www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(4): 883-887

www.thepharmajournal.com
Received: 06-01-2022
Accepted: 11-02-2022

Kumar E
Ph.D., Scholar and Assistant Professor, Department of veterinary Public Health & Epidemiology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Krishnaiah N
Retired Professor, Department of Veterinary Public Health& Epidemiology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Shashi Kumar M
Associate Dean, College of Dairy Technology, Kamareddy, Telangana, India

Kalyani P
Assistant Professor, Department of Veterinary Biotechnology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Gopala Reddy A
Controller of Examinations, PVNRTVU, Hyderabad, Telangana, India

Chatalod LR
Senior Scientist, National Research Center on Meat, Chengicherla, Hyderabad, Telangana, India

Dr. Sonali M
Assistant Professor and Head, Department of Veterinary Microbiology, College of Veterinary Science, PVNRTVU Koratla, Telangana, India

Corresponding Author:
Dr. Sonali M
Assistant Professor and Head, Department of Veterinary Microbiology, College of Veterinary Science, PVNRTVU Koratla, Telangana, India

Evaluation of acid fast staining and fluorescence staining methods for detection of *Mycobacterium tuberculosis* complex members from tissue samples collected from bovines from slaughterhouses

Kumar E, Krishnaiah N, Shashi Kumar M, Kalyani P, Gopala Reddy A, Chatalod LR and Dr. Sonali M

Abstract
Tuberculosis in bovines, mainly caused by *Mycobacterium bovis*, an important member of *Mycobacterium tuberculosis* complex, which are having implications for public health and huge economic impact on farming community. In the present study, 94 post mortem samples were collected from 71 bovines, which includes 23 tuberculosis suspected lung tissues and 71 prescapular/ mediastinal/ bronchial/ mesenteric lymph node samples from three slaughterhouses in vicinity of Hyderabad, Telanagana, India the prevalence of tuberculosis in bovines. The prevalence of tuberculosis in slaughtered animals was 73.24% (52/71) by acid fast staining, 85.92% (61/71) by auramine staining and 18.31% (13/71) by PCR. The relative diagnostic sensitivity and specificity of acid fast staining were 100% and 32.76% and for auramine staining were 100% and 17.24% respectively against PCR technique. Among all methods employed, the ZN staining method was found to be more sensitive than auramine O staining and PCR assay. However, only MTBC PCR assay only we could find the presence of MTBC members as microscopic methods could not differentiate the MTBC members from other species of Mycobacteria, which were not amplified due to absence of specific primers in PCR assay. In conclusion, PCR is more reliable diagnostic tool for diagnosis of bovine tuberculosis.

Keywords: Bovine tuberculosis, evaluation of diagnostic accuracy, bovine TB in slaughterhouses

Introduction
Tuberculosis in bovines, mainly caused by *Mycobacterium bovis*, an important member of *Mycobacterium tuberculosis* complex, which are having implications for public health and huge economic impact on farming community (Michel *et al*., 2010) [12]. Worldwide, bovine tuberculosis is recognized as one of the seven most overlooked endemic zoonoses, exhibiting a complex epidemiological pattern with the highest prevalence rates in cattle, found in African countries, part of Asia and Americas (OIE, 2009) [17]. In 1882, Robert Koch discovered *M. tuberculosis* as a causative agent of tuberculosis. Tuberculosis in bovines mainly caused by *M. bovis* and is having zoonotic importance (Tariq *et al*., 2017) [20]. Tuberculosis in bovines is characterized by pulmonary form (Lungs affected) and extra pulmonary form (Other parts of the day) and is a highly contagious disease spread through the sneezing, coughing and talking between the individuals (Beresford and Sadoff 2010). Tuberculosis in bovines is economically important disease of animals as it is drastically affecting the growth rate and reproductive efficiency of the animals (Tariq *et al*., 2017) [20]. The diagnosis of tuberculosis in bovines mainly based on lesion observed during postmortem and acid fast staining (Mittel *et al*., 2014) [13]. Culture of the organism is considered as gold standard test for diagnosis of tuberculosis in bovines (OIE 2012) [16]. Many other diagnostic tools like tuberculin skin test (TST) and interferon gamma release assay (IGRA) are developed to diagnose the tuberculosis in bovines by detecting the cell mediated response (CMI). But all these tests are consuming and requires specialized laboratory settings (Alvarez *et al*., 2012) [2]. Therefore molecular diagnostic tests, such as PCR offers a rapid and sensitive, alternative to available methods for diagnosis of tuberculosis in bovines (Vincent *et al*., 2009) [23]. The present study designed to estimate the sensitivity and specificity of various diagnostic tests currently used in diagnosis of tuberculosis in bovines.

