Time Delay, Temperature Effects and Assessment of Positive Controls on Whole Blood for the Gamma Interferon ELISA to Detect Paratuberculosis

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Summary
Our objective was to evaluate the effects of time and temperature on whole blood used in the gamma interferon enzyme-linked immunosorbent assay (IFN-γ ELISA) for paratuberculosis along with evaluating four potential positive controls, and four different mycobacterial antigens for the ELISA. Nine adult Holstein cattle naturally infected with Mycobacterium avium ssp. paratuberculosis were used in a randomized complete block design. Forty-nine blood tubes were collected from each animal and held at 48.9, 37.8, 26.7, 21.1, 15.6 and 4.4°C for 0, 4, 8, 12, 18, 24, 32, 48 and 72 h. Each blood tube was tested with four mycobacterial antigens (two johnin PPDs, an avain PPD and a whole cell sonicate) and four potential positive controls [concanavalin A (conA), phytohaemagglutinin A (PHA), pokeweed mitogen (PWM) and Staphylococcus enterotoxin A (SEA)]. After incubation for 24 h, the plasma was assayed with a commercial IFN-γ ELISA. Blood stored at 21.1 and 15.6°C maintained the highest ELISA optical densities (OD) over time with severe reduction in OD values at or above 37.8°C. None of the potential positive controls exactly mimicked the antigen response. SEA and PWM were able to elicit a response after the whole blood quit responding to the antigen and conA underestimated the responsiveness. Phytohaemagglutinin A was similar to the antigens on an average, but there was significant disagreement among samples. The PPDs were more potent at stimulating IFN-γ production than the whole cell sonicate. In conclusion, whole blood should be stored/transported at ambient room temperature and stimulated within 12 h of collection.

Introduction
Paratuberculosis or Johne’s disease occurs throughout the world causing significant economic loss. Current serology and organism-based diagnostic tests are insensitive and detect animals after they are infectious (Stabel, 2000). Consequently, more sensitive tests are needed. Because a detectable cell-mediated immune response (CMI) occurs early during infection, diagnostic tests that use the CMI response to detect paratuberculosis have generated significant interest.

One such potential diagnostic test, the gamma interferon enzyme-linked immunosorbent assay (IFN-γ ELISA) has already been approved in the United Stated as an adjunct test for detecting tuberculosis in cattle. The approval for tuberculosis has made this kit readily available and some diagnostic laboratories have begun to offer the test on a limited basis for paratuberculosis.

When an assay, such as the IFN-γ ELISA requires the submission of whole blood with live lymphocytes that are healthy enough to respond to antigen stimulation, it becomes imperative to know quickly the blood needs to arrive at the diagnostic laboratory and what effects hot or cold temperatures have on survival. Several studies have looked at time delay when using the IFN-γ ELISA in tuberculosis (Ryan et al., 2000; Whipple et al., 2001; Gormley et al., 2004; Whelan et al., 2004) and paratuberculosis (Jungersen et al., 2002). All of these studies agree that there was a reduction in IFN-γ production associated with time, but how much of a reduction was not always clear; some studies reported this reduction had little effect on the interpretation of the test which was disagreed by others. Most of these studies lacked sufficient sample numbers to discriminate a small to medium difference in sensitivity.

Less work has been performed with regard to temperature effects. A study found that cells shipped at 37°C had lower IFN-γ production than cells shipped at ambient room temperature (Stabel and Whitlock, 2001).

The FDA has recently approved a IFN-γ ELISA for the diagnosis of latent tuberculosis in humans (Mazurek and Villarino, 2003). For interpretation, this kit includes IFN-γ standards which allow the optical density (OD) values to be converted to international units (IU) of IFN-γ using a standard curve. A positive control, phytohaemagglutinin A (PHA) is also included. The results are reported as a ratio of test sample IU/PHA IU. Phytohaemagglutinin A must also exceed 1.5 IU in order for the test results to be valid.

Although not promoted by the manufacturer of the bovine IFN-γ ELISA kit, many researchers have suggested using a mitogen or super antigen as a positive control (Stabel and Whitlock, 2001; Jungersen et al., 2002; Manning et al., 2003; Stabel et al., 2003). Two primary reasons have been suggested. First, positive controls help identify anergic animals not capable of responding because of end-stage paratuberculosis or possible tampering with immunosuppressive agents. The second is to ensure the lymphocytes in the whole blood have survived transport to the laboratory. A proper positive control would allow an animal that has a response to the mitogen, but not the antigen, be considered negative. An animal that failed to have a response to the mitogen should by definition also be negative to the antigen and an inconclusive result would be given.
Some researchers have documented that concanavalin A (conA) often fails to elicit a positive response when the lymphocytes are still able to generate a measurable response to an antigen (Jungersen et al., 2002). Other than this, there is little work published that actually validates any positive controls for the bovine IFN-γ ELISA for tuberculosis or paratuberculosis.

