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BIO-SAFETY OF MILK PRODUCTS AND *Mycobacterium avium* SUBSPECIES *paratuberculosis* AS MAJOR MICROBIAL CONTAMINANT USING MULTIPLE TESTS INCLUDING CULTURE AND SYBR GREEN REAL-TIME ASSAY

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KEYWORDS

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Indigenous ELISA kit

Indirect fluorescent antibody test

Latex agglutination test

Mycobacterium avium subspecies *paratuberculosis*

ABSTRACT

Twelve types of milk products belonging to 22 market brands were purchased from local shops in South Uttar Pradesh and were screened for the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) as major contaminant using six tests (microscopy, Indirect Fluorescent Antibody Test, IS*900* PCR, Indigenous Enzyme linked Immuno Sorbent Assay, dot_ELISA, and Latex agglutination Test). Sample positive in any one of the six tests was considered as positive. Of 276 milk products screened, the cumulative bio-presence of MAP was, 52.8% (146) and was highest in butter (75.0%), followed by curd (66.0%), buttermilk (52.9%), lassi (50.0%), cheese (40.0%) and ice-cream (28.5%). Bio-typing of MAP DNA from milk products using IS*1311* PCR_REA revealed presence of 'Indian Bison Type' as a major biotype. Kappa (0.700 – 0.815) and two-tailed p (<0.0001–1.0) values for six tests were significant for all six tests. This study for the first time revealed large scale contamination of 'milk products' marketed by leading commercial brands in India, with MAP bacilli and therefore not safe for human consumption.

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1 Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the major milk contaminant and is endemic in the domestic livestock population of the country (Singh et al., 2014a; Chaubey et al., 2017) and also globally (Geraghty et al., 2014). Recently, MAP the cause of Johne's disease (JD) in animals has been associated with Crohn's (CD) and other auto-immune disorders in human beings (Singh et al., 2008; Naser et al., 2014; Banche et al., 2015). Johne's disease harms farm economics, low per animal productivity (Shephard et al., 2016; Cho et al., 2012), a serious threat to public health (Singh et al., 2016d), high costs of disease management (Johnson-Ifearulundu et al., 1999), reduced milk production, increased mortality and culling rates (Raizman et al., 2009), the in-utero transmission of MAP (Whittington & Windsor, 2009) and reduced average weaning weight of calves (Bhattarai et al., 2013) leading to huge losses in the dairy industry.

Recently, 'food safety' has become a major concern. Therefore, milk and milk products received maximum attention as a potential source of MAP. In India, studies have limited on the bio-presence of MAP in the milk of cows (Sharma et al., 2008), goats (Singh & Vihan, 2004), in commercial milk (Singh et al., 2009; Raghuvanshi et al., 2010) and milk products (Singh et al., 2018; Shankar et al., 2010). Pasteurization and spray drying used in making of cheese (Faria et al., 2014), powdered infant formula/calf milk replacers (Botsaris et al., 2016; Acharya et al., 2017), yogurt (Cirone et al., 2013), sour cream, and curd (Klanicova et al., 2012; Messelhausser et al., 2012) are unable to kill MAP and viable bacilli have been recovered (Sharma et al., 2008). Pasteurization norms of heating milk to 72°C with a holding time of 15 sec are insufficient to inactivate MAP bacilli. MAP forms clump on heating due to high lipid contents (Mullan, 2015). High bio-presence in raw milk has also been reported by Grant (2006) and Hammer et al. (2006) in the United Kingdom (UK).

Long incubation (4–6 months) and decontamination are major constraints in the culture of MAP (Singh et al., 2007). Molecular methods (IS*900* PCR, Real-time PCR, and f57 quantitative PCR) have frequently been used for rapid detection of MAP with high sensitivity in powdered infant formula (Hruska et al., 2011; Botsaris et al., 2016; Acharya et al., 2017), cheese (Faria et al., 2014), dairy products like yogurt, sour cream, and curd (Klanicova et al., 2012; Messelhausser et al., 2012; Cirone et al., 2013).

Both traditional (Indigenous Enzyme linked Immuno Sorbent Assay Kit (i_ELISA), microscopy and IS*900* PCR) and recently standardized tests (dot-ELISA (d_ELISA), indirect Fluorescent antibody (i_FAT) and Latex Agglutination (LAT)) were used for the detection of MAP (Singh et al., 2016a; Singh et al., 2016b; Singh et al., 2016c) in milk products (Raghuvanshi et al., 2014; Stephen et al., 2016). Information on the bio-presence of MAP in commercially marketed 'milk products' is extremely limited in the country (Sharma et al., 2008; Raghuvanshi et al., 2014; Stephen et al., 2016). Therefore, the present study aimed to estimate the bio-presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection as the major microbial contamination of milk products sold in the Indian markets by the major commercial market brands in India in Indian market using multiple tests {microscopy, serological (i_ELISA, d_ELISA, LAT), molecular (IS*900* PCR), i_FAT and culture}.

2 MATERIALS AND METHODS

2.1. *Profile of milk products*

Samples (276) of 12 types of milk products (Curd, Buttermilk, Ice cream, Lassi, Butter, Cheese, MatkaKulfi, Cream, Coffee milkshakes, Fruit Yoghurt, Makhana Kheer and Rabdi) made from pasteurized milk were purchased from local markets in Mathura (Farah and Kosi) and Agra districts of North India from 2015 to 2017. Milk products were driven from 22 leading commercial market brands (Table 1). Distribution of 276 milk products purchased for screening was: 36.2 (100), 26.0 (72), 15.2 (42), 7.2 (20), 5.8% (16), 3.6 (10) and 1.4% (4) of curd, buttermilk, ice-cream, lassi, butter, cheese, and Matka kulfi, respectively (Table 1). Milk products were of two types: (i) *'Large sample sized'* {Curd (100), Buttermilk (72) and Ice cream (42)} and (ii) *'Small sample sized'* {Lassi (20), Butter (16), Cheese (10), MatkaKulfi (4), Cream (4), Coffee milkshakes (2), Fruit Yoghurt, (2), Makhana Kheer (2), Rabdi (2)}.

2.2. *Screening methods*

Cheese (2.0 gm) was finely grounded in 10–12 ml of autoclaved distilled water and a homogenized solution was used as a 'test sample'. Curd, buttermilk, milkshakes, kheer, yogurt, lassi, rabari, and kulfi were used directly as 'test sample'. Ice-creams were melted at room temperature and the melted liquid was used as 'test sample'. Using tests like Microscopy, IS*900* PCR, i_FAT we targeted bacilli, and using ELISA, d_ELISA, LAT, MAP lactoglobulin were targeted in the milk products.

2.2.1 *Microscopy*

Microscopy was performed as per Singh et al. (2018). The presence of short acid-fast pink staining rods will be taken as positive (Figure 1).

