International Journal of Medicine Research
ISSN: 2455-7404; Impact Factor: RJIF 5.42
www.medicinesjournal.com
Volume 1; Issue 2; May 2016; Page No. 133-135

Tuberculosis diagnosis by staining, isolation and PCR from bovine milk samples

[1] Shivaraj S Aralikatti, [2] Rathnamma D, [3] Renuka Prasad C, [4] Shri Krishna Isloor, [5] Chandranaik BM, [6] Narayan Bhat. M.

[1, 2, 3, 4, 6] Department of Veterinary Microbiology, KVAFSU, Bangalore, Karnataka, India.
[5] Institute of Animal Health and Veterinary Biologicals, KVAFSU, Bangalore, Karnataka, India.

Abstract
Tuberculosis is a age old disease in humans and animals. Being zoonotic disease plays a role establishing a considerable impact in maintaining the health of nation in terms of healthy human resource and profitable (healthy) animal resource. A complete eradication needs a comprehensive approach from both human and animal sector. Milk is a major source for large scale spread of tuberculosis infection to humans next only to spread among infected and healthy individuals. Thanks to Louis Pasteur and Pasteurization on advent of which the tuberculosis cases and reports from milk might have reduced considerably. But still due to some myths about raw milk consumption posing the susceptible population to milk borne infections made the work and article to focus on the aspect. In India after eradicating polio, central health ministry is concentrating on TB eradication by advertising on public platforms regarding the disease and the need of eradication endorsed by Mr. Amithab Bachan who once suffered the disease, recovered after proper diagnosis and scheduled treatment. Age old Gold standard staining and isolation techniques along with PCR was performed on milk samples with BCG as positive control for organisms belonging to Mycobacterium Tuberculosis Complex (MTC) group. Effective quarantine measures, diagnosis of disease, concept of clean milk production with wiping of udder with clean cloth before applying milking machines in a dairy herd might have enabled the control of tuberculosis infection in the selected farm.

Keywords: Tuberculosis, Milk, PCR, Isolation and Staining

1. Introduction
Milk is a source of nutrition to humans. Infants are often feed with cow's milk. Raw milk may contain many infectious bacteria like Mycobacterium, Brucella and Scarlet fever [1, 5] Pasteurization of raw milk before consumption have reduced the risk of getting infected and infant mortality in developing countries have been reduced considerably after advent of pasteurization [6]. In developing countries like India and Africa daily milk is pasteurized and boiled at households and used for consumption for infants, adults and age old to avoid milk borne diseases. In spite of such practises in developing countries there are myths in developed countries that raw milk consumption is good for health as boiling or pasteurization reduces nutrients in milk [7] raw milk kills pathogens, good for infant feeding than pasteurized milk, homogenisation produces dangerous changes in milk [8] documented by millions of people till today purchasing raw milk every year in United States.

Tuberculosis has posed great public health concern due to consumption of raw milk in some parts of the world. Cheese consumption has direct impact on transmission of tuberculosis from bovines to humans. *Mycobacterium bovis* has been isolated from milk and udder tissues of cows by many workers [9, 17]. Milk from dairy animals which is used for human consumption pose the public health concern with respect to spread of tuberculosis infection in humans and discourage TB eradication programmes undertaken by Health ministry with WHO assistance. Parallel National TB eradication programmes in animal husbandry sector strengthened with TB diagnosis, treatment, quarantine measures in bovines and susceptible wild life may accelerate being helpful in putting an end to human TB infection in India.

2. Materials and methods
2.1Milkcollection
A total of 24 milk samples were collected from 24 milking cows from UAS collage dairy farm Bangalore. About 2 ml of milk from each animal was collected from all the four teats into two aliquots before morning routine milking practise and stored at -4^0 C until used for DNA extraction.

2.2 Isolation of mycobacterium
About 2 ml of milk was centrifuged at 3500 rpm for 30 minutes. The supernatant was discarded after retaining the cream, which was then mixed thoroughly with sediment. Smears were prepared from cream-sediment mixture a were air dried and heat fixed. 1.5 ml of 4% NaOH was added to the original suspension for decontamination of other organisms and the mixture was allowed to stand for 30 minutes at 37^0C with occasional shaking. The suspension was then diluted with 2 ml of sterile normal saline and centrifuged at 3500 rpm for 30 minutes. The clear supernatant was discarded and the sediment was again with 20 ml of sterile normal saline and centrifuged as above retaining 1.5 ml of sediment the rest of the supernatant was discarded. This sediment was inoculated in duplicates onto Lowenstein Jensen (LJ) medium containing glycerol and another without glycerol. A positive control- BCG vaccine was inoculated into LJ media slant and incubated at 37^0C at 5% CO_2 tension for 6- 8 weeks.

