Diagnosis of *Mycobacterium bovis* infection in livestock using gamma interferon assay and single intradermal comparative tuberculin test in Assam and Meghalaya

Acheenta G. Barua*, Himangshu Raj, Ashok Kumar[1], Chandana C. Barua[2], Arundhati Purkayastha[2] and Pompy Patowary[2]

Department of Veterinary Public Health, College of Veterinary Science,
Assam Agricultural University, Khanapara-781 022, Guwahati, Assam, India.

ABSTRACT

The present study was carried out to investigate the diagnostic potential of gamma interferon (IFN-γ) assay and single intradermal comparative tuberculin test (SICTT), including species specification of bovine tuberculosis infection in different livestock farms of Assam and Meghalaya. A total of 199 animals (cattle and buffalo) were examined for bovine tuberculosis symptoms and swab samples were cultured. Biochemical tests and PCR were used for species specification of bovine tuberculosis. Out of 199 cases examined, 33 (16.58%) showed positive for SICTT, 39 (19.59%) for IFN-γ and 35(17.59%) for PCR. Based on PCR targeting p*ncA* region, the confirmation was done for *M. Bovis*. IFN-γ thus ensures a sensitive and specific detection of early bovine tuberculosis infection together with SICTT and hence may be considered as a screening method of choice.

Key words: IFN-γ, *Mycobacterium bovis*, SICTT.

INTRODUCTION

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis* (*M. bovis*), is one of the most serious economic animal health problems affecting livestock industry. In many countries BTB is a major infectious disease having zoonotic importance affecting human, livestock and certain wild animals. It cannot be controlled in human unless it is controlled in animals. An infected animal can spread the disease to 10-15 men in a year. In India, the disease is endemic and its prevalence in cattle was reported from 14.3% - 34.2% (Thakur.*et al*., 2010). Lall (1969) reported BTB varying from 1.6-16% in cattle and 3-25% in buffaloes. The raising tendency of tuberculosis (TB) in India is due to intensive dairying and cross breeding of indigenous breed with less resistant exotic breed of cattle.

Cell mediated immune response, which dominates in the early stages of tuberculosis, activates and recruits T-cells to the site of infection (Pollock *et al*., 2005). With the development of several diagnostic assays like tuberculin skin test that measures immune response, have proven effective in diagnosing tuberculosis in cattle (de la Rua-Domenech *et al*., 2006). Although various tuberculin products with different potencies could be expected to influence the sensitivity and specificity of the skin test (Good *et al*., 2011), that may result in failure to detect all *M. bovis* infected animals (Monaghan

et al., 1994). The tuberculin used in cattle contains a crude mixture of predominantly secreted mycobacterial proteins derived from specified strains of *M. bovis* (Inwald *et al*., 2003) and varies widely both in protein content and antigenic profile (Tameni *et al*., 1998). However, many of these antigens are also found in non-pathogenic environmental mycobacterial species and this cross reactivity to common antigens can result in a reduced specificity of the test, giving rise to non-specific reactors *i.e.* false positive (Monaghan *et al*., 1997). For this reason, *M. avium* tuberculin is included in the single intradermal comparative tuberculin test (SICTT). In *M. bovis* infected cattle, CD4 T-cells produces IFN-γ leading to the activation of macrophage, with CD8 T-cells greater involvement in the lysis of infected cells (Skinner *et al*., 2003). Polymerase chain reaction (PCR) of *pncA* gene was evaluated for species specification for *M. bovis* and *M. tuberculosis*.

The present study was undertaken to investigate the infection of BTB in organized dairy farms in some parts of Assam and Meghalaya. Till date very few information are available on the actual prevalence of BTB in these areas of North East India. In this study, we investigated the efficiency of IFN-γ assay for diagnosis of early infection and SICTT performance based on bacterial culture, biochemical tests and polymerase chain reaction (PCR) for species specification.

*Corresponding author's e-mail: acheentagbarua@gmail.com
[1]Veterinary Public Health Division, IVRI, Izatnagar, India.
[2]Department of Pharmacology and Toxicology, College of Veterinary Science, AAU, Khanapara, Guwahati, India.

