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Seasonal and Age Distribution of *Toxoplasma gondii* in Milk of Naturally Infected Animal Species and Dairy Samples

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PRIMARY infection by *Toxoplasma gondii* is habitually food borne and ingestion of foods with animal origins is measured as a risk factor. The current survey was performed to assess the seasonal and age distribution of *T. gondii* in diverse kinds of raw milk and traditional dairy products. Three-hundred and seventy raw milk and traditional dairy samples were collected and subjected to DNA extraction. *B1* specific gene of *T. gondii* was perceived by means of the molecular technique. Eighteen out of 370 (4.86%) studied samples were positive for *T. gondii*. Molecular incidence of *T. gondii* in raw milk was 6.08%. *T. gondii* was also perceived in 2.85% of studied traditional dairy samples, respectively. Sheep milk (10%) and cheese (6.66%) had the uppermost molecular incidence of parasite. Molecular incidence of *T. gondii* in raw camel milk and cream and butter samples were 3.33%, 5% and 5%, respectively. Samples which were collected through autumn (15.55%) and summer (9.37%) seasons had the maximum molecular incidence of *T. gondii*. Raw milk samples of older than 4 years animal species (11.39%) had the maximum molecular incidence of *T. gondii*. The existingsurvey is the first report of seasonal and age distribution of *T. gondii* in diverse kinds of dairy samples. Raw sheep, goat, buffalo, bovine and camel milk and also traditional cheese, cream and butter dairy samples may harbor *T. gondii* infection. Seasonal and senile distribution should be measured for molecular incidence of *T. gondii* in milk of primary infected animals and also traditional dairy products.

Keywords: *Toxoplasma gondii*, Raw milk, Traditional dairy products, Season, Age.

Introduction

Milk and dairies have an imperative importance in the human regime. They provide a set of nutrient materials such as fats, proteins, calcium, vitamins, potassium and phosphorus [1, 2]. Milk and traditional dairies are not essentially safe. It is because of numerous surveys demonstrated their consumption as a risk factor for occurrence of diverse kinds of diseases such as toxoplasmosis[3-6].

Toxoplasma gondii (*T. gondii*) is an intracellular cyst-forming Apicomplexan Sarcocystidae protozoan parasite responsible for occurrence of infectious toxoplasmosis in humans and animals [7-10]. *T. gondii* is also responsible for biologically diverse diseases such as abortion in livestock and predominantly sheep, encephalitis in immunocompromised individuals and abortion or congenital flaws in fetuses [7]. Additionally, consumption of contaminated dairies is a significant risk factor for occurrence of toxoplasmosis in human population [7, 8, 11].

Consumption of contaminated milk and other high risks food stuffs is the most substantial route of transmission of *T. gondii* to intermediate hosts [3, 5, 7-10, 12-14]. Gaps in present information about the risk assessment of *T. gondii* by dairy consumption are renowned. Inconsistent information is perceived inside risk assessment of dairy product consumption and occurrence of toxoplasmosis. While some reports described

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positive relationships amid dairy consumption and transmission of *T. gondii* infection to human [8, 11], others specified irrelevant effects of dairy consumption [15, 16]. Thus, it is important to find the portion of raw milk and traditional dairies in transmission of *T. gondii* infections to human. Furthermore, there is no strict data about the role of season and age of animals in the incidence of *T. gondii*. Some scarce reports disclosed that season and age may affect the incidence of toxoplasmosis [17, 18].

Affording to uncertain role of raw milk and traditional dairies in transmission of *T. gondii*, the current survey was performed to evaluate the seasonal and age distribution of *T. gondii* in raw cow, buffalo, sheep, goat, camel and donkey milk and traditional yoghurt, cheese, butter, cream, doogh, kashk, and ice-cream dairy product samples by means of the nested-Polymerase Chain Reaction (nested-PCR).

Materials and Methods

Moral questions

The survey was permitted by the ethical team of the author's institute.Licenses of sampling were taken from the Head of the Faculty.