2.3 Staining methods
Ziehl-Neelsen (ZN) method
The smears were stained by ZN method following the procedures and examined under oil immersion lens.

2.4 DNA extraction via modified heat shock method
Centrifuge all samples after thawing to room temperature about 2 ml of milk in a clean sterile bullet at 3000 rpm for 45 minutes. The sediment/ pellet at the bottom is restored into bullet removing the top fat layer and middle milk layer. Briefly, 1 ml sterile distilled water was added to the pellet, vortexed and

subjected to heating temperature of 100°C for 20 min. The suspension was then cooled immediately to -20°C for 20 minutes, process repeated for 3 times and centrifuged at 13,000 rpm for 3 min before the supernatant was kept in freezer (0 - 5°C) [18-19].

2.5 Polymerase chain reaction

Genomic DNA obtained was subject to PCR for MTB complex specific IS6110 region [20, 21].

Oligonucleotide primers

IS6110

Table 1: Primer sequence for MTC strains.

IS6110F	5'GACCACGACCGAAGAATCCGCTG3'
IS6110R	5'CGGACAGGCCGAGTTTGGTCATC3'

The primers amplify a 445bp sequence on all MTC strains.
Sets of oligonucleotide primers used for the amplification of IS6110 of the MTB complex were synthesized and supplied in lyophilized form by Sumanbio Pvt Ltd., Bangalore. They were reconstituted to 100 pmol/µl stocks in sterile TE buffer. Primers were used at a working dilution of 10 pmol/µl (10 pmol per reaction) in sterile Nuclease Free Water (NFW)

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: Composition of PCR reaction mixture for the detection of MTB complex specific IS6110 region.

Particulars	Quantity
Dream Taq Master Mix	12.5 µl
Forward Primer	2.0 µl
Reverse Primer	2.0 µl
Template DNA	5.0 µl
Nuclease Free Water (NFW)	3.5 µl
Total	25.0 µl

3. Results

3.1 Isolation: No growth on LJ media with glycerol and without glycerol for all 24 milk samples. A positive control -BCG vaccine yielded growth on LJ media with glycerol (Fig 1).

3.2 Staining and microscopy: All the 24 milk samples were negative for acid fast organisms on direct staining of sediment and cream layer from milk samples. On staining of culture from BCG vaccine yielded acid fast organism (Fig 2).

3.3 PCR: All the DNA samples subjected for PCR for MTC group identification, amplifying a region in IS6110 yielded no amplification (Fig 3). Only DNA isolated from culture obtained by inoculating BCG vaccine yielded an amplification of 445 bp used as positive control.

4. Discussion

The raw milk collected from all 24 milking animals from an organized dairy farm appear to be free from organisms belonging to Mycobacterium Tuberculosis Complex (MTC) group. The farm was earlier screened with skin testing and the positive reactors were removed and strict quarantine measure were followed whenever new animals were introduced appear to be effective control measure to ensure the herd free from Mycobacterium infection and further minimizing the risk to human health.

Fig 1: Growth of Mycobacterium from BCG vaccine on LJ media with glycerol.

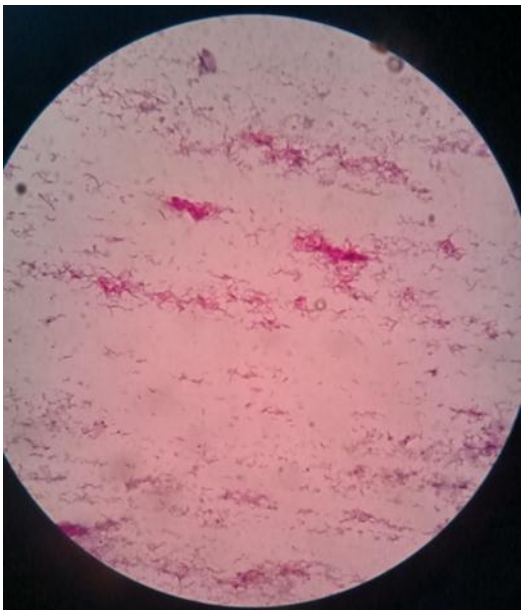

Fig 2: Acid fast organism from BCG vaccine.

