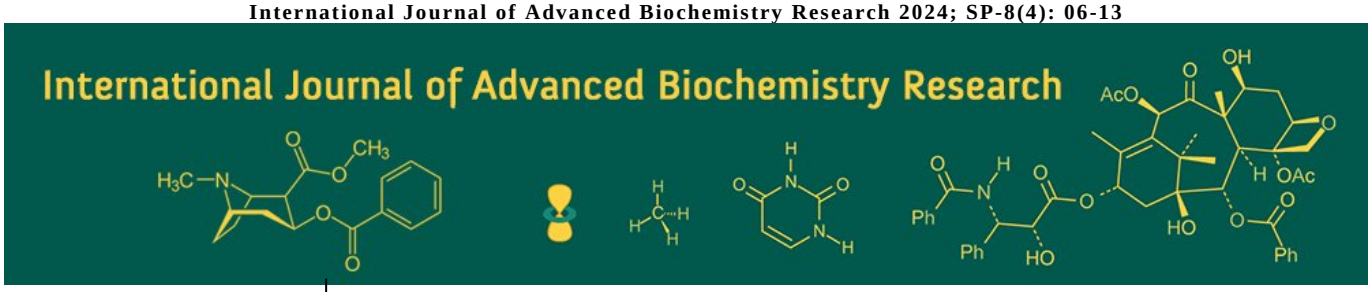

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Comparative analysis of different test for diagnosis of bovine tuberculosis

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Abstract
The aim of this study was comparative analysis of single intradermal comparative cervical tuberculin test (SICCT), immuno-chromatography analysis and polymerase chain reaction for diagnosis of tuberculosis. During the period from January-2019 to February-2020, a total of 800 animals (620 cattle & 180 buffalo) from 20 farms were screened by SICCT test in the different region of Gujarat state. For comparative study between SICCT, LFA and PCR, a total of 40 animals (11 SICCT positive, 09 SICCT negative, 10 Avian positive (PPD-A) and 10 SIT (PPD-B) positive animals) were selected. For PCR, additionally 10 lungs (02 deer and 08 cattle) samples suspected for bTB were also collected from the dead animals. A total of 106 samples comprised of 40 serums for LFA and 56 samples (16 milks, 40 nasal swabs and 10 lungs) were collected for PCR. Prevalence of bovine TB in the cattle and buffalo was 1.37% by SICCT test and 10.62% by SIT test was observed in the Gujarat state. The LFA contains the purified recombinant *M. bovis* specific antigens, all the 40 sera samples were tested by LFA and 08 (20.00%) animals were found to be positive for presence of antibody against *M. bovis*. Out of 40 SICCT selected animals, 23 (57.50%) were found positive in PCR using IS6110 (present in all MTC). Out of 23 MTC positive animals, species-wise, one *M. bovis* and eight *M. tuberculosis* or other members identified using targeting RD4 and RD9 regions. Hence, PCR could detect a greater number of animals as compared to SICCT test and LFA. Sensitivity of PCR was 100.00% and 85.71% when compared with SICCT test and SIT test, respectively. Sensitivity of LFA was 54.54% and 38.09% when compared with SICCT test and SIT test, respectively**.** PCR was found to be more sensitive and less specific than LFA when compared with tuberculin skin test (SICCT or SIT).

Keywords: Bovine tuberculosis, single intradermal comparative cervical tuberculin test (SICCT), single intradermal tuberculin (SIT) test**,** polymerase chain reaction, lateral flow assay (LFA) / immuno-chromatographic assay

1. Introduction
Bovine tuberculosis (bTB) is an important chronic bacterial zoonotic disease of cattle caused by *M. bovis*, a member of the *Mycobacterium tuberculosis* complex (MTC). Other members of MTC includes *M. tuberculosis* (MTB), *M. bovis*, *M. africanum*, *M. microti. M. caprae*, *M. pinnipedii*, *M. mungi*, *M. canettii*, *dassie bacillus* and *M. orygis* (Afzal *et al*., 2016) [1]. The organism with major concern worldwide distribution because of its high economic impact on livestock industry due to mortality, decreased production, carcass condemnation, and zoonotic potential (Thakur *et al*., 2016) [20]. bTB is considered a significant zoonotic disease of great socioeconomic and public health significance by the World Organization for Animal Health (OIE), with an impact on international trade of animals and animal products. Cattle infection with tuberculosis is typically chronic, and may remain sub-clinical for a long time. Cattle are regarded as *M. bovis* true hosts (Fitzgerald & Kaneene, 2013) [11].
Bovine TB is a disease that primarily activates a cell-mediated immunity (CMI) at an initial stage, and a change from Th1 to Th2 is associated with a reduction in CMI and the production of serological (humoral) responses. For antibody detection in bTB, cross-reactions to other bacteria (environmental mycobacteria) can make interpretation of the test complicated (Casal *et al*., 2014) [5]. The methods of polymerase chain reaction (PCR) offer great sensitivity and have been used successfully to diagnose bTB in different types of naturally infected components such as tissue, blood, milk and nasal exudates (Figueiredo *et al*., 2010) [9]. *M. bovis* mainly causes extra-pulmonary forms of TB and they excreted in exhaled air, sputum, faeces, urine, milk, vaginal and uterine discharges (Verma *et al*., 2014) [22].

