



Comparison between intradermal tuberculin skin testing and interferon gamma ELISA for diagnosis of bovine tuberculosis in Jabalpur region

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ABSTRACT

Interferon gamma ELISA was compared with the conventional tuberculin testing for the antemortem diagnosis of bovine tuberculosis. A single intradermal tuberculin skin test (SID) was carried out as per the standard protocol on animals from which 10 ml blood was collected earlier and *Mycobacterium bovis* gamma interferon ELISA for cattle was performed. Out of the 100 animals tested, 36 animals (36%) were found positive by tuberculin testing and 24% (22% bovine and 2% avian tuberculin) were found positive for interferon gamma. From 36 tuberculin positive cases only 16 (44.44%) were positive for interferon gamma. Interestingly, from 64 tuberculin negative cases, 8 (12.50%) were determined to be positive for interferon gamma. It can be concluded that SID has a high rate of false-positive as well as false-negative results and the specificity of interferon gamma assay was more than SID.

Key words: Bovine tuberculosis, INF gamma ELISA, *Mycobacterium bovis*, Tuberculin testing

Throughout the history of the eradication program of bovine tuberculosis (bTB), a disease of zoonotic and economic importance, tuberculin skin tests have been the only tests officially approved for diagnosis of tuberculosis in live cattle (Whipple *et al.* 2001). The single intradermal tuberculin test (SID) is the primary test used in many countries including India. A supplemental diagnostic test that is used in several countries is gamma interferon (γ -IFN) assay, which is an *in vitro* assay commercially available as a kit. The γ -IFN assay is conducted by stimulation of blood with bovine PPD and avian PPD followed by detection of γ -IFN in the plasma using an enzyme immunoassay (EIA). Preferential production of γ -IFN in response to stimulation with bovine PPD is considered a positive test result. The strategic use of the gamma-interferon (IFN-gamma) assay can provide a means for the early identification of *Mycobacterium bovis* infected cattle, thus ensuring their removal from an infected herd (Gormley *et al.* 2004). Pusic *et al.* (2009) suggested that the γ -IFN assay should not be used as a screening test in routine surveillance, but its strategic use should be targeted in herds and regions with high bTB incidence, in parallel with the tuberculin test to lower the possibility of bTB infected cattle to be

misdiagnosed as being clear. However, Okafor *et al.* (2013) documented that IFN- γ response is sufficient to classify cattle as positive for tuberculosis. The present study was conducted to compare the single intradermal tuberculin test (SID) with the γ -IFN in cattle of Jabalpur region.

MATERIALS AND METHODS

For the antemortem diagnosis of tuberculosis female cattle were selected from Livestock Farm Adhartal and Dayadoya, between the age group of 1 and 10 years. Blood (10 ml) was collected in heparinized vial aseptically from jugular vein of the animal. The blood was collected randomly from 125 animals taking care to include both clinically healthy and unhealthy animals showing respiratory distress.

Intradermal tuberculin skin test: A single intradermal tuberculin test was carried out as per *OIE Manual of Standards* (2008) on 125 animals from which blood was collected earlier at the farms. The injection site, the mid neck region was clipped and cleaned. A fold of skin within each clipped area was measured by digital vernier caliper and the site was marked with ink; 0.1 ml of bovine PPD tuberculin (2,000 IU/animal; 1 mg protein/ml) was inserted obliquely into the deep layers of the skin of the mid neck with a tuberculin syringe.

The injected site was examined 72 h later and the extent of indurations was measured with digital vernier caliper. The reaction was considered to be negative if only limited swelling was observed, with an increase of not more than

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2.00 mm and without clinical signs such as diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymphnodes. The reaction was considered inconclusive/doubtful if none of the clinical signs were observed and if the increase in the skin folds thickness was more than 2.00 mm but less than 4.00 mm. The reaction was considered to be positive if clinical signs were observed or if there was an increase of 4.00 mm or more in skin fold thickness.

Grouping of animals: Based on their *in vivo* tuberculin reactivities and clinical criteria, the cattle were divided into 4 groups:

Group A: Tuberculin positive with clinical signs of tuberculosis (n=15).

