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Assessment of critical parameters in blood processing for the bovine interferon-gamma ELISPOT assay to detect *Mycobacterium bovis* infected cattle in India

Maroudam Veerasami, Loganathan Moorthy, Dipankar Das, Sugumar Parthasarathy, Dev Chandran, Rajendra Lingala & Villuppanoor Alwar Srinivasan

Summary

In vitro production of bovine interferon gamma (BoIFN-γ) cyokine from bovine peripheral blood mononuclear cells (PBMCs) can be detected using the most sensitive enzyme-linked immunosorbent spot (ELISPOT) assay. ELISPOT assays are dependent on the quantity and quality of PBMC preparations and hence contribute significantly to the performance of this assay. In order to standardise the methods for isolation of PBMCs, we compared two methods for the processing of bovine blood which included aliquots of blood that were stored in a horizontal position without dilution or agitation and aliquots of blood that were immediately diluted 1:1 with complete Rosewell Park Memorial Institute (RPMI) 1640 medium and stored in a horizontal position with gentle agitation. PBMCs were isolated at 2, 4, 6, 8 and 24 h and at 4°C and at 22°C ± 2°C. They were stimulated using tuberculosis-specific antigens, after which the ELISPOT assay was performed. Quantities of spot-forming cells (SFC) created by the release of BoIFN-γ in ELISPOT assays were significantly greater in the samples stored at 22°C ± 2°C than those held +4°C and the intensity of the signals dropped following processing after 6 h. A further drop in SFC was observed in those samples that had been stored undiluted and without agitation. These findings demonstrated that optimisation of PBMC isolation procedures can lead to increased sensitivity in the detection of BoIFN-γ using the ELISPOT assay, thus contributing to an enhanced diagnosis of bovine tuberculosis.

Keywords

Bovine tuberculosis, ELISPOT assay, Enzyme-linked immunosorbent spot, IFN-γ, India, Interferon gamma, *Mycobacterium bovis*, Peripheral blood mono-nuclear cells, Tuberculosis.

Valutazione di parametri critici nell'analisi ematica tramite il test ELISPOT gamma interferon bovino, al fine di rilevare infezioni da *Mycobacterium bovis* nel bovino in India

Riassunto

La produzione in vitro *dell'interferone gamma bovino (BoIFN-γ) a partire da cellule mononucleari del sangue periferico (PBMC) bovino può essere rilevata utilizzando il più sensibile dei dosaggi ovvero ELISPOT. I dosaggi ELISPOT dipendono dalla quantità e qualità delle preparazioni delle PBMC e contribuiscono quindi in maniera significativa alle prestazioni di questo dosaggio. Al fine di*

Research and Development Centre, Indian Immunologicals Limited, Rakshapuram, Gachibowli Post, Hyderabad 500 032, India
srini@indimmune.com

standardizzare i metodi per l'isolamento delle PBMC abbiamo confrontato due metodi per la lavorazione del sangue bovino. Uno con aliquote di sangue conservate in posizione orizzontale senza diluizione e senza agitazione e uno con aliquote di sangue diluite immediatamente 1:1 con terreno di coltura Rosewell Park Memorial Institute *(RPMI) 1640 completo e conservate in posizione orizzontale con leggera agitazione. Le PBMC sono state isolate a 2,4, 6, 8 e 24 ore, ovveero a 4 °C e 22 °C ±2 °C. Le PBMC sono state stimolate con antigeni specifici per la tubercolosi, e successivamente è stato eseguito il dosaggio ELISPOT. Le quantità di cellule formanti spot (SFC), create dal rilascio di BoIFN-γ nei dosaggi ELISPOT erano significativa-mente maggiori nei campioni conservati a 22 °C ±2 °C rispetto a quelle mantenute a +4 °C e l'intensità dei segnali è caduta in seguito alla lavorazione dopo 6 ore. Un'ulteriore caduta delle SFC è stata osservata nei campioni che erano stati conservati non diluiti e senza agitazione. Questi risultati hanno dimostrato che l'ottimizzazione delle procedure di isolamento delle PBMC può determinare una maggiore sensibilità nella rilevazione di BoIFN-γ per mezzo del dosaggio ELISPOT, contribuendo così a migliorare la diagnosi della tubercolosi bovina.*

Parole chiave

Dosaggio ELISPOT, IFN-γ, India, Interferone gamma, Cellule mononucleate del sangue periferico, *Mycobacterium bovis*, Tubercolosi, Tubercolosi bovina.

