Development of a serodiagnostic test for sheep scab using recombinant protein Pso o 2

Francesca G. Nunn, Stewart T.G. Burgess, Giles Innocent, Alasdair J. Nisbet, Peter Bates, John F. Huntley

Division of Parasitology, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, EH26 0PZ, Scotland, UK
Biomathematics & Statistics Scotland JCMB, King’s Buildings, Edinburgh, EH9 3JZ, Scotland, UK
VMEC, Chertsey, Surrey, KT16 8DR, UK

A B S T R A C T

Early stages of sheep scab, the disease caused by the non-burrowing mite Psoroptes ovis, are often sub-clinical, or can be mis-diagnosed. A diagnostic test capable of detecting early disease and latent infestations is therefore highly desirable in disease control. This paper describes the design and validation of an ELISA, which incorporates a recombinant P. ovis antigen (Pso o 2), for the early detection of anti-P. ovis serum antibodies in sheep. This ELISA was evaluated using sera from sheep infested with P. ovis (n = 58) and sheep (n = 433) with no P. ovis infestation as well as sheep infected with other parasites including gastrointestinal nematodes (GIN), or chewing lice. A receiver operating characteristic (ROC) curve analysis was generated using the ELISA results for 491 sheep sera with the area under the curve (AUC) being 0.97. An optimal OD450 cut-off of >0.06 absorbance units gave a test sensitivity of 0.93 and specificity of 0.90. The Pso o 2-based ELISA was able to detect specific antibodies to P. ovis during early experimental infestation prior to disease latency, indicating its utility for detecting sub-clinical infestation.

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1. Introduction

Sheep scab is caused by the non-burrowing ectoparasitic mite, Psoroptes ovis. The disease occurs worldwide and is notifiable in many countries. Infestation is characterised by yellowish lesions (scabs) that can spread over the entire body of the sheep, which are usually highly pruritic and are initiated through the presence and feeding of the mites on the skin surface [1]. Ulceration and secondary bacterial infections are common, and infestation can also induce epileptiform fits which can be fatal [2]. Low birth weight of lambs born to infested ewes has also been demonstrated [3]. This parasite is therefore of major welfare concern and, although current treatments (injectable endectocides or dipping with acaricides) are effective, it is highly likely that widespread resistance to these chemicals will occur in the future. Indeed, resistance to synthetic pyrethroids in P. ovis has been documented previously in the UK [4], and resistance against doramectin confirmed in Argentina [5].

One of the major problems in controlling sheep scab is the rapid spread of infestation, which is normally through direct contact or the transfer of mites from, for example, infested fleece strands, fence posts, machinery and workers [6]. The early stages of infestation are not obvious, and sheep can appear clinically normal during this ‘early’ or ‘lag phase’ of infestation [7]. This period of apparent normality may be several weeks in duration during which the sheep can transfer infection. The identification of sub-clinical cases is thus likely to be crucial in disease control. Therefore, the present study focused on evaluating a serological test (ELISA) for the detection of specific antibodies for the diagnosis of sub-clinical infestation and disease. This antigen, Pso o 2, was first described as an immunogen in cattle infested with P. ovis [8] and later characterised as a homologue of the house dust mite allergen Der p 2 [9]. The present study indicates that this ELISA, using a recombinant form of Pso o 2, provides a specific and sensitive tool to aid future control strategies for sheep scab.

2. Materials and methods

2.1. Expression of recombinant Pso o 2 (rPso o 2)

A cDNA representing the gene encoding Pso o 2 was identified within an expressed sequence tag (EST) (contig POC00006) from
the dataset held at: http://www.nematodes.org/NeglectedGenomes/ARTHROPODA/wwwPartiGene.php [10]. The cDNA fragment representing amino acid residues 18 to 142 of Pso o 2 was sub-cloned into pET-22b(+ (Novagen, UK) following amplification by polymerase chain reaction (PCR) using first strand cDNA (generated from mRNA extracted from mixed stages of P. ovis [11]) as template. Oligonucleotide primers were designed to omit the termination codon and the putative signal peptide (identified using SignalP 3.0 [12]). Primer sequences are available from the authors on request. The PCR was performed using the Advantage2® PCR Kit (Clontech, UK) under the following cycling conditions: 30 cycles of 94 °C/30 s, 57 °C/30 s, 72 °C/90 s, followed by a cycle of 72 °C for 5 min. The PCR product was digested with HindIII and BamHI restriction enzymes (Roche, UK), purified using the QIAprep PCR cleanup kit (Qiagen, UK), and used to transform E. coli BL21-CodonPlus® (DE3)-RIL competent cells (Stratagene, UK). Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 6 weeks. At this time point, the bacterial cells were harvested, washed with washing buffer, pH 9.6, and solubilised in 8 M urea. Subsequent purification by nickel column affinity chromatography using HisTrap™ HP columns (GE Healthcare, UK) was performed using a step-wise imidazole gradient in the presence of 8 M urea.

