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Evaluation of different diagnostics tests for detection of tuberculosis in cattle

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ABSTRACT

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Single intradermal cervical tuberculin skin testing (TST) is only available diagnostic test for detection of tuberculosis in bovines in India. TST may miss some positive tuberculosis cases due to its limited sensitivity. Present study was aimed to evaluate different diagnostic tests (ELISA and PCR) for detection of tuberculosis in cattle. A total of 123 randomly selected cattle were examined for tuberculosis with TST, ELISA and PCR. Detection rate of tuberculosis by TST, ELISA and PCR were 8.94%, 13.82% and 5.69% respectively. This detection rate could be increased up to 18.69% by using multiple diagnostic tests serially. ELISA and PCR could detect the disease condition in advance stage, failing to detect by TST. Use of multiple diagnostic tests may minimize chance of missing the positive cases.

Keyword: Tuberculosis, tuberculin skin test, ELISA, PCR

INTRODUCTION

Bovine tuberculosis caused by *Mycobacterium bovis* is a chronic, infectious and progressive disease causing granulomatous inflammation, necrosis, calcification and encapsulation of lung, intestines, lymph nodes and other tissues[1, 2]. In dairy cattle it causes huge economic losses to dairy industry in resource poor and developing nations[3]. Globally, 3 billion US$ economic losses annually have been estimated due to bovine tuberculosis[4]. In India, the disease is endemic and its prevalence in cattle was reported from 14.3% - 34.2%[5]. Tuberculosis in cattle poses a serious threat to human health due to consumption of unpasteurized milk, dairy products, raw meat and meat products from infected animals. Incidence of tuberculosis in human beings due to *M. bovis* is not uncommon and strong association between animals and human beings has been reported. Single intradermal cervical tuberculin skin testing (TST) is most commonly used diagnostic test for screening of bovine tuberculosis[6] but its sensitivity is limited which may lead to some false negative results even after minimizing human error in tuberculin inoculation and observation in skin thickness measurement[7,8]. These undetected cases may be potential threat to other susceptible cattle and human beings as well. Interpretation criteria for determining cut off value in TST is subjective to various conditions such as endemicity of infection, host species and purpose of diagnosis which may invariably lead to errorneous and equivocal results. Each diagnostic test for bovine tuberculosis are not suitable to detect every phase of disease (early, late and latent). Diagnostic tests based on CMI response are well suited to detect early and latent phases of the disease while serological tests are more suited in diagnosis of advanced stage of disease. The objective of this study is to determine efficacy of different diagnostic tests in detection of bovine tuberculosis in cattle and identifying the positive cases which were missed in single intradermal skin testing (TST). These cattle may be potentially *M. bovis* shedders and play a critical role in transmission of zoonotic *M. bovis* to human beings.

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MATERIALS AND METHODS

A total of 123 cattle comprising all age groups (male and female) were included in this study. All cattle (n=123) were properly restrained and 1 cm^2 skin area was shaved. Skin thickness was measured with vernier caliper. Approximately 2000 IU of bovine PPD (0.1 ml) was injected intradermal into the mid cervical region of the cattle. Before PPD inoculation, blood was collected in vacutainer (BD-Vacutainer) for serum and heparinized vacutainer for plasma. Lymph node aspirates (n=123) were collected from the cattle. After 72 hrs of PPD inoculation, increase in skin thickness or any sign of inflammation (pain, erythema and swelling) at point of inoculation was observed. Increase in skin thickness by more than 4 mm or any sign of inflammation was considered positive for bovine tuberculosis. Sera samples (n=123) were analyzed in duplicate and repeated twice with Anigen BTB ELISA

(Bionote, Korea) kit following manufacture's guidelines. Briefly, 50 µl positive and negative control sera were added to each well in triplicate. 50 µl serum (test sample) was added to each well in duplicate followed by 50 µl of *M. bovis* antigen-HRP conjugate. Plate content was mixed thoroughly on vibrating mixer and incubated at 37°C for 60 minutes. Plates were washed 6 times with 350 µl of diluted washing solution. Liquid was aspirated from wells and added 100 µl of mixed substrate solution (Ready to use), incubated at room temperature (18~25°C) for 15 minutes, added 100 µl of stopping solution and absorbance was recorded with a bichromatic spectrophotometer at 450 nm. ELISA cut off value (S/P) was calculated as: (sample OD – average OD of negative control serum)/average OD of positive control serum – average OD of negative control serum). S/P value e" 0.5 was considered as positive while d" 0.5 S/P value was considered as negative.

MTB complex specific and M. bovis specific PCR assay

DNA was extracted from lymph node aspirates (n=123) using genomic DNA isolation kit (Fermantas, USA) by following manufacturer's guidelines with some modifications. MTB complex specific IS1081 sequence common to all MTB complex bacteria was used for primary screening[9]. After initial screening, species identification and differentiation was done by *M. bovis* specific PCR targeting 12.7 Kb region[10]. Positive and negative controls (without target DNA) were also run simultaneously with test samples.

