Comparative Immunology, Microbiology and Infectious Diseases 64 (2019) 53–60

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Profiling of *Mycobacterium avium* subspecies *paratuberculosis* in the milk of lactating goats using antigen-antibody based assays

Manju Singh[a,b], Saurabh Gupta[a], Kundan Kumar Chaubey[a], Shoor Vir Singh[a,*], Jagdip Singh Sohal[b,*]

[a] Department of Biotechnology, Institute of Applied Sciences & Humanities, GLA University, Mathura, Uttar Pradesh, India
[b] AIMT & AIB, Amity University Rajasthan, Jaipur, India

ARTICLE INFO

Keywords:
Mycobacterium avium subspecies *paratuberculosis*
Milk
Indigenous plate ELISA
Dot-ELISA
Latex Agglutination test
Indirect Fluorescent Antibody test
IS*900* PCR
'Indian Bison type'

ABSTRACT

Johne's disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* is endemic in the domestic livestock population, still it is not priority for control in the country. First time we used 'multiple assays' for screening raw milk of 465 goats (farm/farmer's herds) to estimate bio-load and bio-type profile of bacilli. Each sample was screened by six tests and compared their sensitivity and specificity. Of 465 raw milk samples screened, bio-load of bacilli was 65.3% by six assays. Assay-wise bio-load was 49.4 and 62.7% in antigen and antibody detection tests, respectively. Bio-load was 48.8, 46.6, and 13.9% in Indirect Fluorescent Antibody Test (i_FAT), microscopy and IS*900* PCR and 39.1, 57.4 and 55.6% in Indirect Enzyme Linked Immuno Sorbant Assay (i_ELISA), Dot Enzyme Linked Immuno Sorbant Assay (d_ELISA) and Latex Agglutination Test (LAT), respectively. Dot-ELISA was most sensitive followed by LAT, i_FAT, microscopy and i_ELISA. Milk DNA samples positive in IS*900* PCR on bio-typing using IS*1311* PCR_ Restriction Enzyme Analysis (IS*1311* PCR_REA) revealed, 72.3% (47/65) were 'Indian Bison Type'. Milk was easy to collect sample and first time we used 'whole milk' as 'test sample' without centrifugation. High bio-load of MAP in milk underlined need for urgent control of disease in lactating goatherds. Bacilli was important 'Milk born' infection and on the basis of sensitivity, specificity, resources and requirements, of the 'six assays' most appropriate assay/s (single or in combination) can be chosen for the screening and diagnosis of Johne's disease in lactating goatherds using whole milk as sample.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP), the cause of chronic incurable Johne's disease (JD) is endemic in the domestic livestock population of the country [1]. MAP has been associated with auto-immune disorders [2] of human population, where inflammation is primary feature e.g., inflammatory bowel disease (IBD), Crohn's disease (CD), Ulcerative Colitis (UC) etc., [3–11]. MAP infection to serious economic losses to the livestock industry globally [11–13]. Farm-level losses to the tune of EUR 35–165 per cow [14,15] have been reported from Europe. In the first long term study, Singh et al. [1], reported consistent increase in the bio-load of MAP in the domestic livestock population of country in past 33 years (1985–2018) [16]. At > 132.43 million tonnes of milk production per year [17], India is leading milk producer in the world, but per animal productivity is very low (1/6th in Asian countries). Country lacks information on the bio-load of MAP in the domestic livestock at National level [1]. Many European countries, reported surveys using milk as sample to estimate National bio-load of MAP in the cattle population. However, in India this information is extremely limited. This is mainly due to the reluctance of farmers in providing samples specially blood. Therefore, in this study we focussed on alternative samples (milk) to estimate the bio-load of MAP in lactating goats. Raw milk is an important carrier of MAP bacilli from livestock to human population [18–20]. Raw milk is frequently used as important constituent in traditional medicines [21].

In the absence of control programs, MAP bacilli is excreted in the milk of infected goats and since it is not inactivated during pasteurization, continues to infect new born kids through feeding of milk and colostrums. Since MAP bacilli is not in-activated during pasteurization, therefore milk and milk products made from pasteurized milk are important source of infection to human population. Early and accurate diagnosis of MAP is critical for the control of JD in dairy herds [22]. In chronic diseases e.g., tuberculosis and paratuberculosis, multiple assays are essential to say that particular sample is infected (pos

- Conversion(M.E.)

* Corresponding authors.
E-mail addresses: shoorvir.singh@gla.ac.in (S.V. Singh), jssohal@jpr.amity, jssohal@jpr.amity.edu (J.S. Sohal).

https://doi.org/10.1016/j.cimid.2019.02.006
Received 7 August 2018; Received in revised form 5 February 2019; Accepted 7 February 2019

itive) or non-infected (negative). No single test can accurately detect MAP infection in animals [1,23]. Early and "silent" infections can be detected only by culturing the organisms either from samples (feces, milk, blood, vaginal secretions, semen etc.) of sub-clinically or clinically infected goats or from necropsied tissues (intestine and mesenteric lymph nodes) and rarely by histopathology. Paratuberculosis being spectral disease variety of assays are recommended for the diagnosis, however, the choice of test varies with the stage of disease, requirement and purpose of study and resources available. Indigenous ELISA (i_ELISA) and IS*900* PCR are most frequently used assays [24,25], have shown good sensitivity and specificity [20,23,26]. Culture though highly specific but is time-consuming and laboratory intensive. Collection of blood samples need expertise and farmers show reluctance, therefore, we have standardized, indirect Fluorescent Antibody test (i_FAT), dot-ELISA (d_ELISA) and Latex Agglutination test (LAT) for the screening of raw milk samples. [27–29]. In the present study, six (3 antigen and 3 antibody) assays were used to estimate bio-load of MAP in raw milk samples of lactating goats from farm and farmer's herds in North India. Bio-type profile of MAP was also analyzed using IS*1311* PCR_RE in IS*900* PCR positive milk samples. However, representative milk samples were also cultured to confirm the presence of live acid fast MAP bacilli in the milk of lactating goats.

2. Materials and methods

2.1. Collection and processing of milk

Table 1, shows profile of 465 raw goat milk samples collected from 465 lactating goats {158 goats from farmer's herds in South Uttar Pradesh (Mathura-114, Agra-10, Gwalior-5, Jaipur-9 and Gurgaon-20) and 307 farm goats {Central Institute for Research on Goats (CIRG), Mathura district} in North India from January, 2015 to November, 2017. Un-like earlier studies (Stephan et al., 2016), wherein milk samples were first centrifuged for partitioning and concentrate MAP bacilli into fat and sediment layers by spinning at 4000 rpm for 45 min., first time 'whole milk' was used directly (without centrifugation) as 'test sample'. Three routinely used (microscopy, IS*900* PCR, i_ELISA) and 3 newly standardized (d_ELISA, LAT and i_FAT) assays were employed for the screening of lactating goatherds using milk samples. Each of the 465 goat milk samples were screened six times. Limited IS*900* PCR positive milk samples were analyzed using IS*1311* PCR_RE and culture to estimate bio-type profile and presence of live acid fast bacilli in raw milk samples of lactating goats endemically infected with MAP, respectively. Each milk sample represented one individual goat.

2.2. Acid fast staining (microscopy)

Smears made from 20 µl of 'whole milk', were heat fixed, stained (Ziehl Neelsen - ZN) [31] and examined under oil immersion (×100) for acid-fast bacilli (AFB) indistinguishable to MAP.

