2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(3): 260-266, September 25, 2020
ISSN 2322-4568
DOI: https://dx.doi.org/10.36380/scil.2020.wvj34

Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran

Ali Fattahi Bafghi[1,2], Mohammad Reza Yavari[2,3], Farzaneh Mirzaei[1*], Abolghasem Siyadatpanah[4], Watcharapong Mitsuwan[5], Maria de Lourdes Pereira[6], Veeranoot Nissapatorn[5], Roghayeh Norouzi[7] and Seyed Abdollah Hosseini[8]**

[1]*Department of Parasitology and Mycology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*
[2]*Infectious Diseases Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*
[3]*Department of General, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*
[4]*Ferdows school of Paramedical and Health, Birjand University of Medical Sciences, Birjand, Iran*
[5]*School of Allied Health Sciences and Research Excellence Center for Innovation and Health Products (RECIHP), Walailak University, Nakhon Si Thammarat, Thailand*
[6]*CICECO-Aveiro Institute of Materials and Department of Medical Sciences, University of Aveiro, Aveiro, Portugal*
[7]*Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran*
[8]*Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Iran, Department of Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

*Corresponding author's *Email: mirzaei.farzaneh2015@yahoo.com; ORCID: 0000-0001-8652-7556; **Email: nissapat@gmail.com; ORCID: 0000-0001-8652-7556

ORGINAL ARTICLE
pii: S232245682000034-10
Received: 02 Jul 2020
Accepted: 10 Aug 2020

ABSTRACT

Consumption of raw vegetables is an important route of parasites transmission. It is an important source for foodborne outbreaks in both developed and developing countries, and outbreaks of parasitic diseases in humans. The objective of the present study was to detect the presence of *Cryptosporidium* oocysts in raw fresh vegetables in Yazd city, Iran, from 2017 to 2018. A total of 275 fresh vegetable samples were collected and tested using a sucrose flotation medium of 1.21 specific gravity and a Modified Ziehl-Nielsen staining procedure. Of the 275 vegetables examined, 85 (31.5%) samples were positive for *Cryptosporidium* oocysts. Lettuce had the highest rate (n= 16, 47.1%) of contamination with *Cryptosporidium* oocysts while basil and parsley showed the lowest rates of contamination (n= 6, 20%). There was a significant association between the occurrence of *Cryptosporidium* oocysts and the investigated vegetable types. According to the locations of the vegetable field, Amir Abad and Bahaman Hospital area had the highest (n: 16, 59.3%) and lowest (n= 5, 18.5%) rates of *Cryptosporidium* oocysts contamination, respectively. The plant part showed that the root vegetables had the highest contamination rates (n= 41, 45.6%), followed by leafy vegetables (n= 44, 24.4%). The analysis further indicated a significant association between the occurrence of *Cryptosporidium* oocysts and the route of vegetable consumption. Based on these results, the edible vegetables in Yazd city are one of the potential sources of *Cryptosporidium* infections in humans. Moreover, the vegetable fields within the city of Yazd are contaminated with *Cryptosporidium* oocysts which can pose public health problems.

Keywords: *Cryptosporidium,* Oocysts, Raw vegetables, Yazd city, Iran

INTRODUCTION

Cryptosporidium is a protozoan coccidian intestinal parasite that causes a diarrheal disease called cryptosporidiosis, a major public health problem in both developed and developing countries, and is an opportunistic infection among patients with AIDS and responsible for outbreaks of gastrointestinal disease (Desai et al., 2012). Many species of *Cryptosporidium* infect both humans and animals. This parasite has an outer layer that protects it from harsh external conditions as some disinfectants such as chlorine (Rossle and Latife, 2013). *Cryptosporidium* is one of the most frequent microbial causes of diarrhea, accompanied by pain and abdominal colic and a notable loss of weight. Since the 1980s, cattle have considered the most important source of zoonotic cryptosporidiosis (McDaniel et al., 2014). Although it is typically an opportunistic and short-term acute infection, the parasite can cause severe and unresolved condition in immune-compromised individuals such as the patients with AIDS, whose its presence remains in the lower intestine, and can remain for up to one month (Rossle and Latif, 2013; Mohaghegh et al., 2017).