Introduction

A fundamental feature of the immune system is the ability of antigen-specific T-lymphocytes to mount an immune response against invading pathogens which may prevent or limit re-infection for extended periods of time (2). Therefore, the study of various T-cell functions may provide insights into immune-mediated diseases, pathogenesis of infectious agents, characterisation of epitopes which are specifically T-cell targets (9) and immunological memory. Understanding the underlying principles of protective immunity following a mycobacterial infection would play a key role in the development of protective vaccines and diagnostic assays. The advent of different immunoconjugates and assays, such as flow cytometry and enzyme-linked immunosorbent spot (ELISPOT), has increased the sensitivity, reliability and accuracy in evaluating T-cell specific responses. Flow cytometry has the ability to analyse cell function and phenotypic characterisation of complex cells, including T-cells, whereas the ELISPOT assay relies on a functional readout of *in situ* interferon-gamma (IFN-γ) release following antigen stimulation to enumerate T-cell response (10).

Researchers have recently begun to explore technologies on different platforms to study the cell mediated immune response against bovine tuberculosis (15), paratuberculosis (5) and *Brucella* (4). The parameters involved in the separation of peripheral blood mononuclear cells (PBMCs) from blood samples is critical for the performance of T-cell assays such as ELISPOT and flow cytometry (1). In addition, the integrity, purity, viability and functional ability of PBMCs without red blood cells, granulocytes and platelet contamination is critical for the performance of ELISPOT (6).

Several key parameters in PBMC separation which include the type of anticoagulant used, optimal time delays between withdrawal of blood and processing and cryopreservation conditions have been reported in the literature and used for the development of standard protocols and recommendations for good laboratory practice. ELISPOT assays critically depend on the quality of the blood sample; however standard procedures for handling blood samples prior to assay set-up have not been fully evaluated to date. In field conditions, shipping constraints often delay the processing of blood samples which can range from 24 h or longer, thereby resulting in a decrease in the IFN-γ release (3).

In this study, we attempted to fill in the lacunae that exist with respect to the preparation of bovine PBMCs prior to performance of ELISPOT assay for bovine tuberculosis (TB) diagnosis which include conditions for storage of blood samples, with or without dilution and agitation, and the processing of blood at different time points. The functionality of PBMCs was further evaluated using the bovine IFN-γ (BoIFN-γ) ELISPOT assay.

Materials and methods

Immunochemicals, reagents and kits

Bovine purified protein derivative (BoPPD) was purchased from the Indian Veterinary Research Institute (IVRI) at Izatnagar, Uttar Pradesh. The Bovigam® IFN-γ ELISA kit, BoPPD and avian PPD were purchased from Prionics AG (Schlieren, Zurich). Recombinant *Escherichia coli* expressed TB-specific antigens early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) were used for stimulation of PBMCs. Rosewell Park Memorial Institute 1640 (RPMI 1640), horse serum, an antibiotic and antimycotics were purchased from Gibco (New York). Concanavalin–A (Con-A) was purchased from Bangalore-Genei (Bangalore). Phorbol 12 myristate 13 acetate (PMA), ionomycin, dimethyl sulfoxide (DMSO), 3-3-5-5-tetramethyl-benzidine peroxidase (TMB) substrate were purchased from Sigma (St Louis, Missouri). Lymphoprep™ solution and Lymphoprep™ tubes (the Lymphoprep™ tube is a sterile tube in which the Lymphoprep™ is contained below a plastic porous filter disc) were purchased from Axis-Shield PoC (Rodeløkka, Oslo). Endogen's BoIFN-γ ELISPOT kits were purchased from Pierce (Thermo Scientific, Rockford, Illinois). Golgistop™ (containing brefeldin A), stain buffer, fix/perm buffer and perm/wash buffer, 96-well enzyme-linked immunosorbent assay (ELISA) plates and vacutainer tubes containing sodium heparin were purchased from BD Biosciences (San Jose, California). Bovine IFN-γ antibodies, anti-human cluster of differentiation protein 3 (CD3) fluorescein isothiocyanate (FITC) and anti-bovine IFN-γ R-phycoerythrin (RPE) cells were purchased from AbD Serotec (Kidlington). All plastics supplies were purchased from Nunc (Rochester, New York).

