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## Comparison of Single Intradermal Test, Gamma Interferon Assay and Indirect ELISA for the Diagnosis of Tuberculosis in a Dairy Farm

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### ABSTRACT

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* which belongs to *Mycobacterium tuberculosis* complex (MTC), is a globally distributed zoonotic disease in cattle. The present study was conducted in a dairy herd with the history of prevalence of bovine tuberculosis. Two Cell Mediated Immunity (CMI) based tests, Single Intradermal Test (SID), gamma interferon (IFN- $\gamma$ ) assay and a serological test (enzyme linked immunosorbant assay, ELISA) were employed for the diagnosis of tuberculosis in 45 animals. Of these, 8 (17.77%) were positive by SID test, 10 (22.22%) by interferon gamma assay and none of the animals were found positive by ELISA. Both the CMI tests performed were found better than the antibody detection. Between the CMI tests, IFN- $\gamma$  assay showed better sensitivity than the SID test; combination of tests showed better detection of the bTB infection in animals. ELISA results indicated that animals were still in progressive stages of infection and the sensitivity of the tests depends on the stage of infection in the study subjects. As a whole, 26.67% of the animals tested in the farm were found to be positive and/or reactors to tuberculosis. It indicates that both the CMI tests were better than those targeting antibody detection. ELISA did not detect even a single animal as positive. These results indicate that no single test is able to detect all the infected animals and also not having 100 % sensitivity and specificity. So combination of tests always can be better employed for the diagnosis of bovine tuberculosis.

**Key words:** Bovine tuberculosis, *Mycobacterium bovis*, diagnosis, single intradermal test, IFN- $\gamma$  assay, indirect ELISA

### INTRODUCTION

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* which belongs to *Mycobacterium tuberculosis* complex (MTC), is a globally distributed zoonotic disease in cattle (Michel *et al.*, 2010). The MTC include *Mycobacterium bovis*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. canetti* and *M. caprae* (Cousins *et al.*, 2003; Good and Duignan, 2011; Atkins and Robinson, 2013). The bTB is a chronic debilitating and highly contagious disease of cattle, buffaloes, goats, pigs and several wild species. The disease is characterized by nodular granuloma

or tubercle formation with resultant caseations and calcification in many of the vital organs especially in the lungs, lymph nodes, intestine and kidney except skeletal muscles (Hardstaff *et al.*, 2013; Le Roex *et al.*, 2013). In developing countries, bTB is enzootic and has an impact on human health and causing huge economic loss to animal industry (Raghvendra *et al.*, 2010). The bTB spreads from animal to human and causes significant economic loss due to high cost of eradication programs and has serious consequences for movements of animals and their products, biodiversity, public health and significant economic effects (Dhama *et al.*, 2013; Le Roex *et al.*, 2013; Rodriguez-Campos *et al.*, 2014). Early and accurate diagnosis of bTB in cattle is very important, since the disease can become chronic with prolonged incubation periods leading to intermitted spread of infection to other animals and humans through aerosolization and consumption of unpasteurized milk (Churbanov and Milligan, 2012). Cell Mediated Immunity (CMI) based tests like *in vivo* single intradermal test and *in vitro* interferon gamma assay detect infection in early stages (Pollock *et al.*, 2001) and are commonly used than serological tests like indirect Enzyme Linked Immunosorbant Assay (ELISA) (Coad *et al.*, 2008; Whelan *et al.*, 2008).

Single intradermal test is widely used due to ease with which it can be applied at herd level and it is an OIE prescribed ante mortem test for international trade of cattle (Costello *et al.*, 1997; Anonymous, 2004; Praud *et al.*, 2014). The bTB has been eradicated from many developed countries such as Australia, Canada, Switzerland, most EU member states and from different states in USA (Cousins, 2001) by regular skin testing and slaughtering of reactors. Another CMI test is gamma interferon (IFN- $\gamma$ ) assay, used as an ancillary test to SID test (Antognoli *et al.*, 2011; Praud *et al.*, 2014). Antibody-based assays may facilitate the detection of infected cattle in advanced phases of infection and in anergic animals (Cousins and Florisson, 2005; Coad *et al.*, 2008; Whelan *et al.*, 2008; Da Silva *et al.*, 2011).