Materials and Methods

Study population: 94 post mortem samples were collected from 71 bovines which includes 23 tuberculosis suspected lung tissues (Fig 1) and 71 pre scapular/ mediastinal/ bronchial/ mesenteric lymph node samples from three slaughter houses in vicinity of Hyderabad, Telanagana, India. Aseptic precautions were taken while collecting the samples and all the samples were packed and transported in a triple packaging system consists inner airtight primary screw capped and sealed container, secondary water tight container and tertiary robust outer container. The processing of all the samples was done according to OIE standard procedure (OIE 2009) [17] in BSL-II. Decontamination of all the samples was done by using 5ml of 1% CPC and 2% NaCl as mentioned in the RNTPC 2010.

Diagnostic tests

Acid fast staining: Smears were prepared from decontaminated samples, after heat fixation, smears were placed in a sequential order on staining rack with smear side up. Smears were flooded with concentrated carbol fuschin and steamed and then allowed for 5-6 min for staining. Washed with distilled water. Smears were de colorized by using acid alcohol (20% Sulphuric acid) for 2 min. washed with distilled water. Smears were counterstained with Loeffler methylene blue for 1 min and washed with distilled water. Smears were blotted carefully and allowed to air dry and examined by subjecting the oil immersion objective (RNTCP manual, 2010) [19].

Fluorescence microscopy staining: by Phenol- Auramine stain solution as per the recommended protocol (RNTCP manual, 2010) [19]. Smears were flooded with phenol-auramine then allowed to stand for 7 to 10 minutes. Stain was removed by washing with running tap water. Decolorization was done by using acid alcohol (20% Sulphuric acid) for two minutes. Washed with running tap water. Counter staining was done with 0.1% potassium permanganate for 45 seconds. Washed with running tap water. Smears were blotted carefully and allowed to air dry and examined at 250X or 450X magnification within 24 hours of staining because of stain fading (RNTCP manual, 2010) [19] using fluorescence microscope.

Polymerase Chain Reaction procedure

DNA isolation from tissue samples: DNA was extracted from all the tissue samples by using DNeasy Blood and Tissue kit (Qiagen, USA) as per the manufacturer's instructions. Identification of MTBC members was done using published primers designated to amplify an insertion sequence *IS6110* present in all members of MTBC (Verma *et al.*, 2011) [22]. The forward primer used was 5'-CCTGCGAGCGTAGGCGT-3' and reverse primer used was 5'- TCAGCCGCGTCCACGCC-3'. The primers were custom-synthesized and supplied by Bioserve Biotechnologies (India) Pvt. ltd, Hyderabad. Standard *Mycobacterium* strain for *M. tuberculosis* was used as positive control in this study and was provided by Microbial Type Collection Centre (MTCC), Chandigarh, India. The standard *M. tuberculosis* strain was H_{37}Ra maintained by Microbial Type Collection Centre (MTCC), Chandigarh. PCR amplification was performed as per the procedure recommended by Verma *et al.*, (2011) [22] with some modifications by using readymade 'Emerald Amp GT PCR Master mix' in thin-walled 0.2 ml PCR tubes containing approximately 12.5 µl of PCR Master Mix, 10 µl of bacterial genomic DNA (approximately 100ng), 0.5 µl of IS6110 Forward (10 pmol/µl), 0.5 µl of IS6110 Reverse (10 pmol/µl) and the reaction volume was made up to 25 µl with Nuclease free water (NFW). The PCR amplification was achieved in a Gradient Thermal cycler (Prime Duo, Himedia, India) and programmed of initial denaturation at 95° C for 10min, followed by 34 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min and extension 72 °C for 1 min 10 sec, followed by a final extension at 70°C for 10 min. A non-template control was run in all the PCR experiments to rule out the possibility of contamination in the samples. The PCR amplified products were resolved in 1.5% agarose gel electrophoresis with ethidium bromide and visualized under UV light in a gel documentation system (Mini Transilluminator, Bio-Rad, USA).