2.2. Serum samples

A detailed description of the samples used in this study is given in Table 1. After having taken individual blood samples from experimental donor sheep (Group A), they were each infested by placing a bolus of ~20–50 mites directly on to the skin surface [13]. The experimental sera samples were collected from sheep (n = 18) infested to maintain the mite colony at the Moreden Research Institute; this was done before infestation and at weekly intervals for 6 weeks. At this final sampling point, scabs ranged in size from 250 to 500 cm² (Group B). Sera from sheep without P. ovis infestation (Group C) were collected from the Institute’s own flock maintained at Firth Mains Farm, Scotland.

Samples were also collected during a sheep scab outbreak following diagnosis by a qualified veterinarian. Samples (Groups D and E) were taken from sheep during outbreaks on separate farms during 2007–2009, from farms in the Central Belt and the Highland region of Scotland. Blood was also taken from ewes (Group F) maintained on pasture (Firth Mains Farm) heavily contaminated with gastrointestinal nematodes endemic in Scotland (GIN; including Teladorsagia circumcincta and Trichostrongylus vitrinus). Samples from lice-infested but P. ovis-naive sheep (Group G) were provided by Professor Neil Sargison, R(D)SVS. Samples (Group H) were also taken from a free-roaming, but closed, flock of Soay sheep maintained on the Isle of St Kilda, Scotland. These sheep were known to be exposed to a wide range of endemic parasites, including nematodes, Eimeria spp., and Melophagus ovinus [14] but there have been no reports of sheep scab on these islands for many years (Prof. J Pemberton, personal communication). The receiver operating characteristic (ROC) curve was generated from data derived from P. ovis-infested sheep from the Moreden Research Institute, Firth Mains Farm, known to have been P. ovis-free for a number of years prior to sampling (Group C; n = 433) plus P. ovis-infested sheep (Group D (n = 40)) from confirmed natural outbreaks (by physical examination and skin scraping performed by a qualified veterinarian), combined with terminal bleeds from experimental donor sheep (Group B (n = 18)). The additional 40 samples used to validate the ROC curve analysis consisted of an additional 20 P. ovis-infested samples from experimental terminal bleeds (Group J) and 20 P. ovis-uninfested samples from P. ovis-free field samples and experimental pre-bleds (n = 14 and n = 6, respectively, Group I). Groups K0–K6 serum samples were obtained from a group of sheep (n = 6), infested with P. ovis over a 6-week time course (K0 = pre-infestation (baseline serum samples) and K1–K6 = 1–6 weeks post-infestation sera samples, respectively).

### Table 1

<table>
<thead>
<tr>
<th>Group Summary</th>
<th>Number of samples</th>
<th>Range of OD₄₅₀</th>
<th>Mean OD₄₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18</td>
<td>0.02–0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>0.15–1.1</td>
<td>0.43</td>
</tr>
<tr>
<td>C</td>
<td>433</td>
<td>0.01–0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>0.01–1.08</td>
<td>0.38</td>
</tr>
<tr>
<td>E</td>
<td>18</td>
<td>0.01–0.44</td>
<td>0.13</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>0.00–0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>G</td>
<td>23</td>
<td>0.02–0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>H</td>
<td>52</td>
<td>0.00–0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>0.00–0.03</td>
<td>0.003</td>
</tr>
<tr>
<td>J</td>
<td>20</td>
<td>0.02–0.77</td>
<td>0.33</td>
</tr>
<tr>
<td>K₀</td>
<td>6</td>
<td>0.01–0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>K₁</td>
<td>6</td>
<td>0.02–0.23</td>
<td>0.13</td>
</tr>
<tr>
<td>K₂</td>
<td>6</td>
<td>0.02–0.38</td>
<td>0.16</td>
</tr>
<tr>
<td>K₃</td>
<td>6</td>
<td>0.06–0.47</td>
<td>0.22</td>
</tr>
<tr>
<td>K₄</td>
<td>6</td>
<td>0.17–0.45</td>
<td>0.27</td>
</tr>
<tr>
<td>K₅</td>
<td>6</td>
<td>0.18–0.48</td>
<td>0.30</td>
</tr>
<tr>
<td>K₆</td>
<td>6</td>
<td>0.32–0.78</td>
<td>0.45</td>
</tr>
</tbody>
</table>