Impression smear staining and Histopathology

Impression smear from lungs and mediastinal lymph nodes were stained with Ziehl-Neelsen staining technique. For histopathological examination, representative tissue pieces from lungs and mediastinal lymph nodes were fixed in 10% formalin, processed and stained with haematoxylin and eosin[11]. Agreement among TST, ELISA and PCR were analyzed by kappa statistics using GraphPad QuickCals (San Diego, CA, USA) and results were interpreted as per standard guidelines.

RESULTS

Eleven out of 123 cattle (8.94%) showed moderate to higher response (increase in skin thickness >3.5 mm to 4.5 mm) in TST. These cattle were classified as positive in TST. 5 out of 123 cattle (4.06%) showed less to moderate response (increase in skin thickness 2-3 mm) and 107 out of 123 (87%) cattle did not show any significant increase in skin thickness and sign of inflammation at the site of PPD inoculation. These were considered as non-responders in TST (Table.1). 17 out of 123 cattle (13.82%) were detected positive in bovine TB ELISA based on S/P value. S/P cut off value for positive case was considered e" 0.5. 7 out of 17 ELISA positive cattle were also detected positive in MTB complex and *M. bovis* specific PCR. 6 out of 17 cattle (35.29%) was also detected positive with TST. 7 out of 123 cattle (5.69%) were found positive with IS1081 MTB complex specific PCR, produced 135 bp amplicon (Fig. 1). These positive samples were further analyzed by *M. bovis* specific PCR targeting 12.7 kb region produced 168 bp amplicon in *M. bovis* while 262 bp amplicon in *M. tuberculosis* control sample (Fig.2). Acid fast bacilli were observed in ZN stained lung and mediastinal lymph node impression smears (Figure 3a). Lung parenchyma showed few small multiple granulomas distributed randomly which were characterized by central necrotic area surrounded by macrophages, lymphocytes and few giant cells. Mediastinal lymph node also showed similar lesions with giant cells formation, macrophage and lymphocytes infiltration (Figure 3b). A fair agreement (0.359) between TST and ELISA, good agreement between TST and PCR (0.642) and moderate agreement (0.547) between ELISA and PCR were observed (Table 2).

Table 1. Different diagnostic tests for detection of tuberculosis in cattle.

		Tuberculin reaction		
		Skin test positive	Skin test negative	Total
		11	112	123
ELISA	Positive	6	11	17
	Negative	5	101	106
PCR	Positive	6	1	7
	Negative	5	111	116

Table 2. Kappa statistics (agreement) between different diagnostic tests.

		ELISA				PCR			
		+	-	Kappa	SE	+	-	Kappa	SE
Tuberculin	+	6	5	0.359	0.125	6	5	0.642	0.134
	-	11	101	(Fair)		1	111	(Good)	
PCR	+	7	0	0.547	0.122				
	-	10	106	(Moderate)					

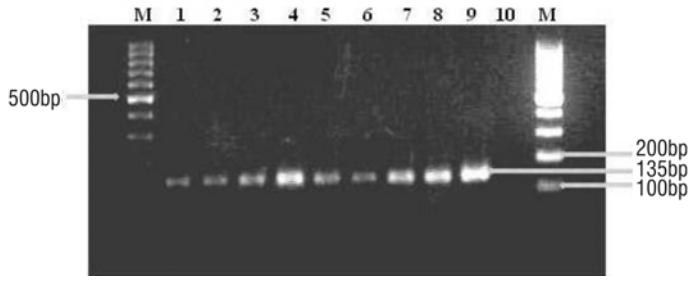

Fig. 1. PCR targeting MTB complex specific IS1081 genomic region. Lane M: 100 bp DNA ladder; Lane 1-8: MTB complex PCR amplicon using lymph node aspirate samples (test samples); Lane 9: Positive control; Lane 10: Negative control.

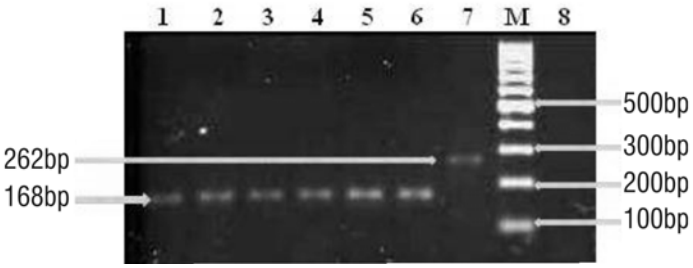

Fig. 2. PCR targeting *M.bovis* specific 12.7kb genomic region. Lane 1-5: 12.7 kb PCR amplicon (*M.bovis*) using lymph node aspirate; Lane 6: M. bovis positive control; Lane 7: *M. tuberculosis* positive control; Lane M: 100 bp DNA ladder; Lane 8: Negative control (without DNA template).

