Original Article

A fluorescence polarization assay using recombinant protein *ESAT-6* for the detection of antibodies against pathogenic *Mycobacterium bovis* in bovine

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Abstract

Background: Bovine tuberculosis (bTB) is a major bacterial disease that causes significant economic disruption across the globe. **Aims:** Our study was based on using a fluorescence polarization assay (FPA) that used fluorescein-labeled ESAT-6 protein to detect *Mycobacterium bovis* antibodies in bovine serum. **Methods:** The ESAT-6 protein was used in a FPA. Positive TB reactors were determined by the comparative intradermal test (CID) and interferon gamma test (IFN-γ). Antibodies against *M. bovis* were detected using a fluorescein isothiocyanate (FITC) labeled tracer and a whole culture FITC labeled tracer in the positive cattle. **Results:** Of the 192 animals tested for bTB, 37 were found to be positive by either the CID or IFN-γ assays. Using the mP values from five culture-positive serum samples, a cutoff value of more than >127 mp provided the best discrimination between positive reactors and negative bTB animals. The ESAT-6 results of FPA in comparison with CID results revealed sensitivity of 92.9% and specificity of 64.6%, and in comparison with results IFN-γ, showed sensitivity of 95.7% and specificity of 49%. FPA using FITC labelled ESAT-6 as a tracer has better sensitivity (95.7%) and specificity (49.1%) than IFN-γ test in humoral immune response in animals. **Conclusion:** This work revealed that the ESAT-6 protein as an antigen can be used in diagnosing bTB using a practical and sensitive humoral test.

Key words: ESAT-6, FITC, Fluorescence polarization assay, Gamma interferon assay, *M. bovis*

Introduction

Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex. This disease is still widespread in poor countries, causing major economic losses and trade restrictions. The gold standard diagnostic assay for bTB is indeed the comparative intradermal (CID) test (skin test) utilizing pure protein derivative (PPD). The CID test and other CMI-based diagnostics (e.g., gamma interferon and lymphocyte proliferation tests) have a multitude of disadvantages in the diagnosis of bTB. The limitations are the poor sensitivity and specificity of the delayed-type hypersensitivity in response to the antigen. It's unclear whether *M. bovis*, *M. avium* subsp. *paratuberculosis*, or environmental mycobacteria like *M. avium* subsp. *avium* are responsible for the PPD response. Another, there is a substantial CMI response during the initial phases of infection with low bacillary loads, which may be scarce or deficient in animals at late stages of sickness with high bacterial loads. Tests based on CMI cause the animals to become anergic (Harboe *et al*., 1990; Mirsky *et al*., 1992). The ESAT-6 system produces ESAT-6, a 6-kDa early secretory antigenic target of *M. tuberculosis* encoded in region of difference 1 (RD1) and generated by *M. tuberculosis*. Esx-1 is a tuberculosis-specific antigen with a significant immunodominance (Kerry *et al*., 2011). It is one of the most essential features that contributes in TB immunodiagnosis and vaccine development. Infected calves respond strongly to the mycobacterial antigen ESAT-6, a low molecular weight protein present in virulent *M. bovis* culture filtrate, whereas non-infected animals do not (Buddle *et al*., 1999; Vordermeier *et al*., 1999). Furthermore, it is absent from the majority of environmental mycobacterial species (Harboe *et al*.,

1996). The skin response to CMI-based tests is thought to be a marker of TB in cattle, but a second test should be done to confirm the diagnosis. This test evaluates an animal's responses to PPD-A and PPD-B to determine if it is positive or negative. However, susceptibility to environmental mycobacteria or coinfection with *(Mycobacterium aviam paratuberculosis*) lowers specificity in both tests (CID and IFN-) (Schiller *et al*., 2010). In our research, the ESAT-6 protein was used in enzyme-linked immunosorbent assays (ELISAs) to detect antibodies in the serum of *M. bovis* infected mice. ELISAs, on the other hand, are lengthy tests designed mainly for laboratory use that take at least one working day to complete (Lin *et al*., 1996).

Materials and Methods

192 animals (cattle and buffaloes) were chosen at random from an organized dairy farm in Ludhiana from various age groups (3-14 years). CID and IFN-γ tests were used to screen all of the animals. Samples containing blood were collected from above mentioned TB reactor animals.

Ethics approval

All authors appreciate the ethical committee for permitting the use of animals, and the iaec/cpcsea clearance may be accessed under reference number iaec/2015/26/013.

