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Molecular Incidence of *Toxoplasma gondii*, *Neospora caninum*, and *Cryptosporidium parvum* in Dissimilar Kinds of Raw and Pasteurized Milk Samples of Naturally Infected Animal Species

(Kejadian Molekul *Toxoplasma gondii, Neospora caninum* dan *Cryptosporidium parvum* dalam Sampel Susu Mentah dan Terpasteur Tak Serupa daripada Spesies Haiwan yang Dijangkiti secara Semula Jadi)

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ABSTRACT

Milk of naturally infected animal species is considered an essential reservoir of *Toxoplasma gondii*, *Neospora caninum*, and *Cryptosporidium parvum*. An extant survey was performed to measure the *T. gondii*, *N. caninum*, and *C. parvum* incidence and periodic frequency amongst milk samples. One-thousand and one-hundred and sixty raw and 400 pasteurized milk samples were collected. Milk samples were used for DNA extraction. The nested-polymerase chain reaction was applied to diagnose *B1*, *NP,* and *hsp70* genes of the *T. gondii*, *N. caninum*, and *C. parvum*, respectively. The total incidence of *T. gondii, N. caninum,* and *C. parvum* in examined milk samples were 2.69%, 10.51%, and 2.94%, respectively. Co-contamination of examined milk samples with all three protozoa was 0.38%. *T. gondii* had the highest distribution amongst raw sheep (5.00%) milk samples, while *N. caninum*, and *C. parvum* had the highest distribution in raw buffalo (23.68% and 6.31%, respectively) milk samples. Marked periodicity with the higher distribution of all protozoa among raw milk samples collected during autumn and summer seasons were found (P <0.05). Raw milk of animal species and pasteurized cow milk may be reservoirs of *N. caninum*, *T. gondii*, and *C. parvum*. Accurate monitoring of raw and pasteurized milk, particularly in autumn and summer seasons, can diminish the risk of human and animal toxoplasmosis, cryptosporidiosis, and neosporosis. However, further investigations are essential to understand the exact epidemiological aspects and other risk factors associated with the *N. caninum*, *C. parvum*, and *T. gondii* presence in raw and pasteurized milk.

Keywords: *Cryptosporidium parvum*; incidence; milk; *Neospora caninum; Toxoplasma gondii*

ABSTRAK

Susu spesies haiwan yang dijangkiti secara semula jadi dianggap sebagai takungan penting *Toxoplasma gondii, Neospora caninum* dan *Cryptosporidium parvum*. Tinjauan sedia ada telah dilakukan untuk mengukur insiden *T. gondii, N. caninum* dan *C. parvum* serta kekerapan berkala di antara sampel susu. Satu-ribu seratus enam puluh sampel susu mentah dan 400 sampel susu pasteur telah dikumpulkan. Sampel susu tersebut digunakan bagi pengekstrakan DNA. Tindak balas rantai polimerase berantai (PCR) telah digunakan untuk mendiagnosis gen B1, NP, dan *hsp70* masing-masing *T. gondii, N. caninum* dan *C. parvum*. Jumlah insiden *T. gondii, N. caninum* dan *C. parvum* dalam sampel susu yang diperiksa masing-masing ialah 2.69%, 10.51%, dan 2.94%. Pencemaran bersama sampel susu yang diperiksa dengan ketiga-tiga protozoa ialah 0.38%. *T. gondii* mempunyai taburan tertinggi dalam sampel susu kambing biri-biri mentah (5.00%), manakala *N. caninum* dan *C. parvum* mempunyai taburan tertinggi dalam sampel susu kerbau mentah (23.68% dan 6.31%, masing-masing). Insiden berkala yang ketara dengan pengedaran yang lebih tinggi bagi semua protozoa dalam kalangan sampel susu mentah yang dikumpul semasa musim luruh dan musim panas ditemui (P <0.05). Susu mentah spesies haiwan dan susu lembu dipasteur mungkin merupakan takungan *N. caninum, T. gondii* dan *C. parvum*. Pemantauan tepat susu mentah dan pasteur, terutamanya pada musim luruh dan musim panas, boleh mengurangkan risiko toksoplasmosis manusia dan haiwan, cryptosporidiosis dan neosporosis. Walau bagaimanapun, kajian lanjut adalah penting untuk memahami aspek epidemiologi yang tepat dan faktor risiko lain yang dikaitkan dengan kehadiran *N. caninum, C. parvum* dan *T. gondii* dalam susu mentah dan pasteur.