M. bovis has one of the largest host ranges among the all MTC organisms and can readily spill over into humans or a variety of domestic and wild animals (Fitzgerald *et al*., 2013) [11]. Thus, this study was conducted to detection of bovine TB and detection of *M. bovis* in cows from a different organized dairy farm.

2. Materials and Methods

2.1 Study area and number of animals selected for study

The study was carried out on intradermal tuberculin screening, sero-detection, and molecular detection of *M. bovis* infection from large ruminants (cattle and buffalo) in various district of Gujarat state as described below in Flow diagramme. Total 800 animals (620 cattle and 180 buffalo) were screened by SICCT test and 40 sera samples were collected for antibodies detection by lateral flow assay. For PCR, nasal swab, milk sample, and dead animal tissue samples were also collected from bTB suspected animals (Table 1).

2.2 Single intradermal comparative cervical tuberculin (SICCT) test

Material was used in SICCT include tuberculin syringes, Vernier calliper**,** straight razor, paint marker, bovine purified protein derivative (B-PPD) and avian purified protein derivative (A-PPD)- both obtained from Prionics, Lelystad, Netherlands. All the animals were subjected to comparative cervical intradermal tuberculin test as per the guidelines from the World Organization for Animal Health (OIE). Briefly, the test was carried out in the middle third of the neck of each animal where avian tuberculin PPD-2500 (PPD-A) (Prionics) and bovine tuberculin PPD-3000 (PPD-B) (Prionics) were injected (i.e., 0.1 ml of PPD) in 10 cm bellow from the crest and 12.5 cm between PPDs sites of the neck Skin thicknesses were measured with calliper before and 72 h after PPD injections. After 72 h, the thickness of the same skin fold at both sites were measured and recorded. Bovine and avian positive reactors were obtained using the formula: {(B72 -B0)- (A72 -A0)}. B0 and A0 indicated skin thickness before injecting bovine and avian tuberculin, respectively, and B72 and A72 to the corresponding skin-fold thickness 72 h post-injection. Interpretation of the SICCT test was carried out in accordance with the OIE guideline (OIE Terrestrial Manual, 2009) [15].

2.3 Single intradermal tuberculin (SIT) test

The SIT test is similar to the SICCT test, but the difference between these two tests is that only bovine PPD (PPD-B) taken from the SICCT analysis, was used for comparative analysis between these two tests (SICCT & SIT), so the interpretation of the SIT test eventually changed as compare to SICCT test. Interpretation of the SIT test was carried out in accordance with the OIE guideline (OIE Terrestrial Manual, 2009) [15].

