Validation of the interferon-γ test for diagnosis of ovine Johne’s disease: sensitivity and specificity field trials


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SUMMARY

Field trials are underway to validate the interferon-γ test for diagnosis of ovine Johne’s disease prior to the test being considered for endorsement and adoption as an official diagnostic test in Australia. The test kits (BOVIGAM™, PARACHEK™) and Johnin PPD for these trials are being provided by CSL Limited. The interferon-γ test specificity trials will involve 6 flocks of Merino sheep on uninfected properties in New South Wales, Victoria, Western Australia and South Australia. Specificity trials on 3 flocks with 120 sheep and 3 age classes (adult ewes, yearlings and weaned lambs) in each flock have been completed. An interferon-γ test sensitivity trial has been conducted in collaboration with NSW Agriculture on an infected commercial Merino flock, containing 145 ewes, with follow-up diagnostic bacterial culture and histopathology at slaughter. Estimated sensitivity was about 67 % and 52 % for the cut-points Johnin PPD minus Nil antigen (PBS) ≥ 0.05 and Johnin PPD minus Avian PPD ≥ 0.05, respectively. The predictive value for a positive result was 55 % and 70 % for these cut-points. In 3 uninfected flocks and the longitudinal trial flock, specificity was > 98.3 %. In the sensitivity trial, the relatively high number of false positives indicated that either the reference standards for detection of infection were relatively insensitive or more probably that the interferon-γ memory response may persist in sheep that have completely eliminated the infection. Since the interferon-γ test had high specificity in unexposed sheep, it is likely that the positive interferon responses, in the tissue culture negative and or histopathologically negative sheep, detected prior exposure in sheep that are no longer infected. The interferon-γ test is also being evaluated, in collaboration with the University of Sydney, in a 3-year longitudinal study on a naturally infected experimental Merino flock of sheep. This trial, which has only just commenced, will provide information on the interferon-γ responses of 429 sheep at 6-monthly intervals during the trial period and their final infection status following autopsy.

INTRODUCTION

The interferon-γ test is being evaluated, in a project funded by Meat and Livestock Australia, to establish the specificity and sensitivity of the assay in unexposed flocks and sheep flocks infected with ovine Johne’s disease (OJD), respectively to determine a possible application for the test in the national control program. The interferon-γ test has the potential to identify infected sheep prior to commencement of bacterial shedding, pasture contamination and exposure of other livestock to infection since a cell-mediated immune response occurs before shedding and seroconversion. The results of research in Spain, Australia and New Zealand provide evidence that the interferon-γ test in its current format may be useful for diagnosis of OJD (1-3,6,7,9). This paper reports preliminary findings on estimates of the sensitivity and specificity for the interferon-γ test in Australian Merino sheep.
MATERIALS AND METHODS

Sensitivity trial.

Animals and collection of samples. The flock consisted of 146 adult pregnant Merino ewes aged approximately 3.5 years which also were part of another project run by NSW Agriculture. The flock was located on a property in the central tablelands of New South Wales near Goulbourn and was a known infected flock exposed since birth. The faecal shedding rate of the flock was approximately 10 %. Heparinised blood for interferon-γ testing and blood for serum for the absorbed ELISA were collected from individual sheep prior to slaughter. Sample collection and slaughter were performed by NSW Agriculture. The blood and tissue samples were then transported to Elizabeth Macarthur Agricultural Institute (EMAI). Tissue samples from 4 sites in the gastrointestinal tract and associated lymph nodes were taken from each sheep for tissue culture and histopathology.

Culture of tissues and histopathology. Culture of tissues was undertaken at EMAI in radiometric medium and the identity of Mycobacterium avium subsp. paratuberculosis (Map) was confirmed by the polymerase chain reaction and growth on modified Middlebrook 7H10 medium with mycobactin J (11,12). The results were then recorded as positive or negative. Histopathology was performed at EMAI and the results were defined as positive or negative according to previous criteria (8). The histopathological lesions were also scored as mild (Perez score 1-2) (5) or moderate (Perez score 3a-3c) (5).

Specificity trial.