Kata kunci: *Cryptosporidium parvum*; insiden; *Neospora caninum*; susu; *Toxoplasma gondii*

Introduction

Milk is a complete source of nutrients useful for the human function (Zhang et al. 2021). Nevertheless, pasteurized milk consumption is common among Iranians, but raw milk consumption is more prevalent amongst others (Kardas et al. 2016; Lucey 2015). However, raw milk is not necessarily safe, as evidenced by higher food-borne illnesses rates associated with its consumption (Quigley et al. 2013; Ranjbar et al. 2019). Raw and even pasteurized milk consumption are considered an imperative risk factor for protozoan food-borne diseases outbreaks, particularly cryptosporidiosis and toxoplasmosis in humans and neosporosis in animals (Dehkordi et al. 2017a, 2014b, 2014c; Koutsoumanis et al. 2018).

Coccidias, Apicomplexan protozoa, are well distributed in the environment globally. Coccidias are responsible for significant diseases with a high economic burden in both humans and animals (Gajadhar et al. 2015). *Toxoplasma gondii* (toxoplasmosis contributing agent), *Cryptosporidium parvum* (cryptosporidiosis contributing agent) and *Neospora caninum* (neosporosis contributing agent) have higher veterinary and medical importance among all known protozoa (Fehlberg et al. 2017). *T. gondii* and *N. caninum* are significantly related cyst-forming intracellular Sarcocystidae protozoa with similar structural, genetic, and immunological structures (Seltmann et al. 2020). *T. gondii* is responsible for infectious abortion in humans and animals and encephalitis in immunocompromised patients (Aguirre et al. 2019). *N. caninum* is a significant cause of mortality in neonate and ruminants stillbirth and abortion and dogs neuromuscular diseases (Gharekhani et al. 2020). There were no recognized data about the *N. caninum* human infection. In contrast, *N. caninum* injection of pregnant monkeys with results in the fatal encephalitis (Oshiro et al. 2015). *C. parvum* is responsible for severe gastrointestinal disorders, including diarrhea, abdominal cramps, nausea, and vomiting (Vanathy et al. 2017). Ingestion of contaminated milk is one of the most important routes of transmission of *C. parvum*, *T. gondii*, and *N. caninum* (Cisak et al. 2017).

T. gondii, *C. parvum,* and *N. caninum* are potential contaminants of foods, particularly those with animal origins (milk and meat). Distrusted cryptosporidiosis, toxoplasmosis and neosporosis food-borne cases, are infrequently established due to the imperfect parasites numbers in the samples, lack of sensitive detection methods adaptable to food, and finally ignoring the role of food in their transmission (Laberge et al. 1996). Thus, an existing investigation was performed to evaluate the molecular incidence of *T. gondii*, *C. parvum,* and *N. caninum* in pasteurized and raw milk samples of naturally infected animal species.

Materials and Methods

Samples collection

From June 2019 to June 2020, 1160 raw cow (n= 210), sheep (n= 200), goat (n= 180), camel (n= 200), buffalo (n= 190) and donkey (n= 180) milk samples were arbitrarily collected from superstores of different parts of Iran. Additionally, 400 pasteurized milk samples produced from the cow milk were collected from the superstores of these areas. Raw milk samples of animal species were collected according to the International Dairy Federation (IDF) (IDF 1995). According to the periodic secretion of milk in sheep, goats, donkeys, and camels, their milk samples were collected during the summer and spring seasons. Other raw milk samples were collected through four seasons of the year. Raw milk samples (80 mL) were transported to the laboratory at 4 °C using separate cool boxes. The first few squirts were overlooked during milk collection.

