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Occurrence of *Cryptosporidium* spp. in Traditional Milk and Dairy Products Supplied in Yazd City, Central Iran

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HIGHLIGHTS

- Among the dairy products, *Cryptosporidium* spp. contamination was 4.0% in milk.
- Each of cream and cheese group samples showed 18% contamination with *Cryptosporidium* spp.
- Occurrence of *Cryptosporidium* spp. was significantly related to the sample types.

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Acronyms and abbreviations
PCR=Polymerase Chain Reaction

ABSTRACT

Background: *Cryptosporidium* is one of the most important agents of food-borne diseases with gastrointestinal symptoms such as diarrhea in either livestock or humans. This protozoon can be transmitted to human through consuming contaminated raw milk and dairy products. The present study aimed to detect *Cryptosporidium* spp. in traditional raw cow milk, cream, and cheese consumed in Yazd city, Central Iran.

Methods: Two hundred traditional (unpasteurized) milk and dairy samples were collected from five different regions of the studied area, including 100 traditional cow milk, 50 cow cheese, and 50 cow cream. DNA extraction was performed. Then, molecular detection was performed using the nested Polymerase Chain Reaction technique. Statistical analysis was performed using SPSS version 23.0.

Results: The findings of this study showed that 11% samples were contaminated with *Cryptosporidium* spp., including 4.0% (4/100) milk, 18.0% (9/50) cream, and 18.0% (9/50) cheese ($p<0.05$). Besides, parasite contamination was 8.8, 12.5, 12.8, 8.3, and 12.8% in the Center, North, East, South, and West of the studied region, respectively ($p>0.05$).

Conclusion: Due to the occurrence of *Cryptosporidium* spp. in unpasteurized dairy samples in Central Iran, developing and designing control and prevention programs is necessary against this parasite.

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Introduction

One of the most important nutrients in human is milk and dairy products, which have critical roles in providing the necessary protein, fat, vitamins, as well as minerals (Chauhan et al., 2021). Human consume various animals' milk; however, cow milk is considered the primary source due to the its effect on humans' growth and

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health (Melini et al., 2017; Schmid, 2009). In addition, dairy products made of raw milk such as cheese, butter, and cream are also demanded. On the other hand, unpasteurized raw cow milk and dairy products may harbor the pathogens' infective agents, resulting in serious infectious diseases (Willis et al., 2018). In other words, any deficiency in the hygienic principles in the preparation and maintenance of milk products affects the consumers' health, especially children (Wochner et al., 2018).

Various microorganisms such as bacteria, fungi, molds, and parasites contaminate milk and dairy products (Oliver et al., 2005). *Cryptosporidium* spp. and *Giardia intestinalis* are the main agents of gastrointestinal symptoms such as diarrhea in human (Lorenzo et al., 2018). *Cryptosporidium* spp. is one of the most common intestinal protozoan parasites which responsible for food-borne infection (cryptosporidiosis) of approximately eight million cases especially in immunocompromised persons and young children in developing countries (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018; Motavalli Haghi et al., 2020; Ryan et al., 2018; Wang et al., 2011). Molecular diagnostic method identified up to 30 species and more than 50 genotypes of *Cryptosporidium* (Dalimi et al., 2017; Mirian et al., 2010; Motavalli Haghi et al., 2017). The main species of *Cryptosporidium* in calves is *C. parvum* but sporadic illness has been reported by other species (Thomson et al., 2017; Wang et al., 2020).

Moreover, around 30 billion oocysts are excreted by the calf over two weeks (Olson et al., 2004). The oocysts are resistant and therefore survive for a long time in environments (Alum et al., 2014). In addition, contaminated unpasteurized cow milk has the risk of infection in human (Fayer and Xiao, 2008; Messner and Berger, 2016). The infectious agent is transmitted to human by consuming contaminated water and food such as raw milk and vegetables (Sleman Ali et al., 2018; Wang et al., 2011). Clinical signs of human infection of *Cryptosporidium* spp. are watery diarrhea, abdominal cramps, nausea, vomiting, weight loss, as well as fever (Certad et al., 2017).