Foodborne outbreaks of cryptosporidiosis have been identified from several food items, mainly fruits, vegetables, and seafood (Fayer et al., 2003; Sherbini et al., 2016). These products are usually consumed raw which raises public concerns. The evidence suggests that the consumption of raw products is a risk factor, at least in outbreak situations. Contamination of vegetables can occur if they are irrigated by contaminated water with human and/or animal feces, due to poor hygienic conditions in the environment in which *Cryptosporidium* is the most likely to be the parasite (Snelling et al., 2007; McDaniel et al., 2014). Although vegetables contain fiber, vitamins, and minerals, and are an essential

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source for maintaining health, the consumption of raw vegetables play an important role in the transmission of parasites, and have been shown to be an important source of foodborne outbreaks in developed and developing countries (Ortega et al., 1997). Therefore, the present study was carried out to investigate the presence of *Cryptosporidium* oocysts in fresh/raw vegetables in Yazd city, Iran.

MATERIALS AND METHODS

Study area

This study was carried out in Yazd city, Iran. It has a total land space of 300 km^2. Yazd is the driest main city in Iran with an average annual rainfall of 55 to 60 millimeters. Shortages of water supply are commonly seen in many areas like Yazd which leads to the scarcity of clean water consumption, and can increase the contamination by waterborne pathogens through water and food supplies including vegetables (DeNicola et al., 2015).

Sample collection and *Cryptosporidium* oocysts detection

A total number of 275 samples of raw vegetables were collected from particular vegetable fields around Yazd city, and subsequently examined for the presence of *Cryptosporidium* oocysts (Table 1). In this study, the whole plant was purchased and transported to the laboratory of Shahid Sadoughi University of Medical Science, Yazd city, Iran. Then, these vegetables were washed with normal saline, and stored until use. In the first stage of sample collection, the survey was primarily done in the selected vegetable fields on the source of vegetables, the source of water used for washing vegetables and the condition of vegetables (fresh and edible) on the farms. Cluster sampling was done in two stages, the city was divided into five sections: north, south, east, west, and central. Simple randomization was carried out in each section. Finally, five vegetable fields were selected. Samples were collected in the clean polythene bags during the spring of 2017 to winter of 2018 and transported to the laboratory at the Department of Medical Parasitology and Mycology at the School of Medicine University of Medical Sciences of Shahid Sadoughi, Yazd, Iran for further examination. In brief, vegetables were prepared and modified according to the protocol provided in study of Abougrain et al. (2010). Two hundred gram of a collected vegetable sample was weighed, washed with 250 mL of saline solution, and left for 10 hours for the sedimentation process. The supernatant was discarded, and the residue was transferred into a centrifuge tube and spun at 1500 rpm for 5 minutes. The supernatant was decanted, and the residue was gently stirred in a sucrose flotation medium with a specific gravity of 1.21. Each test tube was then covered to the edge with the flotation medium to form a meniscus. A coverslip was placed in the test tubes for 3 minutes and was removed and placed on a clean glass slide. Observations for the presence of *Cryptosporidium* oocysts were conducted under a light microscope (Olympus BX41TF, Okayama, Japan). Positive slides for oocysts were allowed to air dry and stained using the standard modified Ziehl-Nielsen technique (Abougrain et al., 2010). Air-dried slides were fixed in methanol for 2-3 minutes. Slides were flooded with cold carbol fuchsin for 5 to10 minutes, and then with 1% hydrochloric acid ethanol until the color stopped flowing and rinsed with tap water. It was then counterstained with 0.25% methylene blue for 30 seconds, rinsed again in tap water and air-dried.

Morphological identification of *Cryptosporidium* oocysts

The prepared slides were examined using a light microscope (Olympus BX41TF, Okayama, Japan) with 40 and 100 magnifications. *Cryptosporidium* oocysts appeared on a pale green background as bright rose-pink spherules (Figure 1). The positives slides identified were used for photomicrographs of *Cryptosporidium* oocysts.

Statistical analysis

All data were analyzed using SPSS 20 (IBM SPSS Statistics 20.0, NY, USA). Pearson chi-square or Fisher's Exact Test were used to analyze the association between the presence of *Cryptosporidium* oocysts and factors such as type, nature, consumption, and source of vegetables. The odds ratio (OR) values greater than the unit denoted association and less than unit denoted that the factor may have a protective effect. The occurrence of *Cryptosporidium* oocysts was estimated by dividing the number of positive samples by the total number of samples. A p value ≤ 0.05 was considered to be statistically significant.