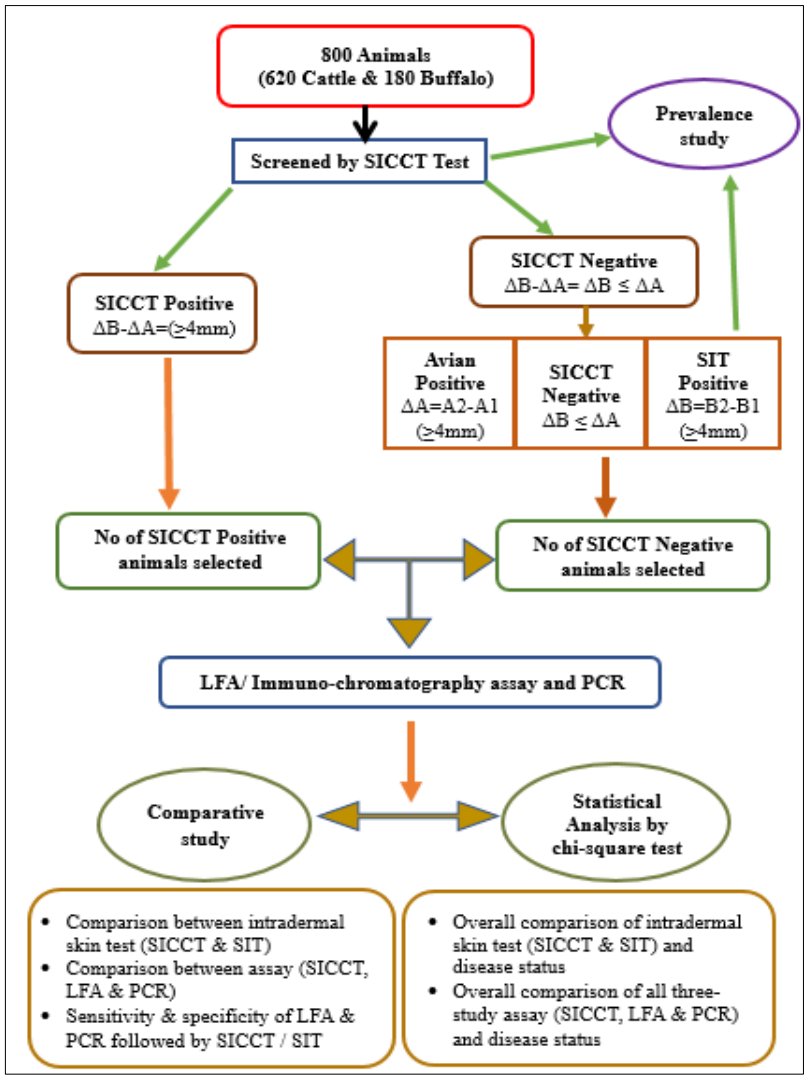

Fig 1: Flow diagram of present study

Table 1: Details of the samples selected for LFA and PCR

	LFA	PCR		
	Serum samples	Nasal swabs	Milk samples	Tissue samples
SICCT positive (11)	11	11	06	-
SICCT negative (09)	09	09	06	-
Avian positive (10)	10	10	03	-
SIT positive (10)	10	10	09	-
Dead animals (10)	-	-		10
Total	40	40	16	10

2.4 Lateral flow assay / immuno-chromatographic assay

The main aim of this study was the qualitative identification of antibodies to *M. tuberculosis* complex antibody in the serum using chromatographic solid phase immune assay. The quickVET Bovine TB Antibody Rapid test kit (Batch NO. Q008011906, Ubio Biotechnology Systems Pvt. Ltd, Kerala, India) was used for immune chromatographic / lateral flow systems. This test utilizes recombinant *M. bovis* antigens to capture the antibody established during infection. The captured IgGs were detected using colloidal gold conjugated detection antibody. Test serum was added to the sample well, with adequate amount of buffer migrate from the sample pad along the conjugate pad, where antibody present in the sample bind to the colloidal gold conjugate. The sample then continues to migrate across the membrane until it reaches the capture zone, where the antibody-antibody conjugate complex bind to the immobilised *M. bovis* antigen (on test line) producing an observable line on the membrane. Using the disposable dropper vertically, added 10µl (1 drop) of the sera into the centre of the sample deposition area (Sample well) on the lateral-flow cassette. Added 2 drop of the assay diluents into the sample well vertically drop wise. The antigens are immobilised on the test line of a sample pad and trap serum antibodies migrating through the test device by capillary forces. The reading was taken at 10 minutes after adding diluents buffer. The result was considered invalid after 15 minutes. All result, where control band does not appear were considered invalid. The interpretation was carryout as per the manufacturer's instruction as: Negative result- Only "C band" was present inside the result window. Positive result- When "T bands" were formed, in addition to the presence of C bands, the test indicates the presence of antibodies against *M. bovis*. Invalid result- If, after conducting the test, the C band was not visible inside the result window, the result was rendered invalid and the specimen was re-tested

2.5 Molecular identification of MTC

Molecular detection of the different spp. of mycobacteria was done by PCR. DNA extraction for PCR were carried out using the DNA extraction kit in both positive and negative samples from nasal swab, milk and dead animals tissue samples (Table 1).

2.5.1 Reference mycobacterial DNA

Bacillus Calmette-Guerin (BCG) vaccine I.P. (TUBERVAC) (Serum Institute of India Pvt. Ltd.) was used as reference strain for standardization of PCR assay. Using QIAamp DNA Mini Kit (Catalogue No. 51306, Qiagen, Germany), DNA was extracted from BCG vaccine.

2.5.2 DNA extraction from clinical samples

DNA extraction protocol QlAamp DNA Mini Kit (Catalogue No. 51306, Qiagen, Germany) for DNA extraction from clinical samples (milk, nasal swab and tissue sample) was carried out as per instruction manual available with kit.

