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Efficacy of Gaseous Chlorine Dioxide as a Sanitizer against *Cryptosporidium parvum, Cyclospora cayetanensis,* and *Encephalitozoon intestinalis* on Produce

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ABSTRACT

The efficacy of gaseous chlorine dioxide to reduce parasite and bacterial burden in produce was studied. Basil and lettuce leaves were inoculated with *Cryptosporidium parvum* and *Cyclospora cayetanensis* oocysts, *Encephalitozoon intestinalis* spores, and a cocktail of two isolates of nalidixic acid–resistant *Escherichia coli* O157:H7. The inoculated samples were then treated for 20 min with gaseous chlorine dioxide at 4.1 mg/liter. *Cryptosporidium* had a 2.6 and 3.31 most-probable-number log reduction in basil and lettuce, respectively. Reduction of *Encephalitozoon* in basil and lettuce was 3.58 and 4.58 CFU/g respectively. *E. coli* loads were significantly reduced (2.45 to 3.97 log), whereas *Cyclospora* sporulation was not affected by this treatment.

Parasites have frequently been identified in fresh fruits or vegetables and have caused several foodborne outbreaks *(7, 11, 22)*. *Cryptosporidium parvum,* a parasite often linked to waterborne transmission, has also been reported to cause disease through the consumption of unpasteurized milk *(7, 13)* or apple cider (either nonpasteurized or ozone treated) *(1, 3, 20)*. Furthermore, in 1997, 54 people acquired cryptosporidiosis by eating unwashed contaminated green onions, and in 1998, 88 students acquired cryptosporidiosis in Washington, due to the ingestion of vegetables and fruits that were contaminated by an ill food handler *(21)*. Cases of cyclosporiasis, on the other hand, have been almost exclusively associated with the consumption of contaminated fresh produce, such as raspberries, lettuce, basil, mixed greens, and snow peas *(2, 5, 8, 14–19, 22)*.

Several species of microsporidia cause diarrheal illness in immunocompetent and immunocompromised individuals *(11)*. The most relevant species associated with gastrointestinal illness are *Enterocytozoon bieneusi,* which to date has not been cultured in vitro, and *Encephalitozoon intestinalis,* which can be easily grown in vitro. Although direct evidence of foodborne microsporidiosis is yet to be reported, spores have been identified in water, produce and environmental samples *(4, 6, 10, 25),* indicating potential transmission through the foodborne route.

In recent studies, chlorine dioxide (ClO_2) gas has been evaluated as a bactericidal and fungicidal on raw produce as an alternative to rinse sanitizers for fruits and vegetables eaten raw. This oxidative treatment is more advantageous than chlorinated water because it can break down phenolic compounds, does not react with ammonia, has high sporocidal activity, its activity is not markedly affected by pH, and has 2.5 times the oxidation capacity of chlorine. Additionally, it can be used in fruits and vegetables whose appearance is damaged by rinse sanitizers.

Effectiveness of ClO_2 gas as a sanitizer has varied with dosage, produce type, and pathogen being evaluated. When ClO_2 was applied to uninjured peppers at 3 mg/liter, *Listeria monocytogenes* was reduced by a magnitude of more than 6 log CFU/5 g, whereas 1.2 mg/liter, on the other hand, resulted in a 6.4-log reduction of *E. coli* O157:H7 *(12)*. High reduction of pathogens has similarly been observed on other smooth-surfaced produce. For example, reductions of 5.15, 5.62, and 5.88 log of *Salmonella, E. coli* O157:H7, and *L. monocytogenes,* respectively, were obtained on treated carrots *(24)*. Likewise, more than a 5-log reduction of *E. coli* O157:H7 occurred on the skin of apples when treated with ClO_2 at 7.2 mg/liter for 10 min or 3.3 mg/liter for 20 to 30 min *(9)*. Effectiveness of ClO_2 as a sanitizer on rough-textured produce items, on the other hand, has been much less. Treatment of cabbage led to a 4.42-, 3.13-, and 3.6-log reduction of *Salmonella, E. coli* O157:H7, and *L. monocytogenes,* respectively, whereas treatment of lettuce led to reductions of only 1.58, 1.57, and 1.53 log, respectively *(24)*. Furthermore, treatment of blueberries or strawberries with ClO_2 gas (4.1 mg/liter) reduced the *Salmonella* and yeast populations by 2.2 to 2.95 and 1.42 to 2.32 log/s, respectively.

