

Human intestinal protozoa in fresh asparagus from different types of markets in northwest Mexico

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Abstract. Caborca is one of the most productive asparagus-growing regions in the state of Sonora in northwest Mexico, an area where some fresh fruits and vegetables are sold at unregulated open-air street markets. This is a cross-sectional study in which fifty bundles of asparagus for exportation, 50 bundles of sub-standard asparagus, and 50 bundles of asparagus from open-air markets were selected randomly and then subjected to Faust, Kinyoun and ELISA testing to detect intestinal parasites. Pearson's chi-square (χ^2) and Student-Newman-Keuls tests were used to estimate differences among the sampling site groups ($P < 0.05$). The pathogens *Cryptosporidium* spp. (29%) *G. intestinalis* (5%) and *Cyclospora* spp. (3%) were found in the asparagus sold in the region. The prevalence of *Cryptosporidium* spp. was higher in both the sub-standard asparagus and the product sampled from the open-air markets than in the samples for exportation ($P < 0.05$). This is the first study to demonstrate contamination by intestinal parasites in asparagus sold in different markets in northwest Mexico.

INTRODUCTION

Currently, consumption of fruits and vegetables continues to increase worldwide (Kirezieva *et al.*, 2015). In Mexico, for example, consumption of fruits and vegetables increased from 110 to 235 g per day from 2006 to 2012 (Olaiiz-Fernández *et al.*, 2006; Gutiérrez *et al.*, 2012). Mexico is an important exporter of vegetables (Matthews *et al.*, 2014) and one of the world's largest asparagus exporters. National asparagus production increased from 84,023 to 144,765 tons between 2013-2017 (SAGARHPA, 2018). Asparagus is one of the principal agricultural products in northwest Mexico (SAGARHPA, 2018), but it is also a potential carrier of gastrointestinal parasitic infections due to improper handling procedures. For example, Matthews *et al.* (2014) reported two outbreaks of

cryptosporidiosis (356 cases) and five outbreaks of gastrointestinal diseases (676 cases) associated with contaminated fruits and vegetables in the United States from 2003 to 2010. Other outbreaks associated with salads contaminated with *Cryptosporidium parvum*, *Cryptosporidium hominis* and *Cyclospora* spp. have been reported in Germany and Denmark (Döller *et al.*, 2002). Sia Su *et al.* (2012) analyzed 80 vegetables (petchay and lettuce) from public and private markets in the Philippines. Using concentration methods, they isolated *Giardia intestinalis* (*G. intestinalis*, 0.6%), *Entamoeba histolytica* (*E. histolytica*, 0.6%), *Entamoeba coli* (*E. coli*, 2.5%), helminths hookworm spp. (2.6%), and *Ascaris lumbricoides* (*A. lumbricoides*, 22.9%). Adenusi *et al.* (2015) used formol – with ether the concentration or the modified Ziehl-Neelsen technique – to detect *A.*

lumbricoides (4.58%), *Trichuris trichiura* (*T. trichiura*, 3.96%), hookworms (1.56%), and *Hymenolepis nana* (*H. nana*, 0.42%) in 250 g of pre-washed fresh vegetables and fruits (lettuce, fluted pumpkin, spinach, jute mallow, tomatoes, carrot and cabbage) in southwest Nigeria. Studies of this kind in Mexico are limited and, even though the prevalence of giardiasis is recognized as being high in the general population (from 3 to 50% in 2009) (Cedillo-Rivera *et al.*, 2009), information on cryptosporidiosis is scarce there. Sánchez-Vega *et al.* (2006) found 41% of *Cryptosporidium* spp. in 100 infants with diarrhea in Mexico City. Earlier research at our study site in Caborca, performed with 120 local school children, found a prevalence of 11.1% of *G. intestinalis*, 7.9% of *Endolimax nana* (*E. nana*), 6.5% of *E. coli*, 1.8% of *E. histolytica*, 0.9% of *Iodamoeba bütschlii* (*I. bütschlii*), 0.5% of *Cryptosporidium* spp., 7.4% of *H. nana* helminths, and 0.92% of *A. lumbricoides* (Lugo-Sepúlveda & Paredes-González, 2011). These findings clearly show that local food can become a vehicle for parasite transmission. In the Caborca region, fruits and vegetables sold in street markets remain unregulated by the Sanitary Control authorities and the Department of Public Health. For example, sub-standard asparagus – that is, product that fails to pass international quality control standards – sold in packing centers or open-air markets in Caborca presents a high risk for contamination by intestinal parasites (Damen *et al.*, 2007). However, there is very little information concerning this at the local level. Therefore, the present study examined the presence of intestinal parasites in asparagus sold at different types of markets in a region of northwest Mexico.