Biological food safety is significant for microbiologists, toxicologists, manufacturers, sanitary, and epidemiological services, government bodies, and consumers. People in many areas prefer to use traditional milk and dairy products. On the other hand, based on our knowledge, there is no study to assess the prevalence of *Cryptosporidium* spp. contamination in cow raw milk and dairy products in Yazd, Central Iran. Therefore, this study assessed the prevalence of *Cryptosporidium* spp. in traditional raw dairy products in Central Iran.

Materials and methods

Ethical statement

This study was approved with the ethical code of IR.SSU.SPH.REC.1398.014 by Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Study area

Yazd city is the capital of Yazd province in Central Iran with an area of 129,285 km^2 at 1,203 m above sea level. Yazd is considered the central dry city in Iran with a hot climate and precipitation amount of 49 mm annually. The summer in this area is sweltering with temperature more than 40 °C and no humidity. The winter has average temperature varying between 11.1 and 2.4 °C.

Sample collection

This study used two hundred samples, including 100 raw milk, 50 homemade cream, and 50 homemade cheese. The samples were collected based on the protocol of the Institute of Standards and Industrial Research of Iran (ISIRI, 2008; no. 326) from five different regions of Yazd, including the Center, North, East, South, and West (Figure 1).

In each region, 15 dairy stores were chosen for sample gathering. The homogenized dairy samples were randomly collected from traditional dairy stores from July to September 2018 and then transferred to the Laboratory of Research Center for Food Hygiene and Safety, Shahid Sadoughi University of Medical Science, Yazd, Iran. Subsequently, they were stored at -20 °C till the next analysis.

Sample preparation

Sample preparation was performed based on the protocol by Machado et al. (2006). Briefly, each sample was homogenized. Then, 5 ml of the sample was mixed with an equal volume of magnesium sulfate (Merck; Germany; density 1.30 g/ml; 750 g/1). Centrifugation was performed at 206 xg for 10 min. Then, the sediment was transferred to the other tube and centrifugation was repeated. The clot was washed with 10 ml of sterile deionized water, transferred to a new sterile tube and centrifuged at 206 xg for 10 min. Each pellet sample was treated with 1 µl of DNase I (CinnaGen, Iran). Each sample was mixed with an equal volume of 1.5% sodium taurocholate (Merck, Germany) and incubated at 37 °C for 4 h.

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DNA extraction

DNA extraction was performed using the boiling-freezing protocol for 5 min (Hawash, 2014). The supernatant was transferred to a new 1.5 ml sterile microtube after centrifugation at 1,000 xg for 5 min, and then stored at -20 °C. The quantification of extracted DNA was done by Nanodrop (Thermo Science, USA).

Molecular identification

Molecular identification of *Cryptosporidium* spp. was performed by nested-Polymerase Chain Reaction (PCR) using the specific primer pair of Cryp-F1: 5′-TTCTAGAGCTAATACATGCG-3′ and Cryp-R1: 5′-CCCATTTCCTTCGAAACAGGA-3′ with the amplicon size of 1,325 bp in the first run; and the specific primer pair of Cryp-F2: 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and Cryp-R2: 5′- AAGGAGTAAGGAACAACCTCCA-3′ for amplification of 819-825 bp fragment in the second run (Feng et al., 2007). In the first round, the reaction was done in a 20 µl volume containing master mix solution (Ampliqon, Denmark), including 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U *Taq* DNA polymerase, 0.5 mM of each primer (Pishgam, Tehran, Iran), and 100 ng extracted DNA as the template. The second round was amplified like the first round that the template was PCR product (1/100 dilution) from the first round. The amplification was done using the thermocycler (ABI, USA). The temperature program for the first round included initial denaturation for 5 min at 94 °C, followed by 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 51.2 °C, and elongation for 1 min at 72 °C. The final elongation was performed for 5 min at 72 °C. The same reaction temperature was conducted for the second run, except for the annealing temperature of 52.9 °C. The PCR products were analyzed using 1% agarose gel electrophoresis (Akhtarian, Iran, Mashhad) with the 50 bp DNA ladder (CinnaGen, Iran, Tehran). The negative and positive controls were run in each reaction using the sterile deionized water and DNA from *C. parvum* received from Pasteur Institute, Tehran, Iran, respectively.