The Merino flocks were selected on the basis of the following selection criteria to ensure that there was a high probability of freedom from OJD. The flocks were to be located in regions of low prevalence of OJD i.e. control, protected or free zones. The flocks should preferably be enrolled in the OJD market assurance program (SheepMAP). There should be no history of OJD in neighboring flocks. The selected flocks should be situated in widely separated geographic locations so as to obtain exposure to cross-reactive microorganisms from different environments. However, the property must be close enough to CSIRO Livestock Industries (CLI), Geelong or a state laboratory to enable processing of samples within 6 h. In order to obtain a 95 % binomial confidence interval of greater than 97 % for a specificity of 99 %, sampling of 6 flocks each with 120 sheep per flock was required, each flock consisting of 40 lambs, 40 yearlings and 40 adult 3-year-old ewes. Three of the flocks were to be located in NSW and one each in Western Australia, Victoria and South Australia. Heparinised blood for interferon-γ testing and blood for serum for the absorbed ELISA were collected from individual sheep on 2 properties in 2 NSW Rural Lands Protection Boards (RLPB)(Riverina and Dubbo) and one property in Western Australia. To assist confirmation of freedom from OJD, pooled faecal cultures (10) were performed on the 120 sheep with 6 pools of 20 sheep by NSW Agriculture (EMAI and the Regional Veterinary Laboratory, Orange) and the Western Australian Department of Agriculture, respectively.

Longitudinal trial

The interferon-γ test is being evaluated in a 3-year longitudinal study on a naturally infected experimental Merino flock in collaboration with the University of Sydney. This trial is part of another project run by the University, examining the effect of age of sheep (lamb/weaners, yearlings, adult ewes) and exposure to different levels of pasture contamination and has just commenced. The infection status of these animals will be monitored during the trial by faecal culture and serology. Interferon-γ responses of 429 sheep at 6-monthly intervals will be monitored during the trial period and their final infection status determined following autopsy. The sheep were bled prior to being placed in their respective paddocks and exposed to infected donors. The bloods were transported by air freight and road transport to the CLI Geelong laboratory arriving approximately 7 h after the completion of bleeding when stimulation of bloods for the interferon-γ test were undertaken.

Interferon-γ test. BOVIGAM kits, phosphate-buffered saline (PBS, nil antigen), M. avium (avian) PPD and M. paratuberculosis (Johnin) PPD were provided by CSL Limited. The Johnin PPD, provided by CSL Limited, was from 2 different sources (CSL A Johnin and CSL B Johnin). Stimulation of 1.5 ml blood samples was done with nil antigen, avian PPD and Johnin. For the specificity trial, CSL A Johnin was used. For the specificity trials, positive control stimulation was also performed with the mitogens, concanavalin A or poke weed mitogen. In 2 flocks (Riverina and WA), CSL A Johnin was compared with CSL B Johnin. For the Dubbo flock and the longitudinal trial sheep, CSL B Johnin was used. The avian PPD was used at a final concentration of 20 µg/ml, the CSL A Johnin (1.5 mg/ml protein nitrogen) was diluted 1/8 and the CSL B Johnin (2 mg/ml protein nitrogen) 1/8 prior to adding 100 µl to the 1.5 ml blood aliquots. The cultures were incubated.
for 19 h at 37 °C and humidified 5 % CO₂ and air or air only. The flock at Dubbo was too far away from the CLI Geelong laboratory for the blood samples to reach the laboratory within 6 h so that the blood stimulation and culture phase was performed at RVL, Orange. The harvested plasma samples were then transported on ice to the CLI laboratory, Geelong. For the WA flock, the interferon-γ test was performed by the WA Department of Agriculture. The interferon-γ ELISA (BOVIGAM™, CSL) was performed in duplicate according to the manufacturer's instructions. The interpretation criteria included; the mean OD values for the Johnin stimulation minus the nil antigen mean optical densities with a difference of 0.05 being required for a positive result (J-N ≥ 0.05); the OD values for J-N minus the mean avian PPD OD following subtraction of nil antigen background OD from the latter (J-A ≥ 0.05); J-N ≥ 0.05 and J-A ≥ 0.05 in parallel.