Table 1. List of vegetables used in the study

Common name	Botanical name	Used Part
Mint	*Mentha spicata*	Leaf
Tarragon	*Artemisia dracunculus*	Leaf
Basil	*Ocimum bacilicum*	Leaf
Lettuce	*Lactuca sativa*	Leaf and stem
Spring onion	*Allium cepa*	Leaf and stem
Coriander	*Coriandrum sativum*	Leaf
Leek	*Allium ampeloprasum*	Leaf
Radish	*Raphanus sativus*	Root
Parsley	*Petroselinum crispum*	Leaf

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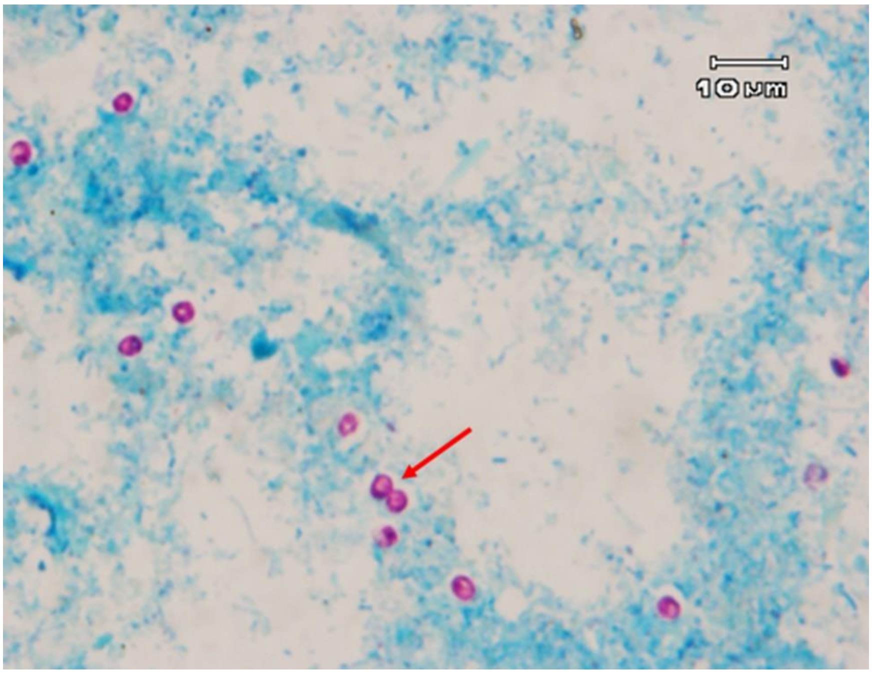

Figure 1. *Cryptosporidium* oocysts stained using the standard modified Ziehl-Nielsen technique. Oocysts appeared as bright rose-pink spherules (arrow).

RESULTS

Contamination of *Cryptosporidium* oocysts in the different vegetables

The oocysts appeared as bright rose-pink spherules with the size of 6-10 µm (Figure 1). Of the 275 samples of vegetables examined, 85 (31.5%) vegetables were positive and 190 (68.5%) were negative for the presence of *Cryptosporidium* oocysts (Figure 2). A total of nine varieties of vegetables were examined, and all were positive for *Cryptosporidium* oocysts. Of these, lettuce had the highest contamination rate (n: 16, 47.1%), followed by radish (n: 14, 46.7%), spring onion (n: 11, 35.5%), leeks (n: 9, 30%), mint and coriander (n: 8, 26.7%), tarragon (n: 7, 23.3%) basil and parsley (n: 6, 20%). There was a statistical significance between the occurrence of *Cryptosporidium* oocysts and types of vegetables examined ($p<0.05$) (Table 2).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to the marketplace

The occurrence of *Cryptosporidium* oocysts in the different vegetable fields showed that Amir Abad area had the highest contamination rate (n: 16, 59.3%), followed by Ghiam square (n: 14, 51.9%), Imam Shahr street (n: 12, 44.4%), Imam Khomeini Avenue (n: 9, 33.3%), Silage (n: 7, 25.9%), Ayatollah Kashani Avenue, Shahid Sadoughi boulevard, Homafar square (n: 6, 2.2%), and Bahaman Hospital area (n: 5, 18.5%) (Figure 3). The results further demonstrated that fresh vegetables from the area of Amir Abad had significantly the highest rate of contamination with *Cryptosporidium* oocysts (OR: 8.36; 95% confidence interval (CI) in OR: 2.25 < OR < 31; p: 0.001), followed by Ghiam square, and Imam Shahr. The lowest contamination was found in the Bahaman hospital area (OR: 1.3; 95% CI on OR: 0.3 < OR< 5.5; p: 0.7), although no statistical significant was found between the association (Table 3).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to the plant parts

