Effects of pre-culture holding time and temperature on interferon-γ responses in whole blood cultures from Mycobacterium bovis-infected cattle

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Abstract

The Bovigam™ assay is approved for use within the United States as a complementary tuberculosis test. Prior to whole blood culture and the ensuing ELISA to detect interferon-γ (IFN-γ), samples are subjected to various holding time/temperature combinations due, in part, to practical constraints associated with shipment of samples to approved laboratories. To evaluate these effects, 5-month-old Holstein calves (n = 7) received $10^3$ cfu Mycobacterium bovis by aerosol. Heparinized blood was collected 2 months after challenge and held at 4 or 22°C for 0, 8 or 24 h prior to culture with mycobacterial antigens or pokeweed mitogen (PWM). Responses of samples held for 8 or 24 h were comparable and lower than responses of cultures prepared immediately after collection, regardless of holding temperature. Differences in responses of samples held at 4°C versus 22°C were also minimal. A subset of samples was held for 2 h at 37°C at the beginning of the holding period. This subset of samples had diminished responses to all stimulants and increased holding times (i.e., 24 h versus 8 h) negatively impacted the response. Pre-processing conditions, particularly delays in set-up and initial high sample temperatures, reduces IFN-γ responses of cells from infected cattle increasing the risk of false negatives in this assay of regulatory importance.

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1. Introduction

The Bovigam™ assay (Wood and Jones, 2001; Prionics AG, Schlieren, Switzerland) is used as a complementary test to traditional skin testing for tuberculosis (TB) surveillance in cattle within the United States. The assay detects interferon (IFN)-γ produced by blood leukocytes exposed to no antigen
(i.e., background response), *M. avium* purified protein derivative (PPD), *M. bovis* PPD, or mitogen (e.g., pokeweed mitogen, PWM). The assay is easily adapted to the diagnostic laboratory setting as whole blood cultures circumvent the need for cumbersome cell separation techniques. Recently, recombinant antigens specific for virulent tubercle bacilli [e.g., early secretory antigenic target (ESAT)-6, culture filtrate protein (CFP) 10, mobility protein bovis (MPB)-64, and MPB-70] have been evaluated for use in IFN-γ-based tests that discriminate between *M. avium*-exposed, BCG-vaccinated, and tuberculous cattle (Fitis et al., 1994; Wilcke et al., 1996; Pollock and Andersen, 1997; Elhay et al., 1998; Lyashchenko et al., 1998; Vordermeier et al., 1999, 2001; Pollock et al., 2000, 2003; Buddle et al., 2001, 2003). Of these antigens, ESAT-6 and CFP10 are leading diagnostic candidates. Despite advances in development of improved antigens and widespread acceptance of the IFN-γ test for use in many national bovine TB eradication campaigns; standard procedures for handling blood samples prior to assay set-up have not been fully evaluated. Under field conditions, shipping constraints often delay processing of blood samples for 24 h or longer resulting in a decrease in the IFN-γ response when compared to responses after holding times of 0, 2, or 8 h (Rothel et al., 1992; Whipple et al., 2001; Gormley et al., 2004). In contrast, others have demonstrated that a 24 h delay does not negatively impact the sensitivity or specificity of the assay (Ryan et al., 2000); however, the probability of a response remaining positive after a 24 h holding period likely depends on the magnitude of that response, temperature of the sample during the holding period, and combined effects of holding time and temperature (Bolin, 2002; Gormley et al., 2004). For diagnosis of cattle infected with *M. avium* subsp. *paratuberculosis*, optimal time/temperature combinations for the holding period are <12 h and 15.6–21.1 °C, respectively (Robbe-Austerman et al., 2006).

Overnight shipment of samples to diagnostic laboratories currently running the Bovigam™ assay (Prionics AG) is possible from most locales within the United States. In certain areas, ≤8 h delivery times are also possible. Depending on location and time of the year, temperatures at the sampling station may vary tremendously. However, control of sample temperature within reasonable ranges is possible by using insulated containers with or without ice packs. Unfortunately, field veterinarians/technicians unaware of temperature constraints may subject samples to holding temperatures of 37 °C for periods of 2 h or more, especially on large farms or when the collection process is delayed. Our objective was to determine affects of the duration and temperature of the pre-processing period on the IFN-γ response by blood leukocytes from cattle experimentally infected with *M. bovis*.