Statistical analysis: The diagnostic sensitivity (DSn) and specificity (DSp) of the tests were determined by a two-sided contingency table (Thrushfield 2007) [21] using open-source software tool MEDCALC (https://www.medcalc.org/calc/diagnostictest.php) as described by Balamurugan *el al.* (2021) [3]. Further, Cohen's Kappa Test [Cohen 1960] [7] was done to determine an agreement between the diagnostic tests used in the present study and Kappa value was estimated at a 95% confidence interval (CI) using the online tool VASSARSTATS (https://vassarstats.net/kappa.html) as described by Balamurugan *et al.*, 2021 [3].

Results and Discussion: In the present study, acid fast staining was employed to study the prevalence of tuberculosis in samples collected from slaughterhouses. The prevalence of tuberculosis in slaughtered animals was 73.24% (52/71) by acid fast staining (Fig 2). Proano-Perez *et al*. (2011) reported that, the prevalence of tuberculosis in postmortem samples as 33.33% (11/33) and acid-fast bacilli were identified in one third of the suspicious cattle. Yahyaoui- Azami *et al.* (2017) [24] have screened the 327 animals at slaughterhouses and reported that 95% (215/225) culture isolates were acid-fast organisms. The present study results were differed with some previous studies and it could be attributed to geographical variations, type of management and husbandry practices employed, genetic variations in the disease resistance and levels of natural immunity among the bovine population and sensitivity and specificity of diagnostic methods employed (Parvez and Faruque 2015). There are very few studies using the acid-fast staining method as an ancillary in parallel test for diagnosis of tuberculosis in animals. By this method, we cannot differentiate the acid fastness of MTBC members and members of nontuberculous mycobacteria or other environmental bacteria. Auramine-Phenol is a fluorochrome stain used to visualize acid-fast structures of microorganisms especially *Mycobacterium tuberculosis* complex members. Prevalence of tuberculosis in bovines slaughtered was 85.92% (61/71) by fluorescence staining (Fig 3). In present study, auramine staining is comparatively more sensitive and reduced the reading time than the ZN staining method. Hendry *et al.* (2009) [9] screened the 71 sputum samples and reported 46.48% (33/71) of samples were positive for fluorescent *Mycobacteria*. They also reported the sensitivity

and specificity of the fluorescence smear microscopy as 82.1% and 96.9%, respectively for the entire samples (n = 71) when compared to culture. Cattamanchi *et al*. (2009) [6] observed that fluorescence microscopy increased the sensitivity of sputum smear microscopy. After evaluation of 426 patients, (82% HIV-infected) by fluorescence microscopy, they identified 11% more smear-positive patients than compound microscopy (49% vs. 38%, *P*< 0.001). Hendry *et al*. (2009) [9] screened the 71 sputum samples and reported 46.48% (33/71) of samples were positive for fluorescent *Mycobacteria*. They also reported the sensitivity and specificity of the fluorescence smear microscopy as 82.1% and 96.9%, respectively for the entire samples (n = 71) when compared to culture. In the present study, the sensitivity and specificity is little more with fluorescence staining than acid fast staining. In the present study, PCR assay was employed for amplification of segments of the *IS6110* element, primarily targeting the 445bp fragments. Genomic DNA was extracted from 71 post mortem tissue samples from three slaughterhouses and subjected for amplification by PCR by using specific primers (INS1/INS2). The amplification of insertion sequence *IS6110* was good at annealing temperature of 54 ^{0}C to identify the MTBC using *M. tuberculosis* template DNA as positive control. The same was used for screening of all the tissue samples. PCR amplification generated specific products of 445bp (Fig. 4). The prevalence of tuberculosis in bovines observed was 18.31% by PCR.

