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Presence, characterization, and genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* from unpasteurized individual and pooled milk, commercial pasteurized milk, and milk products in India by culture, PCR, and PCR-REA methods

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IS*1311* PCR-REA

Summary
Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes Johne's disease in ruminants, a chronic enteritis evocative of human inflammatory bowel disease. In industrialized countries MAP has been cultured from pasteurized milk, compounding the increasing concern that MAP may be zoonotic. The purpose of this study was to evaluate commercially available unpasteurized and pasteurized milk and its products for the presence of viable MAP or MAP DNA from an area of northern India with a population of 150 million people.
Methods: We studied 43 samples (16 unpasteurized, 27 pasteurized) purchased in Mathura, Agra, or New Delhi, for the presence of MAP by culture or by PCR for IS*900* MAP DNA. Positives results were confirmed as MAP by restriction endonuclease analysis and/or DNA sequencing.
Results: Colonies appeared in 1.5—20 months post-inoculation. Of the unpasteurized samples, 44% (7/16) were MAP culture-positive and 6% (1/16) were positive for IS*900* MAP DNA. Of the pasteurized samples, 67% (18/27) were MAP culture-positive and 33% (9/27) were IS*900*-positive. Subsequently, 100% (25/25) of the cultured colonies were IS*900* and IS*1311* MAP DNA-positive.
Conclusions: This is the first report from a developing country of MAP cultured from both pasteurized and unpasteurized milk and milk products. Thus we corroborate the presence of viable MAP in the food chain reported from industrialized countries. With the increasing concern

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that MAP may be zoonotic, these findings have major implications for healthcare in India. The decreased sensitivity in detecting MAP DNA by PCR directly from milk should be ascribed to our employing only one set of PCR primers.
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Introduction

In ruminants, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative organism of Johne's disease (JD),[1] a chronic enteric diarrheal disease that is evocative of human inflammatory bowel disease (IBD).[2] JD is increasing in incidence in both the developed[3] and the developing world, and in India.[4] Apparently unrelated, the incidence of IBD is increasing in the industrialized world[5] as well as in India,[6–10] where gastroenteritis and colitis are common health problems.[11–13]

MAP has been cultured from chlorinated potable municipal water in the USA[14] and Europe,[15] pasteurized milk in the USA[16] and Europe,[17,18] the intestine[19] and stool[20] of patients with Crohn's disease (CD), the breast milk of mothers with Crohn's disease,[21] and from the blood of patients with IBD.[22] MAP is not reproducibly killed by standard pasteurization procedures.[23,24] Although prevailing medical dogma considers MAP to be an innocent commensal,[25] there are increasing concerns that MAP may be zoonotic,[26,27] and at a minimum responsible for IBD[28,29].

The Republic of India has more than 400 million milk producing animals[30] providing approximately 86.9 million tonnes of milk annually. Buffalo milk is the most preferred and costliest, and accounts for 54.5% of the total production; cow milk accounts for 41% and goat milk the remaining 4.5%.[31] Approximately 35% of Indian milk is pasteurized, predominantly by state cooperatives, multinational companies, or government dairy plants.[32] Usually pasteurized products are packaged milk, milk powders, cheese, and ice cream. Of the unpasteurized remainder, 33% remains in the farm or village of production and 51% is distributed by unregulated small vendors, sweet makers, and small dairy units.[33] In India, MAP has been cultured from the unpasteurized milk of goats[4,34] and cattle.[35,36]

We hypothesized that viable MAP may be entering the human food chain in India from both pasteurized as well as unpasteurized milk and its products. We additionally hypothesized that we could identify loco-regional sources of our isolates by performing genotypic analyses on MAP that we isolated. Accordingly, we evaluated milk and milk products for the presence of viable MAP (by culture) and MAP DNA in a region of northern India with a population of 150 million people.

Materials and methods

Origin of milk samples

Forty-three randomly selected commercially available milk samples were purchased in three major cities (Mathura, Agra, and New Delhi) in northern India. Of these, 16 were of unpasteurized milk (500 ml), 18 of pasteurized milk (500 ml), and nine of pasteurized milk products (50 to 500 g). Of the 16 unpasteurized milk samples, six were from dairy farms in the cities of Farah and Mathura that sold milk directly to consumers and 10 were from lactating cows on a dairy farm in the district of Mathura. Milk was aseptically collected from all four quarters of the udder by cleaning and drying each teat and discarding the first expressed samples of milk.