Samples

From April 2016 to April 2017, 370 raw milk and traditional dairy samples including raw buffalo (n= 30), cow (n= 60), sheep (n= 40), goat (n= 50), donkey (n= 20) and camel (n= 30) milk and traditional cheese (n= 30), cream (n= 20), butter (n= 20), yoghurt (n= 30), kashk (n= 20), doogh (n=30) and ice-cream (n=20) samples were arbitrarily obtained through simple random sampling procedure from the retail centers. Samples were collected from diverse provinces located at the center and south-west of Iran. All samples were stored at refrigerator in the retail centers. Sheep, donkey and goat raw milk samples were only collected through the spring and summer seasons. Dairy samples all were derived from cow's milk. Age of targeted livestock was determined by an expert professor of the field of Animal Sciences. Briefly, Samples (10 mL) were collected through hygienic circumstances by means of sterile glass tubes. First few squirts were overlooked during milk collection. Samples were directly transferred to the laboratory by means of sterile cool boxes.

DNA extraction

DNA was extracted from a 200-µL aliquot of samples by means ofthe DNA extraction and purification kit (Thermo Fisher Scientific, Germany) rendering the factory's guidelines. For this purpose, 900 QL of cell lysis solution was added to 300 QL of sample in a cryotube. Samples were subjected to enzymatic pre-digestion by means of proteinase K (20 QL, 20 mg/ml) and incubated at 56°C overnight. Extracted DNA samples were subjected to quantification by NanoDrop device (NanoDrop, Thermo Scientific, Waltham, USA), qualification (2% agarose gel) and purity checking (A260/A280).

Detection of T. gondii B1 gene

DNA thermo-cycler (Eppendorf Master cycler 5330, Hamburg, Germany) was applied in PCR reactions. *T. gondii B1* gene was perceived in DNA samples by means of the method conveyed formerly [19]. Table 1 signifies the PCR circumstances applied for detection of *T. gondii B1* gene. Visualization was performed by means of electrophoresis on agarose gel (2%in 1× TBE buffer stained with SYBR Green (Thermo Fisher Scientific, Germany). Negative control (PCR grade water (Thermo Fisher Scientific, Germany) and positive control (positive DNA for the B1 gene obtained from the Faculty of Veterinary Medicine, University of Tehran) were applied in PCR reactions.

Statistical analysis

Data obtained from the survey were numerically examined by SPSS 21.0 software. Noteworthy relations amid variables were determined by chi-square and Fisher's exact two-tailed tests. P value <0.05 was determined as level of significance.

Results

Three-hundred and seventy dairies were tested for presence of *T. gondii B1* gene by the PCR method. Figure 1 signifies the amplification of *T. gondii B1* gene in first and second (nested) PCR reactions.

Table 2 signifies the molecular incidence of *T. gondii* in diverse kinds of dairies. Eighteen out of 370 (4.86%) raw milk and dairy samples were positive for *T. gondii* B1 gene. Four out of 140 (2.85%) traditional dairy product and 14 out of 230 (6.08%) raw milk samples were also positive for *T. gondii* B1 gene. Sheep milk (10%) was the most commonly contaminated sample amongst the raw milk samples. Additionally, traditional cheese (6.66%) was the most commonly contaminated sample amongst the traditional dairy product samples. Reversely, camel milk (3.33%) and cream (5%) and butter (5%) had the lowermost molecular incidence of *T. gondii*. There were

TABLE 1. PCR circumstances applied for detection of T. gondii B1 gene.