The root vegetables had the highest contamination rate (n: 41, 45.6%), followed by leafy vegetables (n: 44, 24.4%). There was a significant association (OR: 2.58; 95% CI in OR: 1.5 < OR < 4.4; p: 0.001) between the occurrence of *Cryptosporidium* oocysts and the way in vegetables were consumed (Fisher's Exact Test; p: 0.000). There was a statistical significant ($p=0.000$) between the occurrence of *Cryptosporidium* oocysts and the nature of examined vegetables (Figure 4).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to types of vegetables consumption

The present study showed that 108 (39.27%) out of 275 consumed raw vegetables and 167 (60.73%) of those cooked before consuming were positive for *Cryptosporidium* oocysts (Figure 5).

Contamination of raw vegetables with *Cryptosporidium* oocysts according to types of water uses

The present study showed that 40% of retailers had a direct wholesale from vegetables fields (more in the center), while 60% were from middlemen who got vegetables from farms and sold to retailers (more uptown) before being sold to consumers. In addition, the water used in the vegetable farm was from different sources: 40% came from the farmland, 30% from the nearby stream water, and 30% from the tap water. Water is always sprinkled on growing vegetables to maintain freshness, and to attract more consumers. The water, therefore, seemed to expose unhygienic conditions of reuse and containers (data were not shown).

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Table 2. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the types of vegetables

Vegetable	Odd ratio	CI*	P value
Mint	1.455	(.435 4.860)	0.543
Tarragon	1.217	(.355 4.170)	0.754
Lettuce	4.571	(1.452 14.389)	0.009
Spring onion	2.316	(.724 7.407)	0.157
Coriander	1.455	(.435 4.860)	0.543
Leek	1.714	(.523 5.621)	0.374
Radish	3.500	(1.112 11.017)	0.032
Parsley	1.000	(.282 3.544)	1.000

*Confidence Interval. There was a statistical significance between the occurrence of *Cryptosporidium* oocysts and types of vegetables examined (X2: 15.487a, DF: 8; p: 0.049).

Table 3. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the location of vegetables fields

Vegetable field location	Odd ratio	CI*	P value
Amir Abad	8.36	(2.25 31)	0.001
Ayatollah Kashani	1.64	(0.4 6.6)	0.48
Silage	2.01	(0.5 7.8)	0.31
Shahid Sadoughi Blv	1.64	(0.4 6.6)	0.4
Bahaman hospital area	1.3	(0.3 5.5)	0.7
Ghiam square	6.1	(1.6 22.7)	0.006
Imam Shahr	4.6	(1.2 16.9)	0.02
Imam Khomeini Avenue	2.8	(0.7 10.8)	0.1
Homafar Square	1.64	(0.4 6.6)	0.4