DNA extraction and analysis

For DNA extraction, 500 µL of each raw milk sample were transferred to a 1.5 mL microtube. Then, microtubes were centrifuged at 13,700 g for 5 min (Sigma 1-16, Sigma Laborzentrifugen GmbH, Germany). The pellet was incubated with lysis buffer (QIAGEN, Germany) at 90 °C for 2 h and then at 56 °C for 1 h with 20 $mgmL^{-1}$ proteinase K (QIAGEN, Germany). Microtubes were then incubated at 95 °C for 10 min to stop the reaction. QIAamp DNA extraction Kit (QIAGEN, Germany) was directly applied for DNA extraction. The principles of producing manufacture were considered for DNA extraction (Wu et al. 2000). Purified DNA was then diluted in distilled water (DW, Merck, Germany) to a final volume of 60 mL. Extracted DNA purity and concentration (A260/A280) were checked (NanoDrop, Thermo Scientific, MA, USA) (Dehkordi et al. 2012a, 2012b, 2012c). Additionally, the quality of extracted DNA was evaluated on an agarose gel (2%) contained SYBR Green (Thermo Fisher Scientific, St. Leon-Rot, Germany) (Dehkordi et al. 2011a, 2011b, 2013c, 2020).

Detection of *C. parvum, N. caninum,* and *T. gondii*

Nested-Polymerase Chain Reaction (Nested-PCR) was used to detect the *C. parvum*, *N. caninum*, and *T. gondii* in DNA extracted from raw and pasteurized milk samples. A programmable DNA thermo-cycler (Eppendorf Mastercycler 5330, Hamburg, Germany) was applied. All PCR ingredients were obtained from a commercial brand (Thermo Fisher Scientific, Germany). Table 1 shows the PCR circumstances (Burg et al. 1989; Hughes et al. 2006; Rochelle et al. 1997; Yamage et al. 1996). Gel electrophoresis (2% agarose gel) contained SYBR Green

(Thermo Fisher Scientific, Germany) in TBE buffer (1X) was performed at 90V for 30 min. The UVI doc system (Grade GB004, UK) was applied for examination of nested-PCR gels.

TABLE 1. PCR circumstances applied for *C. parvum*, N. *caninum,* and *T. gondii* detection

Target genes		Primer sequence (5'-3')	PCR product (bp)	PCR programs	PCR volume (50 µL)	References
T. gondii B1 gene	Initial reaction	F: GGAACTGCATCCGTTCATGAG R: TCTTTAAAGCGTTCGTGGTC	193	1 cycle: 5 min: 94 °C 40 cycles: 10 s: 93 °C 10 s: 57 °C 30 s: 72 °C 1 cycle: 10 min: 72 °C	10X PCR buffer: 5 µL $MgCl_2$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	(Burg et al. 1989)
	Nested reaction	F: TGCATAGGTTGCAGTCACTG R: GGCGACCAATCTGCGAATACACC	96	1 cycle: 5 min: 94 °C 40 cycles: 10 s: 93 °C 10 s: 62.5 °C 15 s: 72 °C 1 cycle: 10 min: 72 °C	10X PCR buffer: 5 µL $MgCl_2$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U PCR products of initial reaction: 2.5 µL	
N. caninum NP gene	Initial reaction	F: GGGTGTGCGTCCAATCCTGTAAC R: CTCGCCAGTCAACCTACGTCTTCT	328	1 cycle: 3 min: 94 °C 35 cycles: 45 s: 95 °C 15 s: 64 °C 45 s: 72 °C 1 cycle: 8 min: 72 °C	10X PCR buffer: 5 µL $MgCl_2$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	(Hughes et al. 2006; Yamage et al. 1996)
	Nested reaction	F: GGGTGAACCGAGGGAGTTG R: TCGTCCGCTTGCTCCCTATGAAT	198	1 cycle: 5 min: 94 °C 35 cycles: 60 s: 94 °C 2 min: 55 °C 60 s: 72 °C 1 cycle: 10 min: 72 °C	10X PCR buffer: 5 µL $MgCl_2$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U PCR products of initial reaction: 2.5 µL	
C. parvum hsp70 gene	Initial reaction	F: AAATGGTGAGCAATCCTCTG R: CTTGCTGCTCTTACCAGTAC	361	1 cycle: 5 min: 94 °C 35 cycles: 60 s: 94 °C 2 min: 55 °C 60 s: 72 °C 1 cycle: 10 min: 72 °C	10X PCR buffer: 5 µL $MgCl_2$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	(Rochelle et al. 1997)
	Nested reaction	F: TGGTGGTGTTATGACCAAGC R: TGGTACACCTCTTGGTGCTG	199	1 cycle: 5 min: 94 °C 30 cycles: 30 s: 94 °C 45 s: 58 °C 30 s: 72 °C 1 cycle: 10 min: 72 °C	10X PCR buffer: 5 µL $MgCl_2$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U PCR products of initial reaction: 2.5 µL	

