



Load of *Mycobacterium avium* subspecies *paratuberculosis* in Commercial Milk and Milk Products Supplied in Braj Region using Different Diagnostic Methods

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ABSTRACT

Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a specific disease causing agent of Crohn's disease (CD) and Johne's disease (JD) both in humans and animals respectively. Preliminary studies reveal high bio-incidence of MAP in domestic available unpasteurized milk as well as in commercially available pasteurized milk products.

Methods: A total of 46 samples of milk and milk products (butter, milk powder, icecream, flavored lassi, dahi/flavored dahi) were collected from local markets of Mathura, Vrindavan and from canteen in GLA University, Mathura campus. These samples were screened using various diagnostic methods including Ziehl-Neelsen staining (acid fast staining), IS900 PCR, Dot ELISA and Plate ELISA for the presence of MAP.

Result: Out of 46 samples tested, 3, 16 and 37 samples showed the positive results for MAP in Polymerase chain reaction, Microscopy and plate ELISA assay tests respectively. Out of 10 samples screened by means of dot ELISA, 2 were positive for MAP infection. The prime objective of this study was to reconfirm the bioincidence of MAP in commercially sold milk and milk products in Brij region and to establish the fact that MAP is not inactivated through pasteurization.

Key words: Crohn's disease, ELISA, Johne's disease, Milk products, Milk, *Mycobacterium avium* subspecies *paratuberculosis*.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a disease causing agent of incurable Johne's disease (JD)/ Paratuberculosis (pTB) which is chronic granulomatous enteritis disorders that majorly affects small intestine of ruminants. MAP is a slow growing and fastidious organism which can survive in wide environmental conditions (Deb *et al.* 2011). MAP have the ability of causing disease in domestic as well as in wild animals (Beard *et al.*, 1999; Beard *et al.*, 2001; Beard *et al.*, 2001; Chiodini *et al.*, 1983; Greig *et al.*, 1999; Biswal *et al.*, 2020; Audarya *et al.*, 2024; Kumar *et al.*, 2024; Chaubey *et al.*, 2024) free grazing animals (Ayele *et al.*, 2001; Singh *et al.*, 2014) as well as in the primates (McClure *et al.*, 1987; Hines *et al.*, 1995) including humans (Chamberlin and Naser, 2006; Singh *et al.*, 2011). Infection is characterized by decreasing of milk production, diarrhea, infertility, loosening of weight and roughening of hair coat (Thakur and Gupta, 2018; Sharma *et al.*, 2008). In India, bio-load of MAP is increasing vigorously and continuously in domestic available livestock and their milk (Chaubey *et al.*, 2017). Bio-load of MAP has also been reported from milk and milk products (Chaubey *et al.*, 2016; Singh *et al.*, 2007; Singh *et al.*, 2016; Singh *et al.*, 2018; Shankar *et al.*, 2008; Shankar *et al.*, 2010; Raguvanshi *et al.*, 2010; Raguvanshi *et al.*, 2013). Apart from this, MAP has been cultured also from breast milk, stool and intestine of patients with Crohn's disease (CD) (Bull *et al.*, 2003; Chiodini *et al.*, 1986; Naser *et al.*, 2000).

On the daily basis raw milk, pasteurized milk as well as milk products including flavored milk, dahi/ flavored dahi,

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milk powder, butter, chhachh/lassi, ice cream etc is utilized in very high amount by human population in entire world.

Products that are being prepared from pasteurized milk products (cheese used for pizza, sandwich, patties and burger preparation, dahi used for paste preparation of many food items like uttapam and butter is used for frying purpose) consumed on large scale without boiling increasing riskenters to human chain by means of milk and products of milk (Shankar *et al.*, 2010) and causes CD (Singh *et al.*, 2016).

