Excystation of *Cryptosporidium parvum* at temperatures that are reached during solar water disinfection

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SUMMARY

Species belonging to the genera *Cryptosporidium* are recognized as waterborne pathogens. Solar water disinfection (SODIS) is a simple method that involves the use of solar radiation to destroy pathogenic microorganisms that cause waterborne diseases. A notable increase in water temperature and the existence of a large number of empty or partially excysted (i.e. unviable) oocysts have been observed in previous SODIS studies with water experimentally contaminated with *Cryptosporidium parvum* oocysts under field conditions. The aim of the present study was to evaluate the effect of the temperatures that can be reached during exposure of water samples to natural sunlight (37–50 °C), on the excystation of *C. parvum* in the absence of other stimuli. In samples exposed to 40–48 °C, a gradual increase in the percentage of excystation was observed as the time of exposure increased and a maximum of 53·81% of excystation was obtained on exposure of the water to a temperature of 46 °C for 12 h (*versus* 8·80% initial isolate). Under such conditions, the oocyst infectivity evaluated in a neonatal murine model decreased statistically with respect to the initial isolate (19·38% *versus* 100%). The results demonstrate the important effect of the temperature on the excystation of *C. parvum* and therefore on its viability and infectivity.

Key words: *Cryptosporidium parvum*, SODIS-temperatures, excystation, viability, infectivity.

INTRODUCTION

The genus *Cryptosporidium* comprises ubiquitous parasites that infect the epithelial cells at the border of the microvilli in the gastrointenstinal tracts of all classes of vertebrate hosts. Several species parasitize a wide range of hosts, whereas others appear to be restricted to certain groups or even to a single host species. Some species cause acute self-limiting infections, whereas others cause chronic disease. The severity and duration of the infection are also affected by the immune status of the infected host. Immunocompetent individuals may suffer different degrees of acute illness and immunocompromised individuals may develop chronic disease that can be fatal (Fayer, 2007).

Cryptosporidium parvum is a widely distributed species of medical and veterinary importance and affects a large number of hosts that shed in their faeces oocysts that are extremely resistant to environmental conditions and to commonly used disinfectants. The oocysts are infective to hosts via the faecal-oral route, direct contact and contaminated water and foodstuff (Nichols, 2007). Since 1985 when D'Antonio *et al*. (1985) described the first water-related outbreak of cryptosporidiosis, which involved approximately 200 individuals, *Cryptosporidium* has been recognized as a human waterborne pathogen and several studies have demonstrated that *Cryptosporidium* oocysts are found in the environment worldwide, in source and treated drinking water (Fayer, 2004). Solar water disinfection (SODIS) is a simple method that involves the use of solar radiation to destroy pathogenic microorganisms that cause waterborne diseases. Contaminated water is placed in plastic bottles and exposed to full sunlight for a minimum of 6 h (or 2 consecutive days under conditions of >50% cloud cover) (Conroy *et al*. 1996; Sommer *et al*. 1997). Sunlight exerts biocidal effects due to optical and thermal processes, and a strong synergistic effect occurs at temperatures exceeding 45 °C (McGuigan *et al*. 1998, 1999). The effect of batch-process solar disinfection on survival of *C. parvum* oocysts and *Giardia muris* cysts in drinking water has been described (Méndez-Hermida *et al*. 2005; McGuigan *et al*. 2006).

During previous SODIS studies carried out in field conditions at the Plataforma Solar de Almeria (PSA) in Southern Spain, we observed a notable increase in the water temperature and the existence of empty or partially excysted oocysts of *C. parvum* (unpublished observation). As sporozoites thus released are short lived, the aim of the present study was to evaluate experimentally the effect of a range of temperatures (37–50 °C) that can be reached in the

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water bottles during exposure to natural sunlight on the excystation of *C. parvum* and therefore on its viability and infectivity, evaluated by *in vitro* and *in vivo* methods.

MATERIALS AND METHODS

Source of oocysts

Cryptosporidium oocysts were collected from a naturally infected neonatal Friesian-Holstein calf by rectal sampling. Concentration (phosphate-buffered saline (PBS; pH 7·2)/diethyl ether), purification (discontinuous caesium chloride gradients) and quantification (Neubauer haemacytometer) were performed as reported previously (Kilani and Sekla, 1987; Lorenzo-Lorenzo *et al*. 1993). The oocysts were classified as *C. parvum* in accordance with identification of the COWP gene, according to Amar *et al*. (2004). The isolate used was less than 1 month old.

