



A preliminary assessment of new bovine and avian tuberculin in cattle by intra-dermal tests and whole blood interferon gamma assay

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The most effective strategy for the control of bovine TB requires identification and removal of infected animal from herd. The 2 ante-mortem tests recognised and approved for detection of *M. bovis* infected cattle are assay based on cellular *in vitro* immune responses, the tuberculin skin test and interferon- γ test. The IFN- γ was developed to improve the practicality and accuracy of diagnosis of bovine TB (Rothel *et al.* 1992). The interferon gamma (IFN- γ) assay was reported as a test possessing superior sensitivity and specificity compared to the single intradermal skin test, moreover, it had the advantage of being repeated several times since it is an *in vitro* generally encountered with intra-dermal assays (Rothel *et al.* 1992). Conventionally, *M. bovis* strain AN5 and *M. avium* strain D4ER are used for the preparation of bovine tuberculin (PPD) (PPD_B) and avian tuberculin PPD (PPD_A), respectively, for skin tests (DeKantor *et al.* 1984, OIE 2014, Erico *et al.* 1989) and for IFN- γ (Rothel *et al.* 1992) and as described by manufacturers of *Mycobacterium bovis* gamma interferon test kit.

Currently, the official test for screening animals against bovine tuberculosis in India and in many other developing countries is the single intradermal cervical skin test (SIT) (Mukherjee 2006, OIE 2014). The present study reports ante-mortem diagnosis of bovine tuberculosis employing tuberculin test using a new tuberculin PPD_B (*M. bovis*, 3/86) and interferon gamma assay in cattle whole blood culture stimulated with PPD_B and avian PPDA (*M. avium* M-17) using a commercial bovine IFN- γ assay kit.

Mycobacteria: *M. bovis* (3/86) isolated from dairy cattle at Indian Veterinary Research Institute, Izatnagar and *M. avium* (M-17) obtained from Institute of Microbial Technology, Chandigarh were used for production of tuberculins (PPDs).

Antigens: Bovine PPD (*M. bovis*, 3/86) and *M. avium* PPD (M-17) 0.3mg/ml concentration were used to stimulate whole blood culture. PBS (pH 7.2; 0.01 M) was used as control antigen.

Propagation of mycobacterial culture: *M. bovis* (3/86) isolated from dairy cattle at Indian Veterinary Research Institute, Izatnagar and *M. avium* (M-17) obtained from Institute of Microbial Technology, Chandigarh were grown on Dorset Henley's Synthetic Liquid medium.

Preparation of tuberculin antigens: PPDs tuberculin PPD_B and PPD_A from *M. bovis* 3/86 (IVRI, Izatnagar) and *M. avium* strain M-17 (IMTEC, Chandigarh) were prepared by propagating these strain in Dorset Henley's Synthetic Liquid Medium as bulk stationary cultures and using trichloro-acetic acid precipitation of PPDs from cultures. Batches of PPD_B and PPD_A were prepared as per Garg and Verma (2008). The PPDs were standardized by subjecting the PPDs to sterility, safety and potency test (Indian Pharmacopoeia IP 2010). The concentration of PPD_B and PPD_A batches were (2000 IU) 1 mg/ml respectively.

Animals: Cattle were selected from 2 different organized farms and categorized into groups A (30 animals), B (50 animals) and C (7 animals). Cattle (80) from 2 organized farms were screened employing bovine tuberculin PPD (PPD_B) by single intra-dermal (SIT) and caudal fold (CFT) tests; and the controls (7; not tested by skin test) comprised 3 calves sensitized with *M. bovis* strain 3/86 (IVRI) and 4 randomly selected non-sensitized animals not belonging to the 2 farms.

Tuberculin skin test: PPD_B (0.1ml) prepared as above was used for single intradermal skin test and caudal fold test (CFT). The CFT was read after 72 h of the administration of the tuberculin. The injection site was inspected by both visual observation and palpation for indications that the animal has mounted an immune response. If any abnormalities such as discoloration or swelling at the injection site are seen, the animal is classified as CFT test responder (OIE 2009). When tuberculin skin test was used, in addition to subjective palpation and visual observation of the injection site, the skin fold thickness was measured with calipers before and 72 h after the test. The animal with skin thickness of >4mm or more was classified as reactor (OIE 2009).

IFN- γ assay: A sodium heparinised blood sample (5 ml) was collected from each cattle before application of CFT and cervical SIT and brought to laboratory within 8 h of

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collection. Blood samples collected from each animal were dispensed in 3×1.5 ml into 24 well tissue culture plate. Then, 100 µl nil antigen (PBS), as non stimulating control to the first well, 100 µl of PPD_B to the second well and 100 µl of PPD_A to the third well of each sample were added, and the plates were incubated in humidified atmosphere at 37°C for 16–24 h. Both PPD_B and PPD_A were made in-house. The plasma samples were harvested from the cultures and tested with the Bovigam ELISA test according to the instructions provided with the kit. The samples in the ELISA were run in duplicate. Positive and negative controls were used in each plate. The absorbance within 5 min of terminating the reaction was recorded using a 450 nm filter. The mean absorbance values of positive and negative controls were determined for the test validation and compared with positive and negative control values provided with the kit for the validation of the test (negative bovine IFN-γ control < 0.130; positive bovine IFN-γ control > 0.70). The mean of nil antigen, avium and bovine PPD optical density (OD) for each sample were calculated and compared with the mean absorbance values of the nil antigens, avian and bovine PPD controls. A sample was considered as positive when the difference between OD value of the sample stimulated with bovine PPD and OD value of the same sample stimulated with avian PPD and nil antigen was equal or higher than 0.100. A sample was considered as negative when the difference was less than 0.100.

