Effect of water suspended particles on the recovery of *Cryptosporidium parvum* from tomato surfaces

Cristobal Chaidez, Marcela Soto and Nohelia Castro-del Campo

ABSTRACT

An increase in the number of outbreaks of foodborne disease associated with fresh produce consumption has been described. The objective of the present study was to evaluate the effect of water suspended particles during immersing/spraying disinfection processes and the recovery of *Cryptosporidium parvum* oocysts from tomato surfaces. Tomatoes (*Lycopersicum esculetum* Mill.) were immersed/sprayed with chlorinated water with low and high suspended particle content (10 and 1,000 mg/l) containing 100, 1,000 or 10,000 oocysts/l. Tomatoes were evaluated after a contact time of 120 seconds and 30 seconds for immersing and spraying procedures, respectively. The immersing procedure showed a high recovery of *C. parvum* oocysts from the tomato surface when the concentration was 10,000 oocysts/l and 10 mg/l suspended particles (295 ± 94 [mean ± standard deviation]). High particle content affected oocyst recovery and dissolved particles exerted a chlorine demand reducing the disinfectant residual. In the spraying procedure, the highest recovery was observed with 10,000 oocysts/l (225 ± 72). Our understanding is that the association of *C. parvum* oocysts with suspended particles might promote the oocyst deposition in the wash water tanks and that this interaction should be considered when evaluating the quality of the water.

Key words | *Cryptosporidium*, disinfection, recovery, suspended particles, tomato

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INTRODUCTION

In recent years, there has been an increase in the consumption of fresh tomato. Various types of tomato consumed overseas originate from Mexico (CAADES 2004). Geographical and climactic conditions, intensified international trade, improved storage and faster transportation account for this trend. Such products are often eaten raw or with minimal processing. If contaminated with microbial pathogens, they may therefore, pose a health hazard to consumers. An increase in the number of outbreaks of foodborne disease associated with fresh produce consumption has been described (Beuchat 1996); however, the number of reported outbreaks resulting from surface water contaminated crops is relatively low. Protozoan parasites which have been associated with foodborne outbreaks includes, *Giardia intestinalis*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and the helminthes parasites *Fasciola hepatica* and *Ascaris lumbricoides/Ascaris suum* (Orlandi *et al.* 2002).

Tomatoes have not been yet linked to any protozoan-associated foodborne outbreaks (Orlandi *et al.* 2002). However, protozoan parasites have been found on surface water irrigated crops (Bodley-Tickell *et al.* 2002; Thurston-Enriquez *et al.* 2002). Some of the most ubiquitous and resistant human parasites transmitted by water belong to the genera *Cryptosporidium*. Large numbers of *C. parvum* oocysts can enter agricultural water through runoff from areas with dense animal populations, including wildlife, as well as from human populations via wastewater discharge (Slifko *et al.* 2000; Xiao *et al.* 2000). The natural hosts for these parasites have not been identified; however, contamination of produce may take place at all stages during production, both pre- and post-harvest as well as during

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processing. Possible contamination sources are soil, fecal matter (of both human and animal origin), water (irrigation and washing), and product harvesting and handling (FDA 1998). But surface water appears to be an important vehicle for the transmission of these parasites (Medema *et al.* 1998).

Tomatoes harvested from the field are taken to the packinghouse for post harvest processing, which includes a washing procedure in wash water tanks. Water may be contaminated by *Cryptosporidium* oocysts from the source surface water contaminated areas. The water-suspended oocysts might be driven freely in the water flow or attached to suspended particles. In wash water tanks, particle sedimentation velocity might be increased due to water stagnation and high particle concentration. Disturbance of sediments by, for instance, dumping tomatoes coming from the field may give rise to high concentration peaks in the water, yielding a relatively high risk of *Cryptosporidium*-tomato surface attachment.

Isolation and quantification of parasites from fresh produce is considered an important component in the food safety of fruits and vegetables eaten raw. Various methods have been developed to detect *Cryptosporidium* with low and variable recovery efficiency. Robertson & Gjerde (2001) suggested that seed cellular wall components, microflora, bacteria-producing biofilm, among other materials might interfere with the recovery techniques (Flotation and IMS) affecting the elution and isolation procedures. Oocysts might become entrapped in the particulate matter washed from the fresh produce surface. These methods were first developed to detect protozoan parasites from source waters, but recently researchers have adopted them for the recovery of protozoan parasites from fresh produce surfaces (Robertson & Gjerde 2000, 2001).

In view of uncertainties concerning the impact of the presence of *C. parvum* oocysts on the tomato surface, the objective of the present study is to evaluate the effect of water suspended particles during immersing/spraying disinfection processes and the recovery of *Cryptosporidium parvum* oocysts from the tomato surface.