MATERIALS AND METHODS

The study was carried out in different organized cattle and buffalo farms located in various places of Assam and Meghalaya. A total of 199 samples both either from male / female showing symptoms like TB in the age group of 1-8 years were collected for the study.

IFN-γ Assay: All IFN-γ release assays were performed according to kit procedures (RayBio bovine IFN-gamma ELISA kit). Briefly, 100µl standard or sample was added to 96 well microplate coated with anti-bovine IFN-γ and incubated overnight at 4°C. Then 100 µl prepared biotin antibody added to each well and incubated for 1 hour at room temperature. 100 µl HRP-streptavidin was added and incubated at room temperature for 45minutes then 100µl TMB (tri methyl benzidine) one stop substrate reagent was added and incubated 30 min at room temperature at dark. Wells were washed four times with 1X wash buffer. To stop the reaction 50µl stop solution was added. Each blood sample and standard was tested in duplicate. Bovine IFN-γ was used as a standard at 30 ng/ml, 12 ng/ml, 4.80 ng/ml, 1.92 ng/ml, 0.768 ng/ml, 0.307 ng/ml, 0.123 ng/ml, along with the positive and negative controls (RPMI 1640). Samples were read at a wavelength of 450 nm to calculate optical density (OD). A sample was considered as positive when the difference between mean OD value of a negative control with mean OD value of sample is equal or higher than 0.100.

Single intradermal comparative tuberculin test (SICTT): The SICTT was carried out by intradermal injection of cattle with 0.1 mL PPD-bovine (1 mg/mL, 3000 IU, ID Lelystad) and 0.1 mL PPD-avian (0.5 mg/mL, ID Lelystad, 2500 IU) at sites 12 cm apart in the mid-neck region using a tuberculin syringe. Skin thicknesses were measured in mm at both sites before the intradermal injection and after 72 hrs. Test results were interpreted using two different cut-offs for reactors, known as standard or severe interpretation according to Karolemeas et.al. (2012).

Mycobacterial culture and identification: Nasal and throat swab samples were decontaminated using N-Acetyl-L-Cysteine (NALC) and inoculated on to Lowenstein Jensen (LJ) media. Briefly, the supernatant was discarded and the pellet formed resuspended in 300µl of phosphate buffered saline (140mM NaCl, 26mM KCl, 10.0mM Na2HPO4 and 1.7mM KH2PO4). Then the resuspended pellets were inoculated in duplicates onto Lowenstein-Jensen (LJ) slants (one incorporating glycerol and the other pyruvate). LJ slants were incubated at 37°C and observed weekly for eight weeks. Using a sterile 0.1 µl plastic loop, the resuspended pellets were appropriately spread and heat fixed (80°C for 10 min) onto labelled slide. The slides were subjected for staining with modified Zeil Neelson stain.

Biochemical test: Biochemical tests were performed for species specification of mycobacteria. The biochemical tests were done using test kits from Hi-Media.

Nitrate reduction test: (Kubica and Wayne, 1984) A loopful of test culture was emulsified aseptically in nitrate buffer (R056) in glass tube, mixed well and incubated at 37°C on water bath for 2 hrs. Buffer was then acidified with 0.1 N HCL and 2-3 drops of rehydrated nitrate reagent was added. Observation of for red colour was done which indicated the presence of mycobacteria.

Pyrazinamidase test: This test was done as per Wayne, (1974) with slight modification as 1ml of 2-3 week old mycobacterium culture was inoculated in the agar medium (SL121) and incubated at 35°C for 4 days. Pyrazinamidase reagent (R059) was rehydrated with 10 ml of distilled water and kept in refrigerator (2°C to 8°C) for 4 hrs. Observation was made for formation of pink/ red colour band in the reagent layer on the surface of the agar, which indicates positive result.