DATA ANALYSIS

Data analysis was performed by means of the SPSS 21.0 (Chicago, USA). Significant differences were studied according to the results of the Chi-square and Fisher's tests. *P*-value <0.05 was measured as a significant level (Dehkordi et al. 2017b, 2013b; Ghorbani et al. 2016).

RESULTS AND DISCUSSION

T. gondii, N. caninum, and *C. parvum* are important protozoa with high economic, veterinary, and medical importance. Even though the *T. gondii* and *C. parvum* zoonotic aspects are known (Pumipuntu & Pirata 2018; Tenter 2000), there are no accessible methodical papers considering the *N. caninum* zoonotic aspects (Namavari 2020). Humans and all domestic animals, wildlife, and livestock can be potential reservoirs that contribute *T. gondii* and *C. parvum* to food and transmitted to other hosts through the fecal-oral route (Marquis et al. 2019). Permanent distribution of *N. caninum* through raw milk of animal species has been reported, but its zoonotic aspect is indistinctive (Reichel et al. 2020). As a result, understanding foods' potential roles, particularly those with animal origins as potential reservoirs of these parasites, is essential in human and animal health.

Incidence of *C. parvum, N. caninum,* and *T. gondii* in raw cow, sheep, goat, camel and donkey and pasteurized cow milk samples were studied in the present study. Figure 1 shows the PCR and nested-PCR gel electrophoresis of *T. gondii* (A)*, N. caninum* (B), and *C. parvum* (C). Table 2 shows the molecular incidence of *T. gondii, N. caninum,* and *C. parvum* in raw and pasteurized milk samples of animal species. Findings showed that the incidence of *T. gondii, N. caninum,* and *C. parvum* in raw and pasteurized milk samples of all examined animal species were 2.69%, 10.51%, and 2.94%, respectively. Simultaneous incidence of *T. gondii* + *N. caninum*, *T. gondii* + *C. parvum*, *N. caninum* + *C. parvum,* and finally *T. gondii* +*N. caninum* + *C. parvum* in inspected samples were 1.28%. 0.57%,