Alleviation of disease is very difficult in asymptomatic carriers and sub clinically infected animals. Lack of potent diagnostic method that detects MAP infections at early stages of infections is major obstacle in controlling JD at National level (Whitlock *et al.*, 1996). This is bane for farmer that none of the available test (fecal culture, fecal microscopy, milk microscopy, milk culture and blood PCR) is potential field test (Singh *et al.*, 2016). Although culture is gold standard test but it is time consuming as take approx 6-8 weeks (Cocito *et al.*, 1994) while PCR is rapid confirmatory test for MAP detection due to high sensitivity and specificity (Singh *et al.*, 2014; Whitlock *et al.*, 1996; Singh *et al.*, 2013; Nielsen *et al.*, 2014; Garg *et al.*, 2015). There is an intense need of screening every herd for MAP infection so as to control JD.

MATERIALS AND METHODS

Sample collection and processing

This study has conducted in GLA University, Mathura, U.P. between the 2020 to 2021. In this study, 46 Samples of pasteurized milk (liquid milk and flavored milk) and milk products (milk powder, butter, cheese, dahi/flavored dahi, icecream, lassi) were collected from local markets of Mathura and Vrindravan (Table 1). Samples were also purchased from canteen and fruit shop in GLA University Mathura campus. These samples were associated with 11 leading market brands. Samples were stored in 15 ml of sterilized centrifuge tubes. Packet, sachets and boxes were teared and 10-12 ml of sample was filled in centrifuge tubes. Few samples were stored in eppendorf tubes (Fig 1). Samples of cheese and butter were first processed before storage. For this purpose firstly mortar and pestle were wiped properly with 70% ethanol and then 2-3 g of sample were crushed using 5 ml distilled water to made fine paste. Paste was then filled in centrifuge tubes. All the samples were stored at 4°C for testing. All tests were performed from whole milk.

Ziehl neelsen staining (microscopy)

Smears were prepared on the clean slides using 1-2 drops of milk and milk product samples. Two smears were prepared for each sample. Smears were then air dried, heat fixed and stained by Ziehl Neelsen (ZN) staining and examined under oil emersion at 100 X (Ecoline Binocular Corded LED Microscope) for acid fast *bacilli* identical to MAP (Fig 2). As per Singh *et al.* (2013), 8-10 fields were screened and samples were categorized as +1, +2, +3 and +4.

DNA isolation

DNA isolation from commercial milk and milk product samples were performed with minor modifications using whole milk protocol Van Soolingen *et al.* (1991). For this purpose 200 µl of milk lysis buffer was added to 500 µl of commercial milk sample in each sterile eppendorf tube and vortexed for 2-4 minutes. Then 200 µl of 10% (SDS) sodium dodecyl sulphate was added and then incubated for 10 minutes at room temperature. After that tubes were putted in boiling water bath for 10 minutes at 80°C and then 40 µl of lysozyme (20 mg/ml) was added to each sample, followed by incubation at 37°C for an hour. Then, 10 µl of proteinase K (20mg/ml) was added and then incubated at 56°C for 2 hours. After that 100 µl of 5M NaCl were added, followed by addition of 64 µl of CTAB - NaCl and incubated at 65°C for 30 minutes. Later, Chloroform : Isoamyl alcohol (24 : 1) were added in equal volume and centrifuged at 10000 rpm for 15 minutes (40°C). Aqueous layers obtained after centrifugation were then transferred to sterile eppendorf tubes and then DNA was precipitated by adding 0.6 volume of chilled isopropanol and then these eppendorf tubes were kept for overnight incubation at -20°C. DNA pellet was obtained by centrifugation at 10000 rpm for 20 min (4°C). Pellet was then washed with 1 ml of 70% ethanol and again centrifuged for 10 min at 10000 rpm (4°C). Finally, 30 µl of TE buffer/Nuclease free water was added to tubes and stored at -20°C. Isolated DNA was



Fig 1: Collection of milk and milk products from different brands.