Niacin detection test: Distilled water (2ml) was added to > 3 week old culture grown on LJ slant. Slant was cut or stabs with a spade of needle and incubated at 37°C for 2 hrs on a water bath. Further slant was retained for 30 min and 1 ml from this slant was added to the reagent solution using syringe and observation for yellow colour was done (Gadre et. al., 1995).

Species specification by pncA PCR: DNA was isolated from bacterial culture and subjected to PCR targeting pncA gene. Primer pair of pncA gene with forward primer pncAMTB-1.2 (5'-ATGCGGGCGTTGATCATCGTC-3') which is same for both M. Bovis and M. tuberculosis and reverse primer pncAMT-1 (5'-CGGTGTGCCGGAGAAGCGG-3') specific for M. tuberculosis not M. Bovis and reverse primer pncAMB-2 (5'-CGGTGTGCCGGAGAAGCCG-3') specific for M. Bovis not M. tuberculosis (De Los Monteros et al. 1998), which amplify a product size of 185 base pair.

Statistical analysis: Those animals that show positive for IFN-γ / SICTT but negative in culture are taken as false positive and animals that show negative for IFN-γ/ SICTT but positive in culture were false negative. Animals that test positive for all assays are true positive and negative for all assays are true negative.

Sensitivity (in percentage) = (true positives / (true positives + false negatives)) x 100

Specificity (in percentage) = (true negatives / (true negatives + false positives)) x 100.

The inter-rater agreement (weighted kappa) among IFN-γ assay, SICTT and PCR were analysed using MedCalc Statistical Software (trial version 15.8 MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2015). Kappa values were interpreted according to Altman (1991).

RESULTS AND DISCUSSION

In this study, a total of 199 animals from different herds were subjected for IFN-γ assay, SICTT and swab culture and subsequent PCR. Of these, 33 (16.58%) were found

Table 1: Animal wise response to different screening methods

Animal	No.s tested	IFN-γ+ve	SICTT +ve	PCR +ve
Cattle	127	28 (22.05%)	23 (18.11%)	26 (20.47%)
Buffalo	72	11 (15.28%)	10 (13.89%)	9 (12.50%)
Total	199	39 (19.59%)	33 (16.58%)	35 (17.59%)

Table 2: Calculation of sensitivity and specificity of IFN-γ and SICTT based on *M. bovis* PCR positive results

	IFN-γ	SICTT	PCR
positive	39	33	35
negative	167	166	164
All test positive (true positive)	24		
All test negative (true negative)	153		
PCR negative, IFN positive (false positive)	8		
PCR positive, IFN negative (false negative)	4		
PCR negative, SICTT positive (false positive)	6		
PCR positive, SICTT negative (false negative)	8		
Sensitivity	75.00%	80.00%	-
Specificity	97.45%	95.03%	-

Table 3: Inter-rater agreement (weighted kappa) between different pairs of screening methods

	PCR +ve	PCR -ve	Kappa	SE	SICTT +ve	SICTT -ve	Kappa	SE
IFN +ve	31	8	0.801	0.055	27	12	0.695	0.067
IFN -ve	4	156			6	154		
SICTT +ve	27	6	0.752	0.063				
SICTT -ve	8	158						

positive by the SICTT and 39 (19.59%) of the animals were found reactive by the IFN-γ assay and 35(17.59%) were culture and PCR positive (Table 1). Out of 33 SICTT positive 30 (15.07%) were specific to *M. bovis* and 3 (1.51%) were found to avian reactive. Interestingly 3 SICTT inconclusive reactors were found positive for IFN-γ but negative in culture. The sensitivity and specificity of IFN-γ assay were determined as 75.00% and 97.45%, respectively and that of SICTT were 80.00% and 95.03%, respectively (Table 2). A very good agreement was recorded (0.801) between PCR and IFN-γ. However, SICTT shows only good agreement with PCR (0.752) and IFN-γ (0.695) (Table 3).