2.5.3 Polymerase chain reaction (PCR)

Specific primer used targeting gene are given in the (Tables 2). List of thermal cycling primers and step & conditions for specific primer pairs in PCR as given in tables 3 and 4 respectively**.** Visualization of PCR products by agarose gel electrophoresis

Table 2: Primer role in PCR

Primers		Primer role in PCR
INS1 (F) & INS2 (R)		Primers were amplified insertion segments of the IS6110 (present in all MTC species) element amplification of a 245 bp fragment (Figueiredo *et al*., 2010) [9]
RD4	F & R	RD4 region present in *M. tuberculosis*, *M. caprae*, *M. microti*, *M. africanum*, *M. pinnipedi*, and *M. canetti* they amplify 172bp fragment using RD4-F & RD4-R primers
	F & I	RD4 region absent in *M. bovis* and *M. bovis* BCG, they amplify 268bp fragment using RD4-F & RD4-I primers
RD9	F & R	RD9 region absent in *M. bovis* and *M. bovis* BCG, they amplify 206bp fragment using RD9-F & RD9-R primers
	F & I	RD9 region present in *M. tuberculosis* and *M. canetti* they amplify 333bp fragment using RD9-F & RD9-I primers

(F) = Forward primer; (R) = Reverse primer; (I) = Internal prime

Table 3: Primer details for detection of MTC by PCR

Primer Name	Primer Sequence (5'-3')		Product Size (bp)	Reference
INS1	F	CGTGAGGGCATCGAGGTGGC	245bp for *M. tuberculosis* complex	(Filia *et al*., 2016) [10]
INS2	R	GCGTAGGCGTCGGTGACAAA		
RD4	F	ATGTGCGAGCTGAGCGATG	172bp for *M. tuberculosis*, *M. caprae*, *M. microti*, *M. africanum*, *M. pinnipedi*, and *M. canetti*	(Warren *et al*., 2006) [23]
	R	TGTACTATGCTGACCCATGCG		
	F	ATGTGCGAGCTGAGCGATG	268bp for *M. bovis* and *M. bovis* BCG	
	I	AAAGGAGCACCATCGTCCAC		
RD9	F	GTGTAGGTCAGCCCCATCC	333bp for *M. tuberculosis*, and *M. caneeti*	(Das *et al*., 2007) [6]
	I	GCTACCCTCGACCAAGTGTT		
	F	GTGTAGGTCAGCCCCATCC	206bp for *M. bovis* and *M. bovis* BCG	
	R	CAATGTTTGTTGCGCTGC		

(F) = Forward primer; (R) = Reverse primer; (I) = Internal primer

Table 4: Steps and conditions of thermal cycling for different primers in PCR

Primers	Cycling conditions				
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
INS1 & INS2 F & R	94 °C 5 min	94 °C 1min	63 °C 1 min	72 °C 1 min	72 °C 7 min
	1	Repeated for 30 cycles			1
RD4 F, R and I	95 °C 15 min	94 °C 1min	62 °C 1 min	72 °C 1 min	72 °C 10 min
	1	Repeated for 45 cycles			1
RD9 F, R and I	95 °C 5 min	95 °C 1 min	54 °C 1 min	72 °C 1 min	72 °C 10 min
	1	Repeated for 30 cycles			1

(F) = Forward primer; (R) = Reverse primer; (I) = Internal primer

3. Results and Discussion

3.1 Tuberculin skin testing

Out of 800 animals, 1.37% (11/800) were found positive, 32.25% (258/800) were inconclusive and 66.37% (531/800) were negative by SICCT test. Overall prevalence of bTB in SICCT test was 1.37%. Out of 800 animals, 10.62% (85/800) were found positive, 37.37% (299/800) were inconclusive and 52.00% (416/800) were negative by SIT test. Overall prevalence of bTB in SIT test was 10.62% (Tables 5).

Table 5: Overall information about SICCT and SIT test

		SICCT test results (%)		
		Positive ΔB-ΔA=(≥4mm)	Inconclusive ΔB-ΔA=(1-4mm)	Negative ΔB-ΔA=(<1mm)
Cattle=620 Buffalo=180	Total	11 (1.37%)	258 (32.25%)	531 (66.37%)
		SIT (PPD-B) test results (%)		
		Positive ΔB=B2-B1=(≥4mm)	Inconclusive ΔB=B2-B1=(2-4mm)	Negative ΔB=B2-B1=(<2mm)
	Total	85 (10.62%)	299 (37.37%)	416 (52.00%)

3.2 Immuno chromatographic assay/ lateral flow assay

Lateral flow assay applied on 40 SICCT selected animals (Table 1). The quickVET Bovine TB AB Rapid Test Kit results (Table 6) (Fig. 2). Out of total (N=40) animals, 8 (20.00%) were found positive and 32 (80.00%) were negative by LFA. Among these 8 LFA positive animals, 07 were HF cross cattle and 01 were kankrej cattle. All the SICCT test negative animals and Avian positive animals were negative by LFA. While five SICCT test positive animals, were negative in LFA. However, two animals negative by SICCT test and positive by SIT test were also found positive by LFA assay (Table 6).

