



Comparative analysis of intradermal tuberculin test and γ -interferon assay for diagnosis of bovine tuberculosis

S S VYAVAHARE¹, M K JHALA¹ and B B BHANDERI^{1✉}

Anand Agricultural University, Anand, Gujarat 388 001 India

Received: 26 October 2020; Accepted: 21 July 2022

ABSTRACT

The present study was carried out in and around Anand district of Gujarat with the aim to assess the sensitivity and specificity of IFN- γ assay as compared to intradermal tuberculin test for diagnosis of bovine tuberculosis during the period 2011-12. The prevalence of bovine TB in the cattle was 26.19% by TST and 38.49% by IFN- γ assay. Breed wise, highest prevalence of bovine TB was found in Gir (38.96%), followed by Triple cross (23.95%) and Kankrej (16.45%) by TST; while by IFN- γ assay, highest prevalence was observed in Triple cross (42.70%). Age wise, highest prevalence of bovine TB was found in adults (37.90%) followed by calves (26.66%) and heifers (11.22%) by TST; while by IFN- γ assay, highest prevalence was observed in calves (66.66%) followed by adults (44.35%) and heifers (22.44%). Sex wise, more prevalence of bovine TB was found in males (56.25%) than in females (21.81%) by TST; while by IFN- γ assay, more prevalence was observed in females (39.25%) than in males (31.25%). Sensitivity and specificity of TST in detecting bovine TB were 27.27% and 57.52% respectively, compared to IFN- γ assay. ELISPOT assay showed 34.78% animals were found positive for bovine TB. IFN- γ assay showed better sensitivity in detecting bovine TB at younger age compared to TST, and hence can be useful in timely removal of the infected animals from the herd.

Keywords: Bovine tuberculosis, ELISPOT, IFN- γ , Tuberculin test

Bovine Tuberculosis (BTB) is a chronic bacterial disease caused by *Mycobacterium bovis*, a member of *Mycobacterium tuberculosis* complex involved in major animal health problems with zoonotic and economic implications (Dhaliwal *et al.* 2020). Infected animals are main source of infection for other animals and, wildlife species act as reservoirs of infection which presents a complex epidemiological picture (El-Sayed *et al.* 2016). The diagnostic tests currently approved and practiced for BTB include the single intradermal tuberculin test (TST) using purified protein derivative (PPD) antigens and/or *in vitro* IFN- γ release assay (IGRAs) (de Lisle *et al.* 2017, Olea-Popleka *et al.* 2017 and Coad *et al.* 2019). TST is widely used due to its cost effectiveness, easy availability, long history of use, and lack of alternative methods to detect BTB (Bezoz *et al.* 2018). TST has many limitations including difficulties in administration and interpretation of results, need for second visit to the farm, low degree of standardization, and imperfect test accuracy (Duignan *et al.* 2019). Use of crude PPD results in low sensitivity and specificity of the test (Domenech *et al.* 2006, Casal *et al.* 2017).

Early secretory antigenic target-6 (ESAT-6) and Culture Filtrate Protein-10 (CFP-10) are important TB

specific diagnostic target proteins in the whole blood IFN- γ assay (Praud *et al.* 2019, Srinivasan *et al.* 2019), due to their absence in many environmental, non-tuberculous mycobacteria as well as in the BCG vaccine strain (Mahairas *et al.* 1996). The IGRAs enzyme linked immunospot (ELISPOT) assay was introduced in 1983 to detect cellular immunoglobulin production and then adapted for measuring the cytokine response against a defined antigen (Czerkinsky *et al.* 1983). ELISPOT is a fundamental tool used in cellular immunology, providing both qualitative and quantitative information on cellular cytokine responses to defined antigens. ELISPOT assay was approved and adopted as confirmatory diagnosis in human latent TB (Lalvani *et al.* 2001). Present study was carried on comparative diagnosis of BTB using TST, IGRAs (IFN- γ) and ELISPOT in the cattle.

MATERIALS AND METHODS

A total of 252 cattle of three breeds, viz. Kankrej [n=79, adult (39), heifer (35), calves (5)], Gir [n=77, adult (41), heifer (25), calves (11)] and Triple cross [n=96, adult (44), heifer (38), calves (14)] of various age groups who were known to be TB infected during the previous regular annual tuberculin skin testing were included (Supplementary Table 1).

