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Study on *Cryptosporidium* contamination in vegetable farms around Tehran

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Abstract. In recent years, an increase in the number of cases of food-borne illnesses linked to fresh vegetables has been reported. One of the causative agents of these infections is *Cryptosporidium* and it appears that one route of transmission to humans is food-borne, so fruits and vegetables have important roles. The goal of this study was to determine the level of *Cryptosporidium* contamination in vegetable farms around Tehran, Iran. A total of 496 samples from 115 vegetable farms in different regions around Tehran (Capital city of Iran) were collected and different types of vegetables were investigated for the parasite in June and July, 2012. A sediment concentration method followed by modified Ziehl-Neelsen's acid-fast staining was used to determine the presence of *Cryptosporidium* oocysts. Our findings revealed that 6.6% of studied samples were contaminated with *Cryptosporidium* species. The highest rate of contamination was reported in Bagher Abad (South of Tehran) (11.1%), and green onions were more commonly contaminated (14.8%) than any other vegetables tested. Furthermore, when waste water was used to irrigate vegetable farms, the contamination rate was (33.3%). Statistical analysis showed a correlation between contamination with *Cryptosporidium* spp. and studied risk factors including: different regions around Tehran, type of vegetables, and type of water used for farm irrigation. Therefore, vegetables may provide a route by which *Cryptosporidium* can be transmitted to humans, and control strategies should be considered.

INTRODUCTION

Cryptosporidium is a protozoan parasite that causes severe diarrhoeal disease (cryptosporidiosis) in mammals, including humans and livestock and is responsible for many food and waterborne outbreaks in many countries (Ajonina *et al*., 2012). This protozoan is transmitted by oocysts, passed in the faeces (Fayer *et al*., 2000) and can be transmitted via the faecal-oral route (Robertson & Gjerde, 2000). Humans appear to be the sole host for the latter and a distinct seasonality has been observed in endemic areas around the world (Ortega *et al*., 1997).

Oocysts are remarkably resistant to common disinfectants and they can survive for several months in foods (Gornik & Exner, 1991; El Zawawy *et al*., 2010). Generally, Food-borne parasites can be divided in two main groups according to the route of transmission to humans. These parasites reach human beings through the consumption of raw infected food such as muscle tissues of different animal species or vegetables, and contaminated food and water resources (Pozio, 2008). Cryptosporidiosis is produced by ingestion of food and water contaminated with oocysts, which are known to be highly resistant to treatment procedures (Ethelberg *et al*., 2009). Findings of an investigation showed that *Cryptosporidium* oocysts are sensitive to heat and, to some extent, to blast freezing, but are resistant to chlorine (Duhain *et al*., 2012). Therefore, the use of chlorine during vegetable processing is not a critical control point for oocysts. Moreover, even now, the low infection dose of less than 30 oocysts and its resistance to chlorination makes it, also a challenge for drinking water (Füchslin *et al*., 2012). Haas & Rose (1995) defined an action level for *Cryptosporidium* in drinking water: "Whereas a *Cryptosporidium* concentration exceeding 0.1 oocysts/l can cause sporadic cryptosporidiosis cases in population, concentrations over 0.3 oocysts/l will almost certainly cause a cryptosporidiosis outbreak." Protozoans can contaminate vegetables via various routes, including: direct application of contaminated manure or irrigation of vegetables (Duhain *et al*., 2012). Some research conducted in many regions in the world implicated wastewater irrigated plants as a major potential source of transmission of protozoan diseases especially cryptosporidiosis (Ajonina *et al*., 2012; Koompapong & Sukthana, 2012).

Therefore, the aim of this study was to estimate the risk of *Cryptosporidium* infections in humans associated with vegetables grown in the agricultural areas around Tehran.

MATERIALS AND METHODS

Study period and sample collection

A survey was carried out which included 496 samples from 115 vegetable farms in different regions around Tehran (Capital city of Iran) where different types of vegetables were investigated for *Cryptosporidium* spp. Samples were collected from five different regions from southeast to southwest of Tehran that were major agricultural areas around this metropolitan region. The frequency of selected samples is seen in Table 1. Samples were collected in June and July, 2012, directly from vegetable farms. After collection and the recording of some information including: region, type of vegetables, and type of water for farm irrigation, samples were transferred separately in transparent nylon bags to the Parasitology laboratory.

Sampling procedure

The current USFDA method to recover parasites from fruits and vegetables is derived from procedures used to isolate parasitic protozoa from water. In the laboratory, 200 g of the collected samples were processed in a cleaning bath with 1.5 liters of detergent solution (1% sodium dodecyl sulfate, 0.1% Tween 80) and were stirred for 10 min. Then, vegetable samples were removed and the wash water was collected in a polypropylene beaker. Following 30 min deposition of the fluid, we emptied the supernatant fluid and the sediment was transferred to 50 ml polypropylene centrifuge tubes and centrifuged for 15 min at 270xG. If a large quantity of extraneous matter is contained in the sediment it was reduced by layering on Sheather's fluid and centrifuging at 270xG for another 15 min. Again, the supernatant was collected and washed twice in distilled water.

