Research Article

Development and validation of sandwich quantitative ELISA prototype based on the bovine IFNg for the detection of cellular immunity

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Abstract: Immunodiagnostic tests based on in vitro enzymatic methods are mostly preferred to in vivo methods for the detection of cellular immunity due to their cost, applicability, repeatability, and higher sensitivity. Therefore, the development of a quantitative sandwich ELISA prototype detecting bovine IFNg (bIFNg) was planned. In this study, two clone originated monoclonal antibodies were used for capturing the plates, and biotin conjugated monoclonal Ab was used as a secondary antibody in sandwich ELISA. Different approaches such as inter- and intraassay precision and dilution parallelism were evaluated for test validation. The correlation of coefficient variation that is over 95% and 5% variations in precision detection, with an estimated concentration of 98.7%, makes this prototype an alternative test for detection and quantitation of bIFNg. Trials intending to detect the shelf-life of the prototype for 6 months demonstrated that the detection limit was found to be approximately 100 pg/mL of the capturing Ab from clone I with a detection range of 10% CV throughout that period. Preliminary results show that the prototype can be applied to in vitro bovine tuberculosis detection for plasma samples at least for the validation period of 6 months.

Key words: Bovine IFNg, sandwich ELISA, tuberculosis, paratuberculosis, cellular immunity

1. Introduction

Tests based on classical cultural methods and the amplification of specific genes by polymerase chain reaction (PCR) are not sufficient for the diagnostics of some clinical and subclinical intracellular infections. Immunological methods detecting the cellular immunity are also required for diagnosis. These types of in vivo and in vitro cellular tests are based on cytokines produced specifically by these types of immune responses or reactions to antigens in tissues. In vitro applications are preferred to the counterpart in vivo tests for their higher sensitivity at the initial stage of infection and their repeatability, availability, and applicability to a variety of antigens. They are also better in terms of logistic facilities and shorter test intervals (shorter desensitization time). Therefore, in vitro applications are utilized in the detection of tuberculosis (TB) and paratuberculosis (1–5). Different enzyme linked immunosorbent assay (ELISA) models are implemented for the development of a sandwich quantitative ELISA (qELISA) for measuring proteins or cytokines (6–8).

Although the skin test is still widely used as a clinical indicator for evidence of previous or active tuberculosis infection in vivo, the European Union authorized a test known as Bovigam in vitro in 2002, and the test received approval by the USDA as an ancillary test in the US bovine TB surveillance program in 2003 (9,10). Since then it has been successfully adopted in Australia, New Zealand, South America, and Asia (7,9).

Cellular immunity can also be monitored in vitro either by cytokine release tests such as IFNg sandwich ELISA or by lymphoproliferation test based on a radioactive substance (11–13). There are a variety of commercial kits and protocols that are able to detect IFN gamma induced by specific protein antigens, especially for the detection of TB, brucellosis, paratuberculosis, and various parasitic and viral infections (2–4,7,14). Problems with the availability of these kits, their cost, and the requirement of standardization of the test for each event led us to develop the sandwich qELISA.

In this study, the production and the validation of the sandwich qELISA prototype were designed as an ancillary diagnostic test to be used for tuberculosis infections. Therefore, two kinds of monoclonal antibodies out of 5 commercially available ones with different clone origins and different ELISA reagents were used for the development of the quantitative sandwich ELISA to

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measure IFN gamma quantity. In the study, performance characteristics and stability tests of the prototype were performed and validation studies were completed with bovine TB confirmed plasma samples.

2. Materials and methods

2.1. Induction of blood cells and separation of plasma

Plasma samples used in this study were obtained from tuberculosis-suspicious blood samples and healthy calves. The induction and separation of plasma were performed in accordance with the instructions of Bovigam tuberculosis detection kit as follows. Initially, blood samples in heparin-containing tubes were transferred to cell-culture plates and then PBS, avian PPD, and bovine PPD were added. The mixture was incubated at 37 °C in 5% CO_2 incubator for 18 h to stimulate lymphocytes to produce IFNg. In addition to PPDs, Concanavalin A (ConA, Sigma C5275) was used as a mitogenic stimulator. Plasma samples separated from blood were used for detection of tuberculosis using the commercial ELISA kit (Bovigam), and then stored in the freezer until use. In the second stage, IFNg detection was tested using the sandwich ELISA as suggested by the manufacturer. Different criteria were used for the interpretation of the test results due to the difference and proportion of OD values in bovine, avian, and control samples (7). Results were interpreted as positive when OD values of bovine PPD-nil antigen and bovine PPD-avian PPD were found ≥0.1, and as negative when differences were ≤0.1 (9). Plasma samples induced with bovine PPD and found positive were used for detection of IFNg quantitation for the development of the prototype.