2.2.2 *Indirect Fluorescent antibody test*

Indirect Fluorescent antibody test (i_FAT) developed for tissues (D'Haese et al., 2005), was modified for milk samples (Singh et al., 2016b). Positive samples exhibiting green fluorescence were considered positive (Figure 2).

Table 1 Comparative evaluation of six, five, four, three, two and one tests combinations for the screening of *Mycobacterium avium* subsp. *paratuberculosis* infection in milk products

Tests (Samples)	Six tests combinations - % (*n*), 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Positives % (*n*)
1. Microscopy	+	-	+	+	+	+	-	-	+	-	-	-	+	+	+	+	-	-	41.3 (114)
2. i_FAT	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	+	-	39.8 (110)
3. IS*900* PCR	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	7.9 (22)
4. i_ELISA	+	-	+	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	22.4 (62)
5. d_ELISA	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	33.3 (92)
6. LAT	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	23.1 (64)
Total-*n* (276) %	14	130	30	6	2	6	6	2	8	4	2	2	26	2	2	18	8	8	52.8 (146)
	5.0	47.1	13.0			5.0			5.0				12.3				12.3 (34)		
			35.5 (98)																

Total samples n-276; (-): Denotes the negative samples in an individual test of that particular test combination; (+): Denotes the positive samples in an individual test of that particular test combination; 1-18: Maximum permutation and combinations possible in 6 test regimen; Total-n: Represents only total positive samples in that particular test combination.

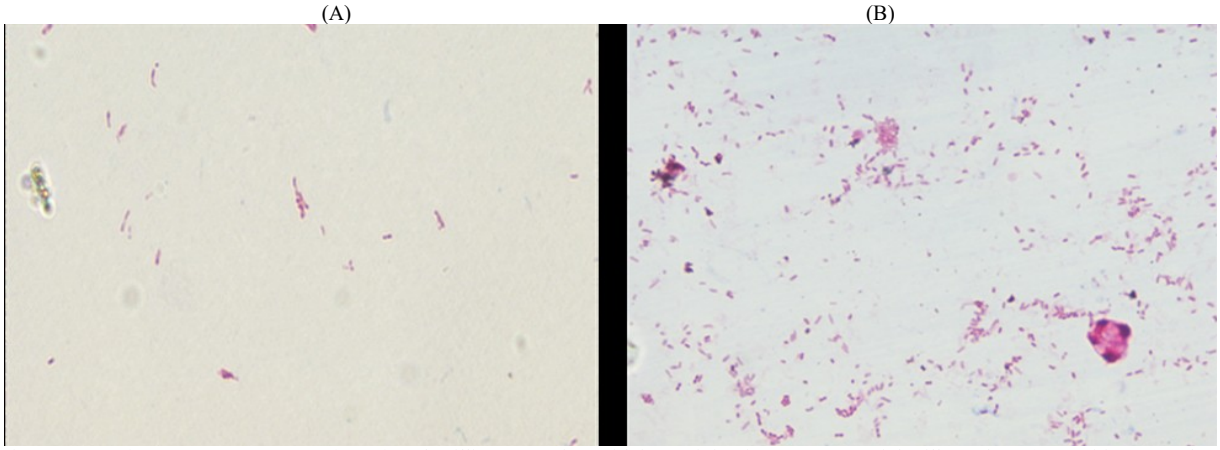

Figure 1 *Mycobacterium avium paratuberculosis* bacilli as seen after acid-fast staining in (A) commercial milk product; (B) positive control

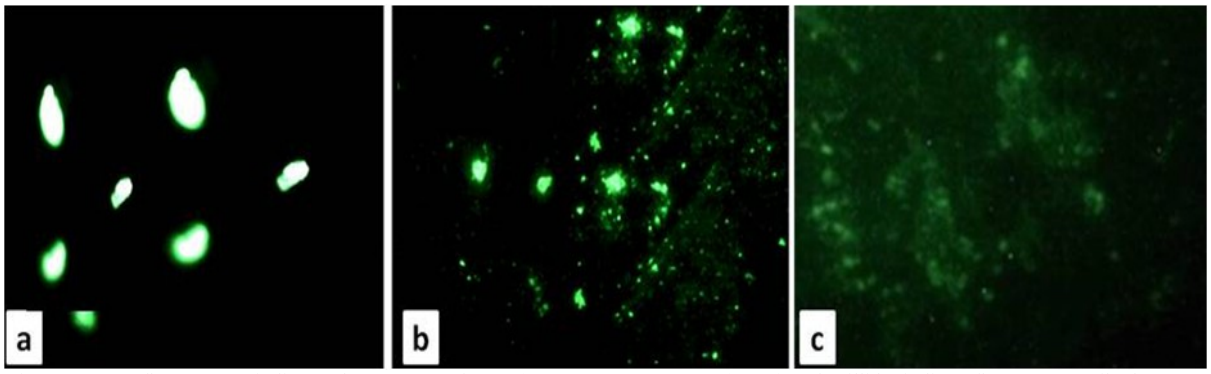

Figure 2 Green fluorescence indicates the presence of *Mycobacterium avium paratuberculosis* bacilli by indirect fluorescent antibody test; a: positive control; b: commercial milk product; c: negative control

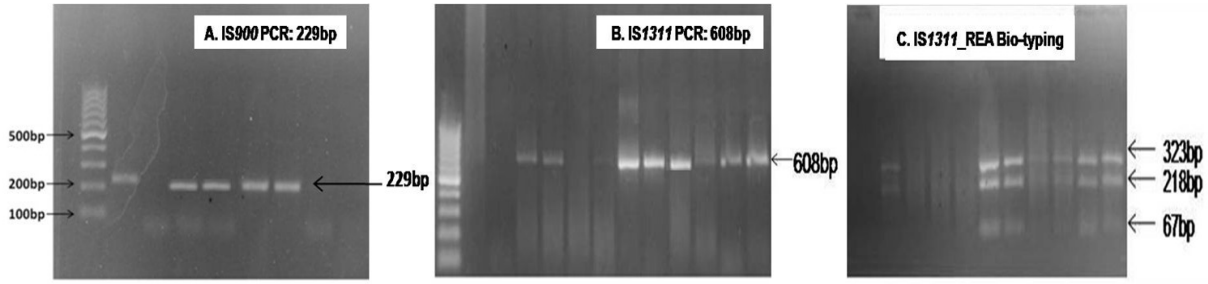

Figure 3 Agarose gel electrophoresis of amplicons obtained by IS*900 assay*(A) performed on commercial milk products; molecular characterization of IS*900* positive *Mycobacterium avium paratuberculosis* Deoxyribonucleic acid by IS*1311assay*(B) and IS*1311*_Restriction Enzyme analysis (C)