### 2. Materials and methods

#### 2.1. Calves, *M. bovis* challenge, and necropsy

Male, TB-free, Holstein calves (*n* = 7) were housed according to institutional guidelines at the National...
Animal Disease Center, Ames, Iowa (NADC) in a biosafety level 3 (BL-3) facility. Calves received *M. bovis* by aerosol at 3 months of age. The *M. bovis* aerosol inoculum (Schmitt et al., 1997) and challenge was prepared and delivered, respectively as described previously (Palmer et al., 2002). Briefly, inoculum (2 ml *M. bovis* in PBS) was delivered to restrained calves by nebulization into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. Upon inspiration, inoculum was inhaled through a one-way valve into the mask and directly into the lungs via the nostrils. The process continued until the inoculum, a 1 ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered, a process taking ~12 min. Biosafety level 3 protocols were followed to protect personnel from exposure to *M. bovis*.

2.2. Pre-culture conditions and IFN-γ assay

Two months after challenge, blood was collected by jugular venipuncture into tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, N.J.). Animals were not injected with PPD for skin test prior to blood collection for IFN-γ analysis. Blood in collection tubes was stored at 4 °C (i.e., to mimic containers with ice packs) or 22 °C (i.e., to mimic containers at room temperature) for 0, 8, or 24 h. A pre-incubation condition of 37 °C for 2 h was included to evaluate effects of early elevated temperature on subsequent responses at various time/temperature treatments. This pre-incubation time and temperature was used to mimic conditions encountered when larger herds are sampled on hot days and ice or other refrigerants are not available at the sampling station. To keep the total incubation period constant, this pre-incubation step was included in the initial 2 h of each of the 8 and 24 h holding time frames (e.g., 2 h at 37 °C and 6 h at 4 °C, etc.). After being held at the various times and temperature, blood was dispensed in 1.5 ml aliquots into individual wells of a 24 well plate (Falcon 353047, Becton Dickinson). Wells contained whole blood plus 20 μg/ml *M. bovis* PPD (Prionics AG), 20 μg/ml *M. avium* PPD (Prionics AG), 1 μg/ml rESAT-6:CFP-10 (Waters et al., 2004; a kind gift from Dr. C. Minion, Iowa State University), 1 μg/ml PWM, or medium alone (no stimulation). Cultures were incubated for 24 h, plasma harvested and subsequently

![Fig. 1. Effects of holding time and temperature on IFN-γ responses to mycobacterial antigens. Blood in collection tubes were stored at 4 or 22 °C for 0 h (i.e., Immediate), 8 h, or 24 h prior to culture with (A) 1 μg/ml PWM, (B) 20 μg/ml *M. bovis* PPD (Prionics AG), 20 μg/ml *M. avium* PPD (Prionics AG), (C) 1 μg/ml rESAT-6:CFP-10, or medium alone (no stimulation). Blood cultures were incubated for 24 h, plasma harvested and analyzed by a commercial ELISA-based kit (Bovigam™, Prionics AG). Duplicate samples for individual treatments were analyzed and data are presented as mean optical density (OD) values of responses to antigen/PWM minus the response to no stimulation (i.e., ΔOD). Comparison of treatment (time × temperature) effects on responses to *M. avium* PPD were similar to those of *M. bovis* PPD (data not shown). ***P < 0.001.](image-url)
IFN-γ concentrations were determined using a commercial ELISA-based kit (Bovigam™, Prionics AG) within a single lot (i.e., a 30 plate kit). Optical densities (OD) of kit standards and test samples were read at 450 nm using an ELISA plate reader (Molecular Devices, Menlo Park, Calif.). Duplicate samples for individual treatments were analyzed. Data are presented as mean OD values of responses to antigen/PWM minus the response to no stimulation (i.e., ΔOD). Optical density was used for evaluation as this is the unit of measure used by diagnostic laboratories and is recommended by the kit manufacturer.

2.3. Statistics

Data were assessed for normality prior to statistical analysis and analyzed as a split-plot with repeated measures ANOVA using Statview software (version 5.0, SAS Institute, Inc., Cary, NC). The statistical model included effects of time, temperature and the interaction of time and temperature on the IFN-γ response in whole blood cultures. Fisher’s protected-LSD test was applied when effects (P < 0.05) were detected.