2.2. IFNg sandwich quantitative ELISA

In this study, modifications recommended by Rothel et al. (6), Rue-Domenech et al. (7), and Genç et al. (8) were used for the development of sandwich qELISA for measuring bIFNg in plasma and sera. Sandwich ELISA assays based on 2 types of conjugates and monoclonal antibodies were used to determine the IFNg quantity in plasma and sera; blocking buffer was the control. For this purpose, mouse monoclonal antibodies of bIFNg, originated from two different clones (bIFNg-I, Mabtech and CC302, Thermo), were used as primary antibodies and were absorbed to the wells of ELISA plates (Immulon, 2HB) at a concentration of 2 μg/mL in coating buffer (1X PBS, pH 7.4). After overnight incubation at 4 °C, the plates were blocked with 0.1% bovine serum albumin (BSA, Sigma) in 1X PBS (pH 7.4) for 1 h at 37 °C. The plates were washed three times with 50 mM Tris- HCl containing 0.05% Tween 20 (pH 7.0–7.4). Standard r-bIFNg (Thermo, RBOIFNGI, MF158073) was added into the reagents (blocking buffer, plasma, and sera) at different concentrations as triplicate and incubated for 2 h at 37 °C. After the plates were washed three times, biotinylated anti-mouse bIFNg (Mabtech,

PAN-biotin) was added at a concentration of 0.5 μg/mL as secondary antibodies. After 1 h of incubation at 37 °C, the plates were washed, and streptavidin conjugates with alkaline phosphatase (AP) (Sigma, S2890) and horse radish peroxidase (Sigma, S2438) were added to the wells and the plates were incubated at 37 °C for 1 h. Following washing, pNPP substrate (Amresco) solution for AP conjugate and OPD (Calbiochem, 523120) solution for PO conjugate were added and the plates were incubated for 60 min. The reaction colors were measured using a microplate reader (Thermo, Multiscan EX). The concentration of bIFNg was calculated using a linear-regression equation obtained from the absorbance values of the standards ($R^2 > 0.95$). In addition to test optimization, validation trials, assay precisions, detection limit comparisons, and shelf-life testing of the prototype were performed.

2.2.1. Validation of qELISA in the blocking solution, plasma, and serum samples

The evaluations of quantitation of recombinant bIFNg (r-bIFNg) in the blocking buffer, plasma, and serum samples were performed in the same plate. Results were expressed as pg/mL for specifying IFNg concentrations and the detection limit in samples.

2.2.2. Assay precisions

A standard curve was created on each plate by linear regression calculations with respect to the bIFNg standard calibrator in the blocking buffer, plasma, and serum samples. The results of the inter- and intraassays were interpreted after obtaining a regression value of $R^2 > 0.95$.

2.2.2.1. Interassay precision

Five different concentrations (50, 62.5, 125, 250, and 500 pg/mL) of reference r-bIFNg were evaluated in 5 separate plates in 4 repeats using blocking buffer, sera, and plasma. The coefficient of variation of each sample was calculated at 5 concentrations in the blocking buffer.

2.2.2.2. Intraassay precision

Each test, using 6 samples in 6 replicates, was performed to determine the coefficient of variation in two plates with 10–1000 pg/mL (10, 50, 100, 200, 400, and 1000 pg/mL) concentrations.

2.3. IFNg quantity with the confirmed plasma samples

In the study, sandwich qELISA was applied for the detection of IFNg quantity in plasma samples that were confirmed by Bovigam detection kit. For this purpose, a total of 122 plasma samples consisting of 72 tuberculosis positive and 50 tuberculosis negative samples were evaluated in terms of IFNg quantity. The results of the sandwich qELISA are presented in Table 1.