2.3. Indirect Fluorescent Antibody test (i_FAT)

Tissue based i_FAT [30] was standardized for screening of milk samples [28,31]. Briefly, smears prepared on clean slides (20 µl milk), were air dried and heat fixed. Slides were dipped in solution of 30.0% H_2O_2 in 90.0% methanol (3:7 ratio) and incubated for 10 min at 37 °C, followed by second dipping in phosphate-citrate buffer (2.1% citric acid and 3.56% disodium hydrogen phosphate in 100 ml triple distilled water, pH- 5) and were heated to boiling in microwave for 30 s (15 cycles) with 20 s rest after each heating cycle (total time 10 min). To air dried slides whey (ratio of 1:4 as primary antibody) or serum (in ratio of 1:50) in serum dilution buffer (1% BSA in PBST) was added. Slides were incubated for 1 h at 37 °C followed by washing in 1X PBS (3 times). Anti-species secondary antibody (FITC conjugate) added in the ratio 1:750 in 1X PBS (pH-7.6) and incubated in dark for 1 h at 37 °C followed by washing of slides 5 times in 1X PBS in dark. Slides were air dried in dark at room temperature and mounted in glycerine with cover slip and observed immediately under fluorescent microscope. Slides positive for MAP exhibited green fluorescence.

2.4. DNA isolation

Isolation of DNA from raw milk was carried out as per van Soolingen et al. [32] with some modifications [28,31]. Briefly, to 500 µl milk sample, 100 µl of lysis buffer (50 mM NaCl, 125 mM EDTA, 50 mM Tris-HCl; pH 7.6) was added and incubated for 15 min at RT. Then 100 µl of 24% sodium dodecyl sulfate (SDS) was added and incubated at RT for 10 min., followed by heating at 80 °C for 10 min. Proteinase K (325 µg) was added to sample and incubated at 55 °C for 2 h., followed by addition of 115 µl of 5 M NaCl and 93 µl CTAB-NaCl with proper mixing and incubated at 65 °C for 30 min. Equal volume of phenol:-chloroform:isoamyl alcohol (25:24:1) was added to sample and centrifuged at 15,800 *g* for 5 min. After centrifugation, aqueous phase from sample was transferred to sterilized eppendrof tube and DNA was precipitated by adding 0.8 vol of chilled iso-propanol and kept at −20 °C for 2 h. DNA was pelleted out by centrifuging at 15,800 *g* for 10 min at 4 °C and supernatant was discarded. Pellet was washed with 500 µl of 70% ethanol and re-suspended in 30 µl TE buffer/ nuclease free water and stored at −20 °C.

Table 1
Profile of raw milk samples (*n* - 465) collected from lactating goatherds (farm and farmer's) in North India for screening against *Mycobacterium avium* subspecies *paratuberculosis* infection.

Sn	Geographical Regions / districts & State	Places	Goats / milk samples, *n*	Positive / Total *n* (%)	
				Individual goatherds	Farm Management
1.	Mathura,	Mathura city	10	08 (80.0)	Farmer's goatherds 129/158 (81.6)
	Uttar Pradesh	Kurkunda & Bhai village	26	24 (92.3)	
		Farah & nearby villages	56	44 (78.5)	
		Makhdoom village	22	17 (77.2)	
2.	Agra, Uttar Pradesh	Keetham village	10	08 (80.0)	
3.	Gwalior, Madhya Pradesh	Sadanwara village	05	05 (100.0)	
4.	Jaipur, Rajasthan	Jaipur city	09	09 (100.0)	
5.	Gurgaon, Haryana	Kharkhari village	20	14 (70.0)	
6.	CIRG, Mathura,	Experimental unit	60	40 (66.6)	Farm175/307 (57.0)
	Uttar Pradesh	Jakhrana unit	109	57 (52.2)	
		Barbari unit	127	70 (55.1)	
		Jamunapari unit	11	08 (72.7)	
Total		465		304 (65.3)	

2.5. IS900 PCR

DNA isolated from raw milk samples were screened by specific IS*900* PCR using 150C and 921 primers [33] and 229 bp PCR product was positive for MAP.

2.6. IS1311 PCR and restriction endonuclease analysis (IS1311 PCR _REA)

IS*1311* PCR was performed on IS*900* PCR positive DNA samples using M56 and M119 primers as per Sevilla et al. [34]. PCR products of 608bp were taken as positive and was further genotyped using HinfI and MseI enzymes (Fermentas) [34].

2.7. Culture

IS*1311* PCR positive milk samples were cultured by decontaminating 1.0 mL of raw milk in 0.9% hexa-decylpyridinium chloride for 18–2hr at room temperature. From 1.0 mL of sediment, 0.02 mL residual mucilaginous sludge was inoculated on modified Herrold's egg yolk medium (HEYM) [35,36] with Mycobactin J (Allied Monitor Inc., MO, USA). Culture tubes were screened at weekly interval up to 20 months for the appearance of typical MAP colonies [37,38].

2.8. Indigenous ELISA (i_ELISA)

Assay was performed as per Singh et al. [28,31]. Briefly, each well of flat bottom 96 well ELISA plate was coated with 0.1 µg of protoplasmic antigen in 100 µl of carbonate-bicarbonate buffer, (pH 9.6) per well and incubated at 4 °C overnight. Plates were washed thrice with PBST (PBS with 0.05% Tween 20) followed by blocking in 100 µl of 3.0% skimmed milk in PBS, incubated for one hour at 37 °C. Plates were washed three times with PBST and then 100 µl of whole milk diluted in PBST with 1.0% BSA in ratio of 1:1 was added as sample in duplicate wells and incubated for 2 h at 37 °C. Plates were washed thrice followed by addition of 100 µl of optimally diluted rabbit anti-bovine (1:6000 in 1X PBS) / caprine (1:5000 in 1X PBS) conjugate and again incubated for one hour at 37 °C. Finally after five times washing, 100 µl of freshly prepared OPD substrate was added and incubated till colour developed (3–5 min) at 37 °C. Absorbance was read at 450 nm in ELISA reader (i-Mark micro-plate reader, Biorad). Milk from weak and culture positive and healthy and culture negative and weak goats were positive and negative controls, respectively. Optical densities (OD) were expressed as sample-to-positive (S/P) ratios [39].

2.8.1. Analysis of OD values

S/P ratio = [(Sample OD – Negative OD) / (Positive OD - Negative OD)]. Sample-to-positive ratios indicating corresponding status of bovine JD in serum samples [39] was used for goat milk in this study. Samples in low positive (LP), positive (P) and strong positive (SP) categories were considered positive for MAP infection.

2.9. Dot- ELISA (d_ELISA)

Test was performed as per Singh et al. [28]. Briefly, tips of 12 legged immune-diffusion combs (Advanced Microdevices pvt. ltd., Ambala, Haryana) fixed with nitrocellulose membrane were coated with 1 µl (2 µg of sPPA in 1 µl of carbonate-bi-carbonate buffer, pH 9.6) of sPPA spot in middle were incubated for 2 h at 37 °C. Combs were dipped in blocking solution (3.0% skimmed milk powder in PBS) for 1 h at 37 °C. After washing in PBST combs were dipped in test samples (100 µl whole milk in 1:2 dilution in 1% BSA in 1XPBST) for one hr followed by again washing. Combs were incubated with 200 µl of rabbit anti-caprine HRP conjugate solution at 37 °C for 30 min., and finally dipped in 200 µl of 3, 3′-Diaminobenzidine (6 mg / 10 ml of 1X PBS), at room temperature till development of brown colour (1–2 min) (Fig 9). Once brown spot was visible to stop the reaction combs were dipped in water. Positive and negative controls used in i_ELISA were coated on two legs of each comb.

2.10. Latex Agglutination test (LAT)

Goat raw milk (n-465) samples were screened by LAT as per Singh et al. [29,31]. Briefly, MAP antigen coated latex beads were prepared using 10 µl of polystyrene latex beads (3.0 µm mean size, Sigma Aldrich). Beads were washed four times in distilled water and re-suspended in 20 µl of 0.5 M glycine saline buffer (1.4 gm glycine, 0.07 gm Sodium Hydroxide, 1.7gm Sodium Chloride, 0.1 gm Sodium Azide in 100 ml of triple distilled water) (pH- 8.6), then 20 µl of antigen (4 mg / ml) was added and incubated for 3 h at 37 °C in shaker incubator. Mixture was centrifuged at 5000 rpm for 10 min and after aspirating the supernatant, mixture was re-suspended in blocking buffer (1% BSA in 1 X PBS) and mixed in shaker incubator for 45 min at 37 °C. Finally beads were washed twice in 1 X PBS. Milk sample (4 µl) was mixed with 2 µl of antigen coated latex beads on a glass slide. Slide was shaken gently and milk sample was considered positive, if agglutination was observed within 2 min and negative if no agglutination.