Gormley *et al.*, (2013) found the sensitivity of IFN-γ varied between 73.0% and 100% and specificity with a range of 85.0–99.6%. In another study, BTB free cattle herd showed the specificity and sensitivity of the SICTT ranged in 78.8-100% and 75-95% (de la Rua-Domenech *et al.*, 2006). Recently modelling work has estimated the sensitivity and specificity ranges for SICTT to be 58.6-77.3% and 99.2-99.7% (Clegg *et al.* 2011). IFN-γ assay can detect latent infection (Palmer *et al.*, 2006). In the present study, we found 3 animals positive for IFN-γ assay, culture and PCR but negative for SICTT. This result may be due to human errors, such as inappropriate injection. Young animals (< 6 months) can have high levels of IFN-γ, due to the presence of higher proportion natural killer cells (Clegg *et al.*, 2011). Therefore,

younger animals (<6-months of age) were not included in our study. Co-infection with *Mycobacterium avium* subsp. *paratuberculosis* can reduce the sensitivity of the IFN-γ test (Alvarez *et al.*, 2009). The underlying mechanisms responsible for false positive reactions are unknown, although variations in the types and potency of tuberculin used and the criteria used for interpretation of the test results can affect sensitivity and specificity of the test (Cagiola *et al.*, 2004).

Various biochemical tests *viz.* Nitrate Reduction test, Pyrazinamidase test, Niacin Detection test were performed for identification and species specification of positive mycobacterium culture. All the culture positive samples (Fig1) isolated from different farms showed negative for nitrate, pyrazinamidase, niacin test which indicated positive for *M. bovis*. In PCR, all the culture samples were found to be positive for BTB, showing band at 185bp (Fig 2) specific for *M. bovis*. The gene like *pncA* is used for genotypic identification of *M. bovis* species that distinguishes it from the other members of the *M. tuberculosis* complex.

Very good agreement between PCR and IFN-γ assay indicates positive correlation with molecular detection and antibody titre specific to *M. bovis*. Good agreement between SICTT with both PCR and IFN-γ assay indicates SICTT a good differential screening method for bovine tuberculosis infection with *M. Bovis* and non-pathogenic environmental mycobacterial species.
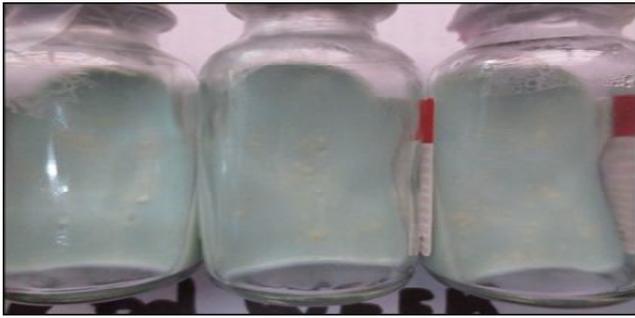
Fig 1: Positive culture of *M. bovis* in LJ slants

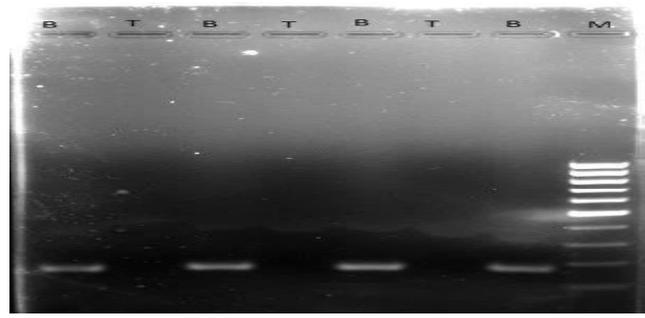

Fig 2: Lanes marked 'B; indicates *M. bovis* specific *pncA* with 185bp and lanes marked 'T' with no bands indicates negative for *M. tuberculosis*. Lane 'M' indicates 100bp marker

However good agreement between IFN-γ and SICTT indicates both tests are reliable for early detection of *M. bovis* infection.

CONCLUSION

It can be concluded that IFN-γ assay can be used for detection of latent tuberculosis infection together with SICTT and may be included in eradication programmes of bovine tuberculosis.

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