TST: The tuberculin test was performed on the mid-neck of the 252 cattle. Bovine purified protein derivative (2000 International Units, IVRI, Izatnagar) was used for TST. The procedure and interpretation of TST was carried out as

Present address: ¹College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat.
 ✉Corresponding author email: bbbhanderi@gmail.com

Table 1. Number of the animal's positive by TST and IFN- γ assay

Positive only by TST	Positive only by IFN- γ	Positive by both	Negative by both
48 (19.04%)	79 (31.35%)	18 (7.15%)	107 (42.46%)
Total	252		

per the OIE Terrestrial Manual (2009).

IFN- γ assay: Whole blood stimulation assay was carried out from the blood samples from 252 cattle within 5 h for IFN- γ assay as per the method described by Bhavani *et al.* (2011). Purified antigen ESAT-6 (5 μ g/well) and CFP-10 (5 μ g/well) were used (*E. coli* expressed *Mycobacterium bovis* specific recombinant antigen codon optimized for *E. coli* expression obtained from IIL, Hyderabad). After stimulation, obtained plasma was stored at -20°C till the IFN- γ assay was performed. IFN- γ assay is an enzyme linked immunosorbent assay (ELISA) to measure bovine IFN- γ derived from stimulated whole blood supernatant samples. Following appropriate antigen-specific stimulation, lymphocytes rapidly express and secrete cytokines. The secreted-out cytokines from bovine cells were quantified using the sandwich ELISA developed by IIL, Hyderabad. IFN- γ assay was performed as per Schiller *et al.* (2010) for the 252 blood supernatant samples obtained after whole blood stimulation. Stimulation index was calculated as optical density of antigen stimulation divided by optical density of media. Stimulation index ≥ 2 is indicative of TB +ve and < 2 is indicative of TB -ve.

ELISPOT assay: Blood samples from 23 animals (Supplementary Table 1) were collected separately for peripheral blood mononuclear cell (PBMC). PBMCs separated from 23 animals and stored in liquid nitrogen were processed for ELISPOT as per Veerasami *et al.* (2011). 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.

Statistical analysis: For statistical analysis of breed-wise, age-wise and sex-wise prevalence of TB, results of positivity obtained by both the tests, viz. TST and IFN- γ assay were combined, to get the overall estimates of TB prevalence. This was done as per the OIE guidelines. To compare the breed-wise, age-wise and sex-wise proportion of diseased animals chi-square test was carried out. The level of chi-square test was 5% (carried as per Thrusfield *et al.* 2005)

RESULTS AND DISCUSSION

Tuberculin skin testing: Out of 252 animals, 66 (26.19%) animals were found to be positive by skin testing. The positive reactors showed increase in skin thickness with ≥ 4 mm along with diffused or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts and lymph nodes. Breed-wise, 13 (16.45%) animals of Kankrej (n= 79), 30 (38.96%) of Gir (n=77) and 23 (23.95%) of Triple cross (n=96) breed were positive by TST. Age-wise, 47 (37.90%) adults (n=124),

11 (11.22%) heifers (n=98) and 8 (26.66%) calves (n= 30) were positive by TST. Sex-wise, 18 (56.25%) males (n=32) and 48 (21.81%) females (n=220) were positive by TST (Supplementary Table 2). Breed-wise, highest prevalence was observed in Gir, followed by Triple cross and Kankrej. Earlier, Ameni *et al.* (2006) reported that zebu cattle are relatively resistant to TB infection than exotic cattle. Age-wise, highest prevalence was observed in adults, followed by calves and heifers. Regarding age-wise prevalence, our results were in accordance with the previous study reported by Linton and Dorshkind (2004) and Frasca and Blomberg (2011). Sex-wise, more percentage of males were positive than females by TST. Similar findings for sex-wise prevalence were previously reported by Kazwala *et al.* (2001). Our study shows that prevalence was only found in calves and heifers of Gir and Triple cross but not of Kankrej by TST, indicating that the Kankrej animals are relatively resistant to TB infection. Only adults of Kankrej were found positive. The high prevalence in adult animals possibly suggests their long-term exposure to the infection and the declining immune defense with advancement of age (Linton and Dorshkind 2004, Frasca *et al.* 2011). Our study showed that Kankrej animals are relatively resistant to TB infection compared to Gir and Triple cross.