Table 6: Comparison of LFA result with SICCT selected animals

SICCT/ LFA	SICCT Positive (N=11)	SICCT Negative (N=29)			Total (N=40)
		SICCT Negative(09)	Avian Positive(10)	SIT Positive(10)	
LFA Positive	6	-	-	2	8 (20.00%)
LFA Negative	5	9	10	8	32 (80.00%)

Fig 2: LFA positive sera samples

In present study, total 20% (8/40) animals were found positive by LFA assay. These results were comparatively higher than those observed by Ameni *et al*. (2010) [2]; 10.40% (73/701) and lower than El-Mahrouk *et al*. (2010) [8]; (70.00%) (7/10) and Bermúdez *et al*. (2012) [4]; (45.79%) (49/107) with different LFA test devices. Very recently successfully use of immune-chromatographic detection of anti-*M. tuberculosis* complex antibodies in the sera of infected wild animals have been reported in deer, elephants and camels (Koo *et al*., 2005; Lyashchenko *et al*., 2006; Wernery *et al*., 2007) [12, 13, 24]. Bermúdez *et al*. (2012) [4] concluded the high false positive rate for both tissue PCR and LFA observed in study, the LFA is not a useful test, even in combination with tissue PCR, and it would be better to use additional diagnostic methods, such as bacteriological culture or tuberculin skin test.

3.3 Molecular Detection of MTC

PCR assay performed on 40 SICCT selected animals and 10 bTB suspected dead animals (2 deer and 8 cattle). Using DNA extraction kit, (QlAamp DNA Mini kit) DNA was extracted from 40 nasal swab; 16 milk samples and 10 suspected lung samples (Table 1). All the samples were first screened for PCR by using specific primer (IS6110) for the detection of *Mycobacterium tuberculosis* complex and then identification and differentiation of *M. tuberculosis* and *M. bovis* by targeting RD4 and RD9 regions. In present study, all the 66 DNA were excreted using QlAamp DNA Mini kit for accordance with de Souza Figueiredo *et al*. (2012) [9] who also used similar technique for DNA extraction from nasal swab, milk samples and tissue samples

3.3.1 Detection of MTC Targeting Insertion Sequence (IS6110) by PCR

In present study, all the 66 extracted DNA of mycobacteria were subjected to PCR for detection of insertion segment IS6310 (INS1 and INS2 primers) present in all MTC. Out of 66 samples, 24 samples were found positive by PCR amplified a 245bp fragments specific for all MTC members (Fig. 3). Out of 40 SICCT test selected animals, 23 nasal swabs were found positive for MTC by IS6110. Out of 10 lung samples, one (10.00%) lung sample from deer also found positive for MTC by IS6110. Amongst the 23 MTC positive samples, 11 (100%) were SICCT positive, 7 (70.00%) were SIT positive and 5 (50.00%) were Avian positive (Table 7). These finding are accordance with (Thakur *et al*., 2016; Filia *et al*., 2016) [20, 10] who also used same primer pair and found that positive samples contain insertion segment IS6110 present.

Table 7: Overall PCR result by IS6110 PCR

PCR/ SICCT test	PCR	
	PCR Positive	PCR Negative
SICCT Positive (N=11)	11 (100.00%)	-
SICCT Negative (n=09)	-	9 (100%)
SIT Positive (n=10)	7 (70.00%)	3 (30.00%)
Avian positive (n=10)	5 (50.00%)	5 (50.00%)
Total (40)	23	17
Tissue sample (n=10)	1 (10.00%)	9 (90.00%)
Grand total (50)	24	26

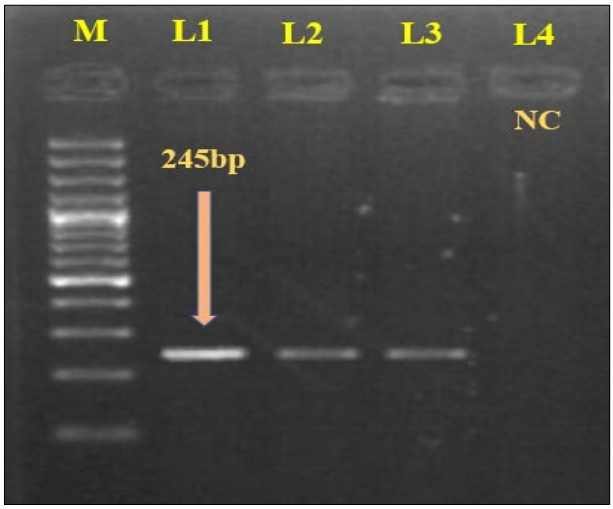

Fig 3: Agarose gel showing amplified products by IS6110 primer, (M: 100 bp plus molecular weight marker, L1: Positive control for M. bovis BCG, L2 & L3: Samples positive for M. tuberculosis complex, L4: Negative control)