Reactions	Primer sequence (5'-3')	PCR product (bp)	PCR programs	PCR volume (50 µL)
First PCR	F: GGA ACT GCA TCC GTT CAT GAG R: TCT TTA AAG CGT TCG TGG TC	193	1 cycle: ^{0C} ------ 5 min. 94 40 cycle: ^{0C} ----- 10 s 93 ^{0C} -----10 s 57 ^{0C} ----- 30 s 72 1 cycle: ^{0C} -------5 min 72	5 µL PCR buffer 10X 2 mM $Mgcl_2$ 150 µM dNTP 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase 3 µL DNA template
Second PCR	F: TGC ATA GGT TGC AGT CAC TG R: GGC GAC CAA TCT GCG AAT ACA CC	96	1 cycle: ^{0C} -----5 min. 94 40 cycle: ^{0C} -----10 s 93 ^{0C} -----10 s 62.5 ^{0C} -----15 s 72 1 cycle: ^{0C} -------5 72	5 µL PCR buffer 10X 2 mM $Mgcl_2$ 150 µM dNTP 0.75 µM of each primers F & R 1.5 U Taq DNA polymerase 3 µL DNA template

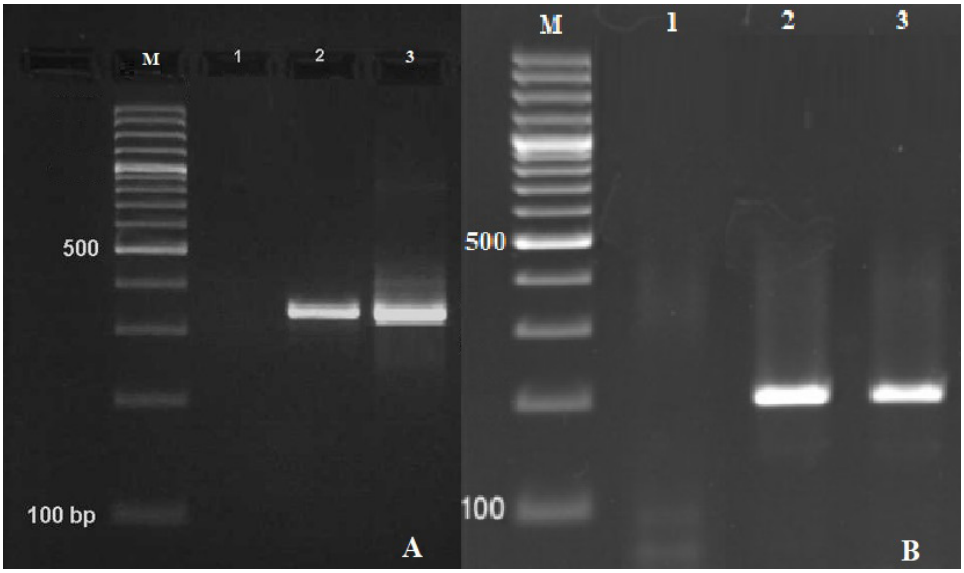

Fig.1. Results of the gel electrophoresis of the *B1* gene of the *T. gondii*. A: the first step PCR amplification: M: 100 bp ladder (Thermo Fisher Scientific, Germany), 1: Negative control (negative DNA control consisting of PCR grade water (Thermo Fisher Scientific, Germany)), 2: Positive control (positive DNA for the *B1* gene) and 3: positive sample (328 bp). B: The nested-PCR amplification. M: 100 bp ladder (Thermo Fisher Scientific, Germany), 1: Negative control (negative DNA control consisting of PCR grade water (Thermo Fisher Scientific, Germany)), 2: Positive control (positive DNA for the *B1* gene) and 3: positive sample (198 bp).

TABLE 2. Molecular incidenceof *T. gondii* in diverse kinds of raw milk and traditional dairy product samples.

Type of samples (raw milk and traditional dairy products)	N samples collected	N (%) samples positive for *T. gondii*
Buffalo milk	30	2 (6.66)
Cow milk	60	3 (5)
Goat milk	40	3 (7.50)
Sheep milk	50	5 (10)
Donkey milk	20	-
Camel milk	30	1 (3.33)
Total raw milk	230	14 (6.08)
Cheese	30	2 (6.66)
Cream	20	1 (5)
Butter	20	1 (5)
Yoghurt	20	-
Kashk	20	-
Doogh	30	-
Total dairies	140	4 (2.85)
Total	370	18 (4.86)

no detectable *T. gondii B1* gene in donkey raw milk and yoghurt, kashk and doogh samples. Statistically noteworthy variances were gotten amid kinds of samples and molecular incidence of *T. gondii* ($P<0.05$).