The optimal antigen concentration employed to coat 96-well ELISA plates (Greiner, UK) was determined by titrating dilutions of the stock rPso o 2, and testing against a positive control sample which consisted of a pool of hyperimmune sera (collected from sheep (n = 6) six weeks after secondary infestation with P. ovis). Optimal antigen coating of the stock rPso o 2 in sodium carbonate buffer, pH 9.6, resulted in an OD₄₅₀ of 0.85 absorbance units (AU) for the positive control sample (as described above). Blank wells contained 50 µl of sodium carbonate buffer only (pH 9.6). Plates were sealed and left overnight at 22–24 °C (room temperature; RT). Plates were then washed 6 times in wash buffer [WB, phosphate buffered saline (PBS) incorporating Tween 20 at 0.05% vol/vol], then blocked with PBS containing 3% fish gelatine (Sigma, UK) at RT for 90 min or overnight (16 h) at 4 °C. Sera were diluted 1 in 200 in sample buffer (SB: PBS-NaCl 0.5 M Tween 80 0.1%) and 50 µl of each sample were added per well, to one blank well and two test wells, and incubated at RT for 60 min. Following washing, rabbit anti-sheep IgG HRP conjugate (DAKO, PO163) was diluted 1 in 2000 in SB, 50 µl were added per well and plates were then incubated at RT for 30 min, followed by addition of 50 µl of P. ovis-infested sheep ‘in contact’ with P. ovis-infested cases (Group E); samples from lice-infested but P. ovis-uninfested sheep (Group G); and samples from the MRI Firth Mains Farm that were P. ovis-uninfested (Group C); samples from the MRI flock at Firth Mains Farm that were infected with GINs (Group F); samples from a closed flock of Soay sheep on St Kilda that are known to be free of Psoroptic mange but known to be infected with endoparasites and other ectoparasites (Group H); samples used to validate the ROC curve which consisted of further samples from the MRI Firth Mains Farm flock uninfested with P. ovis (n = 14) and MRI experimental pre-bleds (n = 6) (Group I); further experimental terminal bleeds from the MRI experimental flock (Group J) and samples from the longitudinal infestation (time course) study from baseline (Group K₀, r = 0) through weeks 1–6 post-infestation with P. ovis (Groups K₁–K₆, respectively). Although several of the P. ovis-uninfested groups demonstrated a range with values above the 0.06 OD₄₅₀ cut-off, these represented very low numbers of sheep; 29 in Group C, 3 in Group F, and 2 each for Groups G and H.
for 60 min. Plates were washed 6 times before adding 50 μl tetramethylbenzidine (TMB) substrate (KPL, UK) and allowing the assay to develop for 10 min at RT. The reaction was stopped by adding 25 μl of sulphuric acid (2.5 M) per well and absorbance read at 450 nm. The standard positive control was diluted 1 in 400 in SB to give an OD₄₅₀ value of approximately 0.85 and was included on each plate as an interplate control. Plate readings were normalised to the same OD₄₅₀ reading and any control value outside the range ± 20% of OD₄₅₀ 0.85 led to the plate being repeated. Adjusted sample test readings were averaged, normalised to the control, and the average background reading for each plate was subtracted from the test readings to generate a final adjusted OD₄₅₀ value.

2.4. Statistical analyses

2.4.1. Specificity data
Specificity data were analysed and graphs generated in GraphPad Prism (GraphPad v5.01, San Diego, USA). The data were not well described by a normal distribution after transformation; as such the means of the pre- and terminal- bleed samples for the longitudinal study were compared using the non-parametric Mann–Whitney test and the specificity data using a Kruskal–Wallis one-way analysis of variance test with Dunn’s post-test to look at pairs of groups. Formal statistical significance was specified as a p-value of <0.05.