3.3.2 Detection of RD4 Region by PCR

Warren *et al*., (2006) [23] used three-primers system, RD forward (RD4F), RD reverse (RD4R) and RD internal (RD4I). RD4 region present in *M. tuberculosis*, *M. caprae*, *M. microti*, *M. africanum*, *M. pinnipedi* and *M. canetti* they amplified a 172bp fragment by RD4-F and RD4-R primers. RD4 region absent in *M. bovis* and *M. bovis* BCG, they amplified a 268bp fragment by RD4-F and RD4-I primers. In the present study, samples identified as MTC by IS6110 primers were subjected at species level identification by targeting RD4 primers. Out of 24 MTC positive samples, one nasal swab amplified a 268bp fragment specific for *M. bovis* and one tissue (deer-lung) sample amplified a fragment of approximately 172bp specific for *M. tuberculosis* or other members (Fig.4 and Table 8)

3.3.3 Detection of RD9 Region by PCR

In the present study an attempt was made using MTC specific primers, for identification of MTC at species level targeting RD4 and RD9 by multiplex PCR. Out of 24 MTC positive samples, seven nasal swabs amplified a 333bp fragment specific for *M. tuberculosis* or *M. canetti* and one nasal swab from {A.ID-(C-11)} amplified a 206bp fragment specific for *M. bovis*. One tissue (deer-lung) sample amplified a 333bp fragment specific for *M. tuberculosis* or *M. canetti* (Fig 4 and Table 8). Out of 24 MTC positive sample by IS6110, one sample identified as *M. bovis* and eight samples identified as *M. tuberculosis* or other members. During the study, 57.50% (23/40) nasal swab were found positive in PCR by IS6110.

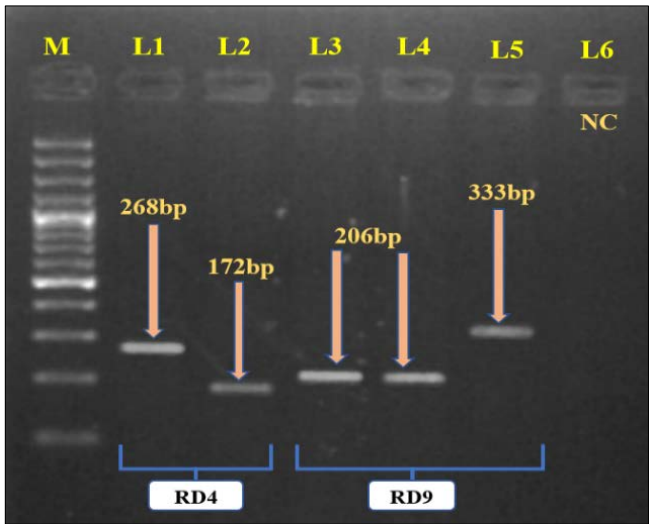

Fig 4: Agarose gel showing amplified products for RD4 region and RD9 region (M: 100 bp plus molecular weight marker, RD4:- L1: Positive control for *M. bovis* BCG, L2: Sample positive for *M. tuberculosis* or others, RD9:- L3: Positive control for *M. bovis* BCG, L4: Samples positive for *M. bovis*, L5: Samples positive for *M. tuberculosis* or *M. canetti*, L6: Negative control)

Table 8: Showing PCR result for various samples

	IS6110	RD4 primers		RD9 primers	
	F & R (245bp)	F & R (172bp)	F & I (268bp)	F & R (206bp)	F & I (333bp)
Referance strain (*M. bovis* BCG)	+	-	+	+	-
Nasal swab (40)	(23) +	(01) +	-	(01) +	(07) +
Milk (40)	-	-	-	-	-
Tissue (10)	(01) +	(01) +	-	-	(01) +

{(+) = Positive and (-) = Negative}

During the study, 57.50% (23/40) nasal swab were found positive in PCR by IS6110. These results were comparatively higher than those observed by Figueiredo *et al*. (2010) [9]; 5.90% (2/34) and Senthil *et al*. (2014) [19]; 9.80% (9/92) using same primers. Romero *et al*. (1999) [17] verified that nasal mucus samples were better for *in vivo* PCR based detection of microorganism than other fluid such as milk or blood. Out of 16 milk sample, none of the samples were found to be positive by PCR. Similarly, in examining milk samples of cows from infected herds, earlier studies have not detected any positive animals (Perez *et al*.,

2002) [16]. The use of the PCR technique in spiked milk samples does not promise that it would perform equally effectively in the examination of naturally infected samples. One could expect that in the last, the collaboration between the bacilli and the milk matrix could be more complex, and even that bacilli in milk might have already been killed by mammary macrophages (Zumarraga *et al*., 2005) [26] and the DNA may be degraded. In present study, 10% (1/10) tissue (lung) samples were found positive in PCR using IS6110. Our results are comparatively lower than those observed by Figueiredo *et al*. (2010) [9]; 88.23% (15/17) in culture positive isolates and Mehdikhan *et al*. (2012) [14]; 57.70% (23/40) in cattle lymph-node. It should be declared that the PCR was sensitive enough to detect *M. bovis* in a higher proportion (59.00%) of those samples that failed to grow in culture, as also reported by Zanini *et al*. (2001) [25] and Araújo *et al*. (2005) [3].

