Comprehensive study on the molecular prevalence and seasonal and age distribution of *Mycobacterium avium subspecies paratuberculosis* in raw milk and traditional dairy products

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Abstract: Due to the high consumption rate of milk and dairy products, they should have a high microbial quality. *Mycobacterium avium subspecies paratuberculosis* is one of the emerging zoonotic bacteria with high economic importance. The present study was done to assess the prevalence of the MAP in different types of raw milk and traditional dairy samples. Seven-hundred and eighty-five raw milk and traditional dairy samples were collected. DNA was extracted using a DNA extraction kit. Detection of MAP was done using the *IS900* nested-PCR method. Eighty-eight out of 785 (11.21%) raw milk and traditional dairy product samples harbored MAP. Prevalence of MAP in raw milk and traditional dairy samples were 12.38% and 8.84%, respectively. Raw buffalo (25%) milk samples had the highest prevalence of MAP, while raw donkey (6.66%) had the lowest. Traditional cheese (24%) samples had the highest prevalence of MAP, while traditional kashk (2.50%) had the lowest. Seasonal and age distributions were also observed for the prevalence of MAP. *Raw milk and traditional dairy samples may be the sources of MAP* transmission into the human population. Marked seasonality and age distribution were seen for the prevalence of MAP. Further studies are required to find more information about MAP infection in raw milk and traditional dairy products.

Keywords: Age distribution, *Mycobacterium avium subspecies paratuberculosis*, Raw milk, Seasonal distribution, Traditional dairy

Introduction

Milk and dairy products are full from valuable nutritional factors such as proteins, lipids, minerals, and vitamins. There are several kinds of dairy products such as cheese, cream, butter, yoghurt, and ice cream with high beneficial effects on human health (Momtaz et al. 2012; Rahimi et al. 2014a; Ranjbar et al. 2018a; Ranjbar et al. 2018b; Safarpour Dehkordi et al. 2013a; Safarpour Dehkordi et al. 2014b). Kashk and dough are two important traditional dairy products in Iran and some other parts of the world. Kashk comes in liquid or dried forms and is traditionally made with the milk left over from cheese-making. Dough is a popular salty yogurt-based beverage amongst an Iranian people (Momtaz et al. 2012; Rahimi et al. 2014a; Ranjbar et al. 2018a; Ranjbar et al. 2018b; Safarpour Dehkordi et al. 2013a; Safarpour Dehkordi et al. 2014b). Based on the high consumption rate of milk and dairy products, it is important to ensure from the microbial quality of these types of food samples (Momtaz et al. 2012; Rahimi et al. 2014a; Ranjbar et al. 2018a; Ranjbar et al. 2018b; Safarpour Dehkordi et al. 2013a; Safarpour Dehkordi et al. 2014b). However, the microbial quality of raw milk and traditional dairy products has been decreased and several foodborne infections have been occurred (Atapoor et al. 2014; Ghorbani et al. 2016; Hasanpour Dehkordi et al. 2017; Hemmatinezhad et al. 2015; Madahi et al. 2014; Momtaz et al. 2013a; Momtaz et al. 2013b; Nejat et al. 2015; Rahimi et al. 2014b; Ranjbar et al. 2017; Safarpour Dehkordi et al. 2017; Safarpour Dehkordi et al. 2013b; Safarpour Dehkordi et al. 2014a; Safarpour Dehkordi et al. 2012).

Mycobacterium avium (*M. avium*) *subsp. paratuberculosis* (MAP) is the causal agent of Johne's disease (a chronic granulomatous enteritis of cattle and other ruminants) and chronic infectious enteritis and especially Paratuberculosis of domestic and wild ruminants. Paratuberculosis is characterized by a long incubation period, weight loss, diarrhea, progressive cachexia, and death (Chamberlin et al. 2001; Manning 2001). Furthermore, MAP has also been isolated in intestinal tissues of Crohn's disease (a chronic inflammatory condition of the gut) patients,

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thus giving rise to a hypothesis of there being a possible link between these two diseases (Chamberlin et al. 2001; Manning 2001). Milk plays an important role in transmission of infection and above all the excretion via milk is viewed with caution since raw milk may be a possible source of human infection (Chamberlin et al. 2001; Gill et al. 2011; Grant 2003; Manning 2001). It has long been suggested that MAP may cause or be otherwise involved in Crohn's disease, but the available evidence of this involvement has been inconclusive. Additionally, the concern about presence of MAP in milk and dairy products is about its heat resistance nature and also its controversial role in occurrence of Crohn's disease in human (Chamberlin et al. 2001; Gill et al. 2011; Grant 2003; Manning 2001).