Comparative intradermal (CID) test

As per the World Organization for Animal Health Office International des Epizooties (OIE) Terrestrial Manual (2009) following blood collection for IFN-γ assay, desired animals (n=192) were subjected to CID. The test was performed using bovine tuberculin PPD from *M. bovis* (strain AN5, 3000 IU) and avian tuberculin PPD from *M. avium* subspecies *avium* (strain D4ER, 2500 IU) cultures purchased from Prionics (Netherlands). The bovine PPD was administered in the caudal region, whereas the avian tuberculin PPD (0.1 ml) and the bovine tuberculin PPD (0.1 ml) were injected intradermally. The animal was declared positive if the rise in skin thickness at the bovine injection site was more than 4 mm larger than the response seen at the avian injection site. If a slight variation of 1 mm variation in skin fold thickness was detected, the response was reported as negative. Results are declared doubtful if the difference lies between 1-4 mm.

Gamma interferon (IFN-γ) test

BOVIGAM (Prionics, Switzerland), IFN-γ kit for cattle, was used to perform this test. It is a fast in-vitro blood-based test of *M. bovis* PPD cell-mediated immune response. Before interpreting the sample data, the control results were checked for test validity. The animal was deemed positive when the difference between the OD of bovine PPD and the OD of avian PPD, and the difference between the OD of bovine PPD and the OD of nil antigen was less than 0.1. When the difference between the OD of bovine PPD and the OD of avian PPD, as well as the difference between the OD of bovine PPD and the OD of nil antigen, was less than 0.1, the animal was ruled negative.

Detection of antibodies against *M. bovis* using fluorescein labelled ESAT-6 protein in FPA

Materials required

ESAT-6 protein (Prospec, USA), sephadex (G-25 super fine) (MP Biomedicals, India), borosilicate column, fluorescein isothiocyanate (FITC) (MP Biomedicals India), and buffers (phosphate buffer, normal saline). Blood samples (n=192) were obtained from the same animals which were earlier screened for CID and IFN-γ assays. These blood samples yielded sera and were preserved at -20°C. They were then thawed at room temperature before being tested.

Preparation of FPA antigen

As per method, the ESAT-6 protein (20 g) was labelled with FITC (MP Biomedicals, India) (Surujballi *et al*., 2002). 20 g of the protein was added to nah_2po_4-naoh (0.15 m, ph 9.5) and combined with fitc (0.15 ml, 1 mg/ml). After 1 h of incubation at 37°C, the mixture was put in a sephadexG-25 column (MP biomedicals Ltd., India) (1 cm × 23 cm). Phosphate buffer (0.1 M, pH 7.0) was used to pre-equilibrate the column (Radostits *et al*., 2010). A total of 25 fractions from the column were extracted, and the protein concentration was determined using a NanoDrop. A fluorescence polarization analyzer was used to measure the fluorescence intensity of all the fractions (Model Tecan infinite F-200 pro, India). As a final tracer, the fraction with the greatest fluorescence intensity was utilized. The fractions were then kept in test tubes at 4°C until they were required.

Fluorescence polarization assay

In a 12 mm × 75 mm borosilicate glass test tube, each serum sample was diluted 1:5 (final volume of 1 ml) in phosphate buffered saline (Sigma, India) supplemented with 0.1 percent sodium azide and 0.05% lithium dodecyl sulphate (MP Biomedicals, India). A fluorescence polarization analyzer was used to get a blank result after full mixing. The test was performed in a 96-well Nunc® black plate. Each well had 180 µL of PBSALDS (PBS, pH 7.2) (Sigma, India) supplemented with 0.1% sodium azide and 0.05% lithium dodecyl sulphate (MP Biomedicals, India) buffer, 10 µL of serum, and 10 µL of tracer for 2 min incubation. With a gain optimal, number of flashes = 25, settle time =100 s, and filters of 485 nm for excitation and 533 nm for emission, the fluorescence polarization of the sample was observed. The result was expressed in millipolarization values and indicated the amounts of antibodies present in the serum sample.

Statistical analysis

Receiver operating characteristic curve analysis was used to examine the data (MedCalc Software, Maria kerke, Belgium). A dot blot was generated first, then a

receiver operator characteristic analysis was performed (ROC). The FPA findings were analysed to establish the best cutoff point for discriminating between positive and negative results (at which the sum of sensitivity and specificity values is maximum). The area under the ROC curve, which indicates the test's accuracy, is calculated using ROC analysis. The sensitivity and specificity values for CID and IFN-γ as the reference test were computed in the second technique.