**Absorbed ELISA.** The ELISA antibody assay was performed on sera using PARACHEK™. The absorbed ELISA cut-point was evaluated as the OD of the negative control plus 0.2 (4).

### RESULTS

**Sensitivity trial**

Testing with the interferon-γ and absorbed ELISA assays were performed blind. The results for these tests were then provided to an independent decoder for matching with the key-list of sheep tag numbers and the results for culture and histopathology. Results for 145 instead of 146 sheep were analysed since there were no culture/histopathology results for one sheep.

**Culture and histopathology.** Of 145 animals examined, 28 (19.3 %) sheep were positive on both tissue culture and histopathology, 8 (5.5 %) were negative on tissue culture and positive on histopathology and 18 (12.4 %) were positive on tissue culture but negative on histopathology. There were 46 (31.7 %) culture test positives, 36 (24.8 %) histopathological test positives and 54 (37.2 %) sheep that were positive to either reference test (Table 1). A total of 91 (62.7 %)

**Table 1. Estimated sensitivity of the interferon test in an OJD infected Merino flock of 145 sheep.**

<table>
<thead>
<tr>
<th>Reference standard</th>
<th>Number</th>
<th>J-N &gt;= 0.05</th>
<th>J-A &gt;= 0.05</th>
<th>J-N or J-A &gt;=0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture +ve</td>
<td>46</td>
<td>30</td>
<td>65</td>
<td>49.8 - 78.6</td>
</tr>
<tr>
<td>Histology +ve</td>
<td>36</td>
<td>29</td>
<td>81</td>
<td>64.0 - 91.8</td>
</tr>
<tr>
<td>Culture or histology +ve</td>
<td>54</td>
<td>36</td>
<td>67</td>
<td>52.5 - 78.9</td>
</tr>
<tr>
<td>Perez score 1-2</td>
<td>15</td>
<td>11</td>
<td>73</td>
<td>44.9 - 92.2</td>
</tr>
<tr>
<td>Perez score 3a-3c</td>
<td>18</td>
<td>15</td>
<td>83</td>
<td>58.6 - 96.4</td>
</tr>
<tr>
<td>Total IFN +ves</td>
<td>66</td>
<td>40</td>
<td>67</td>
<td>52.0 - 80.5</td>
</tr>
</tbody>
</table>

CI 95% binomial interval confidence.

For J-N ≥ 0.05, 66 (45.5 %) of the 145 samples were positive, compared to 40 (27.6 %) for J-A ≥ 0.05 (Table 1). J-N values were generally greater than the corresponding J-A values, except at very low readings. Only one sheep was negative for J-N ≥ 0.05 and positive for J-A ≥ 0.05, compared to 27 samples negative for J-A ≥ 0.05 and positive for J-N ≥ 0.05. This sheep was culture-positive for OJD but negative on histology, and had borderline readings of 0.045 and 0.05 for J-N and J-A, respectively. There was a strong correlation between the results for J-N and J-A (r² = 0.90, 95 % CI = 0.86 – 0.92, F statistic = 1246).

The reference standard for estimating sensitivity of the interferon-γ test was the comparison with culture and histopathology test positives interpreted in parallel (Table 1). Estimated sensitivity was higher for J-N ≥ 0.05 (67 %) than for J-A ≥ 0.05 (52 %). Sensitivity was only 39 % for J-N ≥ 0.05 in animals that were positive on culture only (culture positive/histopathology negative), compared to 65 % for all culture positives (culture positive/histopathology negative and culture positive/histopathology positive) and 81% for all histopathological positives (histopathology positive/culture positive and histopathology negative).
positive/culture negative). The sensitivity estimate of 69 % if either J-N or J-A or both ≥ 0.05 were used as the cut-point was almost identical to that for J-N ≥ 0.05 alone (Table 1).

