixodid ticks). Antibodies to *S. scabiei* were present in 26 out of 169 sera (15.4%) obtained by clinically healthy chamois within a scabies outbreak area, indicating that asymptomatic infestations by *S. scabiei* can be revealed by serological methods in the studied Caprinae hosts.

Sarcoptes / chamois / ELISA / avidin-biotin

1. INTRODUCTION

Scabies is a contagious skin disease caused by the mite *Sarcoptes scabiei*. Man, domestic animals and several captive and free-ranging wildlife are affected worldwide [31]. Wild Caprinae are particularly sensitive to *Sarcoptes* infestation and severe outbreaks of a generalised form of crusty scabies have been reported in chamois (*Rupicapra rupi-*

capra rupicapra and *Rupicapra pyrenaica parva*) and ibex (*Capra ibex ibex* and *Capra pyrenayca*) in southern Europe [15, 24, 33]. Effects on the population are obvious, in fact crude mortality exceeds 90% on occasions [24, 33]. This is a matter of concern for local authorities and resource managers and there is a demand for careful monitoring of the spread and severity of the outbreaks as well as, possibly, forecasting

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future scenarios. Despite considerable effort by wildlife veterinarians, biologists and field personnel, monitoring scabies outbreaks by means of current methods of investigation (active search for carcasses, observation from distance, direct counts) is a time-consuming, difficult and somewhat indefinite task because of environmental conditions and problems associated with sampling and identifying mite-infested free-ranging animals [28].

In 1991, Boyce et al. [12] showed that antibodies to *Psoroptes* spp. may be detected in naturally and experimentally infested bighorn sheep (*Ovis canadensis*) by means of a kinetic immunosorbent assay. The test was subsequently used to determine the distribution of mite-exposed bighorn sheep herds in California [26]. The aim of the screening was to avoid accidental spreading of psoroptic scabies during active relocation programmes. Based on the above experiences, a new serological test – a labelled avidin-biotin immunosorbent assay (LAB-ELISA) – was developed as an aid to epidemiological and pathogenetic studies of *Sarcoptes* scabies in wild Caprinae in southern Europe.

2. MATERIALS AND METHODS

2.1. Antigen

Living *S. scabiei* mites at different stages of development and of both sexes were collected from the crusty lesions of naturally infested red foxes (*Vulpes vulpes*), according to Fisher [17]. Mites were transferred to Eppendorf tubes and stored at -20°C until required.

Mite samples were thawed and washed in 0.1 M carbonate buffer pH 7.2 (Na_2CO_3 0.1 M 33%, Na_2HCO_3 0.1 M 67%) containing 1:1000 Tween 20 to eliminate incidental impurity. Then they were filtered out, air dried and weighed. Aliquots of 20 mg mites were homogenised at room temperature in a Griffiths tube with 0.1 M carbonate buffer pH 9.6 and subsequently sonicated five

times for 30 s. The ratio of mites to saline in this preparation was approximately 10 mg/15 mL. The suspension was stirred at 4°C for 16 h and the supernatant was harvested after centrifugation at 18 000 g for 30 min.

The soluble extract was filtered at $0.2\ \mu\text{m}$ and the protein concentration, determined by the Lowry method, was adjusted to a concentration of $200\ \mu\text{g}/\text{mL}$ in 0.1 M carbonate buffer pH 9.6. The antigen was stored at -30°C in 1 mL aliquots.

2.2. Chamois sera

Serum samples, obtained from 5 scabietic plus skin-scraping positive chamois from a longtime scabies-endemic area (Eastern Alps, Italy) and 5 healthy unexposed chamois from a scabies-free area (Western Alps, Italy), were initially used to determine optimal assay conditions. According to the repeatability estimate, these sera were later used as control samples during routine runs of the assay.

