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Tuberculosis diagnosis in bovines by single intradermal testing, gamma interferon assay and PCR

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Abstract
Tuberculosis is a chronic bacterial disease affecting both humans and animals. Staining and microscopy of acid fast bacilli using zeil-Neelson technique for human sputum samples is a common routine first line diagnostic test followed till today in India. In bovines intra dermal skin testing using bovine PPD for herd screening and gamma interferon assay with PCR increases the sensitivity and specificity in diagnosis of tuberculosis in bovines. Skin testing using tuberculin PPD is the test recommended by OIE for herd screening of bovines worldwide for tuberculosis diagnosis. Many countries have successfully eradicated the tuberculosis in bovines only using Skin testing for herd screening. Two important species affecting humans and animals are *Mycobacterium tuberculosis* and *Mycobacterium bovis* belonging to Mycobacterium Tuberculosis Complex Group (MTC).

Keywords: Tuberculosis, diagnosis, Bovines, Mycobacterium *tuberculosis*, *Mycobacterium bovis*

1. Introduction

Tuberculosis is a chronic debilitating disease affecting both humans and animals. Tuberculosis is caused by an acid fast organism belonging to mycobacterium species. In humans chronic cough and debility is associated with the disease. Radiography of chest, sputum staining, microscopy, culture and nucleic acid amplification tests are carried out for routine tuberculosis diagnosis in human patients. In human there is a ban on serum based tests. Sputum samples are screened routinely in TB diagnostic centers followed by sputum culture on LJ slants and CB-NAAT. Use of antibody based tests are banned nationally and internationally for detection of tuberculosis in humans as these tests are non-reliable and provide spurious results. Director of WHO Stop TB Department. "A blood test for diagnosing active TB disease is bad practice. Test results are inconsistent, imprecise and put patients' lives in danger." The research by WHO revealed "low sensitivity" in commercial blood tests which leads to an unacceptably high number of patients wrongly being given the 'all clear' (i.e. a false-negative when in reality they have active TB). This can result in the transmission of the disease to others or even death from untreated tuberculosis. It also revealed "low specificity", which leads to an unacceptably high number of patients being wrongly diagnosed with TB (i.e. a false-positive when in reality they do not have active TB). Those patients may then undergo unnecessary treatment, while the real cause of their illness remains undiagnosed, which may then also result in premature death. More than a million of these inaccurate blood tests are carried out annually to diagnose active TB, often at great financial cost to patients. The transmission of organisms mainly *Mycobacterium bovis* and *Mycobacterium tuberculosis* from humans to animals and from animals to humans is very common.

In bovines skin test for tuberculosis diagnosis is commonly followed. Radiography of chest of bovines remains impractical and inconclusive for diagnosis of tuberculosis. The symptoms in bovines are not as conclusive as in humans in suspecting the disease by symptoms. The animals may harbor the disease and act as source of transmission or remain localized due to good immune response in bovines. Skin testing using tuberculin PPD in bovines remains herd screening test as standard test by oie for tuberculosis diagnosis followed by many countries in Bovine tuberculosis eradication program me. Tuberculin skin testing is a measure of Cell mediated immune response measuring the skin thickness after 72 hrs of inoculation of tuberculin PPD at the neck of bovine which is Type IV hypersensitive reaction-Delayed type.

Gamma interferon assay is recent and more sensitive test than Tuberculin skin testing commonly followed in developed countries [1]. In herd screening of bovines for tuberculosis using gamma interferon assay appears costly and not cost effective for developing countries like India, but remains helpful when skin testing remains inconclusive. Gamma interferon assay uses cell mediated immune response where blood is collected, lymphocytes are stimulated using bovine PPD, after 16 hrs of lymphocyte stimulation the plasma collected are subjected for sandwich ELISA to determine the amount of gamma interferon released.

Nucleic acid amplification test- Mainly PCR targeting different conserved regions in Mycobacterium species are been under research for diagnosing the Mycobacterium Tuberculosis Complex and to species level identification and diagnosis using specific primers amplifying a specified amplified product size.

Culture, staining and microscopy remain a gold standard test for tuberculosis diagnosis. Rapid and accurate diagnosis is need of hour requirement in tuberculosis diagnosis as the organism being slow grower takes months to appear on LJ slants.

2. Materials and methods

2.1 Skin Testing

Cattle from semi-organized farm and goshala were identified. Around 40 animals were screened with intradermal skin testing using bovine PPD supplied by IVRI Izatnagar U.P. About 0.1 ml was inoculated at neck of the cattle using TB gun. Initial reading was taken before inoculation and after 72 hrs final reading was taken using digital calipers.

2.2 Gamma interferon Assay

Blood was collected from all 40 animals into EDTA vials and subjected to gamma interferon assay by stimulating lymphocytes in blood within 8hrs of collection at the rate of 20 microgram/ml into round bottom tissue culture plates [2]. After overnight stimulation the plasma carefully collected was subjected to sandwich ELISA. The ELISA kit procured by Ray Bio Inc Ltd was used following the protocol supplied by manufacturer.

2.3 PCR Assay of Nasal swabs

The nasal swabs were collected aseptically into sterile swab tubes from all the forty animals and genomic DNA was isolated using qiuzen kit following kit protocol. DNA from swabs was amplified using published primers. [4, 5]. Initially primer sequence IS6110F and IS6110R to amplify a 445 bp sequence on all MTC strains was amplified with a total mix of 25 micromoles, with 5 microliters of the sample, 10 Pico moles each of forward and reverse primer, 12.5 microliters of Dream Taq master mix (1.5 units of Taq DNA polymerase) and remaining Nuclease Free Water to make total volume of 25 microliters. The thermal cycler conditions were: initial denaturation at 94^0 C for 10 minutes followed by denaturation step at 94^0 C for 1minute, primer annealing at 54^0 C for 1 minutes, extension at 72^0 C for 1 minute and the reaction continues for 30 cycles followed by a final extension at 72^0 C for 5 minutes. The amplicon analyzed on agarose gel electrophoresis. The genomic DNA which yielded 445 bp amplicon for MTC strains were further subjected to duplex PCR with single forward primer and two reverse primers with the same cycling conditions. The amplicon size of 389 bp with reverse primer for *Mycobacterium tuberculosis* and size of 823 bp with reverse primer for *Mycobacterium bovis*.

