MULTIPLE DIAGNOSTIC TESTS BASED BIO-LOAD AND BIO-TYPE PROFILES OF MYCOBACTERIUM AVIUM SUB-SPECIES PARATUBERCULOSIS INFECTION IN RAW MILK OF BUFFALOES

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ABSTRACT

Raw milk samples of 503 individual buffaloes were collected from dairy farms located in Agra and Mathura cities in North India. Multiple tests {Indirect Fluorescent Agglutination test (i_FAT), IS*900* PCR, Microscopy, Indigenous ELISA kit (i_ELISA), Dot-ELISA (d_ELISA) and Latex agglutination test (LAT)} based bio-load and bio-type profile of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was studied. Cumulatively average bio-load was 61.2% using three antigen and three antibody based. In i_FAT, IS*900* PCR and microscopy, 43.5, 13.3 and 40.9% milk were positive for MAP, respectively. Whereas, 32.8, 49.3 and 44.1% milk samples were positive in i_ELISA, d_ELISA and LAT, respectively. Bio-typing of representative milk samples using IS*900* PCR positive raw milk (67), 13.4% were infected with 'Indian Bison Type' biotype using IS*1311* PCR_REA. Study concluded that 'Indian Bison type' was the predominant bio-type infecting lactating buffaloes of this region. Raw milk was highly convenient sample in buffaloes and 'milk samples' were first time screened without initial processing of milk samples. Detection limits of each tests was improved. Results of five tests (d_ ELISA, LAT, i_ELISA, microscopy, i_FAT were comparable, except IS*900* PCR. High bio-load of MAP in milk of buffaloes was major health hazard for human health. High bio-load of MAP was alarming and calls for initiation of Johne's disease control programs in the country.

Keywords: *Bubalus bubalis*, buffaloes, *Mycobacterium avium* subspecies *paratuberculosis*, buffalo milk, indigenous plate ELISA kit, Dot-ELISA, latex agglutination test, indirect fluorescent antibody test, IS*900* PCR, Indian Bison Type

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP), the cause of Johne's disease (JD) in ruminants is endemic in domestic livestock population and is also a strong candidate for inflammatory bowel disease (Crohn's disease) in humans (Singh *et al*., 2008a; Chiodini *et al*., 2012). Early estimation of the bio-load of MAP is critical for the control of disease in the dairy farms (Eamens *et al*., 2000) and bovine population of the country. MAP infection leads to serious economic loss to the dairy industry by decreased milk yield, low feed conversion efficiency, lowered fertility,

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etc., (Garcia and Shalloo, 2015), which leads to low per animal productivity and is a major problem faced by Indian livestock (Singh *et al*., 2016a).

Bovine milk is major source of human exposure to MAP (Gill *et al*., 2011) and raw milk is frequently used in herbal medicines in this part of the world (Tursun *et al*., 2015). Studies have showed that pasteurizational processes can lead to a significant decrease of MAP in milk and dairy products (Gill *et al*., 2011; Okura *et al*., 2012). However, few reports addressed the survival abilities of MAP bacilli in raw milk, pasteurized milk and dairy products (Shankar *et al*., 2010; Raghuvanshi *et al*., 2014) since MAP is not in-activated during pasteurization (D'haese *et al*., 2005). High bio-load of MAP in raw milk could lead to contamination of dairy products (Gill *et al*., 2011). Many workers (Eltholth *et al*., 2009; Okura *et al*., 2012; Botsaris *et al*., 2016) reported presence of live MAP in raw and pasteurized liquid milk, cheese, and other dairy products made from bovine milk and sold in retail market, is serious risk to the consumers. Consumption of milk and milk based products made from milk of infected animals is the source for transmission of MAP to human population (Chaubey *et al*., 2017; Corti and Stephan, 2002; Stabel *et al*., 2002). Dormant MAP bacilli or dead cells and structural components of MAP also reported to cause health risks (Malli, 2010).

India is the highest milk producer in the world with >143 million tonnes of milk per year (FAO, 2016). Buffalo population in the country has shown increasing trend in last 70 years. All low or un-productive buffaloes go for slaughter for meat production. Despite high slaughter rate per buffalo production is very low. Since buffalo is Asian animal, there is not a single study that report economic losses due to MAP infection. Though, several studies (Tiwari *et al*., 2008; Fodor *et al*., 2014) have reported high economic losses (EUR 35-165 per cow) in cows. Singh *et al.* (2014) reported consistent increase in the bio-load of MAP in the domestic livestock population every 5 yearly interval in past 32 years (1985 to 2017). In the absence of rapid, cost effective and sensitive 'indigenous diagnostic' test or kits MAP continues to infect large number of naive buffaloes (Chaubey *et al*., 2016).

