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Quantitative Assessment of Contamination of Fresh Food Produce of Various Retail Types by Human-Virulent Microsporidian Spores[▽]

Szymon Jedrzejewski,[1] Thaddeus K. Graczyk,[2,3,4]* Anna Slodkowicz-Kowalska,[1] Leena Tamang,[2] and Anna C. Majewska[1]

Department of Biology and Medical Parasitology, Poznan University of Medical Sciences, Poznan, Poland[1]; Department of Environmental Health Sciences, Division of Environmental Health Engineering, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205[2]; Johns Hopkins University Center for Water and Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205[3]; and Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205[4]

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This study demonstrated that fresh food produce, such as berries, sprouts, and green-leafed vegetables, sold at the retail level can contain potentially viable microsporidian spores of human-virulent species, such as *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Encephalitozoon cuniculi*, at quantities representing a threat of food-borne infection.

Fresh produce is considered an essential part of a healthy diet; however, a growing trend of food-borne outbreaks associated with consumption of berries, sprouts, and vegetables indicates that consuming fresh food products is not always risk free (17, 22). Microsporidia are obligate eukaryotes parasitizing a wide range of hosts, with *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi* being the most common microsporidian opportunistic pathogens in humans (5, 6, 28, 29). Microsporidia are on the contaminant candidate list of the U.S. Environmental Protection Agency (EPA) (27) due to unknown transmission routes (5), technologically challenging identification and inactivation of waterborne spores (11), and difficult treatment of human infections (6). Microsporidian spores have been reported to be found in samples from groundwater and surface water (3, 4, 7–10, 13, 24), including water used for irrigation of fresh-produce operations (26). Although berries, sprouts, and vegetables have been contaminated with a variety of human pathogens (12, 17, 19, 21, 22, 25), reports on contamination with microsporidian spores are scant (1). Because *E. intestinalis* has been identified in irrigation water used for ready-to-eat crop production (26) and since unspecified spore species have been found in samples from strawberries, lettuce, and parsley (1), we initiated testing of commercial fresh produce at the retail level to quantitatively assess contamination with human-virulent microsporidian spores.

The fresh food products were purchased in the Wielkopolska region of western Poland. A total of 80 products, i.e., berries ($n = 25$), sprouts ($n = 20$), and vegetables ($n = 35$), were tested. These included 15 containers of strawberries and 10 of raspberries, 20 containers of sprouts (5 each of mung beans, alfalfa, radishes, and mixed), 15 heads of lettuce (5 each of iceberg, curly, and arugula), and five bunches of each of the following: red radishes, leeks, parsley leaves, and dill. Each food item was purchased at the smallest retail size available and originated from commercial groceries, supermarkets, street vendors, and food stall markets (Table 1). Most of the food items were produced locally. Each food item was eluted by vigorous agitation for 30 min in 1 liter of sterile phosphate-buffered saline (pH 7.4), to which 50 ml of 0.01% Tween 80 was added. The eluent was filtered through gauze and centrifuged at 4°C (2,000 × *g*; 30 min), the supernatant discarded, and the pellet washed of Tween 80 by centrifugation (2,000 × *g*; 30 min) with sterile phosphate-buffered saline. The resulting pellet was examined using Chromotrope-2R, calcofluor white M2R (28), and fluorescence in situ hybridization (FISH), as described previously (23), except that all oligonucleotide probes were labeled with the same fluorochrome, i.e., monofluorochrome FISH (Table 1). All spore-positive samples were retested by use of a multiplexed FISH assay (16) (Table 1).

Human-virulent microsporidian spores were detected in 9 of 80 (11.3%) food items, i.e., in 6 of 25 (24.0%) berry units, 1 of 20 (5.0%) sprouts, and 2 of 35 (5.7%) vegetable units (Table 1). All retail types had at least one (and at maximum four) spore-contaminated product(s) (Table 1). *E. intestinalis* was identified in four units of berries (i.e., three units of strawberries and one of raspberries); *E. bieneusi* in two units of raspberries, one unit of mung bean sprouts, and one unit of curly lettuce; and *E. cuniculi* in parsley leaves (Table 1). *E. bieneusi* spores were recovered from multiple food items (i.e., raspberries, sprouts, and lettuce), whereas all strawberry units were positive for a single microsporidian species, i.e., *E. intestinalis* (Table 1). As spores can originate from a variety of nonhuman vertebrate as well as invertebrate hosts (5, 6, 28, 29), food items positive by conventional staining but negative by FISH (Table 1) most likely contained spores of other microsporidian species. On average, 8.5×10^2 of human-virulent microsporidian spores were recovered from fresh food produce (Table 1). The number of spores in berries varied from 5.6×10^2 to 1.6×10^3 (mean, 9.2×10^2) and from 1.2×10^2 to 1.9×10^2 (mean,

* Corresponding author. Mailing address: Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205. Phone: (410) 614-4984. Fax: (410) 955-0105. E-mail: tgraczyk@jhsph.edu.