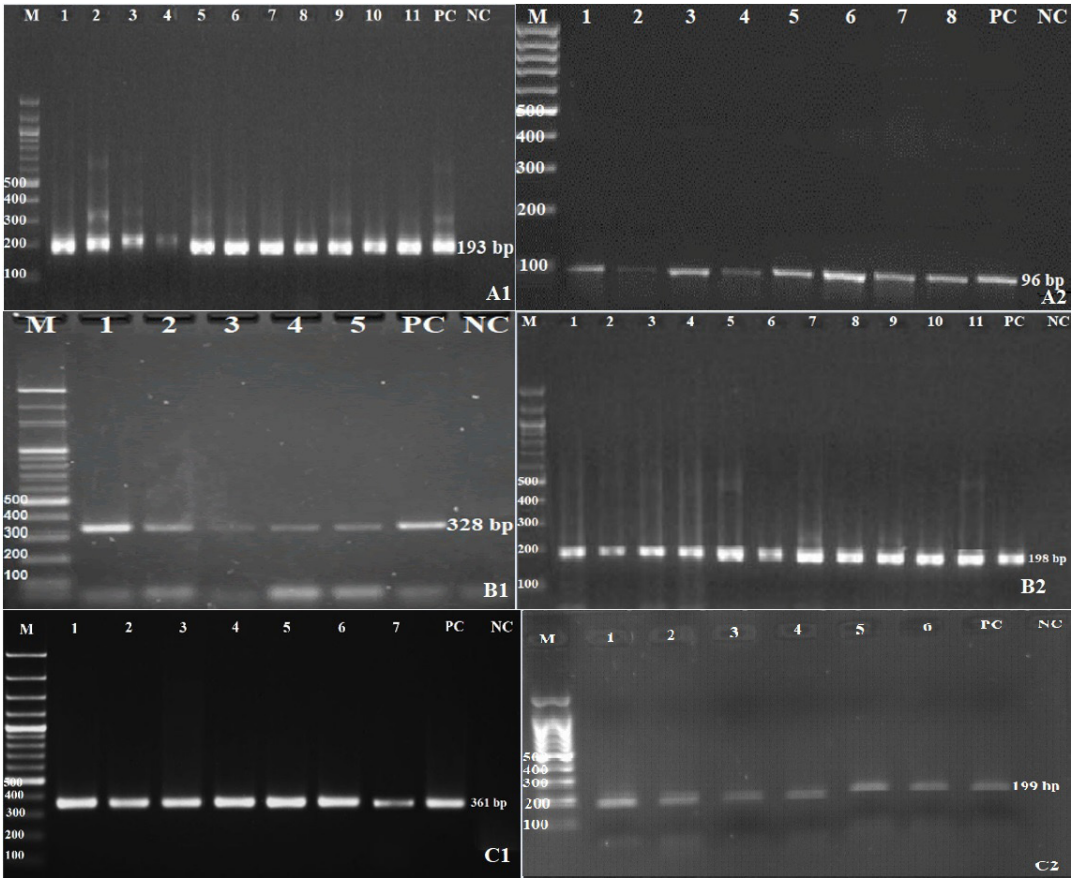

FIGURE 1. PCR and nested-PCR gel electrophoresis of *T. gondii* (A)*, N. caninum* (B), and *C. parvum* (C). A1: *B1* gene of *T. gondii* PCR amplification (193 bp) and A2: *T. gondii B1* gene Nested-PCR amplification (96 bp); B1: *N. caninum NP* gene PCR amplification (328 bp) and B2: *N. caninum NP* gene Nested-PCR amplification (198 bp); C1: *C. parvum hsp70* gene PCR amplification (361 bp) and C2: *C. parvum hsp70* gene Nested-PCR amplification (199 bp). M: ladder (100 bp), PC: Positive control and NC: Negative control, and other clear lines indicate positive samples

1.28% and 0.38%, respectively. Raw sheep milk samples had the maximum incidence of *T. gondii* (5.00%). Raw buffalo milk samples had the maximum incidence of *N. caninum* (23.68%). Raw buffalo milk samples harbored the highest incidence of *C. parvum* (6.31%). Pasteurized milk samples had the maximum incidence of *N. caninum* (6.25%). Raw sheep milk samples had the maximum simultaneous contamination rate of all three protozoa (1.00%). *T. gondii* and *N. caninum* incidences were significant among samples (P <0.05). *C. parvum* and *N. caninum* incidences were significant among samples (P <0.05). Incidences of three examined protozoa were significant amongst the samples (P <0.05). Incidences of three examined protozoa were significant amongst raw and pasteurized milk samples (P <0.05).

TABLE 2. *C. parvum*, *N. caninum,* and *T. gondii* molecular incidence in raw and pasteurized milk samples

Type of milk samples	N samples collected	Number (%) of samples positive for each protozoan						
		T. gondii	*N. caninum*	*C. parvum*	*T. gondii*+ *N. caninum*	*T. gondii*+ *C. parvum*	*N. caninum*+ *C. parvum*	*T. gondii*+ *N. caninum*+ *C. parvum*
Raw cow	210	5 (2.38)	44 (20.95)	10 (4.76)	3 (1.42)	2 (0.95)	6 (2.85)	1 (0.47)
Raw sheep	200	10 (5.00)	12 (6.00)	5 (2.50)	5 (2.50)	3 (1.50)	2 (1.00)	2 (1.00)
Raw goat	180	8 (4.44)	11 (6.11)	4 (2.22)	4 (2.22)	2 (1.11)	2 (1.11)	1 (0.55)
Raw camel	200	3 (1.50)	20 (10.00)	3 (1.50)	1 (0.50)	-	1 (0.50)	-
Raw buffalo	190	4 (2.10)	45 (23.68)	12 (6.31)	1 (0.52)	1 (0.52)	4 (2.10)	1 (0.52)
Raw donkey	180	6 (3.33)	7 (3.88)	2 (1.11)	2 (1.11)	-	-	-
Total raw milk	1160	36 (3.10)	139 (11.98)	36 (3.10)	16 (1.37)	8 (0.68)	15 (1.29)	5 (0.43)
Pasteurized milk	400	6 (1.50)	25 (6.25)	10 (2.50)	4 (1.00)	1 (0.25)	5 (1.25)	1 (0.25)
Total	1560	42 (2.69)	164 (10.51)	46 (2.94)	20 (1.28)	9 (0.57)	20 (1.28)	6 (0.38)