3. Results

Under field conditions, blood samples may be exposed to high temperatures prior to packaging for over-night delivery to diagnostic laboratories. Pre-incubation of blood samples at 37 °C for 2 h prior to holding at 4 or 22 °C for 2 h decreased (P < 0.001) antigen- and mitogen-induced responses relative to responses after holding at 4 or 22 °C for 24 h (Table 1). Similarly, pre-incubation for 2 h at 37 °C decreased (P < 0.05) IFN-γ responses in samples held for 8 h (data not shown). The one exception was the marginally lower (P = 0.12) M. bovis PPD elicited response of samples pre-incubated to 37 °C for 2 h before holding at 4 °C for the remaining 8 h period versus those of samples held at 4 °C for the entire 8 h period.

antigen/PWM minus the response to no stimulation (i.e., ΔOD). Comparison of treatment (time x temperature) effects on responses to M. avium PPD were similar to those of M. bovis PPD (data not shown). ***P < 0.001.
Interferon-γ responses of samples held at 4 or 22 °C were similar with 2 exceptions. Responses to *M. bovis* PPD were marginally lower (*P* = 0.09) in 24 h samples held at 4 °C versus 22 °C (3.02 ± 0.07 versus 2.80 ± 0.09). In samples pre-incubated to 37 °C for 2 h, responses to *M. bovis* PPD were lower (*P* = 0.05) in 22 h samples held at 4 °C versus 22 °C (2.1 ± 0.08 versus 1.85 ± 0.06).

Responses to antigens and mitogen were lower (*P* < 0.05) in samples held for 8 or 24 h versus samples cultured immediately after blood collection, regardless of holding temperature (Figs. 1 and 2). The only exception was the comparable rESAT-6:CFP10 response when held at 4 °C for 8 h versus samples cultured immediately (Fig. 1C). In regards to samples held for 8 h versus 24 h, responses to *M. avium* and *M. bovis* PPD did not differ (*P* > 0.05). Responses of rESAT-6:CFP10 and PWM stimulated cultures, however, were lower (*P* < 0.01 and *P* = 0.06, respectively) in samples held for 24 h at 4 °C than in samples held for 8 h at 4 °C (Fig. 1). Effects of holding time (i.e., 6 h versus 22 h) were significant (*P* < 0.001) in samples pre-incubated for 2 h at 37 °C, regardless of temperature (i.e., 4 or 22 °C) and stimulant (Fig. 2).

### 4. Discussion and conclusions

Within the United States, TB is nearly eradicated and tests are increasingly applied for identification of recently infected animals. Strict protocols for test application are particularly important in the current diagnostic climate. Once a sample is delivered to the laboratory, the testing protocol is easily controlled for optimal reproducibility as laboratories undergo frequent mandated evaluation to ensure consistency in testing. Before reaching the laboratory, the sample encounters multiple levels of handling including blood collection, storage prior to packaging (i.e., in the field and at the packaging station), packaging, delivery, and storage upon receipt at the laboratory. Delays and temperature fluctuations at each of these levels may affect the condition of the sample resulting in variability in the diagnostic test. Variations in temperature during the delivery, particularly with air couriers, may not be anticipated yet often occur due to circuitous routes of delivery.

Present findings indicate that delays in set-up of blood cultures for stimulation negatively impact the IFN-γ response to mycobacterial antigens and mitogen. Pre-incubation of samples to 37 °C for 2 h, as might be encountered in field situations or upon delivery, diminishes the response. Responses are similar when samples are held at 4 °C (i.e., mimicking containers with ice packs) or 22 °C (i.e., room temperature). In contrast, increased holding periods (i.e., 8 h versus 24 h) negatively impact on the response to *M. bovis* PPD and rESAT-6:CFP10 when samples are warmed to 37 °C for the initial 2 h. Thus, it may be advantageous to package samples with ice packs to decrease the likelihood of high temperatures, either in the field or at any level of the delivery process. A limitation of the present study was the use of samples with high responses to mycobacterial antigens generated by experimental inoculation, albeit a low challenge dose. It may be anticipated that responses by naturally infected animals would be lower and more seriously diminished by inhibitory time × temperature treatments. Also, it is not certain if time × temperature treatments would impact responses by non-infected cattle (i.e., specificity of the test). Regardless, present findings indicate that current-testing strategies using IFN-γ-based tests should employ strict control measures to ensure optimal sample quality as this test relies on the maintenance of the functional capacity of cells for the production of the readout parameter. When immediate processing is not practical, samples should be immediately stored between 4 and 22 °C and maintained at this temperature until processed at the diagnostic laboratory.

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### References