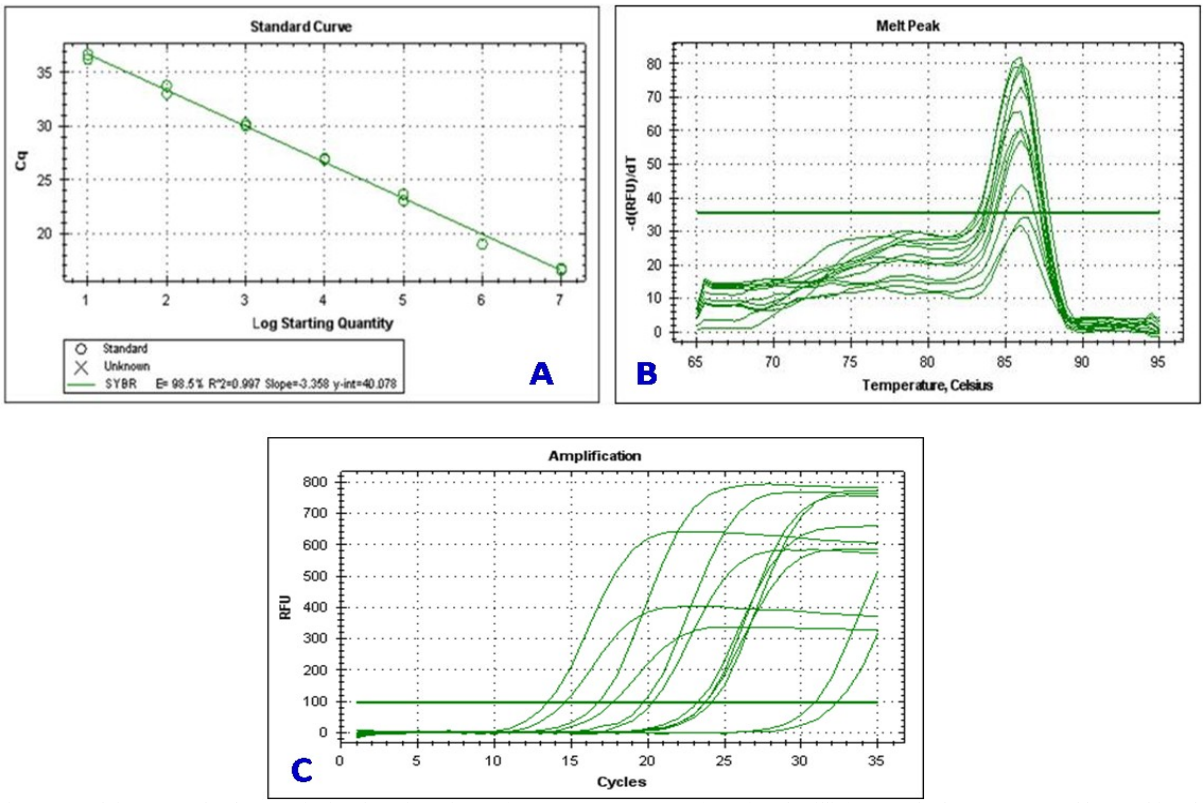

Figure 4 Real time assay for the quantitative detection of *Mycobacterium avium paratuberculosis* bacilli {A: Standard curve generated by graphing the log of the Deoxyribonucleic acid concentration (serial dilutions from 0 to 10^5 folds) of *Mycobacterium avium paratuberculosis* Deoxyribonucleic acid (10ng/µL) vs. the CT value showing 98.5 efficiencies (R^2=0.997) for detection of *Mycobacterium avium paratuberculosis*; B: Melting peak of IS*900* amplification product for tested *Mycobacterium avium paratuberculosis* Deoxyribonucleic acid samples; C: Amplification plot of milk products showing positive for the *Mycobacterium avium paratuberculosis* Deoxyribonucleic acid using Real Time assay based assay targeting IS*900* gene}

2.2.3 *Deoxyribonucleic acid isolation*

Commercial milk product's DNA was isolated as per van Soolingen et al. (1991) with some modifications (Singh et al., 2018).

2.2.4 *IS900 Polymerase Chain Reaction*

Isolated DNA was amplified by specific IS*900* PCR (Singh et al., 2008) using Vary et al. (1990) primers. The yield of a specific PCR product (229 bp) will be taken as positive (Figure 3).

2.2.5 *IS1311 assay and restriction endonuclease analysis*

IS*900* PCR positive DNA samples were subjected to bio-typing using *M56* and *M119* primers (Sevilla et al., 2005) (Figure 3).

2.2.6 *SYBR Green Real Time assay Targeting IS900 Gene*

MAP DNA from milk products (n=34) was screened by IS*900* SYBR green Real time PCR (SYBR RT_PCR) following the Gupta et al. (2017) protocol (Figure 4).

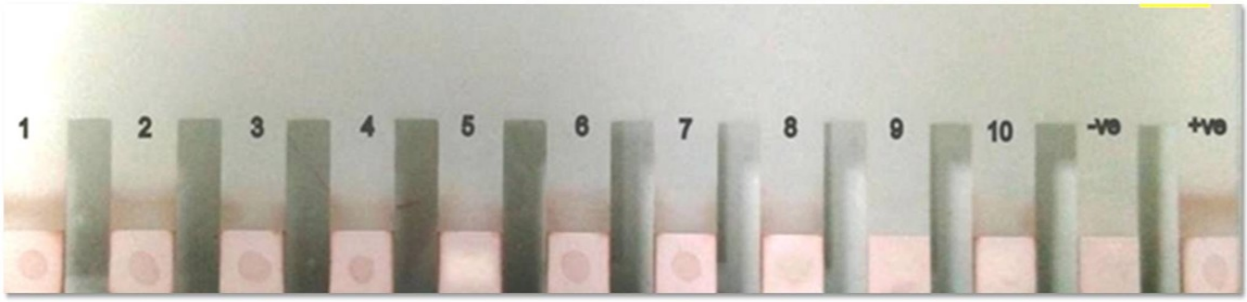

Figure 5 Dot-Enzyme Linked Immuno Sorbent Assay of commercial milk products (1-10) showing the presence of *Mycobacterium avium paratuberculosis* antibodies as a positive brown dot; +ve: positive control (brown dot); -ve: negative control (no brown dot)