Experimental design

Microcentrifuge tubes of 1·5 ml vol. were filled with 1 ml of distilled water and contaminated with 2×10^6 purified *C. parvum* oocysts. All vials were maintained at room temperature (18–20 °C) during 1 h to avoid sharp changes in temperature before exposure to elevated temperatures in a dry block heat bath (Uniequip GmbH, Dresden, Germany), i.e. 37 °C, 40 °C, 42 °C, 44 °C, 46 °C, 48 °C and 50 °C, for 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h. The dry block heat bath in which samples were incubated was fitted with a lid, which protected the samples from ultraviolet light. Following incubation, each sample was centrifuged (10 000 ***g*** for 5 min), the supernatant aspirated to 0·5 ml and the sediment resuspended. Aliquots of 10 μl of the samples were viewed under phase-contrast optics (400× magnification) in an Olympus BH2 microscope (Olympus Optical Co., Ltd, Tokyo, Japan), and the percentage of excystation was calculated according to Robertson *et al*. (1993). The proportions of totally excysted (empty), partially excysted and intact oocysts were identified and enumerated twice. Between 200 and 400 oocysts were counted for each enumeration and the percentage excystation was calculated as follows: excystation (%) = [(no. of empty oocysts + no. of partially excysted oocysts) ÷ total no. of oocysts counted] × 100. The number of oocysts present in the rest of the sediment was recounted in a Neubauer haemocytometer before viability and infectivity assays were carried out, as described below.

Viability assays

The viability of *C. parvum* oocysts was determined by inclusion/exclusion of the fluorogenic vital dye propidium iodide (PI) (Sigma P 4170, Madrid, Spain), in accordance with Campbell *et al*. (1992) and a further modification that includes an immunofluorescence antibody test to verify oocyst identification (Dowd and Pillai, 1997). Briefly, aliquots of 5×10^4 oocysts were suspended in 100 μl of Hanks' balanced salt solution (HBSS) (Sigma H 6136, Madrid, Spain) and then incubated with 10 μl of PI working solution (1 mg/ml in 0·1 M PBS, pH 7·2) at 37 °C for 10 min. After PI staining, oocysts were washed twice in PBS at 10 000 ***g***, and 4 °C, for 5 min and incubated with 20 μl of monoclonal antibodies labelled with fluorescein isothiocyanate (FITC) (Aqua-Glo G/C Direct, Waterborne, Inc., New Orleans, LA, USA). Oocysts were firstly identified under FITC filter (excitation at 450–480 nm; barrier at 515 nm) before being examined for PI inclusion/exclusion under PI filter (excitation at 510–550 nm; barrier at 590 nm). The proportions of PI-positive (dead) and PI-negative (viable) oocysts were quantified in an epifluorescence microscope equipped with Nomarski differential interference contrast, FITC and PI filters (Olympus AX70, Olympus Optical Co., Ltd.).

Taking into account that only intact oocysts were considered in the assays involving inclusion/exclusion of the vital dye PI, and that totally or partially excysted oocysts are not viable, the global viability of the isolate was calculated as follows: global viability (%) = [(intact oocysts (%) × PI-negative oocysts (%)) ÷ 100].

Infectivity assays

Two litters of CD-1 Swiss mice (10–15 mice/litter; 3–4 days old; 2·5–3·0 g weight) were inoculated intragastrically with 0·1 ml of water containing $2{\cdot}5 \times 10^4$ intact oocysts for each temperature tested and an exposure time of 12 h. The mice were sacrificed 7 days later (Peeters *et al*. 1989) and the entire small and large intestines were removed and placed in 5 ml of PBS (pH 7·2). The intestines were then homogenized in an Ultra-Turrax® T10 homogenizer (Ika®-Werke GmbH & Co.KG, Staufen, Germany), and the oocysts were counted (Neubauer haemacytometer). The intensity of infection was defined as the number of oocysts per homogenized intestinal tissue.

Statistical analysis

Results are the means of counts performed in at least 2 separate experiments. Differences in excystation, inclusion of PI, and the infectivities and intensities of infection were compared by pairwise multiple comparison procedures (Student-Newman-Keuls method) and one-way ANOVA, with GraphPad Instat® version 3.05 statistical software (©1992–2000 GraphPad Software, La Jolla, CA, USA).