Results

CID and IFN-γ

CID was implemented to screen 192 animals for bTB, and after injecting both the bovine and avian PPD antigens, a four-fold increase in skin thickness was found. CID identified 14 animals (7.29%) as positive for bovine tuberculosis out of 192 animals examined for the disease. The findings revealed that 16 animals were inconclusive, whereas the remaining 162 animals had negative reactions. The CID test is the gold standard for diagnosis of bTB in animals (live) in the field, and at least a 4 mm rise in skin thickness should be visible after 72 h of performing the test to confirm it as positive (OIE, 2009). Secondly, the tests were based on hypersensitive responses that were delayed (OIE, 1992). This test has a number of documented flaws, including issues administering and interpreting the findings, the necessity for a second step visit, a lack of standardization, and test accuracy that is not perfect (Gutierrez *et al*., 1998). False negative responses may happen just after an infection, late in the infection, in animals with weakened immune systems, and in animals that have recently given birth (Radostits *et al*., 2010). The strength and dosage of tuberculin delivered, desensitization, purposeful interference, post-partum immune suppression, and observer variance all alter the test's sensitivity. Specificity is influenced by sensitization to *M. avium*, *M. paratuberculosis*, and other environmental mycobacteria as reported by (Collins *et al*., 1994). CID can discriminate animals infected with *M. bovis* and those sensitized to tuberculin after exposure to *M. avium* complex organisms to non-pathogenic mycobacteria more readily than SIT (Monaghan *et al*., 1994). Bovine tuberculin reactivity is generally highest in cattle infected with *M. bovis* or *M. tuberculosis* (Francis, 1958; Pollock *et al*., 2005). Using CID, infected cattle might be recognized as early as three weeks after infection (Kohler *et al*., 2001). Desensitization to a second tuberculin injection in infected calves due to the depressing impact of the prior injection after a period of skin reactivity may result in the inability to identify infected animals as reactors (Lepper *et al*., 1997). Errors due to malfunctioning equipment or operator errors, such as when tuberculin is not lodged into the skin, the amount injected is insufficient, or the test findings are evaluated too soon or deferred after tuberculin injection, could perhaps result in false negative skin test results, as with any diagnostic test (Mihret *et al*., 2013).

Gamma interferon assay

Gamma interferon assay was conducted on the same animals. 23 animals (11.9%) were positive for IFN-γ from 192 animals assessed for tuberculosis. Twenty of these animals had an equivocal response to the IFN-γ assay, displaying a pale yellow hue in the well, whereas the other 149 animals had a negative reaction (Table 1). In the identification of *M. bovis* infection, IFN-γ is a potent biomarker. In most tuberculosis eradication and control programs, the IFN-γ assay is used in the detection of bovine tuberculosis and is now mostly used as a complement to the skin test *in vitro*. IFN-γ is generated in large amounts, making it an excellent biomarker for TB diagnostic testing (Wood *et al*., 1991). The IFN-γ test is often used to assess cell viability by stimulating whole blood for 16 to 24 h with *M. bovis* PPD B and PPD A (Rua-Domenech *et al*., 2006). The IFN-assay has a higher sensitivity and a slightly lower specificity than the skin test, needs only one visit to the farm, and eliminates the observational variability associated with measuring skin test responses (Schiller *et al*., 2010). The sensitivity and specificity of the IFN-γ test are expected to be 73 to 100% and 87.7 to 99.2%, respectively, when PPDs are used (Harboe *et al*., 1990).

Table 1: List of animals positive for CID and IFN-γ

Number of animals (192)	Positive	Negative
CID	14 (7.29%)	178
IFN-γ	23 (11.97%)	169

Fluorescence polarization assay was performed using ESAT-6 protein (Prospec TechnoGene Ltd., USA) as antigen labeled with FITC. Figures 1 and 2 represent the frequency distributions of the FPA results from 192 serum samples and 5 culture positive animals. The histograms presented that the majority of the serum samples had cutoff values of >127. This indicated cutoff value of 127 for differentiating between positive and negative samples. According to the approximate value of 127, our results were categorized into positive and negative samples.