The *T. gondii* incidence in raw and pasteurized milk samples of the present study were 3.10% and 1.50%, respectively. Moreover, the *N. caninum* incidence in raw and pasteurized milk samples were 11.98% and 6.25%, respectively. *N. caninum* and *T. gondii* incidences in milk samples collected from Greece (Anastasia et al. 2013), Romani (Iovu et al. 2012), Czech Republic (Bártová et al. 2015), and Iran (Razmi et al. 2017) were 5.40% and 12.40%, 16.80% and 53.71%, 12.00% and 26.00%, 2.30% and 52.80%, 0.50% and 9.70%, and 35% and 11.38%, respectively. Reversely, scarce reports were conducted about the *C. parvum* detection in milk. Krascsenicsová and Siekel et al. (2007) successfully detect 10^1-10^3 *C. parvum*'s oocysts per mL milk samples according to the type of the PCR technique. Additionally, Di Pinto and Tantillo (2002) successfully used PCR method for *C. parvum* detection in raw and pasteurized cow milk. Shakerian et al. (2015) reported that 2 out of 38 (5.26%) raw cow's milk samples collected from Iranian dairy farms were contaminated with *C. parvum,* which was higher than our findings. However, the higher *N. caninum* incidence in the milk of naturally infected animal species in this study and other investigations, detecting *T. gondii* and *C. parvum* due to their confirmed zoonotic aspects has higher importance. However, no previously published reports about the incidence of *C. parvum* in raw camel, goat, sheep, buffalo, and pasteurized milk samples. Oocysts of *C. parvum* are regularly existed in dairy herds and might be conveyed to the human population by raw and even pasteurized milk. Nevertheless, *C. parvum* detection in milk is problematic owing to the oocysts small numbers and low sample, which entails specific and sensitive detective methods. Furthermore, milk is denser, containing proteins, fat, lactose, and solid particles such as somatic cells, making it challenging to detect oocytes of the *C. parvum*. In keeping with this, previous research showed that *N. caninum* caused significant infection in two rhesus monkeys (*Macaca*

