



Short Communication

Identification of *Mycobacterium Avium* Subspecies *Paratuberculosis* in Fresh Cheese (Paneer) from Goat Herds Endemic for Johne's Disease

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ABSTRACT

Live *Mycobacterium avium* subspecies *paratuberculosis* bacilli have been cultured from the pasteurized milk and cheese, compounding public health concern and food safety. Map is known to survive pasteurization and commercial paneer is usually made from pasteurized milk. Therefore it is essential to screen milk products especially made from the milk of animals endemic of Johne's disease. In India, first time Map has been identified in the samples (n = 24) of paneer made from milk of goatherds endemic for Johne's disease. Of the 24 samples screened, 4 (16.6%) and 0 (0%) were positive in microscopy and IS900 PCR, respectively. And of the 4 positive samples, 3 (12.5%) and 1 (4.2%) were from the fat and sediment fractions of paneer, respectively. Since Map free milk and milk products would have minimum risk for consumers, therefore it is important to control disease in animals. Besides paneer prepared from properly boiled milk instead of pasteurized milk may decrease the risk of transmission of Map to human beings through food chain.

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Key Words: Goat milk product; *Mycobacterium avium* subspecies *paratuberculosis*; IS900 PCR

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India possesses huge population of domestic ruminants and at 117 million tones, is leading milk producer in the world (FAOSTAT, 2012). Fresh cheese (paneer) is important milk product enriched with high quality animal fat, proteins, minerals and vitamins and is dietary favourite of the Indians specially vegetarians. Therefore, fresh cheese is in high demand in urban and peri-urban areas of country. In the industrialized countries *Mycobacterium avium* subspecies *paratuberculosis* (Map) has been cultured from pasteurized milk and cheese, compounding increasing public health concern. Commercially made paneer from pasteurized milk, where Map bacilli are not in-activated, may be health risk for consumers. Risk not only results from viable Map but also from inactive or dead cells and even from their structural components (Malli, 2010).

Goat rearing is an economical preposition for the landless rural poor and small marginal farmers in India. Goat milk (41 lakh MT) and meat product (4 million tonnes) in India constitutes about 26.45 and 8.16% of world's milk and meat production (FAOSTAT, 2012). Map is the cause of incurable Johne's disease (JD), which is endemic in the domestic livestock and also reported from wild ruminants including primates (Singh et al., 2010; Singh et al., 2013a). High prevalence of Map has been reported from the raw milk of cows (Sharma et al., 2008), buffaloes (Yadav et al., 2008), goats (Raghuvanshi et al., 2010), commercial dairy milk (Singh et al., 2009) and milk products (Shankar et al., 2010). Recently Map has been associated with inflammatory bowel disease (IBD) or Crohn's disease (CD) in human beings (Chamberlin and Naser, 2006; Singh et al., 2008). Clinically and sub-clinically infected animals excrete viable Map bacilli in their milk (Ayele et al.,

2001). High prevalence of live Map bacilli has been reported in raw milk, pasteurized milk and milk products in many countries and has potential zoonotic and public health concern (Grant et al., 2002; Slana et al., 2008; Shankar et al., 2010; Hruska et al., 2011; Ikononopoulos et al., 2005).

Earlier studies reported moderately high prevalence of Map by milk-ELISA in the raw milk of goat herds located at Central Institute for Research on Goats (CIRG), Makhdoom (Kumar et al., 2008; Raghuvanshi et al., 2010), however, information on the presence of Map in the paneer (fresh cheese) prepared from pasteurized goat milk is not available in the country. This pilot study is the first attempt to investigate the presence of Map in the samples of paneer made from the goat milk using microscopy and IS900 PCR.

Five goat units consisting of five different goat breeds (Jamunapari, Barbari, Jakhana, Sirohi and Barbari type goats of experimental farm unit) were maintained at CIRG, Makhdoom, since 1977, in order to conserve the pure germplasm of these native breeds of the Northern region. Milk produced at these five goat units is daily collected in the morning and evening and is pooled in the Goat Product Technology (GPT) laboratory, from there goat milk is distributed for human consumption and surplus milk is converted to paneer (as per Sharma et al., 1998), which is again sold. These goat units being endemic for the Map infection are monitored for Johne's disease since 1980 (Singh et al., 2013a) and still many goats suffer from clinical JD. Goats suspected for JD (weak, unthrifty, stunted, low growth rate, poor body condition and suffering from intermittent diarrhea) are regularly screened for JD by fecal microscopy. Goats found positive in fecal microscopy are culled from the stock as part of

management strategy for the control of JD. Before this study, paneer made from goat milk has never been screened for the presence of Map. A sample of paneer made from pooled milk was collected daily for twenty four days from the GPT laboratory for the screening of Map.

Table 1: Presence of Map in the paneer made from milk of goatherds endemic for Johne's disease

Samples (n)	Microscopic examination n (%)		
	Fat	Sediment	Fat+sediment
24	3 (12.5%)	1 (4.2%)	4 (16.6%)
Samples (n)	PCR (IS 900) n (%)		
	Fat	Sediment	Fat+sediment
24	0 (0.0%)	0 (0.0%)	0 (0.0%)

Approximately, 2.0 grams of paneer sample was finely grounded in 10–12 ml of autoclaved distilled water and centrifuged at 3500 rpm for 45 min at room temperature. After centrifugation, three layers (fat, whey and sediment) were formed. Fat and sediment layers were subjected for microscopic examination. Fat and sediment layers were decontaminated in 1.0% hexadecyl pyridinium chloride (HPC) for 24–36 hrs. After decontamination upper fluid layer was removed and smears were prepared from sediment of each of the sample (fat and sediment), stained by Ziehl Neelsen (ZN) method and were examined under oil immersion (100X) for the presence of acid-fast bacilli (AFB) indistinguishable to Map (figure 1)