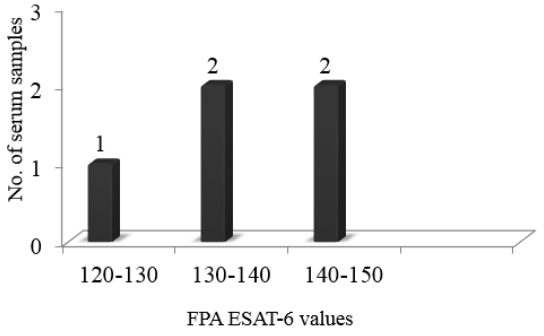

Fig. 1: Frequency distribution of the FPA results that were obtained for the serum samples with a cutoff value of >127 mP. Maximum discrimination between positive reactors and negative bTB animals was established by using the mP values from 5 culture positive serum samples. The x-axis shows mP values and the y-axis shows number of serum samples

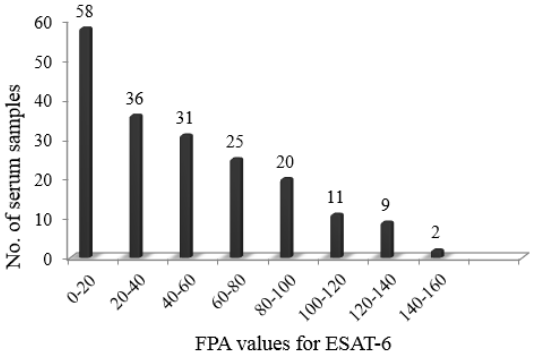

Fig. 2: Frequency distribution of the FPA results that from 192 serum samples of cattle/buffalo. The x-axis shows mP values and the y-axis shows number of serum samples

FPA in comparison to CID and IFN-γ

The results of the FPA test on the ESAT-6 protein were juxtaposed to the findings of the CID and IFN-γ tests. The MedCalc programme enabled to determine the cutoff. ROC analysis created a dot plot to begin with. FPA developed a dot plot and ROC curve analysis for cow and buffalo sera, respectively. Figures 3 and 4 showed a cut-off value of 29 mp for maximum, discriminating between positive and negative animals when compared to CID. When comparing FPA's ESAT-6 findings to CID's, the AUC curve (area under the curve) was 0.715, with a sensitivity of 92.9% and specificity of 64.6%. With an AUC of 0.715, a randomly chosen individual from the population will have a higher test value than a randomly selected individual from the negative population. Final results from FPA were also compared with IFN-γ results. IFN-γ results revealed a cutoff value of ≤44 with AUC curve (Figs. 5 and 6) of 0.729 (sensitivity = 95.7%, specificity = 49%).

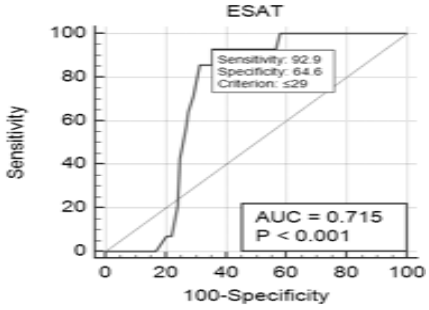

Fig. 3: ROC analysis of serum samples

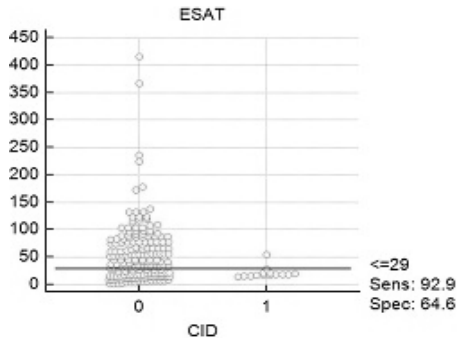

Fig. 4: Dot plot of the antibodies of serum samples

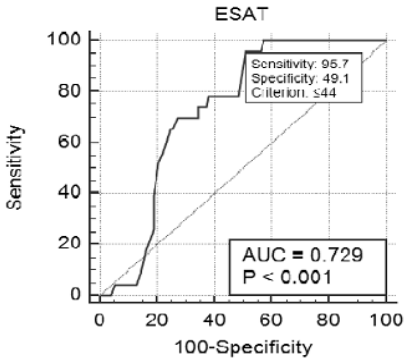

Fig. 5: ROC analysis of selected serum samples using ESAT-6 protein with IFN-γ

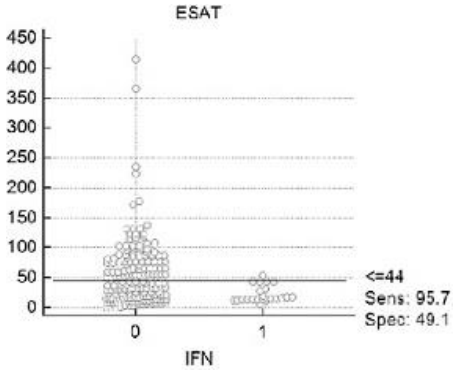

Fig. 6: Dot plot of selected serum samples using ESAT-6 with IFN-γ obtained from MedCalc software