2.4.2. Evaluation of intra- and inter-assay variability
Consistency between operators was analysed using Fleiss’s Kappa statistic. This allows the comparison of the agreement between a number of different raters (n = 3). Results from any of the three operators were classified as positive if the OD was >0.06 AU, and negative if ≤0.06 AU. A further analysis was conducted using three classes of outcome, defined as negative (OD < 0.05 AU), possibly positive (OD between 0.05 and 0.10 AU) and definitely positive (OD > 0.10 AU). This analysis will be somewhat conservative since Fleiss’s Kappa assesses the agreement between operators in placing individuals into one of two or more classes. It does not take account of any ordering in the classes such as that seen here. This analysis was carried out using the R statistical package [15].

2.4.3. Receiver operator characteristic (ROC) curve analysis
The 433 known P. ovis-infested sheep (Groups C and D combined) were tested using the ELISA and their adjusted corrected OD₄₅₀ values calculated as described above. As the clinical status of the sheep was known, this information was used to determine the test sensitivity and specificity at a selected cut-off. Receiver operator characteristic (ROC) curves (sensitivity vs. 1-specificity) were plotted to compare the OD₄₅₀ for each set of samples [P. ovis-uninfested (Group C) and P. ovis-infested (Groups B and D combined)]. ROC curves are routinely used in clinical diagnostics to assess the true positive (TP) rate against false positive (FP) rate for a range of different cut-off points of a diagnostic assay; they illustrate the trade-off between sensitivity and specificity. During this study, ROC curves were used to define a threshold value above which an unknown serum sample could be defined as being positive for P. ovis infestation. This threshold was obtained by plotting a ROC curve of the OD₄₅₀ values for the known P. ovis-infested samples (n = 58) against the known P. ovis-uninfested samples (n = 433). The area under the ROC curve (AUC) was calculated to provide a good representation of how well the test performed [16]. The optimal threshold was then calculated from the ROC curve by selecting the OD₄₅₀ value that maximized the sum of sensitivity and specificity. The ROC analysis described above was performed using the Analyse-it plug-in within Microsoft Excel (Analyse-it, v 2.21, Analyse-it Ltd).

In order to ensure that this sample of individuals was representative of the population as a whole, a further 20 known P. ovis-infested and 20 P. ovis-uninfested sheep were sampled and tested with the ELISA (Groups J and I, respectively). The statistical properties of these corrected OD₄₅₀ values were compared to those of the known P. ovis-uninfested (Group C) and P. ovis-infested sheep (Groups B and D combined) using both a two-sample Wilcoxon test and a Kolmogorov–Smirnov test. Both tests compare distributions non-parametrically; the first compares position only, the second, both position and distributional shape. These analyses were performed in the statistical analysis package R [15].

3. Results

3.1. Receiver operator characteristic (ROC) curve analysis

Table 1 describes the samples used for each set of analyses, with 433 P. ovis-uninfested samples (Group C) and 58 P. ovis-infested samples (Groups B and D combined). The ROC curve is presented in Fig. 1a and test sensitivity and specificity (at a range of cut-off values) can be seen in Fig. 1b. The accuracy of the ELISA depends on its ability to delineate test-positive from test-negative samples. Accuracy is measured by the area under the ROC curve (AUC), with an AUC of 1 representing a perfectly performing test with 100% sensitivity and 100% specificity; in contrast, an AUC of 0.5 represents a worthless test with discrimination between positive and negative values no better than a random guess. Traditionally, the accuracy of a diagnostic test is classified using the following criteria: AUC = 0.9–1.0 (Excellent), 0.8–0.9 (Good), 0.7–0.8 (Fair), 0.6–0.7 (Poor), 0.5–0.6 (No discrimination) [17]. The AUC for the rPso o 2 ELISA was 0.97, indicating that the test has an excellent level of discrimination between test-positive and test-negative results.

Diagnostic tests based on continuous outputs, such as optical density (OD), require a cut-off value to be defined which optimises some utility function [16]. In this study we sought to minimise (1-specificity) or false negative (FN) rate + (1-sensitivity) or false positive (FP) rate, where se and sp are the sensitivity and specificity, respectively, at a defined cut-off. This can be thought of as minimising the proportion of misdiagnosis, when the numbers of the truly test-positive and truly test-negative sheep are identical. We are implicitly assuming that the cost of each type of misclassification (false positive or false negative) is identical. Using this definition, the optimal cut-off for the present assay is at an adjusted OD₄₅₀ value of >0.06 AU. At this point, the sensitivity of the test is 0.93, and the specificity is 0.90, meaning that in a sample which is equally “balanced” between test-positive and test-negative sheep, 3.5% would represent false negative (FN) and 4.9% false positive (FP) test results. For comparison, if we were to increase the cut-off to an OD₄₅₀ value of 0.1 AU, then the specificity would be 0.83, and the sensitivity 0.98, meaning that 8.5% of test results would be FN and 0.8% would be FP.