2.4. Dilution parallelism

Recombinant bIFNg in four samples within the range of 50–400 pg/mL was diluted at serial 2-fold dilutions, from 1:4 to 1:16, to ensure the recovery rate of IFNg (Table 2).

2.5. Shelf-life testing

Plates coated with clone I originated mAb blocked with BSA were packed with aluminum foil with desiccant and incubated at 4 °C for a period of 6 months. After the blocking step, 10 freshly coated qELISA plates and 15 out of the 50 IFNg coated plates kept at 4 °C were evaluated for 6 months. Mean OD and CV (%) comparisons of the sandwich qELISA based on different concentrations of bIFNg (100–800 pg/mL) were performed with freshly prepared ELISA prototypes for various time periods of 1,3, and 6 months (Table 3).

2.6. Statistics

Regression values and correlation coefficients were calculated, and comparisons of percentages and CVs were done with MedCalc-version 15.2.2 (http://www.medcalc.org).

3. Results

3.1. Quantitative enzyme linked immunosorbent assay

The results were 100% compatible with those of Bovigam, when the detection limit was evaluated as the cut-off value. All 50 positive plasma samples (50/72, 69.4%) approved with Bovigam tuberculosis test were found to contain over 5 ng/mL IFNg with the primary monoclonal antibody of the clone CC302. However, the detection limit with the primary antibody of clone I was determined as 100 pg/mL and it was evaluated as sensitive. The IFNg quantity results of 72 positive and 50 negative plasma samples are shown in Table 1.

3.1.1. Validation of qELISA with plasma and sera

Fifty negative and 72 positive plasma samples as detected by sandwich ELISA (Bovigam) were found compatible in 100% and 69.4%, respectively, with the antibodies of clone I and clone CC302 when the cut-off point was accepted as the detection limit. However, as clone I based qELISA has a cut-off value of 100 pg/mL, all 72 positive plasma samples were detected in different concentrations in a range of 125 pg/mL to 5 ng/mL. When a comparison of the detection limit was performed in connection with bIFNg in the blocking buffer, plasma, and serum samples, the blocking solution was significantly different (P < 0.05) from the other samples. The detection limit of bIFNg in the blocking solution was 50 pg/mL, but it was 100 pg/mL in both plasma and serum. Besides, the inhibitions were determined mostly in serum, but it did not make any difference in the detection limit (P > 0.05). The prototypes were tested in order to determine inter- and intraassay precision of the test for validation trials. Comparisons with both inter- and intraassay tests indicated a less than 5% coefficient of variation.

3.1.2. Sensitivity of the test

The lowest concentration of the test was detected as 100 pg/mL in plasma and sera and was used as the cut-off value throughout the test. At the same time, OD values of the negative controls were obtained with lower limits of 10–50 pg of r-bIFNg, so these values were accepted out of the detectable range. As the detection limits of plasma and sera were found to be 100 pg/mL, the concentration of r-bIFNg in negative samples was <100 pg/mL; this was concordant (90%, 45/50) with the negative samples obtained from tuberculosis free samples shown in Table 1.

3.1.3. Intraassay and interassay comparisons

The average coefficient of variation was less than 5% for each concentration within the detection limits. In interassay comparisons, the mean CV results of less than 5% were determined similar to the results in the intraassay studies (not illustrated).

Table 1. Sensitivity, specificity, accuracy, and detection range of the sandwich qELISA based on two primary antibodies with different clone origins.

Sandwich qELISA tests with two different primary Abs		Bovigam ELISA		Sensitivity	Specificity	Accuracy
		positive (n = 72)	negative (n = 50)			
Clone I		50 (>5 ng)*	5 (<500 pg)*	100	90	95.9
	positive (n = 77)	12 (125–500 pg) *				
		10 (125 pg) *				
	negative (n = 45)	0	45 (<100 pg)*			
Clone CC302	positive (n = 50)	50 (>5 ng)**	0			
	negative (n = 72)	22 (<5 ng)**	50 (<5 ng)**	69.4	100	82

A total of 122 plasma samples tested by Bovigam ELISA were used for comparison of the assays performed with clone I and clone CC302 monoclonal antibodies. *: plasma concentrations measured by clone I, and **: clone CC302 based ELISAs.