Table 1. Frequency of samples based on different regions and types of vegetables

	*Region 1	*Region 2	*Region 3	*Region 4	*Region 5	Total
Mint	15	15	15	30	7	82
Leek	15	15	15	30	15	90
Cress	15	15	15	30	15	90
Green onion	15	6	3	18	12	54
Coriander	15	15	15	30	15	90
Basil	15	15	15	30	15	90
Total	90	81	78	168	79	496

* Region 1) Shahr'e Ray (South of Tehran), Region 2: Bagher Abad (South of Tehran), Region 3: Varamin (South of Tehran), Region 4: Yaft Abad (Southwest of Tehran), Region 5: Aloak (Southeast of Tehran)

This procedure is adequate for protozoa (Bier, 1991; Calvo *et al.*, 2004; Vuong *et al.*, 2007). Then, slides were prepared from the remaining sediment. After fixation by methanol, all slides were stained by modified Ziehl-Neelsen's acid-fast. The Ziehl-Neelsen stained smears were examined by light microscopy under 1000X magnification for the presence of *Cryptosporidium* oocysts. A sample was considered positive for cryptosporidiosis, if an oocyst was observed with the correct morphology including optical properties, internal structure, size, and shape.

Statistical analysis

SPSS 10.0 for Windows was used to do the statistical analyses. Chi square (χ^2) method was used to detect significant differences between the various groups at 5% level of significance.

RESULTS

Results showed that 33 out of 496 samples (6.6%) were contaminated with *Cryptosporidium* spp. and the highest contamination rate was found in Bagher Abad region (South of Tehran) with 9 out of 33 contaminated samples (11.1%), whereas no protozoan oocysts were reported in Aloak (Southeast of Tehran) (Table 2). Statistical analysis showed a significant correlation between contamination with *Cryptosporidium* spp. and different regions around Tehran (χ^2 =16.48) (p=0.002).

Moreover, our findings showed that the highest rate of contamination was observed in green onions (14.8%) and the lowest was in basil (1.1%) (Table 3). Statistical analysis showed that the differences in contamination with *Cryptosporidium* were significant in different types of vegetables (χ^2 =13.03) (p=0.023).

Furthermore, the type of water used on vegetable farms was associated with contamination rates. Results showed that when waste water was used on vegetable farms, the incidence rate of oocysts was (33.3%) higher than when well water was used (Table 4) (χ^2 =14.09) (p=0.006).

DISCUSSION

In recent years, many researchers have revealed that clinical cryptosporidiosis exists globally and the most important infection route for *Cryptosporidium* spp. is anthroponotic transmission (Ranjbar-Bahadori *et al.*, 2011). The most important ways for transmission of cryptosporidiosis to human are waterborne and person to person, but fruits and vegetables are also important (Bier, 1991).

Hence, the present study carried out on 496 vegetable samples from 5 agricultural regions around Tehran showed that 6.6% of samples were contaminated with *Cryptosporidium* spp. Daryani *et al* (2008) showed that 25% of market vegetables and 29% of garden vegetables were contaminated with pathogenic parasites in Ardabil, Iran and parasites detected were *Giardia* cysts (7%), *Dicrocoelium* eggs (6%), *Fasciola* eggs (5%) and *Ascaris* eggs (2%). Razavi *et al.* (2009) revealed that 23.5% of collected lettuce from different areas in Shiraz (center of Iran) was contaminated with *Cryptosporidium*. Both of two recent studies in Iran were conducted

Table 2. Frequency and percentage rate of vegetables contaminated with *Cryptosporidium* in different regions around Tehran

	*Region 1	*Region 2	*Region 3	*Region 4	*Region 5	Total
Non-contaminated	85 (94.4%)	72 (88.9%)	77 (98.7%)	150 (89.3%)	79 (100%)	463 (93.4%)
Contaminated	5 (5.6%)	9 (11.1%)	1 (1.3%)	18 (10.7%)	0 (0)	33 (6.6%)
Total	90 (100%)	81 (100%)	78 (100%)	168 (100%)	79 (100%)	496 (100%)

*Region 1) Shahr'e Ray (South of Tehran), Region 2: Bagher Abad (South of Tehran), Region 3: Varamin (South of Tehran), Region 4: Yaft Abad (Southwest of Tehran), Region 5: Aloak (Southeast of Tehran)

Table 3. Frequency and percentage rate of *Cryptosporidium*-contaminated vegetables

Type of vegetables	Mint	Leek	Cress	Green onion	Coriander	Basil	Total
Non-contaminated	75 (91.5%)	87 (96.7%)	82 (91.1%)	46 (85.2%)	84 (93.3%)	89 (98.9%)	463 (93.4%)
Contaminated	7 (8.5%)	3 (3.3%)	8 (8.9%)	8 (14.8%)	6 (6.7%)	1 (1.1%)	33 (6.6%)
Total	82 (100%)	90(100%)	90 (100%)	54 (100%)	90 (100%)	90 (100%)	496 (100%)

Table 4. Frequency and percentage rate of *Cryptosporidium*-contaminated vegetables based on type of water used

Type of water	Well water	Waste water	Total
Non-contaminated	455 (94%)	8 (66.7%)	463 (93.4%)
Contaminated	29 (6%)	4 (33.3%)	33 (6.6%)
Total	484 (100%)	12 (100%)	496 (100%)

in rural areas, but our study was done on vegetable farms around Tehran. The more industrial nature of these regions may be a main cause for the lower contamination rate in this study.