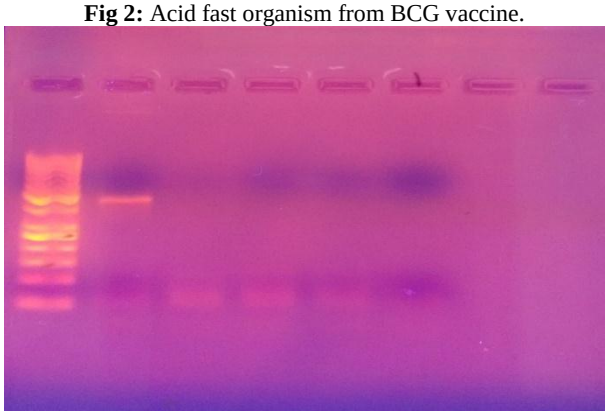

Fig 3: PCR of milk samples with BCG vaccine control. 445bp 50 bp ladder

5. References

1. Jaros P, Cogger N, French N. A systematic review of the human disease evidence associated with the consumption of raw milk and raw milk cheeses. A report prepared for the New Zealand Food Safety Authority (NZFSA), 2008. Available at: http://www.nzfsa.govt.nz/science/research-projects/final-report-rawmilk.pdf
2. World Health Organization. Populations at Risk, page 3. Available at: http://www.who.int/foodsafety/publications/general/en/fos_brochure1999_2en.pdf
3. Kaplan MM, Abdussalam M, Bilgona G. Diseases transmitted through milk. Available at: http://whqlibdoc.who.int/monograph/WHO_MONO_48_%28p11%29.pdf
4. Weisbecker A. A legal history of raw milk in the United States. J. Environ Health 69. 2007. Available at: http://www.marlerclark.com/pdfs/raw-milk-jeh.pdf
5. Steele JH. History, trends, and extent of pasteurization. J. Am Vet Med Assoc 2000; 217:175-8.
6. Warriner K. Raw Milk. Political Football or Food Safety Issue. Raw milk webinar, 2011.
7. Macdonald LE, Brett J, Kelton D, Majowicz SE, Snedeker K, Sargeant JM. A systematic review and meta-analysis of the effects of pasteurization on milk vitamins, and evidence for raw milk consumption and other health-related outcomes. J. Food Prot. 2011; 74:1814-32.
8. Homogenization of milk and milk products. Guelph University. Available at: http://www.foodsci.uoguelph.ca/dairyedu/homogenization.html
9. Lesslie IW, Birn KJ. Tuberculosis cattle caused by the avian type tubercle bacilli. Vet. Rec. 1967; 80:559-564.
10. Ilukevich, Anerik A. Experimental study of diagnosis of bovine tuberculosis by culture and animal inoculation methods. Ciencias Veterinarias Venezuela, 1974; 4:137-200(45:1014).
11. Kamel YY, Ismail AA, Ahmad AA, Moustafa TH, Mahamoud MS. Sanitary conditions of market milk in Assiut city. Incidence of M. bovis and a modified scheme for typing. Assiut. Vet. Med. J. 1975; 2:169-181:48(483).
12. Nassal J. Infection of a cow with human type tubercle bacilli. Excretion of the organisms from the apparently normal udder. Rinder Tuberkulose, 1956; 51:189-198. (Vet. Bull., 27, 1333.
13. Chapman JS, bernards JS, Speight M. Isolation of Mycobacteria from raw milk. Am. Rev. Resp. Dis. 1965; 91:351-355.
14. Jones RH, Jenkins DEV, Hsu KHK. Raw milk as a source of mycobacteria. Can. J. Microbiol., 1966, 12.
15. Mathews PRJ, Collins P, Jones PW. Isolation of Mycobacteria from diary creamery effluent. Sludge. J. Hyg., 1976; 76:718-721.
16. Aich AC, Haidar SK, Khanna PN, Sen GP. Acid-fast bacilli in milk in relation to tuberculin reaction in cattle. Indian J. Anim. Sci., 1981; 51:838-842.
17. Dunn BL, Hodgson DG. Atypical mycobacteria in milk. J. Appl. Bacteriol, 1982; 52:373-376.
18. Keegan H, Boland C, Malkin A, Griffin M, Ryan F, Lambkin H. Comparison of DNA extraction from cervical cells collected in Preserv Cyt solution for the amplification of Chlamydia trachomatis. Cytopathology, 2005; 16:82-87.
19. Quigley L, O'Sullivan O, Beresford TP, Paul Ross R, Fitzgerald GF, Cotter PD. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. J. Appl Microbiol. 2012; 113(1):96-105.
20. Thangaselvam M, Kidangam A, Rishbendra Verma, Ramana SP. Molecular detection and differentiation of Mycobacterium tuberculosis complex in human sputum samples using PCR assays. A preliminary report Indian. J. Vet. Res, 2009; 18(2):50-54.
21. Alex Kidangam and Rishendra Verma. PCR-SSCCP analysis in detecting point mutations targeting rpoB, kat G and inh A genes for determining multi-drug resistance in Mycobacterium bovis and Mycobacterium tuberculosis strains. Indian. Journal of Animal Science. 2014; 84(12):1256-1260.