The aims of this study were to evaluate the effects of time delay and temperature on the IFN-γ response to paratuberculosis antigens, evaluate three mitogens and one super antigen as to their appropriateness as positive controls, and to assess the potency of four different mycobacterial antigens.

Materials and Methods

Study design
A randomized complete block design was used. Nine naturally infected adult Holstein cattle (eight cows, one steer) that were part of the NADC Johne’s disease herd and had a history of positive IFN-γ results were bled twice 1 week apart. For each bleeding, 30 ml heparinized blood collection tubes were filled. One tube from each animal was processed immediately and the nine other tubes were held at the prescribed temperature for the appropriate times. The tubes were held at temperatures 48.9, 37.8 and 26.7°C the first week and 21.1, 15.6 and 4.4°C the next week. To evaluate time delay, tubes were held at their prescribed temperatures for 4, 8, 12, 18, 24, 36, 48 and 72 h post-bleeding.

IFN-γ ELISA
After the allotted time delay, the blood tube was gently mixed and 1 ml was added to nine wells on a 24-well tissue culture plate. One well was left as a non-stimulated control with no additive. Other wells were treated with the following antigens: 10 μg Johnin PPD (PPDj3; National Veterinary Services Laboratory, USDA, Ames, IA, USA) made from MAP ATCC type strain 19698, 10 μg johnin PPD (PPDj4; National Veterinary Services Laboratory) made from a recent cattle MAP isolate, 10 μg whole cell sonicate (MpS; National Animal Disease Center, ARS, USDA, Ames, IA, USA) made from strain 19698 and 10 μg avian PPD (PPDav; Pfizer Animal Health, Omaha, NE, USA). Four different positive controls were also used: 10 μg pokeweed mitogen (PWM; Toxin Technology, Sarasota, FL, USA), 1 μg Staphylococcus enterotoxin A (SEA; Sigma Chemical, St Louis, MO, USA), 10 μg PHA (Toxin Technology) and 10 μg concavalin A (conA; Toxin Technology).

All plates were incubated for 24 h in 5% CO₂ humidified atmosphere and then centrifuged at 1296 g for 5 min. Approximately 500 μl of plasma were removed and stored at −80°C until assayed.

A commercial IFN-γ kit (Bovigam, Pfizer Animal Health, Omaha, NE, USA) was used according to manufacturer’s instructions. Samples were run in duplicate and wells were read at 450 nm. As previously reported by others, there was a wide range of OD values in the positive controls supplied by the kit for plates run at different times. This was most likely due to laboratory ambient temperature variations. The kit only required the positive control be >0.7. In order to reduce plate-to-plate variation, the plates were standardized using the method suggested by others which was ODₜ = [(sample OD - NegC) x (mean Pos - mean Neg)/(PosC - NegC)] + mean Neg, where ODₜ is the calibrated OD, PosC and NegC are the positive and negative kit controls on the plate and mean Pos and mean Neg are the mean values of the positive and negative kit controls from all plates run (Jungersen et al., 2002). All results reported are calibrated OD of the test well – calibrated results of the non-stimulated negative control.

Samples that had a non-stimulated well with a corrected OD values of above 0.150 were eliminated from the analysis. A total of 5.2% of blood samples fit this criterion. These samples were seemingly randomly scattered throughout the times and temperatures, but one cow did contribute 60% of high non-stimulated samples.

Statistical analysis
Separate regression equations were calculated for each antigen and positive control treatment for OD response as a function of time and temperature (TABLECURVE 3D, Version 3.01, SPSS, Chicago, IL, USA). The mean OD responses of the nine cows were used in the regression analyses. General linear model F-tests were used to determine if there were any overall equation differences between the four antigens, between the four positive controls and between the overall antigens and positive controls. If the F-test showed that there were differences between the equations (treatments) tested, the 95% confidence intervals on the equation parameters for time and temperature were calculated and used for finding treatment comparison differences.

Repeated measures mixed model ANOVA were conducted to compare mean OD responses of the four antigens, the four positive controls and between overall antigens versus positive controls (PROC MIXED, SAS Version 9.1.3, SAS Institute, Cary, NC, USA). Time and temperature variables were considered to be random effects in these models. If the ANOVA F-tests were significant at P ≤ 0.05, then differences of least-squares mean values (LSMeans) was used as a multiple comparison test for determining mean differences among the four antigen mean values and among the four positive control mean values.

Results
All nine cattle had IFN-γ responses to all mycobacterial antigens (mean OD = 1.51) and positive controls (mean OD = 2.01) at time 0. While there was some variation in OD values of the antigens initially (range: 0.320–2.753), there were no significant differences between cattle at the rate (or slope) at which the cells lost the ability to produce IFN-γ. Consequently cattle which had lower IFN-γ responses initially became ELISA-negative earlier (data not shown).