IFN- γ assay: Out of 252 animals, 97 (38.49%) animals were found positive by IFN- γ assay (indirect ELISA) conducted after whole blood stimulation assay. If the Stimulation index was ≥ 2 , then the animal was considered positive and if the stimulation index was < 2 , then the animal was considered negative. Breed-wise, 24 animals (30.37%) of Kankrej (n=79), 32 (41.55%) of Gir (n=77) and 41 (42.70%) of Triple cross (n=96) were positive by IFN- γ assay. Age-wise, 55 (44.35%) adults (n=124), 22 (22.44%) heifers (n=98) and 20 (66.66%) calves (n=30) were positive by IFN- γ assay. Sex-wise, 10 (31.25%) males (n=32) and 87 (39.54%) females (n=220) were found positive by IFN- γ test (Supplementary Table 3). Breed-wise, highest prevalence was observed in Triple cross, which was marginally higher than in Gir and lowest in Kankrej by IFN- γ assay. Age-wise, highest prevalence was observed in calves followed by adults and heifers. In our study, IFN- γ assay was able to detect 12 more calves and 11 more heifers than the TST, positive for TB infection indicating that IFN- γ assay was able to detect TB infection at an earlier age with better sensitivity. This may help in taking proper preventive measures which can be taken to reduce further progression of the disease. These findings were in accordance with the previous study reported by Huda *et al.* (2003). Sex-wise, more prevalence was observed in females as compared to males, which was contrary to the TST results for sex-wise prevalence. It is difficult to explain these contradictory findings as the sample size for male and female were very much different. Higher prevalence of bovine TB in females was also observed by Nwanta *et al.* (2011). Buddle *et al.* (2000) conducted a study to evaluate a single mycobacterium antigen, ESAT-6 in the IFN- γ test for use in skin test-positive cattle. The test based

on ESAT-6 had a higher specificity than the test based on PPD tuberculin, but this was offset by a small decrease in sensitivity, while still maintaining a very high specificity. In present study, two *M. bovis* specific antigens ESAT-6 and CFP-10 were used for improving the sensitivity and specificity of the assay.

ELISPOT assay: Out of 252 animals, PBMCs of 23 animals were collected and stored in liquid nitrogen until they were processed for ELISPOT. The assay was performed in triplicates; results were represented as mean of triplicate wells and expressed as spot forming cells (SFC) per million cells. If 20 or greater than 20 spots were recorded per well, then the animal was considered as positive. If less than 20 spots were recorded per well, then the animal was considered as negative (Supplementary Fig. 1). The ELISPOT assay detects the number of IFN- γ producing cells rather than the total amount of IFN- γ as is measured by cytokine ELISA assays. ELISPOT has been reported to be 10–200 times more sensitive in the detection of cytokines than the ELISA (Parthsarthy *et al.* 2012). In the present study, out of 23 animals, 8 (34.78%) animals were found positive for bovine tuberculosis by ELISPOT assay (Supplementary Fig. 1). Earlier, Zhang *et al.* (2009) tested the hypothesis that the poor results of IFN- γ and ELISPOT assays are due to lack of assay standardization, rather than the inherent complexity of T-cell assays. They found that ELISPOT assay provides reproducible results when the assay procedure and data analysis were standardized. Their study showed that ELISPOT assay is the ideal candidate for the robust and reproducible monitoring of T-cell activity *in vivo*, indicating ELISPOT assay as sensitive tool for the diagnosis. Use of ELISPOT assay in HIV-AIDS vaccine research was reported by Streeck *et al.* (2009). Vordermeier *et al.* (2012) demonstrated that due to the exquisite sensitivity of ELISPOT assay, it became an important tool in vaccine development programme.