Animals used in the study and the single intradermal tuberculin test

Holstein/Friesian crossbred cattle aged 10-11 years (cattle 1) and 6-9 months (cattle 2) were used. One animal (cattle 1) on a local farm was suspected to be naturally infected with *Mycoplasma bovis* and a healthy crossbred animal (cattle 2) maintained in Indian Immunologicals Limited, Hyderabad, India holding farm was used for this study. The single intradermal tuberculin test (SITT) for cattle was performed using bovine tuberculin PPD in accordance with recommendations by Mukherjee (11). Briefly, 0.1 ml of bovine tuberculin PPD was injected intradermally and a delayed type hypersensitivity reaction to tuberculin was recorded 72 h post injection.

Collection and processing of blood samples, isolation of peripheral blood mononuclear cells and cryopreservation

Peripheral blood samples were collected from the animals using standard protocol by jugular venipuncture into vacutainer tubes containing sodium heparin. Following blood collection, the animals were injected with tuberculin BoPPD for the SITT. The blood was further used for whole blood assays or was processed for the isolation of PBMCs.

After collection, the blood samples were processed immediately using two different methodologies, as follows:

- blood sample aliquots of 2.5 ml in 5 ml tubes were stored in a horizontal position at two different temperatures (4°C and 22°C ± 2°C) without dilution or agitation
- the blood sample was diluted at the ratio of 1:1 with complete RPMI 1640 medium (c-RPMI 1640) supplemented with 10% inactivated horse serum and 1% antibiotic and antimycotic) in 5 ml tubes and stored in a horizontal position at two different temperatures (4°C and 22°C ± 2°C) with gentle agitation at 300 rpm on a rocking platform.

PBMCs were isolated by density gradient centrifugation using either Lymphoprep™ solution or Lymphoprep™ tubes. These tubes not only allow the careful overlay of small volumes of blood over the Lymphoprep™ phase but also help in the separation of the PBMCs layer/buffy coat from the red blood cell pellet after density gradient centrifugation, thereby leading to better PBMC quality and quantity. Briefly, 4 ml of diluted blood samples were slowly overlayed on 2 ml of Lymphoprep™ or in Lymphoprep™ tube and

centrifuged at 805 × g for 20 min at 22°C ± 2°C (deceleration zero). The buffy coat was collected and washed three times with c-RPMI medium and centrifuged at 500 × g for 8 min at 24°C. The recovery of PBMCs was assessed by counting the cells using the trypan blue dye exclusion method and a haemocytometer. Cells were resuspended at a concentration of 5×10^6 cells/ml of freezing medium (90% inactivated horse serum and 10% DMSO) in cryovials which were then placed in a biofreezer (Mr Frosty 1°C cryogenic freezing container) (Thermo Fisher Scientific, Rochester, New York) at –70°C overnight. The vials were transferred to a liquid nitrogen (LN_2) container for long-term storage.

Just before use, the cryopreserved vials were thawed rapidly in a 37°C water bath (<1 min), the contents transferred to 15 ml tubes containing 9 ml c-RPMI 1640 and centrifuged at 800 × g for 7 min. Pellets were resuspended, washed twice in 9 ml of c-RPMI 1640 and recovered PBMCs were enumerated using the trypan blue dye exclusion method with a haemocytometer to determine the concentration of viable cells.

Detection of bovine interferon-gamma cytokine

Peripheral whole blood culture as an *in vitro* tuberculosis diagnostic assay

Peripheral whole blood cultures were performed as described by Parida *et al.* (12). Briefly, 0.2 ml of heparinised blood samples were stimulated with 5 µg/well of ESAT-6 and CFP-10 in a 96-well tissue culture plate. A 5 µg/well of Con-A and 50 µl c-RPMI 1640 stimulated samples were used as positive and negative controls, respectively. The samples were incubated for 24 h at 37°C with 5% CO_2 in a humidified incubator; supernatants were harvested by centrifugation at 500 × g for 7 min at 22°C ± 2°C. The secreted IFN-γ concentration was determined using the Bovigam® IFN-γ ELISA kit in accordance with the instructions of the manufacturer.