Once assay conditions were established, 104 sera collected from healthy unexposed chamois (Western Alps, Italy) were used as the negative panel, and 40 sera from scabietic plus skin-scraping positive chamois (21 from the Eastern Alps, Italy, and 19 from the Cordillera Cantabrica, Spain) were used as the positive panel. Out of the scabietic chamois, 14 were mildly affected (less than 10% of the skin surface showing alopecia and focal hyperkeratotic areas), 18 were moderately affected (diffuse hyperkeratosis and localised thick crusts over 10–25% of the skin surface) and 8 were severely affected (thick fissured crusts over up to 40% of the skin surface). Sera were obtained also from: (i) 32 chamois from scabies-free zones (Western Alps, Italy), showing patent infections of one or more of the following skin pathogens: chiggers (*Trombicula* spp.; N = 12), Ixodid ticks (*Ixodes ricinus* and/or *Dermacentor marginatus*; 15), biting lice (*Damalinia alpina*; 16), keds (*Melophagus rhipicephalus*; 4), *Trichophyton verrucosum* (2), *Dermatophilus*

congolensis (1) and the Contagious Ecthima virus (3); (ii) 169 healthy chamois from a scabies outbreak area in Auronzo di Cadore (Eastern Alps, Italy). For the sake of brevity, the last ones will be lately cited as "healthy exposed".

Blood was collected from the heart of 246 culled animals (40 healthy unexposed, 25 scabietic, 12 affected with other skin pathologies, 169 healthy exposed); and from the jugular vein of 99 animals (15 scabietic, 64 healthy unexposed, 20 affected with other skin pathologies) captured either chemically or physically using drive nets. After clotting, blood samples were centrifuged at 200 g for 15 min and sera were stored at -20°C in 1 mL aliquots until required.

2.3. ELISA method

A 100 μL aliquot of the antigen was added to each well of a microtiter plate (TPP[®] Gamma sterilized), except for the first column where 100 μL of carbonate buffer pH 9.6 were added. The plate was left for 40 min at 37°C , and subsequently at 4°C overnight. Coated like this, the plate was stable for 8 h at 4°C ; otherwise the plate, incubated for 1 h in PBS containing 3% Bovine Serum Albumin and 0.01% Thiomersal and stored at 4°C , is stable for a week.

Each coated well was washed five times in phosphate buffer pH 7.2 containing 0.05% Tween 20 (SAF-T: $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ 0.18%, Na_2HPO_4 1.24%, NaCl 0.85%, Tween 20 0.05%). A 100 μL aliquot of serum diluted 1:100 in SAF-T containing 2.0% Bovine Serum Albumin was added to each well; the plate was then shaken for 1 h at 37°C on a Bellco Mini-Orbital Shaker (Bellco Glass Inc., Vineland, NJ, USA). After five additional washings in SAF-T, 100 μL of biotinylated Protein G (Immunopure[®] Biotinylated Protein G, Pierce Chemical Co., Rockford, IL, USA) diluted 1:1000 in sodium acetate buffer 0.1 M, pH 5.0 ($\text{C}_2\text{H}_3\text{O}_2\text{Na}\cdot 3\text{H}_2\text{O}$ 1.4%) was added. The plate was again left one hour at 37°C , and then washed three times in SAF-T.

A 100 μL aliquot of horseradish-peroxidase-labelled Avidin (Immunopure[®] Avidin Horseradish Peroxidase Conjugated, Pierce Chemical Co., Rockford, IL, USA) diluted 1:10 000 in phosphate buffer containing 0.01% gelatine pH 7.2 was added to each well, and incubated for one hour and washed as before. The plate was washed one time in citrate buffer 0.4 M before the addition of 100 μL of substrate solution [2 mM H_2O_2 0.2 M ABTS (2,2'-diazino (3 ethylbenz-thiazoline-6-sulfonate), Sigma-Aldrich Co., St. Louis, MO, USA) 0.4 M citrate pH 4.0] to each well. The plate was then incubated for 30 min at room temperature.

Optical density was read at 405 nm with a spectrophotometer (Anthos 2010, Anthos Labtec Instruments, Wals, Austria).

