Blood culture and stimulation conditions for the diagnosis of tuberculosis in cervids by the Cervigam assay


Mitogen- and antigen-induced interferon-γ (IFN-γ) responses of peripheral blood leucocytes from cervids were evaluated by a commercial whole-blood assay. The assay was applied to Mycobacterium bovis-infected white-tailed deer and reindeer, M. bovis BCG-vaccinated white-tailed deer and elk, and unvaccinated, uninfected white-tailed deer, fallow deer, elk and reindeer. The responses of the M. bovis-infected white-tailed deer to pokeweed mitogen (PWM) varied with time and between individuals. The responses of the M. bovis-infected reindeer to PWM and M. bovis purified protein derivative (PPD) were positively associated. Samples from tuberculosis-free captive herds in various parts of the USA were also evaluated. Four per cent of fallow deer, 20 per cent of elk, 44 per cent of white-tailed deer, and 91 per cent of reindeer had responses to PWM exceeding 0.25 Δ optical density, that is, PWM stimulation index no stimulus. The specificity of the responses to M. bovis PPD and a Mycobacterium tuberculosis complex-specific antigen (RTS6-CFP10), excluding animals not responding to PWM, ranged from 78 per cent to 100 per cent and was dependent upon the species and the positive response cut-off value. The results show that the commercial assay is valid for the detection of TB in reindeer; however, further development of the assay will be required before it is used in surveillance programmes for white-tailed deer, fallow deer, and elk.

Farmed deer constitute a significant alternative livestock industry, with numbers exceeding two million in New Zealand, one million in China, 500,000 in the USA, 400,000 in Russia and 100,000 in Canada (Canada and New Zealand, Canada, New Zealand, Europe and Asia). In the USA, the spread of bovine TB in captive herds is strongly associated with inter-herd and cross-country movements of infected deer (Essey and Koller 1994). M. bovis was diagnosed in 41 captive herds of deer in the USA between 1991 and 2003 (Massengill 2005, in 35 captive herds in Canada between 1989 and 1998 (Essey and Koller 1994, Massengill 2004) and in 41 of 14,842 farmed deer slaughtered in Ireland between 1993 and 1996 (Quigley and others 1997). TB has also been diagnosed in captive axis deer (Cervus axis) in India (Baska and others 1975), in elk (Cervus elaphus nelsoni) in Korea (Kim and others 2002) and sika deer in Japan (Itoh and others 1992) and China (Shiliang and Shanzi 1985).

As a result of a series of confirmed cases in the mid 1980s, TB was designated a 'notifiable' disease in farmed deer in Great Britain (R. de la Rua, personal communication). Similarly, a 1991 outbreak in captive elk, attributed to the importation of an infected elk, encouraged the government and industry leaders in the USA to draft a Uniform Methods and Rules for the Eradication of bovine TB in captive cervids (Essey and Koller 1994). In conjunction with the Deer Farmers Association and the Deer Branch of the New Zealand Veterinary Medical Association, the New Zealand Ministry of Agriculture and Fisheries adopted a voluntary TB control scheme in 1984 followed by a compulsory national TB control scheme in 1991 (de Lisle and others 2001). Such TB control campaigns have generally relied on test and removal, slaughterhouse surveillance, movement restrictions and/or wildlife reservoir control strategies. In the UK, USA and Canada, farmed deer are monitored by a combination of slaughter surveillance and skin-testing strategies. In the USA the programme is voluntary, but required for interstate transport and herd accreditation. In Canada, a negative TB test for each deer is required for a change of ownership or any movement, except to slaughter. In New Zealand, captive deer more than six months of age are monitored regularly by skin testing (approximately 700,000 were tested in 2004) and all deer are inspected for lesions.
at slaughter (approximately 600,000 were tested in 2004). In contrast with New Zealand, compliance with TB control strategies in many other countries is poor. In 2003, less than 6 per cent of approximately 500,000 captive deer in the USA were tested for TB and less than 1 per cent of 363 captive herds in the UK submitted to voluntary testing. The reasons for such poor compliance include inadequate handling facilities, injuries resulting from the handling required, the poor perception by owners of the specificity of skin testing in cervids, and in the USA, the reduction in interstate movements due to the restrictions imposed as a result of chronic wasting disease. Estimates of the sensitivity and specificity of skin tests for naturally infected red deer, fallow deer and white-tailed deer vary according to the study and species, with ranges from 80 to 84 per cent for sensitivity and 46 to 86 per cent for specificity (Palmer and others 2001); no estimates are available for naturally infected reindeer (Rangifer tarandus).