Intracellular cytokine staining of tuberculosis-specific interferon-gamma by flowcytometry

The cryopreserved PBMCs were thawed rapidly in a 37°C water bath and resuspended in 5 ml c-RPMI 1640. Cells were washed, counted and plated at a concentration of 1×10^6 viable PBMCs per well in 24-well plate. Cells were rested in a 5% CO_2 incubator at 37°C for 2 h. ESAT-6 and CFP-10 were used individually as stimulants at different concentrations (0.5, 1, 2, 4 and 5 µg/well/10^6 cells) as well as in combinations of 1:1 of ESAT-6: CFP-10 (0.5, 1, 2, 4, and 5 µg/well/10^6 cells). PMA (25 ng) + ionomycin (1 µg) were added for positive stimulation and c-RPMI 1640 media was used as the negative control. Cells were incubated with antigen for different durations (0, 2, 4, 6, 8 and 24 h) in a 5% CO_2 incubator at 37°C. For cultures held between 2 h and 4 h, brefeldin A (1 µl per million cells) was added after 1 h. For those held for 6, 8 and 24 h, brefeldin A (1 µl per million cells) was added during the last 4 h of stimulation. brefeldin A inhibits the cytokine secretion from the Golgi apparatus into the cytoplasm and thus facilitates the intracellular accumulation of cytokines. Cells were harvested by centrifugation at 500 × g for 8 min at 24°C and washed with 500 µl of phosphate-buffered saline (PBS) containing 20 µg/ml bovine gamma globulin (BGG). The cells were centrifuged at 500 × g for 8 min at 24°C and the pellet resuspended in 200 µl of PBS containing 20 µg/ml BGG. A total of 10 µl of 1:10 diluted anti-human CD3 FITC conjugate was added and incubated for 30 min at 4°C to surface stain the lymphocytes. The cells were centrifuged, resuspended and washed with 400 µl of stain buffer. The cells were centrifuged and the pellet was resuspended in 100 µl of fix/perm buffer and incubated for 20 min at 4°C. After fixation, the cells were washed with 300 µl of stain buffer and pellet was resuspended in 400 µl of perm/wash buffer and washed once. The pellet was resuspended in 100 µl of perm/wash buffer and 10 µl of 1:2 diluted anti-BoIFN-γ RPE conjugate was added and incubated for 30 min at room temperature. Cells were then washed with 400 µl of perm/wash buffer and the cell

pellet resuspended in 200 µl of stain buffer for data acquisition. All washing steps were conducted at 500 × g for 8 min at 24°C. The cells were acquired using FACSCanto II (BD, San Jose, California). A total of 20 000 events were recorded in list mode for all samples and these were analysed with FACS Diva 6.1 software.

ELISPOT assay for detection of bovine interferon-gamma

Selection of tuberculosis-specific antigen concentration for bovine interferon-gamma ELISPOT assay

To determine the optimal antigen concentration for stimulation of ESAT-6 and CFP-10 either alone or in various combinations, the antigens were titrated at different concentrations *viz.*, 0.5 µg, 1 µg, 2 µg and 4 µg per well. Con-A, at an optimal concentration of 5 µg per well, was used as a positive control and c-RPMI 1640 was used as a negative control. ELISPOT assay was performed in accordance with the instructions of the manufacturer, as described below.

ELISPOT optimisation and measurement of bovine interferon-gamma cytokine

The BoIFN-γ ELISPOT assay was performed using the Endogen kit with a slight modification of the instructions of the manufacturer. PBMCs ranging in counts from 1-3 × 10^6 cells per well were stimulated with ESAT-6 and CFP-10 and mitogen (1 ng per ml of PMA + 500 ng per ml of ionomycin or 50 µg per ml of Con-A) and the plates were incubated at 37°C in a CO_2 incubator for the 24 h. The plates were washed six times with ELISPOT wash buffer incubated with 50 µl of detection antibody per well for 1 h at 22°C ± 2°C. The plates were washed six times with ELISPOT wash buffer and 100 µl of streptavidin-alkaline phosphate conjugate was added per well and incubated for 1 h at 22°C ± 2°C. The plates were washed six times with ELISPOT wash buffer and 100 µl of nitro blue tetrazolium/5-bromo-4-chloro-3 indolyl phosphate (NBT/BCIP) substrate was added per well and incubated at 22°C ± 2°C for 9 min. The reaction was stopped using 200 µl/well of ultra pure distilled water. The ELISPOT plate was scanned for the development of spots and assay results were analysed on an Immunospot® Series 5 UV Reader (CTL, Shaker Heights, Ohio) using its automated software features Immunocapture® and Immunospot® for user-independent settings of counting parameters (SmartCount®) and the gates (Autogate®). The assays were performed in triplicate, results were represented as a mean of triplicate wells and expressed as spot-forming cells (SFC) per million cells. This process was repeated for PBMC samples from various time points and the results were expressed as mentioned above.