MATERIALS AND METHODS

Study area

Caborca is a city with 81,309 inhabitants (INEGI, 2018) located 280 meters above sea level. Its weather is semi-warm and extremely dry, with an average annual temperature of 22.3°C, though in December-

January this falls to 12.4°C (Climate-Data.Org, 2018).

Study design and sample collection

This was a cross-sectional study. Asparagus sampling was performed between January and April 2015. A total of 150 bundles of asparagus were randomly sampled as follows: fifty of export asparagus for and 50 of sub-standard asparagus (that is, of inappropriate size or with visible damage) were collected from three packing centers, while 50 more bundles were obtained in unregulated open-air markets ($n = 10$). The export and sub-standard bundles were taken from different conveyor belts, selected randomly, and then numbered. Bundles of asparagus from display racks in open-air markets were also selected randomly and numbered. All sampling sites were inside the study region.

Sample-processing

After collection, each bundle of asparagus was placed, hygienically, in sterile sampling bags, labeled (Nasco, Whirl-Pak, 2015), and transported to the Centro de Investigación en Alimentación y Desarrollo, A.C., where they were kept at a temperature of 4-6° for fewer than 24 hours until analysis. For processing, a modification of the Ezatpour *et al.* (2013) was used, as follows: a number of asparagus spears were taken randomly from each bundle to complete a weight of ≈ 250 g. All the turions were washed in 1 L of 0.95% NaCl in sterile sampling bags, then the wash water was filtered by a vacuum pump using Whatman filters (1 μ m). Following each procedure, all the glassware utilized was washed to prevent contamination. following the United States Environmental Protection Agency Method 1623.1 (USEPA, 2012). Filtration was performed at a flow rate of 0.01 L/s (USEPA, 2012). The material that remained in the filter was processed and analyzed using the proper diagnostic techniques to detect species of parasites that may be present in human gastrointestinal infections in the study region, while another portion was re-suspended and homogenized in 3.0 mL of sterile distilled water, and then

transferred to 5.0-mL Eppendorf tubes, and stored at -20° until analysis by ELISA.

Faust technique

In the Faust technique (Markell *et al.*, 1999), each sample of sterile distilled water used during the washing process of the asparagus was poured into a round-bottom tube (100 by 13 mm) to within 20 mm of the rim. The sediment was suspended and centrifuged for 10 min at 2500 rpm (700 x *g*). All centrifugations were performed without mechanical breakage. The supernatant was decanted, and the last drop was drained onto a clean section of paper towel. This washing procedure was repeated 3 times. Next, 3.5 mL of aqueous ZnSO₃ solution (1.180 specific gravity) were added to within 50.8 mm of the rim of the tube. The packed sediment was re-suspended using applicator sticks until no coarse particles remained. This suspension was centrifuged for 5 min at 2500 rpm (700 x *g*) and transferred without agitation to a rack that maintained it upright, where the suspension was allowed to stand for 20 min. With a wire loop 5 mm in diameter and bent at a right angle to the stem, two loops of the surface film were transferred to a drop of iodine solution (Weigert's solution) on a glass slide (76.2 by 50.8 mm) for wet-mount examination using 10x and 40x objectives to identify cysts of *G. intestinalis*. The specific gravity of the zinc sulfate solution was checked every 7 days throughout the study using a calibrated hydrometer with a specific gravity range of 1.00–1.20. This technique can detect the pathogens *G. intestinalis*, *Entamoeba histolytica* (*E. histolytica*) and *Blastocystis hominis* (*B. hominis*), and the non-pathogens *Endolimax nana* (*E. nana*) and *Iodamoeba bütschlii* (*I. bütschlii*) (CDC, 2018).