2.4. Performance criteria of the assay

Assay results were expressed as Optical Density (OD) percentage obtained by the Percent Positivity (PP) method [21, 35], where the OD of each sample tested is expressed as a percentage of a high positive reference standard. This relative measurement results in a uniform and continuous scale of 0–100 percent positivity, calculated according to the following formula:

OD percentage: $\text{OD}(\times) - \text{OD}(-) / \text{OD}(+) - \text{OD}(-)$.

Each sample was assayed in triplicate, to obtain a mean V_{max} value; because multiple plates were used, V_{max} values on each plate were multiplied by a correction factor, so that between plates comparisons were valid [34]. A correction factor was determined by the following formula: Correction Factor: $\text{mean } V_{\text{max}} \text{ of SPCS across plates} / \text{mean } V_{\text{max}} \text{ of SPCS within a given plate}$, where SPCS means the strong positive control sera.

The intra-plate variability (CV_1) was estimated by testing the control samples 10 times in triplicate within a particular plate; inter-plate variability (CV_2), was assessed by

Table I. Distribution of the % OD values of sera obtained from 345 chamois (*Rupicapra rupicapra* and *Rupicapra pyrenaica*). The examined cohorts were: healthy unexposed, originating from scabies-free areas (N = 104); scabietic (40), grouped in three classes according to the severity of skin lesions (mildly affected = less than 10% of the skin showing alopecia and focal hyperkeratotic areas; moderately affected = diffuse hyperkeratosis and localized thick crusts over 10–25% of the skin; severely affected = thick fissured crusts over up to 40% of the skin); affected with skin pathologies other than scabies (32); healthy exposed, originating from a scabies outbreak area (169).

%OD	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	101–110
Healthy unexposed	7	25	31	25	10	3	1	2	0	0	0
Scabietic	0	0	0	0	2	3	8	12	11	1	1
Mildly affected					1	2	1	6	4		
Moderately affected					1	2	6	3	4	2	
Severely affected							1	3	3	0	1
Skin diseases other than scabies	2	8	10	8	3	1	0	0	0	0	0
Healthy exposed	14	40	33	30	21	10	9	5	5	1	1

comparing the results of the same control samples in five separated assays.

Repeatability was also determined by calculating the amount of agreement using different batches of antigen.

Determination of the cut-off between the positive and negative samples was achieved using the VIFD (Visual Inspection of Frequency Distributions) method, based on frequency distributions of normalised test results from the two panels of samples, positives and negatives. The cut-off value was set at the intersection of the results of the two distributions [20, 27].

The sensitivity and specificity of the assay were calculated according to the following formulae: Sensitivity: True Positive \times 100 / True Positive + False Negative; Specificity: True Negative \times 100 / True Negative + False Positive.

3. RESULTS

The distribution of the LAB-ELISA OD values for the sera examined is reported in Table I. Values from the scabietic chamois were significantly higher than those from the healthy unexposed chamois (74.3 ± 13.6

vs. 27.3 ± 13.4 ; $t = 8.96$, $P < 0.001$, Mann-Whitney U-test). No difference occurred between the sera of 40 chamois allocated to three classes based on scabies severity ($H = 11.59$; $P = 0.48$, Kruskal-Wallis test).

The cut-off value of the test, found at the intersection of the frequency distributions of sera ODs from healthy unexposed and scabietic chamois, was 55% (Fig. 1). Based on the selected cut-off, 101 of the 104 sera collected from healthy unexposed chamois had negative OD values (true negatives) and 3 showed positive OD values (false positives); 37 of the 40 sera from scabietic plus skin-scraping positive chamois had positive OD values (true positives) and 3 showed negative OD values (false negatives). The resulting sensitivity was 93% and the specificity 97%.

A high degree of repeatability was indicated by $CV_1 = 10\%$ for triplicate within plates, and a $CV_2 = 5.8\%$ for SPCS V_{max} across the plates. Correction factors derived from the SPCS values varied from 0.91 to 1.09.