For both the J-N ≥ 0.05 and J-A ≥ 0.05 cut-points, respectively, estimated sensitivity of the interferon-γ test was higher for histopathologically positive animals (81 % and 64 %) than for culture positives (65 % and 54 %), although differences were not statistically significant (Chi-squared test, p > 0.10)(Table 1). Sensitivity of the interferon-γ test was slightly higher for moderate-severe histopathological lesions, compared to mild lesions for both cut-points, although these differences were also not statistically significant (Fisher’s exact test, p = 0.10). The proportion of ELISA antibody test positives in lesion score categories 1, 2, 3a, 3b and 3c were 1/9, 0/6, 2/7, 1/5 and 3/6, respectively. Three sheep, one of which was antibody positive, had histopathological lesions in the mesenteric lymph nodes only and were not scored. There were 2 antibody test positive and culture positive sheep that were histopathologically negative and 4 antibody test positive animals that were negative on culture and histopathology. Apparent specificity of the ELISA was 95.6 % for the cut-point used. Some or all of the 4.4 % antibody false positives may be truly infected due to failure of the reference tests to detect infection.

Table 2. Estimated sensitivity for the absorbed ELISA in an OJD infected Merino flock of 145 sheep.

<table>
<thead>
<tr>
<th>Reference standard</th>
<th>Number</th>
<th>ELISA +ve</th>
<th>Sensitivity %</th>
<th>CI1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture +ve</td>
<td>46</td>
<td>8</td>
<td>17</td>
<td>7.8 - 31.4</td>
</tr>
<tr>
<td>Histology +ve</td>
<td>36</td>
<td>8</td>
<td>22</td>
<td>10.1 - 39.2</td>
</tr>
<tr>
<td>Culture or histology +ve</td>
<td>54</td>
<td>10</td>
<td>19</td>
<td>9.3 - 31.4</td>
</tr>
<tr>
<td>Perez score 1-2</td>
<td>15</td>
<td>1</td>
<td>7</td>
<td>0.2 - 31.9</td>
</tr>
<tr>
<td>Perez score 3a-3c</td>
<td>18</td>
<td>6</td>
<td>33</td>
<td>13.3 - 59.0</td>
</tr>
<tr>
<td>Total ELISA +ves</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 CI 95% binomial confidence interval.

Specificity trial

Interferon-γ test. The results are shown in Table 3 for the 3 flocks so far tested. With CSL A Johnin and using the cut-points J-N ≥ 0.05 or J-A ≥ 0.05 for the Riverina, NSW flock the specificity of the interferon-γ test was 99.2 % and 100 %. Specificity was slightly lower for this flock with CSL B Johnin (J-N ≥ 0.05 98.3 %, J-A ≥ 0.05 98.3 %). A total of 88 % of the 120 sheep had a positive interferon-γ response to poke weed mitogen (Mitogen-Nil ≥ 0.05). For the Western Australian flock, with CSL A Johnin, the corresponding specificities were 92.5 % and 99.2 %, respectively but with CSL B Johnin, specificity was slightly higher for the J-N ≥ 0.05 cut-point (95.8 %) and lower for J-A ≥ 0.05 (98.3 %). Of the 120 sheep, 55 % responded to the mitogen, concanavalin A (Mitogen-Nil ≥ 0.05). Only CSL B Johnin was available for the Dubbo trial. Specificity for J-N ≥ 0.05 or J-A ≥ 0.05 was 98.3 % or 99.2 %. The poke weed mitogen response rate was 93 %.

Absorbed ELISA. In the 2 uninfected flocks so far tested, specificity of the absorbed ELISA
was 99.2 % (119/120) and 100 %. The reactor in the first flock was in the yearling ewe group and had a mean OD of 0.362.

**Longitudinal trial**

*Interferon-γ test.* CSL B Johnin was used for the longitudinal trial. Specificity for J-N ≥ 0.05 or J-A ≥ 0.05 was 99.1 % and 100 %, respectively (Table 3). The poke weed mitogen response rate (Mitogen-Nil ≥ 0.05) was 63 %.