Fig 1: Postmortem lung tissue showing tuberculosis lesions collected from Slaughterhosues.

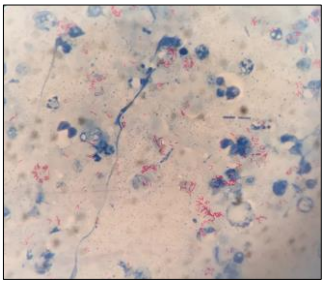

Fig 2: ZN stained smear showing Acid-fast bacilli (pink slender rods) in Lung tissue impression smears (1000X).

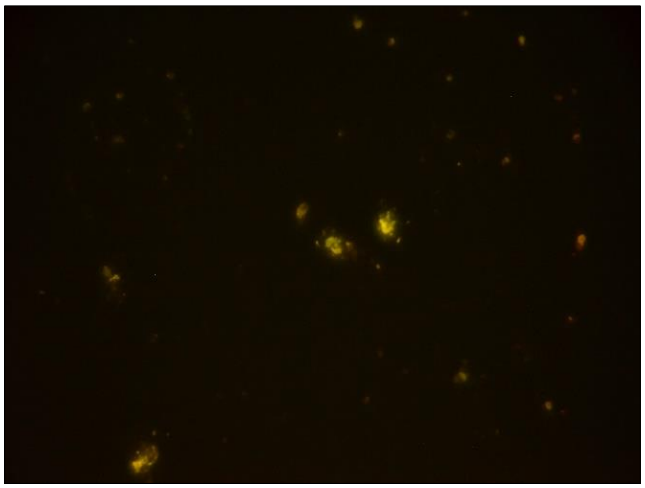

Fig 3: Fluorescent bacilli (yellowish bright bacilli) present in the lung tissue impression smear (400X)

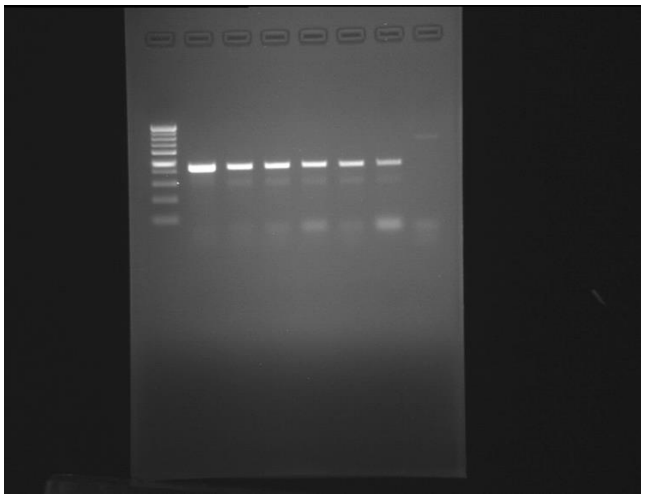

Fig 4: Agarose gel electrophoresis (1.5%) image showing amplification of IS6110 gene

Lane M- DNA 100bp Marker; **L1**-Postive control (*M. tuberculosis* H37Ra);
L2-L6- PSLG aspirates and Lung tissue sample; **L7**- Negative control

Comparison of diagnostic tests used in the present study

In the present study, a total of 94 Post mortem tissue samples were collected from 71 animals and diagnosed by using acid fast staining, auramine staining and MTBC PCR by targeting *IS6110* sequence (Fig 5). The diagnostic sensitivity and specificity of acid fast staining (Table 1) and auramine staining (Table 2) was estimated against the known positive and known negative samples obtained by PCR assay as gold standard test.

