Int J Pharm Bio Sci 2012 Oct; 3(4): (B) 740 - 749

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

DETERMINATION OF THRESHOLD STIMULATION INDEX FOR IGRAS USING ESAT-6 AND CFP-10 ANTIGENS FOR BOVINE TB DIAGNOSTICS IN INDIA

BHAVANI V. J[1], MAROUDAM VEERASAMI[2], DEV CHANDRAN[*2] AND DIPANKAR DAS[2]

[1] Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, India.
[2] Research and Development Centre, Indian Immunologicals Limited, Gachibowli, Hyderabad 500 032, India

ABSTRACT

Bovine tuberculosis (bTB) leads to morbidity, mortality and economic losses worldwide and can be controlled by detection and removal of infected animals. Bovigam™, based on IFN-γ release assays, (IGRAs) has been recently approved as an ancillary diagnostic test to the conventional tuberculin skin test (TST). The assay, which is costly kit is based on the use of purified protein derivative (PPD) as antigen, and thus leads to low specificity. In the present study, the IFN-γ response to bTB was evaluated *in vitro* with bTB specific antigens ESAT-6 and CFP-10 and the secreted IFN-γ was estimated using in-house standardized bIFN-γ IC-ELISA. The TST negative cattle (n=100) from a bTB low incidence farm were screened to determine the assay stimulation index cut-off for IGRAs. The achieved cut-off was applied in the three reference positive and three reference negative samples for estimation of assay sensitivity and specificity.

KEY WORDS: Bovine tuberculosis, bIFN-γ, IGRAs, IC-ELISA.

DEV CHANDRAN
Research and Development Centre, Indian Immunologicals Limited, Gachibowli, Hyderabad 500 032, India

INTRODUCTION

Bovine Tuberculosis (bTB) is a chronic; debilitating bacterial disease caused by *Mycobacterium bovis* and it primarily infects cattle[1]. It is a zoonotic disease leading to adverse effect on socioeconomic, public health and trade of animals and animal products[2]. Infected animal are an important source of infection for other animals but the wild life species act as reservoirs of infection and lead to a complex epidemiological picture[3, 4]. The main component of defense against tuberculosis infection is the cell mediated immunity (CMI) [5] and thus CMI based Tuberculin skin test (TST) has been used conventionally over hundred years to detect and control bTB[6]. The test involves the measurement of CMI in form of delayed type hypersensitivity reaction to the intradermal injection of Purified Protein Derivative (PPD) [7, 8]. The *in vitro* estimation of the IFN-γ released by sensitized T-cells upon stimulation with antigens like PPD using an enzyme linked immunosorbent assay using Bovigam™ kit[9] was used as an ancillary test to supplement the TST. The PPD based Bovigam™ kit has been reported to be more sensitive than the TST but lacks specificity because of the undefined nature of PPD antigens. To overcome the loss of specificity a number of studies have been done using various recombinant TB antigens. Early secretory antigenic target (ESAT)-6 and Culture filtrate protein (CFP)-10 are specific towards virulent mycobacteria and have been extensively used in *in vitro* IFN-γ assays that help discriminate between *M. avium*-exposed, *M. bovis* BCG vaccinated, and tuberculous cattle[10].

Thus in the present study the purified ESAT-6 and CFP-10 antigens were used to study the IGRAs and cut-off achieved using the TST negative animals (n=100) from a bTB low incidence farm. The achieved cut-off in the present study was evaluated for the sensitivity and specificity using the reference positive (n=3) and negative animals (n=3). Owing to the disease status of the countries which are endemic to bTB it is of utmost importance that cut-offs determined individually for each country and then should be checked for their diagnostic sensitivity and specificity in the TB prevalent and TB low incidence farms with reference set of animals[9] before implementing the bTB control programmes.

MATERIALS AND METHODS

(i) Chemicals, reagents and instruments

The Sartobind Q 75 catridge was purchased from Sartorius stedim, Germany. The Limulus Amebocyte Lysate (LAL) Test kit was purchased from Kan Health care, India. Sodium heparin vacutainer tubes were purchased from BD Biosciences, Franklin lakes, New Jersey, USA. 96 well tissue culture plates (flat bottom plates and U bottom plates) were purchased from Corning, New York, USA. Rose well Park Memorial Institute 1640 (RPMI1640), horse serum, antibiotic and antimycotic mix were purchased from Gibco, Grand Island, New York, USA. The concanavalin-A (Con-A) was purchased at Genei, Bangalore, Karnataka, India. The bIFN-γ monoclonal antibodies and the recombinant bIFN-γ were purchased from AbDSerotec, UK. The carbondioxide (CO_2) incubator used during stimulations was purchased from Thermo SCIENTIFIC, USA. The Optical density was measured at 450nm using PowerWave™ HT Microplate ELISA reader Reader, BioTek, UK with its associated Gen5™ Data Analysis Software.