Kinyoun technique

Three mL of each sample of sterile distilled water recovered from washing the asparagus were centrifuged for 10 min at 2500 rpm (700 x *g*). The sediment was smeared on a slide and left to dry after applying methanol to fix the smear on the slide. *Cryptosporidium* and *Cyclospora* oocysts in the samples are acid-fast under carbol-fuchsin staining. Diluted

sulfuric acid (1–3%) was used as the decolorizer, and methylene blue was applied to stain the decolorized smears once again. When the colored smears were dry, immersion oil was added and the 100x objective was used for microscopic observation. A contrast between the pale-stained oocysts (pink) and the heavily-stained background (blue to dark blue) was observed (Harrington, 2008). The modified acid-fast stain has demonstrated good performance in detecting *Cryptosporidium* spp. and *Cyclospora* spp. using wet preparations and trichrome stains (Ribes *et al.*, 2004; Laatamna *et al.*, 2018).

Detection of *Cryptosporidium* by ELISA

To detect *C. parvum* spp. antigens, 1 mL of wash water was homogenized, then transferred to cryogenic vials (2 mL), properly-labeled and stored at -20°C until analysis. Samples were allowed to thaw at room temperature (24°C), and then 5 mL of anti-*C. parvum* spp. solution was added to each vial. The contents was homogenized and 200 µL of a second anti-*C. parvum* spp. solution was added to form a “sandwich” with the *C. parvum* spp. antigen bound by the first antibody. The reaction was visualized by adding a second *C. parvum* antibody bound to a peroxidase conjugate with chromogenic tetramethylbenzidine against the second *C. parvum* antibody. The blue color revealed the presence of the *C. parvum* antigen bound to the anti-*C. parvum*. The reaction was stopped using 1M phosphoric acid. A yellow color developed and was read using a model 680 microplate reader (Bio-Rad Laboratories, Hercules, USA) in an absorbance range of 450-650 nm. Positive and negative standard references for quality control were included in each run. A positive result was considered when the reading was ≥0.150, in concordance with the manufacturer's instructions. The DRG ELISA kit used had a sensitivity of 93% and a specificity of 98% for detecting *C. parvum* antigens (*Cryptosporidium* Ag Stool, DRG International, Mountainside USA).

Statistical analysis

The response variable was the presence of at least one species or genus of parasite

per bundle of asparagus. This variable was represented as parasitism (1) or non-parasitism (0). The parasitic prevalence was calculated as the percentage of positive cases with respect to the total number of bundles of asparagus sampled. Differences in the prevalence among sampling sites was estimated using Pearson's chi-square test (χ^2) followed by a Student-Newman-Keuls test. Data were analyzed using STATA/SE version 12.0 with significance set at $P \leq 0.05$.

RESULTS

In this study, 42% (n = 63) (CI = 0.32–0.48) of the 150 bundles of asparagus sampled contained at least one type of parasite (Table 1); however, no difference was found in the prevalence of *G. intestinalis* (5%) vs. *Cyclospora* spp. (3%); or *G. intestinalis* vs. *I. bütschlii* (3%) ($P = 0.376$). *Cryptosporidium* spp. (29%) was the intestinal parasite most often isolated ($P < 0.05$), followed by *E. nana* (9%). Regarding the bundles of asparagus from the open-air markets, analyses revealed a prevalence of intestinal parasites of 68% (34/50), while the incidence in the samples of sub-standard and export asparagus were 54% (27/50) and 4% (2/50), respectively (Table 2). No difference was found in the prevalence of parasites between the asparagus from open-air markets and the sub-standard product ($P = 0.151$, 95% CI = -0.329, 0.049), but the prevalence of parasites in both the open-air

and sub-standard asparagus was higher than in the product for exportation ($P = 0.001$). The prevalence of *Cryptosporidium* spp. (50%) (25/50) was higher in the asparagus from the open-air markets than in the sub-standard (32%) (16/50), ($P = 0.032$, 95% CI = -0.344, -0.016) and export asparagus (4%) (2/50) ($P = 0.001$, 95% CI = -0.656, -0.264). The prevalence of *Cryptosporidium* spp. in the open-air market and sub-standard asparagus was also higher than in the export product (Table 2), while the prevalence of *E. nana* was higher (10/50) in the asparagus from the open-air markets than in the sub-standard asparagus (3/50), ($P = 0.011$, 95% CI = -0.247, -0.032), but this parasite was not detected in the export product, nor was *G. intestinalis*, *I. bütschlii* and *B. hominis*