Results

Status of cattle used in the study

Cattle 1 and cattle 2 were subjected to the skin test using BoPPD according to standard protocol described by Mukherjee (11). The animals were kept under observation for a period of 72 h and swelling was measured using a vernier. Cattle 1 and cattle 2 showed swellings of 8 mm and 2.5 mm indicating that the animals were a reactor and non-reactor, respectively. The presence of BoIFN-γ in both the animals following stimulation of their whole blood with the BoPPD and avian PPD was measured using the Bovigam® kit. A 20-fold increase was observed in the stimulation index of cattle 1 when compared to cattle 2 which indicated that cattle 1 was presumably positive for bovine TB infection.

Flow cytometry confirmation of tuberculosis-specific bovine interferon-gamma secreting cells

PBMCs isolated from cattle 1 and cattle 2 were subjected to cytokine flow cytometry at different time points (0, 2, 4, 6, 8, and 24 h) to determine the optimal time of incubation. Different concentrations of ESAT-6 and CFP-10 were also used separately or as a cocktail to stimulate PBMCs and the resultant BoIFN-γ cytokine secreting cells were quantitated using flow cytometry.

A rest period of 2 h of cells at 37°C followed by stimulation with 0.5 µg of a combination of ESAT-6 and CFP-10 for 6 h was found to be

optimal for the detection of TB-specific cytokine secreting lymphocytes. Cattle 1 showed 1.2% TB-specific IFN-γ secreting cells whereas cattle 2 showed only 0.1% TB-specific IFN-γ secreting cells.

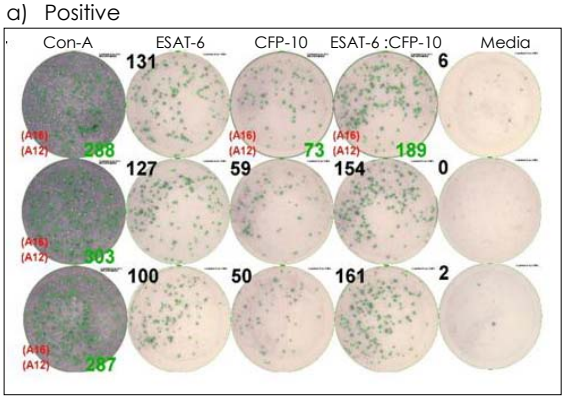
Determination of optimal concentrations of ESAT-6 and CFP-10

The BoIFN-γ ELISPOT assay was performed as indicated in the instructions provide with the Endogen kit with slight modifications. The frozen PBMCs were thawed rapidly and cell density adjusted to 1×10^6 cells/well and stimulated with different concentrations of ESAT-6, CFP-10 antigens and a combination of ESAT-6-CFP-10 (1:1) antigens and incubated at 37ºC at 5% CO_2 for 24 h. The number of TB-specific SFC in different antigen combinations is presented in Figures 1a and 1b. Results indicated that the use of a combination of antigens resulted in a synergistic effect in detecting the secretion of TB-specific IFN-γ in comparison to individual antigens. The cocktail of antigen at a concentration of 0.5 µg per well was optimal for the generation of the TB-specific SFC and it yielded an average of 165.34 SFC/10^6 cells which was much higher than other concentrations (Table I). Con-A mitogen at a concentration of 5 µg per well, in addition to PMA (1 ng/ml) and ionomycin (500 ng/ml) were used as positive controls.

Standardisation of methodology for purification of peripheral blood mononuclear cells and their use in the ELISPOT assay

The variables analysed were as follows: dilution of blood (i.e. dilution of blood with RPMI or without dilution), mode of blood storage until processing (gentle agitation vs without agitation), using Lymphoprep™ vs Lymphoprep™ tubes. The yields of PBMCs from blood samples were analysed at different time points (0, 2, 4, 6, 8 and 24 h), with or without dilution and agitation and at two different temperatures (22°C ± 2°C and 4°C). Results indicated that the PBMC yield dropped ~2-fold (59 million to 30 million following 6 h of storage at 22°C ± 2°C whereas the decrease was more significant ~5.5-fold

a) Positive



b) Negative

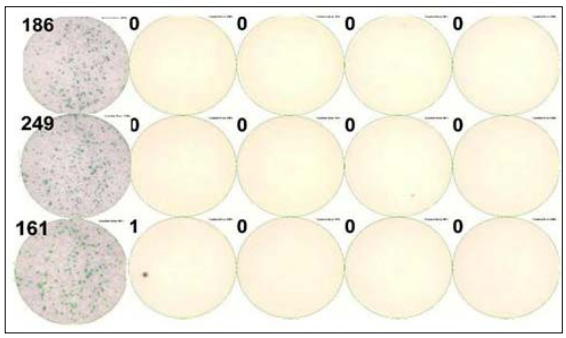


Figure 1
ELISPOT IFN-γ responses after stimulation with Concanavalin–A (5 µg), ESAT-6 (0.5 µg), CFP-10 (0.5 µg) and ESAT-6: CFP-10 (0.5 µg) and media in cattle 1 and cattle 2 presenting positive and negative skin test reactors, respectively

