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Comparing the Efficacy of Chlorine, Chlorine Dioxide, and Ozone in the Inactivation of *Cryptosporidium parvum* in Water from Parana State, Southern Brazil

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Abstract In the present work, assays were performed to compare the efficacy of hypochlorous acid, chlorine dioxide, and ozone in the inactivation of *Cryptosporidium* oocyst in public water supply from Brazilian South conditions. Experiments were carried out in samples containing 2×10^4 oocysts/ml of *C. parvum* purified from feces of experimentally contaminated calves. An in vitro excystation method was used to evaluate oocysts' viability and to determine the inactivation rates of hypochlorous acid at 2 ppm, chlorine dioxide at 1, 2, and 5 ppm, and ozone at the doses of 0.18, 0.24, 0.36, 0.48, and 1.44 mg/l. By using hypochlorous acid, the maximum inactivation rate obtained was 49.04% after 120 min. Chlorine dioxide at 5 ppm inactivated 90.56% of oocysts after 90 min of contact. Ozone was the most effective product, rendering an inactivation of 100% with the concentration of 24 mg/l. Resistance of *Cryptosporidium* to the usual disinfectants and the need for more effective water treatments to prevent waterborne diseases in Brazil are discussed in this manuscript.

Keywords Chlorine dioxide · *Cryptosporidium parvum* · Disinfection methods · Hypochlorous acid · In vitro excystation · Ozone

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Introduction

Among the protozoan that causes waterborne diseases, *Cryptosporidium* spp. is considered one of the most important worldwide [1]. The parasite has a wide range of hosts, including humans, domestic animals, wild mammals, birds, and reptiles [2]. The transmissible stages (oocysts) are released with the feces and can contaminate both soil and water. An infected host can excrete between 10^9 and 10^{10} per gram of feces [3]. Cryptosporidiosis is usually self-limiting in immunocompetent individuals, but infection determines severe clinical pictures in immunodeficiency hosts, causing symptoms as diarrhea, malabsorption, nutritional deficit, and weight loss [4].

Several waterborne outbreaks of cryptosporidiosis have been reported in developed countries [5]. The largest one occurred in Milwaukee in which 403,000 people became infected by ingesting potable water with oocysts [6]. The frequent reports of *Cryptosporidium* waterborne outbreaks stimulate efforts to develop treatments for removing and inactivating *Cryptosporidium* in public water supply [1, 7].

Although the conventional water treatment processes involving coagulation, flocculation, sedimentation, and filtration can attain a 99% removal of *Cryptosporidium* [3], disinfection stages are basic to guarantee the quality of treated water, considering the low infective dose of the parasite (1 to 1,024 oocysts) [8]. Chlorine is the most widely used disinfectant in conventional water treatment processes [9]. However, it does not present effective inactivation rates for *Cryptosporidium* spp. in the concentrations and contact times applicable to treatment [10, 11]. *Cryptosporidium* oocysts have been demonstrated in 54% of water treated with filtration and chlorination processes [12]. Thus, more efficient disinfecting products must be tested to inactivate the parasite. Chlorine dioxide and ozone are alternative substances to be considered so as to improve conventional water treatment systems [9]. As the operational parameters vary with temperature, pH, and quantity of organic substances present in water, the present work aimed at evaluating the efficacy of hypochlorous acid, chlorine dioxide, and ozone in the inactivation of *Cryptosporidium* in experimentally contaminated water.

Material and Methods

Cryptosporidium parvum Oocysts

C. parvum samples were obtained from feces of newborn calves which were orally infected with an aqueous solution of 10^6 oocysts. The animals were fed with cow's skim milk (UHT) all along the study, and their feces were examined daily for the *C. parvum* oocyst research. The positive samples were diluted in water, successively sieved in 0.150-, 0.053-, and 0.037-mm meshes, and then stored in 2.5% potassium dichromate ($K_2Cr_20_7$) at 4 °C. Oocyst purification was carried out using the protocols described by Ortega-Mora et al. [13] with modifications according to Silva [14]. Parasites were quantified in a Neubauer counting chamber, stored under refrigeration (4 °C), and used in the experiments for a maximum period of a month.