To date, the microbicidal efficacy of ClO_2 gas towards parasites has not been investigated. Therefore, the objective of this study was to determine the efficacy of ClO_2 gas as a sanitizer against *Cryptosporidium, Cyclospora,* and *E. intestinalis* inoculated onto lettuce and basil. As a basis of comparison, the response of *E. coli* O157:H7 to the treatments was also conducted.

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MATERIALS AND METHODS

***C. parvum* oocysts.** *C. parvum* oocysts were obtained from the Parasitology Laboratory, University of Arizona, Tucson. The oocysts were stored at 4°C in 1 ml of antibiotic solution with 0.01% Tween 20, 100 U of penicillin, and 100 μg of gentamycin until ready to use.

Human adenocarcinoma (HCT-8) cells (ATCC CCL 244, American Type Culture Collection, Manassas, Va.) were maintained in 25-cm^2 tissue culture flasks (Corning, Inc., Corning, N.Y.). The HCT-8 cells were grown in RPMI 1640 medium (GIBCO/Invitrogen Corp., Grand Island, N.Y.) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Ga.), 2 mM L-glutamine (Sigma-Aldrich Corp., St. Louis, Mo.), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich), 0.1 mM nonessential amino acids (Sigma-Aldrich), 1.0 mM sodium pyruvate (Sigma-Aldrich), and antibiotic-antimycotic (100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate, and 0.25 μg/ml amphotericin B as Fungizone; GIBCO/Invitrogen). The cells were incubated at 35°C with 5% CO_2 and 100% humidity. For routine cell passage (every 2 or 3 days), 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, Ga.) was used as the maintenance medium, whereas 7% FBS was used as a growth medium whenever parasites were grown.

Forty-eight hours prior to parasite treatment, HCT-8 cells were seeded onto sterile tissue culture 8-well chamber slides (Lab-Tek, Nalge Nunc International, Naperville, Ill.) at a concentration of 7×10^4 to 10^5 for each well. Slides were incubated at 35°C in a 5% CO_2 atmosphere with 100% humidity.

Once confluency of cells was achieved, monolayers were incubated with either treated or nontreated oocysts at 4×10^6/50 μl. Forty-eight hours postinoculation, slides were fixed with 100% methanol for 10 min and then stained with HEMA 3 (Fisher Scientific Co., L.L.C., Kalamazoo, Mich.). Each well of the chamber slide was then examined at ×40 and ×100 magnification by bright field microscopy (Olympus CX31 microscope, Olympus America, Inc., Hauppauge, N.Y.) (Fig. 1B) and compared with noninfected cell monolayers.

E. intestinalis. *E. intestinalis* spores (ATCC 50651), were maintained in 75-cm^2 flasks (Corning) containing RK-13 (ATCC CCL-37) rabbit kidney cells. The RK-13 cells were grown in RPMI medium 1640 with 7% fetal FBS, 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (Sigma), and antibiotic/antimycotic solution (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B; GIBCO/Invitrogen). The cells were incubated at 35°C, with 5% CO_2 and 100% humidity. Spores were harvested by removing the culture medium from the cell monolayer and filtered through a 5-μm filter to remove host cell debris, and centrifuged at 1,000 × *g* for 10 min at room temperature in 50-ml polypropylene tubes (Falcon, Becton Dickinson, Franklin Lakes, N.J.) by using an Eppendorf model 5810 centrifuge. The pellet was washed twice with sterile phosphate-buffered saline (PBS) pH 7.2 (0.01 M $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl, 0.01 M $Na_2HPO_4 \cdot 7H_2O$) and resuspended in 1 ml of sterile PBS. An aliquot of the spore suspension was stained with Calcofluor White (REMEL, Lenexa, Kans.), observed with a UV microscope (Olympus BX60, Olympus America, Inc., Melville, N.Y.), and counted using a Neubauer hemacytometer (Leavy Double Counting Chamber, Hausser Scientific, Horsham, Pa.) at ×400 magnification. Spores were stored at 4°C and used within 2 to 3 days.