2.11. Statistical analysis

Statistical significance between two assays was measured. Mc Nemar's test and kappa agreement methods were applied using Graph Pad software, USA. Sensitivity and specificity of the assays were measured by Med-Calc software, Belgium.

3. Results

Each of the raw milk sample representing one goat, if found positive in any one of the six assays was considered positive for MAP infection. Screening of 465 lactating goats from farm and farmer's hreds in North India showed high bio-load (65.3%) of MAP using six assays, and tests had 95.3% agreement. Exclusively 4.5% milk were positive in individual assays {microscopy (0.2%), d_ELISA (3.0%) and LAT (1.2%)} (Table 4). Therefore, of 65.3% (302) positive raw goat milk using six assays, majority 60.8% (283) were positive in combination of more than one assay. In combination of six, five, four, three and two assays, 9.4, 21.9, 11.3, 9.0 and 9.0% milk samples were detected positives, respectively, (Table 4). Bio-load was higher (81.6%) in farmer's goats (70.0–100.0%), as compared to farm (57.0%) goats (52.2–72.7%), located at CIRG, Mathura (Table 1), Of the 465 goat milk screened by antigen based assays, 48.8, 46.6 and 13.9% were positive by i_FAT, microscopy and IS*900* PCR, respectively (Table 2). Together, antigen based assays detected 49.4% bio-load, exhibiting 96.5% agreement. However, small percent (3.4%) were positive in single test (2.8 - i_FAT, 0.6% - microscopy) (Table 3a). In antibody based assays, 57.4, 55.7 and 39.1% milk were positive in d_ELISA, LAT and i_ELISA, respectively (Table 2). Together antibody based assays detected, 62.7% bio-load, exhibiting 90.7% agreement and only 9.2% were positive in single test (d_ELISA-3.8% and LAT-5.3%) (Table 3b).

Bio-typing of representative (IS*900* PCR positive) raw milk showed majority (72.3%, 47/65) were bio-typed as 'Indian Bison Type' and rest could not be bio-typed. Of 47 milk cultured, live acid fast MAP bacilli were recovered in 27.6% (13/47) at the end of 6 months incubation at 37 °C (Table 2).

Statistically, kappa values and two-tailed p values were calculated (Table 5). In antigen based assays, i_FAT and IS*900* PCR had very good and fair level of agreement with respect to microscopy. Using antibody assays, d_ELISA and LAT had good and moderate level of agreement with respect to i_ELISA, respectively. With respect to microscopy, i_FAT and IS*900* PCR had sensitivity of 98.6 and 29.9%, respectively. Specificity of i_FAT and IS*900* PCR with respect to microscopy was 94.7 and 100.0%, respectively. With respect to i_ELISA, sensitivity of d_ELISA and LAT was 100 and 91.7%, respectively. d_ELISA and LAT had specificity of 69.9 and 67.4% with i_ELISA, respectively (Table 5).

Table 2
Profile of diagnostic assays for screening of milk samples: Standardized for detection of MAP infection at CIRG, Makhdoom.

Assays	Sample	Test		Period / sample	Dev. Year	Detects	Specificity %	Sensitivity %
1. Culture	Feces	Quali-	Confir-	120 days / 65	1988	Live bacilli	100.0	67.5
2. IS*900*PCR	Feces	tative	matory	2 days / 40	2004	Anti-		23.0
3. i_FAT			Screen	4 hrs / 20	2015	gen	93.5	83.5
4. Microscopy			-ing	2 hrs / 20	1984		60.0	80.5
5. LAT	Milk			2 min / 01	2015	Antib-	70.0	82.2
6. Dot-ELISA	Serum			4-5 hrs / 80		ody	70.0	89.7
7. i_ELISA kit		Quanti- tative		5 hrs / 90	1985		**90.0**	**83.3**

Dev - Developed / standardized in year.

Table 3a
Comparison of 'Antigen based' assays for detection of MAP in raw milk of individual goats (n-465)*.

Diagnostic Assays	Antigen Detection assays, *n* (%)								Total Positives *n* (%)
	Test Combinations								
	1	2	3	4	5	6	7	8	
i_FAT	+	–	+	+	–	+	–	–	**227 (48.8)**
Microscopy	+	–	+	–	+	–	+	–	**217 (46.6)**
IS*900* PCR	+	–	–	+	+	–	–	+	**65 (13.9)**
Total - 465	**65 (13.9)**	**235 (50.5)**	**149 (32.0)**	**0**	**0**	**13 (2.8)**	**3 (0.6)**	**0**	**230 (49.4)**

Antigen based assays detected **49.4**% (230/465) raw milk positive or goats positive for MAP infection; Perfect Agreement - 64.5% (300/465), Mis-match - 35.4% (165/465).

4. Discussion

Association of *Mycobacterium avium* subspecies *paratuberculosis*, with number of human disorders of auto-immune nature including Crohn's disease [4,11] has renewed interest in screening of milk samples. Percent share of milk from goats in 2014-15 was 3.54% (Annual report 2015-16, page no. 4-5, Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmers Welfare, Government of India, New Delhi). Low per goat productivity was primarily due to high bio-load and endemicity of MAP [1]. In the absence of attention and control measures, MAP has become endemic in the domestic livestock population of the country [40].

Serum and fecal samples have been widely used as 'test sample for the screening of goatherds against Johne's disease, [41,1]. But in this study, raw milk has been used as 'test sample' to estimate bio-load of MAP in lactating goatherds. MAP bacilli present in milk, are not inactivated during pasteurization [42,43], therefore besides raw milk [44], liquid milk and milk products made from pasteurized milk [45,46] also pose serious public health hazard [47–49]. Milk is important vehicle for the transmission of MAP (food/milk borne pathogen) from goatherds to human population. Though milk is usually not 'preferred sample' for screening of herds since only lactating females in a population can be screened. But when available, milk is the most convenient and easy to collect 'clinical sample' especially in large ruminants. Other workers in India [50–52] and globally [53,54] too used milk as 'test sample' for the screening of lactating animals against JD.

In our earlier studies, before screening milk samples were processed to concentrate MAP bacilli by centrifugation. On centrifugation MAP bacilli were partitioned in fat and sediment layers [55]. But in this study, raw milk samples were screened directly by multiple assays. Direct screening of milk helped in simplifying test protocols and results were obtained in quick time and it was essential step in the development of field based test for the screening milk samples. Milk was convenient sample to collect from lactating large ruminants. Bulk milk (pooled milk) tank test has been employed as the herd screening [20] and for the estimation of National prevalence of MAP by many European countries.

Present study using multiple assays reported very high bio-load (65.3%) of MAP in lactating goatherds of North India (Table 2). Bio-load was lower (57.0%) in farm lactating goatherds (CIRG, Mathura), as compared to farmer's goatherds (81.6%) from different parts of North India. Bio-load was low in CIRG goatherds, due to regular screening and culling of positive goats. Goats negative in microscopy but exhibited clinical symptoms of stunting, loss of body weight, recurrent diarrhoea) were also culled from CIRG goatherds [1]. Though by using 'test and cull', methodology, we could not control JD. Culling in farmer's goatherds is seldom based on health or on the status of JD. Screening of farmer's goatherds for JD was 'non-existence' in the country [55,56]. Similar situation may exist in other developing and poor countries [57–59] also. Screening and culling of goats for JD at CIRG did not help

Table 3b
Comparison of 'Antibody based' assays in detection of MAP in raw milk of individual goats (n-465)*.