Comparison of the original data from experimental infestations with two additional groups of samples (Groups I and J) consisting of 20 P. ovis-uninfested and 20 P. ovis-infested sheep indicated that there was no evidence that the position and distribution of the OD₄₅₀ values from positive sheep were not identical (Wilcoxon test p = 0.91, Kolmogorov–Smirnov test p = 0.999). However, the P. ovis-uninfested sheep in the original experimental group (Group B) had statistically significantly higher OD₄₅₀ values, on average, than the group of 20 (Wilcoxon test p < 0.001, Kolmogorov–Smirnov test p < 0.001). This result indicates that the test was at least as discriminatory in this second confirmatory group of 40 sheep, compared with the larger group used for the ROC analysis. Using the optimised OD₄₅₀ cut-off of >0.06 AU, all 20 P. ovis-uninfested sheep (Group I) were correctly...
diagnosed, whilst 18 of 20 P. ovis-infested sheep (Group J) were correctly identified. These results were not statistically significantly different from those expected using the estimates of sensitivity and specificity stated above (p = 0.65 and 0.25, respectively, using an exact binomial test).

3.2. Longitudinal sampling of experimental sheep

The results of longitudinal sampling of a pilot group of six experimentally-infested sheep are shown in Fig. 2a. Sheep were sampled prior to infestation with P. ovis (t = 0, Group K0) and then weekly (weeks 1–6 post-infestation, Groups K1–K6 respectively). Antibody levels represented by OD450 values >0.06 AU were demonstrated in four of the six sheep just one week post-infestation (Group K1, mean OD450 = 0.13, range = 0.02–0.23), in four out of the six sheep two weeks post-infestation, with one sheep being marginal (Group K2, mean OD450 = 0.16, range = 0.02–0.38), in four sheep from three weeks post-infestation, with two sheep being marginal (Group K3, mean OD450 = 0.22, range = 0.06–0.47), and in all sheep by four weeks post-infestation (Group K4, mean OD450 = 0.27, range = 0.17–0.45). Two sheep demonstrated a decrease in OD450 values between weeks 2 and 3, but all had higher OD450 levels at week 6, with OD450 values ranging from 0.32 to 0.78 AU and a statistically significant increase in mean OD450 levels (p = 0.04). The group mean increased each week post-infestation (Fig. 2a).

3.3. Specificity

Further samples from experimentally P. ovis-infested sheep (Group B), clinically confirmed P. ovis-infested sheep from natural
outbreaks (Group D). *P. ovis*-uninfested sheep associated with those outbreaks (‘in contact’, Group E), confirmed *P. ovis*-naïve sheep (Group C) and sheep infected with other parasites, but *P. ovis*-naïve from the field (Groups F, G and H) were assayed, as detailed in Table 1, with the specificity of the ELISA illustrated in Fig. 2b. Statistically significant differences in ELISA OD450 values (p < 0.05) were demonstrated between the *P. ovis*-infested (Groups B and D) and *P. ovis*-uninfested groups (Groups A, C, G and H). OD450 values for *P. ovis*-infested field samples (Group D, mean = 0.38, range = 0.09–0.97) were lower than those from experimentally infested sheep (Group B, mean = 0.43, range = 0.15–1.1) although this might be accounted for by differences between natural and experimental infestations, where a fixed number of mites (n = 20–50) are placed directly on to the skin, in a controlled environment and in an area that prevents their removal through self grooming behaviour, thus leading to well-defined lesions. No statistically significant differences were demonstrated between the OD450 values of *P. ovis*-free lambs (Group C) and those infected with GIN or chewing lice and samples from Soay sheep. Although sera from these groups (F, G and H) exhibited OD450 readings of up to 0.09 AU (Group maximum values = 0.09, 0.07 and 0.08, respectively) this only occurred in two (G, H) or three (F) sheep per group, indicating a lack of cross reactivity to antigens from other common endo- and ecto-parasites of sheep. There was, however, a significant difference (p < 0.05) between the OD450 values of samples from known *P. ovis*-free lambs and those taken from lambs from an infested flock but not showing clinical signs of infestation at the time of sampling (Group E). OD450 values for these “in contact” sheep were generally lower (with a mean of 0.13) than those of the clinical cases (mean = 0.34), but exhibited a wider range of OD450 values than the *P. ovis*-free samples (ranges = 0.01–0.44 and 0.01–0.1, respectively). The differences in OD450 values between this ‘in contact’ group (Group E) and those exhibiting clear clinical signs of infestation, from either experimental or field cases (Groups B and D, respectively), were therefore not statistically significant. Nor were there statistically significant differences between Group E and the samples from lice and GIN-infected sheep (Groups F and G). However, there was a statistically significant difference between Group E and the samples from the Soay sheep (Group H), since these latter samples resulted in particularly low OD450 values (range = 0.00–0.08).