(ii) Animals

TST negative animals (n=100) and the reference positive and reference negative animals were selected as mentioned in Sugumar et al 2012[11] and Maroudam et al 2011[12]. Holstein Friesian (HF) healthy and naive calves which were 6 to 12 month old (n=100) were randomly selected from the TB low incidence farm. Animals (n=3) which were TST positive, IFN-γ ELISA positive by

Bovigam™, TB culture positive by BD BACTEC 960, and Multi antigen print immunoassay (MAPIA) positive were used as reference positive animals. Animals (n=3) which were TST negative, IFN-γ ELISA negative by Bovigam™, TB culture negative by BD BACTEC 960, and MAPIA negative were used as reference negative animals. Whole blood samples were collected from the farm animals into heparinized vacutainers tubes and transported within 30 minutes to laboratory for IGRAs.

METHODS

(i) Cloning, expression and purification of bTB specific antigens

The recombinant proteins ESAT-6 and CFP-10 specific for bTB were produced as per Bhavani et al 2011[13]. Briefly, the synthetic, codon optimized, recombinant ESAT-6 and CFP-10 genes encoding culture filtrate proteins of *M. bovis* were purchased at GENEART, Germany. The recombinant antigens were cloned into expression vectors, expressed in the *E. coli* BL-21 pLysS cells as poly histidine tagged proteins and were purified to near homogeneity by using Ni-NTA IMAC.

(ii) Endotoxin removal

The endotoxin was removed from the antigens by using Sartobind Q 75-Catridge following manufacturers' instructions.

(iii) Limulus Amebocyte Lysate (LAL) Test

LAL reagent purchased from Kan Health care was used to test for the endotoxin level of the proteins according to the manufacturers' instructions.

(iv) Cut-off determination in the bTB free animals: (iv a) Whole blood stimulation

Peripheral whole blood cultures were performed according to Vordermeier et al., 1999[10] with slight modifications. Briefly, 0.2 ml of heparinised blood samples (n=100) from animals of bTB low incidence farms were stimulated with antigens ESAT-6 or CFP-10 in a concentration ranging from 20 µg/well to 0.5 µg/well in a 96 well Costar™ tissue culture plates. Concanavalin-A at a concentration of 1 µg/well and 50 µl/well RPMI were added as positive and negative control respectively. The samples were cultured at 37°C, 5% CO_2, for 22 h to 24 h and then the supernatants were harvested by centrifugation at 500 x g for 7 minutes at 22 ± 2°C and stored at -20°C until IC-ELISA was performed.

(iv b) Bovine IFN-γ estimation: In-house bovine IFN-γ IC-ELISA was optimized using commercial bovine IFN-γ MAb pair (Data Unpublished). The recombinant bovine IFN-γ was used as standard for the validation of the assay and the assay performed to detect the release of antigen stimulated bovine IFN-γ by the T-cells in the peripheral blood. The results were recorded as optical density at 450nm (OD_{450nm}). The stimulation index was calculated as OD_{450nm} of the antigen stimulated samples divided by the OD_{450nm} of their respective media controls[14]. The threshold value was fixed by calculating the mean of the stimulation index added to thrice the standard deviation of all the negative samples[15].Sensitivity and Specificity determination: The whole blood stimulation (with an optimized average antigen concentration of 5µg/well) and bovine IFN-γ estimation were performed on three reference positive samples and three reference negative samples.

RESULTS

(1) Cloning, expression and purification of bTB specific antigens

The purity of ESAT-6 and CFP-10 proteins was analyzed by running 5 µg of the purified, endotoxin free, recombinant proteins on 15% SDS-PAGE. The results indicated homogeneity of the purified ESAT-6 and CFP-10 proteins (Figure 1).

SDS-PAGE analysis of the purified recombinant proteins ESAT-6 and CFP-10.