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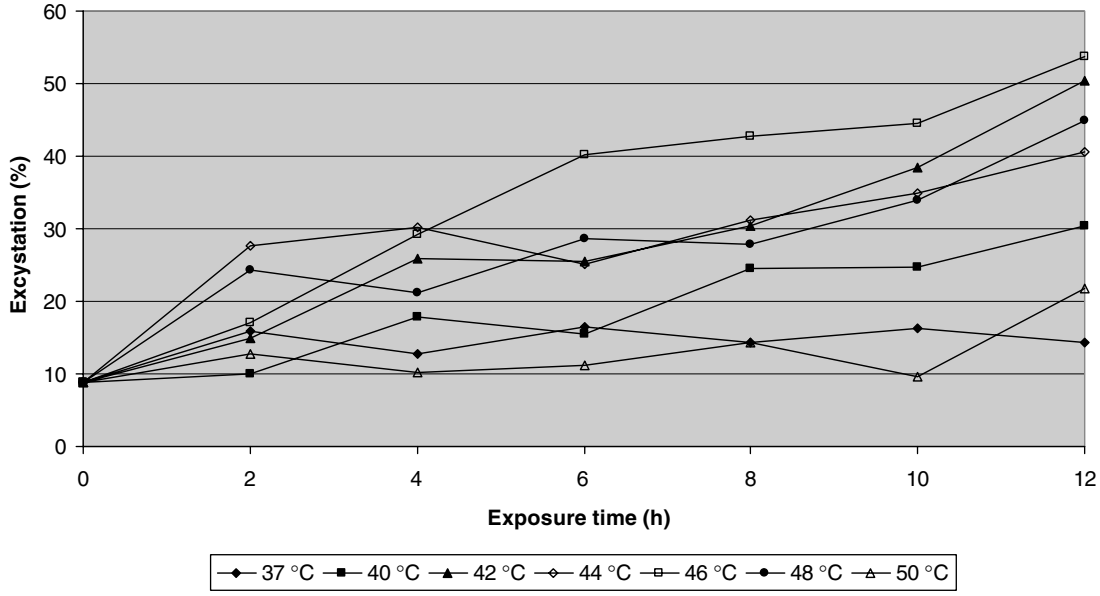

Fig. 1. Evolution of *Cryptosporidium parvum* excystation at temperatures that can be reached during SODIS procedures.

Differences were considered statistically significant at a probability level of $P<0{\cdot}05$.

RESULTS

The isolate of *C. parvum* used in the experiments showed initial values of 8·80% excysted oocysts and 8·74% oocysts permeable to the vital dye PI, and the global viability of the isolate was 83·30%. The assay in neonatal mice showed 100% infectivity and an intensity of infection of $5{\cdot}08\times10^6$ oocysts/homogenized intestinal tissue.

The results for *C. parvum* excystation and inclusion/exclusion of PI obtained in the different assays are shown in Fig. 1 and Fig. 2, respectively. The rates of excystation showed 2 types of responses: exposure temperatures of 37 °C, 40 °C and 50 °C did not have a statistically significant effect on spontaneous excystation, although there was an increase in the percentage of excystation after 6 h of exposure of the water samples at 40 °C (15·46%) and a further increase after 12 h of exposure (30·46%). At intermediate temperatures of 42–48 °C there was a gradual increase in the percentage excystation ($P<0{\cdot}05$) as the time of exposure increased, with the maximum reached at 46 °C after 12 h (53·81%) (Fig. 1).

Likewise, temperatures of 37 °C and 40 °C did not have a statistically significant effect on oocyst viability. At 42 °C, and after 6 h of exposure (15·28% of PI-positive oocysts), there was an increase in the percentage of oocysts that were permeable to the PI dye, which reached 30·01% after 12 h of exposure. At higher temperatures, a higher percentage of oocysts was permeable to the dye ($P<0{\cdot}05$), even with shorter times of exposure. In this way, 41·65% of oocysts were PI-positive after exposure at 50 °C for 4 h, and approximately 50% at the maximum time of exposure considered (12 h) (Fig. 2).

The global viability of oocysts, calculated with the previously described equation and taking into account the percentage excystation and the viability determined by inclusion/exclusion of the fluorogenic vital dye PI, is shown in Fig. 3. At 37 °C, the global viability was not statistically different from that of the initial isolate. A decrease (to 53·39%) in the global viability of oocysts subjected to a temperature of 40 °C was observed; the decrease became statistically significant as the temperature increased. However, the minimum global viability (24·71%) corresponded to an exposure temperature of 46 °C.