Diagnostic Assays	Antibody Detection assays, *n* (%)								Total Positives *n* (%)
	Test Combinations								
	1	2	3	4	5	6	7	8	
i_ELISA	+	–	–	+	+	–	–	+	**182 (39.1)**
d_ELISA	+	–	+	+	–	+	–	–	**267 (57.4)**
LAT	+	–	+	–	+	–	+	–	**259 (55.7)**
Total -n 465	**167 (35.9)**	**173 (37.2)**	**67 (14.4)**	**15 (3.2)**	**0**	**18 (3.8)**	**25 (5.3)**	**0**	**292 (62.7)**

Antibody based assays detected, **62.7**% (292/465) milk positive or goats positive for MAP infection; Perfect Agreement - 73.1% (340/465), Mis-match - 26.8% (125/465).

*Total samples ***n*** -465; **(-):** Denotes the negative samples in individual test of that particular test combination; **(+):** Denotes the positive samples in individual test of that particular test combination; **1-8:** Maximum permutation and combinations possible in 3 test regimen; **Total-n:** Represents only total positive samples in that particular test combination.

Table 4
Comparison of Antigen and Antibody based assays for detection of MAP in raw milk of individual goats (n-465).

Diagnostic Assays	Test Combinations, *n* (%)											
	1	2	3	4	5	6	7	8	9	10	11	12
i_FAT	+	–	+	+	+	+	+	+	+	+	+	–
Microscopy	+	–	+	+	+	+	+	–	–	+	–	–
IS*900* PCR	+	–	–	+	+	–	+	–	+	+	–	–
i_ELISA	+	–	+	–	+	–	–	+	+	–	–	+
d_ELISA	+	–	+	+	–	+	–	+	–	–	+	+
LAT	+	–	+	+	–	+	+	+	+	–	+	+
Total- 465	**44**	**161**	**91**	**11**	**2**	**35**	**7**	**8**	**1**	**1**	**4**	**24**
n (%)	**(9.4)**	**(34.6)**	**(19.5)**	**(2.3)**	**(0.4)**	**(7.5)**	**(1.5)**	**(1.7)**	**(0.2)**	**(0.2)**	**(0.8)**	**(5.1)**
	44	**161**	**102**		**53**					**42**		
	(9.4)	**(34.6)**	**(21.9)**		**(11.3)**					**(9.0)**		

Diagnostic Assays	Test Combinations, *n* (%)										Total Positives *n* (%)
	13	14	15	16	17	18	19	20	21	22	
i_FAT	–	+	–	+	–	–	+	–	–	–	**227 (48.8)**
Microscopy	–	+	+	–	–	–	–	–	+	–	**217 (46.6)**
IS*900* PCR	+	–	–	–	–	–	+	–	–	–	**65 (13.9)**
i_ELISA	+	–	–	–	+	–	–	–	–	–	**182 (39.1)**
d_ELISA	+	–	+	+	–	+	–	+	–	–	**267 (57.4)**
LAT	–	+	–	–	+	+	–	–	–	+	**259 (55.6)**
Total-	**1**	**12**	**1**	**1**	**16**	**14**	**10**	**14**	**1**	**6**	**304 (65.3)**
n (%)	**(0.2)**	**(2.5)**	**(0.2)**	**(0.2)**	**(3.4)**	**(3.0)**	**(2.1)**	**(3.0)**	**(0.2)**	**(1.2)**	
			42 (9.0)					**21 (4.5)**			**304 (65.3)**

LAT- Latex Agglutination test; i_FAT- Indirect Fluorescent Antibody test.
Total samples ***n*** -465; **(-):** Denotes the negative samples in individual test of that particular test combination; **(+):** Denotes the positive samples in individual test of that particular test combination; **1-22:** Maximum permutation and combinations possible in 6 test regimen; **Total-n:** Represents only total positive samples in that particular test combination.

to reduce the bio-load of MAP, instead it continued to increase [1]. In four farm goatherds at CIRG, bio-load was minimum in lactating goats of Jakhrana breed due to the vaccination of goats against JD [60]. 'Indigenous vaccine' developed at CIRG was both therapeutic and preventive [61].

Present study showed that screening of lactating goats using six assays, the bio-load of MAP was significantly high (65.3%). This high bio-load may be due to the negative energy balance caused by 'milking stress' on these goats. In simultaneous screening of other lactating domestic livestock species, similar results were reported, wherein bio-load of MAP was very high {sheep (95.2%, 20/21), cows (78.5%, 252/321) and buffaloes (61.2%, 308/503)}. In a recent study based on 28 years (1985–2013) of research on the bio-load of MAP in India, reported high to very high bio-load (25.0–60.0%) of MAP in domestic livestock using multiple assays. However, in goats the bio-load was low (average-20.1%) and ranged from 11.1 to 35.1% in 28 years by multiple assays [1]. It showed that lactation stress was the single biggest reason for increase in the bio-load of MAP. Similar studies on the bio-load of MAP in raw milk of dairy cattle showed MAP was endemic in the cattle herds [40]. Before this study information on recovery of MAP from goat milk were fewer and were based on limited number of milk samples [37,41]. High and continuous rise in bio-load of MAP was primarily due to absence of National programs for the control of Johne's disease [62].

Of the three, 'Antigen detection' assays, highest positivity was recorded in i_FAT (48.8%), followed by microscopy (46.6%) and IS*900* PCR (13.9%) (Table 2). Microscopy was simple to perform, cost effective and user friendly and could be developed as good 'field based test' for the screening of milk samples. Limitation of the test was that it was highly dependent on the expertise of person using it. Poor sensitivity of IS*900* PCR was due to low count of MAP bacilli in milk and also due to the presence of inhibitory substances in milk, as reported by Chui et al. [63]. In IS*900* PCR, 13.9% milk samples were positive and were also found positive by all 3 antigen detection assays. Therefore, IS*900* PCR could be 'confirmatory test' and had better detection rate (sensitivity) in samples having high bio-load of MAP (heavy shedders). Other studies also reported lower bio-load of MAP in milk in low shedder animals using IS*900* PCR [26,55]. I_FAT and microscopy had potential to be developed as test kits. Besides i_FAT had good sensitivity (84.7%) and specificity (90.4%) with respect to microscopy. Only few studies had reported the effectiveness of i_FAT for the diagnosis of JD in tissue samples [64–66] and in milk samples specifically [27,67,68].

In 'antibody based assays', d_ELISA was most sensitive (57.4%) followed by LAT (55.6%) and i_ELISA (39.1%) (Table 2). i_ELISA has been extensively evaluated in past > 25 years, since first developed at CIRG

Table 5
Sensitivity and specificity of diagnostic assays for the screening of raw milk of individual goats (n-465).

Assays Type	Diagnostic Test	Comparative Test	Two tailed P value	Kappa ± SE	Strength of agreement	Sensitivity (%)	Specificity (%)
Antibody based	LAT	Indigenous plate ELISA	< 0.0001	0.551 ± 0.036	Moderate	91.7%	67.4%
	d_ ELISA		< 0.0001	0.646 ± 0.032	Good	100%	69.9%
Antigen based	i_FAT	Microscopy	< 0.0001	0.931 ± 0.017	Very good	98.6%	94.7%
	IS*900* PCR		< 0.0001	0.313 ± 0.033	Fair	29.9%	100%

*LAT- Latex Agglutination Test; i_FAT- Indirect Fluorescent Antibody Test; * < 0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial (good); and 0.81–1.00, very good/almost perfect.

Table 6
Global bio-load of MAP in Goats raw milk: Country-wise.