For estimating bio-load of MAP, use of multiple tests has been recommended since no single test can accurately detect MAP infection in animals (Collins *et al.,* 2005; Singh *et al.,* 2014). Microscopy, indigenous ELISA (p-ELISA) and IS*900* PCR have been frequently used for the diagnosis (Nielsen and Toft, 2014; Garg *et al.,* 2015) and all three tests have shown high sensitivity and specificity (Stabel *et al.,* 2002; Collins *et al.,* 2005; Sharma *et al.,* 2008). Recently we have standardized, Indigenous dot-ELISA (d_ELISA), indirect Fluorescent antibody test (i_FAT) and Latex agglutination test (LAT) for the detection of MAP in milk samples (Singh *et al.,* 2016b; Singh *et al.,* 2016c; Singh *et al.,* 2016d) and were employed for the screening of raw milk samples in this study. Tests were useful in confirming the presence of MAP in lactating buffaloes. Present study estimated bio-load and bio-type profile of MAP in raw milk of lactating buffaloes using multiple diagnostic tests (microscopy, IS*900* PCR, indigenous ELISA kit, IS*1311* PCR, culture) and newly standardized tests (dot-ELISA, Latex agglutination test and indirect Fluorescent antibody test). Bio-type profile of MAP was studied using IS*1311* PCR_RE in IS*900* PCR positive milk in milk samples. Culture of representative milk samples was performed to know the presence of live MAP bacilli in the milk of lactating buffaloes.

MATERIALS AND METHODS

Collection and processing of milk

Table 1, shows profile of 503 raw milk samples collected from buffaloes belonging to farm and farmer's herds in Uttar Pradesh (Mathura-349, Agra-154) in North India. Un-like earlier studies (Stephan *et al*., 2016), wherein milk samples were first centrifuged to concentrate the MAP bacilli by spinning at 4000 rpm for 45 minutes. First time in this study, 'whole milk' of buffaloes was used as 'test sample'. Using traditional (microscopy, IS*900* PCR, i_ELISA) and newly standardized tests (d_ELISA, LAT and i_FAT), 465 milk samples were screened by each of six tests. To know bio-type profile and presence of live bacilli in raw milk of goats, IS*1311* PCR_RE and culture were used, respectively. Each individual buffalo was represented by one milk sample.

Acid Fast Staining (Microscopy):

Smears made from 20 µl of whole milk, heat fixed, stained by Ziehl Neelsen method (Singh *et al*., 2008b) and examined for acid-fast bacilli (AFB) indistinguishable to MAP (Figure 1).

Indirect fluorescent antibody test (i_FAT) Tissue based i_FAT (D' Haese *et al.,* 2005) was modified and standardized in milk samples by Singh *et al.* (2016b and 2018). Slides positive for MAP infection exhibited green fluorescence (Figure 2).

DNA isolation DNA isolation from buffaloes' raw milk was carried out as per van Soolingen *et al*. (1991) with some modifications (Singh *et al*., 2018).

IS*900* PCRDNA isolated from whole milk was subjected to specific IS*900* PCR using 150C and 921 primers of Vary *et al.* (1990). Presence and yield of specific PCR product (229bp) was considered as positive for MAP infection (Figure 3).

IS*1311* PCR and restriction endonuclease analysis (IS*1311* PCR _REA)IS*1311* PCR of IS*900* PCR positive DNA samples was performed using M56 and M119 primers as per Sevilla *et al.* (2005) (Figure 4).

CultureIS*900* PCR positive milk samples were cultured by decontaminating 1.0 mL of whole milk in 0.9% hexa-decylpyridinium chloride (HPC) for 18 hours at room temperature. From 1.0 mL sediment, 0.02 mL residual mucilaginous sludge was inoculated on the modified Herrold's egg yolk medium (HEYM) (Merkal and Richards, 1972; Whipple *et al*., 1991) with Mycobactin J (Allied Monitor Inc., MO, USA). Tubes were screened weekly up to 20 months for the appearance of typical MAP colonies (Singh and Vihan, 2004).

Indigenous plate ELISA test (i_ELISA)

Test was performed as per Singh *et al.* (2016c and 2018) and sample (100 µl of raw milk diluted in 1X PBST with 1.0% BSA in ratio of 1:1) was added in duplicate wells. Absorbance was read at 450 nm in micro-plate reader (i-Mark, Biorad). Milk from weak and culture positive and healthy and culture negative buffaloes were used as positive and negative controls, respectively.