***C. cayetanensis* oocysts.** *C. cayetanensis* oocysts were obtained from feces of naturally infected individuals. Feces were sieved and stored in 2.5% potassium dichromate. A modified ethyl acetate method was used for initial concentration. Pellets were diluted in distilled water and layered over a primary discontinuous sucrose gradient and centrifuged at 1,500 × *g* for 20 min. Oocysts were stored in 2.5% potassium dichromate solution at 4°C.

Unsporulated *Cyclospora* oocysts were washed three times with distilled water and centrifuged at 1,500 × *g* for 5 min to remove all potassium dichromate. *Cyclospora* isolates from two individuals were used in this study. Oocyst test samples were taken from the stock solution, diluted with distilled water, and enumerated using a Neubauer hemocytometer to prepare inocula of 4×10^6/50 μl.

***E. coli* isolates.** Two isolates of *E. coli* O157:H7 were kindly provided by Dr. Larry Beuchat (Center for Food Safety, University of Georgia, Griffin). These isolates were 932 of human origin from the 1992 Oregon meat outbreak and E 0018 of bovine origin. Isolates were grown separately in MCSAN (Difco, Becton Dickinson, Sparks, Md.) at 37°C for 24 h with 50 μg/ml nalidixic acid (Sigma). Then they were grown separately in tryptic soy broth (Difco, Becton Dickinson) supplemented with 50 μg/ml nalidixic acid at 37°C. After 24 h, population of *E. coli* was determined by serially diluting in sterile 0.1% peptone and plating 0.1 ml on MCSAN. Plates were incubated at 37°C for 24 h, and 2×10^7 cells were inoculated onto vegetable surfaces.

Lettuce and basil tested. Green leaf lettuce and basil were purchased from a local market in Griffin, Ga., and stored at 4°C. Lettuce and basil leaves (2 g) consisting of pieces measuring 4 by 5 cm cut from leaves beneath the wrapper leaves were subjected to inoculation and analysis. Samples were placed separately on single layers in plastic weigh trays (14 by 14 by 2.5 cm) in preparation for inoculation.

Inoculation of produce. Lettuce and basil samples were spot inoculated with 50 μl of *C. parvum, Cyclospora,* and microsporidia (ca. 4×10^6 oocysts per spores) and 20 μl of *E. coli* (ca. 2×10^7 cells) suspension, in droplets distributed randomly on the sample. The vegetables were dried inside the hood for 1 h at 23 ± 2°C. Inoculated samples were placed in a transparent Plexiglas desiccator cabinet (45.7 by 30.5 cm; Fisher Scientific, Pittsburgh, Pa.) on the bottom three shelves of the four-shelf cabinet. A high relative humidity (62 to 81%) was achieved by placing hot water (initially at 98 to 99°C) in a container on the bottom shelf of the cabinet. A brushless 12 V DC fan (6.9 by 6.9 by 2.5 cm; RadioShack, Fort Worth, Tex.) was placed on each of the four shelves to circulate the air. A hygrometer/thermometer recorder (Fisher) was used to monitor humidity and temperature inside the treatment cabinet.

Treatment with gaseous chlorine dioxide (ClO_2 gas). Chlorine dioxide was obtained from ICA TriNova, Inc. (Marietta, Ga.). Before starting treatment, 42 g of dry chemical A (granular porous solid impregnated with sodium chlorite) and 42 g of dry chemical B (granular porous solid impregnated with acid and an acid precursor, ferric chloride) were weighed, mixed, and immediately placed inside a sachet provided by the manufacturer. This proprietary mixture generated chlorine dioxide gas. The sachet was placed in the treatment cabinet. A second cabinet without the sachet was used for treatment control samples. Temperature and relative humidity was determined after 5, 10, 15, and 20 min.