Countries	Sample type	Assays	Bio-load (%)	References
Greece	Raw milk	Culture	7.7	Malli [76]
		ELISA	> 50.0	Angelidou et al. [77] [78],
UK		IMS-PCR	< 1.0	Grant et al. [79]
Norway		IMS-PCR	7.1	Djønne et al. [80]
Italy		PCR	44.8	Nebbia [81]
		ELISA	6.8	Galiero et al. [82]
Brazil		PCR	2.3	Schwarz et al. [83]
Netherlands		ELISA	> 40.0	van Hulzen et al. [84]

(personal observation of corresponding author). Test can be good 'mass screening test' at best. Singh and co-workers initially standardized d_ELISA for the screening of serum samples of domestic livestock at field level and reported higher bio-load (90.3%) as compared to i_ELISA (85.8%) [69]. Later d_ELISA was standardized for the screening of milk samples [28]. Besides, d_ELISA, LAT also had potential to be developed as excellent 'field based diagnostic kit' for 'mass/herd level screenings' [29]. Other researchers employed LAT for the detection of bovine tuberculosis [70,71], cystic echinococcosis [72] and ovine brucellosis [73]. Koo et al. [70] reported better sensitivity (86.7%) and specificity (97.8%) of LAT in bovine tuberculosis.

In this study, newly standardized assays (LAT, i_FAT and d_ELISA) were compared with widely used routine assays (i_ELISA, IS*900* PCR and microscopy) for the screening of MAP infection in clinical samples (Table 6). Screening of goat milk by 'antigen based' assays showed 96.5% agreement whereas number of samples mis-matched were lower (3.4%; i_FAT-2.8%, microscopy-0.6%) (Table 5a). In 'antibody based' assays agreement was 89.8% with lower mismatch of 9.2% (d_ELISA-3.8%, LAT-5.3%). This difference in screening of 'raw milk' was due to fact that 'antigen based' assays were quantitative whereas 'antibody based' assays were qualitative. Considering six assays together, the agreement was 95.3% and mismatch of 4.7% (microscopy-0.2%, d_ELISA-3.0%, LAT-1.2%). Only 3.0% milk samples detected positive exclusively in d_ELISA may be due to the qualitative nature of the test. Study reported that in case of 'raw milk samples', 'antibody assays' were superior to 'antigen based' in screening of milk samples against MAP infection. Of the three antibody assays used, not a single milk sample was detected independently. Results of this study also validated 'i_ELISA kit', as reliable screening test. With respect to choice of assays, study showed that for 'mass screening' of milk samples, depending on purpose, resources and finance any one of the assays (microscopy, i_ELISA, d_ELISA, LAT) can be used. Six test combinations were superior to three test combinations (Table 5a, 5b and 6). Therefore in chronic infections like MAP, it was prudent to use multiple assays. Singh and co-workers in 2014 recorded 7.6–69.8% and 23.0–84.4% bio-load of MAP in the milk of goats and cattle, using 'Indigenous milk-ELISA' and 'IS*900* PCR, respectively [1]. In another study, Raghuvanshi et al. [50] reported 30.7, 13.8 and 7.6% milk samples positive for MAP by i_ELISA, microscopy and IS*900* PCR, respectively. Co-relatively previous and present studies reported poor sensitivity of IS*900* PCR in the detection of MAP bacilli in raw milk samples. Variable bio-load of MAP was reported in small ruminants using routine assays [1]. No single test can be recommended as 'confirmatory' for the screening of goatherds [23] and use of multiple assays improved the probability of detecting a sample positive (detection rate) in chronic infections like JD.

High sensitivity of 'antibody assays' in goats may be due to the fact that source of antigen (PPA) was from a MAP strain 'S 5' ('Indian Bison Type') recovered from a goat suffering from clinical JD at CIRG [34]. Recent studies by Singh and co-workers co-related all the six antigen and antibody based detection assays for the screening of milk and milk products made from pasteurized milk [31]. Wherein, 'antigen based' assays together had higher' detection rates' as compared to 'antibody based' detection assays. This is clearly due to modifications in the protein moiety present in clinical sample, which gets destroyed due to heating in pasteurization or due to denaturing of the proteins during pasteurization. Therefore, in liquid milk and milk products made from pasteurized milk, it may be prudent to select microscopy over 'i_ELISA kit' as screening test [31]. Comparison of two assays combinations in screening of raw milk showed that i_FAT confirmed the cases detected by microscopy (considered having poor sensitivity) were 46.0% (214/265) samples and only 0.6% (3/265) samples were detected independently by microscopy. Similarly in antibody detection assays, LAT and d_ELISA showed better results and two assays together detected 50.3% positive (perfect agreement) and there was 9.2% mis-match.

All six assays were compared statistically and strength of agreement was estimated to be 'good' for d_ELISA with respect to i_ELISA with a kappa value of 0.646. For LAT with respect to i_ELISA, strength of agreement was 'moderate' with kappa value of 0.551. The strength of agreement was 'very good' for i_FAT with respect to microscopy with a kappa value of 0.931 (Table 5).

Culture though 'Gold standard' cannot be a routine screening test therefore was not included for the comparison purpose in this study. However, six test combinations were used to replace culture in order to establish presence of MAP infection. Representative samples were screened by culture to confirm presence of live acid fast MAP bacilli in milk samples. Of the 47 milk cultured, 27.6% were positive (Table 5). Bio-type profiles of MAP were estimated using IS*1311* PCR_RE. Only 175 MAP DNA could be bio-typed and out of which, 44.5% were 'Indian Bison Type' as reported in earlier studies in dairy animals [26,74]. Outside India, 'Indian Bison Type' has not been reported so far, however, there is only one report of 'Bison type' MAP from wild bison in Montana, USA [75], indicated 'Indian Bison type' was dominant biotype' in our domestic livestock.

Economy of dairy animals is linked to trading of high quality milk and milk products and requires a sanitary control program with the purpose of eradicating this disease. This is important especially when viable MAP carried with milk and milk products escape current pasteurization standards, presenting potential threat to human population. High bio-load of MAP as reported in the lactating goatherds in this study, points to the fact that infection of MAP is rampant in domestic livestock of the country and calls for immediate need for the control of Johne's disease. Since JD is also protein loosing enteropathy is also responsible for low per animal productivity of Indian domestic livestock breeds [40]. It also causes negative energy balance in the body leading to further stress on already scarce nutritional resources and the need to keep large number of animals to meet domestic needs for milk and milk products [69].

5. Conclusion

Bio-load of MAP was high in goats on the basis of screening of raw milk using six assays. Study compared efficacy of six assays and identified better diagnostic test / test combination ('testing strategy') for detecting presence of MAP in goat milk / goats. Study established the role of milk as carrier of MAP to newborn animals and human population. Study showed that MAP was the major, 'Milk born pathogen'. Thereby, helping in perpetuation of MAP infection generations to generations in animals and from animals to human population. The choice of test for screening of a population depends on the purpose and resources available. LAT, d_ELISA and microscopy had potential to be good screening assays for screening of 'raw milk'. Whereas, i_FAT and i_ELISA could be used as confirmatory assays. Milk was convenient and good clinical sample for estimating bio-load of MAP in lactating animals.

Conflict of interest

No conflict of Interest to declare.

Acknowledgements

Authors are thankful to MoFPI, New Delhi for providing the funds and Director, CIRG, Mathura for providing necessary facilities.

References

[1] S.V. Singh, P.K. Singh, A.V. Singh, J.S. Sohal, N. Kumar, Bio-load and bio-type profiles of *Mycobacterium avium* subspecies *paratuberculosis* infection in the domestic livestock population endemic for Johne's disease: a survey of 28 years (1985-2013) in India, Trans. Emerg. Dis. 61 (2014) 43–55.

[2] K.K. Chaubey, S.V. Singh, S. Gupta, M. Singh, J.S. Sohal, N. Kumar, M.K. Singh, A.K. Bhatia, K. Dhama, *Mycobacterium avium* subspecies *paratuberculosis*–an important food borne pathogen of high public health significance with special reference to India: an update, Vet. Q. 37 (2017) 282–299.

[3] A.V. Singh, S.V. Singh, G.K. Makharia, P.K. Singh, J.S. Sohal, Presence and characterization of *Mycobacterium avium* subspecies *paratuberculosis* from clinical and suspected cases of Crohn's disease and in the healthy human population in India, Int. J. Infect. Dis. 12 (2008) 190–197.

[4] R.J. Chiodini, W.M. Chamberlin, J. Sarosiek, R.W. McCallum, Crohn's disease and the mycobacterioses: a quarter century later. Causation or simple association? Crit. Rev. Microbiol. 38 (2012) 52–93.