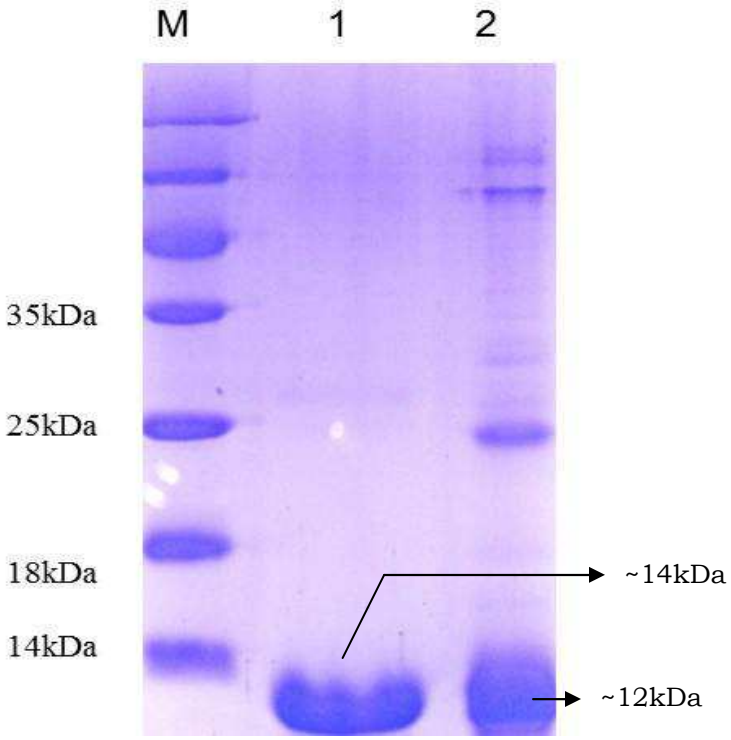

Figure 1
SDS-PAGE analysis of the purified recombinant proteins ESAT-6 and CFP-10.

5 µg of purified, endotoxin free, recombinant proteins ESAT-6 and CFP-10 were run on a 15% SDS-PAGE gel. Lane M: Unstained Protein molecular weight Marker, Lane 1: ESAT-6 purified protein at 14kDa and Lane 2: CFP-10 purified protein at 12kDa. The results indicate the purified bands of the proteins with > 95% homology for the proteins.

(2) Determination of cut-off for IC-ELISA

bIFN-γ IC–ELISA was performed on TST negative samples (n=100) from bTB low incidence herds after stimulation with the bTB specific recombinant antigens ESAT-6 and CFP-10 in different concentration (ranging from 20 µg/well to 0.5 µg/well). The cut-off value was calculated as mean of the stimulation index added to thrice the standard deviation of the stimulation index in all different concentration using the TST negative animals (Figure 2). The basal level bIFN-γ secretion using TB specific antigens in the TST negative animals (n=100) showed the threshold below 3 stimulation index.

Determination of cut-off for IGRAs by IC-ELISA

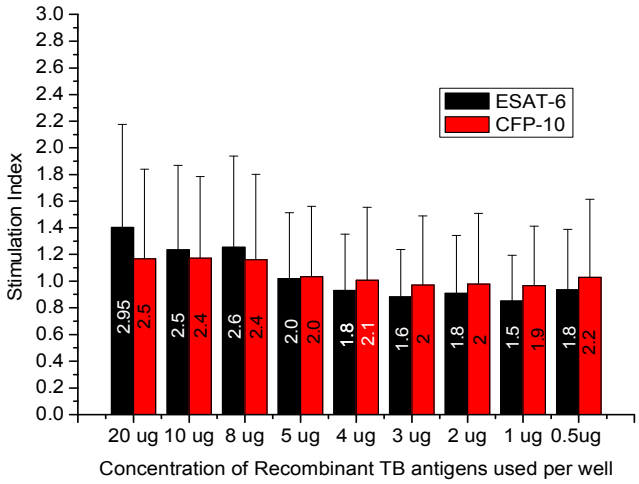

Figure 2
Determination of cut-off for IGRAs by IC-ELISA.

100 samples from a bTB low incidence farm were stimulated with 20 µg to 0.5 µg of ESAT-6 and CFP-10 recombinant antigens and the bIFN-γ released was estimated by IC-ELISA. The cut-off was calculated as mean of the stimulation index added to thrice the standard deviation of the stimulation index of all the negative samples. Based on the results a cut-off of 3.0 stimulation index was achieved.ug in figure represents µg.