MATERIALS AND METHODS

Tomatoes

Fresh, ripe tomatoes were obtained at a local packinghouse. They were washed with sterile tap water and stored at 5 ± 2°C until approximately 1 h before the inoculation procedure.

Test strain

Cryptosporidium parvum oocysts were acquired from Waterborne Inc. Purified oocysts were supplemented with 1,000 U of penicillin and streptomycin per ml at 4°C.

Inoculum preparation

Hemocytometer counts of *C. parvum* oocysts were made, and serial dilutions were conducted to obtain working suspensions containing 100, 1,000, and 10,000 oocysts/l. The concentration was determined by placing 10 μl aliquots on glass well microscope slides and staining the preparations with monoclonal antibodies (A200 FL-1X Aqua Glo G/C indirect, Waterborne Inc., New Orleans, LA). The slides were examined by using an epifluorescence microscope as described in USEPA Method 1622.

Particle soil preparation

The soil characteristics are listed in Table 1. Natural soil particles were collected from Culiacan Valley, which is located at 30 km south of the City of Culiacan, Mexico. To remove large particles, the soil was wet sieved through a standard testing sieve (425 μm). The soil that passed through the mesh was collected and concentrated by sedimentation. The resulting suspension was stored in a sealed container at 4°C.

Water conditions

The physical and chemical conditions of the water utilized in this study are listed in Table 2. Purified water was obtained from a local distributor to conduct the study. The water was inoculated with *C. parvum* oocysts at concentrations of 100, 1,000, 10,000 oocysts/l and spiked with sterile soil to generate a high suspended particle condition (100 NTU, nephelometric units). It should be noted that a high suspended particle condition was taken from the immersing water tanks evaluation performed at local packinghouses (Chaidez *et al.* 2003). Chlorine concentrations commonly used in packinghouse operations were also employed (100 and 300 mg/l of

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Table 1 | Soil employed to simulate high suspended particles on test water

Parameters	Result
pH (25°C)	7.38
Conductivity (ds/m)	0.39
Organic matter (%)	2.07
Nitrogenous N-NO_3 (ppm)	4.0
Phosphorus P-PO_4 (ppm)	38.5
Sodium (ppm)	117.038
Potassium (ppm)	403.534
Calcium (ppm)	5194
Magnesium (ppm)	1936.29
Iron (ppm)	49.03
Manganese (ppm)	177.66
Zinc (ppm)	3.39
Copper (ppm)	2.27
ClC (me/100 g)	43.65
Texture	Clay
Clay(%)	46.48
Limo (%)	27.28
Sand (%)	26.24
Particle Size[a]	425 μm

[a]Standard testing sieve, No. 40.

chlorine as NaOCl). Concentrations of chlorine and water turbidity were determined with the spectrophotometer 2010 and the turbidmeter 2100P (Hach Co., Ames, IA, USA) as described in *Standard Methods for the Examination of Water & Wastewater* (1998), respectively.

Immersing procedure simulation

Tomatoes were placed in a 4-liter beaker containing separately working suspensions of parasites (100, 1,000, 10,000 oocysts/l), chlorine concentrations (100 and 300 mg/l) and suspended particles concentrations (10 and 1,000 mg/l). The immersed tomatoes were agitated for 120 seconds to evenly distribute the oocysts over the tomato surface. The tomatoes were removed from the 4-liter beaker with tweezers, placed on a paper towel in a laminar flow hood to dry for 2 minutes, and then carefully placed in a sterile Ziploc bag and stored at 4°C until used. Control tomatoes were exposed only to phosphate buffer solution.

Table 2 | Chemical and physical parameters of the inoculated water

	Procedure			
Parameters	Immersing		Spraying	
pH	7.0	7.0	7.0	7.0
Chlorine (mg/l)[a]	100	300	100	300
Temperature (°C)	25	25	25	25

[a]Total chlorine as sodium hypochlorite.

Spraying procedure simulation

One-liter manual sprayers containing separately working suspensions of parasites (100, 1,000, 10,000 oocysts/l) and chlorine concentrations (100 and 300 mg/l) were employed for the spraying procedure. Tomatoes were sprayed with the inoculated water at a distance of 15 cm during 30 seconds, immediately after the contact time, tomatoes were stored and processed as previously described. Control tomatoes were exposed only to phosphate buffer solution (PBS).

Sample processing

Sample processing was divided into four distinct stages: (i) elution of the parasites from the tomato surface, (ii) concentration of the suspension, (iii) separation by flotation, and (iv) screening by microscopy. Details of these procedures are outlined below.