After 20 min, vegetables from both chambers were transferred to 50-ml tubes. Leaves were washed with 10 ml Dey-Engley neutralizing broth and 40 ml of sterile E-Pure water to re-

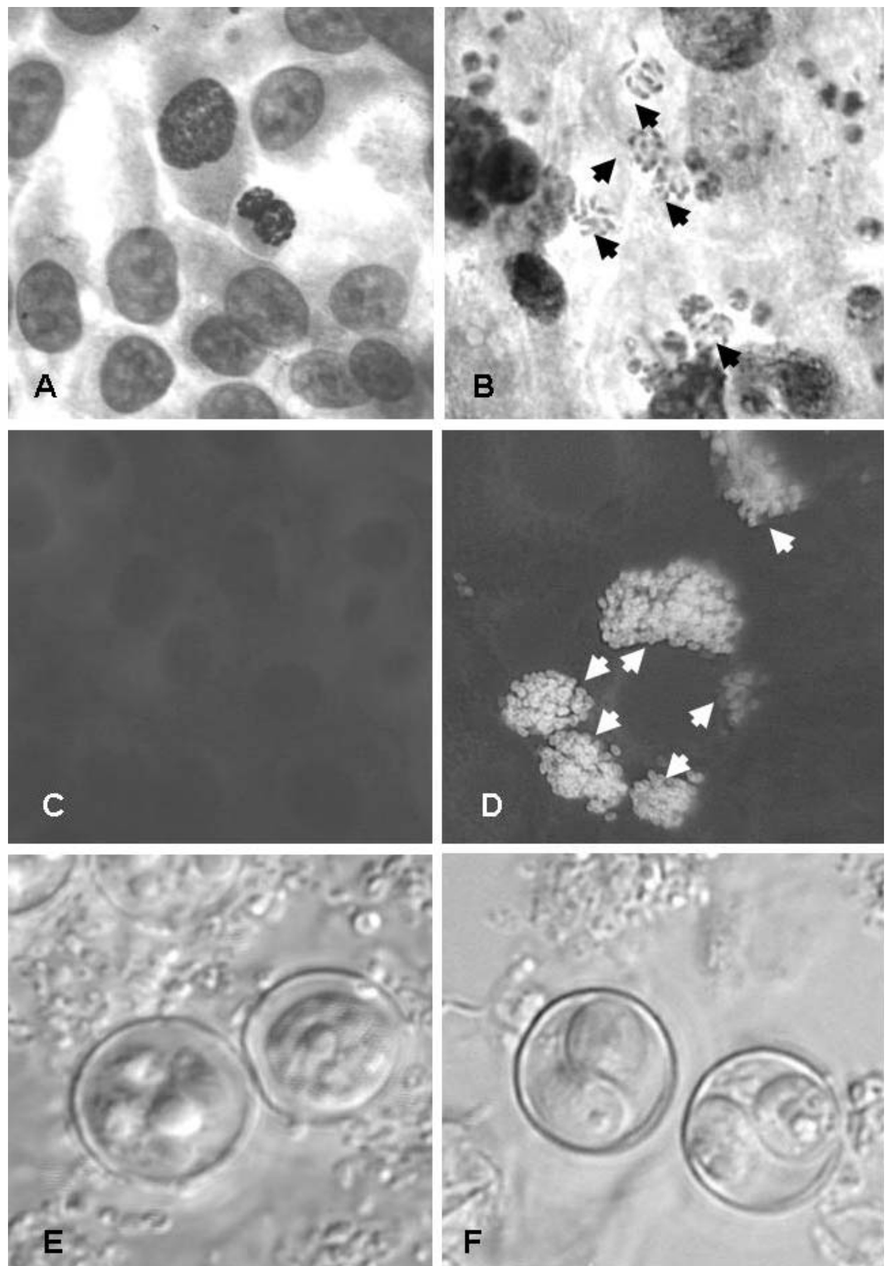

FIGURE 1. *In vitro cultivation of* C. parvum, *(A) noninfected HCT-8 cells and (B) infected cells, meront type I (arrows).* E. intestinalis, *(C) noninfected cells and (D) infected cells, parasitic vacuoles containing spores (arrows).* C. cayetanensis *oocysts, (E) nonsporulated oocysts and (F) sporulated oocysts.*