(3) Sensitivity and specificity

IGRAs were applied in the reference positive (n=3) and reference negative samples (n=3) using the TB specific antigens ESAT-6 and CFP-10 (Figure 3). The reference positive samples (animal IDs-1 to 3) showed stimulation index value clearly above the pre-estimated cut-off of 3.0 whereas the reference negative samples showed stimulation index values below the cut-off (animal IDs 4 to 6).

Sensitivity and specificity of IC-ELISA.

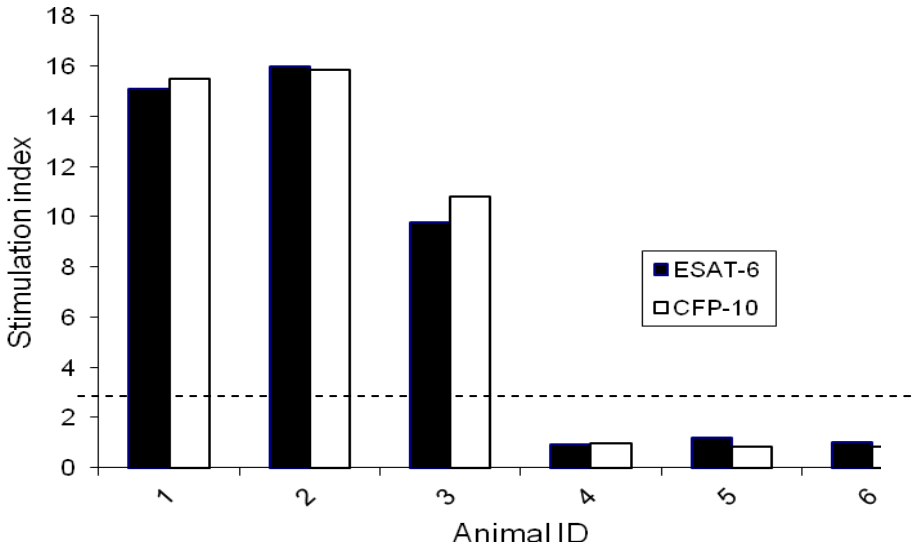

Figure 3
Sensitivity and specificity of IC-ELISA.

Three reference positive animals and three reference negative animals were stimulated with 5 µg of ESAT-6 and CFP-10 and the bIFN-γ released was estimated by IC-ELISA as stimulation index value. The cut-off is indicated by a dotted line. The stimulation index value clearly above the cut-off of 3.0 for the reference positive animals (animal IDs 1 to 3) whereas the reference negative samples showed stimulation index values below the cut-off (animal IDs 4 to 6).

DISCUSSION

bTB is a chronic bacterial disease causing major animal health problem with global zoonotic and economic implications[16] and the early detection of bTB is the cornerstone in the control programmes as in cases of human TB[17,18]. The diagnostic tests currently approved and practiced for global bTB control programme include the TST using PPD[19] and/or *in vitro* assays for IFN-γ produced in response to PPD stimulation using Bovigam™ kit [20]. Advantages of TST and reasons for its wide use are low costs and, for a long time, the lack of alternative methods to detect bTB. The TST has many known limitations including difficulties in administration and interpretation of results, the need for a second visit, low degree of standardization, and imperfect test accuracy[21]. The Bovigam™ IFN-γ assay is OIE listed as an alternative test for international trade [22, 23] and approved as a complementary bTB diagnostic test by the United States Animal Health Association as described in USFDA, APHIS, Bovine Tuberculosis Eradication, Uniform Methods and Rules[24] and by the European Union[25]. But the main limitation of TST and the expensive Bovigam™ kit is the use of PPD for the tests. PPD is a crude water-soluble protein extract from a heat-treated culture of *M. bovis* and shows cross-reactivity with environmental mycobacteria[19] and BCG vaccination. Results in lower sensitivity and specificity of bTB diagnostics opened up the quest for more characterized antigens that could help to develop highly sensitive and specific diagnostic tests[26, 27].