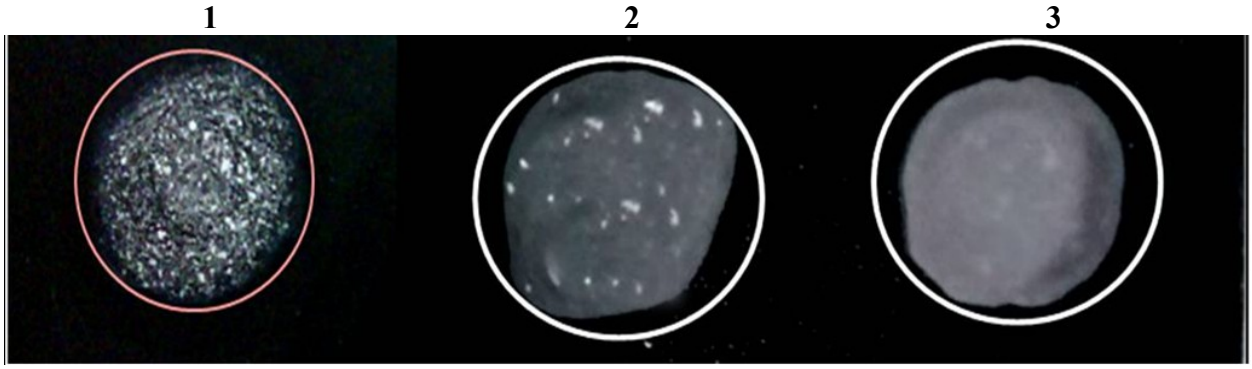

Figure 6 Latex agglutination test result for the detection of *Mycobacterium avium paratuberculosis* antibodies in commercial milk products (1: Milk positive control; 2: Positive commercial milk product; 3: Negative commercial milk product)

2.2.7 *Culture*

IS*1311*PCR positive samples were cultured as per Merkal & Richards (1972) and tubes screened at weekly intervals for the appearance of typical MAP colonies (Singh & Vihan, 2004).

2.2.8 *Indigenous Enzyme Linked Immuno Sorbent Assay kit*

Milk-based indigenous ELISA' kit (Singh et al., 2007) used for screening with modifications (Singh et al., 2018). OD values were expressed as sample-to-positive (S/P) ratios as per Collins (2002). Sample in low positive (LP), positive (P), and strong positive (SP) categories were considered positive for MAP infection.

2.2.9 *Dot-Enzyme Linked Immuno Sorbent Assay*

Dot-ELISA was performed as per Singh et al. (2016a) (Figure 5).

2.2.10 *Latex Agglutination test*

The Latex Agglutination test (LAT) was performed as per the method of Singh et al. (2016c) and positive results were noted within 2 min (Figure 6).

2.3 *Statistical analysis*

Statistical significance was measured between two tests, McNemar's test and Kappa agreement statistical analysis methods applied by Graph Pad software, California, United States of America (USA). Sensitivity and specificity were measured by Med-Calc software, Acacialaan 22 8400 Ostend, Belgium.

3 Results

Samples from Cream (4), Coffee milkshakes (2), Fruit Yoghurt (2), MakhanaKheer (2), Rabdi (2) were negative in all diagnostic tests performed. Of 276 milk products screened, cumulative bio-presence was 52.8% (146) by six tests (Table 1). Product-wise, bio-presence of MAP was the highest in butter (75.0%), followed by curd (66.0%), buttermilk (52.9%), lassi (50.0%), cheese (40.0%) and ice-cream (28.5%). The sensitivity of the test for the detection of bio-presence of MAP was, 41.3 (114), 39.8 (110), 33.3 (92), 23.1 (64), 22.4 (62) and 7.9% (22) in microscopy, i_FAT, dot_ELISA, LAT, i_ELISA and IS*900* PCR, respectively. None of the 47.1% (130) milk products (perfect negative) were detected by any of the six tests (Table 1). Samples (22) positive in IS*900* PCR were bio-typed and

bio-typing of seven types of milk products revealed the presence of 'Indian Bison Type' biotype in 5.7% (16) in five milk products (curd, buttermilk, ice-cream, butter, and cheese) (Table 2).

3.1 Large sample-sized milk products

3.1.1 *Curd*

The Bio-presence of MAP was very high (66.0%) in curd samples (100) of 14 brands. The 50.0, 40.0, and 6.0% samples were found to be positive for the bio-presence of MAP using microscopy, i_FAT and IS*900* PCR (antigen detection), and 34.0, 44.0 and 34.0% in i_ELISA, d_ELISA and LAT (antibody detection) tests, respectively. The 6.0, 18.0, 6.0, 6.0, 8.0, and 20.0%, samples were positive in six, five, four, three, two, and one test, respectively (Table 3). Microscopy was most sensitive followed by d_ELISA, i_FAT, i_ELISA, LAT, and IS*900* PCR. Except, Namaste India and Nestle, the rest of 14 market brands, 85.7% (12) were positive and bio-presence was 76.9 and 46.1% in Mother dairy and Amul brands, respectively (Table 4). Bio-typing of 46.1% positive of Amul revealed 7.6% were 'Indian Bison Type'.

3.1.2 *Buttermilk*

Buttermilk from eight brands was next largest sample {37.5% (*n*-72)} and 50.0% were positive (Table 2). Bio-presence was 52.9, 28.5, and 50.0% in Amul, Mother Dairy, and Ananda brands, respectively (Table 4). All samples from Namaste India (4), Parag (2), and Sanchi dairy (2) were positive. Two samples each from Gyan and Payas diary were negative. The i_FAT was most sensitive (44.4%) followed by microscopy (38.8%), d_ELISA (33.3%), LAT (22.2%), i_ELISA (19.4%), and IS*900* PCR (19.8%) for the detection of bio-presence of MAP in milk products. In all the six tests, 8.3 and 50.0% samples were positive and negative, respectively (Table 3). Except, 8.3% of samples detected by single test (microscopy, i_FAT, and d_ELISA), rest 41.6% were detected by multiple tests (two to six tests). Bio-typing of 36 (50.0%) positive samples showed, 16 (22.2%) were 'Indian Bison Type' where Amul (5.8%), Mother dairy (14.2% and Namaste India (100.0%) (Table 4).

3.1.3 *Ice cream*

Of 42 ice-cream samples, 12 (28.5%) were positive (Table 2). The Bio-presence of MAP was 37.5, 25.0, 33.3, and 100.0% in Cream well, Sudha, Havmor and Say Natural brands, respectively. None of the samples from Amul, Vadilal, and Madhu brand was positive (Table 4). i_FAT was most sensitive (23.8%) followed by microscopy (19.0%), d_ELISA (9.5%), and in three tests (LAT, i_ELISA, and IS*900* PCR-4.7% each). In i_FAT, 9.5% of samples were positive and 19.0% by multiple tests (two to six tests) (Table 3). Of two (4.7%) positive ice-cream (Say Natural brands) in IS*900* PCR, both were 'Indian Bison Type' (Table 4).