The results of the experimental infections carried out with the oocysts exposed to the different temperatures for 12 h revealed that the infectivity decreased slightly at 42 °C (to 95·00%, compared with 100% for the initial isolate), decreased further at 46 °C (to 19·38%) and was finally reduced to nil at 48 °C. However, there was a statistically significant decrease ($P<0{\cdot}05$) in the intensity of infection, with respect to the control mice at 42 °C ($0{\cdot}87\times10^6$ compared with $5{\cdot}08\times10^6$) (Fig. 4). In mice inoculated with oocysts exposed at 48 °C for 6 h and 8 h, the infectivities were 100% and 57·14%, respectively, and the corresponding intensities of infection were statistically lower ($P<0{\cdot}05$) than those obtained in the control mice ($0{\cdot}97\times10^6$ and $0{\cdot}41\times10^6$, respectively).

DISCUSSION

The mechanisms involved in the excystation of *Cryptosporidium* are not yet known, and although *in vitro* excystation protocols mimic host-derived

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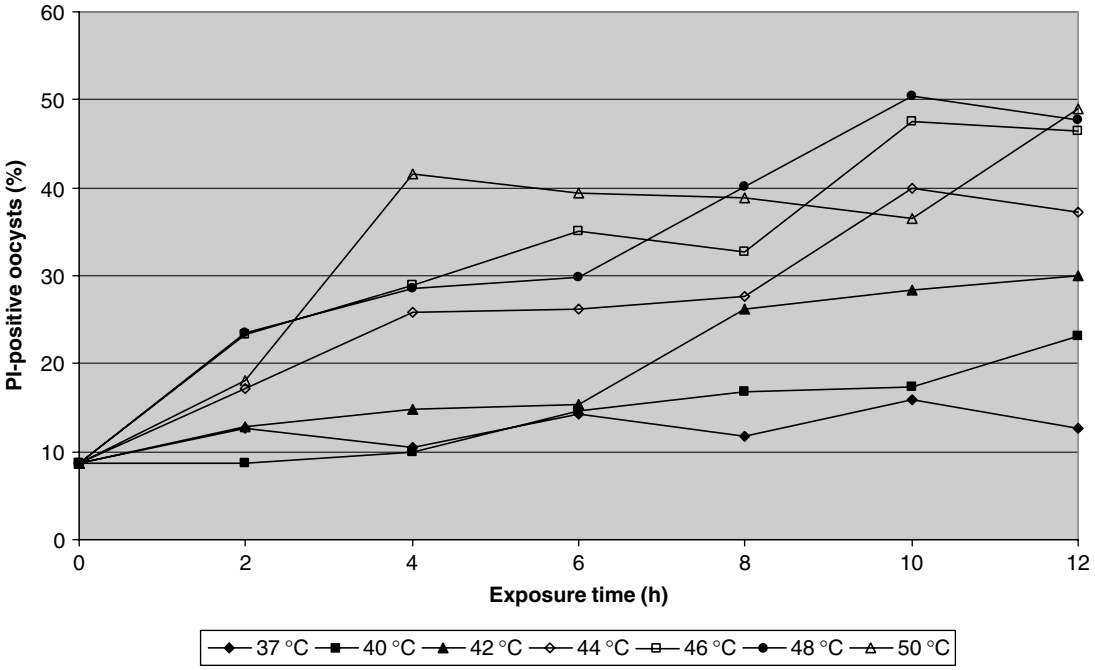

Fig. 2. Evolution of the inclusion of the fluorogenic vital dye propidium iodide (PI) by *Cryptosporidium parvum* oocysts at temperatures that can be reached during SODIS procedures.