[5] R.J. Greenstein, Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease, Lancet Infect. Dis. 3 (2003) 507–514.

[6] M. Feller, K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G.E. Pfyffer, T. Jemmi, A. Baumgartner, M. Egger, *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis, Lancet Infect. Dis. 7 (2007) 607–613.

[7] J.L. Mendoza, R. Lana, M. Díaz-Rubio, *Mycobacterium avium* subspecies *paratuberculosis* and its relationship with Crohn's disease, World J. Gastroenterol. 15 (2009) 417–422.

[8] P.S. Rani, L.A. Sechi, N. Ahmed, *Mycobacterium avium* subsp. *Paratuberculosis* as a trigger of type-1 diabetes: destination Sardinia, or beyond? Gut Path 2 (2010) 1–6.

[9] G. Rosenfeld, B. Bressler, *Mycobacterium avium paratuberculosis* and the etiology of Crohn's disease: a review of the controversy from the clinician's perspective, Can. J. Gastroenterol. Hepatol. 24 (2010) 619–624.

[10] S.A. Naser, S.R. Sagramsingh, A.S. Naser, S. Thanigachalam, *Mycobacterium avium* subspecies *paratuberculosis* causes Crohn's disease in some inflammatory bowel disease patients, World J. Gastroenterol. 20 (2014) 7403–7415.

[11] S.V. Singh, J.T. Kuenstner, W.C. Davis, P. Agarwal, N. Kumar, D. Singh, S. Gupta, K.K. Chaubey, A. Kumar, J. Misri, S. Jayaraman, Concurrent resolution of chronic diarrhea likely due to Crohn's disease and infection with Mycobacterium avium paratuberculosis, Front. Med. 3 (2016) 49.

[12] Y.J. Johnson-Ifearulundu, J.B. Kaneene, Management-related risk factors for *M. Paratuberculosis* infection in Michigan, USA, dairy herds, Prev. Vet. Med. 37 (1998) 41–54.

[13] A.B. Garcia, L. Shalloo, Invited review: the economic impact and control of paratuberculosis in cattle, J. Dairy Sci. 98 (2015) 5019–5039.

[14] A. Tiwari, J.A. VanLeeuwen, I.R. Dohoo, G.P. Keefe, A. Weersink, Estimate of the direct production losses in Canadian dairy herds with subclinical *Mycobacterium avium* subspecies *paratuberculosis* infection, Can. Vet. J. 49 (2008) 569–576.

[15] I. Fodor, B. Matyovszky, A. Biczó, L. Ózsvári, The losses due to paratuberculosis and its control in a Hungarian large-scale Holstein-Friesian dairy farm, Magy. Allatorvosok Lapja. 136 (2014) 213–222.

[16] K.K. Chaubey, R.D. Gupta, S. Gupta, S.V. Singh, A.K. Bhatia, S. Jayaraman, N. Kumar, A. Goel, A.S. Rathore, J.S.Sohal Sahzad, B.J. Stephen, M. Singh, M. Goyal, K. Dhama, A. Derakhshandeh, Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock, Vet. Q. 36 (2016) 203–227.

[17] FAOSTAT, Food and Agriculture Organization of the United Nations, (2016).

[18] C.O. Gill, L. Saucier, W.J. Meadus, *Mycobacterium avium* subsp. *Paratuberculosis* in dairy products, meat and drinking water, J. Food Protect. 74 (2011) 480–499.

[19] S. Corti, R. Stephan, Detection of *Mycobacterium avium* subspecies *paratuberculosis* specific IS*900* insertion sequences in bulk tank milk samples obtained from different regions throughout Switzerland, BMC Microbiol. 2 (2002) 15.

[20] J.R. Stabel, S.J. Wells, B.A. Wagner, Relationships between faecal culture, ELISA and bulk tank milk results for Johne's disease in US dairy herds, J. Dairy Sci. 85 (2002) 525–531.

[21] D. Tursun, Y.Z. Shi, Q.L. Yang, Y. Liu, W.C. Xue, A. Abas, W.J. Yang, Influence on strychni semen's analgesic effect and toxicity of milk-impregnated- processing-technology of traditional uighur medicine, Zhong Yao Cai 38 (2015) 267–270.

[22] G.J. Eamens, R.J. Whittington, I.B. Marsh, M.J. Turner, V. Saunders, P.D. Kemsley, D. Rayward, Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease, Vet. Microbiol. 77 (2000) 357–367.

[23] M.T. Collins, S.J. Wells, K.R. Petrini, J.E. Collins, R.D. Schultz, R.H. Whitlock, Evaluation of five antibody detection assays for diagnosis of bovine paratuberculosis, Clin. Diag. Lab. Immun. 12 (2005) 685–692.

[24] S.S. Nielsen, N. Toft, Bulk tank milk ELISA for detection of antibodies to *Mycobacterium avium* subsp. *paratuberculosis*: correlation between repeated assays and within-herd antibody-prevalence, Prev. Vet. Med. 113 (2014) 96–102.

[25] R. Garg, P.K. Patil, S.V. Singh, S. Sharma, R.K. Gandham, A.V. Singh, G. Filia, P.K. Singh, S. Jayaraman, S. Gupta, K.K. Chaubey, R. Tiwari, M. Saminathan, K. Dhama, J.S. Sohal, Comparative evaluation of different test combinations for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infecting dairy herds in India, Biomed Res. Int. (2015), https://doi.org/10.1155/2015/983978.

[26] G. Sharma, S.V. Singh, I. Sevilla, A.V. Singh, R.J. Whittington, R.A. Juste, V.S. Vihan, Evaluation of indigenous milk ELISA with m-culture and m-PCR for the diagnosis of Bovine Johne's disease (BJD) in lactating Indian dairy cattle, Res. Vet. Sci. 84 (2008) 30–37.

[27] S.V. Singh, B.J. Stephen, M. Singh, S. Gupta, K.K. Chaubey, S. Jayaraman Sahzad, T.K. Sachan, G.K. Aseri, M. Jain, J.S. Sohal, K. Dhama, Evaluation of Indirect Fluorescent Antibody Test (i_FAT) as the 'mass screening test' for the diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in the milk samples of lactating domestic livestock, J. Exp. Bio. Agric. Sci. 4 (2016) 533–540.

[28] S.V. Singh, B.J. Stephen, M. Singh, S. Gupta, K.K. Chaubey, S.Jayaraman Sahzad, G.K. Aseri, J.S. Sohal, A.K. Bhatia, J. Chauhan, K. Dhama, Evaluation of milk dot-ELISA as field based test vis a vis milk plate ELISA for the detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in lactating domestic livestock, Indian J. Biotech. 15 (2016) 166–171.

[29] S.V. Singh, B.J. Stephen, M. Singh, S. Gupta, K.K. Chaubey, S.Jayaraman, Sahzad, T.K. Sachan, J.S. Sohal, K. Dhama, S.Y. Mukartal, Z. Hemati, Comparison of newly standardized 'Latex milk agglutination test', with 'Indigenous milk ELISA' for 'on spot' screening of domestic livestock against*Mycobacterium avium* subsp. *paratuberculosis* infection, Indian J. Biotech. 15 (2016) 511–517.

[30] I. D'Haese, H. Dumon, V. Werbrouck, Jonghe De, L. Herman, Improved detection of *Mycobacterium paratuberculosis* in milk, J. Dairy Res. 72 (2005) 1–4.

[31] M. Singh, S. Gupta, S.V. Singh, K.K. Chaubey, J.S. Sohal, K. Dhama, Bio-incidence of *Mycobacterium avium* subspecies *paratuberculosis* in the pasteurized liquid milk, flavoured milk and milk powder commercially sold by leading market brands in India, J. Exp. Bio. Agri. Sci. 6 (2018).

[32] D. van Soolingen, P.W. Hermans, P.E. de Haas, D.R. Soll, J.D. van Embden, Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis, J. Clin. Microbiol. 29 (1991) 2578–2586.