Table 1: Comparison of results of acid fast staining and PCR assays for diagnosis of bovine tuberculosis

AFS	PCR			Sensitivity	Specificity	Accuracy	PPV	NPV
	Positive	Negative	Total					
Positive	13	39	52	100% (75.29%-100.0%)	32.76% (21.01-46.34%)	45.07% (33.23-57.34%)	25.00%(21.78-28.52)	100%
Negative	0	19	19					
Total	13	58	71					

Table 2: Comparison of results of auramine staining and PCR assays for diagnosis of bovine tuberculosis

FS	PCR			Sensitivity	Specificity	Accuracy	PPV	NPV
	Positive	Negative	Total					
Positive	13	48	61	100% (75.29%-100.0%)	17.24%(8.59-29.43)	32.39% (21.76%-44.55%)	21.31% (19.41-23.53%)	100%
Negative	0	10	10					
Total	13	58	71					

The acid fast staining method has given a relative diagnostic sensitivity of 100 per cent (95% confidence interval (CI):75.29 to 100%) and specificity of 32.76 per cent (95% CI: 21.01% 46.34%) with an accuracy of 45.07 per cent (95% CI: 33.23% to 57.34%), PPV is 25.00per cent (21.78-28.52) and NPV is 100 per cent and slight agreement of Cohen's kappa value 0.151 ± 0.0912SE (95% CI: 0.0 to 0.33) against PCR assay while testing with results of known PCR positive and negative animals (n=71) for bovine tuberculosis.

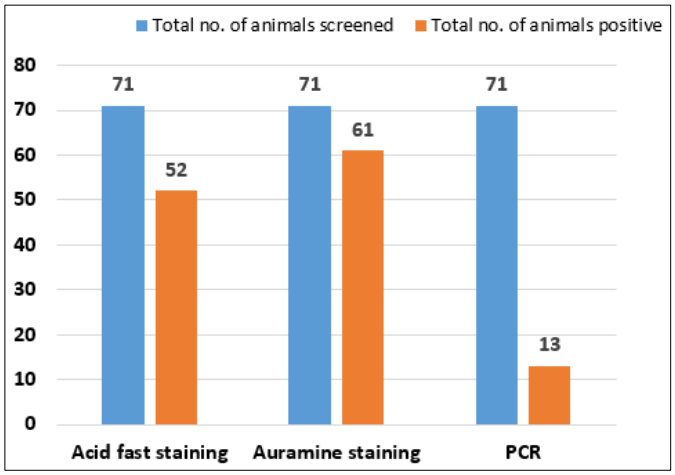

Fig 5: Prevalence of tuberculosis in bovines in slaughterhouses of Telangana state using various diagnostic tests in Telangana state