Oocyst Viability

To evaluate the viability of the oocysts, samples were submitted to the excystation method [15]. In short, about 10^5 oocysts were transferred to a 1.5-ml microtube, which was

centrifuged at 11,000×*g* for 10 min. The sediment was incubated with 1.0 ml phosphate-buffered saline (PBS), pH 3.8, at 37 °C for 30 min. Sample was centrifuged again, and the sediment containing oocysts was incubated in the excystation solution (200 μl Hanks solution, 400 μl bovine bile at 1%, and 50 μl $NaHCO_3$ at 0.44% solution) at 37 °C overnight. After centrifugation, the sediment was examined by light phase contrast microscopy (Olympus BX41). A total of 200 to 300 parasitary forms were counted, applying the following formula for viability calculation and later expressed in percentages: OD + PD/*T*, where OD stands for the number of excysted oocysts; PD is the number of partially excysted oocysts; and *T* is sum of all forms, including OD, PD, and intact oocysts.

Disinfection by Hypochlorous Acid

The effect of the 2 ppm concentration of free chlorine on the viability of *C. parvum* oocysts in water was tested. The chlorine solution was prepared from a hypochlorous acid solution and added to the phosphate buffer (0.01 M, pH 7.5) prepared with ultrapure water. Free chlorine was checked with a colorimetric method using *N*,*N*,*N*-diethylphenylenediamine (DPD free chlorine reagent, PermaChem Reagents) [16]. The disinfection assay was carried out in a 2-l volumetric balloon to which 1 ml of the oocyst suspension containing 4×10^7 parasites (final concentration of 2×10^4 oocysts/ml) was added. A control sample was kept in buffered solution without chlorine application.

After 0, 15, 30, 45, 60, and 120 min, 200 ml of the suspension was collected and transferred to a beaker, 30 ml of which was reserved for measuring pH, temperature, and free chlorine. At each dosage, a correction of chlorine demand was carried out so as to restitute the concentrations to 2 ppm level in the original flask. In the remaining sample (170 ml), the chlorine action was finalized by adding a 1% sodium thiosulfate solution in a ratio of 10 ppm to each 1 ppm of chlorine present to discontinue the chlorine action.

Disinfection by Chlorine Dioxide

The effect of 1, 2, and 5 ppm concentrations of chlorine dioxide in water on the viability of the *C. parvum* oocysts was tested. The chlorine dioxide solutions were prepared from a concentrated solution of the product (1,300 to 1,500 ppm)—kindly provided by the EKA Chemicals do Brasil S.A.—diluted in phosphate buffer (0.01 M, pH 7.0) prepared in ultrapure water. The chlorine dioxide concentration was checked by a colorimetric method using *N*,*N*,*N*-diethylphenylenediamine (DPD free chlorine reagent, PermaChem Reagents) [16]. Each disinfection assay was carried out separately in a 1-l volume in a volumetric balloon to which 1 ml oocyst suspension containing 2×10^7 parasites (2×10^4 oocysts/ml final concentrations) was added. A control sample was kept in buffered solution without chlorine dioxide.

After 5, 15, 30, 45, 60, 90, and 120 min, 100 ml of the solution was taken out of the flask, 30 ml of which was reserved for measuring temperature, pH, and chlorine dioxide. A 1% sodium thiosulfate solution was added to the remaining 70 ml in a ratio of 10 ppm of sodium thiosulfate to each 1 ppm of chlorine to discontinue the chlorine action.

Disinfection by Ozone

Ozone was generated from pure oxygen using an ozone generator (type OKTEC®) which was kindly made available by the Diagtech company.

The disinfection assays by ozone were carried out in a 1-l Pirex® volumetric balloon (closed system) with agitation inside the reactor. The ultrapure water volume was 500 ml containing 2×10^4 *C. parvum* oocysts/ml. Ozone was applied in 0.18, 0.24, 0.36, 0.48, and 1.44 mg/l concentrations. In each experiment, the ozone concentration was measured by the trisulphonate blue method [17]. In this case, a control sample was kept in ultrapure water without ozone application.