Analysis of OD values

Optical densities (OD) were expressed as sample-to-positive (S/P) ratios (Collins, 2002), indicating corresponding status of JD in buffaloes milk used in this study. Samples in low positive (LP), positive (P) and strong positive (SP) categories

were considered as positive for MAP infection.

Dot- ELISA (d_ELISA)

Test was performed as per Singh *et al.* (2016c). Positive and negative controls used in i_ ELISA were coated on two legs of each comb to help in reading the results (Figure 5).

Latex agglutination test (LAT)

LAT was used as per Singh *et al.* (2016d and 2018). Milk sample was considered positive, if agglutination was observed within 2 minutes and negative if no agglutination in 2 minutes (Figure 6).

Statistical analysis

Buffalo positive in any one of the six tests was considered positive for MAP infection. Statistical significance between two tests was measured. Mc Nemar's test and kappa agreement methods applied using Graph Pad software, USA. Sensitivity and specificity of the tests was estimated by Med-Calc software, Belgium.

RESULTS

Of 503 buffaloes milk samples screened, cumulatively average bio-load was 61.2% using six (three antigen and three antibody detection tests) tests (Table 1).

Of the 503 milk samples from buffaloes screened by antigen detection tests, 40.9, 43.5 and 13.3% were positive in microscopy i_FAT, and IS*900* PCR respectively. Together 45.3% milk samples were positive in atleast one of the three tests and 6.1% were detected exclusively in single test (i_FAT - 4.3% and microscopy - 1.7%). Agreement in the three test combination was 93.9% (Table 2).

In antibody detection tests, 32.8, 49.3 and 44.1% milk were positive in i_ELISA, d_ELISA and LAT, respectively. Three tests together detected 57.0% milk samples positive and 11.5% were positive exclusively by single test (d_ELISA - 3.7%, LAT - 7.7%). Agreement in three test combination was 88.5%, (Table 3).

Total samples *n* -503; (-): Denotes the negative samples in individual test of that particular test combination; (+): Denotes the positive samples in individual test of that particular test combination; 1-27: Maximum permutation and combinations possible in 6 test regimen; Total-n: Represents only total positive samples in that particular test combination.

Together 61.2% milk were positive in atleast one of the six tests and 5.1% were positive exclusively in single test (i_FAT - 0.4%, microscopy - 0.6%, d_ELISA - 2.3% and LAT - 1.6% and 0.2% in i_ELISA) (Table 4). Agreement in six test combination was 94.9%. Study showed that of 61.2% positive raw buffaloes milk samples positive in six tests combinations, majority (56.1%) were detected positive in more than one test combination. Combinations of six, five, four, three and two tests combinations detected 6.5, 17.9, 12.3, 7.7, 15.1 and 5.1% milk samples positive, respectively (Table 4).

Culture though 'Gold standard' can neither be a routine screening test nor was included for comparison purpose in this study. However, of the total 503 raw milk representative (67) buffaloes were screened by culture for primary isolation of MAP on HEY medium supplemented with Mycobactin J. Typical MAP colonies were observed in 17.9% milk of buffaloes at the end of 6 months of incubation at 37ºC.

Of 503 milk screened statistically, kappa

Table 1. Profile of raw milk samples (n - 503) collected from farm and farmer's herds in North India for screening against *Mycobacterium avium* subspecies *paratuberculosis* infection.

Sn	Geographical Regions / Districts	Places	Buffaloes milk, n	Positive / % (n)
1	Mathura, Uttar Pradesh	Jhandipur and Shahpur	40	60.0 (24)
		Mathura city	34	61.7 (21)
		Farah and nearby villages	189	60.8 (115)
		Makhdoom village	86	59.3 (51)
2	Agra, Uttar Pradesh	Agra city	154	62.9 (97)
Total			503	61.2 (308)

Table 2. Comparison antigen detection tests in raw milk of individual buffaloes (n-503).

Diagnostic tests	Antigen detection tests								Total
	Test combinations								
	1	2	3	4	5	6	7	8	
i_FAT	+	-	+	+	-	+	-	-	43.5 (219)
Microscopy	+	-	+	-	+	-	+	-	40.9 (206)
IS*900* PCR	+	-	-	+	+	-	-	+	13.3 (67)
Total n (%)	13.3 (67)	54.6 (275)	25.8 (130)	0	0	4.3 (22)	1.7 (9)	0	45.3 (228)

*Figures in parenthesis are percent, Total samples (n) =503, (-) - negative, (+) – Positive.

Antigen based tests detected 45.3% (228/503) milk samples positive or buffaloes positive for MAP infection.