### DISCUSSION

The sensitivity of the interferon-γ test was relatively high with estimates approximating 67 % and 52 % for the cut-points of J-N ≥ 0.05 and J-A ≥ 0.05, respectively, in comparison to the combined reference standards, histopathology and tissue culture. In a previous comparison with histopathological findings, Perez *et al.* (6) obtained a sensitivity of 52.5 % for the interferon-γ test with an index value of A/N ≥ 1.5. In the present study, estimated sensitivities for J-N ≥ 0.05 and J-A ≥ 0.05 were 81 % and 64 %, respectively using histopathology test positive results as the reference standard.

Perez *et al.* (6) reported an association between cell-mediated immune (CMI) responses and histopathological lesion type. Sheep with type 1 lesions (focal and without acid fast bacteria) and type 3c lesions (diffuse with high numbers of lymphocytes and few or no AFB) were mostly positive to the interferon-γ and skin tests. There was a substantially lower percentage of CMI positive sheep with 3b lesions (diffuse and large numbers of macrophages in the lamina propria with numerous AFB). In the present study there was a higher proportion of interferon-γ test positive sheep with severe histopathological lesions (3a-3c) than mild (score 1 and 2) suggesting a higher test sensitivity in animals with more severe lesions. However, this difference was not statistically significant and further studies are required to confirm an association. There was also a relatively low number of animals in each lesion score category as well as different criteria for interpretation of the interferon-γ test for a valid comparison with that of Perez *et al.* (6).

Depending on the interpretation criteria, the results of specificity trials in 3 uninfected flocks and in a pre-exposed flock in

<table>
<thead>
<tr>
<th>Cut-point</th>
<th>Riverina, NSW flock</th>
<th>WA flock</th>
<th>CI</th>
<th>Dubbo, NSW flock</th>
<th>CI</th>
<th>Longitudinal trial</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-N &gt;= 0.05</td>
<td>100</td>
<td>97-100</td>
<td>95</td>
<td>89.4-98.1</td>
<td>99.2</td>
<td>95.4-100</td>
<td>98.3</td>
</tr>
<tr>
<td>J-N &gt;= 0.05</td>
<td>99.2</td>
<td>95.4-100</td>
<td>92.5</td>
<td>86.2-96.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>J-A &gt;= 0.05</td>
<td>100</td>
<td>97-100</td>
<td>99.2</td>
<td>95.4-100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>J-N &gt;= 0.05</td>
<td>98.3</td>
<td>94.1-99.8</td>
<td>95.8</td>
<td>90.5-98.6</td>
<td>98.3</td>
<td>94.1-99.8</td>
<td>99.1</td>
</tr>
<tr>
<td>J-A &gt;= 0.05</td>
<td>98.3</td>
<td>94.1-99.8</td>
<td>98.3</td>
<td>94.1-99.8</td>
<td>99.2</td>
<td>95.4-100</td>
<td>100</td>
</tr>
<tr>
<td>Mitogen-N &gt;= 0.05</td>
<td>88</td>
<td>55</td>
<td>92.9</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 CSL A Johnin PPD.
2 CSL B Johnin PPD.
3 CI 95% binomial confidence interval.
culture or histopathology. It is assumed that the sensitivity of the combined reference tests approaches 100% but this has not been established due to the absence of an appropriate gold standard. It is also assumed that histopathologically positive lesions with no AFB (Perez type 1 and 3c) are initial or latent infections although this may overestimate the number of true positives if the cellular accumulations remain following elimination of Map from these sheep.

Secondly, it is highly probable that the CMI recall response may persist in sheep that have completely eliminated the infection. Thus this CMI memory may indicate not only current infection but exposure to past infection which has completely resolved. Since the interferon-γ test had high specificity in unexposed sheep, it is likely that the positive interferon responses in the tissue culture negative and/or histopathologically negative sheep detected prior exposure in sheep which are no longer infected.

CONCLUSIONS

The interferon-γ test has relatively high sensitivity (52-69 % depending on the cut-point) and specificity (>98 %) for Map infection and thus shows promise as a screening test for detection of exposure to OJD and for certifying flock freedom from disease. Further sensitivity and specificity trials are being undertaken to provide additional information on the diagnostic accuracy of the test and the most appropriate cut-point before consideration of its standard use in OJD control programs.

ACKNOWLEDGEMENTS

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REFERENCES