Table 2 Test-wise bio-presence and bio-type profile of *Mycobacterium avium s*ubsp. *paratuberculosis* in 'commercial milk products' of leading Indian market brands

Milk products *n* (%)	Positives[a] / Samples screened, % (n)						Bio-type[b]
	Microscopy	i_FAT	IS*900* PCR	i_ELISA	d_ELISA	LAT	
1. Curd - 100 (36.2)	50.0 (50/100)	40.0 (40/100)	6.0 (6/100)	34.0 (34/100)	44.0 (44/100)	34.0 (34/100)	6.0 (6)
2. Butter milk- 72 (26.0)	38.8 (28/72)	44.4 (32/72)	13.8 (10/72)	19.4 (14/72)	33.3 (24/72)	22.2 (16/72)	5.5 (4)
3. Ice cream - 42 (15.2)	19.0 (8/42)	23.8 (10/42)	4.76 (2/42)	4.76 (2/42)	9.52 (4/42)	4.76 (2/42)	4.7 (2)
4. Lassi - 20 (7.2)	40.0 (8/20)	40.0 (8/20)	0.0 (0/20)	20.0 (4/20)	20.0 (4/20)	20.0 (4/20)	00
5. Butter - 16 (5.8)	87.5 (14/16)	87.5 (14/16)	12.5 (2/16)	37.5 (6/16)	75.0 (12/16)	25.0 (4/16)	12.5 (2)
6. Cheese - 10 (3.6)	40.0 (4/10)	40.0 (4/10)	20.0 (2/10)	20.0 (2/10)	40.0 (4/10)	40.0 (4/10)	20.0 (2)
7. MatkaKulfi- 4 (1.4)	50.0 (2/4)	50.0 (2/4)	0.0 (0/4)	0.0 (0/4)	0.0 (0/4)	0.0 (0/4)	00
Total[c](276)	41.3 (114)	39.8 (110)	7.9 (22)	22.4 (62)	33.3 (92)	23.1 (64)	5.7 (16)

LAT: Latex Agglutination Test, i_FAT: Indirect Fluorescent Antibody Test, Cheese (fresh paneer); [a]Positive in at least one of six tests; [b] 'Indian Bison Type' using IS*1311* PCR_REA; [c]None of these 6 samples {8. Cream (4), 9.Coffee milkshakes (2), 10.Fruit Yoghurt (2), 11.Makhana Kheer (2), 12. Rabdi (2)}

Table 3 Market brands and milk product-wise bio-presence of *Mycobacterium avium s*ubsp. *paratuberculosis* in six tests, combinations

Sn	Milk products screened (*n*)			Positives in 'test combinations', % (*positive samples*)				
	Market Brands	Sample Type		6 tests	2 - 5 tests	Single test (%)		
						AFB	i_FAT	d_ELISA
1	Mother Dairy (56)	Butter milk	14	00	28.5 (4)	00	00	00
		Curd	26	00	61.5 (16)	15.3 (4)		
		Lassi	8	00	25.0 (2)	00		
		Paneer and Fruit yogurt	4	00	00			
		Butter	4	50.0 (2)	50.0 (2)			
2	Amul (84)	Butter milk	34	5.8 (2)	29.4 (10)	5.8 (2)	5.8 (2)	5.8 (2)
		Curd	26	23.0 (6)	7.6 (2)	15.3 (4)	00	00
		Butter	8	00	75.0 (6)	00	00	25.0 (2)
		Cheese	4	00	100.0 (4)	00	00	00
		Cream and Ice cream	8	00	00			
		Lassi	4	00	50.0 (2)	50.0 (2)		
3	Ananda (14)	Butter milk	12	00	50.0 (6)	00		
		Curd	2	00	100.0 (2)			
4	Cavins(2)	Coffee milkshake	2	00	00			
5	Cream well (18)	Ice cream	16	00	12.5 (2)	00	25.0 (4)	
		Matkakulfi	2	00	100.0 (2)	00	00	
6	Gopal ji (4)	Curd	2	00	00	00	00	100.0 (2)
		Lassi	2	00	00	00	00	00
7	Gyan Dairy (12)	Buttermilk	2	00	00			
		Curd	10	00	60.0 (6)			
8	Havmor (6)	Ice cream	6	00	33.3 (2)			
9	Madhu(2)	Ice cream	2	00	00			
10	Madhusudan (2)	Curd	2	00	100.0 (2)			
11	Mahanand (4)	Curd	4	00	100.0 (4)			
12	Namaste India (8)	Curd	4	00	00			
		Butter milk	4	100.0 (4)	00			
13	Nestle (4)	Curd	4	00	00			
14	Pankaj Paneer (2)	Paneer	2	00	00			
15	Parag Dairy(8)	Butter milk	2	00	100.0 (2)			
		Curd	4	00	50.0 (2)	00	00	50.0 (2)
		Lassi	2	00	100.0 (2)	00	00	00
16	Payas (2)	Buttermilk	2	00	00			
17	Sanchi Dairy(8)	Hung curd	2	00	100.0 (2)			
		Butter milk	2	00	100.0 (2)			
		Lassi	2	00	100.0 (2)			
		Rabdi	2	00	00			
18	Saras(10)	Curd	4	00	50.0 (2)	00	50.0 (2)	
		Butter	2	00	100.0 (2)	00	00	00
		Lassi and Paneer	4	00	00			
19	Say Natural (2)	Ice cream	2	00	100.0 (2)			
20	Sheer (6)	Curd	6	00	00	100.0 (6)	00	
21	Sudha(16)	Curd	4	00	50.0 (2)	00		
		Ice cream	6	00	33.3 (2)			
		Cream and Makhana Kheer	4	00	00			
		Butter	2	00	100.0 (2)			
22	Vadilal(6)	Ice cream and Matkakulfi	6	00	00			
	Total		276	5.0 (14)	35.5 (98)	6.5 (18)	2.8 (8)	2.8 (8)

Table 4 Cumulative percent bio-presence' and bio-type profile of *Mycobacterium avium s*ubsp. *paratuberculosis* in the screening of milk products using six tests combinations