Table 3. Comparison antibody detection tests in raw milk of individual buffaloes (n-503).

Diagnostic Tests	Antibody detection tests								Total
	Test combinations								
	1	2	3	4	5	6	7	8	
i_ELISA	+	-	-	+	+	-	-	+	32.8 (165)
d_ELISA	+	-	+	+	-	+	-	-	49.3 (248)
LAT	+	-	+	-	+	-	+	-	44.1 (222)
Total: n (%)	23.6 (119)	42.9 (216)	12.7 (64)	9.1 (46)	0	3.7 (19)	7.7 (39)	0	57.0 (287)

*Figures in parenthesis are percent, Total samples (n) =503, (-) - negative, (+) – Positive

Antibody based tests detected 57.0% (287/503) milk samples positive or buffaloes positive for MAP infection.

Table 4. Comparison of antigen and antibody tests in raw milk of individual buffaloes (n-503).

Diagnostic tests	**Test combinations**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
i_FAT	+	-	+	+	+	+	+	+	+	+	-	-	+	-
IS*900* PCR	+	-	-	+	+	-	+	-	+	-	-	-	-	-
Microscopy	+	-	+	+	+	+	+	-	+	-	-	+	+	+
ip_ELISA	+	-	+	-	-	-	-	+	-	-	+	-	-	+
d_ELISA	+	-	+	+	-	+	+	+	-	+	+	+	-	+
LAT	+	-	+	+	+	+	-	+	-	+	+	+	+	-
Total % (n)	6.5 (33)	38.7 (195)	14.3 (72)	3.5 (18)	2.1 (11)	4.3 (22)	0.6 (3)	1.6 (8)	0.4 (2)	0.8 (4)	0.9 (5)	0.6 (3)	3.1 (16)	0.2 (1)
			17.9 (90)		12.3 (62)				7.7 (39)					

Diagnostic tests	**Test combinations**													**Total**
	15	16	17	18	19	20	21	22	23	24	25	26	27	
i_FAT	+	+	-	+	+	-	-	+	+	-	-	-	-	43.5 (219)
IS*900* PCR	-	-	-	-	-	-	-	-	-	-	-	-	-	13.3 (67)
Microscopy	-	+	+	-	-	-	-	+	-	-	+	-	-	40.9 (206)
ip_ELISA	+	+	-	-	-	-	+	-	-	-	-	-	+	32.8 (165)
d_ELISA	+	+	+	+	-	+	+	-	-	+	-	-	-	49.3 (248)
LAT	-	-	-	-	+	+	-	-	-	-	-	+	-	44.1 (222)
Total % (n)	0.4 (2)	1.2 (6)	0.4 (2)	0.4 (2)	0.8 (4)	3.3 (17)	7.3 (37)	0.4 (14)	0.4 (2)	2.3 (12)	0.6 (3)	1.6 (8)	0.2 (1)	61.2 (308)
			15.1 (76)						5.1 (26)					

LAT- Latex Agglutination test; i_FAT- Indirect Fluorescent Antibody test.

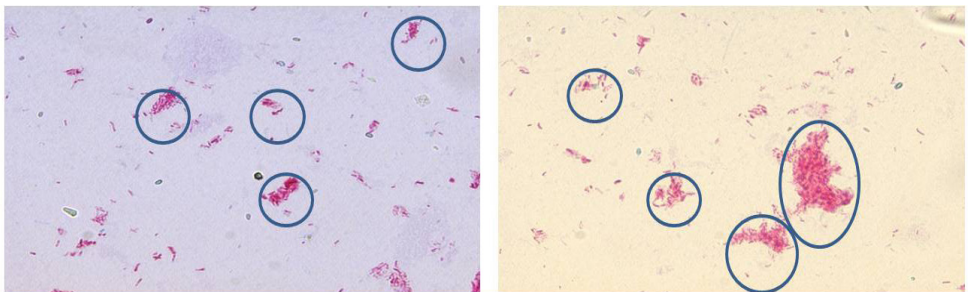

Figure 1. MAP bacilli as seen after acid fast staining in buffalo raw milk sample.

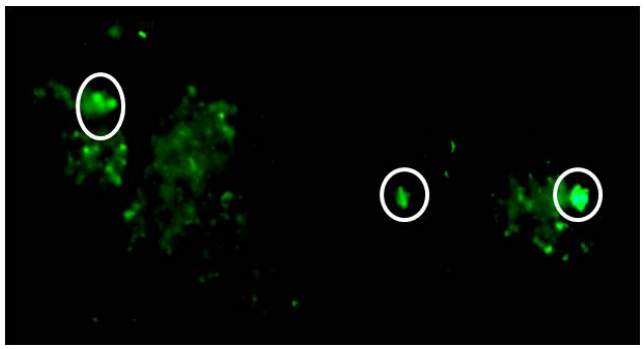

Figure 2. Green fluorescence indicates the presence of MAP bacilli in buffalo raw milk sample by Indirect Fluorescent antibody test.

