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Detection of *Mycobacterium avium* subsp. *paratuberculosis* in commercial milk and milk products of Tamil Nadu

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Abstract
Mycobacterium avium subsp. *paratuberculosis* is drawing attention as zoonotic pathogen in recent times due to its possible role in Crohn's disease of humans *via* consumption of contaminated milk and milk products. About 165 commercial milk and milk products from Tamil Nadu were screened to ascertain the presence of *Mycobacterium avium* subsp. *paratuberculosis* using Ziehl–Neelsen staining and Polymerase chain reaction targeting IS900 and F57 region. None of the milk products found positive for *Mycobacterium avium* subsp. *paratuberculosis*, which indicates negligible bio-load of organisms among commercial milk and milk products of Tamil Nadu. However, extensive sampling and screening using battery of diagnostic tests may delineate the contamination of this hazardous organism in human food chain.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, Milk products, IS900, Ziehl–Neelsen (ZN) staining

1. Introduction
Mycobacterium avium subsp. *paratuberculosis* (MAP) is an intracellular pathogen that causes paratuberculosis (Johne's disease), a debilitating chronic granulomatous enteritis of wide range of domestic as well as wild ruminants [1, 2]. Alarmingly *Mycobacterium avium* subsp. *paratuberculosis* drawing more attention as a zoonotic pathogen due to its possible association in Crohn's disease of humans [3, 4]. Zoonotic importance of MAP is still debatable due to the resemblances between pathological lesions of paratuberculosis and Crohn's disease, intermittent isolation of MAP organisms and frequent detection of MAP-specific DNA from Crohn's disease lesions [3, 4].
Bio-contamination of MAP bacilli in various milk products has been reported globally. Infected animals excrete MAP organisms in the milk, faeces and environment (feed, water, bedding and soil) which act as possible vehicles for the transmission of MAP to humans [4, 5]. Presence of MAP in animals and their products increases likelihood of exposure of humans to MAP organisms *via* oral route [5]. Thick waxy cell wall of MAP enables survival of organisms at pasteurization temperature and facilitates the occurrence of MAP organism in milk products [3, 6]. Furthermore, certain types of cheeses prepared from the raw milk or milk that has been exposed sub optimal temperature of pasteurization also favours the existence of MAP organisms in milk products [7]. Presence of MAP in dairy products increases the likelihood of exposure as well as development of CD in genetically predisposed persons [5, 8]. Further, most of milk products in India not exposed to proper pasteurization [9, 10]. Therefore assessing the occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in milk products is essential particularly in paratuberculosis endemic regions including India.
Though paratuberculosis is widespread in India, information regarding occurrence of MAP in commercial dairy products is limited to certain geographical locations [10, 11, 12] and there is no report from Tamil Nadu state, India. Hence, a pilot scale study was designed to ascertain the presence of MAP in commercial milk and milk products from Tamil Nadu.

2. Materials and Methods
About 165 commercial milk products including pasteurized milk (30), Buttermilk (30), Curd (30), Paneer (15), Butter (15), Ghee (15), Ice cream (15) and Skim milk (15) were screened for

MAP in the present study. Approximately 50 g of milk products or 50 ml of liquid samples collected from retail markets of Chennai and Vellore districts of Tamil Nadu. Samples were immediately transported to the laboratory under refrigerated conditions and stored at -20 °C until further processing.

The solid milk products were processed as per [13] while liquid products processed as described by [14] with slight modifications. About ten-gram of each solid dairy products were homogenized for two minutes with 30 mL of buffer solution containing 25% Sodium citrate and 4% Polyethylene Glycol 8000. Thereafter, about 10 ml homogenate was centrifuged for 15 min at 1500×g and supernatant was discarded. The pellets were suspended in 500 µL of PBS and second centrifugation step was performed (10 min at 13,000×g). About 10 ml of each liquid milk and milk products centrifuged at 3000 rpm for 15 minutes and the supernatant discarded. The rest of the sediment subjected to Ziehl–Neelsen (ZN) staining (Hi-Media, Mumbai) and DNA extraction using commercial kit (DNA extraction kit, Qiagen biotech services, Germany) as per the manufacturer's instructions.

DNA isolated from commercial milk and milk products samples was subjected to specific IS900 and F57 PCR using primers of [15] and [16] respectively. Presence specific PCR amplicons at 279 bp and 424 bp were considered as positive for IS900 and F57 segment of MAP respectively, which indicates contamination of MAP in commercial milk and milk products.