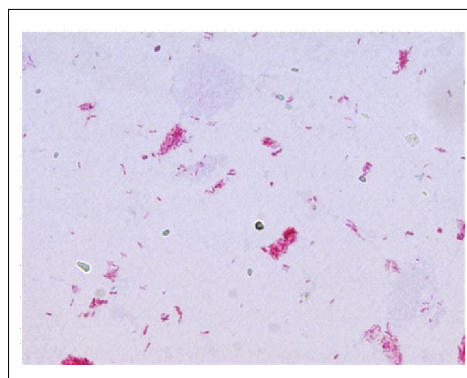


Fig 2: Acid fast bacilli in milk and milk products.

run on agarose gel electrophoresis to see whether the isolated DNA is intact or not (Fig 3).

IS900 PCR

Isolated DNA was then amplified via specific IS900 PCR using 150C primer (forward primer) and 921 as reverse primer designed by Vary *et al.* (1990) with minor modifications. Briefly, 20 µl reaction mixture was prepared in 0.5 ml of sterile eppendorf tubes (10 µl MM, 0.25 µl of each primer, 2.5 µl Nuclease free water and 7 µl of template DNA). Amplifications were performed (denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min) with 37 cycles for 2 hours in a Thermal cycler. Finally, PCR products (8 µl sample) with 2 µl tracking dye were subjected to 1.8% agarose gel electrophoresis and visualized under Biorad Gel Doc XR system (Crescent Lab Equipments). PCR product of 229 bp is specific and was considered to be as positive to MAP infection. DNA isolated from MAP culture was used as positive control (Fig 4).

d-ELISA was performed with minute modifications as per Singh *et al.* (2016). For this purpose 12 legged immune

diffusion combs were used (Advanced Micro Devices Pvt. Ltd., Ambala, Haryana. Knobs of comb fixed with nitrocellulose membrane were coated with antigen (1µg of semi-protoplasmic antigen in 1 µl of carbonate-bicarbonate buffer, pH 9.6) and incubated at 37°C for two hours followed by washing in 250 µl of PBST (0.05% tween 20 in PBS). Strip was then dipped in washing buffer (3% skimmed milk in 1X PBS) and again incubated for an hour at 37°C. Then comb was washed with PBST and then dipped in 100 µl commercial milk samples (100 µl of commercial milk sample in 1:4 dilution in 1% BSA in 1X PBS) and incubated for 2 hours at 37°C. Positive serum was taken as positive control (0.5% positive serum in serum dilution buffer *i.e.* 1% BSA in 1X PBS) and negative serum was taken as negative control. After further washing combs were then incubated with 100 µl anti-bovine conjugates solution (1:4000) for 30 min at 37°C. Finally washed strips were dipped in 100 µl of substrate buffer (H₂O₂ + 3 mg of 3,3-Diamino benzidine in 5 ml 1X PBS) at room temperature in dark till color development. Lastly, strips were dipped in triple distilled water to stop the reaction and then controls taken were confirmed by PCR.

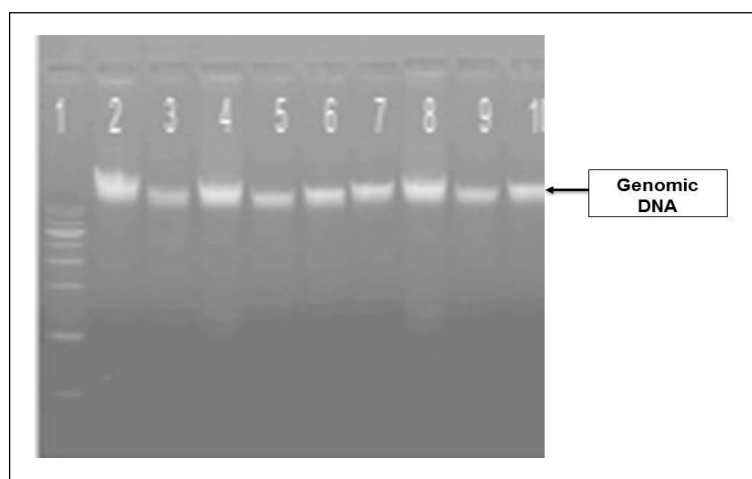


Fig 3: Genomic DNA run on agarose gel electrophoresis.