move oocysts, spores and bacteria from leaves. The wash was centrifuged for 10 min at 1,000 × *g* (Eppendorf Centrifuge 5810, Brinkmann Instruments, Inc., Westbury, NY). Resulting pellets were rinsed twice with sterile E-Pure water. After the third wash, the pellet was transferred into labeled microcentrifuge tubes, centrifuged at 5,724 × *g* for 4 min, using Hereaus Baxter Biofuge 13 centrifuge and a type 3743 rotor (Heraeus Instruments, Hanau, Germany). Approximately 100 μl of the sample was resuspended with 1 ml of RPMI containing 3.5% FBS and 2× antibiotics, vortexed, and centrifuged at 5,724 × *g* for 4 min. The supernatant was aspirated, and 100 μl was left for viability testing.

Viability testing. The treatment and recovery process for *Cryptosporidium, E. intestinalis,* and *C. cayetanensis* was similar and tested for pathogen viability. For *E. coli* O157:H7, washes were serially diluted in sterile 0.1% peptone and plated on MCSAN.

Cell culture for *Cryptosporidium* and *Encephalitozoon.* *C. parvum* oocysts pellets from each treatment were resuspended in 300 μl (final volume 400 μl) of growth medium that had been prewarmed to 37°C, mixed well, and aliquoted in triplicate (100 μl to each tube). Serial dilutions (10-fold) were prepared in prewarmed growth medium. One hundred microliters from each dilution was further diluted with 100 μl of taurocholic acid (Sigma) dilution 1/8 (1.5% [wt/vol], diluted with RPMI base medium) and immediately inoculated onto HCT-8 cell monolayers (sterile tissue culture 8 well chamber slides). The inoculated cells were incubated for 48 h at 35°C, with 5% CO_2 and 100% relative humidity. After incubation, the culture medium was removed, and the cells were fixed with 100% methanol for 20 min. The slides were air dried and stained with HEMA 3 and examined by bright field microscopy at a magnification of ×1,000 to determine whether *C. parvum* infected the HCT-8 cells. A well was scored as positive when an infection focus, representing secondary infections and numerous stages, was observed. No infection was observed in negative sample controls (Fig. 1A and 1B).

The RK-13 cell line was used to determine infectivity for microsporidia. These cells were grown on chamber slides in RPMI

TABLE 1. *Viability of* Cryptosporidium, *microsporidia, and* E. coli *O157:H7 when inoculated on the surface of lettuce or basil and treated with gaseous chlorine dioxide (4.1 mg/liter)*

	Population			
	E. intestinalis (log CFU/g)[a,b]		*C. parvum* (log MPN/ml)[c]	
Produce	Recovered (SD)	Reduction	Recovered (SD)	Reduction
Basil	3.58 (0.10) B	3.58 C	3.51 (3.78–3.20)	2.60
Lettuce	4.58 (0.10) A	3.62 C	3.62 (3.92–3.30)	3.31

[a] Mean values not followed by the same letter are significantly different at $P < 0.005$.
[b] Recovery (SD) of *E. coli* (log CFU/g) in lettuce leaves in each trial was 5.0 (1.58) and a log reduction of 3.97.
[c] Recovery (SD) of *E. coli* (log CFU/g) in lettuce leaves in each trial was 8.20 (0.70) and a log reduction of 2.45.

medium 1640 with 7% FBS. RK-13 cell monolayers containing the microsporidial spores (treated and controls) were fixed with 100% methanol (VWR, West Chester, Pa.) for 10 min and stained with Calcofluor white (Remel) at a 1:3 dilution with sterile PBS (pH 7.2) for 3 min at 20 ± 2°C. The slides were rinsed with slow-running water and counterstained with 0.005% Evans Blue (Sigma-Aldrich) in PBS for 25 s. The slides were rinsed under slow-running water, allowed to air dry, and viewed under a UV microscope. The organism appeared as a bright light blue oval structure with a purple/bluish background when compared to uninfected control cell monolayers (Fig. 1C and 1D).