Parasite Concentration and Disinfection Evaluation

After the disinfection assays, recovery of oocysts was performed by filtration and elution methods [18] modified as follows: each sample was filtered in a Millipore™ equipment (Millipore Filter Holder part no. 4) containing a cellulose acetate filtering membrane (47-mm diameter and 0.8-μm pore size) under 10 to 15 cmHg negative pressure. The membrane was taken out and placed into a plastic wrapping with 10 ml of PBS-T. After manual friction of the membrane, the eluate was collected in a conic tube (15 ml) and centrifuged at 1,000×*g* for 15 min. The sediment was transferred to a 1.5-ml microtube, and the excystation protocol [15] was carried out. The viability reduction was evaluated by comparison between the viability of the oocysts submitted to disinfectants and oocysts control, considered 100% viable.

The disinfection methods' efficacy was estimated by *CT* (ppm concentration × time) and by linear regression of the number of viable oocysts in relation to the *CT* values. The *CT* calculation was performed through the multiplication of the necessary contact time of disinfectant with the oocysts and the disinfectant concentration. The linear regression was carried out using the Origin 7.0 Program.

Results and Discussion

Evaluating Oocyst Viability

To check the efficacy of chemical products, an excystation technique [15] was used to determine the viability of *C. parvum* oocysts. The intact oocysts were considered potentially not viable, and the excysted and partially excysted oocysts were considered potentially viable. All of them could be clearly identified at phase contrast microscopy (Fig. 1). The viability of the purified oocysts used in the disinfection experiments varied from 69% to 95%. Such variation can be related to factors such as oocysts' maturity and even to individual biological differences between samples, resulting in different degrees of excystation. For security, an individual control was used for each disinfection experiment.

The induction of in vitro excystation is one of the alternatives to evaluate protozoan viability. In this technique, oocysts are exposed to an environment that simulates the host gastrointestinal system, and then, if excystation occurs, infectivity could be assumed. Some reports have demonstrated that this technique is better than those based on the use of vital dye such as fluorescein diacetate, 4′,6-diamidino-2-phenylindole, and propidium iodide [19, 20]. Other method to evaluate *Cryptosporidium* inactivation is animal infectivity [10, 21, 22]. Although there are authors who suggest that it is the most reliable method for the evaluation of parasite viability [23–25], a positive correlation between experimental infection and excystation has been demonstrated in other reports [26, 27]. Dealing with laboratory animals is a complex procedure, as it is considered expensive, time-consuming, and involves ethical issues [28], which are disadvantages in comparison with excystation. Besides,

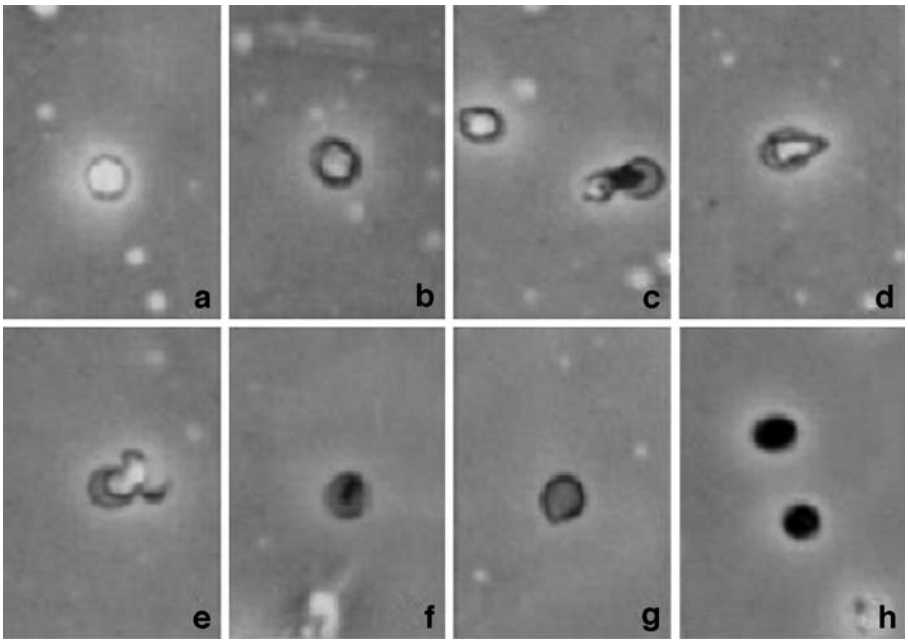

Fig. 1 In vitro excystation for *C. parvum* as seen through phase contrast microscopy. **a**, **b** Intact oocysts; **c**–**e** partially excysted oocysts; **f**–**g** excysted oocysts (only the oocyst's membrane); **h** intact oocysts (with internal structures destructed by exposition to ozone). Magnification, 800×

the maintenance of a conventional biotery is required, which is not feasible for the treatment public water supply because of the high costs and the need of qualified employees.