A blood-based TB test for initial surveillance might increase the compliance of deer farmers. Ancillary blood-based tests currently used in TB control programmes around the world include the fluorescence polarisation assay, ELISA and lateral flow antibody-based tests, in addition to assays based on interferon-γ (IFN-γ) for example, the Cervigam assay (Prionics) and lymphocyte-blastogenesis. Their use varies between countries and they are not widely utilised with cervids. The Cervigam assay is particularly attractive owing to the widespread acceptance of other assays based on IFN-γ, for example, Bovigam (Prionics) for cattle and Quantiferon Gold (Cellestis) for human beings.

The aim of this study was to evaluate the Cervigam assay for TB surveillance by applying it to samples from four species of captive cervid of significance to the national programme in the USA. Its objectives were to evaluate a positive standard as an indicator of the performance of the assay and to determine its specificity with samples from TB-free herds. Antigens used for the specificity studies include M bovis purified protein derivative (PPD), a complex antigen mixture and a fusion of recombinant Early Secreted Antigenic Target-6 kDa protein and Culture Filtrate Protein 10 (ESAT-6-CFP-10), generally a Mycobacterium tuberculosis-complex-specific antigen. Both antigens are commonly used in TB tests based on IFN-γ.

### MATERIALS AND METHODS

#### Animals, M bovis infection, and M bovis BCG vaccination

Eleven yearling white-tailed deer were given 300 colony forming units (cfu) M bovis strain 95-1315 by intratracheal instillation as described by Thacker and others (2006) and Palmer and others (2006), and 13 reindeer were given 1 x 10⁶ cfu of the same strain by the same route. Blood was collected monthly during the six months after the challenge for evaluation of responses to mitogen and antigen. Infection was confirmed postmortem in each animal by pathological examination and bacteriological culture of tissues (Palmer and others 2006; Thacker and others 2006). All the inoculated reindeer were classified as reactors to a comparative cervical skin test six weeks after the vaccination to evaluate the effects of delays in setting up the assay (to mimic overnight delivery) on the IFN-γ responses. All the vaccinated white-tailed deer were classified as reactors to a comparative cervical skin test four-and-a-half months after they were vaccinated, and all the elk reacted similarly after eight months.

For the specificity studies, that is the response of uninfected deer to M bovis antigens, samples were collected from TB-monitored herds with no history of TB. Samples were obtained from fallow deer in Iowa, North Carolina and Texas, from elk in Iowa, Minnesota, North Carolina, Wisconsin and Ontario Canada, from white-tailed deer in Iowa and Wisconsin, and from reindeer in Alaska, Colorado, Iowa, Michigan, Nebraska, Ohio, North Dakota, Oregon, Pennsylvania, Tennessee and Wisconsin. The samples from Iowa were processed immediately and those from the other states within 24 hours of collection.

#### Whole blood cultures and analysis of IFN-γ production

Heparinised blood was dispensed in 1.5 ml aliquots into individual wells of a 24-well plate ( Falcon 353047; Becton Dickinson). Treatments included no stimulation (phosphate-buffered saline (PBS) only), and stimulation with 20 μg/ml Mycobacterium avium purified protein derivative (PPD) (Prionics), 20 μg/ml M bovis PPD (PPDbovis) (Prionics), 10 μg/ml ESAT-6-CFP-10, or mitogen (Table 1) in PBS as described by Palmer and others (2004) and Waters and others (2006). Several mitogens, including phytohaemagglutinin, concanavalin-A and pokeweed mitogen (PWM) were evaluated for use as polyclonal stimulators of IFN-γ secretion by blood leucocytes from elk and reindeer (Table 1). The mitogens were not titrated for the samples from fallow deer owing to the limited volume of blood available for testing.