Table 1. Overall prevalence of intestinal parasites in 150 sampled bundles of asparagus from ten open markets and three packing centers located in Caborca, Sonora, Mexico during January-April 2015.

Parasite	% (n)	CI
* <i>Cryptosporidium</i> spp. ^α	29 (43)	21.5–35.6
<i>E. nana</i> [§]	9 (14)	4.6–14.4
<i>G. intestinalis</i> ^α	5 (7)	1.9–9.3
<i>Cyclospora</i> spp. ^α	3 (5)	0.7–6.6
<i>I. bütschlii</i> [§]	3 (5)	0.7–6.6
<i>B. hominis</i> ^α	1 (2)	0.1–3.6

CI = Confidence Intervale.

* = 10 (23%) of 43 cases were identified as *C. parvum*.

§ = *E. nana* and *I. bütschlii* are considered as non-pathogenic protozoa.

α = *B. hominis*, *G. intestinalis*, *Cyclospora* spp., *Cryptosporidium* spp. are considered as pathogenic protozoa.

Table 2. Prevalence of intestinal parasites isolated of bundles of asparagus sampled from different collection sites located in the region of Caborca, Sonora, Mexico during January-April 2015.

Parasites	Open-aired Market	Lag	Export	Total	*P
	n = 50	n = 50	n = 50	n = 150	
% (contaminated)					
<i>Cryptosporidium</i> spp.	50 (25)	32 (16)	4 (2)	28.7 (43)	0.001
<i>E. nana</i>	20 (10)	6 (3)	0 (0)	8.7 (13)	0.001
<i>G. intestinalis</i>	8 (4)	6 (3)	0 (0)	5 (7)	0.162
<i>Cyclospora</i> spp.	6 (3)	2 (1)	0 (0)	2.7 (4)	0.324
<i>I. bütschlii</i>	0 (0)	8 (4)	0 (0)	2.7 (4)	0.034
<i>B. hominis</i>	0 (0)	2 (1)	0 (0)	0.7 (1)	1.000

* = Pearson's chi-square test (χ^2) followed by the Student-Newman-Keuls test.

were only detected in the sub-standard asparagus (Table 2). No difference was found in the prevalence of *Cyclospora* spp. between the asparagus from the open-air markets and the sub-standard product ($P = 0.324$), and this parasite was not detected in the export asparagus. The study identified *C. parvum* in 10 of 43 cases of *Cryptosporidium*. It is important to note that no other species of *Cryptosporidium* were identified. *B. hominis* (1%) was the least common intestinal parasite found. Finally, no helminths were detected.

DISCUSSION

Cryptosporidium spp. (29%) was the intestinal parasite most often isolated from the 150 samples of asparagus in our study. In contrast, a study in Norway detected *Cryptosporidium* spp. at a much lower prevalence (4%) in 475 vegetables sampled (mostly lettuce and mung bean sprouts) using the USEPA Method 1623 (Robertson & Gjerde, 2001). The other pathogen, *G. intestinalis*, showed a general prevalence of 5%. In Norway and Iran, *Giardia intestinalis* was found at similar prevalences to those in our study (2% and 1.8%, respectively) from 475 and 218 vegetables sampled (Robertson & Gjerde, 2001; Shahnazi & Jafari-Sabet, 2010). Shahnazi and Jafari-Sabet used the indirect fluorescent antibody. However, Mohamed *et al.* (2016) found a higher prevalence of *Giardia* (28.6%) isolated from 260 samples of fresh vegetables in Sudan using a sedimentation technique with formol saline and iodine. They suggested that the water used to keep the vegetables fresh might be contaminated. Returning to our study, the bundles of asparagus from the open-air markets had a higher prevalence of intestinal parasites (68%) than those of the sub-standard and export asparagus. Similarly, Bekele *et al.* (2017) found that over half (54.4%) of 360 samples of fresh fruits and vegetables sampled from open-air markets in Ethiopia had intestinal parasites. However, Mohamed *et al.* (2016) found a lower prevalence (13.5%) in their study of intestinal