## MATERIALS AND METHODS

**Study population:** A rural dairy farm in Bangalore district (Karnataka), India with 45 cross bred milch cattle with a few reactors (4/40) to SID test about a year and half ago, which were subsequently removed, was selected for the study. Blood samples were collected for gamma interferon assay *in-vitro*; serum samples separated from an aliquot of the same blood sample were used for indirect ELISA and *in-vivo* SID test was carried out on all the animals (4 animals were declared as reactors in the previous year by SID, which were subsequently removed from a herd with 40 animals).

**Single intradermal test:** Single intradermal test was performed on all the 45 cattle in the herd by injecting 0.1 mL of bovine tuberculin Purified Protein Derivative (PPD) intradermally (2,000 tuberculin units per 0.1 mL, obtained from the Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh). Initial and final skin thickness was measured with Vernier Caliper. An animal with an increase of 4 mm or more in skin thickness was considered as reactor.

**IFN-  $\gamma$  assay:** Heparinized blood samples were collected from all the 45 animals before skin testing and transported to laboratory within 30 h of collection at room temperature. Stimulation of blood was carried out with Phosphate Buffered Saline (PBS), avian and bovine PPD followed by sandwich ELISA for analyzing the plasma samples for IFN- $\gamma$  as described in AniGen TB-feron kit instructions supplied by Bionote Company, Korea.

**Indirect ELISA:** Serum samples were collected from all the 45 animals before skin testing and tested for the presence of antibodies by using Anigen bTB ELISA kit supplied by Bionote Company, Korea.

**Statistical analysis:** Single intradermal test, IFN- $\gamma$  assay and ELISA were compared by applying paired 't' test in making the bTB diagnosis. By keeping SID test as standard, the other two tests (IFN- $\gamma$  assay and ELISA) were compared. There was no significant difference between SID and IFN- $\gamma$  assay with a p value 0.4204 and correlation co-efficient of 0.5903. However, there was a significant difference between SID and ELISA with a p value of 0.0035; with no correlation between SID and ELISA. In between CMI and humoral response there was a significant difference with p value of 0.0023 with no correlation.

**RESULTS AND DISCUSSION**

Out of the 45 animals, 08 (17.77%) were positive by SID test, 10 animals (22.22%) were positive for interferon gamma assay and none of the animals were found positive by indirect ELISA, however one animal showed sample to positive ratio (S/P) value of 0.418 which was nearer to cut off (0.5) as shown in Fig. 1.

Only 6 animals were commonly detected as reactors/positives when SID test and IFN- $\gamma$  assay were applied. Whereas, on individual basis, SID test and IFN- $\gamma$  assay detected 2 and 4 more animals, respectively, as reactors/positives, which were otherwise negative for the other test. In other words, SID test and IFN- $\gamma$  assay were able to detect 8 and 10 animals, respectively, as reactors/positives. However, the combination of both the tests could detect 12 out of 45 animals as tuberculosis animals, thus increasing the sensitivity of the diagnosis (Table 1).

Table 1: Combination of results of SID test and IFN- $\gamma$  assay for 45 cattle for the diagnosis of tuberculosis

	SID test		Total
	Positive	Negative	
<b>INF-<math>\gamma</math> assay</b>			
Positive	6	4	10
Negative	2	-	2
Total	8	4	12

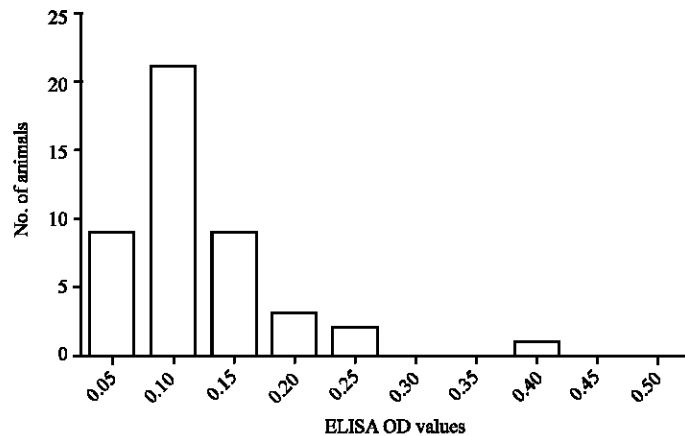


Fig. 1: Sample to positive ratio (S/P) values plotted in frequency graph obtained in indirect ELISA