Sensitivity and specificity: The relative sensitivity and specificity were calculated using the method of McDiarmid and Hellstrom (1987).

Comparison of results of skin tests and whole blood IFN-γ: Cattle (80) from 2 organized farms were screened using bovine tuberculin PPD (PPD_B) by single intra-dermal cervical (SIT) and caudal fold (CFT) tests revealed that 44 (55%) tested as positive and 21 as negative reactors. The skin test results were inconclusive for 15 animals. Compared to skin test results, the whole blood interferon gamma (IFN-γ) assay identified 36 (45%) and 11 as positive and negative reactors, respectively. The IFN-γ results were inconclusive for 34 animals. In the controls (7; not tested by skin tests) consisting of 3 calves sensitized with *M. bovis* strain 3/86 (IVRI) and 4 non-sensitized animals, 4 reacted positively in the IFN-γ assay.

Bovine tuberculosis is a devastating infectious disease caused by *M. bovis*, which also affect several wild animals and man. Bovine tuberculosis poses 2 important aspects, viz. (i) bovine may act as reservoir of *M. bovis*, (ii) zoonosis. Therefore, it is imperative, to control bovine TB, accurate identification and removal of infected animals from herd is necessary. The 2 ante-mortem tests recognised and approved for detection of *M. bovis* infected cattle were assay based on cellular immune responses, the tuberculin skin test and interferon -γ test. Out of these 2 ante-mortem tests, only the tuberculin test is performed in cattle in India.

Worldwide, intra-dermal tuberculin test is used as standard method for detection of bovine tuberculosis. The

CFT is reported to be slightly less sensitive (however the difference was not found statistically significant) than the simple cervical skin test with 0.1 mg of PPD_B with high biological activity was used for each test (DeKantor *et al.* 1984); the difference in skin thickness before and after inoculation of antigen along with cardinal sign of inflammation was used as criteria for declaration of results. However, a similar report (Norby *et al.* 2004) had compared the sensitivities of the CFT (using 0.1ml of biologically balanced PPD_B @ 1mg/ml for USDA), and CFT and comparative cervical skin test (using 0.1ml of biologically balanced PPD_B and PPD_A from USDA at a concentration of 1mg/ml) in series (CFTCCT_{SER}), wherein for declaration of CFT results after intra-dermal 0.1 ml of PPD_B from USDA (1mg/ml) was based on visual examination and palpation. They reported a sensitivity of 93.02% for the CFT and 88.37% for CFTCCT_{SER}; on stratification of data based on low and moderate prevalence in herds. The sensitivities were reported to be 96.77 and 93.55% in low prevalence herds and 83.33% and 75 in moderate prevalence herds. Further, Whelan *et al.* (2010) reported that the single intra-dermal comparative cervical skin test (SICCT) is more specific than either CFT had been recommended (Erico *et al.* 1989). The CFT was used effectively to screen bovine tuberculosis in small herds in control regions of low prevalence (Portacci *et al.* www.sciquest.org.nz/.../T6-3.3.5). In the present study, we used 0.1ml of PPD_B from *M. bovis* strain 3/86 (IVRI) at a concentration of 1mg/ml for testing Indian cattle by CFT and cervical skin test (equivalent to SIT as mentioned above) and used the interpretation criteria adopted by Norby *et al.* (2004) for the declaration of the results of CFT. According to Norby *et al.* (2004), caudal fold test (CFT) had the highest sensitivity (93.2%) in CFT, comparative cervical skin test and PCR. We found high sensitivity (63.88%) in IFN-γ test compared to sensitivity (52.27%) in the tuberculin test performed either by SIT or CFT.

Middle of neck is the choice for inoculation of PPD intradermally, whereas, in most of the developed and other countries, caudal fold test is preferred. In both of these methods, the handling of animals can not be ruled out, though caudal fold test is easier to perform and interpretation is based on just visual palpable swelling and discoloration after 72 h. The skin test strategy is based on the intention (rule out or rule in the disease) and the prevalence rate (low, high or moderate). The potency of the PPDs used can also affect the outcome when 2 tests are being compared. Generally, the CFT is considered to be more sensitive compared to the cervical skin test. However, the comparative intradermal cervical skin test (SICCT) is reported to be more specific than CFT. So, an initial screening by CFT followed by SICCT on CFT positive or doubtful animals had been recommended (Erico *et al.* 1989, De Kantor *et al.* 1989, Norby *et al.* 2004, Whelan *et al.* 2010).