3.4 Comparative Study

3.4.1 Comparison between SICCT, LFA and PCR

Out of 40 SICCT test selected animals, 08 (20.00%) animals were found positive for LFA which includes 06 (54.50%) SICCT positive and 02 (20.00%) SIT test positive and 23 (57.50%) animals were found positive for PCR which includes 11 (100.00%) SICCT test positive, 07 (70.00%) SIT test positive and 05 (50.00%) Avian positive (Table 9 and Fig.5).

Table 9: SICCT test selected animals compare with LFA and PCR

LFA/PCR/SICCT test		SICCT test				Total (N=40)
		SICCT Positive (N=11)	SICCT Negative (N=29)			
			SICCT Negative (n=09)	SIT Positive (n=10)	Avian Positive (n=10)	
LFA Results	Positive	6 (54.50%)	-	2 (20.00%)	-	8 (20.00%)
	Negative	5 (45.50%)	9 (100.00%)	8 (80.00%)	10 (100.00%)	32 (80.00%)
PCR Result	Positive	11(100%)	-	7 (70.00%)	5 (50.00%)	23 (57.50%)
	Negative	-	9 (100.00%)	3 (30.00%)	5 (50.00%)	17 (42.50%)

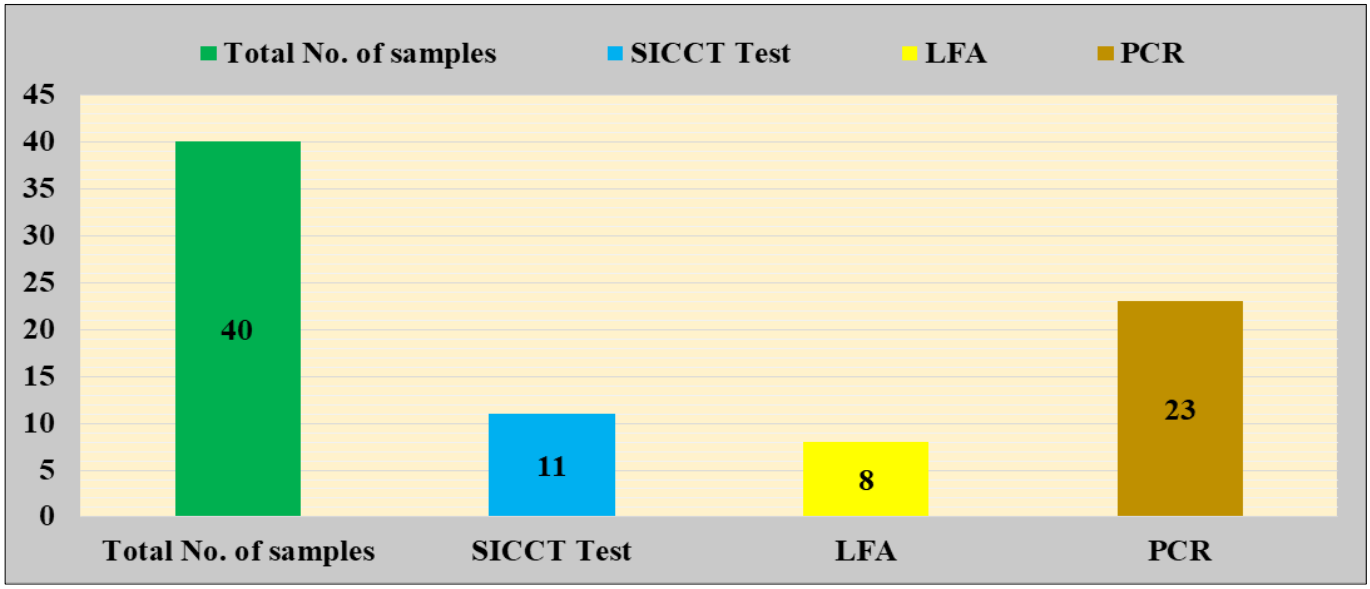

Fig 5: Comparison of SICCT test, LFA and PCR for detection of bovine TB

3.4.2 Sensitivity and Specificity of LFA and PCR followed by SICCT / SIT test

The statistical formulation given by Samad *et al*. (1994) [15] was used as described below table 10 to compare the sensitivity, specificity and overall agreement between the study test. Sensitivity and specificity of LFA and PCR were assessed by comparing with SICCT or SIT test positive results. Out of 40 SICCT test selected animals, 11 were found positive for SICCT test, 21 were found positive for SIT test, 8 were found positive for LFA and 23 were found positive for PCR.