Single intradermal test detected 02 more animals as reactors which were negative for IFN- $\gamma$  assay. Of these 2 animals, 1 has sample to positive ratio (S/P) value of 0.418 which is nearer to cut off of 0.5 in Anigen bTB Ab ELISA indicating that animal was infected and it was in progressive stage of infection. The other animal may be false positive due to complex nature of PPD (De La Rua-Domenech *et al.*, 2006). But there are reports that small proportion of bTB animals were found as reactors to SID test but not detected by IFN- $\gamma$  assay (Costello *et al.*, 1997; Pollock *et al.*, 2005; Praud *et al.*, 2014).

Among the 04 IFN- $\gamma$  assay positive and SID test negative animals, 1 animal was recently calved, it may be due to that SID test was not able to detect the infection. The other 03 animals were detected as positives the could be due to low specificity of IFN- $\gamma$  assay and not detected in SID test due to low sensitivity of SID test (Llamazares *et al.*, 1999). Here, the chances of false positives with IFN- $\gamma$  assay are rare because of comparative nature of assay in which animal's IFN- $\gamma$  response to avian and bovine PPD are compared. According to Alvarez *et al.* (2012), the IFN- $\gamma$  assay positives are having high probability of being truly infected; despite *Mycobacterium* recovery rate is less culturing and they stated that, IFN- $\gamma$  assay always detects more positives than SID test due to good sensitivity and may also be due to time taken to elicit IFN- $\gamma$  test response after infection (1-5 weeks) is marginally shorter than for the SID test (3-6 weeks) (De La Rua-Domenech *et al.*, 2006). Application of both CMI based tests helps to increase the diagnostic sensitivity while keeping a reasonable specificity (Wood *et al.*, 1992; Gormley *et al.*, 2006; Antognoli *et al.*, 2011). So IFN- $\gamma$  assay is used as an ancillary test to SID test.

Indirect ELISA detected none of the 45 animals as positives. But sample to positive ratio (S/P) value of 01 animal was nearer to cut off value at 0.5. These results indicated that ELISA is not able to detect even single infected animal and no correlation could be established between antibody response on one side and two of the CMI tests on the other side.

Immune response to *Mycobacterium* infections in cattle is dominated by CMI responses which occurs as early as three weeks post infection (Pollock *et al.*, 2001). So diagnosis of bovine tuberculosis in live animals is primarily based on the detection of specific CMI responses (De La Rua-Domenech *et al.*, 2006; Gormley *et al.*, 2006). In Mycobacterial infections, CMI and humoral immunity are inversely related. In initial stages, CMI is dominating and in advanced stages humoral immunity is dominating (De La Rua-Domenech *et al.*, 2006). So antibody response can be seen only when animal is in advanced stages of infection and accordingly ELISA can be applied to animals which are in advanced stages of infection or in anergic state (Cousins and Florisson, 2005; Da Silva *et al.*, 2011). So indirect ELISA acts as a complementary/alternative test to SID test and interferon gamma assay (Veeregowda *et al.*, 2005) but it does not serve as sole diagnostic test for tuberculosis.

## CONCLUSION

As a whole, 26.67% of the animals tested in the dairy farm were found to be positive and/or reactors to tuberculosis. It indicates that both the CMI tests performed were better than those targeting antibody detection. Between the CMI tests, IFN- $\gamma$  assay was able to detect more positives indicating that it is more sensitive than the SID test. ELISA did not detect even a single animal as positive owing to the late appearance of antibodies/appearance of antibodies at advanced state of tuberculosis and by that time animal would have shown clinical signs, so obviously clinical diagnosis would have been made. These results indicate that no single test is able to detect all the infected animals and also no single test is having 100% sensitivity and specificity. Sensitivity of tests depends on the stage of infection in study subjects. So, combination of tests can always be helpful in better diagnosis of bovine tuberculosis.

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