A single seroreactor was found amongst the 32 chamois affected with skin pathologies other than scabies (a chiggers infested

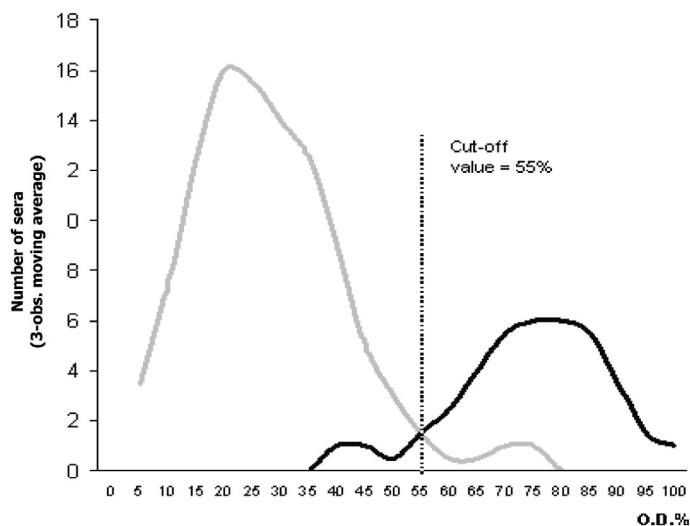


Figure 1. Frequency distribution of % Optical Density (OD) of sera obtained from scabietic (positive) and healthy unexposed (negative) chamois. The Y-axis represents the number of sera in each 5% OD interval on the X-axis. The grey line refers to negative sera and the black line to positive sera (3-observations moving average). The broken line represents the cut-off value (55%) selected from data analysis.

female, which showed a 59% OD value). OD values from this cohort were similar as those from healthy unexposed ones (26.4 ± 12.5 vs. 27.3 ± 13.4 ; $z = 0.455$, $P = 0.649$, Mann-Whitney U-test).

A relatively high number of seroreactors was found amongst healthy exposed chamois (26 out of 169; 15.4%). The prevalence of seroreactors significantly differed between these chamois, the healthy unexposed ones and those affected with skin pathologies other than scabies ($\chi^2 = 13.16$; $P = 0.001$, Chi-square test). OD values from healthy exposed chamois were higher (33.0 ± 21.4) than values from any of the above two cohorts of unexposed individuals but the difference was not significant.

4. DISCUSSION

Antibody responses to *S. scabiei* infestation have been documented in domestic and wildlife hosts [1, 3, 8] and standardised immunosorbent assays are currently available for diagnosis and monitoring of sca-

bies in dogs and swine [9, 10, 19]. To the best of our knowledge, the LAB-ELISA described here is the first immunosorbent assay designed for the detection of specific antibodies against *S. scabiei* in a free-ranging ruminant host.

A recognised problem when evaluating a serological test is the availability of one or more gold standards with which to compare the results of the test. This aspect is particularly delicate in the case of dogs and swine, which are prone to develop a hyper-sensitive form of scabies where large mite populations are unlikely to occur [13, 29]. Besides a limited proportion of mite positive individuals, in the above hosts, positives are a heterogeneous group comprising either dogs positively responding to specific treatment [10] or pigs derived from infected farms [19].

In the present study, the positive (infested) and negative (unexposed) status of chamois was objectively reliable. All positives were represented by scabietic chamois testing positive to *S. scabiei* at the skin scraping.

Negatives were healthy individuals captured in recognised scabies-free zones of northern Italy, some 450 km west of the closest focus of chamois scabies. It is worth stressing that scabies has a dramatic epidemic course in previously unexposed chamois populations [33]; therefore, there is no chance that such a condition may long remain undetected in a host which is of the outmost interest for thousands of mountain hikers and sport hunters across alpine Europe.