Table 1: Oligonucleotide primers used to detect MAP by PCR

Target gene	Type	Primer sequence (5' – 3')	Product size	Reference
IS 900	Forward	CGTCGTTAATAACAATGCAG	279 bp	[15]
	Reverse	GGCCGTGCGTTAGGCTTCGA		
F57	Forward	CCTGTCTAATTCGATCACGGA CTAGA	432 bp	[16]
	Reverse	TCAGCTATTGGTGTACCGAAT GT		
	Nested Reverse	TGGTGTACCGAATGTTGTTGT CAC	424 bp	

Fig 1: Agarose gel showing amplicons for IS900 PCR of MAP in milk products. M: DNA marker (100 bp ladder), Lane 1- Positive control; Lane 2- Negative control; Lanes 3-7: DNA test samples.

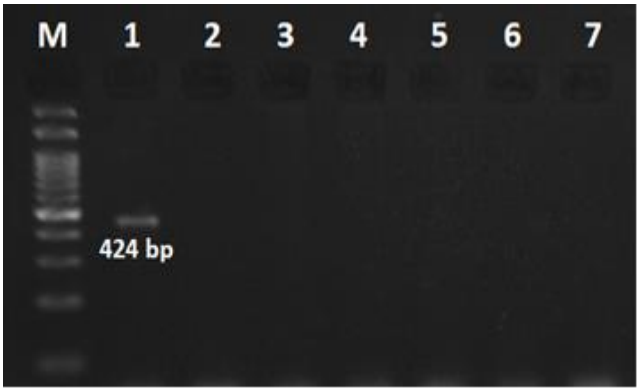

Fig 2: Agarose gel showing amplicons specific for F57 PCR of MAP in milk products. M: DNA marker (100 bp ladder), Lane 1- Positive control; Lane 2- Negative control; Lanes 3-7: DNA test samples.

3. Results and Discussion

Pathogens shared between animals (domestic or wild), humans and environment are emerging as serious challenge for the animal as well as human health. *Mycobacterium avium* subsp. *paratuberculosis* is one such animal pathogen causes Johne's disease (paratuberculosis) in wide range of ruminants [1, 2]. In recent times, MAP also evolving as a zoonotic pathogen of public health threat transmitted via consumption of unpasteurized milk or other dairy products [3, 4, 17].

IS900 sequence is a common target gene for PCR that routinely used for rapid detection of slow-growing MAP organisms in milk, faeces and clinical samples [14, 18]. However, carriage of IS900-like elements in some closely related environmental mycobacteria impedes the diagnostic specificity of IS900 PCR [19, 20]. Hence, other sequences such as IS*Map*02, IS*Mav2*, *hsp*X, locus 255 and F57 also recommended as a target for MAP identification to avoid the uncertainty [21]. The present study targeted IS900 along with F57 region to improve the diagnostic specificity of MAP detection from the milk and milk products.

Paratuberculosis is endemic in bovines of Tamil Nadu that causes huge economic and production losses to the dairy farmers [22]. However, our study revealed that none of the commercial milk and milk products found positive for MAP contamination by ZN staining as well as PCR targeting IS900 and F57 region. Our results were agreement with Biswal *et al.* [11] where no MAP DNA detected from the dairy products of Odisha, India. Contrarily, Shankar *et al.* [10] found MAP DNA in 39% (7/18) of pasteurized milk and 22% (2/9) of commercial milk products while Singh *et al.* [12] reported the presence of MAP in 9.0% (9/100), 0% (0/19), 21.4% (3/14) of the commercial milk, flavoured milk and milk powder samples respectively in India by IS900 PCR. Stephen *et al.* [23] showed the MAP contamination in 32.7% (18/55) of paneer samples. Negative results of our study could be due to low sample size, sensitivity of the diagnostic tests, PCR inhibitors in milk and sampling protocols, which needs to be refined.

This is the first pilot study in Tamil Nadu that revealed none of the commercial milk and milk products of bovine origin found positive for MAP contamination. However, negative results not exclude the MAP infection since success of the diagnostic test depends upon the sensitivity of the test and quantum of MAP bacilli in samples [11, 24]. Therefore, our study recommends further epidemiological studies with extensive systematic sampling and using battery of diagnostic tests to delineate the exact status of MAP contamination in dairy products. Furthermore, regulatory measures including

early detection of subclinical carriers, culling of infected animals, creating awareness among the farmers, improved of hygienic practices and vaccination should implemented strictly to enhance the national food security.

4. Conclusion

The present study revealed that the bio-load of MAP is negligible among commercial milk and milk products of Tamil Nadu. However, screening of larger number of samples with battery of diagnostic tests, herd health management, creating awareness among producers and enforcement of stringent regulations could help in elimination of this hazardous pathogen from human food chain.

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