Traditionally, MAP culture from ruminant's milk is a low sensitive, time consuming and dangerous diagnostic method (Chamberlin et al. 2001; Gill et al. 2011; Grant 2003; Manning 2001). Highly sensitive molecular techniques such as different types of Polymerase Chain Reaction (PCR) have greatly contributed to identify MAP strains in food samples with animal origins and especially milk (Pillai & Jayarao 2002). As yet there is no literature concerning the detection of MAP by PCR in raw milk and traditional dairy products in Iran. Therefore, the present investigation was done to study the prevalence rate and seasonal and age distributions of MAP in raw bovine, ovine, caprine, buffalo, camel and donkey milk and traditional cheese, dough, kashk, yoghurt, butter and cream dairy product samples using the nested-PCR reaction.

Materials and Methods

Ethics approval and consent to participate

The study was approved by the Ethical Council of Research of the Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran. Verification of this research project and the licenses related to sampling process were approved by the Prof. Ebrahim Rahimi and Prof. Amir Shakerian.

Samples collection

From June 2016 to June 2017, a total of 785 various types of dairy samples including raw bovine (n= 120), ovine (n= 120), caprine (n= 110), camel (n= 85), buffalo (n= 60) and donkey (n= 30) raw milk samples and traditional cheese (n= 50), yoghurt (n= 40), dough (n= 50), cream (n= 40), butter (n= 40) and kashk (n= 40) dairy product samples were randomly collected from different

parts of Iran. Before collection, teats were thoughtfully cleansed with alcohol to avoid samples contamination with the skin. Milk (40 mL) was collected in a sterile 50-mL centrifuge tube from the 4 quarters by milking, discarding the first 10-15 mL. Samples were immediately transferred to the Food Hygiene Research Center of the Islamic Azad University of Shahrekord in cooler with ice-packs. All samples showed normal physical characters including odor, color and consolidation.

Milk preparation and DNA extraction

Prior to DNA extraction, milk samples were centrifuged at 1000 g for 15 min and the supernatant discarded. The resultant pellet was washed thrice in phosphate buffer saline (PBS, pH 7.3) and centrifuged at 500 g for 15 min. the pellet was suspended in 1 mL of PBS, centrifuged and resuspended in 100 mL of 0.2 N NaOH. DNA extracted using a commercial kit (DNeasy tissue kit, Qiagen, Hilden, Germany) preceded by an enzymatic digestion with lysozyme buffer (lysozyme 18 mg/ml, 15 mM Tris-HCl pH 8.0, 1 mM EDTA and 1% Triton X100) and proteinase K (Qiagen, Hilden, Germany). Procedure was done rendering to the manufacturer's guidelines. Purity (A260/A280) and concentration of extracted DNA were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA). The truth of the DNA was assessed on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany).

Nested PCR-based detection of *M. avium subsp. paratuberculosis*

M. avium subsp. paratuberculosis was detected using the specific nested-PCR reaction according to method described previously (Englund et al. 1999; Nebbia et al. 2006). Four primers were selected from the *M. avium subsp. paratuberculosis* specific insertion sequence IS900 (Accession No. X16293 in GenBank). Table 1 represents the list of primers used in primary and nested PCR reactions.

The first and the second amplification reaction were optimized individually regarding the concentration of MgCl₂, nucleotides, primer, and Taq DNA polymerase as well as the annealing temperature and number of cycles in the amplification. In the reactions 1 pg to 50 pg DNA from milk and traditional dairy samples were used. Finally, the 50-mL reaction mixture contained 60 mM Tris-HCl buffer (pH 8.8), 2.0 mM MgCl₂, 0.2 mM of each of the four dNTPs (Thermo Fisher Scientific, St. Leon-Rot, Germany), 10 pmol of each of the primers, 0.5 U Taq DNA

Table 1 List of primers used for detection of the *M. avium subsp. paratuberculosis* in raw milk and traditional dairy product samples