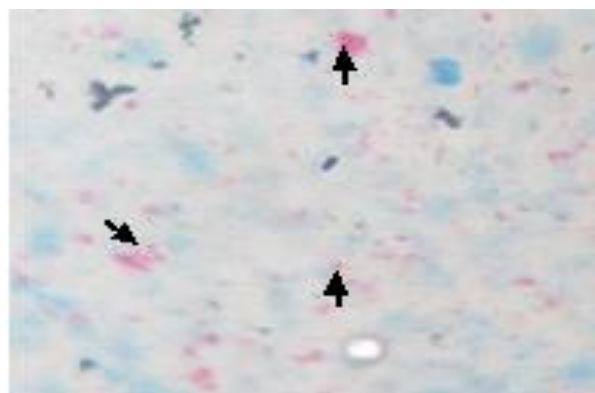


Figure 1: Acid fast Map bacilli as seen in microscopy of goat milk paneer

Sediment of both samples (Fat and sediment layer) was centrifuged and pelleted. Pellets were washed twice with PBS and subjected for isolation of DNA as per van Embden et al. (1993) with some modifications. DNA was amplified by PCR using IS900 primers as per Vary et al. (1990). Briefly, PCR was set in volume of 50 µl using 1 µl of forward–reverse primer (10 pmol) as per Vary et al. (1990). The 25 µl of 2X red dye master mix (Genex, Bangalore) and 1.0–5.0 ng sample DNA were used as a template. Thermal cycling conditions were: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 10 sec, annealing at 61°C for 30 sec, extension at 72°C for 10 sec, and final extension at 72°C for 10 min. Amplicon sizes of 229 bp were considered positive, after separation on 1.8% agarose gel stained with ethidium bromide. Same conditions of PCR were used for positive (Map DNA) and negative (sterilized milliQ water) controls.

In the present study, 24 fat and sediment layers of paneer were screened wherein 16.6% (4) and 0.0% (0) were positive using microscopy and IS900 PCR, respectively. Presence of Map was higher in microscopy of fat layer (3 or 12.5%) as compared to sediment (1 or 4.2%). Kumar et al. (2008) has reported 43.3

and 45.2% samples positive by Map culture from fat and sediment layers, respectively. Sharma et al, (2008) reported higher recovery of Map in sediment layer as compared to fat in the milk of cows. In another report higher presence of Map in the fat layer of paneer from goat milk may be due to the smaller size (2 micrometer) of fat globules; which may remain suspended in liquid phase of goat milk as compared to milk of cows (fat globulin size, 2.5–3.5 micrometer) (Chandan et al., 1992). Present findings indicated that processing of both fat and sediment layers each separately was necessary for better recovery of Map from paneer and assessment of diagnostic tests (microscopy and PCR). Earlier studies have also reported that moderately higher lacto-prevalence of Map in raw milk of goats (Kumar et al., 2008) and 33.8% goats were found positive for lacto-prevalence of Map infection in these goat herds endemic for Johne's disease (Raghuvanshi et al., 2010).

IS900 PCR was carried out using DNA isolated from all the 24 paneer samples prepared from pooled goat milk and all were found negative for the presence of Map. Low sensitivity PCR in detecting Map DNA directly from milk product (paneer) may be attributed to low bacillary count of Map (<1 in microscopy) in pooled milk, employing only one set of IS900 PCR primers and presence of PCR inhibitors in the samples (Singh et al., 2013b). Though several methods have been used for the detection, isolation and identification of Map in the milk samples in past decades, however, in the detection of Map, preparation of samples is basic and critical step. Basic procedure for the detection of Map in milk includes centrifugation to collect the pellet fraction, chemical decontamination, and subsequent processing of samples by detection method / methods (cultivation, PCR etc.) and most often used method of Map detection is culture. Second most frequent method of Map detection in milk is PCR (single, nested, Real-Time PCR). However, in few cases methods of visual detection of Map such as bioluminescence and indirect Map detection methods-ELISA/M-ELISA have been used. Recent studies suggested that Map may survive pasteurization temperature have led to increase in the testing of milk and milk products for the presence of Map (Shankar et al., 2010). Milk is the primary route of transmission of Map infection from mother to offspring since milk is the single significant element of diet of children and many adults in India. Since most milk is consumed after pasteurization, focus has been on the identification of Map in raw milk and pasteurized milk and in milk products. Published documents record the detection of Map in colostrum, non-pasteurized and pasteurized milk, baby milk powder and all types of cheese. Generally, detection of any organism from these matrices depends upon a good isolation method. Sensitivity and detection rate increases with the improvement of the detection and isolation methods of Map in these matrices. Map has been most frequently reported from the milk and milk products made from cow's milk however, potential Map contamination of milk from other ruminant species remains to be elucidated. In sheep and goats, Map was detected by IS900 PCR in 23% of bulk tank milk samples from 403 different farms throughout Switzerland in the year 2003 (Muehlherr et al., 2003). Present study is the first record for the presence of Map in paneer (fresh cheese) samples.

In conclusion, the study is the first report on the presence of Map in the paneer (fresh cheese) samples prepared from pooled milk of goats endemic for Johne's disease. Consumption of paneer made from improperly heated goat milk or pasteurized milk may increase the risk of transmission of Map to human population through food chain.

CONFLICT OF INTEREST

No Conflict of Interest to declare.

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