Sn	Market brands, Area of operation	Milk products (positives / screened) % (*n*) Milk products		Positives[(a)]	Bio-Type[(b)]
1	Mother Dairy (56), Delhi	Butter milk	14	28.5	14.2
		Curd	26	76.9	00
		Lassi	8	25.0 (2)	00
		Paneer	2	0.0 (0)	00
		Fruit yoghurt	2	0.0 (0)	00
		Butter	4	100.0 (4)	50.0 (2)
2	Amul (84), Gujrat	Butter milk	34	52.9 (18)	5.8 (2)
		Curd	26	46.1 (12)	7.6 (2)
		Butter	8	100.0 (8)	00
		Cream	4	0.0 (0)	00
		Cheese (Paneer)	4	100.0 (4)	50.0 (2)
		Ice cream	4	0.0 (0)	00
		Lassi	4	100.0 (4)	00
3	Ananda (14), New Delhi and Uttar Pradesh	Butter milk	12	50.0 (6)	00
		Curd	2	100.0 (2)	00
4	Cavins (2), India	Coffee milk shake	2	0.0 (0)	00
5	Cream well (18), India, Delhi	Ice cream	16	37.5 (6)	00
		Matka Kulfi	2	100.0 (2)	00
6	Gopal ji (4), Uttar Pradesh	Curd	2	100.0 (2)	00
		Lassi	2	0.0 (0)	00
7	Gyan Dairy (12), Lucknow	Butter milk	2	0.0 (0)	00
		Curd	10	60.0 (6)	00
8	Havmor(6), New Delhi and Rajasthan	Ice cream	6	33.3 (2)	00
9	Madhu(2), Agra	Ice cream	2	0.0 (0)	00
10	Madhusudan (2), New Delhi and Uttar Pradesh	Curd	2	100.0 (2)	00
11	Mahanand(4), Maharashtra	Curd	4	100.0 (4)	00
12	Namaste India (8), Uttar Pradesh and Lucknow	Curd	4	0.0 (0)	00
		Butter milk	4	100.0 (4)	100.0 (4)
13	Nestle (4), Switzerland	Curd	4	0.0 (0)	00
14	Pankaj Paneer (2), Agra	Paneer	2	0.0 (0)	00
15	Parag Dairy (8), Uttar Pradesh	Butter milk	2	100.0 (2)	00
		Curd	4	100.0 (4)	00
		Lassi	2	100.0 (2)	00
16	Payas(2), Rajasthan	Butter milk	2	0.0 (0)	00
17	Sanchi Dairy (8), Madhya Pradesh	Hung curd	2	100.0 (2)	00
		Butter milk	2	100.0 (2)	00
		Lassi	2	100.0 (2)	00
		Rabdi	2	0 (0.0)	00

Sn	Market brands, Area of operation	Milk products (positives / screened) % (*n*) Milk products		Positives[(a)]	Bio-Type[(b)]
18	Saras (10), Rajasthan	Curd	4	100.0 (4)	00
		Butter	2	100.0 (2)	00
		Lassi	2	0 (0.0)	00
		Paneer	2	0 (0.0)	00
19	Say Natural (2), India	Ice cream	2	100.0 (2)	100.0 (2)
20	Sheer (6), Catch, Noida	Curd	6	6 (100.0)	00
21	Sudha(16), Bihar	Curd	4	2 (50.0)	00
		Ice cream	8	2(25.0)	00
		Cream	2	0 (0.0)	00
		Makhana Kheer	2	0 (0.0)	00
		Butter	2	2 (100.0)	00
22	Vadilal (6), Gujrat, Ahemdabad	Ice cream	4	0 (0.0)	00
		Matkakulfi	2	0 (0.0)	00
Total samples			276	52.8 (146)	5.7 (16)

None of the 6 milk products {Cream (4), Coffee milkshakes (2), Fruit Yoghurt (2), Makhana Kheer (2), Rabdi (2)} was positive in any of the six tests; [(a)]Positives in at least one of 6 tests; [(b)]IS*1311* PCR_REA

3.2 Small sample size milk products

3.2.1 *Lassi*

Out of 11.7% (20) lassi samples (six brands), 50.0% (10) were positive (Table 2). Bio-presence was 25.0, 100.0, 100.0, and 100.0% in samples from Mother dairy, Amul, Parag, and Sanchi dairies, respectively. None from Gopalji and Saras were positive (Table 4). i_FAT and microscopy were most sensitive (40.0%) followed by d_ELISA, LAT, and i_ELISA (20.0%). In IS*900* PCR, none was positive. Except, 10.0% samples (positive in one test), rest 40.0% were detected by multiple tests (two to six tests) (Table 3).

3.2.2 *Butter*

In the case of butter, cumulatively, 12.5% and none samples were positive and negative respectively, in all six tests. Out of 9.6% (16) butter samples (four brands), 75.0% (12) were positive (Table 2) and bio-presence was 100.0% in Amul, Mother dairy, Saras, and Sudha brands (Table 4). i_FAT and microscopy were most sensitive (87.5%) followed by d_ELISA (75.5%), i_ELISA (37.5%), LAT (25.0%) and IS*900* PCR (12.5%). Except, 12.5% (positive in one test), rest 87.5% were positive by multiple tests (two to six tests) (Table 3). Screening of 16 positive samples in IS*900* PCR, only two samples from Mother dairy were 'Indian Bison Type' (Table 4).

3.2.3 *Cheese and paneer*

Of the total, 6.5% (10) cheese samples (four brands), four (40.0%) were positive in multiple tests (i_FAT, microscopy, d_ELISA, and LAT) (Tables 2 and 3) and bio-presence was 100.0% in Amul brand, and samples from Mother dairy, Pankajpaneer and Saras) were negative (Table 4). i_ELISA and IS*900* PCR detected 20.0% samples. Bio-typing of four IS*900* positive samples, 50.0% (2) from Amul were 'Indian Bison Type' (Table 4).

3.2.4 *Matkakulfi and other milk products*

Of four Matkakulfi (Cream well and Vadilal), two (Cream well) were positive by i_FAT and microscopy. Fruit yogurt (Mother dairy), cream (Amul), coffee milkshake (Cavins), Rabdi, and Makhana Kheer(Sudha) were negative (Tables 3 and 4).

3.3 *Screening by culture and IS900 Assay*

Of 276 milk products screened, 2.8% (lassi-1, ice-cream-1, butter-2, curd-2, and butter milk-2) were positive for live MAP bacilli. Culture positive milk products (2.8%) were also detected positive by six tests except for one sample of lassi which was negative in IS*900*PCR. Further, among the 7.9% (22/276) milk products positive in IS*900*PCR, 6.0 (6/100), 13.8 (10/72), 4.76 (2/42), 12.5 (2/16) and 20.0% (2/10) belonged to curd, butter-milk, ice cream, butter, and cheese, respectively (Table 2).

3.4 *Sensitivity and specificity of six tests*

Based on kappa and two-tailed p values, the sensitivity and specificity of each test were calculated and has been provided in Table 5. d_ELISA had highest (100.0%) and IS*900* PCR had lowest (19.3%) sensitivity while IS*900* PCR found to be most

specific (100.0%) and d_ELISA least specific (85.9%). Based on a statistical comparison of six tests, the strength of agreement was 'good' for d_ELISA concerning i_ELISA, with a kappa value of 0.734. A comparison of LAT with i_ELISA showed the strength of agreement was 'very good' with a kappa value of 0.815. Similarly, the strength of agreement was 'good' for i_FAT concerning microscopy with a kappa value of 0.700 (Table 5).