Elution

For each experiment, three treated tomatoes were eluted in a homogenizer bag containing 200 ml of detergent solution

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(1X PBS; 1% Sodium dodecyl sulfate; 0.1% tween 80), 150 ml of distilled water and 50 ml of membrane-filter elution buffer. The bags containing the protozoan inoculated tomatoes were processed in a shaker bath and agitated intermittently for 15 minutes. The homogenizer bag content was concentrated by centrifugation at 1,050 × *g* for 10 minutes in a centrifuge (Centra CL3X). The supernatant was aspirated, and the filter sediment was resuspended in elution solution. All samples were then stored at 4°C until flotation purification.

Concentration

The concentrates were further purified by re-suspending the pellet in 30 ml Percoll-sucrose solution (1.1 gravity) to make a final volume of 50 ml with eluting solution. The pelleted water concentrate was centrifuged at 1,050 × *g* for 10 minutes. A 20 ml-volume was aspirated with the interface including 5 ml below the interface. Eluting solution was added to the conical tube to bring the final volume to 50 ml and the sample was centrifuged again at 1,050 × *g* for 10 min. The supernatant was aspirated and discarded leaving a final volume of 5 ml. The sample was resuspended by vortexing.

Separation

The sample concentrates were pipetted directly onto prewetted 25-mm cellulose acetate filters (0.22 μm pore size; Millipore) to ensure the even distribution of the sample. A 25 mm filter (grade 1) cellulose acetate filter was used in each sample. Each filter was rinsed with 2 ml of 1% bovine serum albumin. Volumes of 0.5 ml of monoclonal antibodies (A200 FL-1X Aqua Glo G/C Indirect, Waterborne, New Orleans, LA) of *Cryptosporidium* and *Giardia* were diluted according to the manufacturer's instructions using 1X phosphate buffer solution; then incubated for 25 min/37°C and rinsed five times with 2 ml of 1X PBS. 0.5 ml of monoclonal antibodies were added to the filters and allowed to react for 25 minutes at 37°C. The slides were then examined by epifluorescence microscope as described in Method 1622.

Epifluorescence microscopy

The samples were screened by fluorescence microscopy (Leica DME) at X200 and X400 to enumerate the monoclonal antibody labeled oocysts.

Statistics

An experiment with repeated measures with four and two replicates for immersing and spraying application was conducted, respectively. The results were presented as a mean recovery and the analysis of variance (ANOVA) was performed in separate for immersing and spraying applications. Multiple comparisons among means were performed using Tukey test with $P < 0.05$ of significant differences. Data were subjected to the MINITAB system for analysis of variance (Version 14).

RESULTS

Immersing procedure

Results shown in Table 3 correspond to the mean of *C. parvum* oocysts recovered from tomato surfaces after treatment with low and high suspended particles content (10 and 1,000 mg/l) and chlorinated water (100 and 300 mg/l). Differences in suspended particles concentration affected the numbers of oocysts recovered from tomato surfaces ($P < 0.05$), while chlorine concentrations did not show any significant effect ($P < 0.05$). The mean recovery of oocysts from tomato surfaces in water with low suspended particles content resulted on average between 295 and 27 of spiked oocysts, while the high suspended particle content recovered on average between 114 and 40 (Table 3). Mean *Cryptosporidium* recoveries with low suspended particle

Table 3 | Recovery of *Cryptosporidium parvum* oocysts from tomato surface after immersing procedure

Particle concentration mg/l	Oocyst concentration[a]	Mean recovery[b] ± SD	
10	A	27	18
	B	141	91
	C	295	94
1000	B	40	47
	C	114	153

[a]Number of oocysts inoculated to a 4-liter beaker (**A**, 100; **B**1,000; **C**10,000 oocysts/l).
[b]Number of experiments ($n = 4$).

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content and initial concentrations of 100 oocysts/l were 27 ± 18 (mean ± standard deviation), with 1,000 oocysts/l 141 ± 91 and with 10,000 oocysts/l 295 ± 94 (Table 3). Mean *Cryptosporidium* recoveries with high suspended particle content and initial concentration of 100 oocysts/l were not detected, with 1,000 oocysts/l 40 ± 47 and with 10,000 oocysts/l 114 ± 153 (Table 3).

Spraying application

Results for the spraying application are presented in Table 4. Differences in tomato surfaces recovery of *C. parvum* oocysts were dependant on the inoculums size ($P < 0.05$) while the chlorine concentrations did not show any significant effect ($P < 0.05$). The mean recovery of protozoan oocysts from tomato surfaces were on average between 225 and 68 of spiked oocysts. Mean *Cryptosporidium* recoveries with initial concentrations of 100 oocysts/l were not detected, with 1,000 oocysts/l were 68 ± 135 and with 10,000 oocysts/l 225 ± 72 (Table 4).