*Confidence Interval

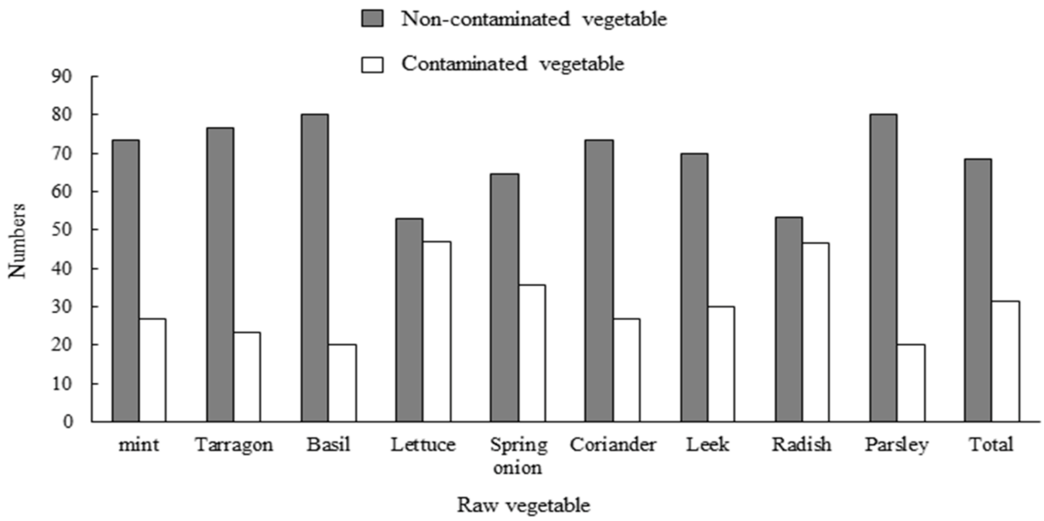

Figure 2. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the types of vegetables

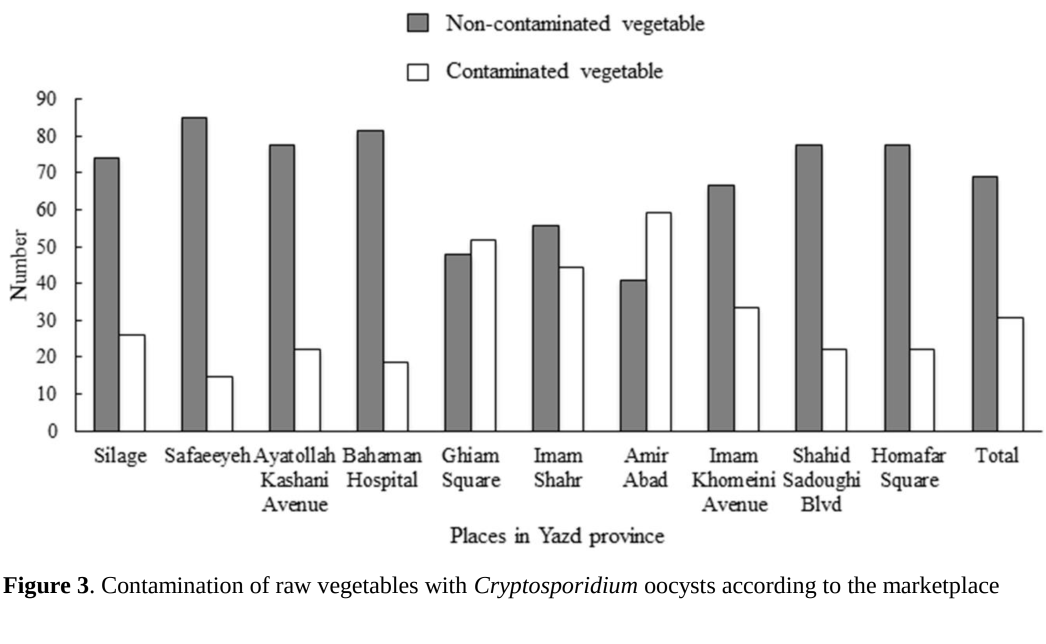

Figure 3. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the marketplace

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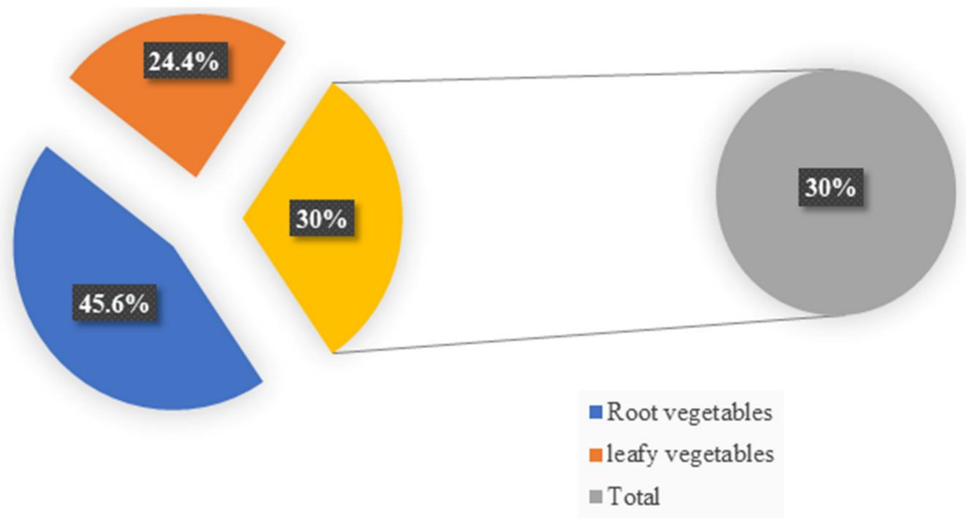

Figure 4. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the plant parts.