Sequencing and analysis

The PCR products were sent to the Pishgam Company (Tehran, Iran) for purification and sequencing using Sanger method.

Statistical analysis

The statistical analysis was conducted using SPSS software version 23.0 (Chicago, II, USA) via chi-squared test . The differences were considered significant at a *p*-value of <0.05.

Results

Molecular detection indicated that out of 200 samples, 11% (22/200) samples were contaminated with *Cryptosporidium* spp., including 4% (4/100) milk, 18% (9/50) cream, and 18% (9/50) cheese. The statistical analysis showed that parasite occurrence was significantly related to the sample types (p=0.007). *Cryptosporidium* spp. contamination rates of dairy products were 8.8, 12.5, 12.8, 8.3, and 12.8% in Center, North, East, South, and West of the studied region, respectively (Table 1). Statistical analysis revealed no significant relationship between parasite occurrence in dairy products and investigated regions. Sequencing had noise annoying the species identification may be due to mixed occurrence of species.

Table 1: Prevalence of *Cryptosporidium* spp. in traditional milk and dairy products in different geographical areas of Yazd, Central Iran

Geographical area	Positive (%)	Negative (%)	Total (%)	*P* value
North	5 (12.5)	35 (87.5)	40 (20.0)	
South	4 (8.3)	44 (91.7)	48 (24.0)	
East	5 (12.8)	34 (87.2)	39 (19.5)	0.95
West	5 (12.8)	34 (87.2)	39 (19.5)	
Center	3 (8.8)	31 (91.2)	34 (17.0)	
Total	22 (11.0)	178 (89.0)	200 (100)	

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Figure 1: Location of sampling areas in the country and Yazd province

Discussion

Cryptosporidiosis is one of the main zoonotic diseases worldwide especially in developing and low-income countries (Pumipuntu and Piratae, 2018). The prevalence of this infection varies between countries and regions. For example, some reports indicate that the prevalence of cryptosporidiosis ranges from 1.5 to 40.6% in cattle in different parts of the world (Fayer et al., 2005; Koyama et al., 2005; Nguyen et al., 2007; Sevinc et al., 2003; Trotz-Williams et al., 2006). Similarly, in human, it ranges from 2.7 to 22 per 100,000. Furthermore, based on the study was conducted by Motavalli Haghi et al. (2020), the occurrence of *Cryptosporidium* in cattle and calves of Iran ranges from 2.1% in Ahvaz to 28.3% in Mashhad. So, these studies show that *Cryptosporidium* should be considered as one of the most important parasitic diseases.

In this study, we found that 11% of the dairy samples were contaminated with *Cryptosporidium*. Various studies have investigated this issue. A study in Iraq showed that 32% of raw ovine and 46% of caprine milk were contaminated with *Cryptosporidium* (Hasan et al., 2018) that it is higher than our study. It may be due to their differences in susceptibility to *Cryptosporidium*, special the behavioral pattern and the animals' nutritional habits. Also, other previous studies reported higher infection rates of *Cryptosporidium* in sheep and goat milk samples (20% to 35.1%; Bakir, 2005; Mosa, 2016). However, there is another study was conducted in Montreal that found no positives in bulk milk (Lasprilla-Mantilla et al., 2019). Also, in compared to our results, a higher contamination rate of *Cryptosporidium* (5.26%) was reported in raw milk in Isfahan, Iran (Shakerian et al., 2015). In general, our findings and previous reports fall within the global infection prevalence estimation of this infection in cattle, i.e. 1.5%-40.6% as per the report from China (Cai et al., 2019), Japan (Koyama et al., 2005), Vietnam (Nguyen et al., 2007), Turkey (Sevinc et al., 2003), Canada (Trotz-Williams et al., 2006), and USA (Fayer et al., 2005).

The results of this study also revealed that the contamination rate of samples obtained from North, East, Center, West, and South of studied regions was 12.5, 12.8, 8.8 12.%, and 8.3%. However, no significant association was observed between geographical area and the frequency of *Cryptosporidium*. This may be due to the limited traditional dairy producer centers in Yazd, Central Iran.