In the present study, the findings are in accordance with that of Basit *et al*. (2014) [4] who reported the prevalence of TB by microscopy was 7.5% (15/200) followed by the PCR 6.5% (13/200). Although PCR was a more sensitive technique than ZN staining, the results obtained in the present study showed a higher number of positive samples with ZN staining as compared to PCR. These variations could be attributed to the presence of other Mycobacterial species in the samples and in our study only MTBC members were identified by PCR. Similarly Khan *et al*. (2010) [10] reported that ZN staining showed the AFB presence in 17.4% intestinal and 16.4% in lymph node tissue of buffalo, while PCR confirmed 12.8% in intestinal and 12.4% in lymph node sample positive for Paratuberculosis, which show higher number of positive results by microscopy than PCR. Moreover staining microscopy could not permit differentiation between species of MTB complex, only PCR methods can differentiate between them. In contrary, Ndugga *et al.* (2004) [15] compared the sensitivity and specificity of PCR with ZN staining and culturing techniques and concluded that PCR assay was considered as high sensitive technique and can be used as an alternative to ZN staining for diagnosis of TB. In another study by Al- Saqur *et al*. (2009) [1], compared the performance of AFB staining, culturing, histopathology and PCR for *Mycobacterium tuberculosis* revealed that PCR has high sensitivity and specificity and has a potentially important role in improving the diagnostic accuracy of extra pulmonary tuberculosis. Further in the present study the PPV value of acid fast staining is poor and NPV is excellent which indicates that whenever animals were screened with acid fast staining method, if the sample results negative, the probability of the sample to be negative is high. Whereas if the test result gives positive, it needs to recheck with another confirmatory tests (Nagalingam *et al*. 2015) [14]. The results obtained by phenol auramine fluorescence staining were compared with results of known positive and known negative animals by PCR assay for estimating the diagnostic sensitivity and specificity. The auramine staining technique has exhibited a relative diagnostic sensitivity of 100 per cent (95% confidence interval (CI): 75.29% to 100%) and specificity of 17.24 per cent (95% CI: 8.59% to 29.43%) with an accuracy of 32.39 per cent (95% CI: 21.76% to 44.55%), PPV is 21.31 per cent (95% CI: 19.41 to 23.53%) and NPV 100 per cent and slight agreement of Cohen's kappa value 0.0709 ±0.076SE (95% CI: 0.0 to 0.220) against PCR assay while testing with results of known PCR positive and negative animals (n=71) for bovine tuberculosis. The present study findings are in agreement with the findings of Llamazares *et al*. (1999) [11] who reported the sensitivities of Culture isolation- PCR compared with those of post mortem examination and auramine O staining method were 92·7% and 85·7%, respectively. Out of 412 samples, 113 (27.43%) samples were found positive for fluorescent bacilli by auramine O staining method and among 113 samples, 96 samples showed typical tuberculous lesions (success rate of 78.0%). Further in the present study the PPV value of auramine staining is poor and NPV value is excellent which indicates that whenever animals were screened with acid fast staining method, if the sample results negative, the probability of the sample to be negative is high. Whereas if the test result gives positive, it needs to recheck with another confirmatory tests (Nagalingam *et al*. 2015) [14]. In conclusion, acid fast staining and auramine staining were can be used as preliminary diagnostic tests for diagnosis of tuberculosis in bovines in resource limited laboratories especially at field level.

Acknowledgement
The authors are grateful to acknowledge the Principle Investigator, NAHEP-II, PVNRTVU for providing funds through NAHEP project.

References

1. Al-Saqur M, Al-Thwani AN, Al-Attar IM. Detection of *Mycobacterial* spp. in cow's milk using conventional methods and PCR. Iraqi Journal of Veterinary Sciences. 2009;23:259-262.
2. Alvarez J, Perez A, Bezos J, Marqués S, Grau A, Saez JL, *et al*. Evaluation of the sensitivity and specificity of bovine tuberculosis diagnostic tests in naturally infected cattle herds using a Bayesian approach. Veterinary microbiology. 2012;155(1):38-43.
3. Balamurugan V, Thirumalesh SR, Alamuri A, Sowjanya Kumari S, Vinod Kumar K, Linshamol L, *et al*.

