Original Research

Serological Detection of *Mycobacterium bovis* from Suspected Cattle by Fluorescence Polarization Assay

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Abstract

A Fluorescence polarization assay (FPA) was optimized and validated to detect antibodies to Mycobacterium bovis in cattle sera. A total 192 (cattle and buffalo) blood samples were screened for the CMI response by Comparative Intradermal (CID) tests and Gamma interferon (IFN-γ) tests as per the standard protocol of OIE, World Organization of Animal Health 2009. The presence of antibodies is indicative of prior infection with Mycobacterium bovis. Out of 192 animals screened, 14 and 23 animals were positive by CID and IFN-γ respectively. The diagnostic test revealed the results as out of 33 samples (positive samples by either of the two tests) and subjected to FPA, 7 samples were positive by confirmatory protocol given by the kit (milli-polarisation >50). A total of 53 animals (33 of which were positive by either of the CID and IFN tests, while 20 were negative by either of the CID and IFN-γ tests) were subsequently selected for the FPA. The FPA detected 12/53 (22.64%).

Key words: Antibody, CID, FPA

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Introduction

Mycobacterium bovis, is the causative agent for bovine tuberculosis (bTB), a chronic bacterial disease which infects and causes disease in animals, well as man. The disease is common in developing countries and causes severe economic losses. It has been included in the "LIST B" diseases of World Organisation for Animal Health (OIE, 2008). The disease can be transmitted in several ways. It can be spread by exhaled air, sputum, direct contact with the excreta of an infected animal or by inhalation of aerosols, depending on

the species involved. The infection leads to a decrease of milk production, loss of weight and reduction of fertility. The most frequently used diagnostic methods for bovine tuberculosis rely on the cell mediated immune (CMI) response to test for exposure to *M. bovis*. Diagnostic tests for CMI include the caudal fold test (CFT), CID and IFN gamma. The intradermal tuberculin test, which is most commonly used test in our country, is recognized by the World Organization of Animal Health (OIE) and the European Commission as the primary screening test for detection of tuberculosis in cattle (Karolemeas, 2012; Schillar, 2010). A disadvantage of diagnostic tests that are based on the CMI response is specificity, as false positive tests occur in cattle exposed with organisms related to *M. bovis* (Hope *et al.,* 2005). In addition to problems with specificity, the CMI diagnostic methods have a relatively high total cost per test. For all three of the diagnostic methods that rely on the CMI response there is the risk that as *M. bovis* disease advances, a severely infected animal may become anergic and fail to mount a detectable CMI response (Ritacco, 1991; Surujballi, 2002). Thus, conventional CMI methods may fail to detect infected animals with long standing *M. bovis* infection.

It is also reported that there is an inverse relationship between the cell-mediated and the humoral immune responses to *M. bovis* infection. There is also evidence which suggests that when animals are exposed to relatively high numbers of organisms, antibodies may be produced as early as two weeks post-infection Hanna *et al.* (1989). Serological assays provide an important tool for large volume testing for exposure to *M. bovis*. They offer the important advantages of ease of use, assay speed and relatively low cost. A serological assay for *M. bovis* testing would complement the other established *M. bovis* diagnostic methods and facilitate diagnosis of the disease (Lin, 1996; Domenech, 2006).There have been several reports of the development of low cost serological tests that might provide a more definitive diagnosis of *M. bovis* infection (Wood, 1992; Wood and Rother1994; Lin ,1996 ; Surajballi, 2002).

Fluorescence polarization assay (FPA) detects the binding of a fluorescent low molecular weight moiety (tracer) to its high molecular weight binding partner by determining the tracer's fluorescence polarization. FPA has been applied to serological immunodiagnostics for various veterinary diseases (Jolley and Nasir, 2003). FPA is well suited for animal disease general surveillance because of its rapidity, ease of use and high sensitivity and specificity (Jolley and Nasir, 2003). The FPA reported here is a direct binding primary screening assay using a fluorescence labeled small polypeptide derived from the *M. bovis* MPB70 protein. A secondary inhibition assay confirms suspect or presumed positive samples.

The MPB70 protein, secreted by *M. bovis* and other members of the *M. tuberculosis* complex, is a major immunodominant antigen (Wood, 1988; Lin, 1996). This protein is absent in *Mycobacterium avium* subsp. *avium or Mycobacterium avium* subsp. *paratuberculosis* which are other members of the genus having epidemiological importance. MPB70 protein has been shown to elicit both T and B-cell responses, and has been used in enzyme-linked immunosorbent assays (ELISAs) to detect antibodies in the sera of animals

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infected with *M. bovis* (Harboe, 1990; Griffin, 1991; Ritaccoet, 1991; Wood and Rothel, 1994; Gaborick, 1996; Sugden, 1997; Lightbody, 1998). A FPA for the detection of antibodies to *M. bovis* using the whole MPB70 protein has been described (Lin, 1996; Surujballi, 2002). There is very scarce report from our country where in FPA has been utilized for diagnosis of bovine tuberculosis. Here, we describe the use of a FPA employing a polypeptide-based tracer, derived from the whole MPB70 protein (Jolley and Nasir, 2003) as a diagnostic tool for *M. bovis* surveillance in bovine populations.