Antigen responses
In general, there was a loss of IFN-γ production that correlated with the time delay prior to stimulation. IFN-γ responses were maintained at levels comparable with time 0 for 4 h except for the most extreme temperature (48.9°C) and then began to drop (Fig. 1). Blood kept at 48.9°C was unable to mount an IFN-γ response after 4 h. Blood kept at body
Effects of Time and Temperature on Whole Blood used in the IFN-γ ELISA for Paratuberculosis

Positive control responses

Phytohemagglutinin A was the positive control that mimicked the mycobacterial antigen responses most accurately across the range of times and temperatures tested. Pokeweed mitogen and SEA were able to elicit strong IFN-γ responses after many antigen responses were not detectable. ConA lost its ability to elicit a response even when the antigens still consistently had detectable responses. Figure 4 demonstrates the typical relationship between an antigen (PPDj3) and the positive controls for all the times except 72 h where there is little response to any of the antigens.

Regression analysis

The regression equations used for predicting OD over time and temperature for each treatment were:

\[ Z = a + b\ln(X) + cY \]

where \( Z \) = mean OD response, \( a \) = OD intercept, \( b \) = time parameter, \( c \) = temperature parameter and \( Y \) = temperature. All equations were statistically significant in explaining OD over time and temperature (\( P < 0.0001 \)). Parameter estimate slopes and intercept coefficients in the models were also significant (\( P < 0.0001 \)), indicating true, non-zero contributions to the prediction equation. The only exception was the temperature coefficient for conA positive control, which was significant at \( P = 0.023 \). Table 1 contains the intercept, time and temperature parameters for the antigens and Table 2 for the positive controls. There were not enough data points at colder temperatures to accurately characterize the behaviour of the blood stored below 15.6°C with the regression equations.

Discussion

The nine cows chosen for this study were selected based on previous history of consistent positive IFN-γ ELISA responses. Consequently most of these cows had strong IFN-γ responses and this biased our results causing weak and
moderately positive IFN-γ ELISA cows to be underrepresented.

Nonetheless, there was enough variation in initial OD to evaluate differences in rates of IFN-γ production loss over time and temperature between individual cows. The rates were remarkably similar regardless of initial OD results suggesting that rates of IFN-γ loss were independent of the strength of initial OD response. This important finding suggests that regression equations can be accurately used to predict the potential time delay and temperature effects on whole blood. It is important to note that even though the cow’s individual slopes did not vary, there were significant differences in slopes among the antigens. Care must be taken when extrapolating these equations and slopes to other antigens not used in this study.

There was a significant difference in IFN-γ responses to antigens in this study. The PPDs were more potent than the MpS antigen at stimulating IFN-γ. Similar results were found in sheep in a previous study by Robbe-Austerman et al. (2006). If more cattle had been tested, the johnin PPDs would have likely also been statistically more potent than PPDav. This is contrary to others who reported higher responses to PPDav than to Johnin PPD (Jungersen et al., 2002). There are several reports in the literature about the variability of potency and lack of lot of consistency in all the PPDs (Johnson et al., 1949; Landi et al., 1975). Because of this lack of consistency and cross-reactivity with other mycobacteria, there is strong interest in developing specific antigens for use in the IFN-γ ELISA for paratuberculosis. In bovine tuberculosis IFN-γ assay the specific antigens, ESAT 6 and CFP 10, are not as potent as tuberculin PPD (Waters et al., 2004). This relative lack of potency of these specific proteins could increase the consequences of a delay in setting up blood samples for the IFN-γ ELISA (Whelan et al., 2004). It is likely similar consequences were noticed in the FDA approved human tuberculosis IFN-γ kit as samples are required to be set up with in 12 h (Mazurek and Villarino, 2003).

It is surprising that the majority of the positive controls used in the literature failed to mimic the IFN-γ production loss over time of the mycobacterial antigens. Pkoweed mitogen and SEA are inadequate positive controls and give a false sense of security by maintaining a positive response long after the cells could no longer respond to the mycobacterial antigens. This study also confirms other work, which reported that conA often failed to elicit a response when cells still had the ability to respond to the antigen (Jungersen et al., 2002). Phytohemagglutinin A on average mimicked the antigen curves most accurately. This is likely the reason why PHA is the positive control used in the human tuberculosis IFN-γ ELISA kit. However, PHA should be validated for each species tested as it fails to stimulate IFN-γ production in sheep whole blood.

The IFN-γ ELISA has potential as a diagnostic test for paratuberculosis as well as tuberculosis. However, specimen handling is of utmost importance. To maximize the accuracy of the test, whole blood should be stimulated within 12 h of sampling, and the storage/transport temperature prior to stimulation should be maintained between 15.6 and 21.1°C.

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References