It should be mentioned that this parasite is a major cause of diarrhoeal disease in humans, worldwide and is a major cause of protozoan waterborne diseases. *Cryptosporidium* has a life cycle which is suited to waterborne and food-borne transmission, and *Cryptosporidium parvum* is the major zoonotic *Cryptosporidium* species (Smith *et al.*, 2007). Ortega *et al* (1997) showed that of the total vegetables examined in markets of an endemic region in Peru, 14.5% contained *C. parvum* oocysts. The first food-borne outbreak caused by *C. parvum* was reported in Finland. The outbreak occurred among persons who had eaten in a canteen, and a salad mixture was suspected (Pönka *et al.*, 2009). Also, *Cryptosporidium* sp. oocysts were detected in 4.69% of vegetable samples, but not in any fruit samples (Rzezutka *et al.*, 2010). But in Norway, samples of various fruits and vegetables were analyzed and only 4% of samples were contaminated with protozoans, of which 26% were lettuce, and 74% were mung bean sprouts. Moreover, mung bean sprouts were significantly more likely to be contaminated than the other fruits and vegetables. This is the first time that parasites have been detected on vegetables and fruit obtained in a highly developed wealthy country, without there being an outbreak situation (Robertson & Gjerde, 2001). Gharavi *et al.* (2002) showed that the highest rate of contamination in studied vegetable samples was in leek and parsley and the lowest one was detected in tarragon. Also, the presence of *Cryptosporidium* was determined in lettuce, parsley, cilantro, strawberries and blackberries acquired in a local agricultural market of Costa Rica, in order to establish the possible transmission risk of these microorganisms and other pathogens from the consumption of these raw products.

The parasite was present in all products but strawberries (Calvo *et al.*, 2004). In another study in Costa Rica, *Cryptosporidium* sp. oocysts were found in 5.2% of cilantro leaves, in 8.7% of cilantro roots and 2.5% of lettuce samples. A 1.2% incidence was found in other vegetable samples (carrot, cucumber, radish and tomatoes). Oocysts of this parasite were absent in cabbage (Monge & Arias, 1996). Our findings showed that the highest rate of contamination was observed in green onions (14.8%), possibly because it is a root vegetable and it makes more contact with the ground. Moreover, basil (1.1%) had the lowest rate of contamination because of its being a stem form and its elevation from the soil. Therefore, it appears that shape and appearance of vegetables is important in the contamination rate of parasites, and root vegetables are likely contaminated due to contact with the soil and contaminated irrigation water. The highest contamination rate was determined in Bagher Abad (South of Tehran) (11.1%) and when waste water was used in vegetable farms (33.3%). It appears that lack of a proper sewage system in Tehran may cause a mix of underground water resources with sewage and the use of these water resources in farm irrigation is an important factor in the contamination of vegetables in southern areas around Tehran. Furthermore, the presence and grazing of some animals, including small ruminants, on or close to vegetable farms and excretion of their infected faeces may lead to the contamination of fruits and vegetables. Robertson & Gjerde (2000) have mentioned the important role of sheep and goats in transmission of the infection to human and have emphasized preventative measures including avoiding contamination of water resources with animal faeces.

Some studies also suggest that washing vegetables does not completely remove the parasite (Ortega *et al.*, 1997). Robertson & Gjerde (2000) published techniques for recovering parasites from fruit and vegetables, and they mentioned that these are generally inadequate, with low and variable recovery efficiencies. For example, recovery efficiencies from lettuce, Chinese lettuce, and strawberries were found to be approximately 42% for *Cryptosporidium*. On the other hand, none of the tested washing methods succeeded in completely removing oocysts from apples. The most efficient removal (37.5%) was achieved by rigorous manual washing in water with a detergent and by agitation on an orbital shaker with Tris-sodium dodecyl sulfate buffer (Macarisin *et al.*, 2010).

Thus, market vegetables may provide a route by which *Cryptosporidium* can be transmitted (Pozio, 2008). Therefore, as a general role, control strategies should be based on the public health education of farmers, shepherds and consumers, the development or improvement of more sensitive methods to detect these parasites in slaughtered animals and in foodstuff, control of waste water on pastures, control of drinking water resources, and the reduction of contact between livestock and wild animals which frequently represent the most important reservoir of this pathogen

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