[33] P.H. Vary, P.R. Andersen, E. Green, J. Hermon-Taylor, J. McFadden, Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease, J. Clin. Microbiol. 28 (1990) 933–937.

[34] I. Sevilla, S.V. Singh, J.M. Garrido, G. Aduriz, S. Rodriguez, M.V. Geijo, R.A. Juste, Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions, Rev. - Off. Int. Epizoot. 24 (2005) 1061–1066.

[35] R.S. Merkal, Laboratory diagnosis of bovine paratuberculosis, J. Am. Vet. Med. Asso. 163 (1973) 1100–1102.

[36] D.L. Whipple, D.R. Callihan, J.L. Jarnagin, Cultivation of *Mycobacterium paratuberculosis* from bovine fecal specimens and a suggested standardized procedure, J. Vet. Diagn. Invest. 3 (1991) 368–373.

[37] S.V. Singh, V. Vihan, Detection of *Mycobacterium avium* subsp. *Paratuberculosis* in goat milk, Small Rumin. Res. 54 (2004) 231–235.

[38] P.K. Singh, S.V. Singh, A.V. Singh, J.S. Sohal, Caprine paratuberculosis in farm and farmer's goat herds: an assessment of prevalence in target tissues, comparison of culture, PCR and indigenous ELISA kit and genotypes of *Mycobacterium avium* subspecies *paratuberculosis*, Indian J. Small Rumin. 14 (2008) 211–217.

[39] M.T. Collins, Interpretation of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay by using likelihood ratios, Clin. Diag. Lab. Immun. 9 (2002) 1367–1371.

[40] K.D. Rawat, S. Chaudhary, N. Kumar, S. Gupta, K.K. Chaubey, S.V. Singh, K. Dhama, R. Deb, Economic losses in a commercial dairy farm due to the outbreak of Johne's disease in India, Res. J. Vet. Pract. 2 (2014) 73–77.

[41] P. Kumar, S.V. Singh, A.K. Bhatia, I. Sevilla, A.V. Singh, R.J. Whittington, R.A. Juste, V.K. Gupta, P.K. Singh, J.S. Sohal, V.S. Vihan, Juvenile Capri-Paratuberculosis (JCP) in India: incidence and characterization by six diagnostic assays, Small Rumin. Res. 73 (2007) 45–53.

[42] R.W. Sweeney, M.T. Collins, A.P. Koets, S.M. McGuirk, A.J. Roussel, Paratuberculosis (Johne's disease) in cattle and other susceptible species, J. Vet. Int. Med. 26 (2012) 1239–1250.

[43] A. Serraino, P. Bonilauri, F. Giacometti, M. Ricchi, G. Cammi, S. Piva, V. Zambrini, A. Canever, N. Arrigoni, Investigation into *Mycobacterium avium* ssp. *Paratuberculosis* in pasteurized milk in Italy, J. Dairy Sci. 100 (2017) 118–123.

[44] S.V. Singh, A.V. Singh, R. Singh, K.S. Sandhu, P.K. Singh, J.S. Sohal, V.K. Gupta, V.S. Vihan, Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal-PCR for the diagnosis of bovine Johne's disease (BJD) in India, Comp. Immun. Microbiol. Infect. Dis. 30 (2007) 175–186.

[45] H. Shankar, S.V. Singh, P.K. Singh, A.V. Singh, J.S. Sohal, R.J. Greenstein, Presence, characterization, and genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* from unpasteurized individual and pooled milk, commercial pasteurized milk and milk products in India by culture, PCR and PCR-REA methods, Int. J. Infect. Dis. 14 (2010) e121–126.

[46] B.J. Stephen, M. Singh, S.V. Singh, S. Gupta, K.K. Chaubey, M.Jain Sahzad, J.S. Sohal, S.Y. Mukartal, K. Dhama, Bio-contamination' estimates of *Mycobacterium avium* subspecies *paratuberculosis* in fresh cottage cheese (Paneer) sold in rural, semi-urban and peri-urban regions of South Uttar Pradesh using multiple diagnostic tests, Adv. Anim. Vet. Sci. 4 (2016) 441–448.

[47] G. Banche, V. Allizond, R. Sostegni, A. Lavagna, M. Bergallo, F. Sidoti, M. Daperno, R. Rocca, A.M. Cuffini, Application of multiple laboratory tests for *Mycobacterium avium* ssp. *Paratuberculosis* detection in Crohn's disease patient specimens, New Microbiol. 38 (2015) 357–367.

[48] I.A. Carvalho, A. Silva, V.E. Campos, M.A. Moreira, Short communication: detection of *Mycobacterium avium* subspecies *paratuberculosis* by polymerase chain reaction in bovine milk in Brazil, J. Dairy Sci. 92 (2009) 5408–5410.

[49] S.R. Pillai, B.M. Jayarao, Application of IS*900* PCR for detection of *Mycobacterium avium* subsp. *Paratuberculosis* directly from raw milk, J. Dairy Sci. 85 (2002)