The whole blood IFN-γ assay is reported to be more sensitive and specific than either CFT or cervical skin test,

moreover, since it is not an *in vivo* invasive assay, it is used frequently on the same animal for testing, circumventing the issue of hyper-sensitization or desensitization noticed in skin tests using PPDs when repeated within 42 days (Rothel *et al.* 1992, Monaghan *et al.* 1994, Vordermeier *et al.* 2006). In this study, we tested the performance of new PPDs-PPD_B and PPD_A in a whole blood IFN- γ assay using a commercial kit to check its efficiency in terms of sensitivity compared to the CFT and the cervical skin test. The dose of PPDs used in the assay was as per the dose recommended by the kit.

The protocol of interferon- γ test kit was used. From two organized dairy farms, three *M. bovis* (3/86) sensitized calves and some random calves were selected for interferon- γ test and tuberculin test. Tuberculin test on the middle of neck was included as a control test to investigate relevance of caudal fold test (CFT). The IFN- γ response measured was not a non-specific response, but an antigen-stimulated response. That is, the response of heparinised whole blood over 24 h to antigens such as bovine tuberculin and avian tuberculin- a measure of exposure to environmental mycobacteria. The IFN-gamma test was sensitive and may detect infection sometimes before the skin test shows positive, and before lesions appear (i.e. in the absence of positive skin test and lesion data). The BOVIGAM™ IFN- γ test, a blood-based assay to detect cattle infected with bovine tuberculosis (TB) is a fully-validated test that is recognised by the EU and OIE as an ancillary test to the tuberculin skin test to maximise the detection of infected animals. Many developed countries like Australia, New Zealand, Canada, and USA have used IFN- γ test for control and eradication of bovine tuberculosis (Rothel *et al.* 1992, DEFRA 2011, Strain *et al.* 2012).

Our investigation revealed that the production of IFN- γ when stimulated with bovine PPD was significantly greater than with avian tuberculin as it was evident from the OD values in positive animals. Palmer *et al.* (2004) who reported the production of IFN- γ greater in response to bovine PPD than that seen in response to avium PPD in infected deer. Whipple *et al.* (2001) recorded significantly higher OD values for samples for bovine PPD than for sample stimulated with avium PPD in positive *M. bovis* animals. Rothel *et al.* (1992) suggested that IFN- γ responses in infected cattle were affected by the prior application of tuberculin skin tests. They observed gradual increase in the OD values for the IFN- γ test between 7 and 59 days after a caudal fold SIT. Different views on the influence of tuberculin testing on response of IFN- γ were described earlier (Gormley *et al.* 2004, Whelan *et al.* 2004). We did tuberculin test on animals and blood samples of animals were collected prior to tuberculin test. This study also showed that in some blood samples release of IFN- γ in response to avian PPD was greater than the bovine PPD as was evident from the OD values. This result might be due to sensitization to *M. avium* or other nontuberculous environmental mycobacterial species. Vervenne *et al.* (2004) reported the same finding and they suggested that to avoid

misdiagnosis, animals showing high avian PPD responses should be retested more than 2 weeks after the original test. We also found that relative sensitivity of IFN-gamma (63.88%) was higher than the SIT test (52.27%), which was supported by Wood *et al.* (1991). They found that that IFN- γ test had a higher sensitivity (93.6%) than the CFT (65.6%). In conclusion the preliminary study indicated that the new tuberculin antigens PPD_B and PPD_A possessed the potential to be used in skin tests and IFN- γ assay. However, further validation of these antigens is necessary in larger production comprising herds that have been classified as positive and negative for bovine tuberculosis.

SUMMARY

The present study reports ante-mortem diagnosis of bovine tuberculosis employing tuberculin test using a new tuberculin PPD_B (*M. bovis*, 3/86) and interferon gamma assay in cattle whole blood culture stimulated using a commercial bovine IFN- γ assay kit. Dairy cattle (87) categorized in groups A, B and C were tested using tuberculin test and gamma interferon (IFN- γ). Fresh batches of bovine PPD (from *M. bovis* 3/86) and avian PPD (*M. avium* N-17) were prepared and were standardized by conforming to sterility, safety and potency. For tuberculin testing cervical single intradermal test (SIT) and caudal fold test (CFT) were performed using tuberculins at concentration of 1mg/ml and at the concentration of 0.3mg/ml as a stimulating antigens in IFN- γ test. Out of 80 cattle (group A + B), 44 (55%) were reactor to tuberculin, 21 (26.25%) non reactor and 15 (18.75%) were inconclusive animals and by IFN- γ test 36 (45%) were *M. bovis* positive, 10 (12.5%) negative, 13 (16.25%) inconclusive and 21 (26.25%) were *M. avium* positive animals. In group C (not tuberculin tested) only 4 (57.14%) out of 7 cattle were positive. Relative sensitivity of IFN- γ test (63.88%) was higher than the tuberculin test (52.27%). Our study indicated that skin tests (CFT and SIT) and IFN- γ test can be used for successful control and eradication of bovine tuberculosis as IFN- γ test had higher sensitivity.

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