3.5 *Screening of IS900 assay negative samples by SYBR green Real Time assay*

Screening of 34 samples, {negative in IS*900*PCR and positive in microscopy (*n*-18), in i_FAT (*n*-8) and d_ELISA (*n*-8)}, 20.6% (7/34) were positive using RT_PCR, which improved the detection rate of MAP infection in milk products (Table 6).

4 Discussion

India has 17.0% of the world's population of domestic livestock and JD is endemic in the country (Singh et al., 2014a; Gupta et al., 2019). Chaubey et al. (2017) in past 31 years (1985-2017) showed consistent increase in the bio-load from 11.4% (464/4057) in 1985–1990 to 44.2% (3832/8658) in 2011-2017, using multiple tests; {(average – 26.8% (6976/26009)}. Studies further showed that bio-presence increased from 44.2% (3832/8658) in 2011-2017 to 48.6% (5440/11180) in past nine months (April 2017-December 2017) (unpublished data). This is mainly due to low priority accorded to JD therefore there is absence of control measures. Though at 132.43 million tonnes of milk production (FAOSTAT, 2018), India is the leading milk producer in the world, however, per animal productivity is very low mainly due to the high bio-presence of MAP in the domestic livestock. Studies from Shankar et al. (2010) reported 22.0 – 100.0% bio-presence of MAP in milk products and 56.0 – 78.0% in ice-creams and flavored milk in 2010, Raghuvanshi et al. (2014) observed 0.0 to 16.6% in paneer samples from goat milk and Stephen et al. (2016) reported, 32.7 – 74.5% in paneer samples from bovine milk revealed high bio-presence of MAP in milk and milk products sold in India for human consumption.

The 276 milk products (12 types and 22 leading commercial brands) from Agra and Mathura were screened and seven (curd, buttermilk, ice cream, lassi, butter, cheese, and Matka kulfi) were positive for MAP. Milk products (cream, coffee milkshakes, fruit yogurt, makhana kheer, Rabdi) negative for MAP may be due to low sample size (12/276 or 4.3%) or required high temperature or long time heating (coffee milkshakes, makhana kheer, rabdi) during manufacturing. Makhana kheer a native porridge requires a long time boiling of milk. Rabdi is concentrated milk made by slow long time heating. Coffee milkshakes need boiling of milk. The cream is not boiled but in fruit yogurt, fruits are added to the cream.

In large sample-sized milk products, the highest samples were positive in curd (66.0%), followed by buttermilk (50.5%) and ice-cream (28.5%), posing a serious threat to human health since products are of mass consumption. Human patients suffering from severe MAP infection had a weakness for ice-creams (Singh et al., 2016d). In small sample size products, the highest bio-presence was in butter (100.0%), followed by lassi (50.5%), Matka kulfi (50.0%), and cheese (40.0%). High bio-presence in milk products may be due to high bio-presence in domestic livestock (Singh et al., 2014a; Chaubey et al., 2017) and continue to increase (un-published data up to December 2017). A similar high bio-presence was reported in the human population of this region (Singh et al., 2014b), Study showed

Table 5 Sensitivity and specificity of diagnostic tests for the screening of commercial milk products (*n*-276)

Tests type	Diagnostic test	Comparative test	Two tailed P value	Kappa ± SE	Strength of agreement[(b)]	Sensitivity (%)	Specificity (%)
Antibody based	d_ ELISA	i_ELISA	0.0003	0.734± 0.063	Good	100.0	85.9
	LAT[(a)]		1.000	0.815± 0.059	Very good	87.1	95.3
Antigen based	i_FAT[(a)]	Microscopy	0.8231	0.700± 0.062	Good	80.7	88.8
	IS*900* PCR		<0.0001	0.219± 0.059	Fair	19.3	100.0

[(a)]LAT- Latex Agglutination Test; i_FAT- Indirect Fluorescent Antibody Test; [(b)]<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 Comparative table of the presence of MAP bacilli in 34 milk products using Real Time IS*900* PCR vis a vis microscopy, i_FAT, traditional IS*900* PCR and d_ELISA

Species(n)	Status of MAP; N (%)(a)				
	RT_PCR	Microscopy	i_FAT	IS900PCR	d_ELISA
Milk products(n= 34)	20.6 (7/34)	52.9 (18/34)	23.5 (8/34)	0.0	23.5 (8/34)

[(a)]Figures in parenthesis are numbers; N- Positive samples in each test

milk products sold by leading market brands were 'unsafe' for human consumption. Of 23,196 human beings reported for screening at pathology laboratories in Mathura, 28.8% against auto-immune disorders, 40.5% infectious diseases, and 40.9% non-infectious disorders in 2013 (Singh et al., 2014b). Screening of 23196 patients by i_ELISA, MAP was active in 34.0% population (Singh et al., 2014b). Screening of 3093 blood by IS*900* blood PCR, 8.4% were positive for human paratuberculosis. These studies corroborated the high bio-presence of MAP in the human population as a consequence of the consumption of milk products contaminated with high bio-presence of MAP (Singh et al., 2014b).

Highest positivity was in butter (100.0%), followed by curd (66.0%), buttermilk (50.5%), lassi (50.5%), Matka kulfi (50.0%), cheese (40.0%) and ice-cream (28.5%). Thick waxy wall of MAP bacilli has an affinity for fat molecules in butter, curd, lassi, buttermilk, and Matka kulfi (Mullan, 2015). Similarly, cheese (40.0%) has high-fat contents, wherein bacilli get partitioned with fat. Though fat contents are high in ice-creams due to heating during making MAP bio-presence may be low. Of the milk products screened, butter was most unsafe for human consumption. In the absence of control programs, MAP bio-presence was high in domestic livestock (Singh et al., 2014a). This study first time revealed that consumption of milk products not safe, though the human population including babies (infant milk formula), largely depends on milk products as food items of mass consumption and are continuously getting exposed to MAP. Depending on stress conditions several cases are culminating as patients of 'human paratuberculosis'.

Test-wise, the sensitivity of i_FAT, microscopy, and d_ELISA was highest and parallel. i_FAT was most sensitive followed by microscopy and d_ELISA. i_ELISA and LAT had similar sensitivity in curd, buttermilk, ice-cream, and lassi. i_ELISA was superior for the screening of butter as compared to cheese, where LAT was better. The study highlighted the selection of a test for screening milk products after considering the nature of milk products. Depending on resources, purpose, and sample we can choose the screening test(s). The use of RT_PCR and culture led to further confirmation of MAP in milk products.

High bio-presence of MAP (28.5–100.0%) in the milk products in present findings were similar to our earlier reports of high bio-presence in domestic animals (Singh et al., 2014a) raw milk (personal communication) and commercial pasteurized liquid milk (Singh et al., 2018) and human population (Singh et al., 2014b) in North India. Unless MAP infection is controlled in animals, the human population will continue to get exposed to MAP. Chaubey et al. (2017) correlated that high bio-presence of MAP in the human population may have resulted in a sharp rise in auto-immune disorders (Crohn's disease, inflammatory bowel disease, ulcerative colitis, diabetes type 1, thyroiditis, rheumatoid arthritis, Allergic rhinitis, milk allergies, etc.). MAP infection is incurable both in animals and human beings.