1052–1057.
[50] T.S. Raghuvanshi, R.B. Sharma, A.V. Singh, B. Singh, S.V. Singh, K. Dhama, 'Indigenous milk ELISA kit' vis a vis multiple test regime for the estimation of lacto–prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in goatherds endemic for Johne's disease, Indian J. Comp. Microbiol. Immun. Infect. Dis. 31 (2010) 41–43.
[51] O.R. VinodhKumar, L. Gunaseelan, B.S. Ronald, S.M. Sakthivelan, Slaughterhouse prevalence of ovine paratuberculosis in Southern India, Trop. Anim. Health Prod. 45 (2013) 1063–1069.
[52] P. Kaur, G. Filia, S.V. Singh, P.K. Patil, K.S. Sandhu, Molecular detection and typing of *Mycobacterium avium* subspecies *paratuberculosis* from milk samples of dairy animals, Trop. Anim. Health Prod. 42 (2010) 1031–1035.
[53] I.A. Carvalho, P.A. Pietralonga, D.G. Schwarz, A.C. Faria, M.A. Moreira, Recovery of viable *Mycobacterium avium* subspecies *paratuberculosis* from retail pasteurized whole milk in Brazil, J. Dairy Sci. 95 (2012) 6946–6948.
[54] I.R. Grant, A.G. Williams, M.T. Rowe, D.D. Muir, Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *Paratuberculosis* in milk, Appl. Environ. Microbiol. 71 (2005) 2853–2861.
[55] S. Kumar, S.V. Singh, I. Sevilla, A.V. Singh, R.J. Whittington, R.A. Juste, G. Sharma, P.K. Singh, J.S. Sohal, Lacto-prevalence, genotyping of *Mycobacterium avium* subspecies *paratuberculosis* and evaluation of three diagnostic tests in milk of naturally infected goatherds, Small Rumin. Res. 74 (2008) 37–44.
[56] S.V. Singh, N. Kumar, K.K. Chaubey, S. Gupta, K.D. Rawat, Bio-presence of *Mycobacterium avium* subspecies*paratuberculosis* infection in domestic livestock farms, Res. Opin. Anim. Vet. Sci. 3 (2013) 401–406.
[57] M. Soltani, M.R. Nassiry, F.E. Shahroudi, M.R. Bassami, Detection of *Mycobacterium paratuberculosis* in feces and milk samples from holstein dairy cows by PCR, Biotech. 7 (2008) 582–595.
[58] W.Y. Ayele, P. Svastova, P. Roubal, M. Bartos, I. Pavlik, *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic, Appl. Environ. Microbiol. 71 (2005) 1210–1214.
[59] F.A. Paolicchi, M.J. Zumarraga, A. Gioffre, P. Zamorano, C. Morsella, A. Verna, A. Cataldi, A. Alito, M. Romano, Application of different methods for the diagnosis of paratuberculosis in a dairy cattle herd in Argentina, Zoonoses Public Health 50 (2003) 20–26.
[60] S.V. Singh, S. Gupta, K.K. Chaubey, S. Bhushan, K.D. Rawat, N. Kumar, H.A. Tiwari, V. Chaturvedi, J.S. Sohal, K. Dhama, Z. Hemati, 'Therapeutic management of incurable paratuberculosis using' Indigenous vaccine' in goatherds, endemically infected with Johne's disease, Int. J. Pharmacol. 13 (2017) 145–155.
[61] S.V. Singh, P.K. Singh, A.V. Singh, J.S. Sohal, V.K. Gupta, V.S. Vihan, Comparative efficacy of an indigenous 'inactivated vaccine' using highly pathogenic field strain of *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' with a commercial vaccine for the control of Capri-paratuberculosis in India, Vaccine 25 (2007) 7102–7110.
[62] S. Jayaraman, M. Jain, K. Dhama, S.V. Singh, M. Datta, N. Jain, K.K. Chaubey, S. Gupta, G.K. Aseri, N. Khare, P. Yadav, A.K. Bhatia, J.S. Sohal, Diva technology: indispensable tool for the control of johne"s disease, J. Exp. Biol. Agri. Sci. 4 (2016) 17–25.
[63] L.W. Chui, R. King, P. Lu, K. Manninen, J. Sim, Evaluation of four DNA extraction methods for the detection of *Mycobacterium avium* subsp. *Paratuberculosis* by polymerase chain reaction, Diag. Microbiol. Infect. Dis. 48 (2004) 39–45.
[64] O.P. Paliwal, B.S. Rajya, S.G. Krishna, Fluorescent antibody technique (FAT) in the diagnosis of *Mycobacterium johnei* infection in goats, Indian J. Vet. Path. 7 (1983) 6–14.
[65] B. Abbas, H.P. Riemann, B. Lonnerdal, Isolation of specific peptides from *Mycobacterium paratuberculosis* protoplasm and their use in an enzyme-linked immunosorbent assay for the detection of paratuberculosis (Johne's disease) in cattle, Am. J. Vet. Res. 44 (1983) 2229–2236.
[66] S. Chaturvedi, S.V. Singh, A.K. Srivastava, N.K. Gangwar, N. Kumar, K.D. Rawat, S. Gupta, K.K. Chaubey, R.A. Singh, R. Singh, K. Dhama, Comparative evaluation of FAT, IS*900* PCR and microscopy vis a vis histo-pathology for the detection of *Mycobacterium avium* subsp *paratuberculosis* infection in tissues of goats naturally died in herds endemic for Johne's disease, Indian J. Anim. Sci. 87 (2017) 18–26.
[67] D.J. Dean, M.K. Ableseth, P. Atanasiu, The fluorescent antibody test, in: F.-X. Meslin, M.M. Kaplan, H. Koprowsky (Eds.), Laboratory Techniques in Rabies, 4th ed., WHO, Geneva, Switzerland, 1996, pp. 88–96.
[68] T.M. Foral, R.A. French, H.J. Van Kruiningen, A.E. Garmendia, Fluorescent antibody test for rapid detection of West Nile Virus antigen in avian tissues, Avian Dis. 51 (2007) 601–605.
[69] S.V. Singh, S.D. Audarya, M. Singh, B.J. Stephen, D. Chhabra, Development of new 'Indigenous Dot-ELISA Kit'as sensitive field based herd screening test for the diagnosis of Johne's disease in the domestic buffalo population, Asian J. Anim. Vet. Adv. 11 (2016) 44–52.
[70] H.C. Koo, Y.H. Park, J. Ahn, W.R. Waters, M.V. Palmer, M.J. Hamilton, G. Barrington, A.A. Mosaad, K.T. Park, W.K. Jung, I.Y. Hwang, Use of rMPB70 protein and ESAT-6 peptide as antigens for comparison of the enzyme-linked immune-sorbent, immune-chromatographic, and latex bead agglutination assays for sero-diagnosis of bovine tuberculosis, J. Clin. Microbiol. 43 (2005) 4498–4506.
[71] L. Ganju, H.V. Batra, G.P. Talwar, R. Mukherjee, A rapid latex agglutination test for detection of antibodies in tuberculosis and Hansen's disease, J. Immun. 12 (1991) 579–595.
[72] A. Sheeba, A. Sangaran, B.R. Latha, Diagnosis of cystic echinococcosis in buffaloes by native 8 kDa antigen using latex agglutination test (LAT), J. Para. Dis. 40 (2016) 1401–1405.
[73] A.B. Ismael, A.A. Swelum, S.A. Mostafa, A.R. Alhumiany, Latex agglutination using the periplasmic proteins antigen of *Brucella melitensis* is a successful, rapid, and specific serodiagnostic test for ovine brucellosis, Int. J. Immunopathol. Pharmacol. 29 (2016) 480–487.
[74] D. Yadav, S.V. Singh, A.V. Singh, I. Sevilla, R.A. Juste, P.K. Singh, J.S. Sohal, Pathogenic' Bison–type' *Mycobacterium avium* subspecies *paratuberculosis* genotype characterized from riverine buffalo (*Bubalus bubalis*) in North India, Indian J. Comp. Microbiol. Immun. Infect. Dis. 31 (2008) 373–387.
[75] R.J. Whittington, J.B. Lloyd, L.A. Reddacliff, Recovery of *Mycobacterium avium* subsp. *Paratuberculosis* from nematode larvae cultured from the faeces of sheep with Johne's disease, Vet. Microbiol. 81 (2001) 273–279.
[76] Z. Dimareli-Malli, Detection of *Mycobacterium avium* subsp. *Paratuberculosis* in milk from clinically affected sheep and goats, Int. J. Appl. Res. Vet. Med. 8 (2010) 44–50.
[77] E. Angelidou, P. Kostoulas, L. Leontides, Bayesian validation of a serum and milk ELISA for antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in Greek dairy goats across lactation, J. Dairy Sci. 97 (2014) 819–828.
[78] E. Angelidou, P. Kostoulas, L. Leontides, Flock-level factors associated with the risk of *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) infection in Greek dairy goatherds, Prev. Vet. Med. 117 (2014) 233–241.
[79] I.R. Grant, L.M. O'Riordan, H.J. Ball, M.T. Rowe, Incidence of *Mycobacterium paratuberculosis* in raw sheep and goats' milk in England, Wales and Northern Ireland, Vet. Microbiol. 79 (2001) 123–131.
[80] B. Djønne, M.R. Jensen, I.R. Grant, G. Holstad, Detection by immunomagnetic PCR of *Mycobacterium avium*subsp.*paratuberculosis* in milk from dairy goats in Norway, Vet. Microbiol. 92 (2003) 135–143.
[81] P. Nebbia, P. Robino, S. Zoppi, D. De Meneghi, Detection and excretion pattern of *Mycobacterium avium* subspecies *paratuberculosis* in milk of asymptomatic sheep and goats by Nested-PCR, Small Rumin. Res. 66 (2006) 116–120.
[82] A. Galiero, B. Turchi, F. Pedonese, R. Nuvoloni, C. Cantile, G. Colombani, M. Forzan, D. Cerri, P. Bandecchi, F. Fratini, Serological, culture and molecular survey of *Mycobacterium avium paratuberculosis* in a goat flock in Tuscany, Folia Microbiol. 62 (2017) 471–477.
[83] D.G. Schwarz, M.C. Lima, M. Barros, F.L. Valente, T.M. Scatamburlo, N. Rosado, C.T. Oliveira, L.L. Oliveira, M.A. Moreira, Passive shedding of *Mycobacterium avium* ssp. *Paratuberculosis* in commercial dairy goats in Brazil, J. Dairy Sci. 100 (2017) 8426–8429.
[84] K.J. van Hulzen, A.P. Koets, M. Nielen, J. Hoeboer, J.A. van Arendonk, H.C. Heuven, Genetic variation for infection status as determined by a specific antibody response against *Mycobacterium avium* subspecies *paratuberculosis* in milk of Dutch dairy goats, J. Dairy Sci. 95 (2012) 6145–6151.