Group B: Tuberculin positive but apparently healthy (n=21).

Group C: Tuberculin negative with clinical signs similar to tuberculosis (n=35).

Group D: Tuberculin negative and apparently healthy (n=29).

Mycobacterium bovis gamma interferon ELISA for cattle: The AntiGen TB-feron is an indirect ELISA for the qualitative detection of interferon gamma (IFN- γ) in plasma. The assay was performed by using AntiGen TB-feron kit and interpreted according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Single intradermal tuberculin skin testing (SID): Out of the 100 animals tested, 36 animals (36%) were found positive by tuberculin testing with an increase of 4.00 mm or more in skin fold thickness in our study.

The test is based on the intradermal injection of purified protein derivative (PPD), a crude mixture of mycobacterial antigens. The tuberculin (delayed hypersensitivity) test is the standard method of diagnosis in live cattle and the prescribed test for international trade. The comparative intradermal tuberculin test can be used to distinguish between infections with *M. bovis* and sensitization to other *Mycobacterium* spp. Variations such as the thermal test and Stormont test have also been used. False negative reactions may occur in animals that have poor immunity or are anergic, old, or have recently calved.

High prevalence rates of bovine tuberculosis on the basis of SID were reported from southern Indian states (Nalini *et al.* 1998). The variable results of tuberculin testing in cattle were observed by Desmecht (1980), Wilson and Howes (1980), De Kantor *et al.* (1984) and Kovacic and Lackovic (1984). Authors concluded that the caudal fold test performed with bovine PPD with high biological activity was more sensitive than the comparative neck test with bovine and avian PPD's.

Delayed hypersensitivity may not develop for 3 to 6 weeks following infection. Thus, if a herd/animal is suspected to have been in contact very recently with infected animals,

delaying testing should be considered to reduce the probability of false-negatives. As the sensitivity of the test is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test. It should be recognized that when used in chronically infected animals with severe pathology, the tuberculin test may be unresponsive. However, SID has many drawbacks including a high rate of false-positive results (because it includes mycobacterial antigens that are not specific for *M. tuberculosis/bovis*), a high rate of false-negative results among immune-suppressed animals, and subjectivity and interpersonal variability when interpreting the results. SID also has the potential disadvantage of boosting an anamnestic response with successive tests (Miguel *et al.* 2004). Oztork *et al.* (2010) have emphasized that early stages of infection, allergy and weak immune system can be responsible for low sensitivity and specificity of tuberculin testing in animals. A number of alternative methods of interpreting the skin test responses have been adopted, recognising that false-positive reactions may be caused by sensitisation by other mycobacteria and by local inflammation. It is important to recognize that there is a balance between sensitivity and specificity and achieving high concurrent values may not be possible.

Mycobacterium bovis gamma interferon ELISA for cattle: The IFN-gamma assay is the first *in-vitro* cellular assay to be used as a routine diagnostic test in veterinary medicine. In the present study an indirect ELISA for the qualitative detection of interferon gamma (IFN- γ) in plasma was performed. Gamma interferon (γ -IFN) assay detects the cytokine γ -interferon, which is predominantly released by T-cells after *in vitro* stimulation with bovine PPD and avian PPD. IFN- γ is thought to be involved in immunity to mycobacterial infections and is released *in vitro* in quantities that are readily measurable by enzyme immunoassay. A positive result is seen where there is a preferential release of IFN-gamma to constituents of *M. bovis* compared to other mycobacteria (Wood and Jones 2001). IFN-gamma is regarded as a pivotal cytokine released during the immune response to TB. It is produced by a wide variety of cell types but primarily by T-cells, which on exposure to mycobacterial antigens release IFN-gamma that in turn leads to a cascade of immune responses, most notably the activation of macrophages. Therefore, the assay is a measure of the responsiveness of T-cells to the organism which is part of the acquired immune responses i.e. the presence of reactive T-cells implies exposure to the organism.