An in vitro assay that has been proposed to assess oocysts inactivation is cell culture infectivity [29]. After the infection, asexual reproduction occurs in the cells; parasite stages are labeled with specific antibodies and then detected by epifluorescence microscopy. Rochelle et al. [30] reported that cell culture is equivalent to mouse infectivity to evaluate oocysts inactivation, but both methods presents variability, being appropriated to discerning relatively large differences in disinfection tests. Also, in cell culture, specialized equipments, high cost reagents, and trained staff are drawbacks to use it for disinfection kinetics experiments or by the water supply companies. In fact, there is no test able to reproduce perfectly the oocysts inactivation, as all of them have advantages and disadvantages. Based on the feasibility and the correlation of in vitro excystation with infection shown in other reports [26, 27], this method was used in the present work.

Disinfection by Hypochlorous Acid

Hypochlorous acid at 2 ppm presented low efficacy in the inactivation of *Cryptosporidum* oocysts, with viability reduction indexes of 6.71% at 15 min, around 37% at 30–60 min, and 49.04% at 120 min (Table 1). The temperature and average pH during the experiments were 20.1 °C and 7.5, respectively. In Fig. 2, data are presented as viability reduction (*N*/No) according to the disinfectant concentration and its contact time with the parasite's oocysts (*CT*).

It is widely known that *C. parvum* is very resistant to chlorine, showing a *CT* of 240 in 120 min to inactive 50% of parasites in the present work. Different authors reported the

Table 1 Efficacy of hypochlorous acid (2 ppm) in the inactivation of *C. parvum* oocysts in buffered solution (pH 7.5, 20 °C).

Time (min)	Chlorine[a] (ppm)	Viability reduction (%)	*CT*
0	2.08	0	
15	2.06	6.71	
30	2.03	38.81	
45	1.96	36.72	
60	1.83	37.92	
120	1.83	49.04	240[b]

[a] Chlorine concentration restituted at 2 ppm, adding hypochlorous acid after each checking at preset times.
[b] *CT* for the highest inactivation index (49.04%)

inefficacy of the chlorine to inactivate *Cryptosporidium* oocysts [10, 25]. Korich et al. [25] have reported that 80 ppm concentrations of free chlorine for 90 min were necessary to promote the inactivation of *C. parvum* oocysts at 25 °C, pH 7, with *CT* value 7,200 to eliminate 1 log. Fayer et al. [10] treated oocysts with sodium hypochlorite solution at 5.25% for 2 h and, even then, they were still capable of infecting laboratory animals. Keegan et al. [31] obtained less than 0.1 log of inactivation using chlorine at 10 mg/l for 1 h. With a 5-mg/l concentration of chlorine and 24-h incubation, there was no significant inactivation either.

In water treatment stations, the use of higher concentrations of hypochlorous acid is either not viable or recommended because of the possibility of toxic subproduct formations such as trihalomethanes (THMs) [32]. Besides, retention time in a conventional station varies from 60 to 120 min (Company Sanitation of Paraná). Considering these facts, disinfection of water requires the use of other substances rather than chlorine or those which can act synergically with it to promote higher inactivation rates of *Cryptosporidium*.