Comparative analysis of the tests employed: Sensitivity and specificity of TST were assessed by comparing with IFN- γ assay, as IFN- γ assay yielded more positive results than the TST. Forty-eight (19.04%) and 79 (31.35%)

animals were found positive only by TST and IFN- γ assay, respectively; 18 (7.15%) animals were found positive by both TST and IFN- γ assay and 107 (42.46%) animals were negative by both TST and IFN- γ assay (Table 1). The sensitivity and specificity of TST was found 27.27% and 57.52%, respectively with reference to IFN- γ assay, whereas the overall agreement between both the tests was 49.60 % (Table 2). Overall comparison between TST and IFN- γ assay revealed that out of 252 animals, 66 (26.19%) were positive by TST and 97 (38.49%) by IFN- γ assay (Supplementary Table 4). Out of 66 animals positive by TST, 18 were positive while 48 were negative by IFN- γ (Table 3). In our study, IFN- γ assay was able to detect 31 (12.30%) more animals positive for bovine TB indicating that IFN- γ assay has more sensitivity than TST (Table 3). Out of 124 adult animals, 47 (34.90%) and 55 (44.35%) animals were positive by TST and IFN- γ assay, respectively. The IFN- γ assay was able to detect TB infection in 8 (6.45%) more animals than TST. Out of 98 heifers, 11 (11.22%) animals were found positive by TST, and 22 (22.44%) animals were found positive by IFN- γ assay. IFN- γ assay detected 11 (11.22%) more heifers positive for TB infection (Supplementary Table 4). Out of 30 calves, IFN- γ assay detected 20 (66.66%) calves which were positive for TB infection. This shows that IFN- γ assay can detect TB infection at earlier stage so that we can formulate the measures to prevent further progression of disease. On the contrary, TST was able to detect only 8 (26.66%) calves positive for TB infection (Supplementary Table 4). Looking at the comparison between TST and IFN- γ assay, we can say that IFN- γ assay has more sensitivity in detecting the TB infection as compared to TST. In our study, IFN- γ assay was able to detect the TB infection at earlier stage with better sensitivity. IFN- γ assay was in fact able to detect a greater number of animals positive for TB infection in each age group as compared to TST indicating its higher sensitivity for detecting the infection.

Comparison between TST, IFN- γ and ELISPOT assay: Out of 23 animals subjected for ELISPOT, 8, 3 and 7 were positive by ELISPOT, TST and IFN- γ respectively. Three of

Table 2. Sensitivity and specificity analysis of TST for diagnosis of bovine TB with reference to IFN- γ assay

Test to be compared (TST)	Standard test (IFN- γ assay)		Total	Sensitivity	Specificity	Overall agreement
	Positive	Negative				
Positive	18	79	97			
Negative	48	107	155	27.27 %	57.52 %	49.60 %
Total	66	186	252			

Table 3. Details of the animals positive by TST and IFN- γ assay

Breed	Adults (n=124)			Heifers (n=98)			Calves (n=30)		Total	
	Only by IFN- γ assay	Only by TST	Both	Only by IFN- γ assay	Only by TST	Both	Only by IFN- γ assay	Only by TST	IFN- γ assay (%)	TST (%)
Kankrej	12	07	06	04	—	—	02	—	24 (30.37%)	13 (16.45%)
Gir	11	14	06	07	03	02	06	05	32 (41.55%)	30 (38.96%)
Triple cross	16	10	04	09	06	—	12	03	41 (42.70%)	23 (23.95%)
Total	39	31	16	20	09	02	20	08	97 (38.49%)	66 (26.79%)

the ELISPOT positive animals were negative by both TST and IFN- γ , 3 were also positive by IFN- γ assay but negative by TST, one animal was positive by TST but negative by IFN- γ , while one animal was positive by all the three tests (Supplementary Fig. 1). Although it is difficult to conclude the ELISPOT results with the limited number of samples processed, it is logical to imply that ELISPOT can detect positive animals in more sensitive way. Limited number of documentations available in literature on ELISPOT applied for TB diagnosis also suggests its meaningful application for this purpose, especially when TB is an important zoonosis and has severe consequences with public health point of view. Present study was attempted to standardize and apply this advanced technique, and the results obtained should encourage further studies involving large number of samples.

Overall breed, age and sex-wise BTB prevalence by chi-square test: The chi-square value for breed-wise prevalence obtained was 16.72 (p value 0.0002), which indicates that difference in the breed wise disease prevalence is highly significant. The result of this study indicates that the Kankrej animals are relatively resistant to TB infection compared to Gir and Triple cross animals. This endorses the finding of Ameni *et al.* (2006). The chi-square value obtained between adults and calves was 7.223 (p value 0.0072) which indicates that difference in age-wise prevalence was significant. The chi-square value obtained between adults and heifers was 31.248 (p value <0.0001) which indicates that difference in age-wise prevalence was significant. The chi-square value obtained between calves and heifers was 35.191 (p value <0.0001) which indicates that difference in age-wise prevalence was significant (Supplementary Table 5). Our study showed that heifers are relatively resistant to TB infection compared to calves and adult. These results agree with the fact that the immune system of the calves is underdeveloped. Similarly, high prevalence in adult animals suggests that immunocompetence declines with the age as suggested by Linton and Dorshkind (2004) and Frasca *et al.* (2011). The chi-square value for sex-wise prevalence obtained was 1.885 (p value 0.1697) which indicates that difference in sex-wise prevalence was not significant. Our results were in accordance with Kazwala *et al.* (2001)