In our study 24% (22% bovine and 2% avian tuberculin) were found positive for interferon gamma out of the 100 animals tested. From 36 tuberculin positive cases (groups A and B) only 16 (44.44%) were found positive for interferon gamma. Interestingly, from 64 tuberculin negative cases (groups C and D) 8 (12.50%) were determined to be positive for interferon gamma. Neill *et al.* (1994) also observed that

the gamma interferon assay detected the *M. bovis* cattle that were SID negative. Lilenbaum *et al.* (1999) found that gamma interferon assay identified animals 60 to 120 days earlier than SID test.

Earlier, Rothel *et al.* (1992) proved that INF-gamma assay system to be a rapid, sensitive and inexpensive method for measuring antigen-specific cell-mediated reactivity as compared to traditional lymphocyte proliferation assay. Domingo *et al.* (1995) observed that the relative sensitivity of the INF-gamma assay varied from 87 to 94% and that of the skin test from 75 to 93%. Whipple *et al.* (1995) found that sensitivity of the caudal fold test (CFT) ranged from 80.4 to 84.4%, while sensitivity of the INF-gamma assay ranged from 55.4 to 97.1%.

Ameni *et al.* (2000) compared the sensitivity and specificity of the comparative cervical tuberculin (CCT) and INF-gamma tests. The sensitivity and specificity of the CCT test were 90.9 and 100%, respectively. Those of the commercial INF-gamma test were 95.5 and 87.7%, respectively. Cagiola *et al.* (2004) showed that the specificity of the INF-gamma test is higher than that of the skin test (96.8%) and ranged from 97.3 to 98.6%.

Thakur *et al.* (2010) found that 14.31% (63/440) tested positive for bovine PPD tuberculin 72 h after administering SID test. Ganesan (2012) recorded that SID test declared 49.20% as reactors and 50.80% as non-reactors whereas the bovine gamma interferon test on the same animals classified 77.70% positive to bovine tuberculin, inclusive of all the reactors to SID test.

The sensitivity and specificity of γ -IFN assay were higher than intradermal tuberculin test in several studies (Wood *et al.* 1992, Ryan *et al.* 2000, Cagiola *et al.* 2004). γ -IFN is approved as an official test for diagnosis of bovine tuberculosis and it can also be used together with intradermal tuberculin test in eradication programmes of bovine tuberculosis in many countries (Whipple *et al.* 2001). It was reported that the use of 2 tests together could assist in the early accurate detection of bovine tuberculosis in infected cattle. While, bovine tuberculosis could be diagnosed by intradermal tuberculin test in 3–6 weeks post infection, γ -IFN assay detects the infection as early as in 14 day post infection (Ryan *et al.* 2000). γ -IFN assay provides the result in 24 h after collection of blood and also removes the operator's errors. It is a test easy to perform; it does not require farm visits to read the test result. Also, there is no time period requirement to wait for repeating the test, but in intradermal tuberculin test there should be 60 days interval to repeat the test. High cost of kits and incubation of heparinised blood with antigen within a few hours of collection are the only disadvantages of γ -IFN assay (Cagiola *et al.* 2004).

On the basis of the literature cited and our present observations, we also recommend that this vastly underutilized diagnostic test should be used for regular

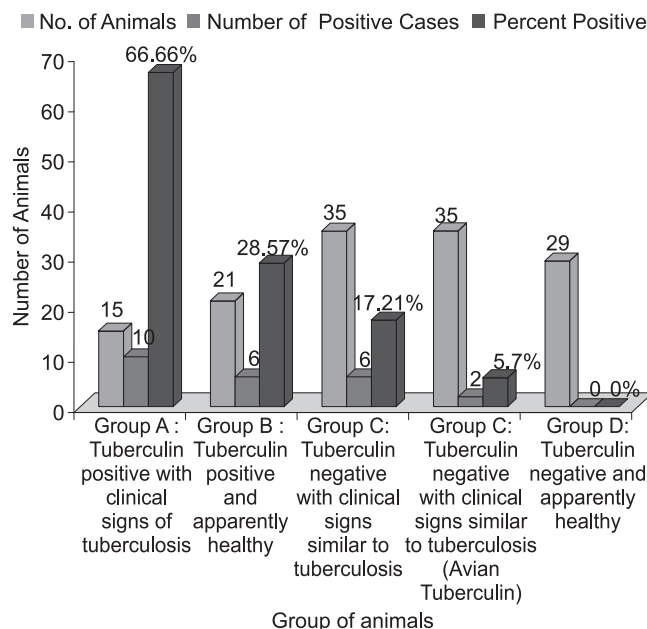


Fig. 1. *Mycobacterium bovis* Gamma interferon ELISA in tuberculin tested animals for diagnosis of bovine tuberculosis.