(59 million to 11 million) at 4°C which further increased (~10-fold) at 24 h (Table II).

Following the isolation of PBMCs, the cells were stored in the vapour phase of liquid nitrogen. Prior to stimulation, the PBMCs were thawed and recovery was estimated by viable cell counts before BoIFN-γ ELISPOT assay. Results indicated that there was maximum recovery of 60%-70% for samples processed after 2 h of blood collection at 22°C ± 2°C and, as time progressed, cell recovery reduced further, to as low as 10% at 24 h (Table II).

Effect of blood storage time and temperature

The various samples obtained as a result of the different processing methods were subjected to the BoIFN-γ ELISPOT assay on kinetic samples (0, 2, 4, 6, 8 and 24 h) with dilution and agitation at two different temperatures (22°C ± 2°C and 4°C) in replicates ($n = 3$) on two different days. The bovine ELISPOT IFN-γ

Table I
Spot-forming cells of tuberculosis-positive skin test reactor cattle 1 in the interferon gamma ELISPOT assay

Concentration of stimulant per well	SFCs in well 1	SFCs in well 2	SFCs in well 3	Mean SFCs	Corrected SFC (mean antigen SFC – media SFC
Con-A	288	303	287	292.66	290
ESAT-6 0.5 µg	131	127	100	119.33	116.67
CFP-10 0.5 µg	73	59	50	60.67	58
ESAT-6+CFP-10 0.5 µg	189	154	161	168	165.34
Media	6	0	2	2.66	

Con-A concanavalin-A
ESAT early secretory antigenic target
CFP culture filtrate protein
SFC spot-forming cells

response of samples fromTB-suspected cattle at different kinetic samples (0, 2, 4, 6, 8 and 24 h) that were processed and stored using two different methods, are presented in Figure 2. There was a declining trend in TB-specific SFCs when blood storage time (after 6 h post collection) increased. However, there was significant decrease in TB-specific SFCs in the samples stored at 4°C compared to 22°C ± 2°C. BoIFN-γ ELISPOT responses of TB-suspected cattle at different kinetic samples that had been stored after dilution and agitation (22°C ± 2°C and 4°C) performed in triplicate with TB-specific antigens (ESAT-6:CFP-10) showed that there was significant drop in the TB-specific SFCs after 6 h in the samples stored at 22°C ± 2°C after dilution and agitation. There was a significant drop in TB-specific SFCs in the samples stored at 4°C after dilution and agitation, and even in those samples monitored at the earlier time points. Samples stored for 24 h showed less SFCs (9-20 SFCs/ million cells) in both methods.

Tuberculosis-specific bovine interferon-gamma ELISPOT respons

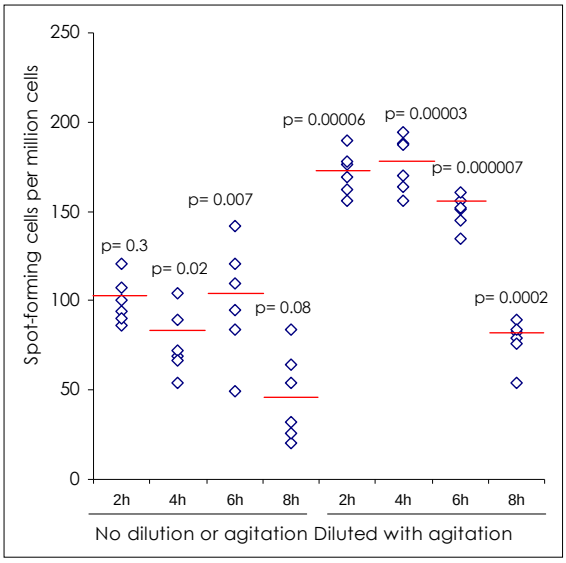
We tested the bovine IFN-γ ELISPOT on kinetic samples (0, 2, 4, 6, 8 and 24 h) undiluted and without agitation at two different temperatures (22°C ± 2°C and 4°C) in replicates ($n = 3$) on two different days. There was poor spot quality and quantity in TB-specific SFCs when blood storage time increased (Fig. 3a and Fig. 3b). Comparative performance of the ELISPOT response in both methods of blood processing clearly indicated that long-term blood storage without dilution or agitation leads to a decrease in the spot