Table 1: Primer sequence for MTC strains.

	IS6110
IS6110F	5'GACCACGACCGAAGAATCCGCTG3'
IS6110R	5'CGGACAGGCCGAGTTTGGTCATC3'

The primers amplify a 445 bp sequence on all MTC strains.

Table 2: PCR conditions for MTC strains**.**

1	Initial Denaturation	94^0C	10 minutes	30 Cycles
2	Denaturation	94^0C	1 minutes	
3	Primer Annealing	68^0 C	1 minutes	
4	Extension	72^0 C	1 minutes	
5	Final Extension	72^0 C	5 minutes	

Table 3: Primer sequence for *M. bovis* and *M. tuberculosis*

	12.7 kb fragment
12.7 kb F	5' CACCCCGATGATCTTCTGTT 3'
12.7 kb R1	5' GCCAGTTTGCATTGCTATT 3'
12.7 kb R2	5' GACCCGCTGATCAAAGGTAT 3'

The primers amplify with 12.7 kb F and 12.7 kb R1 amplify an 823 bp sequence for *M. bovis* and primers 12.7 kb F and 12.7 kb R2 amplify a 389 bp sequence for *M. tuberculosis*

Table 4: PCR conditions for *M. bovis* and *M. tuberculosis*

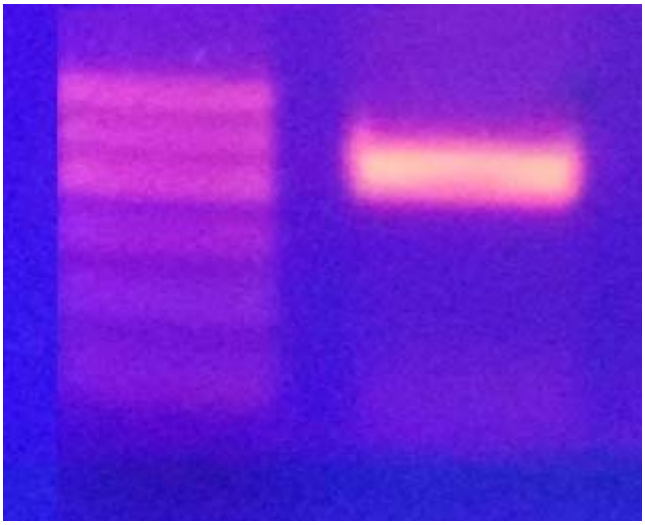
1	Initial Denaturation	94^0C	10 minutes	30 Cycles
2	Denaturation	94^0C	1 minutes	
3	Primer Annealing	54^0 C	1 minutes	
4	Extension	72^0 C	1 minutes	
5	Final Extension	72^0 C	5 minutes	

2.4 Staining and Culture of nasal swabs

The nasal swabs on decontamination with 4% NaOH are inoculated on to LJ slants with and without glycerol and incubated into Co_2 incubator for 6- 8 weeks for growth at 37^0C [3]. The swabs are smeared on glass slide were stained with zeil neelson staining techniques and microscopy was done.

3. Results and Discussions

Most of the animals were nonreactors to skin testing only one animal with evident signs of pneumonia and cough showed suspected reactor to intra dermal tuberculin testing. The animals were found to be under good plain of nutrition in dairy farms maintained by collage and private person. Testing and culling of the animals was also under taken on earlier occasions became evident on collection of history from collage diary farm. Animals were under poor nutritional status in goshals and appeared to be debilitated and positive on gamma interferon assay test. None of the nasal swabs yielded growth on LJ slants with and without glycerol. On PCR of nasal swabs one sample amplified for MTC strains with 445 bp amplification but on duplex PCR yielded neither for *M.bovis* nor for *M.tuberculosis* appear to be some other commensal mycobacterium. Milk collected from the animals on PCR yielded no amplification for MTC strains. Gamma interferon assay of blood samples revealed high optical density for 16 blood samples and appear/considered to be positive for tuberculosis infection by gamma interferon assay test. On staining of nasal swabs none were positive for acid fast organism on microscopy. So Gamma interferon assay test appears to be more sensitive test for tuberculosis diagnosis even under poor plain of nutrition where CMI response may become inconclusive in tuberculosis diagnosis by skin testing.



Lane.1: 100 BP marker Bovine Nasal swab PCR

Fig 1: Agarose gel showing PCR amplification of 445 bp for MTC strains.

4. Conclusions

Tuberculin Skin testing is an OIE excepted test for tuberculosis diagnosis in bovines. Many countries have eradicated tuberculosis infection in bovines by using tuberculin skin testing as herd screening test. Gamma interferon assay is a recent test employed for tuberculosis diagnosis where the sensitivity is very higher than tuberculin skin testing. But Gamma interferon assay appears to be costly for herd screening in developing countries. Isolation and culturing appears to be gold standard test from long past for routine tuberculosis diagnosis but requires months for growth to appears on media. PCR is the rapid test for diagnosis of tuberculosis in humans helpful by increasing the specificity of tuberculosis diagnosis. In short Gamma interferon assay remains the more sensitive test for tuberculosis diagnosis in bovines along with skin testing and sensitivity of the diagnosis can be increased by employing PCR and isolation techniques.

5. References

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