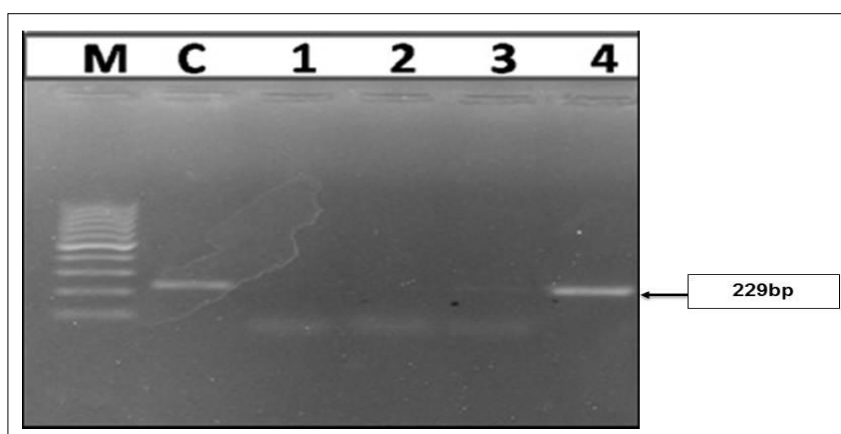


Fig 4: MAP IS900 PCR (229 bp) from DNA isolated from milk and milk products.

Indigenous plate ELISA (i-ELISA)

i-ELISA was performed with subtle modifications as per Singh *et al.* (2016). Each 96 flat bottom well of ELISA plate was coated with antigen (1 µg of protoplasmic antigen in 100µl of carbonate-bicarbonate buffer at pH 9.6 for each well) and plate was then kept at 4°C for overnight incubation. Next day plate was washed thrice with 1X PBST (0.05% tween 20 in PBS) and then blocking was performed using 250 µl of homogenized skimmed milk (3% skimmed milk in 1X PBS), incubated for an hour at 37°C. Again plate was washed thrice with PBST followed by addition of test sample in duplicate wells (100 µl of test sample in 1:4 dilution prepared in 1% BSA in 1X PBST/serum dilution buffer) incubated for 2 hours at 37°C. After further washing with PBST, plate was added with 100 µl of anti-bovine conjugate (1:400) optimally diluted in PBS and then again incubated for an hour at 37°C. Plate was washed five times and finally 100 µl of substrate buffer (pH 5.0) was added to each well of ELISA plate (H₂O + OPD in PBS) followed by incubation at room temperature in dark till color development (approx 20 minutes). Absorbance was taken in ELISA reader at 450 nm (iMark micro-plate reader, Biorad). Milk sample from the positive and negative animals tested by PCR was considered as positive and negative control respectively.

RESULTS AND DISCUSSION

Out of 46 samples tested by microscopy, 16 (34.7%) samples were positive for MAP and 7 and 9 are under +1 and +2 categories respectively and 30 (65.21%) samples were negative (Table 2). Of all 46 samples, 3(6.52%) samples were positive by IS900 PCR. However, Screening by dot ELISA reported that 2 (20%) samples were positive (Fig 5) out of 10 (2 flavored milk, 3 liquid pasteurized milk, 3 dahi/flavored yoghurt and 1 paneer) samples tested. In dot ELISA, flavored milk samples were positive indicating presence of MAP antibodies. All samples screened via indigenous plate ELISA and results of which were characterized on the basis of sample to positive ratio revealed that 80.43% (37/46) samples were in positive (71.73% are in low positive, 6.52% in positive and 2.17% in strong positive categories) and 19.56% (9/46) samples in negative (19.56% in suspected and 0.00% in negative categories) status for bio incidence of MAP (Table 3).