Table 3 signifies the seasonal distribution of *T. gondii* in diverse kinds of dairies. Molecular incidence of *T. gondii* in spring, summer, autumn and winter seasons were 2.10%, 9.37%, 15.55% and 0%, respectively. Moreover, molecular incidence of *T. gondii* in traditional dairies collected through spring, summer, autumn and winter seasons were 0%, 5.26%, 17.64% and 0%, respectively. Statistically noteworthy variances were gotten amid season of sampling and molecular incidence of *T. gondii* ($P<0.05$).

Table 4 signifies the age distribution of *T. gondii* in diverse kinds of raw milk. Molecular incidence of *T. gondii* in raw milk samples of < 2 years old, 2-4 years old and > 4 years old livestock species was 1.81%, 5.26% and 11.39%, respectively. Only

TABLE 3. Seasonal distribution of *T. gondii* in diverse kinds of raw milk and traditional dairy product samples.

Types of samples (N positive samples)	N samples collected in each season				N (%) samples positive in each season			
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter
Buffalo milk (2)	8	9	7	6	-	1 (11.11)	1 (14.28)	-
Cow milk (3)	17	15	14	14	-	1 (6.66)	2 (14.28)	-
Goat milk (3)	20	20	-	-	1 (5)	2 (10)	-	-
Sheep milk (5)	25	25	-	-	1 (4)	4 (16)	-	-
Camel milk (1)	8	8	7	7	-	-	1 (14.28)	-
Total raw milk (14)	78	77	28	27	2 (2.56)	8 (10.38)	4 (14.28)	-
Cheese (2)	6	7	8	9	-	1 (14.28)	1 (12.50)	-
Cream (1)	5	5	5	5	-	-	1 (20)	-
Butter (1)	6	7	4	3	-	-	1 (25)	-
Total dairies (4)	17	19	17	17	-	1 (5.26)	3 (17.64)	-
Total (18)	95	96	45	44	2 (2.10)	9 (9.37)	7 (15.55)	-

TABLE 4. Age distribution of *T. gondii* in diverse kinds of raw milk and traditional dairy product samples.

Types of samples (N positive samples)	N samples collected in different age groups			N (%) samples positive in different age groups		
	<2	2-4	>4	<2	2-4	>4
Buffalo milk (2)	7	10	13	-	1 (10)	1 (7.69)
Cow milk (3)	16	20	24	-	1 (5)	2 (8.33)
Goat milk (3)	12	15	13		1 (6.66)	2 (15.38)
Sheep milk (5)	14	19	17	1 (7.14)	1 (5.26)	3 (17.64)
Camel milk (1)	6	12	12	-	-	1 (8.33)
Total raw milk (14)	55	76	79	1 (1.81)	4 (5.26)	9 (11.39)

7.14% of raw milk samples of younger than 2 years old ovine were positive for *T. gondii*. There were no detectable *T. gondii* in the raw milk samples of younger than 2 years old buffalo, bovine, caprine and camel. Statistically noteworthy variances were gotten amid age of animal species and molecular incidence of *T. gondii* (P<0.05).

Discussion

An existing survey was performed to evaluate the molecular incidence of *T. gondii* in diverse kinds of dairies regarding the age of animal species and season of sampling. As it disclosed, 4.86% of dairies were positive for *T. gondii*. In a same survey[20], incidence of *T. gondii* in raw buffalo, cow, goat, sheep and camel milk samples collected from Iran were 3.65%, 3.50%, 9.44%, 6.48% and 2.50%, respectively. Findings of an existing survey was also like those of Egyptian survey [21]. They exhibited that the incidence of *T. gondii* in raw camel, sheep and goat milk samples were 0%, 5.55% and 3.70%, respectively. *T. gondii* has also been recognized in the milk of numerous hosts, such as goat [22-27], sheep [28-30], donkey [23, 31], cattle [32, 33], camel [21, 34-36], buffalo [11], cat [37], dog [38], rat [39] and even human breast feed [40, 41]. In keeping with this, only consumption of contaminated goat milk was associated with occurrence of human acute toxoplasmosis[42-44].Nevertheless, some sero-epidemiological surveys specified significantly association with ingestion of raw milk of other livestock and *T. gondii* infection [45-49]. Association amid raw milk ingestion and *T. gondii* transmission to human has been conveyed from Iran [11], USA [7], Turkey [50]Germany [51] and Mexico [45]. Other works conducted on Jordan [52] and Kyrgyzstan [16] specified non-significant effect of dairy consumption and occurrence of toxoplasmosis. *T. gondii*'s tachyzoites present in milk are sensitive to gastric digestion.