Disinfection by Chlorine Dioxide

Results of inactivation assay by chlorine dioxide is summarized in Table 2. The average temperature during the experiments was 19 °C, and pH remained 7.0. One part per million

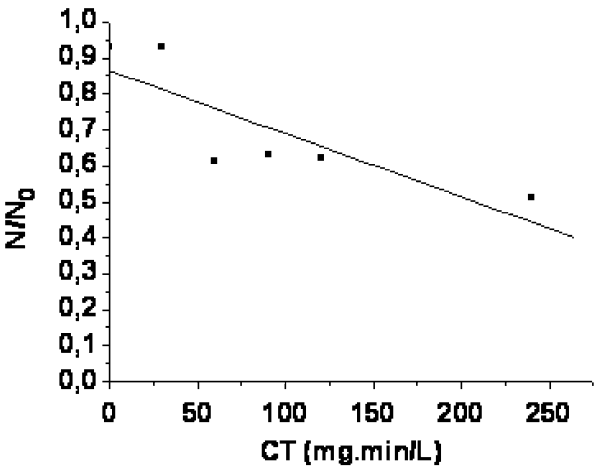

Fig. 2 Linear regression showing the effect of a hypochlorous acid treatment (2 ppm, pH 7.5 at 20 °C) in the inactivation of *C. parvum* oocysts

Table 2 Efficacy of chlorine dioxide in the inactivation of *C. parvum* oocysts in buffered solution (pH 7.0, 19 °C).

Time (min)	Chlorine dioxide (ppm)			Viability reduction (%)			*CT*
	1	2	5	1 (ppm)	2 (ppm)	5 (ppm)	
0	1.10	2.01	5.02	0	0	0	
5	1.05	1.96	4.95	25.97	66.40	67.76	
15	0.99	1.90	4.78	37.42	66.60	65.63	
30	0.96	1.85	4.62	39.83	66.12	72.83	
45	0.93	1.93	4.76	28.04	73.28	74.09	
60	0.90	1.78	4.68	52.55	79.98	83.30	
90	0.87	1.74	4.52	49.32	82.15	90.56	450[a]
120	0.85	1.66	4.46	73.97	87.25	89.78	

[a] *CT* for the highest inactivation index (90.56%)

of chlorine dioxide rendered the lower rates of inactivation (73.97% after 120 min), while the highest rate (90.56%) was obtained when the oocysts were exposed to 5 ppm (*CT*=450). Inactivation kinetic from the three concentrations of chlorine dioxide used is represented in Fig. 3.

Korich et al. [25] reported an inactivation rate of 90% by 1.3 ppm of chlorine dioxide for 60 min, pH 7, at 25 °C (*CT*=78). It is known that the differences in the *CT* values depend on aspects such as pH, temperature, and microorganism lineages. Liyanage [33] has detected a better chlorine dioxide efficacy at pH 11, and Ruffell et al. [34] have demonstrated that *Cryptosporidium* inactivation by chlorine dioxide is faster at pH 10 than at pH 6–8. The latter have also estimated that for each 10 °C decrease in temperature, the *CT* increases at a 3.4 rate approximately. Thus, higher concentrations and/or contact times are necessary for the inactivation of parasites in lower temperatures. Chauret et al. [35] used three different strains of *Cryptosporidium* and have demonstrated the variations in the results of disinfection. Experimental variability could explain why only a 5-ppm concentration promoted a 90% inactivation index in our experiment, whereas other authors have obtained such index with lower concentrations of the product [25, 36].

Comparing the viability reduction in our experiments, it is possible to observe that there are no pronounced differences between 2 and 5 ppm results. Taking into account this

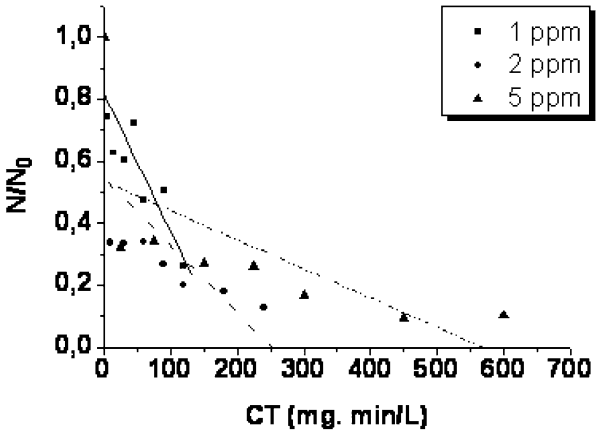

Fig. 3 Linear regression showing the effect of a chlorine dioxide treatment (1, 2, and 5 ppm, pH 7.0 at 19 °C) in the inactivation of *C. parvum* oocysts

observation and the fact that higher than 2 ppm concentrations are not economically viable for conventional water treatment (Company Sanitation of Paraná), chlorine dioxide at 2 ppm can be used in water disinfection with more effective results than hypochlorous acid.