Discussion

Bovines are among the most economically important animals affected by tuberculosis. We conducted ESAT-6 FPA for the identification of *M. bovis* antibodies in the serum. It is reported that like ELISAs, FPA has numerous advantages over many serological tests for detecting antibodies to *M. bovis* (Schiller *et al*., 2010). FPA was reported as an inhibitory test by Jolley *et al*. (2007) that confirmed questionable or suspected positive samples. Total of 4461 assumed negative bovine samples were tested in the specificity trials by different veterinary labs. The FPA was used to analyse if the herd was infected with *M. bovis* or not. The fluorescein-labeled ESAT-6 protein is utilized as the tracer antigen in the FPA test, which assesses the humoral immune response. The substantially bigger fluorescein-labelled immune complex that develops when antibodies in the serum bind to this protein. The estimated molecular weight of 6 kDa, results in an increase in fluorescence polarization. The fluorescein-labeled protein remains free in the absence of particular anti-ESAT-6 antibodies in the serum, leading to a low fluorescence polarization value. The skin reaction to PPD in CID is used as a marker for TB in cattle; however, a second screening test is used to make a more precise diagnosis. The IFN-γ test has been thoroughly investigated, and its usage has been authorized in a number of nations (Aranaz *et al*., 2006). The reactions of culture positive animals are compared in this test to determine if an animal is positive or negative.

The specificity of both tests is affected by sensitivity to environmental mycobacteria or co-infection with MAP (Hanna *et al*., 1989). The IFN-γ test is a highly specific test, but it is unduly expensive, leading to certain restrictions. In this work, IFN-γ and CID responses to the protein ESAT-6 were compared with the goal of determining sensitivity in animals with high and low bovine TB prevalence utilizing CID and IFN-γ formats. The FPA data was put through ROC curve analysis, which calculates a test's sensitivity and specificity at all conceivable cutoff points and offers a high end precision. The MedCalc programme was used to determine the cutoff. The ESAT-6 FPA is a precise indication of *M. bovis* infection, as per ROC curve produced from the study of the FPA findings. The positive sera from the infected animals were limited; so they were collected from bovines from which *M. bovis* could be cultured. The purpose of using a positive sample was to verify that no serum sample was incorrectly categorized, as well as to restrict the sample size since we only had a few of these serum samples. A value of n >127 mp provided the best discrimination between positive and negative animals at the specified cut-off point. When FPA's ESAT-6 findings were compared to CID results, the sensitivity was 92.9% and the specificity was 64.6%. The FPA findings were also compared to those of interferon gamma (IFN-γ). The sensitivity of IFN-γ was 95.7%, while the specificity was 49%. However, due to the feeble status of the bovine immunity, all herds of sick cattle may not demonstrate this high degree of sensitivity. Antibodies may be found as early as two weeks post-infection with a large number of germs, according to research (Costello *et al*., 1997). As a result, there arises a necessity for regular use of an *M. bovis* specific serological test in order to complement the use of the CMI test(s) and gamma interferon testing in order to effectively identify *M. bovis* infections in cattle and control disease transmission. It has been obvious that currently existing serological tests developed for herd testing have limited sensitivity and low specificity due to the use of complicated bacterial extracts including antigens produced by pathogenic and environmental mycobacteria (Ritacco *et al*., 1990; Neil *et al*., 1994). To summarize, the FPA has the ability to identify antibodies in *M. bovis* infected cattle but skin test-negative animals back up the idea of employing serological tests in combination with CMI tests to monitor TB infection. Finally, the use of the ESAT-6 protein helps identify infected animals not detected with CID and IFN-γ, particularly in herds with low frequency. Another evidence supporting the protein's high specificity and sensitivity is that despite the presence of *M. bovis* infection in certain herd animals, no skin reactions were seen. However, a negative result in the CID and IFN-γ assays does not signify that the animal is not infected; it just means that it is in a non-shedding state.

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Conflict of interest

There is no conflict of interest.

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