Though, preceding work disclosed that *T. gondii*'s tachyzoites may sporadically survive in acidic solutions and their ingestion may cause infection in cats and mice [53]. Moreover, *T. gondii*'s tachyzoites may penetrate to mucosal tissue and reach to the blood and lymphatic systems of hosts before reaching the stomach [42, 43, 54]. Furthermore, *T. gondii*'s tachyzoites can survive in milk for about three to seven days at 4°C [55] and in cheese for a about 8 to 10 days [32]. Thus, presence of *T. gondii* in dairies should be measured as a dangerous concern. Serologic surveys conducted in Iran [56], Czech Republic [57], Romani [58], Brazil [59], Greece [60], and finally Thailand [61].

Our findings also disclosed that dairies collected through autumn and summer seasons had the higher molecular incidence of *T. gondii*. Moreover, raw milk samples collected from older animals (>4 years old) had the higher molecular incidence of *T. gondii*. Higher molecular incidence of *T. gondii* in samples collected through autumn and summer seasons, signifying that fresh grasses may harbor *T. gondii* oocysts. Moreover, humid environment exist in these seasons may favor the survival of *T. gondii* oocysts. Our findings were similar to results of previously published works [53, 62, 63]. Though, some surveys have exposed negative portion of season for incidence of *T. gondii* [64, 65].Previous work [66] described that 85 out of 3531 (2.41%) samples collected through autumn seasons were positive for *T. gondii*. Additionally, higher seroprevalence of this parasite was conveyed on samples collected

through autumn (OR 3.462; $P = 0.039$) [66]. Dubey [67] conveyed that access of livestock to outdoors and grazing pasture (in autumn and summer seasons) are imperative risk factors for incidence of *T. gondii*. Role of age of animals has also been investigated [31, 68].Recent survey [69] also disclosed that > 8 months old pigs had the higher seroprevalence of *T. gondii* than young pigs. Previous investigation [70] described that animals and especially sheep and goat can be exposed to *T. gondii* at any stage of their life, while incidence increased in higher ages. Recent report [71] described that incidence of *T. gondii* increased from 37.70% in young sheep to 73.80% in >6 years ewes. Another survey [72] specified the role of age as an substantial risk factor for incidence of *T. gondii*. Totally, portion of food-borne microbes, particularly bacteria, in occurrence of food-borne diseases has been measured in Iran and diverse surveys have been conducted in this field [20, 73-82].

Conclusions

We examined the molecular incidence of *T. gondii* in raw milk and traditional dairy product samples collected from different parts of Iran. Nested-PCR reaction was introduced as a safe and accurate diagnostic method for detection of the *T. gondii B1* gene. *T. gondii* molecular incidence in raw milk and traditional dairies was 6.08% and 2.85%, respectively. Sheep milk and traditional cheese were the most commonly contaminated samples. Camel milk and cream and butter had the lowermost molecular incidence of *T. gondii*. There were no detectable *T. gondii* in donkey raw milk and yoghurt, kashk and doogh samples. Higher molecular incidence of *T. gondii* was found in dairies collected through the autumn and summer seasons. Additionally, higher molecular incidence of *T. gondii* was seen in raw milk samples of older than 4 years old animal species. Our outcomes may disclose that the raw buffalo, cow, goat, sheep, and camel milk samples and traditional cheese, butter and dairies are likely sources of *T. gondii* infection in the community.

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