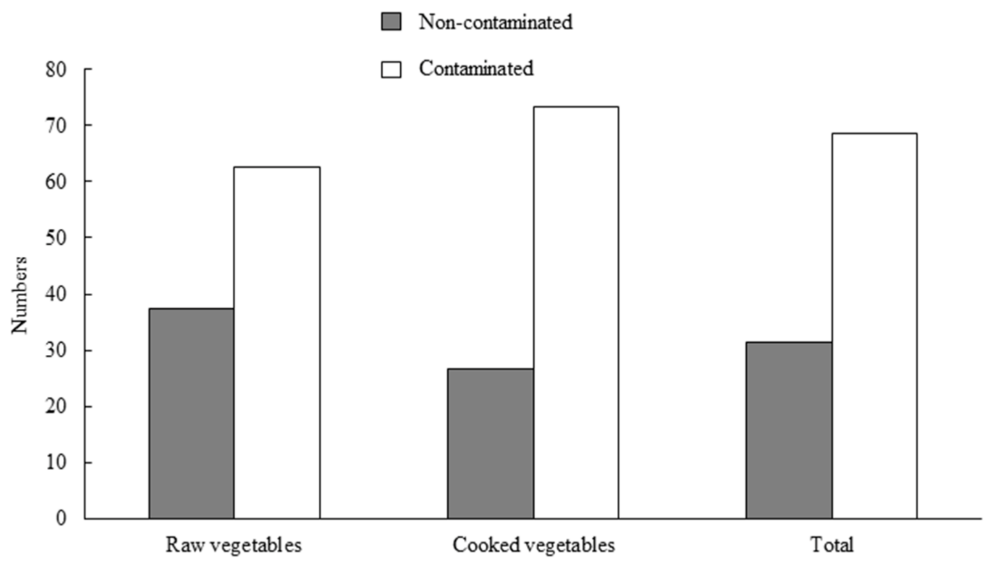

Figure 5. Contamination of raw vegetables with *Cryptosporidium* oocysts according to the types of vegetable consumption

DISCUSSION

Yazd is the driest and the warmest major city in Iran, located in the north of the Persian Gulf coast. A general practice of "Save to Safe Water" is commonly seen among people living in the city of Yazd due mainly to a shortage of water supply. This economic condition of water use, therefore, plays a critical role in water consumption, and can increase the feasibility of contamination in food, including the consumption of vegetables in general. In the current study, the occurrence of *Cryptosporidium* oocysts was high in vegetable farms due to the dry and hot climate in the city of Yazd. This also indicates the lack of hygiene in personal behavioral practices of food and water consumption such as the use of unclean water for washing vegetables. In the fruit and vegetable fields, wholesalers regularly wash vegetables which sprinkle vegetables using the raw water from natural sources to prevent the fading of vegetables (Abedi et al., 2014).

Cryptosporidium is a cosmopolitan protozoan parasite, and water is a significant vehicle for the transmission of this intestinal pathogen. The potential of this parasite for waterborne transmission is further enhanced by the perpetual infectivity of the oocysts, small size (3.5 to 6.0 mM), and low precipitation rate (0.5 mM/s) (Fletcher et al., 2012; Ryan et al., 2014). The surface water is more likely to be contaminated with human and/or animal feces in the environment. It can also be contaminated by feces entering the agricultural run-off of adjacent farm animals or from human sewage (Ryan et al., 2014; Widerström et al., 2014). The proximity of the vegetables to the soil may also played a key role in the contamination of the plants with *Cryptosporidium* oocysts, since the vegetables were kept in an unhygienic condition. Moreover, the reinforcement of horticultural crops, such as vegetables with cattle and sheep dung (dried fecal matters)

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(Rossle and Latif, 2013) with viable oocysts of *Cryptosporidium* represented a significant risk for contamination of vegetables (Snelling et al., 2007; Kinyua et al., 2016).