DISCUSSION

The oocysts recovery efficiency was the key parameter used in this study to assess the effect of water suspended particles during immersing/spraying tomato disinfection processes. The present study demonstrates that oocysts of *C. parvum* are able to attach to tomato surfaces under immersing and spraying water conditions and that suspended particle concentrations may play an important role in the surface attachment. Each protozoan inoculum was challenged individually with chlorinated water and suspended particle levels at different contact time periods. The chlorine concentrations at the contact times tested were found inefficient to avoid the presence of *C. parvum* oocysts on the tomato surfaces when the concentrations of oocysts were high. To the contrary, the amounts of particulate matter surrounding the target microorganisms are likely to reduce the protozoan oocysts attachment to tomato surfaces and might reduce the efficacy of chlorine. Presumably, oocysts were entrapped in soil particles preventing tomato surface attachment and contributing to oocyst deposition. Studies have stated that *C. parvum* oocysts attach to suspended particles changing their settling velocity in a water container (Medema *et al.* 1998). Feng *et al.* (2003) mentioned that *C. parvum* oocysts attach to a particle at concentrations of 105 to 216 mg/l. In the present study the particle concentration necessary to obtain 2 and 100 NTU of water turbidity was 10 and 1,000 mg/l of soil, respectively.

In the immersing procedure, the highest mean level of recovery from the tomato surface was obtained at 10 mg/l of suspended particles, while at 1,000 mg/l, the recovery of oocysts was affected by the particle concentration. When the particle concentration increased further from 10 mg/l to 1,000 mg/l, the mean level of oocysts recovery decreased significantly from 295 ± 94 to 114 ± 153 when the initial oocysts concentration was 10,000 oocysts/l (Table 3). In the spraying procedure, the presence of *C. parvum* oocysts on tomato surfaces increases as the concentration of the inoculums increases (Table 4).

We recognize that the levels of protozoan oocysts used in this study are far greater than those which may be found in immersing/spraying water, however, high numbers of protozoan oocysts were used so that they could be readily detected by the assay used in the present study. As pointed out even low numbers of *C. parvum* oocysts could pose a significant human health risk, since the infective dose, of the environmental resistant oocyst, is very low (DuPont *et al.* 1995; Haas 2000). Kniel *et al.* (2002) showed that oocysts of *Toxoplasma gondii* remained viable and infectious for up to 8 weeks on raspberry and blueberry surfaces.

As described before, the frequency of produce contamination by *C. parvum* is thought to be very small; however, there are no known mitigation strategies which will completely remove the pathogen after contamination has occurred.

The USEPA have proposed two methodologies for the recovery of C. parvum oocysts, the percoll-sucrose method

Table 4 | Recovery of *Cryptosporidium parvum* oocysts from tomato surface after spraying procedure

Particle concentration mg/l	Oocyst concentration[a]	Mean recovery[b] ± SD	
10	B	68	135
	C	225	72

[a]Number of oocysts inoculated to a 4-liter beaker (**A**, 100; **B**1,000; **C**10,000 oocysts/l).
[b]Number of experiments ($n = 2$).

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(ICR protocol) and the immune magnetic separation method (1622 protocol) replaced it (USEPA 1996; USEPA 1999). Although, the Immune Magnetic Separation (IMS) method is a widely used assay, this method still has drawbacks, such as low levels of oocyst recovery and variable results (Rochelle *et al.* 1999). And as stated by Quintero-Betancourt and Botero de Ledesma (2000) both the ICR and the 1622 protocols show no significant differences when evaluated. Robertson & Gjerde (2001) reported that the differences in sample weight, rather than elution techniques or IMS kit, are responsible for the superior results provided by Wilkinson *et al.* (2000). The efficiency of the method employed to conduct the present study showed an overall recovery efficiency of 22.85%.

The presence of suspended particles on the water matrices and the inherent limitations of the percoll-sucrose flotation method might account for the inconsistencies in method reproducibility (low and variable oocyst recoveries) observed in the present study. The recovery efficiencies of oocysts reported in the present study may be highly variable; however, the results still provide information on the oocyst-suspended particle association and/or its tomato surface attachment. Additional modifications for improving method performance such as Envirocheck capsule filter and IMS will undoubtedly increase the oocyst recoveries from either highly turbid wash water or contaminated produce surface.

In conclusion, the results demonstrate that protozoan oocysts of *C. parvum* may attach to tomato surfaces under immersing and spraying chlorinated water applications and that suspended particles present in water could help to reduce the levels of oocysts on the tomato surface. The results also indicate that oocyst-particle associations may favor oocyst sedimentation, and this is expected to reduce the oocysts on the tomato surfaces in the immersing water tanks. However, deposition is also expected to result in the accumulation of oocysts in the sediments of wash water tanks, which can later serve as a source of pathogens since the environmentally resistant oocysts are difficult to eliminate by disinfection methods. Thus, the primary method of avoiding the presence of protozoan oocysts in agricultural water should be the strict adherence to Good Agricultural Practices which means that the best method of reducing the microbial presence on fresh produce surfaces is undoubtedly prevention.

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