3.1.4. Recovery rates

In the study, the recovery rates ranged from 80% to 116%, with an overall recovery of 98.7% (Table 2).

3.1.5. Shelf-life testing

As a result, 25 plates absorbed with clone I originated monoclonal antibodies were used for detection limit comparisons for up to 6 months. The lowest detection limit was 50 pg/mL for the blocking solution, and 100 pg/mL for the plasma and sera samples. The study for determining the shelf-life of the prototype showed that IFNg could be detectable in both blocking and plasma samples for up to 3 months with 5% CV, and for 6 months with 10% CV in plates stored at 4 °C.

4. Discussion

Particularly for intracellular infections, diagnoses based on standard tests are not sufficient. Therefore, improved immunodiagnostic methods for detection of cellular immunity are necessary. In vitro applications are preferred to in vivo assays for their higher sensitivity, repeatability, availability and applicability to different antigens. They are also better in terms of logistic facilities and shorter test intervals (1,7,9). A major breakthrough has been the development of in vitro assays that measure T cell release of IFNg, which have become an alternative to the tuberculin skin test (1,15,16).

The bIFNg test is a well-established method for diagnosis of bovine tuberculosis with a sensitivity of 95.2% (7) and has been used in parallel with the tuberculin skin test in eradication programs (17). It was also demonstrated that the test is able to identify naturally infected but skin-test negative animals (18). Therefore, we aimed to develop a sandwich ELISA for the detection of IFNg response quantitatively in bovine whole blood samples based on commercial antibody reagents. For this purpose, the accuracy and test performance were evaluated using confirmed plasma samples. In this study, only bovine PPD positive plasma samples that were confirmed by Bovigam were used for the measurement of IFNg content. Therefore, 72 positive and 50 negative plasma samples as detected by Bovigam IFNg ELISA were tested for bIFNg content in comparison with the control negative and positive samples for each test. The results showed that IFNg can be detectable in plasma at least at a 125 pg/mL concentration with the use of PPD antigens. As the test was evaluated with different concentrations (not illustrated) and dilutions (Table 2) of r-bIFNg, it was evident that the prototype could detect IFNg in 50, 100, and 100 pg/mL in blocking solutions, plasma and sera, respectively.

In this study, the bIFNg ELISA results with primary antibodies of clone I originated monoclonal antibodies were found sensitive (100%) and accurate (95.9%), but less specific (90%) than those of clone CC302 (Table 1) Therefore, a prototype prepared with clone I originated primary Ab was evaluated for determination of shelf-life. Although the results are satisfactory, a reevaluation with more samples representing tuberculosis status is still needed for this prototype.

Antigen specific stimulation of IFNg production was utilized as a diagnostic tool for the detection of *M. bovis* and *M. paratuberculosis* in cattle (11,18). With this in mind, we designed the present study to evaluate the efficacy of the whole blood home-made bIFNg assay as a detection tool, and also to get some information on the

Table 2. The impact of dilutions on the recovery rate of r-bIFNg in the blocking solution.

Sample no.	Dilution rate	Expected (pg/mL)	Observed (pg/mL)	Recovery of r-bIFNg (%)	Mean recovery (%)
1	1:4	200	185	92.5	
	1:8	100	98	98	102.2
	1:16	50	58	116	
2	1:4	200	206	103	
	1:8	100	94	94	92.3
	1:16	50	40	80	
3	1:4	400	386	96.5	
	1:8	200	207	103.5	101.7
	1:16	100	105	105	
4	1:4	400	405	101.25	
	1:8	200	194	97	98.75
	1:16	100	98	98	

Recombinant bIFNg was diluted at different concentrations with the range of 50–400 pg/mL for detection of the recovery rate of r-bIFNg.

Table 3. Mean OD and CV values of the clone I based qELISA prototype for the detection of shelf life in comparison with simultaneously prepared fresh samples.