Evaluation of the diagnostic potential of recombinant leptospiral OMP A-like protein (Loa22) and transmembrane (OmpL37) protein in latex agglutination test for serodiagnosis of leptospirosis in animals. Letters in Applied Microbiology. 2021;72(6):730-740.
4. Basit A, Hussain M, Ayaz S, Shahid M, Rahim K, Ahmad I, *et al*. Isolation and identification of *Mycobacterium bovis* and *Mycobacterium tuberculosis* from animal tissues by conventional and molecular method. Indian Journal of Animal Research. 2015;49(5):687-693.
5. Beresford B, Sadoff JC. Update on research and development pipeline: tuberculosis vaccines. Clinical Infectious Diseases. 2010;50(3):S178-S183.
6. Cattamanchi A, Davis JL, Worodria W, den Boon S, Yoo S, Matovu J, *et al*. Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. The International journal of tuberculosis and lung disease. 2009;13(9):1130-1136.
7. Cohen J. A coefficient of agreement for nominal scales. Educational and psychological measurement. 1960;20(1):37-46.
8. George G, Mony P, Kenneth J. Comparison of the efficacies of loop-mediated isothermal amplification, fluorescence smear microscopy and culture for the diagnosis of tuberculosis. PloS one. 2011;6(6):e21007.
9. Hendry C, Dionne K, Hedgepeth A, Carroll K, Parrish N. Evaluation of a rapid fluorescent staining method for detection of Mycobacteria in clinical specimens. Journal of clinical microbiology. 2009;47(4):1206-1208.
10. Khan FA, Chaudhry ZI, Ali MI, Khan S, Mumtaz N, Ahmad I. Detection of *Mycobacterium avium subsp. Paratuberculosis* in tissue samples of cattle and buffaloes. Tropical Animal Health and Production. 2010;42:633-638.
11. Llamazares OG, Martín CG, Nistal DA, De La Puente, Redondo VA, Rodríguez LD *et al*. Field evaluation of the single intradermal cervical tuberculin test and the interferon-γ assay for detection and eradication of *bovine tuberculosis* in Spain. Veterinary microbiology. 1999;70(1-2):55-66.
12. Michel AL, Müller B, Van Helden PD. *Mycobacterium bovis* at the animal–human interface: A problem, or not?. Veterinary microbiology. 2010;140(3-4):371-381.
13. Mittal M, Chakravarti S, Sharma V, Sanjeeth BS, Churamani CP, Kanwar NS. Evidence of presence of *Mycobacterium tuberculosis* in bovine tissue samples by multiplex PCR: possible relevance to reverse zoonosis. Transboundary and emerging diseases. 2014;61(2):97-104.
14. Nagalingam M, Thirumalesh SR, Kalleshamurthy T, Niharika N, Balamurugan V, Shome R, *et al*. Comparative evaluation of recombinant LigB protein and heat-killed antigen-based latex agglutination test with microscopic agglutination test for diagnosis of bovine leptospirosis. Trop Anim Health Prod. 2015;47:1329-1335.
15. Ndugga KL, Cleeff MV, Juma E, Kimwomi J, Githuui W, Oskam L, *et al*. Comparison of PCR with routine procedure for diagnosis of tuberculosis in population with high prevalence of tuberculosis and human immunodeficiency virus. Journal of Clinical Microbiology. 2004;42:1012-1015.
16. OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 7th Edition. Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.07BOVINETB.pdf (accessed September 17, 2013). 2012.
17. OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organisation for Animal Health, Paris, France Chap. 2009;2.4.7:1-16.
18. Proaño-Pérez F, Benitez-Ortiz W, Desmecht D, Coral M, Ortiz J, Ron L, *et al*. Post-mortem examination and laboratory-based analysis for the diagnosis of bovine tuberculosis among dairy cattle in Ecuador. Preventive Veterinary Medicine 2011;101(1-2):65-72.
19. RNTCP (Revised National TB Control Programme) Training Manual for Sputum Smear Fluorescence. Central TB DIvison, Directorate General of Health Services, Ministry of Health& Family Welfare, Nirman Bhawan, New Delhi 110011, 2010.
20. Tariq A, Aslam A, Tipu Y, Ahmad M, Sultan R, Anjum AA. preliminary study on prevalence of bovine tuberculosis in cattle and buffalo in outskirts of lahore, pakistan. Wayamba Journal of Animal Science - ISSN: 2012-578X; P1518 - P1526, 2017 First Submitted January 27, 2017; Number 1482427224.
21. Thrusfield M. Veterinary Epidemiology 3a ed. Blackwell_Publishing. Iowa, USA, 2007.
22. Verma R, Sena DS, Sharma N, Alex K, Pamane RS, Singh R. *et al*. Molecular diagnosis of *Mycobacterium bovis* as the cause of tuberculosis in a camel. Indian Journal of Animal Sciences (India), 2011.
23. Vincent V, Brown-Elliot B, Jost KC, Wallace RJ. *Mycobacterium*: Phenotypic and genotypic identification. Manual of Clinical Microbiology, 8th edition. 2009, 560-584pp.
24. Yahyaoui-Azami H, Aboukhassib H, Bouslikhane M, Berrada J, Rami S, Reinhard M, *et al*. Molecular characterization of bovine tuberculosis strains in two slaughterhouses in Morocco. BMC Veterinary Research. 2017;13(1):1-7.