screening of animals in endemic areas of bovine tuberculosis in our state and country. The assay system has proven to be a rapid, sensitive and inexpensive method for measuring antigen specific cell-mediated reactivity when compared with the more traditional lymphocyte proliferation assay. It is very likely that the test will be improved by the further use of mixtures of *M. tuberculosis* complex specific antigens. The primary objective of this will be to increase test specificity as in principle measurable responses to these antigens should be largely TB complex specific. ESAT-6 and CFP-10 remain the most useful specific antigens. However, some workers claim that their use leads to a significant reduction in test sensitivity. Recent work has suggested that the addition of certain other antigens could significantly increase the sensitivity of the test (Schiller *et al.* 2009). However, these now need to be evaluated in the field situation.

It is possible that other immune signaling molecules released following blood stimulation with antigen may be useful either instead of or as an adjunct to interferon-gamma. One promising candidate is the monocyte derived chemokine IP-10. To our knowledge, this target has not been evaluated in cattle and it is at least feasible that it or other similar immune signaling molecules could be better targets or useful as additional targets for future assays of this type.

Statistical analysis

Relative sensitivity, specificity and accuracy: The relative sensitivity, specificity and accuracy of the tuberculin skin testing for the detection of bovine tuberculosis were determined in comparison to the INF-gamma ELISA as described here:

- Sensitivity = $[a/(a+c)] \times 100$, where a is the number of sera positive by tuberculin skin testing and INF-gamma ELISA, c is the number of sera positive by INF-gamma ELISA but negative by tuberculin skin testing.
- Specificity = $[d/(b+d)] \times 100$, where d is the number of sera negative by tuberculin skin testing and INF-gamma ELISA, b is the number of sera negative by INF-gamma ELISA but positive by tuberculin skin testing.
- Accuracy = $[(a+d)/(a+b+c+d)] \times 100$.

The results obtained from the tests were analyzed for the percentage agreement with INF-gamma ELISA with the use of the kappa statistics (Snedecor and Chochran 1994). The kappa statistics is a decimal measure of agreement between two tests, especially in the absence of a standard, and is defined as kappa or κ .

$$\kappa = a + d - P / 1 - P$$

where, $P = (a+b)(a+c) + (c+d)(b+d)$; P is the probability, a is the number of samples positive by both tuberculin skin testing and INF-gamma ELISA, b is the number of samples positive by INF-gamma ELISA but negative by tuberculin skin testing, c is the number of samples negative by INF-gamma ELISA but positive by tuberculin skin testing, and d is the number of samples negative by both INF-gamma ELISA and tuberculin skin testing. A kappa value greater than 0.81 indicates perfect agreement.

The relative sensitivity, specificity and accuracy of the developed tuberculin skin testing against INF-gamma ELISA as a reference standard resulted in a kappa value greater than 0.81 indicating perfect agreement between the 2 tests (Table 1).

Table 1. Relative sensitivity, specificity and accuracy values of SID TT using INF-gamma as a reference standard test.

	INF-gamma assay		Total
	+	-	
SID TT +	16(a)	20(b)	36
-	8(c)	56(d)	64
Total	24	76	100

Sensitivity, 66.00%; specificity, 73.68%; accuracy, 72.00%; Kappa value, 0.987. Kappa value > 0.81 indicates perfect agreement.

It can be concluded that single intradermal test has a high rate of false-positive as well as false-negative results. The specificity of interferon gamma assay was more than SID. Thus, it is recommended that γ -IFN assay can be used together with intradermal tuberculin test in eradication programmes of bovine tuberculosis.

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