As a result of this comparison, PWM at 20 μg/ml was selected for use in subsequent studies. Diagnostic laboratories in the USA generally consider a PWM response of either 0.1 or 0.25 OD as indicative of a positive IFN-γ response, that is, with Bovigam and with a limited number of samples for Cervigam.

In addition to the standard assay procedure, blood tubes were held for eight or 24 hours before culture to determine...
The interpretation of the responses to antigen was based on methods commonly used in the USA for the Bovigam assay, and a limited number of tests with the Cervigam assay. With this method, the responses to PPDt or PWM must exceed the responses to both PPDt and PBS by a given cut-off (0.1 or 0.05 A OD) to be considered positive. The responses to the specific antigen (rESAT-6:CFP-10) were compared with the respective responses to PBS. The data are presented as the percentage of animals with responses to PPDt or rESAT-6:CFP-10 up to 0.1 or 0.5 A OD (Table 2).

**Statistical analysis**

The data were analysed by a one-way analysis of variance followed by a Tukey-Kramer multiple comparisons test, using a commercially available statistics programme (InStat 2.00; GraphPad Software). Pearson's product-moment correlations were computed between the IFN-γ concentrations in plasma stimulated with pokeweed mitogen or PPDt.

**RESULTS**

The magnitude of the responses to PWM was dependent upon the species of deer (Table 3). Ninety-one per cent of the reindeer had responses to PWM exceeding 0.25 A OD, whereas less than 44 per cent of the other deer species had responses to PWM exceeding 0.25 A OD. The response by fallow deer was particularly poor with less than 5 per cent having a response to PWM exceeding 0.25 A OD. The percentages of PWM responses exceeding 0.1 A OD were 22 per cent, 46 per cent, 56 per cent and 98 per cent for the fallow deer, elk, white-tailed deer and reindeer, respectively. The mean PWM responses by the white-tailed deer and reindeer from the TB-monitored herds were similar to those observed by Palmer and others (2004) and Waters and others (2006) in M bovis-infected and control deer.

The OD readings were standardised to units of ng/ml for longitudinal studies. The responses of the M bovis-infected white-tailed deer to PWM varied widely between individual deer and over time (Fig 1). Some deer had PWM responses that never exceeded 10 ng/ml (equivalent to approximately 0.2 A OD) (Fig 1a) and were categorised as low responders, but other deer had responses that exceeded 30 ng/ml (equivalent to approximately 0.4 A OD) (Fig 1c) and were categorised as high responders. Over time, the responses of individual deer to PWM ranged from 0 to 30 ng/ml. Similar variations in the responses of uninfected white-tailed deer to PWM were detected (data not shown).

The responses of the M bovis-infected reindeer and white-tailed deer to PWM and PPDt were positively correlated (P<0.0001) (Fig 2). However, the responses of the white-tailed deer were not as large, with numerous samples in the minimal response range, as indicated by the cluster in the lower left corner of Fig 2a. Thirty-five per cent of the infected white-tailed deer did not have responses to both PWM and M bovis PPD exceeding 0.25 A OD, indicated by the shaded box in Fig 2a.

Under field conditions, samples are routinely shipped overnight, so that there is a delay before they are analysed. However, delays of eight or 24 hours did not significantly affect the response to PWM or PPDt of white-tailed deer or elk vaccinated with M bovis BCG (Fig 3), in agreement with the results of studies with reindeer (Waters and others 2006). However, shipping by overnight courier may result in temperature fluctuations, excessive vibrations and atmospheric pressure changes. Also, caution is needed in the interpretation of these findings owing to limitations in the stimulation by PWM of samples from elk and white-tailed deer, and their modest response to vaccination. For white-tailed deer, fallow deer and reindeer, the ability to respond to PWM was not affected by overnight transport because the responses of samples processed immediately were comparable to those processed after being transported for approximately 24 hours. In contrast, 86 of the 104 samples from elk (83 per cent) that were shipped overnight failed to respond to PWM, compared with 12 of 19 (63 per cent) of those that were processed immediately.