Of the 12 types of milk products screened, consumption of butter, curd, buttermilk, lassi, and cheese was the most likely threat for human infection with MAP. Due to the presence of a thick waxy cell wall, MAP survives pasteurization temperatures by forming clumps (Donaghy et al., 2010). Viable MAP has been detected even after the application of pressure along with pasteurization (Donaghy et al., 2010). The available published reports provided evidence for the existence of live MAP in unpasteurized, pasteurized milk, colostrums, milk powder, and all types of fresh cheese by Bradner et al., (2013) and Eftekhari & Mosavari, (2016).

Studies show high bio-presence of MAP in milk and milk products may be leading to the increased incidence of Crohn's disease in the human population. Botsaris et al. (2010) in their report on the bio-presence of MAP DNA using quantitative real-time PCR (RT_PCR), showed that 25.0% cheese originating from sheep, goat, and mixed milk from farms and products in Cyprus were found positive. However, viable MAP bacilli were not detected either in the combined phage IS*900* PCR or by conventional culture method. Faria et al. (2014) also reported MAP from 10.0 and 3.3% retail Coalho cheese samples in Brazil by PCR and culture, respectively, and sequenced the DNA from positive culture samples showing 99% identity with the insertion sequence IS*900.* Similarly, various researchers tested the efficacy of various tests in the detection of *Mycobacterium avium paratuberculosis* in milk products (Table 7). Using culture in the present study, live MAP colonies were recovered from 2.8% milk products (lassi, ice creams, butter, curd and butter, 0.3, 0.3, 0.7, 0.7 and 0.7%, respectively).

Similar findings were reported by Van Brandt et al. (2011). The authors conducted a study to detect MAP in yogurt and commercial fermented milk products containing probiotic strains. They concluded the easy survivability of MAP in yogurt but MAP numbers decreased in fermented milk products containing probiotic cultures. Their results filled-up the knowledge gap on the behavior of MAP in yogurt and fermented milk products containing probiotic cultures. Though MAP could not be isolated from yogurt or fermented milk, their research was millstone and valuable in the context of the risk of MAP transmission to humans through yogurt and probiotic fermented milk products. Klanicova et al. (2012) and Cirone et al. (2013) confirmed the survival of MAP in fermented milk products (yogurt, acidophilus milk, and kefir) during fermentation conditions, low pH and refrigerated storage for at least 20 days. MAP in raw materials might reach consumers via consumption and emphasize on implementation of good manufacturing practices during the production and storage of fermented milk products.

Table 7 Country-wise global bio-presence of *Mycobacterium avium paratuberculosis* in milk products

Species	Countries	Sample type	Tests	Bio-presence (%)	Reference
Cattle	Switzerland	Semi-hard cheese	PCR	4.0	Stephan et al. (2007)
			Culture	0.0	
	USA	Cheese	Culture	23.0	Clark Jr. et al. (2006)
		Curd	PCR	9.0	
	Brazil	Cheese	PCR	10.0	Faria et al. (2014)
			Culture	3.3	
Goats and Sheep	Czech Republic	Hard cheese	Culture	5.4	Ikonomopoulos et al. (2005)
			PCR	5.4	
		Semi-hard cheese	Culture	5.0	
			PCR	50.0	
		Cheese	PCR	25.0	Botsaris et al. (2010)

Singh & Vihan (2004) screened raw milk of five goats suffering from clinical Johne's disease for the presence of MAP. Consistently high bio-presence of MAP in goat milk samples, authors concluded that MAP was endemic in goat herds located at CIRG, Makhdoom, India. This may be due to repeated transmission of MAP from infected mothers to the new generation through milk and colostrum. Despite regular screening and culling of suspected goats since the establishment of herds in 1979, the bio-presence of MAP could not be reduced and shedding MAP in feaces and milk continued. Screening of raw milk from a large number of goats, cows, and buffaloes the bio-presence was 9.4% in goats (Kumar et al., 2008), 6.0% in cattle (Sharma et al., 2008) and 48.0% in buffaloes (Yadav et al., 2008). Shankar et al. (2010) in 2008 first time screened pasteurized milk and milk products and samples were centrifuged to concentrate MAP bacilli, which got partitioned in fat or sediment layers depending on fat percent. Each layer was processed independently. However, in this study milk products were screened without centrifugation.

The milk used in the preparation of these 12 milk products was mainly of bovine (buffaloes, cattle) origin and goat milk is sold as an adulterant. Therefore, the bio-presence of MAP estimated using milk products reflected the bio-presence of MAP in buffaloes and cattle and goats. After milk and ice-creams, soured milk products (curd, lassi, butter, and buttermilk) and fermented milk products (yogurt, cheese, and cream) were popular milk products. Curd, lassi, and buttermilk due to the presence of lacto-bacilliis prescribed for patients suffering from gastro-disorders and after antibiotic therapy. Mostly fresh curd is prepared in every home, however, in metropolitan cities, people depend on commercial curd, buttermilk, butter, and lassi. Mother dairy was the most popular brand sold in the capital city of New Delhi and Amul all over India.

Bio-typing of milk products (curd, butter-milk, ice-cream, lassi, butter, and cheese) revealed the presence of 'Indian Bison Type' biotype of MAP. It is predominant bio-type reported from animals, milk and milk products (raw and pasteurized), and human population and also wild animals and environment indicating circulation of the bacilli in the 'ecosystem' infecting new population (animals and human beings) and at the same time increasing in the percent bio-presence continuously (Singh et al., 2014a; Singh et al., 2014b; Chaubey et al., 2016; Singh et al., 2018).

Conclusion

The study indicated that the high bio-presence of MAP in milk products was a result of similar high bio-presence of MAP in raw milk used for making these milk products, which enabled MAP to survive heat treatments during pasteurization. High bio-presence of MAP and presence of live MAP bacilli in milk products suggested MAP as high-risk pathogen for the human population and exposure to MAP infection and fragments/components of bacilli. Test combinations were superior to individual tests for the screening of milk products and test combinations can be decided depending on the type of sample, the number of bacilli, purpose study, resources available, etc. In-order to prevent transmission of MAP to the next generation of animals and human population control measures are urgently needed. To prevent the infection of farm animals will lead to a reduction in chances of contamination of milk and milk products, which may lead to infection of the human population. Milk products sold by leading market brands were un-safe for human use. Brands negative in this study may not be a true picture since the sample size was very low in the number of milk products, therefore the need for large scale study encompassing all the regions of the country.

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Conflict Of Interest

No potential conflict of interest to declare.

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