Sporulation of *Cyclospora*. Unsporulated treated oocysts and controls were recovered and incubated in 2.5% potassium dichromate for 15 days at room temperature (20 ± 2°C). Sporulation percentage for each experiment was determined by counting 100 oocysts, using phase-contrast microscopy. Sporulated oocysts were identified as oocysts containing two fully differentiated sporocysts (Fig. 1E and 1F).

***E. coli*.** Seven decimal dilutions in 0.1% peptone were plated in sorbitol MacConkey agar (Difco, Becton Dickinson) supplemented with 50 μg/ml nalidixic acid (MCSAN). Plates were incubated at 37°C for 24 h. Presumptive colonies (five to six) were randomly selected for confirmation using the *E. coli* O157 Test Kit (Oxoid, Ltd., Basingstoke, England).

Statistic analysis. All experiments were repeated in triplicate, each with three repetitions. Mean values of *E. intestinalis* counts and *C. cayetanensis* sporulation were analyzed to determine significant differences ($\alpha = 0.05$) using the Student's *t* test. *Cryptosporidium* calculations were done using the MPN analysis with 95% confidence intervals, using the ICR Most Probable Number Calculator, version 1.00 (USEPA, Cincinnati, Ohio).

RESULTS AND DISCUSSION

Reductions of both *C. parvum* and *E. intestinalis* occurred after treatment of lettuce and basil with ClO_2 gas treatment (4.1 mg/liter) and were comparable to reductions of *E. coli* O157:H7 (Table 1).

Cyclospora sporulation was not affected when inoculated either on lettuce or basil leaves and exposed to the ClO_2 gas treatment (Table 2). These results suggest that overall, gaseous chlorine dioxide at 4.1 mg/liter could inactivate *Cryptosporidium, E. intestinalis,* and *E. coli* at 2 to 3 log; however, *Cyclospora* was not affected by chlorine dioxide. Since limited information is available on the effect of gaseous chlorine dioxide on parasites, *E. coli* O157:H7 was included in these studies to correlate the lethality effect of ClO_2 gas on these parasites. Inactivation of *E. coli* confirms the results of Sy and collaborators *(24)* in which they identified a reduction of 1.53 to 1.58 log CFU/g of a mixture of *Salmonella enterica, E. coli* O157:H7, and *L. monocytogenes* in fresh-cut lettuce. When *Salmonella* was inoculated onto the surface of raspberries, a 0.52-log CFU/g reduction was obtained *(23).* The fact that *Cyclospora* sporulation was not influenced by gaseous chlorine dioxide is not surprising because commercial bleach (5% chlorine) for 15 min has been used in the laboratory for oocyst purification purposes without affecting oocyst sporulation (unpublished data).

TABLE 2. *Sporulation of* Cyclospora *oocysts inoculated on the surface of lettuce and basil when exposed to chlorine dioxide (4.1 mg/liter)*

	% (SD) *Cyclospora* sporulation		
Produce	Treatment	Control	*P* value
Basil	24.90 (7.50)	23.09 (4.83)	0.65
Lettuce	25.15 (6.30)	34.38 (6.06)	0.02

Gaseous chlorine dioxide treatment for apples, tomatoes, and onions at high concentrations seem to be effective at controlling bacterial pathogens, and its effect on the overall appearance of these fruits and vegetables does not seem to change them significantly. However, its use seems limited with certain berries and vegetables because the sensory qualities of these are compromised. In the present study, the overall appearance of the lettuce and basil leaves was compromised, although it was not the objective of the study to perform a sensory evaluation.

In conclusion, *Cryptosporidium* and *E. intestinalis* loads were significantly reduced by ClO_2 gas treatment, whereas *Cyclospora* sporulation was not affected. The ineffectiveness of ClO_2 gas against *Cyclospora* as well as the observed quality deterioration in treated produce warrants that investigation of alternative sanitizing treatment be continued. This area of research is critical for the United States, as more fruits and vegetables are being imported from countries where these parasites are endemic.

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