mulatta) (Barr et al. 1994). Thus, there is a big worry on the *N. caninum* zoonotic potential. However, surveys tried to detect *N. caninum* in raw sheep, goat, camel, buffalo, and pasteurized milk samples were scarce in the literature. In pasteurized milk samples, detection of *C. parvum* has a higher portion because of the protozoan oocytes' high resistance toward hard environmental conditions such as high temperature. Some surveys about the *T. gondii* detection in raw milk of naturally infected animals are available in the literature (Bezerra et al. 2015; Camossi et al. 2011). However, gaps in the *T. gondii* risk assessment in milk are famous. In a specific survey, Dehkordi et al. (2013a) stated that the *T. gondii* incidence in camel, buffalo, goat sheep, and cow milk samples was 2.50%, 3.65%, 9.44%, 6.48%, and 3.50%, respectively, which was reinforced our results. Similarly, several surveys from USA (Jones et al. 2009), Brazil (Moura et al. 2013), and Iran (Fouladv et al. 2010) reported a positive relationship amid human infection and drinking milk by *T. gondii*. Additionally, the role of camel (Dehkordi et al. 2013a; Gebremedhin et al. 2014), donkey (Mancianti et al. 2014; Martini et al. 2014), sheep (Deyhimi et al. 2019; Ossani et al. 2017), buffalo (Dehkordi et al. 2013a) and goat (Amairia et al. 2016; Iacobucci et al. 2019) milk as potential reservoirs of *T. gondii* were reported previously. Abadi et al. (2020) described that the *T. gondii* molecular incidence in raw buffalo, cow, goat, sheep, and donkey milk samples collected from Iran were 6.66%, 5.00%, 7.50%, 10.00%, 0%, and 3.33%, respectively. Raw cow (Barr et al. 1994; Enachescu et al. 2014), sheep (Al-Jomaily et al. 2013; Kyaw et al. 2018), goat (Kyaw et al. 2018; Topazio et al. 2014), and buffalo (Nasir et al. 2018) were also previously considered as reservoirs of *N. caninum*. *N. caninum* incidence among raw milk samples of animal species in Iran ranged between 18.20% and 95.20% (Alipour et al. 2018; Shakerian et al. 2015). Razmi and Barati (2017) reported that the incidence of *N. caninum*, *T. gondii*, and both pathogens in the bulk tank milk samples collected from Iran were 35.00%, 11.38%, and 2.40%, respectively. According to the literature, there were no previously published data about examining *C. parvum*, *N. caninum,* and *T. gondii* protozoa in pasteurized milk samples. Additionally, there was no investigation about the co-contamination of raw and pasteurized milk samples with all three *C. parvum*, *N. caninum,* and *T. gondii* protozoa. It seems that milk testing can be used regularly to assess its protozoan quality in dairy herds because of the easily sampling and rapid findings. The lower incidence of examined protozoa in pasteurized milk samples is maybe due to the reduction of oocyte count due to the thermal process and bactofugation on raw milk samples. However, as only the genome of all three protozoa were detected by nested-PCR in examined pasteurized milk samples, no comments can be made on whether the samples are contaminated or not.

Some differences were obtained for the incidence of examined protozoa between the present survey and other reports. These differences might be because of study design, sample size, sampling methods, types of samples, detection methods, experimental strategies, farm management and hygiene, species of examined animals, and different exposure levels to risk factors. Season of sampling is one of the most critical factors that affected the incidence of infectious diseases (Dehkordi et al. 2014a; Nejat et al. 2015). Our findings showed the higher *C. parvum*, *N. caninum,* and *T. gondii* incidence among autumn and summer raw milk samples. As only raw buffalo and cow milk samples were collected in four seasons, the periodic distribution of protozoa in these samples was more accurate. The findings of periodic distribution of protozoa in raw buffalo and cow milk samples showed the higher distribution of *C. parvum*, *N. caninum,* and *T. gondii* in autumn, rainy, and wet seasons, particularly autumn.

Table 3 shows the *T. gondii* periodic frequency among raw milk samples. *T. gondii* incidence among samples collected through the summer, autumn, winter, and spring seasons was 4.10%, 3.96%, 3.63%, and 2.05%, respectively. Table 4 shows the *N. caninum* periodic frequency among raw milk samples. *N. caninum* incidence among samples collected through the summer, autumn, winter, and spring seasons was 10.36%, 37.62%, 10.00%, and 8.23%, respectively. Table 5 shows the *C. parvum* periodic frequency in raw milk samples. *C. parvum* incidence among samples collected through the summer, autumn, winter, and spring seasons was 3.45%, 10.89%, 1.81%, and 1.64%, respectively. Incidences of three examined protozoa were significant amongst different seasons ($P < 0.05$).

Periodic variation in exposure to protozoa can be associated with environmental conditions that affect the viability and persistence of oocysts in the environment. Nevertheless, slight information is available about the periodic variation of *C. parvum*, *N. caninum,* and *T. gondii* contamination in raw milk of naturally infected animal species. Periodic distribution of *T. gondii* oocytes with higher incidence in autumn and summer was previously reported from Europe (Simon et al. 2017), United States (de Wit et al. 2020), and China (Liu et al. 2017). Higher distribution of *N. caninum* in rainy and wet seasons were also reported by Kamali et al. (2014) (Iran), González-Warleta et al. (2014) (Spain) and Jung et al. (2014)

(Korea). Similar to our findings, periodic distribution of *C. parvum* with higher incidence in wet seasons was reported from United Kingdom (Chalmers et al. 2019), United States (Wolyniak et al. 2010), and Korea (Chai et al. 2001). Probable factors contributing to the periodicity include rainfall, temperature, humidity, contact with animals, and agricultural practices. A likely decrease in the incidence of *C. parvum*, *T. gondii*, and *N. caninum* has been recommended in raw milk samples collected during dry and cold seasons because of a reduction in the number of viable oocysts in the environment.