parasites in 260 samples of fresh vegetables (mostly lettuce) from two open-air markets in Sudan. Similarly, the prevalences of certain species, such as *Cryptosporidium* spp. (50%) (25/50) and *E. nana* (10/50), were higher in the asparagus from open-air markets in our study. Differences in hygiene practices, the types of vegetables sampled, the prevailing environmental conditions in open-air markets and the parasitological techniques used, likely contribute to the differences in the results observed between the study in Sudan and our work. These factors may also explain why *G. intestinalis* and *Cyclospora* were not detected in the export asparagus, or why *I. bütschlii* and *B. hominis* were not detected in the asparagus from the open-air markets. Another relevant finding was the low presence of intestinal parasites in the export asparagus. It is important to mention, as well, that most of the asparagus from Caborca is exported to the US, so it is subject to strict handling regulations by the Animal and Plant Health Inspection Service (APHIS) and the U.S. Department of Agriculture (USDA). However, *Cryptosporidium* spp. was still detected in the export asparagus (4%) even after chlorine treatment. It is well-known that *Cryptosporidium* spp. oocysts are resistant to chlorination (up to 200 ppm) (Duhain *et al.*, 2012). In this study, *C. parvum* was identified in 10 of 43 cases of *Cryptosporidium* contamination, and is often involved in human cryptosporidiosis. Other species, such as *C. hominis*, have also been associated with human infections (CDC, 2018). Significantly, other species of *Cryptosporidium* were not identified, although two studies (Lugo-Sepúlveda & Paredes-González, 2011; Quihui-Cota *et al.*, 2017) carried out with children (120 and 173, respectively) from the same region detected similar intestinal parasite species (namely, *G. intestinalis*, *Endolimax nana*, *Entamoeba coli*, *Iodamoeba bütschlii* and *Cryptosporidium* spp.) to those observed in this study, also in asparagus. Lugo-Sepúlveda & Paredes-González (2011) used Ritchie and Faust flotation techniques, while Quihui-Cota *et al.* (2017) utilized similar parasitological diagnostic techniques to those employed

herein. It is probable that contaminated food is a risk factor for the transmission of gastrointestinal infections in the childhood population of that region. *B. hominis* (1%) was the intestinal parasite least often isolated, but this was likely underestimated in our work. The use of inappropriate techniques for *B. hominis* detection may explain this finding (Dogruman-Al *et al.*, 2010). Finally, no helminths were found in this study, but it is probable that the extreme semi-warm dry climate of the region around Caborca limits the transmission of this kind of parasites (Sia Su *et al.*, 2012). Lugo-Sepúlveda & Paredes-González (2011) detected helminths (*H. nana* and *A. lumbricoides*) in 120 children in their study in Caborca; however, those children lived in rural agricultural communities around the Caborca region that annually receive migrant families from southern Mexico where environmental conditions foster the proliferation of intestinal helminths.

CONCLUSION

This is the first study to reveal a high contamination of intestinal parasites in asparagus sold in different markets in a region of northwest Mexico. Results show that asparagus is a risk factor for the transmission of gastrointestinal diseases associated mainly with *Cryptosporidium* spp., *G. intestinalis* and *Cyclospora* spp., in the population that commonly handles and consumes asparagus, especially from open-air markets and sub-standard asparagus grown in the area. Additional studies of this kind are required to call the attention of health authorities and ensure the application and monitoring of good hygiene practices, including treatment with safe water in the handling of fruits and vegetables in different markets in northwest Mexico.

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Statement of conflict of interest

All authors declare that there is no financial, academic or commercial conflict of interest in this study.

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