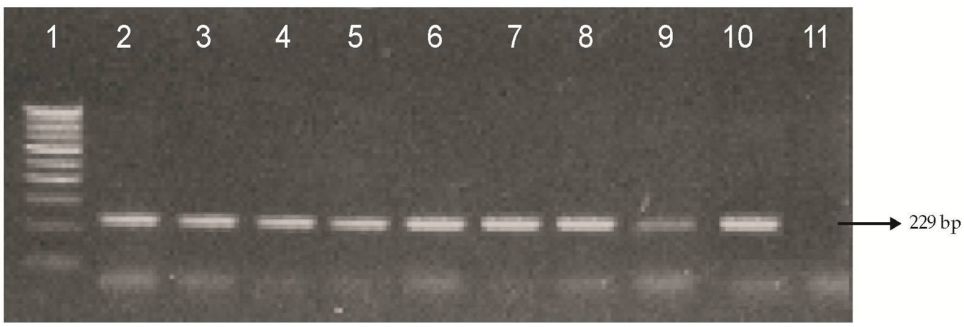

Figure 3. Agarose gel electrophoresis of PCR products obtained by IS*900* PCR (413bp) performed on buffalo raw milk sample.

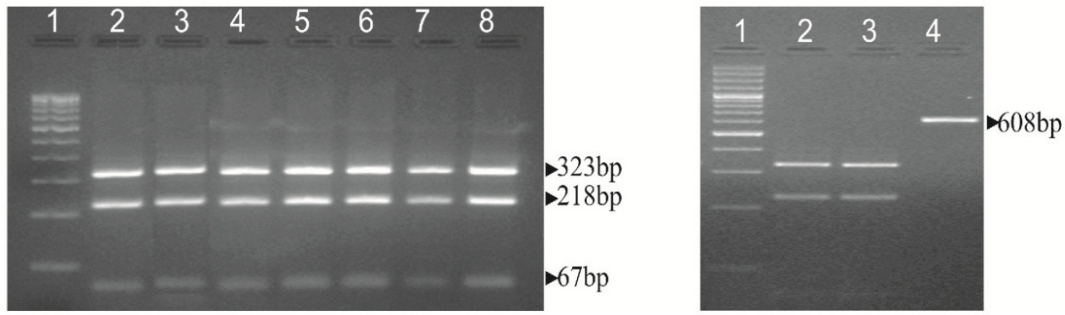

Figure 4. Molecular characterization of IS*900* positive MAP DNA in buffalo raw milk sample by IS*1311* PCR and PCR- IS*1311*_REA analysis.

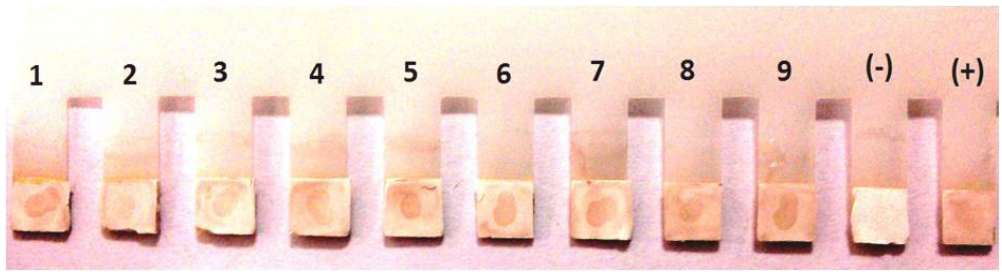

Figure 5. Dot-ELISA of individual buffalo raw milk samples (1-9) showing presence of MAP antibodies as positive brown dot; +ve: Positive control (brown dot); -ve: Negative control (no brown dot).