Target gene	Primer Sequence (5'-3')	PCR product	Size of product (bp)
IS900	GTTCGGGGCCGTCGCTTAGG	Amplicon 1	400
IS900	GAGGTCGATCGCCACGTGA		
IS900	CCGCTAATTGAGAGATGCGATTGG	Amplicon 2	229
IS900	AATCAACTCCAGCAGCGCGGCCTCG		

polymerase, and 5 mL DNA. A positive control, 250 fg DNA from *M. avium* subsp. paratuberculosis strain Linda, and a negative control, sterile water (Thermo Fisher Scientific, St. Leon-Rot, Germany), were included in each amplification set-up. The reaction mixtures were overlaid with two drops of mineral oil, centrifuged briefly and placed in a PCR thermal cycler (Eppendorf Co., Hamburg, Germany) programmed for 94°C for 3 min, and then 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, the last three steps being repeated sequentially for 35 cycles. The second PCR was performed in the same way as the first PCR, but with a 5-mL sample from the first PCR reaction (Englund et al. 1999; Nebbia et al. 2006).

Gel electrophoresis

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 2.5% agarose gel. The gel was stained with 0.1% ethidium bromide (0.4 µg/ml). The UVI doc gel documentation systems (Grade GB004, Jencons PLC, London, UK) was applied for analysis of images.

Statistical analysis

Statistics were subjected to Microsoft office Excel (version 15; Microsoft Corp., Redmond, WA, USA). Statistical analysis was performed by means of the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact two-tailed test were applied to measure any significant relationship. *P* value <0.05 was considered as statistical significant level.

Results and Discussion

Nested-PCR amplification

MAP is responsible for occurrence of dangerous pathogenic diseases such as paratuberculosis and Johne's and Crohn's diseases. Foods with animal origins and especially raw milk and traditional dairy products play an emerging role in transmission of MAP to human population. MAP is present in domesticated ruminants in most regions of the world. Determination of the MAP infection status of individual animals or herds can be difficult. Consequently, estimates of the prevalence of MAP in animals and herds also is difficult (Gill et al. 2011; Grant 2003). Additionally, scarce data have been conducted on detection of MAP from raw milk of naturally infected animals and also traditional dairy products (Gill et al. 2011; Grant 2003). Thus, the present study was done to assess the prevalence of MAP in raw milk of naturally infected animals and also traditional dairy products.

The present study was done to assess the prevalence rate and seasonal and age distributions of the MAP in different types of raw milk and traditional dairy product samples. Figure 1 represents the results of the PCR gel electrophoresis for the nested-PCR reaction used for detection of the *IS900* gene of the MAP.

Prevalence of MAP in studied dairy

Table 2 represents the prevalence of MAP in different types of raw milk and traditional dairy product samples. Eighty-eight out of 785 (11.21%) raw milk and traditional dairy product samples harbored MAP. Sixty-five out of 525 (12.38%) raw milk samples and twenty-three out of 260 (8.84%) traditional dairy product

Table 2 Prevalence of *M. avium* subsp. paratuberculosis in different types of raw milk and traditional dairy product samples

Types of samples	No. samples collected	N (%) samples positive for <i>M. avium</i> subspecies paratuberculosis	P value
Raw bovine milk	120	19 (15.83)	<i>P</i> < 0.05
Raw ovine milk	120	12 (10)	
Raw caprine milk	110	9 (8.18)	
Raw camel milk	85	8 (9.41)	
Raw buffalo milk	60	15 (25)	
Raw donkey milk	30	2 (6.66)	<i>P</i> < 0.05
Total raw milk	525	65 (12.38)	
Traditional cheese	50	12 (24)	
Traditional yoghurt	40	2 (5)	
Traditional dough	50	2 (4)	
Traditional cream	40	3 (7.50)	
Traditional Butter	40	3 (7.50)	
Traditional kashk	40	1 (2.50)	
Total traditional dairy products	260	23 (8.84)	
Total	785	88 (11.21)	

Fig. 1 Results of the gel electrophoresis of the *IS900* gene of the *Mycobacterium avium* subsp. paratuberculosis. A: the first step PCR amplification (400 bp) and B: The nested PCR amplification (229 bp). Lane 1: 100 bp ladder (Thermo Fisher Scientific, Germany), Lane 2: Positive control, Lane 3-6: Positive samples for the *IS900* gene of the *Mycobacterium avium* subsp. paratuberculosis (400 bp in amplicon 1 and 229 bp in amplicon 2) and Lane 7: Negative control

samples were positive for MAP. Raw buffalo (25%) and raw bovine (15.83%) milk samples had the highest prevalence of MAP, while raw donkey (6.66%) and raw camel (9.41%) had the lowest. Traditional cheese (24%) samples had the highest prevalence of MAP, while traditional kashk (2.50%) samples had the lowest. Prevalence of MAP in raw ovine and caprine milk and traditional cream and butter samples were 10% and 8.18% and 7.50% and 7.50%, respectively. Statistically significant differences were seen between types of samples and prevalence of MAP ($P < 0.05$).