Samples were obtained from TB-free herds across the USA and from Canada to evaluate the specificity of the test relative to PPDt and rESAT-6:CFP-10. Animals with PWM responses less than 0.25 A OD were excluded, as well as all the fallow deer because only 4-2 per cent of them had PWM responses exceeding 0.25 A OD. Table 3 shows that 20-3 per cent of elk, 43-9 per cent of white-tailed deer and 91-4 per cent of reindeer had responses to PWM exceeding 0.25 A OD. The inter-

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**TABLE 3: Differences between the interferon-γ responses of blood samples from various cervids stimulated with pokeweed mitogen or not stimulated (that is, phosphate-buffered saline), expressed as the differences in optical density (OD) determined by ELISA**

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Mean (se)</th>
<th>Median (range)</th>
<th>Δ OD &gt;0.25 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fallow deer</td>
<td>213</td>
<td>0.005 (0.02)</td>
<td>0.035 (0.3-0.637)</td>
<td>4.2</td>
</tr>
<tr>
<td>Elk</td>
<td>123</td>
<td>0.174 (0.02)</td>
<td>0.096 (0.1-1.354)</td>
<td>20.3</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>171</td>
<td>0.426 (0.06)</td>
<td>0.168 (0.2-2.862)</td>
<td>43.9</td>
</tr>
<tr>
<td>Reindeer</td>
<td>116</td>
<td>1.517 (0.08)</td>
<td>1.391 (0.120-3.260)</td>
<td>91.4</td>
</tr>
</tbody>
</table>

* Animals were from TB-monitored herds without any previous history of TB.
interpretation of the responses to antigen was based on methods commonly used in the USA for the Bovigam assay, and the results of a limited number of tests with the Cervigam assay. With this method, the responses to PPD-B must exceed the responses to both PPD-A and PBS by a given amount, that is 0.1 or 0.05 Δ OD, to be considered positive. The responses to the specific antigen (ESAT-6:CFP-10) were compared to the responses to PPD-B and no stimulation, that is PBS. Specificities ranged from 78 to 100 per cent depending upon the species of deer, the antigen, and the cut-off value (Table 2). In general, slightly higher specificities were obtained with ESAT-6:CFP-10 than with PPD-B.

DISCUSSION

The initial development of IFN-γ-based tests for TB surveillance requires a consistent stimulant to demonstrate the functional capacity of the sample, a determination of specificity with a reasonable number of viable samples from uninfected animals, and the ability to detect responses to specific antigens by tuberculous animals. Prior studies have demonstrated the potential for IFN-γ-based tests to detect tuberculous white-tailed deer, elk and reindeer (Slobbe and others 2000, Palmer and others 2004, Harrington and others 2006, Waters and others 2006). For reindeer, these findings were extended: PWM was identified as a reliable indicator of IFN-γ protein production, few samples were excluded owing to their inability to produce IFN-γ in response to the positive control stimulant, and the specificity of the assay was determined to be from approximately 83 to 94 per cent depending upon the species and the cut-off value applied. The specificity of the assay was at least comparable to the best estimates for the range of specificities of skin testing of other cervid species, that is, 46 to 86 per cent (Palmer and others 2001). An estimate for the specificity of skin testing in reindeer is not available, but there are anecdotal reports of many false positive reactions. These findings show that the Cervigam assay is potentially useful for the surveillance.