Table II
Peripheral blood mononuclear cell count before and after cryopreservation in the kinetic samples stored after dilution and agitation

Kinetic sample	Temperature	Before cryopreservation (million PBMCs)	After cryopreservation/thawing (million PBMCs)
0 h	22°C ± 2°C	59	35
2 h	22°C ± 2°C	34	21
	4°C	35	13
4 h	22°C ± 2°C	37	15
	4°C	20	8
6	22°C ± 2°C	30	16
	4°C	11	9
8 h	22°C ± 2°C	18	10
	4°C	12	10
24 h	22°C ± 2°C	8	4
	4°C	5	2

PBMC peripheral blood mononuclear cells

a) Effect of temperature (22°C ± 2°C)



a) Effect of temperature 4°C

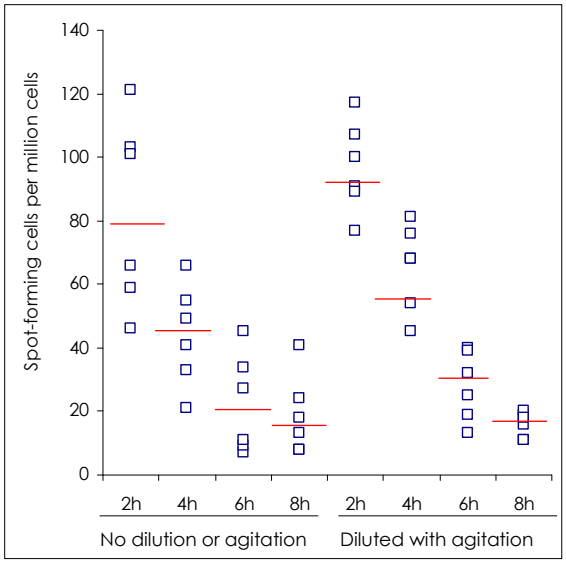


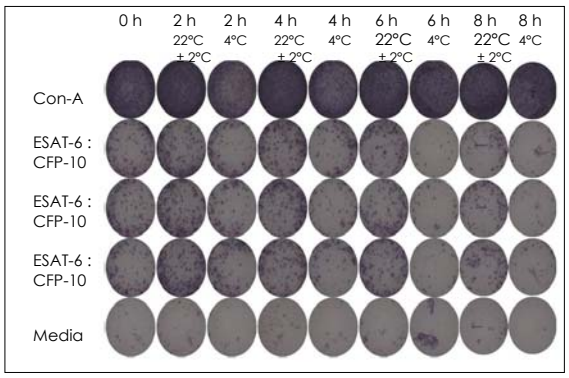
Figure 2
Effect of blood storage time and temperature in spot quality and quantity using ESAT-6: CFP-10 antigens in skin test reactor cattle 1 in diluted/agitated and undiluted/not agitated samples
The *p* values indicated that there was significant difference in spot-forming cells when samples were processed at 22°C ± 2°C after dilution and agitation compared to samples held at 4°C

quality and the drop yield might be due to the contamination of PBMC with granulocytes (9).

Discussion

A 2005 WHO report indicated that bovine TB was a disease of economic significance and

a) Effect on quality



b) Effect on quantity

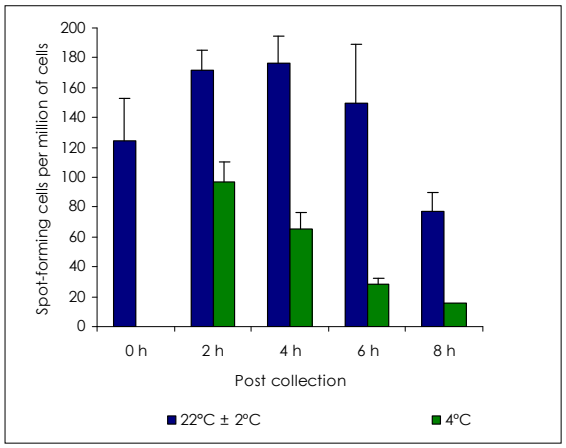


Figure 3
Effect of blood storage time and temperature in ELISPOT IFN-γ spot quality and quantity using ESAT-6: CFP-10 antigens (0.5 µg/well) in skin test reactor cattle 1 PBMCs in diluted and agitated samples