Similarly to Bornstein and Wallgreen [9], the whole body extract of mites obtained from naturally infested red foxes proved a suitable source of antigen. Scabies mites from different host species are morphologically indistinguishable and *S. scabiei* is generally recognised as a unique species split into physiological varieties, adapted to groups of zoologically related host species [14, 30]. Close similarity between *Sarcoptes* strains from nine host species (alpine chamois and red fox included) has been confirmed by means of molecular analyses [5, 36]. Similarly, immunologic cross-reactivity among various strains of scabies mites (var. *hominis*, *canis* and *suis*) has been demonstrated [4]. Scabies is relatively common in red foxes throughout Italy and thousands of mites may be isolated from an individual with generalised crusty lesions. This situation presented us the opportunity to access sufficient quantities of mites for antigen preparation and stock.

The use of recombinant protein G allowed to overcome the problem of unavailable commercial antisera to our target wild hosts. Previously, recombinant protein G proved a valuable alternative to secondary antibody in a kinetic ELISA developed for diagnosis of *Psoroptes* infestation in wild ruminant hosts, *Ovis canadensis*, *Ovis dalli* and *Cervus canadensis* [11, 12, 37].

The avidin-biotin detection system has the potential to increase the sensitivity of the indirect ELISA since avidin has four binding sites for biotin, making an essentially irreversible complex which is stable in later washes and incubations [22]. We

ignore if and how the amplification afforded by the avidin-biotin detection system is crucial to the performance of a *Sarcoptes*-ELISA working with serum samples but the original decision to include this detection system derived from: (i) the relatively low sensitivity shown by a previously validated non-amplified indirect ELISA, when checked in a group of 94 certified *Sarcoptes*-infected pigs [19]; (ii) the perspective of an application of our test to the measurement of lower specific IgG levels in fluids such as the lung extract, which is more easily available than serum of adequate quality in free-ranging wildlife [16].

Other expected advantages of the avidin-biotin detection system are the low background absorbance and the significant saving of specific antibody or protein G [18].

The choice to select a cut-off by means of the VIFD method is an important element for the adaptation of the assay according to epidemiological requirements; depending on epidemiological purposes – estimating the prevalence of a disease, determining if selected herds have been exposed, assessing the risk of disease transmission between wildlife and domestic hosts, etc. – it may be more important to stress specificity rather than sensitivity, or vice versa, and consequently minimise the number of false positives or false negatives. Compared with other methods, such as the receiver-operator characteristic (ROC) curve, VIFD has the advantage of being simple and flexible, and requires no statistical calculations or assumptions about the normality of the distributions of infected and uninfected reference samples [21].

Overall, the LAB-ELISA permitted a clear definition of the scabies infestation status in chamois. Besides good sensitivity leading to correct identification of even mildly scabietic individuals, a high specificity was demonstrated with samples from a reliable scabies-free area and samples from chamois affected with a variety of skin pathologies which have to be considered in differential diagnosis of scabies. Although

sera from *S. scabiei* infested humans and domestic animals are known to cross-react with antigens of other mites, such as the house dust mite *Dermatophagoides pteronyssinus* [2], our data did not support evidence of cross-reaction with *Trombicula* spp., which frequently causes skin damage to chamois and other wild ruminants in Europe [6].

Remarkably, specific antibodies could be detected in healthy exposed chamois, suggesting that asymptomatic scabies infestation in this ruminant host can be revealed by the studied ELISA. In other animal models, the presence of asymptomatic seroreactors has been associated to the incubation phase [7], the subclinical course of the infestation [10] or the persistence of antibodies after therapy [23, 25]. As regards ruminants, persistence of specific antibodies for more than two months after successful treatment with a macrocyclic lactone has been demonstrated in cattle [32]. The possibility to reveal a *S. scabiei* infestation status in asymptomatic chamois is of a great interest for prospective and retrospective studies assessing the distribution and prevalence of *S. scabiei* infestations in this host and other related wild ruminants suffering from epidemic scabies.

Work is continuing in chamois to ascertain the following: (i) how frequently are specific antibodies detectable in subclinical infestations, involving small numbers of mites; (ii) how soon after exposure are specific antibodies detectable in serum; (iii) how long do antibodies persist in this or related hosts after the disease has eventually self cured or been cured. Information on these factors will be necessary to assess the full potential of the test.

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