3.4. Evaluation of intra- and inter-assay variability

Intraplate repeatability, as measured by the coefficient of variation (CV), was assessed by analysing the positive control in duplicate, 30 times on one plate. The estimated CV was less than 3%, demonstrating a high repeatability for the assay. In order to assess the variability of assay performance associated with different operators, 30 serum samples from *P. ovis*-infested and *P. ovis*-uninfested sheep were tested independently by two additional, independent operators. The samples chosen consisted of 10 known *P. ovis*-infested from Group B, 5 ‘in contact’ cases (Group E), and 15 *P. ovis*-uninfested samples (Group A). This validation experiment was performed in a blinded fashion and the two additional operators were not informed of the sample status until after the assay, which was performed according to a Standard Operating Procedure produced by the authors. Fleiss’s Kappa value for the three independent operators was 0.74 (p < 0.001). A Kappa value of between 0.61 and 0.8 represents substantial agreement between operators; therefore a Kappa value of 0.74 indicates a good degree of agreement between the operators for the test set especially given that this estimate confounds any inter-operator and between plate variability.

4. Discussion

Here we describe, for the first time, a sensitive and specific diagnostic ELISA for the detection of antibodies specific to the sheep scab antigen *Pso o 2*, employing a recombinant protein (r*Pso o 2*). The ability to detect specific antibody in the blood of affected sheep demonstrates that this assay will provide a useful tool to monitor the spread and incidence of sheep scab.

The present study has employed a recombinant form of a mite protein *Pso o 2* [9]. Previous assays have been developed for the detection of antibodies against *Psoroptes* spp. mites in sheep, cattle and rabbits [18–24]. These assays have been based on crude extracts of *P. ovis* and therefore carry a potentially greater risk of non-uniformity and reduced specificity due to cross-reactive antigens compared with a test incorporating a single, defined antigen, as described here. These non-specific ‘cross-reactive’ antigens might lead to an increase in FP results, which for sheep scab, would be far from ideal, since even a small number of sheep testing positive could result in the unnecessary treatment of the entire flock. This would increase costs, and add to the pressure for development of resistance for both mites and G1 nematodes. However, in an extensive study using a native antigen-based ELISA, high sensitivity (93.7%) was shown in the detection of *P. ovis*-infested samples from 52 flocks, with a specificity of 96.5% in 254 sheep originating from *P. ovis*-uninfested flocks [23]. Although these values are similar to those reported here using a defined antigen, the major advantage of the ELISA described herein is that reproducibility utilising a recombinant protein is likely to be greater than that achievable with complex mixtures of antigens. In addition, the generation of *P. ovis* extracts for use in ELISA requires the availability of naturally or experimentally *P. ovis* infested sheep to provide a source of antigen, as *P. ovis* cannot currently be maintained in vitro. The in vivo maintenance of mites requires considerable expense and can involve animal welfare issues. In terms of reproducibility, crude extracts of *P. ovis* contain potent proteolytic enzymes [25] which might affect the integrity of diagnostic antigens, resulting in batch variability and reduced assay performance. Thus, the use of a recombinant protein is a major advantage in diagnostic tests, in terms of reproducibility and the potential to generate sufficient quantities for widespread routine and commercial use.