Materials and Methods

The study was carried out in the Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

Sample Collection

Samples were collected from organized dairy farms in and around Ludhiana, GADVASU, which were screened for the CMI response by CID and IFN-γ as per the standard protocol of OIE 2009.

Kits Used for Screening of Animals for Bovine Tuberculosis

All the kits used for testing of animals were procured from standard firm. Tuberculin PPD Kit (Prionics, Netherlands), BOVIGAM assay kit (Prionics, Switzerland) were used in the present study.

Screening of Animals by Comparative Intradermal Tuberculin Test (CITT)

The selected animals were subjected to CID, as per OIE Terrestrial Manual (2009) after blood collection. Bovine tuberculin PPD from culture of *M. bovis* (strain 67 AN5, 3000 IU) and avian tuberculin PPD from culture of *M. avium* subspecies *avium* (strain D4ER, 2500 IU), obtained from Prionics (Netherlands), were used for CID. Two 2 x 2 square inch areas were shaved at the middle third of left side of neck approximately 12-15 cm apart. The zero-hour skin thickness was measured with the help of a vernier caliper. The bovine and the avian tuberculin PPD (0.1ml each) were injected intra-dermally.

Gamma Interferon Assay (BOVIGAM assay)

This test was performed using *Mycobacterium bovis* Gamma Interferon Assay Kit for Cattle (BOVIGAM) procured from Prionics, Switzerland. BOVIGAM is a rapid in-vitro blood-based assay of cell-mediated immune response to *M. bovis* purified protein derivative (PPD). Reagents were reconstituted according to manufacturer‘s instructions before performing the actual test. The Assay was performed in two stages, Stage one was done by Whole blood culture method. A minimum volume of 5 ml of blood from each animal was collected into BD (Becton, Dickinson and Company) vacutainer containing heparin as anticoagulant and gently mixed and transported to the laboratory at ambient temperature (Fig. 1). Three 1.5 ml aliquots of heparinized blood from each animal were dispensed into wells of 24-well tissue culture plate under

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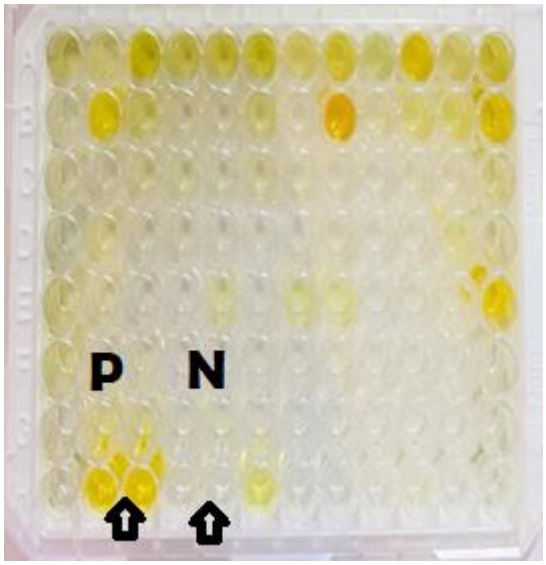
aseptic conditions using sterile pipettes and tips.100µl each of PBS as nil antigen control (pH-7.2), avian and bovine PPD (Prionics, Netherlands) were added aseptically into three wells containing heparinized blood dispensed previously.

Blood mixed with stimulating antigens on a 24-well tissue culture plate



Gamma Interferon assay showing positive reaction.

A-G Row : Test samples

H Row: Negative-N , Positive-P

Fig.1: Gamma Interferon Assay

The antigens were mixed thoroughly were incubated in a humidified atmosphere for 16–24 hours at 37°. After that tissue culture plate containing blood and antigens was centrifuged at 500 g for approximately 10 minutes at room temperature. Then, 500µl of plasma sample was carefully removed and transferred to separate storage tubes were stored at 4°C before being tested within 7 days of collection. Stage two was performed by Bovine IFN-γ Enzyme Immuno Assay (EIA). The absorbance of each well was taken within 5 minutes of terminating the reaction by using a 450 nm filter with a 620 nm reference filter at the end (Thermo Scientific multiscan Go).