Early secretory antigenic target- 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) are outstanding diagnostic target proteins in the whole blood IFN-γ assay[28, 29, 30]. ESAT-6 and CFP-10 are genes encoded by the region of difference-1 (RD1) of *M. bovis* and are

absent in many environmental, nontuberculous mycobacteria as well as in the BCG vaccine[31, 32]. Research supports the fact that in circumstances of exposure to nontuberculous mycobacteria, no background responses were observed for recombinant antigens specific for virulent tubercle bacilli ESAT-6 and CFP-10 stimulation[33]. Owing to the higher specificity and sensitivity of ESAT-6 and CFP-10 over PPD, [34, 35] even in the present study, the proven bTB diagnostic candidates ESAT-6 and CFP-10 antigens were used in the IGRAs. Since India is a country where nontuberculous mycobacteria is more prevalent; testing with *M. bovis* specific antigens (ESAT-6 and CFP-10) would be more adaptive and potent candidates for bTB diagnostics compared to routine TST.

This is the first comprehensive study conducted in Indian cattle population to evaluate the IGRAs as bTB diagnostics. In the present study, ESAT-6 and CFP-10 were cloned, expressed and purified. The purified recombinant antigens were used for the test stimulation of the whole blood samples. Concentrations ranging from 20 µg/well to 0.5 µg/well were attempted to determine the cut-off value. As it was observed that all the ranges of concentration were giving a similar stimulation index value, an optimal concentration of 5 µg/well was used for sensitivity and specificity determination. A positive control (Conconavalin-A) and a negative control (media) for each sample were maintained along with the test stimulations. A positive control was maintained for each sample to understand the functional viability of the cells during whole blood stimulation. A negative control was maintained for each sample for the correction of the media addition in the sample dilution for each sample. The results were further expressed as the stimulation index (SI) values (mean OD_{450} of antigen-stimulated cell supernatants / mean OD_{450} of the negative control) for the correction of the diluents stimulation. Because the coefficient of variation between duplicate wells was found to be less than 5%, culture supernatants were pooled before testing in the IFN-γ ELISA [36]. ELISA was performed in duplicates with plasma supernatants of different cattle samples after antigen stimulation with the recombinant antigens. An average for the duplicate readings was taken for each sample and the stimulation index calculated thereon.

In the present study, we have standardized and evaluated bIFN-γ IC-ELISA assay as a diagnostic tool to enumerate the secreted TB specific bIFN-γ with TB-specific antigens from an organized bTB low incident farm samples and bTB reference animals. The cut-off was obtained for the IGRAs using the in-house TB specific antigens in 100 TST negative animals from bTB low incident farm. The cut-off value was calculated as mean of the stimulation index value of all the control animals added to thrice the standard deviation of the stimulation index of the TB low incidence samples. The cut-off for the positive samples thus achieved was greater than 3.0 stimulation index values. The result in the present study is in agreement with two simultaneous works by Light body et.al., 1998 [37] where the results were deemed positive if IFN-γ specific results was >2.0 and Aagaard et al., 2010[33] where the cut-off of 1.5 for the E+C cocktail and 2.5 for PPD b was achieved by IC-ELISA for the detection of bTB .

The achieved cut-off (>3.0) was evaluated for its sensitivity and specificity in the bTB reference animals. Our results indicated 100% sensitivity and 100% specificity in the assay using limited sample size Viz., The three reference positive samples exhibited stimulation index values above the cut-off and the three reference negative animals had stimulation index values below the cut-off. But to check the versatility of the cut-off achieved in this paper, future work should focus on large sample size. More number of culture positive and culture negative animals have to be evaluated to come to a conclusion on sensitivity and specificity.

The study clearly indicates that the IFN-γ test is highly sensitive and specific, which is not the case with TST [10, 33]. In addition, the IGRAs in the present study do not lead to any

inconclusive results and even if such situations exist, the IGRAs can be repeated whereas reports suggest that TST cannot be repeated before 60 days of performance of the test[38]. The present standardized in-house assay works out cheaper than the Bovigam assay and may prove to be specific even when studied with large sample size also. In Future, the IFN-γ response should be studied among animals with different stages of disease progress, and different exposures to environmental bacteria. The ESAT-6 and CFP-10 proteins should also be supplemented with other *M. bovis* specific proteins to study genetically heterogeneous animals.

CONCLUSION

bIFN-γ IC-ELISA assay can be used as a diagnostic tool to enumerate the secreted TB specific bIFN-γ with TB-specific antigens. We could achieve a cut-off value of > 3.0 stimulation index for the samples. The cut-off when evaluated on a small sample size of bTB reference animals exhibited 100% sensitivity and 100% specificity. In future, the sample size can be increased further to check the versatility of the cut-off.