that it was an important health problem worldwide, ranking among the top seven zoonotic threats to humans (16). Advancement in current diagnostic and vaccination protocols used against bovine TB rely on a thorough understanding of the bovine immunological response. Immunological studies of *M. bovis*-infected cattle have provided ample evidence to suggest that cell-mediated immune response is an essential component of the host's fight against the disease (14). Assays that can detect antigen specific T-cell responses in immune-mediated processes are being increasingly employed to understand disease pathogenesis and immune staging of *M. bovis*. Moreover, recent diagnostic tests for bovine TB rely heavily on the formation of a cell mediated/IFN-γ response, thus helping us to

understand the dynamics of cellular immune response to *M. bovis*.

It has been estimated that designing a quick and accurate diagnostic tool for bovine TB could help in TB control programmes thereby restraining the spread of TB among cattle and other Bovidae. Currently the IFN-γ ELISPOT assay has been used for the detection and functional characterisation of antigen-specific immune response to TB. The sensitivity of the assay enables the direct enumeration of low levels of cytokine-secreting cells, a feature that has been utilised in clinical laboratory practice for the detection of human TB by the novel tuberculosis T-spot assay (7).

The isolation of PBMCs and setting up the ELISPOT assay at the time of sampling is logistically and financially unrealistic in many field situations. PBMCs can be separated, cryopreserved and used later for the ELISPOT assay and is therefore important to investigate the impact of blood processing on storage condition and temperature on cell viability, assay sensitivity, specificity and spot quality.

The aim of our study was to analyse various critical parameters such as the storage and processing of blood samples for isolation of PBMCs in the laboratory and their use in the BoIFN-γ ELISPOT assay. The quality of PBMCs was evaluated by processing at different time points and at different temperatures with or without dilution and agitation. The TB-specific antigens ESAT-6 and CFP-10 were used in this study as they have been identified as potent RD1 region encoded T-cell antigens of *M. bovis* and are absent from all other strains of *M. bovis* Bacillus Calmette-Guérin (BCG) as well as from almost all environmental mycobacteria.

TB-specific ELISPOT IFN-γ responses were optimal up to 6 h and substantially reduced after 6 h post collection when samples were diluted and stored following agitation. It was reported earlier that granulocytes become activated upon prolonged storage of blood and that PBMCs can become contaminated with activated granulocytes 8 h after venipuncture (8). Moreover, activated granulocytes affect the buoyancy profile in density gradient, resulting in poor separation of PBMCs (13) which leads to the reduced relative number of T-cells present in the isolated lymphocytes. This could probably explain the reduction in antigen-specific SFCs after prolonged blood storage observed in this present study.

It was also observed that the TB-specific ELISPOT IFN-γ responses of the blood samples stored undiluted and without agitation were significantly reduced compared to the samples stored diluted and after agitation (Fig. 2). With respect to the storage temperature, it was observed that 22°C ± 2°C gave significantly good TB-specific IFN-γ spots in ELISPOT when compared to those kept at 4ºC. This observation correlates with many previous studies which indicate that blood stored at room temperature leads to better results compared to blood stored at 4°C (8).

The *ex vivo* IFN-γ assays for bovine TB diagnosis to identify the number and the phenotype of the IFN-γ secreting cells have not been validated or studied as thoroughly as those involved in human TB diagnosis where IFN-γ assays showed higher sensitivity than SITT (7). Although the cost of these techniques is high, lacunae in the methodology for blood collection and processing of samples for timely delivery to the veterinary laboratories appear to be the major cause that prevents the use of these techniques in the diagnosis of bovine TB (1).

Conclusions

In conclusion, based on the present study, the following recommendations can be made when PBMCs are isolated for ELISPOT assays:

- blood can be drawn in sodium heparin vacuum-driven tubes and PBMCs separated using Lympoperp™ tubes within 6 h of post collection
- blood is best diluted 1:1 in c-RPMI with gentle agitation at 22°C ± 2°C for ELISPOT assays
- PBMCs can be cryopreserved if ELISPOT assays are not performed immediately
- for optimal detection of TB-specific SFCs, 0.5 µg of a combination of ESAT-6 and CFP-10 antigens can be used per million PBMCs.

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