The researchers showed that cryptosporidiosis outbreak associated with drinking unpasteurized cow milk (Harper et al., 2002; Lal et al., 2015) and the contaminated milk may be occurred by *Cryptosporidium* shedding from the bovine feces, improper handling, and milking practices, or using unclean and unsterilized utensils (Boor et al., 2017). A study conducted by Badoui et al. (2014) has confirmed this issue. They showed that cryptosporidiosis in HIV-infected patients in Morocco have relation with drinking raw milk (Badoui et al., 2014). Also, Ursini et al. (2020) reported that the potential risk of *Cryptosporidium* transmission via raw milk consumption and the risk of cryptosporidiosis cases acquired from raw milk should not be underestimated. The researchers concluded that animal contact is considered the main source for *Cryptosporidium* occurrence in milk (Bouzid et al., 2018). These reports can be due to access to relevant services by patients, healthcare systems, and availability of specific tests. These factors, especially the lack of particular surveillance and reporting practices, are more important in developing countries (Cacciò and Chalmers, 2016). Therefore, some countries maintain restricted

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rules to sell raw milk to consumers, including Germany, France, Holland, Belgium, Denmark, Italy, England, Wales, and Northern Ireland (EFSA BIOHAZ Panel, 2015). Although, specific groups of the population in many countries believe that raw milk is safer and healthier than pasteurized milk, the fact is the raw milk has the potential to transmit many diseases such as cryptosporidiosis. There are restricted rules for milk pasteurization and sanitation in dairy processing plants in Iran, but raw milk sales and dairy products such as cream and cheese are common in many regions (Rahimi, 2013).

Knowledge of the dominant parasite species, genotypes, and subtypes in each area has a critical role in the control and prevention of the disease (Chalmers and Cacciò, 2016) but the lack of laboratory diagnosis respecting identification and genotyping in many parts of the world is considered one of the important factors to fail right reports (European Centre for Disease Prevention and Control, 2018) and discrepancies in the results can be due to the difference in applied diagnostic methods. Some studies reported different methods such as PCR, acid-fast stain, and Enzyme-Linked Immunosorbent Assay (ELISA) to detect *Cryptosporidium* and despite the difficulty of laboratory confirmation in cryptosporidiosis outbreaks (especially to detect cases of cryptosporidiosis in immunocompromised patients), there are studies regarding the use of PCR-based methods to identify *Cryptosporidium* that show PCR technique is more sensitive than the other diagnostic modalities (Rosenthal et al., 2015). Also, a method should include a sample process easily adaptable to milk and causes the elimination of interfering factors. In this study, we used nested PCR, which is a valid method for detection of this parasite with acceptable sensitivity and specificity. However, this method has no capability to detect the viability and infectivity of the parasite and thus pasteurization raw milk is essential.

Generally, it is conclude that proper animal husbandry, emphasis on education and promotion, and provision of industrial animal husbandry infrastructure are effective ways to control cryptosporidiosis (López Torres et al., 2020). Also, researchers showed that one of the important ways to destroy the infectivity of the *Cryptosporidium* oocysts is pasteurization (Harp et al., 1996) because parasite oocytes are sensitive to rising temperatures and are destroyed at 65 °C for 30 min (Razavi et al., 2010).

Conclusion

We studied the *Cryptosporidium* spp. contamination in traditional raw milk and dairy products sold in Yazd, Central Iran. The prevalence of *Cryptosporidium* spp. in milk, cream, and cheese in this area showed that the risk of infectivity in people could be significant, especially if the species are related to the infectious agents for human. To our knowledge, this study was the first report on *Cryptosporidium* spp. occurrence in raw milk and homemade cheese and cream in Central Iran. However, we recommend identification of *Cryptosporiium* spp. in this area.

Author contributions

S.S.: gathered samples and carried out the experiments and wrote the draft of the manuscript; B.H.: supervised the project and edited the manuscript; G.E.: supervised the methods and edited the manuscript; M.H.E.: edited the manuscript; M.H.F: analyzed the data with statistical tests. All authors read and approved the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

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