All samples tested using the *Pso o 2*-based ELISA indicated a high degree of specificity; at the OD450 cut-off value of >0.06 AU few FPs (5 out of 53 tested) were detected in samples taken from sheep known to be infected with gastrointestinal nematodes or lice (Groups F and G combined). Only 2 FPs were detected in 52 samples from Soay sheep maintained on St Kilda (Group H). This suggests that the antigenic epitopes to *Pso o 2* are either restricted to *P. ovis*, or if present in other parasites, are sufficiently different to not be recognised by the ovine immune response. However, it is acknowledged that these tests for specificity were limited due to availability of serum samples from alternative parasitic infestations, and further analysis may be required. In particular, samples are required from sheep infested with *Chorioptes bovis* (Acari: Psoroptidae), since these mites are related to *Psoroptes* spp. and the lesions (chorioptic mange) caused by *C. bovis* can be similar to those of sheep scab [26]. Moreover, an ELISA based on *P. ovis* extracts as the antigen has been shown to cross react with sera from sheep with chorioptic mange [27] demonstrating the existence of cross-reactive antigens. Although less prevalent than sheep scab, the incidence of chorioptic mange in sheep is increasing in areas where sheep scab control has been deregulated and, at present, the only way of distinguishing the two mite species is by their morphology [28]. It is, however, reassuring that the recombinant *Pso o 2* used in the ELISA described here shares only 48% amino acid sequence identity to its closest homologue in the public databases [Lep d 2 from the...
astigmatid mite *Lepidoglyphus destructor* (Acari: *Glycyphagidae*) and that antiseraum raised in rabbits to *P. ovis* o 2 did not cross react with native *Der p* 2 (39% amino acid identity) from the house dust mite (*Dermatophagoides pteronyssinus*) (data not shown).

An important aspect of the present study is the early detection of specific antibodies against *Pso o 2* in experimentally infested sheep. Indeed, these antibodies were detected in the majority of sheep two weeks post-infestation; this is similar to the timings reported in the previously described assay [23]. This early diagnosis is important, since the detection of sub-clinical infestation is an important attribute of any test that is to be employed in the control of sheep scab. Although the assay described here indicates the detection of antibodies in sheep from a field outbreak, which were asymptomatic but from an infested flock (Group E), only limited samples were available and further samples will be sought to confirm this finding. The inference from this result is that the generation of specific anti-*Pso o 2* antibodies precedes overt clinical manifestations in the field. That specific antibodies are generated to sheep scab prior to the appearance of clinical signs has been shown previously in 49% of 70 clinically normal (unaffected) sheep from sheep scab prior to the appearance of clinical signs has been shown with the current ELISA test.

Our study supports the view that antibodies to *Pso o 2* also precede clinical symptoms (e.g. apparent lesions, discoloured fleece, or behavioural indications including scratching or rubbing the infested area and head tossing), because, at two weeks following experimental infestation, these signs were minimal or not apparent. Two weeks after infestation, the developing lesions are small (<2 cm in diameter), and only apparent after close inspection of the sheep. Thus, this assay has the potential to be employed to detect early or sub-clinical sheep scab, and therefore may have wide utility in the monitoring and control of sheep scab. Indeed, a previous study in Switzerland successfully utilised an ELISA to identify and select sheep for treatment for sheep scab, providing good evidence that a targeted approach to control could be beneficial in terms of the reduction in disease and improvement in animal welfare, as well as in limiting the use of endectocides [27].

The movement of sheep, for example those being sent to market or being introduced into other flocks, is a potential source of infestation and the ability to reliably detect sub-clinical infestation with *P. ovis* will be an important step in control. Similarly, with the potential for acaricide resistance to develop, confirmation of successful treatment is required. The latter will almost certainly require a ‘two test’ approach, where a decline in antibody levels is demonstrated, since circulating IgG antibodies may persist for weeks or months and only slowly decline after infestation has been successfully treated [23,29]. In general, although ELISA tests have advantages over traditional methods of sheep scab diagnosis in terms of sensitivity and quantification of antibody, an obvious improvement in terms of ease of use and rapidity of result would be to further develop the current assay to a ‘pen-side’ format where the results could be determined in minutes rather than hours. Lateral flow assays are routinely used for the detection of a number of diseases and conditions in sheep and humans. Recombinant proteins provide an ideal target for such assays, enabling the generation of materials in sufficient quantity, uniformity, purity and stability for a diagnostic test in widespread use. Currently, we are in the process of investigating the potential of using r*Pso o 2* in a lateral flow format, although its value, in terms of diagnosis, will depend on the relative sensitivity, performance and cost compared with the current ELISA test.

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**References**