Preparation of samples for MAP culture and PCR

Each liquid sample was centrifuged (3000 rpm, 20 min) providing three layers: supernatant, pellet, and remainder. The resulting final 215 samples were then tested for the presence of MAP using two methods. The first was direct PCR of the milk. The second was by culture of the milk or milk product for MAP. When a culture manifested growth, the DNA from the colony was subsequently extracted and subjected to MAP-specific (IS*900*) PCR.

Culture of fat and sediment layer

The supernatant and pellet were decontaminated in 0.9% hexadecylpyridinium chloride (HPC) for 18–24 hours at room temperature. The resulting liquid layer was decanted and discarded, and 0.02 ml of the residual mucilaginous sludge was inoculated onto modified Herrold's egg yolk medium (HEYM)[37,38] with and without mycobactin J (Allied Monitor Inc., MO, USA). Culture vials were observed for 1.5–20 months. Colonies were compared for morphological characteristics with MAP previously obtained from the screening of feces,[4] tissues,[39] and milk.[23,34]

Identification of MAP by IS900 on milk products

The remainder of the post-decontamination mucilaginous sludge that was not inoculated was then processed for PCR analysis for MAP DNA[40] with minor modifications.[4] DNA from colonies was isolated[41] and amplified using specific IS*900* primers,[42] which results in a 229-bp amplicon, and subjected to 1.8% agarose ethidium bromide gel electrophoresis. Our positive control was a well-characterized MAP 'bison type' strain.[43] As the negative control we used sterilized Liquipure® water (Sigma) that had been subjected to the identical PCR procedures as the samples and positive control.

IS1311 PCR and restriction endonuclease analysis (REA)

IS*1311* PCR was carried out using M56 and M119 primers as previously described.[43,44] In brief, in a 25 μl volume: 0.5–1.0 ng template DNA, 2.5 μl of 10X PCR buffer (Promega),

Table 1 Culture of MAP, confirmed by IS*900* PCR from unpasteurized milk, pasteurized milk, and milk products

Specimen	Number of samples	Culture-positive			IS*900* PCR	
		Supernatant	Pellet	Combined supernatant and pellet	Original pellets	Culture-positive
		n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Unpasteurized milk samples	16	5 (31)	3 (19)	7 (44)	1 (6)	7 (100)
Pasteurized milk samples	18	11 (61)	9 (50)	13 (72)	7 (39)	13 (100)
Commercial milk products[a]	9	3 (33)	3 (33)	5 (56)	2 (22)	5 (100)
Total	43	19 (44)	15 (35)	25 (58)	10 (23)	25 (100)

MAP, Mycobacterium avium subspecies paratuberculosis.
[a] Milk products include ice cream and flavored milk drinks.

1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs, and 1 unit Taq (Promega). Thermal cycling conditions were: initial denaturation at 94 °C for 3 min followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min. The final extension was 72 °C for 10 min. PCR products were subjected to electrophoresis (2% agarose gel stained with ethidium bromide). Amplicons that co-migrated at 608 bp were considered positive results for IS*1311* PCR.

Differentiating MAP into 'bison type and 'cattle type' using IS1311 PCR-REA

IS*1311* PCR-REA-purified MAP DNA was subjected to *HinfI* and *MseI* (Fermentas) restriction endonuclease digestion at 37 °C for 1.5 hours as previously described.[44] Following incubation, fragments were separated on 4% agarose gel. Genotypes were identified by fragment migration patterns as described.[43]

To confirm that we had isolated MAP, DNA sequencing was performed on 13 isolates and compared to K10.[45]

Results

Positive colonies were detected at between 1.5 and 20 months post-inoculation.

Figure 1 Agarose gel with the IS*900* specific primers, which generate a fragment that migrates at a molecular weight of 229 bp. Lane 1 = markers; lanes 2—10 are all samples that had been grown in culture, had had DNA extracted, and were now tested for the presence of IS*900*-specific MAP DNA sequences. All samples shown here tested positive for the presence of IS*900*.