REFERENCES

1. Pollock JM and Neill SD, *Mycobacterium bovis* infection and tuberculosis in cattle, Vet J, 163: 115–127, (2002).
2. Buddle BM, McCarthy AR, Ryan TJ, Pollock JM, Vordermeier HM, Hewinson RG, Andersen P and de Lisle GW, Use of mycobacterial peptides and recombinant proteins for the diagnosis of bovine tuberculosis in skin test-positive cattle, Vet Rec, 153: 615–620, (2003).
3. Wedlock DN, Skinner MA, de Lisle GW, Buddle BM, Control of *Mycobacterium bovis* infections and the risk to human populations, Microb Infect, 4: 471–480, (2002).
4. Phillips CJC, Foster CRW, Morris PA, Teverson R, The transmission of *Mycobacterium bovis* infection to cattle, Res Vet Sci, 74: 1–15, (2003).
5. Wood PR, Corner LA, Rothel JS, Ripper JL, Fifis T, McCormick BS, Francis B, Melville L, Small K, deWitte K, Tolson J, Ryan TJ, deLisle GW, Cox JC, Jones SL, A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis, Vet Microbiol, 31: 71–79, (1992).
6. Mohan Kumar Shettar, Nalini TS, Anjan Kumar KR, Ravi Kumar P and Azeemulla HR, Hematological and Biochemical studies in Tuberculin test positive reactors, International Journal of Pharma and Biosciences, 2(4): 16-22 (2011).
7. Francis J, Bovine Tuberculosis, Including a Contrast with Human Tuberculosis, Staples Press Ltd., 86–87 (1947).
8. Snider DE Jr, The tuberculin skin test, Am Rev Respir Dis, 125: 108-118, (1982).
9. Wood PR, Corner LA and Plackett P, Development of a simple, rapid *in vitro* cellular assay for bovine tuberculosis based on the production of γ interferon, Res Vet Sci, 49: 46-49, (1990).
10. Vordermeier HM, Cockle PC, Whelan A, Rhodes S, Palmer N, Bakker D, Hewinson RG, Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle, Clin Diagn Lab Immunol, 6: 675-682, (1999).
11. Sugumar P, Maroudam V, Gangadharrao A, Dev Chandran, Dipankar Das and Srinivasan VA, Use of ESAT-6-CFP-10 fusion protein in the bovine interferon-gamma ELISPOT assay for diagnosis of Mycobacterium bovis infection in cattle, J of Microbiol Methods, doi:10.1016/j.mimet.2012.06.001 (Article in Press).