A high occurrence of *Cryptosporidium* oocysts in lettuce is possibly due to its flat leafy nature which may increase it, trapping a great number of oocysts from the contaminated sources. Contamination of lettuce with food toxins and foodborne pathogens can occur in fields resulting from the entry of microorganisms from non-potable water irrigation or fertilizers that still contains viable pathogens (Chau et al., 2014; Bintsis, 2018). Some foodborne pathogens associated with lettuce have been observed, and the ingestion of raw vegetables, such as salad, played a key role in the transmission of parasitic infections (Snelling et al., 2007). In the Amir Abad area, the vegetable field is well supported, more centrally located, and therefore this area can provide more vegetables for the wholesale and retail outlets to customers. However, the greater possibility of obtaining contaminated vegetables from Amir Abad fields was significantly observed. Furthermore, it has been found that this area increases activities that can trigger an easier introduction of infectious agents, including *Cryptosporidium*, into vegetables sold on the market. Consequently, there was a higher rate of recovery of *Cryptosporidium* oocysts in the vegetable field of Amir Abad compared to other places in the city of Yazd.

The findings of this study also suggest that a higher rate of contamination was found in the root part compared to the leafy vegetables. Results of the present study, however, contrary to a previous study, showed a higher rate of contamination in leafy vegetables (Maikai et al., 2013). The reason could be attributed to the nature of the contacted soil-root vegetables which appeared to retain more trapped pathogens than leafy vegetables. Besides, it is obvious that these vegetables were consumed raw or cooked (Snelling et al., 2007). Interestingly, the findings also showed contamination rates in vegetables that are generally eaten raw or cooked. Of these, radish, basil, parsley, and lettuce are normally eaten raw, while spring onion, mint, leek, tarragon and coriander are also eaten raw as well as cooked. Although there was a higher rate of contamination in vegetables normally cooked before consuming, all vegetables should be washed sufficiently with clean/chlorinated water before consuming, whether they are eaten raw or cooked to decrease the chances of contamination with *Cryptosporidium* oocysts.

CONCLUSION

Based on this preliminary study, vegetables sold in Yazd city, were contaminated with *Cryptosporidium* oocysts. The study also indicated a higher occurrence of *Cryptosporidium* oocysts on the root part of vegetables than in the leafy ones. It was found that more densely populated and more supported facilities in the Amir Abad area were more widely contaminated with *Cryptosporidium* oocysts than the remaining vegetable fields in this city. As vegetables are an important part of a healthy diet, and can be consumed either raw or cooked, the findings of the current study raised great public health concerns. Moreover, it is mandatory for the higher authority to strictly and consistently monitor the level of contamination in all edible vegetables and water consumption with *Cryptosporidium* in a larger scale. More comprehensive studies on molecular and phylogenetic analyses are also strongly recommended to confirm the pathogenic strains of *Cryptosporidium* as a zoonotic transmission in this part of Iran.

DECLARATIONS

Acknowledgments

This study was supported by the Shahid Sadoughi University of Medical Sciences (GN: 1394.522-2276) for the cooperation and financial management. The authors would also like to thank our colleagues: Mrs. Marziyeh Beigom Modares Sanavi, Sayyedeh Zahra Hosseini and Zahra Sadeghi for their assistance in data collection. Maria de Lourdes Pereira thanks to project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 & UIDP/50011/2020.

Competing interests

The authors have not declared any conflict of interest.

Author`s contribution

Ali Fattahi Bafghi, Mohammad Reza Yavari, and Farzaneh Mirzaei designed the study. Farzaneh Mirzaei supervised the laboratory examinations. Abolghasem Siyadatpanah performed the experiments. Manuscript preparation and the statistical analysis were conducted by Watcharapong Mitsuwan, Maria de Lourdes Pereira, Veeranoot Nissapatorn, Roghayeh Norouzi, Seyed Abdollah Hosseini. All authors read and approved the final manuscript.

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To cite this paper: Fattahi Bafghi A, Yavari MR, Mirzaei F, Siyadatpanah A, Mitsuwan W, Pereira ML, Nissapatorn V, Norouzi R, and Hosseini SA (2020). Prevalence and Risk Factors Associated with *Cryptosporidium* Infection in Raw Vegetables in Yazd District, Iran. *World Vet. J.,* 10 (3): 260-266. DOI: https://dx.doi.org/10.36380/scil.2020.wvj34