Unpasteurized milk (individual cows and pooled milk)

In our unpasteurized milk samples, 44% (7/16) were MAP culture-positive and 6% (1/16) were MAP IS*900* PCR-positive (Table 1). Of the 7/16 that were MAP culture-positive, all (7/7) were MAP-positive by IS*900* PCR (Figure 1). The proportional agreement[46] between culture and direct PCR showed significant concordance (kappa PA value = 62.0).

Commercially available pasteurized liquid milk

Of the 18 brands of commercially available pasteurized liquid milk samples, 72% (13/18) were MAP culture-positive and 39% (7/18) were directly positive for the presence of MAP by IS*900* PCR (Table 1). Of the samples from which we cultured MAP, all (13/13) were MAP-positive by IS*900* PCR (Figures 1 and 2, Table 1).

Commercially available pasteurized milk products

Of the nine samples of milk products tested, 56% (5/9) were MAP culture-positive and 22% (2/9) were directly positive for

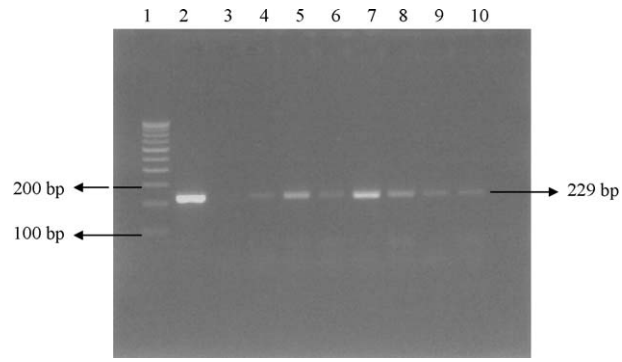

Figure 2 Agarose gel with the IS*900* specific primers, which generate a fragment that migrates at a molecular weight of 229 bp. Lane 1 = markers; lane 2 is the positive MAP DNA control; lane 3 is the negative control (water processed identically with the other samples); lanes 4—10 are all samples that had been isolated from the pellet of samples in this study. All samples show a positive signal.

Table 2 Comparison of concordance of presence or absence of MAP by culture in supernatant and pellet from unpasteurized milk, pasteurized milk, and milk products

	Supernatant MAP culture			
	Positive		Negative	
	n	%	*n*	%
Pellet MAP culture				
Positive	9/43	21	6/43	14
Negative	10/43	23	18/43	42

MAP, Mycobacterium avium subspecies paratuberculosis.
Proportional agreement = 0.62 (substantial).

the presence of MAP by IS*900* PCR. Of the samples that were positive in culture, all (5/5) were MAP-positive by IS*900* PCR (Figure 1, Table 1).

MAP in supernatant and pellet by culture

MAP culture was positive in 44% (19/43) of supernatant (fat) samples and 35% (15/43) of pellet samples (Tables 1 and 2).

Overall detection of MAP by culture and IS900 PCR

For all samples, detection of MAP by culture was 58% (25/43) and by IS*900* PCR was 23% (10/43). Of all 43 samples, 40% (17/43) were only positive by culture and 5% (2/43) were only positive by PCR. Overall, when culture and PCR positivity were combined, we detected MAP in 63% (27/43) of our samples (Table 3).

Genotype profiles of MAP

IS*1311* REA fragment analysis performed on positive MAP cultures showed that 70% (14/20) were 'bison type' and 30% (6/20) were 'cattle type' (Figure 3). When stratified by region of origin, 'bison type' was most prevalent in bovine milk procured from Mathura (100%; 8/8), followed by Agra (71%; 5/7), and New Delhi (20%; 1/5). In contrast, 'cattle type' was more prevalent in the regions of New Delhi (80%; 4/5), followed by Agra (29%; 2/7). Interestingly, in the New Delhi region the 'bison type' MAP was found in one commercial brand that has a chilling plant in the city of Mathura,

Table 3 Comparison of MAP PCR and MAP culture

	Culture			
	Positive		Negative	
	n	%	*n*	%
PCR				
Positive	8/43	19	2/43	5
Negative	17/43	40	16/43	37

MAP, Mycobacterium avium subspecies paratuberculosis.
Twenty-seven positive (63%) in both culture and PCR; 25 live MAP (58%); proportional agreement = 0.55 (moderate).