In contrast, the findings show that the Cervigam assay in its present form is of limited usefulness with samples from white-tailed deer, elk and fallow deer. A major concern was the poor response to mitogen stimulation by more than 50 per cent of the samples from each of these species. With M. bovis-infected white-tailed deer, a poor response to PWM indicated a concurrent poor response to mycobacterial antigen. Only two of 63 samples from infected white-tailed deer had a response to PWM of less than 0.25 Δ OD and a concurrent response to M. bovis PPD of more than 0.05 Δ OD, the lowest recommended cut-off for a positive response. Only 60 per cent of the samples from M. bovis-infected white-tailed deer showed a response to PWM exceeding 0.25 Δ OD and these responses varied widely with time and between individual deer. These findings indicate either that the sample leukocytes were unable to produce IFN-γ consistently, or that the test was unable to detect the cytokine consistently.

The failure of the tests may be related to the biology of the host’s response, the interpretation of the assay, the failure of the assay, and/or the poor quality of the sample. Captive deer are particularly prone to acute stress when handled, which may result in sudden increases in the plasma concentration of cortisol and/or other cytokine response inhibitors (Rehbinder and others 1982, Kock and others 1987). High levels of cortisol may result in reductions in IFN-γ due to the inhibition of transcription factor NF-kB. The mechanisms of the regulation of IFN-γ gene expression, including positive and negative feedback loops of production, are virtually unknown for deer. Translation of the human IFN-γ gene is inhibited by the activation of a RNA-dependent protein kinase via a pseudoknot sensor on the 5’-untranslated region of IFN-γ mRNA, and the subsequent phosphorylation of eukaryotic initiation factor-2α (Kaempfer 2003). Furthermore, T cell activation may be inadequate owing to the presence of inhibitory cytokines, other inhibitory factors, or a lack of co-stimulation. Another reason for the inconsistent detection of IFN-γ in the assay may be the degradation of synthesised IFN-γ as a result of the activation of proteases or inhibitors of glycoprotein-processing enzymes (Kosuge and Toyoshima 2000). Alternatively, changes in the glycosylation of IFN-γ may decrease its half-life. The inhibition of the extracellular release of synthesised IFN-γ, or the upregulation of IFN-γ receptors with immediate binding of synthesised IFN-γ, or the masking of IFN-γ by other factors, could also reduce the availability of free IFN-γ in stimulated plasma.

The mitogens commonly used as positive control stimulants may not consistently induce acceptable quantities of IFN-γ in samples from captive deer, and the interpretation of the assay is difficult without a consistent positive control stimulus. The results of this study emphasise the need for an adequate cut-off value for the assurance of quality control. A minimal cut-off value of 0.1 Δ OD, as used in some laboratories in the USA, is probably insufficient to assure the test’s validity. The unsuccessful stimulation with mitogens may indicate either that there are different mechanisms of activation in cervids, or that there are inhibiting factors in cervid blood. The antibodies used for the Cervigam assay may not recognise IFN-γ from different deer species (as indicated with fallow deer) and polymorphisms in antigen-binding sites may result in variability in detection between individual animals (as indicated with white-tailed deer). Delays in setting up the assay did not significantly affect the response (Fig 3), but samples from deer may be subject to interference resulting from temperature fluctuations and other transport-associated events, such as excessive vibrations and shaking. In the elk, a higher proportion of the samples that were shipped overnight failed to respond to PWM than of the samples that were processed immediately, indicating that transport may
Mycobacterium bovis

white-tailed deer and time on the mean (se) samples from (a) eight on interferon-

been vaccinated with (b) four elk, which had responses of blood

FIG 3: Effects of holding to stimulation

PWM

PPD

PWM

PPD

(a)

(b)

Immediate set-up

8 hours delay

24 hours delay

Immediate set-up

8 hours delay

24 hours delay

caused interference beyond any caused by delays in setting up the assay. For the further development of the test it may be necessary to evaluate additional positive control stimulants, for example, ionomycin, phosphor-12-myristate 13-acetate and recombinant interleukin-2, or cocktails of positive control stimulants, to establish TNF protein sequences from different species of deer (at present only the sequence for red deer has been published) (Sweeney and others 2001), and investigate different combinations of antibodies for the sandwich ELISA (Buchan and Griffin 1990), and/or enhanced sample delivery/stimulation procedures.