Screening of Cattle Sera for *M. bovis* by TB*FPA (ellie) FPA Kit

FPA Testing Procedure

100 µl of serum sample in quadriplets were taken into micro plate (Black Nunc Fluorescence plate) along with known negative control. Then 100 µl of sample diluent was added into each well. After mixing, 10 µl of inhibitor was added to two wells of the four quadruplicates of the same sample. The plate was then incubated for 20 minutes at room temperature. Blank readings of all the samples and controls were also obtained. Then 10µl of tracer was pipetted into all wells containing control and test sample. After proper

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mixing the plate was covered with foil and incubated for 2 minutes at room temperature. In the final step millipolarisation (mP) readings of all samples and controls were taken (Tecan F-200 Infinity FPA reader).

Result and Discussion

Diagnosis of Mycobacterial infections in dairy animals (cattle and buffalo) is of great concern in India. Detection of mycobacterial infections is a tedious job as no single diagnostic tests can currently give 100% sensitive and specific results. Considering the current situation, there is need for the development of newer approaches for the easy and accurate diagnosis for the further treatment, control and prevention programs. The present study was conducted for diagnosis of *Mycobacterium bovis* by fluorescence polarization assay. The results were evaluated based on the screening of animals by CID tests and IFN-γ tests. In CID, a total of 14 animals were found positive whereas 162 animals were found negative and 16 were found inconclusive. Similarly, in IFN-γ assay 23 sera samples were found positive, 169 negative respectively (Table 1).

Table 1: Depicting results Screening of animals by (CID) and (IFN-γ)

Number of Animals (192)	Positive	Negative
Comparative intradermal test (CID)	14 (7.29%)	178
Interferon gamma test (IFN-γ)	23 (11.97%)	169
Total	37	
Positive from both tests (CID& IFN-γ)	33	

Serum samples were taken from animals which were positive from both the screening tests. The analysis was done on the basis of the screening and confirmatory protocol given in the kit. The confirmatory protocol is done by calculating the percentage inhibition value for each sample. A sample is considered positive when the PI (percentage inhibition) of the sample is more than 50%, negative if less than 25 % and a suspect when it is between 25 and 50 %. The kit was standardized to a gain factor of 1.122 and the incubation time varied according to the sample size. The incubation time observed for a microtitre plate was 2 minutes. It varied upon the number of samples tested at one time on a micro titre plate. The negative control must be read between 70 and 90 mP and the positive control must read between 120 and 250 mP as per the kit instructions. The FPA results were compared to the results of the comparative intradermal tests (CID) for (N=192) animals. 33 samples were in common obtained from screening by both the tests and were further subjected to fluorescence polarization assay by (TB*FPA ellie kit), out of which 7 came out to be positive from confirmatory protocol given in the kit. 20 negative samples were also subjected for FPA as control, out of which 5 were found positive for FPA. Hence, a sum total of 53 animals were selected for the FPA respectively. The FPA detected 12/53 (22.64%). Out of the 53 animals tested for FPA, 33 were positive by either of the CID and IFN-γ tests while 20 were found negative (Table 2).

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Table 2: List for TB.FPA Ellie kit

Total no. of animal s positive from both the tests	37/192
No. of Animals [+] for CID & IFN-γ (common + samples)	33/192
No. of Animals [-] for CID	162/192
No. of Animals [-] for IFN-γ	149/192
No. of animals positive for FPA (mp values> 50)	7/33
No. of animals negative for FPA (mp values< (25)	20/20

Application of the FPA might significantly reduce this delay and the associated costs. The technical simplicity, speed and low cost of this serological assay, makes it very attractive for use in conjunction with a test that measures the CMI response, such as the lymphocyte stimulation test, the gamma interferon test or the skin test, or, possibly even as a stand-alone screening test (Surajbali, 2002).

Conclusion

The ability of the FPA to detect antibodies in such a high percentage of the *M. bovis*-infected but skin test-negative animals supports the premise of using serological tests in conjunction with CMI tests to monitor tuberculosis infection. There is evidence that the skin test elicits a strong anamnestic antibody response in infected animals (with no such response in uninfected animals (Harboe, 1990; Hanna, 1992; Lightbody, 1998). Since, we have no information on the pre-skin test serological status of these animals, we cannot comment on whether there was a skin test-induced increase in their antibody levels, and this will need to be studied further. However, if this should prove to be the case, the possibility remains that this FPA could be used as a confirmatory test after a skin test had been administered. Such application may be of use in some countries since animals which react positively to the initial SID test are re-tested with the comparative cervical test (CCT). But it has been recommended that such a re-testing be delayed for a period of approximately 60 days (Radunz and Lepper, 1985 and Doherty, 1995).

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