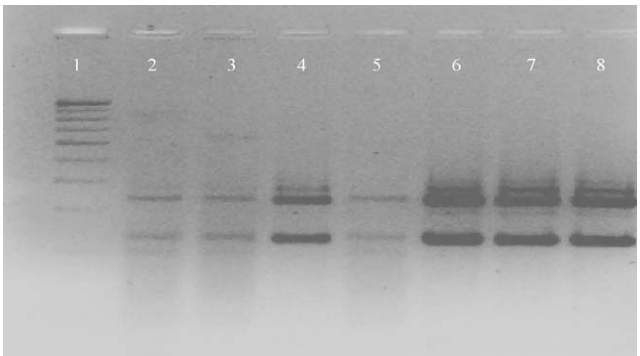

Figure 3 Differentiating MAP into 'bison type' and 'cattle type' using *HinfI* and *MseI* restriction endonuclease analysis (REA) and IS*1311* PCR. Shown is a 4% agarose gel of digested DNA subjected to MAP-specific IS*1311* PCR. Lane 1 = molecular weight markers; 'cattle type' MAP results in three fragments (lanes 2, 3, and 5);[43] in contrast, 'bison type' MAP generates only the two larger bands (lanes 4, 6, 7, and 8).[43].

whereas all the 'cattle type' isolates were from a different commercial brand that has its production facilities in the city of Anand in Gujarat State.

DNA sequencing performed on 13 of our isolates showed 100% homology of a 267-bp fragment of the AV1/AV2 region of IS*900*,[45] confirming that our isolates were MAP (data not presented).

Discussion

India produces more milk products than any other country in the world,[31] the majority of which are not subjected to pasteurization.[32] We cultured MAP from 63% of milk and milk products obtained from an area of northern India that has a population of 150 million. MAP was cultured from both pasteurized as well as unpasteurized samples. Thus, we have shown that present Indian pasteurization standards do not reliably render MAP non-viable. Our data show that the population in India is repetitively exposed to viable MAP, probably more frequently and at higher inoculation counts than are the populations of such countries as the USA[16] and Ireland.[17]

Paradoxically, we identified MAP more frequently by culture (58%) than by IS*900* PCR (23%). The most plausible explanation for the lower detection of MAP DNA that we find is a methodological one. We employed a single set of primers for our IS*900* PCR. Others have used nested primer PCR because of the difficulty of detecting the low copy number of MAP DNA templates.[22] Often in employing the nested primer strategy, the first set of primers fails to detect any signal (RJG unpublished observations). Unfortunately, the probability of contamination-induced false-positives increases exponentially when the nested primer strategy is used. Nevertheless, we suggest that in future studies such as ours, with appropriate precautions against contamination, the use of nested PCR primers may be preferred over the single set of primers that we employed.

We additionally need to consider the possibility that some of our MAP culture results may be ascribable to contamination in culture. However, our data indicate that contamina-

tion does not account for our findings. First, the DNA sequencing analysis on the isolates that we cultured shows that they are not from a single MAP strain. Second, the IS*1311* REA fragment analysis on DNA isolated from the culture-positive colonies shows that there are distinct geographical differences in the incidence of 'bison type' and 'cattle type' strains. For example, when the 'cattle type' genotype was identified in the Delhi area, those isolates were from a processing plant located in Anand in the State of Gujarat. We conclude that our culture data cannot be ascribed to contamination.

The possibility that MAP may be zoonotic[26] remains to be clarified.[27] However, until the MAP zoonosis conundrum is definitively resolved we suggest that the precautionary principle, instituted by the British Government for MAP in the UK[47] should be applied in India and that MAP should be removed from the food chain. We conclude that alternative strategies to diminish the viable MAP burden to which the Indian population is exposed need to be considered and introduced. We suggest that a concerted, Government mandated and directed anti-MAP vaccination program[48] should be amongst the contingencies considered.

Conflict of interest

RJG has submitted provisional patents on hypotheses that were previously tested, and published, concerning MAP. He has no direct conflict of interest on any data published in this manuscript. All other authors declare that they have no conflict of interest.

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