	Incubation time and number of ELISA plates											
IFNg conc.	fresh samples, n: 10			1 mo, n: 5			3 mo, n: 5			6 mo, n: 5		
Plasma (pg/mL)	Mean OD ± SD	CV(%)	MCV(%)	Mean OD ± SD	CV(%)	MCV(%)	Mean OD ± SD	CV(%)	MCV(%)	Mean OD ± SD	CV(%)	MCV(%)
800	3.38 ± 0.17	5.04		3.39 ± 0.13	3.89		3.38 ± 0.15	4.41		3.49 ± 0.32	9.41	
400	2.39 ± 0.11	4.25	3.8	2.40 ± 0.12	5.16	4.6	2.40 ± 0.13	5.20	4.8	2.48 ± 0.24	9.85	10.6
200	1.53 ± 0.04	2.45		1.60 ± 0.07	4.75		1.56 ± 0.08	4.86		1.50 ± 0.16	10.48	
100	0.77 ± 0.03	3.40		0.79 ± 0.04	4.58		0.80 ± 0.04	4.68		0.81 ± 0.10	12.57	
Blocking sol. (pg/mL)	Mean OD ± SD	CV(%)	MCV(%)	Mean OD ± SD	CV(%)	MCV(%)	Mean OD ± SD	CV(%)	MCV(%)	Mean OD ± SD	CV(%)	MCV(%)
800	3.36 ± 0.11	3.45		3.31 ± 0.17	5.10		3.43 ± 0.16	4.75		3.49 ± 0.28	8.05	
400	2.39 ± 0.08	3.39	2.8	2.41 ± 0.11	4.41	4.8	2.47 ± 0.13	5.29	5.6	2.45 ± 0.24	9.64	10.4
200	1.61 ± 0.02	1.72		1.60 ± 0.08	4.68		1.47 ± 0.08	5.61		1.50 ± 0.16	10.4	
100	0.75 ± 0.02	2.66		0.80 ± 0.04	4.99		0.81 ± 0.05	6.74		0.81 ± 0.11	13.5	

Mean OD and CV (%) comparisons of sandwich qELISA prototype based on different concentrations of bIFNg (100-800 pg/mL) in plasma and blocking solution for various time periods of 1,3 and 6 months.

n: the number of plates, mo: month, OD: optical density, CV: coefficient of variation, MCV: mean of CV

IFNg quantity of the evaluated plasma samples that were induced with bovine and avian PPD antigens. There is no precise information on IFNg levels in bovine TB and/or paraTB infected animals, but the detection limit of qELISA measuring IFNg concentrations is about 50–2000 pg/mL. As the animals sensitized with the agent can be detectable within that range, an evaluation of the test results can be performed according to the difference or the proportion of OD values of IFNg in plasma samples induced with a specific protein antigen. But some researchers consider IFNg levels instead of OD value interpretation. Mikkelsen et al. (19) confirmed positive paraTB in PPD johnin stimulated samples with IFNg concentration ≥1 ng/mL when interpreting the results. In our study, confirmed plasma samples stimulated with bovine PPD were evaluated and the results were categorized at a range of 100–1000 pg/mL concentrations. This classification, whether depending on concentration detection or on OD value interpretation, is not strict.

It can be concluded that our home-made prototype can be used for 6 months with 5% variations in precision detection and dilution parallelism with overall recovery rate of 98.7%. In addition, shelf-life studies showed that the prototype can detect bIFNg at a concentration of 100–800 pg/mL for 3 months with 5% CV and for 6 months with 10% CV (Table 3). Although there are many sandwich ELISA kits now available for bIFNg measurement with shelf-life of 1 year with detection capacity of <50 pg/mL (https://www.lifetechnologies.com, https://www.abdserotec.com), such a low detection limit is not required. This supports the use of our home-made sandwich ELISA prototype because our studies performed with the plasma samples, and confirmed by Bovigam test, showed that 13.9% (10/72) of the samples with the lowest IFNg concentration contained 125 pg/mL IFNg, while the lowest detection limit of our assay was 100 pg/mL (Table 1). As a result, this prototype can be applied to different research areas with the advent of novel recognized antigens. This model will facilitate the quantitation of cytokine detection for the cellular immunity determination and will be used as a serial or parallel test as an ancillary for bovine tuberculosis detection. This test can also be proposed for prediction of cut-off values and detection of bIFNg quantity in the range of 100–1000 pg/mL concentrations.

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