We found that the total prevalence of MAP in all studied samples was 11.21%. Additionally, prevalence of MAP in raw milk and traditional dairy product samples were 12.38% and 8.84%, respectively which showed an important public health threat regarding the consumption of un-pasteurized milk and traditional dairy products. MAP isolation from milk was first reported in 1935, in association with advanced clinical paratuberculosis (Taylor et al. 1981). More recent investigations have found MAP isolation rates in milk of up to 45% in clinically affected animals (Giese & Ahrens 2000) and of up to 22% in colostrum or 8% in milk in subclinical cases (Streeter et al. 1995). Available information

reveals that there is wide regional variation in MAP prevalence. For cattle, estimated animal prevalence have ranged from <2 to $<20\%$, and estimated herd prevalence have ranged from <10 to $>50\%$ (Adaska & Anderson 2003; Mohan et al. 2009; Tiwari et al. 2006). In some regions, the prevalence of MAP in beef cattle is apparently about half the prevalence in dairy cattle (Good et al. 2009; Okura et al. 2010). Data on MAP infection of sheep and goats are limited, but prevalence of up to 20% in European herds have been suggested (Nielsen & Toft 2009). Prevalence of MAP in milk of naturally infected cows collected from United States (Sweeney et al. 1992), Canada (Gao et al. 2009), Brazil (Carvalho et al. 2009), Mexico (Favila-Humara et al. 2010), Denmark (Giese & Ahrens 2000), Poland (Szteyn et al. 2008) and United Kingdom (Grant et al. 2002) had a range between 1% to 71%. Prevalence of MAP in milk of naturally infected sheeps and goats collected from European countries had a range of 1 to 100% (Djønne et al. 2003; Grant et al. 2001; Mühlherr et al. 2003; Nebbia et al. 2006). Prevalence of MAP in dairy product samples had a range of 1-78% al-around the world (Hruska et al. 2005; Ikononopoulos et al. 2005; Shankar et al. 2010).

Fig. 2 Seasonal distribution of *M. avium* subsp. paratuberculosis in different types of raw milk and traditional dairy product samples

We found that raw buffalo (25%) and raw bovine (15.83%) milk and traditional cheese (24%) samples had the highest prevalence of MAP. Higher tendency of the MAP bacteria to fat is may be probable reason for the higher prevalence of MAP in buffalo milk. In developed countries, most cheese is made from pasteurized milk. However, many types of cheese continue to be made from raw milk or milk that has been subjected to heating treatments less severe than those used for pasteurization. MAP obviously may be present in raw milk and can survive subpasteurization heating treatments. MAP is also resists against acidic pH and salt concentration present in cheese. Low prevalence of MAP in donkey milk samples is may be due to the fact that equines are not count as a real reservoir of the MAP. We also found that camel milk samples had the low prevalence of MAP. Low prevalence of MAP in kashk samples is may be due to the fact that kashk preparation needs high temperature which will decrease the numbers of MAP bacteria. Gilardoni et al. (2016) reported that the prevalence of MAP in milk in Argentina was 45.42%. Nebbia et al. (2006) reported that the prevalence of MAP in milk in Italy was 44.80%. Gerrard et al. (2018) reported that the prevalence of MAP in milk in England was 10.30%.

Age and seasonal distribution of MAP

Table 3 represents the age distribution of MAP in different types of raw milk samples. We found that milk samples collected from

4-6 years old bovine (42.10%), 2-4 years old ovine (41.66%), 2-4 years old caprine (44.44%), 2-6 years old camel (37.50%), 4-6 years old buffalo (40%) and 4-6 years old and older than 6 years old donkey (50%) had the highest prevalence of MAP. Additionally, milk of younger than 2 years old animals had the lowest prevalence of MAP. Statistically significant difference was seen between age of animal species and prevalence of MAP ($P < 0.05$).