One limitation of this survey may be the lack of sheep, goat, camel, and donkey milk samples in all four seasons. Furthermore, the lack of the pasteurized milk of all animal species to examine parasites' presence is another significant limitation of the present research.

TABLE 3. *T. gondii* periodic frequency in raw milk samples

Raw milk samples (N collected)	Number of collected samples in each season				Number (%) of samples positive for *T. gondii*				
	Summer	Autumn	Winter	Spring	Number of positive	Summer	Autumn	Winter	Spring
Cow (210)	49	53	58	50	5	-	2 (3.77)	2 (3.44)	1 (2.00)
Sheep (200)	96	-	-	104	10	7 (7.29)	-	-	3 (2.88)
Goat (180)	88	-	-	92	8	5 (5.68)	-	-	3 (3.26)
Camel (200)	96	-	-	104	3	3 (3.12)	-	-	-
Buffalo (190)	44	48	52	46	4	-	2 (4.16)	2 (3.84)	1 (2.17)
Donkey (180)	90	-	-	90	6	4 (4.44)	-	-	2 (2.22)
Total (1160)	463	101	110	486	36	19 (4.10)	4 (3.96)	4 (3.63)	7 (2.05)

TABLE 4. *N. caninum* periodic frequency in raw milk samples

Raw milk samples	Number of collected samples in each season				Number of positive	Number (%) of samples positive for *N. caninum*			
	Summer	Autumn	Winter	Spring		Summer	Autumn	Winter	Spring
Cow	49	53	58	50	44	8 (16.32)	18 (33.96)	5 (8.62)	13 (26.00)
Sheep	96	-	-	104	12	8 (8.33)	-	-	4 (3.84)
Goat	88	-	-	92	11	7 (7.95)	-	-	4 (4.34)
Camel	96	-	-	104	20	13 (13.54)	-	-	7 (6.73)
Buffalo	44	48	52	46	45	7 (15.90)	20 (41.66)	6 (11.53)	12 (26.66)
Donkey	90	-	-	90	7	5 (5.55)	-	-	2 (2.22)
Total	463	101	110	486	139	48 (10.36)	38 (37.62)	11 (10.00)	40 (8.23)

TABLE 5. *C. parvum* periodic frequency in raw milk samples

Raw milk samples	Number of collected samples in each season				Number of positive	Number (%) of samples positive for *C. parvum*			
	Summer	Autumn	Winter	Spring		Summer	Autumn	Winter	Spring
Cow	49	53	58	50	10	3 (6.12)	5 (9.43)	1 (1.72)	2 (4.00)
Sheep	96	-	-	104	5	3 (3.12)	-	-	2 (1.92)
Goat	88	-	-	92	4	3 (3.40)	-	-	1 (1.08)
Camel	96	-	-	104	3	2 (2.08)	-	-	1 (0.96)
Buffalo	44	48	52	46	12	3 (6.81)	6 (12.50)	1 (1.92)	2 (4.34)
Donkey	90	-	-	90	2	2 (2.22)	-	-	-
Total	463	101	110	486	36	16 (3.45)	11 (10.89)	2 (1.81)	8 (1.64)

Conclusion

Put all in a nutshell, an existing survey is an initial report of identification and periodic distribution of *C. parvum, T. gondii*, and *N. caninum* in raw milk of naturally infected animal species and pasteurized cow milk. Findings showed that raw milk samples of cow, sheep, goat, camel, buffalo, and donkey, and pasteurized milk might be reservoirs of *T. gondii*, *C. parvum,* and *N. caninum* in the environment. Furthermore, marked periodicity with the higher distribution of examined protozoa in autumn and summer seasons was found in the present research. Periodic variations may be necessary when planning to control toxoplasmosis, neosporosis, and cryptosporidiosis in humans and animals. Further surveys are essential to understand other epidemiological aspects and risk factors for *T. gondii*, *C. parvum,* and *N. caninum* presence of in raw milk of naturally infected animal species and pasteurized cow milk.

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