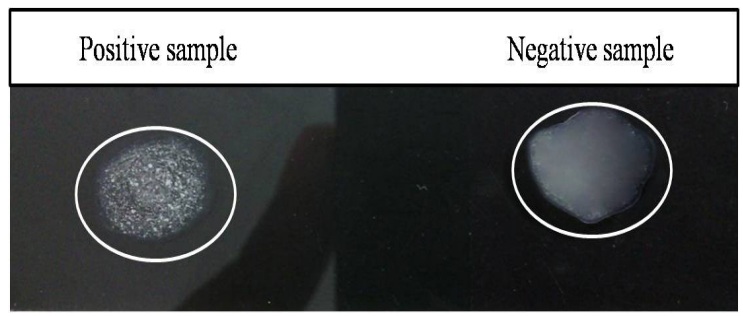

Figure 6. Latex Agglutination test results for the detection of MAP antibodies in buffalo raw milk sample as positive milk showing agglutination and negative milk without agglutination.

values were 0.909, 0.320, 0.661 and 0.480 for i_FAT, IS*900* PCR, d_ELISA and LAT, respectively. In antigen tests, i_FAT and IS*900* PCR had almost perfect and fair level of agreement with respect to microscopy. Using antibody tests, d_ELISA and LAT gave substantial and moderate level of agreement with respect to i_ELISA, respectively. With respect to microscopy, i_FAT and IS*900* PCR gave sensitivity of 97.7 and 30.0%, respectively. Specificity of i_FAT and IS*900* PCR with respect to microscopy was 93.5 and 100.0%, respectively. With respect to i_ELISA, sensitivity of d_ELISA and LAT was 99.7 and 82.2%, respectively. d_ELISA and LAT had specificity of 73.1 and 69.5% with respect to i_ELISA, respectively (Table 5).

DISCUSSION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD) in domestic livestock and has been associated with Crohn's disease in humans (Messelhäusser *et al*., 2012). Shedding of MAP in milk has received more attention MAP is milk borne pathogen and transmit MAP from animals to humans (Carvalho *et al*., 2009). There are several routes by which MAP reach to new buffaloes and infect others buffaloes. From these routes, oral and fecal route is regarded as the main horizontal transmission. The fecal and oral is the main route of transmission by which MAP bacilli spreads from one animal to other animals and to other species (Sweeney *et al*., 1992; Greig *et al*., 1999; Ayele *et al*., 2002). Transmission of MAP was also reported through consumption of colostrum and milk, in which new born calves get MAP infection from infected mothers (Nielsen *et al*., 2008).Studies reported high bio-load of MAP in the raw milk of dairy buffaloes using single tests (milk–ELISA) by Singh *et al.* (2013); Eisenberg *et al.* (2015).

Although Johne's disease was first reported in India in 1913 and is still a major health problem in domestic and wild ruminants (Singh *et al*., 2014) and still Nation-wide estimates on bio-load of MAP infection are not available. In a major study by Singh *et al.* (2014), reported 28.3% bio-load of MAP infection in buffaloes from North India in last 28 years (1985 to 2013). Present study also showed that at least 61.2% can be considered as positive for MAP infection, since these buffaloes were detected positive using six tests combinations (Table 4).

Though India is highest milk producer in the world, per capita milk availability has reached to 290 g / day (2011 to 2012) was higher than world average of 284 gm/day. Milk has been regarded as the main source of transmission of MAP from infected animals to human beings (Banche *et al*., 2015). Milk has been regarded as the important means of transmission of MAP from infected animals to human beings (Banche *et al*., 2015). Milk is the most convenient sample for screening of lactating cattle and buffaloes for MAP infection, in view of zoonotic implications of MAP specially in Crohn's disease, presence of detectable quantities of MAP in milk (Sweeney *et al*., 1992), raw milk has been widely used as clinical sample for screening of MAP from cattle and goats (Pillai and Jayarao, 2002; Kumar *et al*., 2008). Bulk milk test has been employed for herd screening (Stabel *et al*., 2002). Pillai and Jayarao (2002) showed high sensitivity of direct PCR with detection limit of 10 to 100 cfu/ml pertaining to use of large amount of milk samples and found to be more sensitive than culture. Singh *et al.* (1996) observed that MAP colonies took more time to appear in milk culture than in culture of feces and tissues. Previous

studies reported high bio-load of MAP in the raw milk of dairy buffaloes using milk–ELISA (Singh *et al*., 2013; Eisenberg *et al*., 2015). Hendrick et al. (2005) reported moderate agreement between milk and serum ELISA, but milk ELISA results had higher agreement with culture than serum ELISA. MAP share 99% genetic homology with *M. avium* subsp. *avium* (Moss *et al*., 1992), therefore IS*900* PCR was developed to differentiate the two.