India is the largest milk and milk products producer globally. In spite, that productivity of individual animal has been extremely poor in terms of meat and milk as MAP infection is endemic in domestic livestock. Infected livestock starts shedding MAP in milk and is a matter of major health concern as recent studies have revealed that

Table 1: Number of samples taken for testing with brand.

Sample type	No. of samples	Brand
Liquid milk	9	Amul, Mother Dairy
Icecream	7	Creambell, Vadilal
Flavored milk/ Milk shake	9	Cavins, Ananda, Harsey's, Paras
Chhaachh/Lassi	5	Saahaj, Mother Dairy
Dahi/Flavored yoghurt	11	Mother Dairy, Mother Shree Dairy
Paneer	3	Mother Dairy
Butter	1	Amul
Milk powder	1	Nestle
Total	46	

Table 2: Status of MAP bioincidence in different types of sample tested through microscopy.

Samples	Positive % (P)	Negative % (n)
Liquid milk	33.33 (3/9)	66.66 (6/9)
Ice cream	28.57 (2/7)	71.42 (5/7)
Flavored milk/ Milk shake	22.22 (2/9)	77.77 (7/9)
Chhachh/ Lassi	60.0 (3/5)	40.0 (2/5)
Dahi/ Flavored yogurt	27.27 (3/11)	72.72 (8/11)
Paneer	33.33 (1/3)	66.66 (2/3)
Milk powder	100.0 (1/1)	0.00 (0/1)
Butter	100.0 (1/1)	0.00 (0/1)
Total	34.78 (16/46)	65.21(30/46)

*P- Positive, n- Number.

Table 3: Bioincidence of MAP in commercial milk and milk products using i-ELISA.

Category	Negative	Suspected	Low positive	Positive	Strong positive
% (P/n)	0.00(0/46)	19.56(9/46)	71.71(33/46)	6.52(3/46)	2.17(1/46)

n- Number of samples.

MAP infection transmitted in humans through consumption of raw or unpasteurized milk and pasteurized milk.

Study by Singh *et al.* (2018), revealed that 9%, 42.8% and 27% samples of commercial pasteurized milk (liquid milk, flavored milk and milk powder) were positive in IS900 PCR, microscopy and p-ELISA respectively but if compared with present study then 54.54% (6/11) samples of pasteurized milk (liquid milk, Flavored milk and milk powder) samples were tested positive for MAP infection in microscopy, 90.90% (10/11) samples in i-ELISA as infected animal starts to secrete bacilli in milk at much lower levels and therefore samples in low positive categories were considered as positive in case of milk indigenous ELISA kit and no milk sample of liquid milk, flavored milk and milk powder were positive screened by means of IS900 PCR.

Studies that were done to find status of MAP in commercial pasteurized milk and the milk products reported very high bio-load of MAP may be due to use of milk that were collected from infected animal and used for preparation of these products and by means of these pasteurized products MAP enters to human chain and cause incurable chronic CD. As MAP is capable to survive efficiently pasteurization process so milk products should be consumed after boiling. Each and every individual domestic livestock are more prone to JD and must be screened for MAP infection so as to control JD and to prevent this infection in humans by means of consumption of milk and the milk products contaminated with acid fast bacilli (MAP).

CONCLUSION

This study reconfirm high bio-load of MAP in commercial pasteurized milk and the milk products sold in Brij region (Mathura and Vrindavan) using multiple diagnostic assay and also reported the fact that MAP is not inactivated through pasteurization. Results indicate that risk of exposure of human population to MAP infection is very high due to consumption of pasteurized milk and the milk products which harbor MAP bacilli in large amounts. Therefore, these products are no longer safe for consumption and MAP is a potent food borne pathogen in food of animal origin. JD should be controlled at national level in order to prevent incurable chronic infections associated with MAP in human population that transmitted by means of consumption of contaminated milk and the milk products.

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Disclaimers

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Informed consent

Not applicable.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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