Acknowledgements

The authors thank the North American Deer Farmers’ Association, the Reindeer Owners and Breeders’ Association, the North American Elk Breeders’ Association, as well as the numerous veterinary practitioners and captive cervid owners for providing samples for testing. Funds were provided, in part, by the USDAAPHIS for the infection and vaccination trials, Jessica Pollock, Shelly Zimmerman, Bart Olthoff, Mike Howard, Rachel Huegel and Peter Lasley provided excellent technical assistance. The authors are grateful to Dr C. Minion, Iowa State University for a supply of rESAT-6:CFP-10.

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**Autologous platelet concentrates as a treatment for musculoskeletal lesions in five horses**

**D. Argüelles, J. U. Carmona, F. Climent, E. Muñoz, M. Prades**

Two horses with acute tendinopathy of a superficial digital flexor tendon (SDFT) and three horses with chronic proximal desmitis of the suspensory ligament (PSL) were treated by injecting autologous concentrates of their platelets into the lesions. The lesions were monitored ultrasonographically and clinically. There were significant ultrasonographic and clinical improvements in the two horses with SDFT, but no ultrasonographic improvements in the horses with PSL; however, they improved clinically and became less lame. All the horses had returned to their pre-injury level of performance by six months after the completion of the treatment, and none of them had suffered a recurrence after 20 months.

INJURIES to tendons and ligaments heal slowly and inefficiently, and the injured areas do not recover their original biomechanical properties. The presence of catabolic cytokines, such as tumour necrosis factor-alpha and interleukin 1, has been reported in biopsies of tendons from horses suffering from chronic tendinopathy (Hosaka and others 2002, 2004). These cytokines can upregulate matrix metalloproteinases and cyclooxygenase 2, which have detrimental effects on the extracellular matrix and resident cells of tissues such as tendons and ligaments (Tsuzaki and others 2003, Hosaka and others 2004, 2005).

The negative effects of catabolic cytokines on connective tissue may be downregulated by some growth factors (Van Miert 2002). Studies in vitro have demonstrated the beneficial properties of transforming growth factor-beta (TGF-β) (Murray and others 2003, Anitua and others 2004), insulin-like growth factor 1 (IGF-1) (Dahlgren and others 2001), and other growth factors (Murray and others 2003, Wong and others 2003, Zhang and others 2003). Furthermore, Dahlgren and others (2002) reported that IGF-1 had a positive action in a collagenase-induced model of tendonitis in the superficial digital flexor tendon of horses. The results of these studies suggest that growth factors may be beneficial as a complementary treatment of musculoskeletal injuries, by using either pure recombinant proteins or gene therapy (Dahlgren and others 2001, Beredjiklian 2002, Dai and others 2003).

Platelet concentrates are an autologous source of some growth factors, especially TGF-β, and of other molecules that modulate inflammation and the tissue repair process (Anitua and others 2004, 2005, Sutter and others 2004), and they have been used successfully in human medicine for several purposes, including alveolar-maxillary reconstruction (Carlson and Roach 2002, Anitua and others 2004) and plastic (Bhanot and Alex 2002), and orthopaedic surgery (Anitua and others 2004). They have also been used to treat Achilles tendinitis in two athletes and cartilaginous avulsion in a football player (Sánchez and others 2003). Anitua and others (2005) have documented the positive effects of platelet concentrates on human tenocytes; the concentrates induced tenocyte proliferation and upregulation of two angiogenic peptides, vascular endothelial growth factor and hepatocyte growth factor.

This paper describes the results of treating five horses with tendon and ligament injuries with platelet concentrates.

**MATERIALS AND METHODS**

The study was approved by the Ethical Committee of the authors' institution. The horses' owners were informed about the nature of the experiment and the possible complications associated with the injection of platelet concentrates. All the observations and determinations were made by the same clinician (M. P.). Table 1 shows the sex, age, breed and discipline of the five horses, and the limbs that were affected.

Mr Carmona is also at the School of Animal Health, Universidad de Caldas, Calle 65, 26-10, Manizales, Colombia.

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