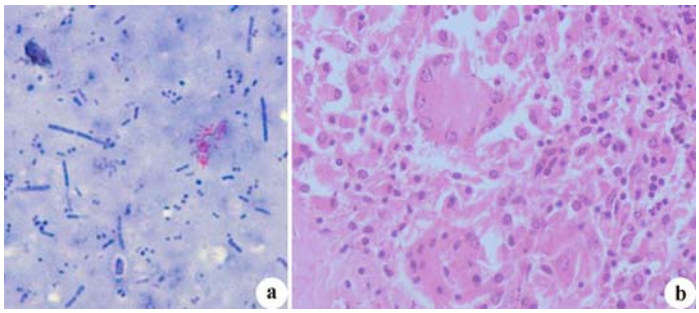

Fig. 3a. Acid fast bacilli in lung impression smear. Ziehl Neelsen x1000; **Fig. 3b.** Giant cell in tuberculous granuloma. H&E x400.

DISCUSSION

Tuberculosis is a zoonotic disease shared by animal and human beings in natural environment. Presence of tuberculosis in cattle and consumption of animal products from infected animal is a serious concern to human health especially in India where animal husbandry is an integral part of human life. Humans usually acquire *M. bovis* infection from their dairy animals due to sharing of environment. In India, Tuberculosis is an endemic disease, reported from different parts of the country[12,13]. Single intradermal cervical skin testing (TST) is globally accepted diagnostic test for the detection of bovine tuberculosis. It does not directly detect causative organism, instead it only detects a signal of *M. bovis* infection in animal. In mycobacterium infected animal, T-lymphocytes react with mycobacterial PPD antigen and inflammatory reaction ensues at the site of inoculation due to complex interaction of macrophages, dendritic cells and release of various pro-inflammatory cytokines mainly INF-ã and TNF-á but the major drawback associated with TST is its moderate sensitivity (65%) which may miss approximately 35% of tuberculosis positive cases[7]. This may be alarming in high endemicity area where chances for missing of positive cases are comparatively higher. TST is based on response to crude *M. bovis* culture filtrate antigen which is mixture of thousand of native antigens sharing antigenic epitopes with non pathogenic environmental mycobacteria. Therefore, it may cause false positive results in case of environmental mycobacterial infection[14,15] and valuable productive healthy animal may be lost if solely rely on TST. This test is based on CMI response towards mycobacterial antigen which may lead to false negative result if animal is immunocompromised or in advanced stage of disease. These infected cattle may be an uninterrupted source of infection to other healthy animals and pose serious health challenges to human beings as well.

In this study, 4.87 % of animals were detected positive with all three diagnostic tests (TST, ELISA and PCR). This reflects both arms of immune system (CMI and humoral) are working synergistically to contain the mycobacteria but pathogen might be able to evade from host immune system and is in progression of establishing disease. 4.06 % of cattle were detected by TST only which were not detected by ELISA and PCR. This might be due to very early stage of disease where cell mediated immune system is actively responding. The other plausible explanation of PCR negativity may be due to low bacterial load to be detected with PCR. ELISA could detect additional 11 out of 123 (8.94%) cattle positive for *M. bovis* antibody in serum. This may be due to transition phase of disease, immunocompromised status of animal, old age of animal, low plane of nutrition or other physiological factors like pregnancy. Although some percentage of false positivity in ELISA cannot be ruled out due to epitopic sharing with environmental mycobacterium. Kappa statistical analysis showed fair agreement between TST and ELISA, which reflect significantly difference between the two tests ($p<.0.01$). It underlines that TST may be suitable to detect early stage of disease only. PCR could detect 7 out of 123 cattle

(5.69%) in which 6 cattle were also found positive in TST and ELISA. This may be due to cattle having active tuberculosis, harboring enough mycobacterium to be detected by PCR. High agreement between ELISA and PCR in kappa statistical analysis indicates positive correlation with antibody titer and secretion of organism[16].This is a serious concern because these cattle are potent source of infection to other susceptible cattle, human beings and environment. Detection rate of tuberculosis by TST, ELISA and PCR were 8.94%, 13.82% and 5.69% respectively. This detection rate could be increased up to 18.69% by using multiple diagnostic tests serially. It will minimize chances of missing false negative cases and it will increase possibility to detect every phase of the disease.

ELISA and PCR could detect tuberculosis in advance stage, failing to detect by TST. This underlines the importance of sero-diagnostic tests and PCR in screening of bovine tuberculosis to rule out any false negative results by TST. Further, there is a need of point of control diagnostics and novel diagnostic techniques to monitor the spread of infection in large areas especially in developing nations[17,18,19]. A bead based microfluidic assay for detection of mycobacterial infections may be used in diagnosis of bovine tuberculosis[20]. Incidence of bovine tuberculosis may be minimized by active surveillance using less invasive and labor intensive biological samples[21]. Molecular epidemiological, phylogenetic analysis and mathematical modeling may be used to understand the origin of new outbreaks and in designing control strategies[22].

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