In conclusion, prevalence of bovine TB in the cattle was 26.19% by TST and 38.49% by IFN- γ assay. Overall prevalence by both these assays was 57.53%. ELISPOT showed promising results for detecting bovine TB and should be explored further involving large number of samples.

REFERENCES

- Ameni G, Aseffa A, Engers H, Young D, Hewinson G and Vordermeier M. 2006. Cattle husbandry in Ethiopia is a predominant factor affecting the pathology of bovine tuberculosis and gamma interferon responses to mycobacterial antigens. *Clinical and Vaccine Immunology* **13**: 1030–36.
- Bezoz J, Roy A, Infantes-Lorenzo J A, Gonzalez I, Venteo A, Romero B, Grau A, Minguez O, Dominguez L and de Juan L. 2018. The use of serological tests in combination with the intradermal tuberculin test maximizes the detection of tuberculosis infected goats. *Veterinary Immunology and Immunopathology* **199**: 43–52.
- Bhavani J V, Dev Chandran, Veerasami M, Parthasarathy S, Vijayan P, Das D, Lakshmi M and Srinivasan A W. 2011. Cloning, expression and purification of ESAT-6 and CFP-10 and their use in the detection of IFN- γ responses in tuberculosis infected cattle. *Society for Applied Biotechnology* **2**: 255–64.
- Buddle B M, Ryan T J, Pollock J M, Andersen P and de Lisle G W. 2000. Use of ESAT-6 in the interferon-g test for diagnosis of bovine tuberculosis following skin testing. *Veterinary Microbiology* **80**: 37–46.
- Casal C, Infantes JA, Rialde MA, Diez-Guerrier A, Dominguez M, Moreno I, Romero B, de Juan L, Saez J L, Juste R, Gortazar C, Dominguez L and Bezoz J. 2017. Antibody detection tests improve the sensitivity of tuberculosis diagnosis in cattle. *Research in Veterinary Science* **112**: 214–21.
- Coad M, Doyle M, Steinbach S, Gormley E, Vordermeier M and Jones G. 2019. Simultaneous measurement of antigen-induced CXCL10 and IFN- γ enhances test sensitivity for bovine TB detection in cattle. *Veterinary Microbiology* **230**: 1–6.
- Czerkinsky C C, Nilsson L A, Nygren H, Ouchterlony O and Tarkowski A. 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody secreting cells. *Journal of Immunological Methods* **65**: 109–21.
- De Lisle G W, Green R S and Buddle B M. 2017. Factors affecting the gamma interferon test in the detection of bovine tuberculosis in cattle. *Journal of Veterinary Diagnostic Investigation* **29** (2): 198–202.
- Dhaliwal N K, Narang D, Chandra M, Filia G and Singh S T. 2020. Evaluation of adenosine deaminase activity in serum of cattle and buffaloes in the diagnosis of bovine tuberculosis. *Veterinary World* **3**(1): 110–13.
- Domenech R, Goodchild AT, Vordermeier H M, Hewinson R G, Christiansen R S and Hadley C. 2006. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, c-interferon assay and other ancillary diagnostic techniques. *Research in Veterinary Science* **81**: 190–210.
- Duignan A, Kenny K, Bakker D and Good M. 2019. Tuberculin PPD potency assays in naturally infected tuberculous cattle as a quality control measure in the Irish bovine tuberculosis eradication program. *Frontiers in Veterinary Science* **6**: 328.
- El-Sayed A, El-Shannat S, Kamel M, Castañeda-Vazquez M A and Castañeda-Vazquez H. 2016. Molecular epidemiology of *Mycobacterium bovis* in humans and cattle. *Zoonoses Public Health* **63**: 251–64.
- Frasca D and Blomberg B B. 2011. Aging impairs murine B cell differentiation and function in primary and secondary lymphoid tissues. *Aging and Disease* **2**(5): 361–73.
- Huda A, Lind P, Kristofferson A B and Jungerson G. 2003. Analysis of repeated tests for interferon-gamma and faecal excretion for diagnosis of subclinical paratuberculosis in anish cattle. *Veterinary Immunology and Immunopathology* **94**: 95–103.
- Kazwala R R, Kambarage D M, Daborn C J, Nyange J, Jiwa S F and Sharp J M. 2001. Risk factors associated with the occurrence of bovine tuberculosis in cattle in the Southern Highlands of Tanzania. *Veterinary Research Communication* **25**(8): 609–14.
- Lalvani A. 2001. Diagnosing tuberculosis infection in the 21st century: New tools to tackle an old enemy. *Chest* **131**: 1898–