Disinfection by Ozone

To evaluate the ozone's inactivation efficacy in *C. parvum* oocysts, a closed system simulating a reactor with applications of 0.18, 0.24, 0.36, 0.48, and 1.44 mg/l was used. The average temperature during the experiments was 20 °C. The oocyst inactivation was 100%, and it remained constant from the 0.24 mg/l concentration on (Fig. 4). Previous studies by Finch et al. [22] had attained 99.9% inactivation with residual ozone dosage of 0.25 mg/l. Also, in water disinfection by ozone, some variables such as temperature, pH, and concentration of organic matter found in water *in natura*, and parasite's lineages can interfere in the *CT* relation. Studies on the inactivation of *C. parvum* oocysts with 1 mg/l of ozone, at pH 8, show values below 90% in winter time and 99% in summer time [37]. Seasonal variations have also been observed by Rennecker et al. [27] who have not obtained a satisfactory inactivation either in the rainy season or in winter. According to the authors, the seasonal difference is due to higher activation energy necessary to inactivate *C. parvum*. Considering the continental size of countries such as Brazil, the accomplishment of experiments with different disinfectants in each region is necessary to determine the parameters to be used in the water treatment of each region.

The disinfection stage must guarantee the inactivation of remaining organisms that pass through physical barriers during the water treatment. Ozone has been proposed as disinfectant in water treatment stations all over the world [25]. Although it is considered more expensive than chlorine [9], ozone is highly efficient and acts in low dosages [38]. Gallard and Von Guten [39] reported that the pretreatment of water with ozone can also result in the formation of by-products such as THM, but the levels are low and demand of chlorine remains unaffected. According to studies of Von Gunten [40], the use of 2.5 mg/l of ozone reduced in 70% THM formation, probably due to a reaction of ozone with organic material that removes partially THM precursors. Production of bromate, which represents risk to the health due to its carcinogenic potential, is another drawback of water treatment with ozone. Addition of ammonia before the application has been investigated as a tool for minimizing bromated production in ozone treatment [41].

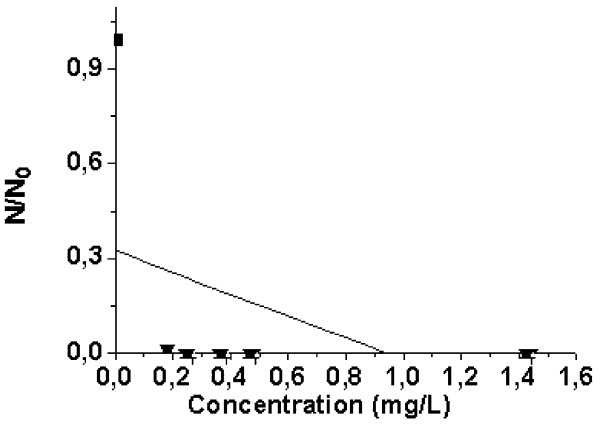

Fig. 4 Linear regression showing the effect of an ozone treatment in the inactivation of *C. parvum* oocysts in ultrapure water (20 °C)

In Brazil, Rule 518 (Brazil Health Minister) suggests that drinking water supplies companies must keep parameters that guarantee the absence of *Cryptosporidium* and *Giardia* in water [42]. However, it does not indicate other disinfectants or established methodologies to verify the removal or inactivation of the parasites. Despite that, it is recognized that more efficient disinfection process must be implemented in the national water treatment. Considering the results obtained here, ozone could be an alternative for water disinfection. It could be used together with chlorine, as the application of different disinfectants increases the efficiency of parasites inactivation [40, 43].

Conclusion

The present work confirmed the higher efficacy of ozone in comparison to chlorine dioxide and hypochlorous acid to inactivate *Cryptosporidium* oocysts, and this treatment could be used as an alternative for water disinfection in Brazil.

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