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TABLE 1. Results of testing of retail fresh food produce for contamination with potentially viable human-virulent microsporidian spores *E. bieneusi*, *E. intestinalis*, and *E. cuniculi*

Food type	Food origin	Result[a] of testing by:				No. of spores
		Conventional staining		FISH		
		Chromotrope-2R	Calcofluor white	Monofluorochrome	Multiplexed	
Berries						
Strawberries	Grocery	+	+	*E. intestinalis*	*E. intestinalis*	5.6×10^2
	Grocery	+	+	*E. intestinalis*	*E. intestinalis*	ND[b]
	Street vendor	+	+	*E. intestinalis*	*E. intestinalis*	1.6×10^3
Raspberries	Grocery	+	+	*E. intestinalis*	*E. intestinalis*	7.8×10^2
	Market stall	+	+	−	*E. bieneusi*	5.7×10^2
	Market stall	+	+	−	*E. bieneusi*	1.1×10^3
Sprouts						
Mung bean	Supermarket	+	+	−	*E. bieneusi*	1.9×10^3
Alfalfa	Supermarket	+	+	−	−	ND
Mixed	Supermarket	+	+	−	−	ND
Vegetables						
Parsley leaves	Grocery	+	+	*E. cuniculi*	*E. cuniculi*	1.2×10^2
Curly lettuce	Market stall	+	+	−	*E. bieneusi*	1.9×10^2
Iceberg lettuce	Market stall	+	−	−	−	ND
Red radish	Market stall	+	−	−	−	ND
Leek	Market stall	+	+	−	−	ND
Dill	Market stall	+	−	−	−	ND

[a] +, positive for spores; −, negative for spores.
[b] ND, not done.

1.6×10^2) in vegetables, and the highest number (i.e., 1.9×10^3 spores) was identified in mung bean sprouts (Table 1). The number of *E. intestinalis* spores varied from 5.6×10^2 to 1.6×10^3 (mean, 9.8×10^2); the number of *E. bieneusi* spores from 1.9×10^2 to 1.1×10^3 (mean, 9.4×10^2); and the number of *E. cuniculi* spores from 1.2×10^2 (Table 1). The percentage of microsporidian spores not showing a FISH reaction by use of a multiplexed approach was very low, not exceeding 2% of FISH-positive spores.

The present study demonstrates that (i) fresh produce can contain potentially viable spores of *E. bieneusi*, *E. intestinalis*, and *E. cuniculi* at the level representing an infection threat (5, 28); (ii) berries can be contaminated with human-virulent microsporidian spores at a significantly higher proportion than sprouts and vegetables (chi-square test; $\chi^2 = 7.68$, $P < 0.03$); and (iii) various fresh-produce retail types can distribute spore-contaminated products. Because microsporidia are emerging human pathogens (29), the 50% infective dose is still unknown; however, animal data indicate that the minimal infective dose is very low (5, 28). Thus, the average of 8.5×10^2 potentially viable microsporidian spores of human-virulent species represents a significant contamination level of products consumed in an uncooked form.

Contamination of fresh produce with microsporidian spores can occur during production, harvesting, packaging, distribution, or sale, and the globalization of the food trade facilitates the spread of contaminated products (1, 17, 22). Inactivation of potential biological contaminants without altering the freshness of the product is impractical, and therefore water of a high microbiological quality is essential for the production of safe fresh food (22). Unfortunately, considerable evidence indicates the involvement of contaminated water in the production of berries, sprouts, and green-leafed vegetables (2, 12, 19, 26). Microsporidian spores resist standard wastewater treatment and can be found in sewage sludge end products commonly used for fertilization of ready-to-eat crops or in runoff-impacted surface water used for irrigation (15).

Fresh food products represent a difficult matrix for laboratory testing and standardization because of their complex surface and porosity, which unfortunately facilitate pathogen attachment and survival (17, 20). As demonstrated in the present study, simultaneous quantitative species-specific identification of potentially viable human-virulent microsporidian spores can be accomplished by use of a multiplexed FISH assay. This offers great benefits for the fresh-produce industry in comparison with conventional stain testing, which lacks specificity (Table 1), or with standard PCR, which does not provide a quantitative assessment (14, 18).

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