- 1906.
- Linton P J and Dorshkind K. 2004. Age-related changes in lymphocyte development and function. *Nature Immunology* **5**(2): 133–39.
- Mahairas G G, Sabo P J, Hickey M J, Singh D C and Stover C K. 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *Journal of Bacteriology* **178**: 1274–82.
- Nwanta J A, Umeononigwe C N, Abonyi G E and Onunkwo J I. 2011. Retrospective study of bovine and human tuberculosis in abattoirs and hospitals in Enugu State, Southeast Nigeria. *Journal of Public Health and Epidemiology* **3**(7): 329–36.
- Olea-Popelka F, Muwonge A, Perera A, Dean A S, Mumford E, Erlacher-Vindeln E, Forcella S, Silk B J, Ditiu L, Idrissi E, Raviglione M, Cosivi O, LoBue P and Fujiwara P I. 2017. Zoonotic tuberculosis in human beings caused by *Mycobacterium bovis*, a call for action. *The Lancet Infectious Diseases* **17**(1): 21–25.
- Parthasarathy S, Veerasami M, Appana G, Chandran D, Das D and Srinivasan A V. 2012. Use of ESAT-6-CFP-10 fusion protein in the bovine interferon-gamma ELISPOT assay for diagnosis of *Mycobacterium bovis* infection in cattle. *Journal of Microbiological Methods* **90**(3): 298–304.
- Praud A, Bourély C, Boschirola M and Dufour B. 2019. Assessment of the specificity of a gamma-interferon test performed with specific antigens to detect bovine tuberculosis, after non-negative results to intradermal tuberculin testing. *Veterinary Record* doi.org/10.1136/vetreco-2019-000335.
- Schiller I, Vordermeier M H, Ray W W, Adam O, Whelan M, Gormley C, Bryce B M, Palmer M, McNair J, Welsh M, Hewinson G R and Oesch B. 2010. Bovine tuberculosis: Effect of the tuberculin skin test on *in vitro* interferon gamma responses. *Veterinary Immunology and Immunopathology* **136**: 1–11.
- Srinivasan S, Jones G, Veerasami M, Steinbach S, Holder T, Zewude A, Fromsa A, Ameni G, Easterling L, Bakker D, Juleff N, Gifford G, Hewinson R G, Martin Vordermeier H M and Kapur V. 2019. A defined antigen skin test has been developed for the reliable diagnosis and vaccine-based control of bovine tuberculosis. *Science Advances* **5**(7): eaax4899.
- Streeck H, Jolin J S, Qi Y, Yassine-Diab B, Johnson R C and Kwon D S. 2009. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. *Journal of Virology* **83**: 7641–48.
- Thrusfield M V. 2005. *Veterinary Epidemiology*. 3rd Edition. Blackwell Science, Oxford. Pp. 234–38.
- Veerasami M, Moorthy L, Das D, Partasarthy S, Chandran D, Lingla R and Srinivasan V A. 2011. 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**: 25–34.
- Zhang W, Caspell R, Karulins A Y, Ahmad M, Haicheur N, Abdelsalam A, Johannesen K, Vignard V, Dudzik P, Georgakopoulou K, Mihaylova A, Silina K, Aptisauri N, Adams V, Lehmann P V and McArdle S. 2009. ELISPOT assays provide reproducible results among different laboratories for T- cell immune monitoring-even in hands of ELISPOT-inexperienced investigators. *Journal of Immunotoxicology* **6**: 227–34.