12. Maroudam V, Loganathan M, Dipankar Das, Sugumar P, Dev Chandran, Rajendra L and Srinivasan VA, Assessment of critical parameters in blood processing for the bovine interferon-gamma ELISPOT assay to detect *Mycobacterium bovis* infected cattle in India, Veterinaria Italiana, 47 (1): 25-34, (2011).
13. Bhavani VJ, Dev Chandran, Maroudam V, Sugumar P, Shahana PV, Dipankar Das, Lakshmi Narasu M and Srinivasan VA, Molecular Cloning, Expression, Purification of novel antigens ESAT-6 and CFP-10 and their application in detection of IFN-γ responses' in Tuberculosis free and Tuberculosis suspected cattle, Biotechnol Bioinf Bioeng, 1(2): 255-264 (2011).
14. Weynants V, Godfroid J, Limbourg B, Saegerman C, and Letesson JJ, Specific bovine brucellosis diagnosis based on *in vitro* antigen-specific gamma interferon production, J Clin Microbiol, 33: 706–712, (1995).
15. Richardson MD, Turner A, Warnock DW and Liewellyn PA, Computer assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis, J Immunol Methods, 56: 201-207, (1983).
16. Michel AL, Muller B and van Helden PD, Mycobacterium bovis at the animal-human interface: A problem, or not? Vet Microbiol, 140: 371-381, (2010).
17. Sooraj SN, Nathiya K, Angayarkanni J and Palaniswamy M, Detection of Mycobacterial infections using filtrate antigen, International Journal of Pharma and Biosciences, 2(3): 378-382, (2011).
18. Humera QF, Ansari and Maimoona Mustafa, Efficacy of Polymerase chain reaction over Ziehl-Neelsen staining in the diagnosis of Mycobacterium tuberculosis, International Journal of Pharma and Biosciences, 2(4): 272-278, (2011).
19. Monaghan ML, Doherty ML, Collins JD, Kazda JF and Quinn PJ, The tuberculin test, Vet Microbiol, 40: 111-124, (1994).
20. Wood PR and Jones SL, BOVIGAM^{TM}: an *in vitro* cellular diagnostic test for bovine tuberculosis, Tuberculosis, 81: 147–155, (2001).
21. de la Rua Domenech R, Goodchild AT, Vordemeier HM, Hewinson RG, Christiansen KH and Clifon-Hadley RS, Ante mortem diagnosis in cattle: A review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques, Res Vet Sci, 81 (2) 190-210, (2006).
22. Rothel JS, Jones SL, Corner LA, Cox JC and Wood PR, A sandwich enzyme immunoassay for bovine interferon-γ and its use for the detection of tuberculosis in cattle, Aust Vet J, 67: 134-137, (1990).
23. Anon, Bovine Tuberculosis: Diagnostic techniques, OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 2.4.7., 2008a, pp. 686–689.
24. Anon, Bovine Tuberculosis Eradication: Uniform Methods and Rules, effective January 1, 2005. United States Department of Agriculture, Animal and Plant Health Inspection Service, Washington (2004a).
25. Anon, Commission Decision of 8 July 2002 amending Annex B to Council Directive 64/432/EEC (2002).
26. Doherty ML, Monaghan ML, Bassett HF, Quinn PJ, Effect of a recent injection of purified protein derivative on diagnostic tests for tuberculosis in cattle infected with *Mycobacterium bovis*, Res Vet Sci, 58: 217–221, (1995).
27. O'Reilly LM. Tuberculin skin tests: sensitivity and specificity, In: C.O. Thoen and J.H. Steele (eds.), *Mycobacterium bovis* infection in animals and humans, Ames, Iowa: Iowa State University Press, 1995, pp.85–91.
28. Pollock JM, Girvin RM, Lightbody KA, Clements RA, Neill SD, Buddle BM, Andersen P, Assessment of defined antigens for the diagnosis of bovine tuberculosis in skin test reactor Cattle, Vet Rec, 146: 659–665, (2000).

29. Waters WR, Nonnecke BJ, Palmer MV, Robbe-Austermann S, Bannantine JP, Stabel JR, Whipple DL, Payeur JB, Estes DM, Pitzer JE, Minion FC, Use of recombinant ESAT-6: CFP-10 fusion protein for differentiation of infections of cattle by Mycobacterium bovis and by M. avium subsp. avium and M. avium subsp. Paratuberculosis, Clin Diag Lab Immunol, 11: 729–735, (2004a).
30. Aagaard CM, Govaerts V, Meikle AJ, Vallecillo JA, Gutierrez-Pabello F, Suarez-Guemes J, McNair A, Cataldi C, Espitia, Andersen P and Pollock JM, Optimizing antigen cocktails for detection of Mycobacterium bovis in herds with different prevalences of bovine tuberculosis: ESAT6-CFP10 mixture shows optimal sensitivity and specificity, J Clin Microbiol, 44: 4326–4335, (2006).
31. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC and Stover CK, Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis,* J Bacteriol, 178: 1274–1282, (1996).
32. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S and Small P, Comparative genomics of BCG vaccines by whole-genome DNA microarray, Science, 284: 1520–1523, (1999).
33. Aagaard C, Govaerts M, Meikle V, Guitiperrez Pabello JA, McNair J, Andersen P, Suarez Guemes F, Pollock J, Espitia C and Cataldi A, Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on PPDB and ESAT-6/CFP10 specificity, Pre Vet Med, 96: 161-169, (2010).
34. Kunst H, Diagnosis of latent tuberculosis infection: the potential role of new technologies, Respir Med, 100: 2098-106, (2006).
35. Pai MP, Riley LW, Colford JM Jr, Interferon gamma assays in the immunodiagnosis of tuberculosis: a systematic review, Lancet Infect Dis, 4:761-6, (2004).
36. Pollock JM and Andersen P, The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis, J Infect Dis, 175: 1251–1254, (1997).
37. Lightbody KA, Skuce RA, Neil SD and Pollock JM, Mycobacterial antigen-specific antibody responses in bovine tuberculosis: an ELISA with potential to confirm disease status, Vet Rec, 142: 295–300, (1998).
38. Radunz BL and Lepper AWD, Suppression of skin reactivity to bovine tuberculin in repeat tests, Aust Vet J, 62: 191-194, (1985).