Since, Johne's disease is endemic in domestic livestock (Singh *et al*., 2014), low positive (44.8% in i_ELISA) can be considered as positive. In a comprehensive study of 28 years (1985 to 2013), bio-load of MAP was moderately high (28.6%) behind cattle (39.3%), sheep (32.7%) and goats (20.1%) (Singh *et al*., 2014). In a ICAR-CIRG study, prevalence was reported 10.0, 100.0, 71.4 and 100% in four buffaloes farms by microscopy and 100% sero-prevalence was estimated in a buffalo farms (Singh *et al*., 2015). Another study by Singh *et al.* (2008b), wherein large scale sero-survey was conducted using this i_ELISA kit and 28.6% in buffaloes from Northern India. Sivakumar *et al.* (2005) reported 70.0 and 30.0% incidence in buffaloes from India using PCR and culture. Lillini *et al.* (2002) reported 13.3% prevalence of MAP in the Latium region of Italy using PCR in fecal samples of water buffalo herds. Molecular epidemiology studies by Kaur *et al.* (2011) revealed that as compared to 'Cattle type' biotype 'Indian Bison type' was the predominant (82.0%) biotype in domestic livestock including buffaloes. Yadav *et al.* (2008) reported 48.0% buffaloes were positive by culture from tissues (MLN and large intestine) and 40% were positive by IS*900* PCR for MAP infection. In another study, Ziehl-Neelsen's stained tissue sections revealed acid-fast bacilli in Grade 3 and Grade 2 buffaloes and acid-fast granular debris were present in grade-1 buffalo. Of 20 buffaloes, 14 (70%) were positive by IS*900* PCR and 6 (30%) were positive by MAP culture (Sivakumar *et al*., 2006).

Singh and co-workers standardized d_ ELISA for screening of milk of domestic livestock (Singh *et al*., 2016b). They screened 156 buffaloes serum samples and recorded high (90.3%) bio- of MAP using d_ELISA and compared with i_ ELISA (85.8%). Authors concluded that d_ELISA had potential to be sensitive and cost effective 'field based herd screening test' for screening of domestic livestock for JD on large scale (Singh *et al*., 2016c). Present study is the first attempt to use LAT and d_ELISA as screening tests using raw milk to detect MAP in domestic livestock species. Few studies on Mycobacterium employed LAT for screening of bovine tuberculosis (Koo *et al*., 2005; Ganju *et al*., 1991), cystic echinococcosis (Sheeba *et al*., 2016) and ovine brucellosis (Ismael *et al*., 2016). Koo et al. (2005) evaluated potential of latex bead agglutination assay for the diagnosis of bovine tuberculosis (bTB) and recorded sensitivity and specificity of 86.7, and 97.8%, respectively. Authors concluded that the assay could be used along with 'skin test' for the accurate detection of bTB by reducing the frequency of mis-diagnosis (Koo *et al*., 2005). Other studies of mycobacterial detection were carried out by using patient's serum samples wherein, study revealed that LAT had a sensitivity of 90.4 and 91.6% for the diagnosis of Hansen's disease (HD) and tuberculosis (TB), respectively.

Performance of newly standardized tests (LAT, i_FAT and d_ELISA) was compared with traditional tests (i_ELISA, IS*900* PCR and microscopy), widely used for the screening of clinical Johne's disease (Table 6). Using 3 antigen, 3 antibody and 6 (antigen and antibody) test combinations, 5.1% mismatch was recorded

in buffaloes milk samples, respectively (Table 4). This may be primarily due to high slaughter rate in buffaloes due to high demand for meat, therefore buffaloes are culled early in life due to reduced productivity and good market price and opening of large number of slaughter houses in country and distribution of their agents in villages or rural areas of country, which leads to lower-exposure of MAP in buffaloes. Singh *et al*. (2007) showed that the sensitivity of i_ELISA using commercial antigen was lower. This was primarily due to use of different strains as the source of antigen in the test (Singh *et al*., 2007). Gamberale *et al*. (2014) also reported low bio-load of MAP in buffaloes over 12 months were subjected to yearly serological examination by i_ELISA and they recorded low prevalence (1.0, 2.0 to 0 and 0%) between 2009 to 2012 (Gamberale *et al*., 2014). In contrast, a recent study reported 41.0% bio-load of Johne's disease in 156 serum samples of the dairy buffaloes in Malwa region using goat based Indigenous ELISA, used in the present study. Study showed that despite of high slaughter rate, bio-load of Johne's disease was high in native population of riverine buffaloes (*Bubalus bubalis*) used for milk production in dairies. This was probably due to poor sanitary and feeding condition of the buffaloes.