Table 10: Methods used for identified the sensitivity and specificity

	Standard test (SICCT or SIT)			
Test to be compared (LFA or PCR)		Positive	Negative	Total
	Positive	a	b	a+b
	Negative	c	d	c+d
	Total	a+c	b+d	a+b+c+d

The notations in the above table are defined as: a+b+c+d= Total number of samples (N), Definitions and formulae of the indices used for comparing SICCT (or SIT) test and LFA (or PCR) are described below, Sensitivity: It is the capacity of the test to detect diseased animals, when compared with the standard test (a/a+c x 100), Specificity: It is the capacity of the test to detect non-diseased animals, when compared with standard test (d/b+d x 100), Overall agreement: Is the proportional similarity of the results of both the tests (a+d/N x 100).

3.4.2.1 Sensitivity and specificity of LFA and PCR followed by SICCT test

The sensitivity and specificity of LFA was found 54.54% and 93.10% respectively with reference to SICCT test, whereas the overall agreement between each test was 82.50%.

The sensitivity and specificity of PCR was found 100.00% and 58.62% respectively with reference to SICCT test,

whereas the overall agreement between each test was 70.00%.

3.4.2.2 Sensitivity and specificity of LFA and PCR followed by SIT test

The sensitivity and specificity of LFA was found 38.09% and 100.00% respectively with reference to SIT test, whereas the overall agreement between each test was 67.50%.

The sensitivity and specificity of PCR was found 85.71% and 73.68% respectively with reference to SIT test, whereas the overall agreement between each test was 80.00%.

3.4.3 Statistical analysis

Comparison of intradermal skin test (SICCT & SIT) and all study assay (SICCT, LFA and PCR) with disease status carried out using chi-square test. The level of chi square test was 5%. The chi square test was carried as per Thrusfield *et al.* (2005) [21].

3.4.3.1 Comparison of intradermal skin test (SICCT and SIT)

To compare the tuberculin skin test proportion of diseased animals, Chi-square test was carried out by analyzing the 2*2 contingency table (disease prevalence row was not considered while applying Chi-square test). The level of chi square test was 5%. The chi square value obtained between skin test and disease status was 60.68 which indicated that difference in skin test and disease status was significant. (χ2tab) < (χ2cal) indicated alternate hypothesis is accepted, hence there is significance difference between both intradermal skin tests and disease status (Table 11).

Table 11: Comparison of skin test by Chi-square test

Intradermal Skin Test/ Disease Status	SICCT test	SIT test
Positive	11	85
Negative	789	715
Disease Prevalence	1.37%	10.62%

(Degree of Freedom:1, Table value of Chi-Square (χ2tab):3.84)

3.4.3.2 Comparison of Study Assay (SICCCT, LFA and PCR)

To compare the study assay (SICCT, LFA and PCR) proportion of diseased animals, Chi-square tet was carried out by analyzing the 2*3 contingency table (disease prevalence row was not considered while applying Chi-square test). The level of chi square test was 5%.

The chi square value obtained between study assay and disease status was 13.83 which indicated that difference between study assay was significant. (χ2tab) < (χ2cal) indicated alternate hypothesis is accepted hence there is significance difference between study assay and and disease status (Table 12).

Table 12: Comparison of Study Assay by Chi-Square Test

Study assay/Disease Status	SICCT test	LFA	PCR
Positive	11	8	23
Negative	29	32	17
Disease Prevalence	27.50%	20.00%	57.50%

(Degree of Freedom:2, Table value of Chi-Square (χ2tab):5.99)

4. Conclusion

Sensitivity and specificity of LFA in comparison to SICCT test was 54.54% and 93.10% respectively whereas in PCR was 100.00% and 58.62% respectively in comparison of both tests with SICCT test. Overall agreement between SICCT test with LFA and PCR were 82.50% and 70.00% respectively. Sensitivity and specificity of LFA was 38.09% and 100.00% respectively whereas in PCR was 85.71% and 73.68% respectively in comparison of both tests with SIT test. Overall agreement between SIT test with LFA and PCR found to be 67.50% and 80.00% respectively. Intradermal skin test (SICCT & SIT) and all study assay (SICCT, LFA and PCR) compared with disease status carried out using statistical analysis (chi-square test).

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