Figure 2 represents the seasonal distribution of MAP in raw milk and traditional dairy product samples. Raw milk samples which were collected through the winter season (46.15%) had the highest prevalence of MAP, followed by autumn (27.69%) and spring (18.46%). Traditional dairy product samples which were collected through the winter season (39.13%) had the highest prevalence of MAP, followed by autumn (30.43%) and spring (21.73%). Samples which were collected through the summer season had the lowest prevalence of MAP. Statistically significant differences were seen for the prevalence of MAP between cold and warm seasons ($P < 0.05$).

We also found that the prevalence of MAP in milk and dairy product samples follows the seasonal distribution. In the other hand, MAP bacteria had the highest prevalence in winter season. The main reason for this finding is the fact that transmission and dissemination of MAP is much higher in the winter than other

Table 3 Age distribution of *M. avium* subsp. paratuberculosis in different types of raw milk samples

Types of samples (No. positive)	Age (year)	N (%) samples positive for <i>M. avium</i> subspecies paratuberculosis	P value
Raw bovine milk (19)	<2	1 (5.26)	$P < 0.05$
	2-4	4 (21.05)	
	4-6	8 (42.10)	
	>6	5 (26.31)	
Raw ovine milk (12)	<2	1 (8.33)	$P < 0.05$
	2-4	5 (41.66)	
	4-6	3 (25)	
	>6	3 (25)	
Raw caprine milk (9)	<2	-	$P < 0.05$
	2-4	4 (44.44)	
	4-6	3 (33.33)	
	>6	2 (22.22)	
Raw camel milk (8)	<2	-	$P > 0.05$
	2-4	3 (37.50)	
	4-6	3 (37.50)	
	>6	2 (25)	
Raw buffalo milk (15)	<2	1 (6.66)	$P < 0.05$
	2-4	4 (26.66)	
	4-6	6 (40)	
	>6	4 (26.66)	
Raw donkey milk (2)	<2	-	$P > 0.05$
	2-4	-	
	4-6	1 (50)	
	>6	1 (50)	

tested seasons. Additionally, we found that milk of older animal species had the highest prevalence of MAP. Otherwise, milk of younger than 2 years old animal had the lowest prevalence of MAP. This finding is may be due to the long incubation period of MAP in animal species. MAP is responsible for Johne's disease and chronic infectious enteritis and especially Paratuberculosis of domestic and wild ruminants. Johne's disease is chronic granulomatous enteritis of cattle and other ruminants. Clinical practices revealed that occurrence of Johne's disease is so rare in younger than 2 years animal species (Adaska & Anderson 2003; Good et al. 2009; Taylor et al. 1981).

Our results might suggest a MAP transmission risk via milk, even from apparently healthy animals. Infection of lambs and kids by this route is likely under normal breeding conditions. As reported for cattle, young animals are more susceptible to MAP infection than adults, with milk consumption from infected ewes being a major source of infection. Control measures similar to those already applied in cattle, such as sanitation, separating newborn calves from their mothers and subsequent artificial feeding, might be useful to small ruminants as well. Consumption of soft and hard cheese and other types of dairy products produced from un-pasteurized cow, sheep and goat milk is becoming increasingly important in Iran and other countries. This

could increase the risk of MAP infection in humans. A link between MAP, Crohn's disease and consumption of dairy products from raw milk is still to be demonstrated (Chiodini 2003), however, even a remote public health risk should contribute to strengthen collaboration between veterinarians, public health officers, livestock breeders and dairy producers, as well as stimulate the development and subsequent routine use of more specific and sensitive diagnostic tools.

Conclusions

To put it in a nutshell, we identified a large numbers of MAP in raw bovine, ovine, caprine, buffalo, camel and donkey milk and also traditional cheese, yoghurt, cream, kashk, dough and butter dairy products. Raw buffalo and bovine milk samples and traditional cheese had the highest prevalence of MAP amongst tested samples. Therefore, raw buffalo and bovine milk and traditional cheese had the higher ability for transmission of MAP into the human populations. Additionally, prevalence of MAP bacteria was related to the seasonal distribution with higher prevalence in winter season. Additionally, prevalence of MAP bacteria was related to the age of animal species with higher prevalence in milk of older animals. To the best of our knowledge, this study is the most comprehensive description study on the

prevalence of MAP in Iran. Although the incidence of MAP infection was relatively low (11.21%), further studies should be conducted to get more information on MAP infection in raw bovine, ovine, caprine, buffalo, camel and donkey milk and also traditional cheese, yoghurt, cream, kashk, dough and butter dairy products, especially in areas where animals are kept close to human populations.

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