Statistical comparisons were evaluated and the strength of agreement was estimated to be 'good' for i_ELISA with respect to d_ELISA with a kappa value of 0.661. For i_ELISA with respect to LAT, strength of agreement was 'moderate' and a kappa value 0.480. The strength of agreement was 'very good' for ZN staining with respect to i_FAT with a kappa value of 0.909. Dot-ELISA has the highest sensitivity of 99.7% vis a vis i_ELISA and IS*900* PCR had the highest specificity with 100% vis a vis microscopy. LAT had the lowest specificity at 69.5% while IS*900* PCR had the lowest sensitivity at 30.0% (Table 5).

Table 5. Sensitivity and specificity of diagnostic tests for the screening of raw milk of individual buffaloes (n-503).

Tests type	**Diagnostic test**	**Comparative test**	**Two tailed P-value**	**Kappa ± SE**	**Strength of agreement**	**Sensitivity(%)**	**Specificity (%)**
Antibody based	LAT	Indigenous plate ELISA	<0.0001	0.480±0.025	Moderate	82.2%	69.5%
	d_ ELISA		<0.0001	0.661±0.020	Substantial	99.7%	73.1%
Antigen based	i_FAT	Microscopy	<0.0001	0.909±0.012	Almost perfect	97.7%	93.5%
	IS*900* PCR		<0.0001	0.320±0.021	Fair	30.0%	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. Profile of bio-load of MAP in raw milk of bovines: National and International scenario.

Countries	Sample type	Tests	Bio-load (%)	References
I. Bio-load of MAP in bovine milk: global studies				
USA	Bulk tank milk	Culture	2.8 – 20.6	Jayarao *et al*., 2004
USA		PCR	13.4-39.0	Jayarao *et al*., 2004; Wilson *et al*., 2010
USA	Individual milk	ELISA	3.3 - 82.4	Sweeney *et al*., 1994; Lombard *et al*., 2006a; 2006b
Canada		ELISA	2.5	Hendrick *et al*., 2006
USA	Raw milk	Culture	2.4 - 28.6	Sweeney *et al*., 1992a; Streeter *et al*., 1995; Stabel *et al*., 2002; Pillai and Jayarao, 2002
Argentina		Culture	8.3	Paolicchi *et al*., 2003
Australia		Culture	35.0	Taylor *et al*., 1981
USA		PCR	33.0	Pillai and Jayarao, 2002
Iran		PCR	14.7	Soltani *et al*., 2008
Iran		PCR Microscopy	32.0 20.0	Anzabi *et al*., 2013
II. Bio-load of MAP in bovine milk: Indian studies				
Rajasthan	Milk	ELISA	31.1	Vinodhkumar *et al*., 2014
Mathura, Agra and New Delhi	Unpasteurized	PCR	100.0	Singh *et al*., 2007b
	Pasteurized	PCR	100.0	
	Individual milk	i_ELISA	38.0	Singh *et al*., 2016
		d-ELISA	46.2	

Results of bio-type profile of MAP studied by restriction analysis using IS 1311 PCR_RE were consistent with previous molecular epidemiological studies from India, wherein buffaloes were exclusively infected with 'Indian Bison type' bio-type of MAP from dairy cows and buffaloes (Sharma *et al*., 2008; Yadav *et al*., 2008). Outside India, 'Bison type' bio-type of MAP has been reported only from wild bison in Montana, USA (Whittington *et al*., 2001). 'Cattle type' MAP has been widely reported from bovines (mainly cows) in other parts of the developed world (Marsh *et al*., 1999; Sevilla *et al*., 2005). In the present study also no bio-type other than 'Indian Bison Type' was detected in the present study. Results of molecular typing indicated the dominance of 'Indian Bison type' bio-type in this study also. Milk is a potential and convenient sample for detection of MAP. The economy of dairy animals is linked to trading of high quality of milk and requires a sanitary and control program with the purpose of eradicating the disease. This is important especially when viable MAP carried with milk and milk products despite pasteurization, MAP truly represents potential pathogen, which is incurable for human population (Singh *et al.,* 2018), therefore should be priority in control of animal diseases.

CONCLUSION

Bio-load of MAP was high on the basis of screening of raw milk of buffaloes using multiple tests. Study not only compared six tests to identify better diagnostic test / test combination (testing strategy) for detecting the presence of MAP in lactating buffaloes but also provided an indication that MAP was important milk borne pathogen both for newly born buffalo calves and human population. Present study underlined MAP as important food (milk) borne pathogen of domestic buffaloes in the country. The choice of test for screening of a population depends on the purpose and resources available. However, LAT, d_ELISA and microscopy had potential to be good screening tests using raw milk directly. Milk was convenient and good clinical sample for estimating bio-load of MAP both in large scale field screenings. This is first study wherein buffaloes milk samples were screened by multiple tests.

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