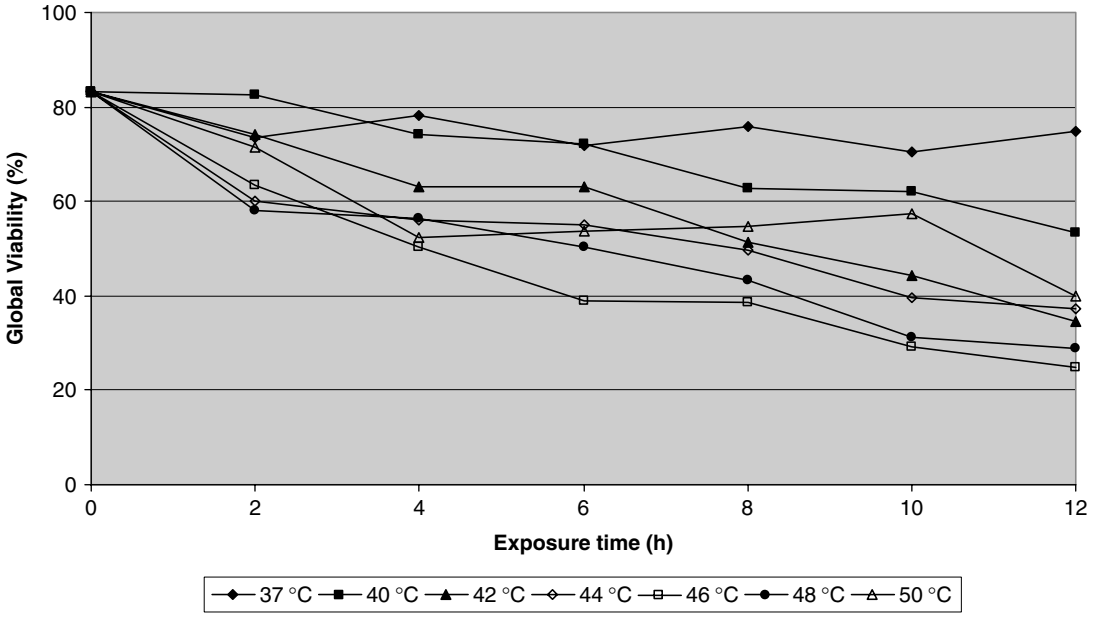

Fig. 3. Evolution of the global viability of *Cryptosporidium parvum* oocysts at temperatures that can be reached during SODIS procedures. Global viability (%) = [(intact oocysts (%) × PI-negative oocysts (%)) ÷ 100].

signals, the hierarchy and synergy of specific triggers are not well understood. However, it has been found that in a small percentage of *C. parvum* oocysts the sporozoites may excyst when incubated at 37 °C in the absence of any of the other major host triggers (< 20%, depending on the age of the oocysts and the type of isolate) (Smith *et al.* 2005); this process is denominated 'spontaneous excystation' and makes survival of the sporozoites impossible as they are outside of the host environment.

On the other hand, it is known that temperature is one of the most important factors that affect the survival of *Cryptosporidium* oocysts in the environment. The oocysts are susceptible to temperatures above 15 °C, which suggests that temperature inactivation is a key abiotic factor that affects oocyst survival and infectivity in the environment (King *et al.* 2005). Temperatures below freezing point may also seriously affect oocyst survival (see King and Monis, 2006).

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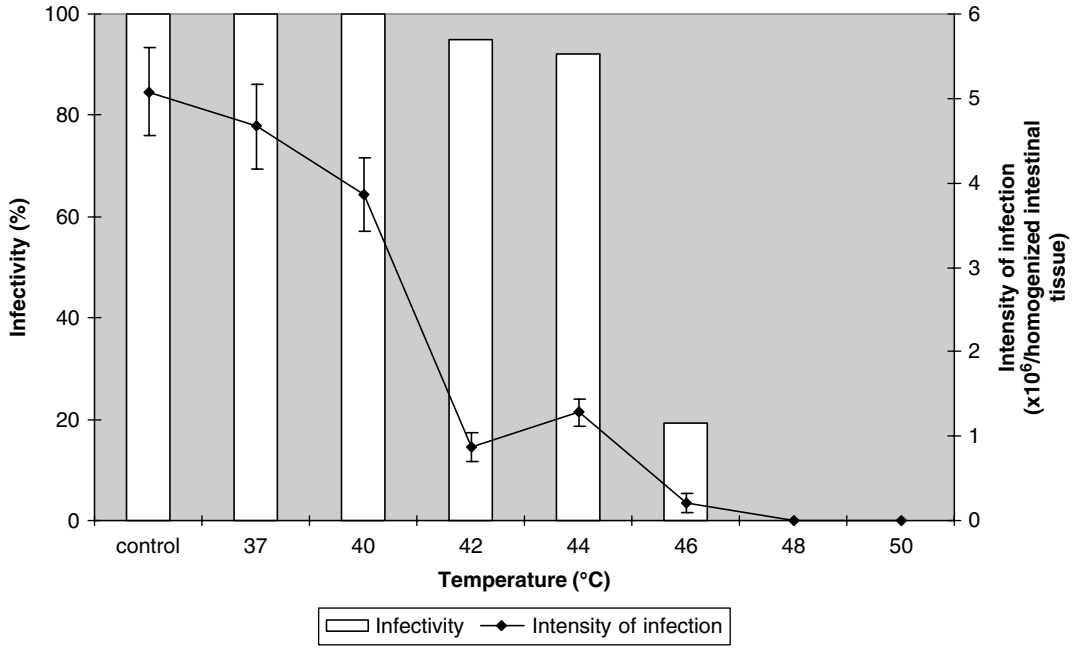

Fig. 4. Infectivities and intensities of infection of *Cryptosporidium parvum* oocysts after 12 h exposure at temperatures that can be reached during SODIS procedures.

The results of the study demonstrate the strong influence of temperature on the process of 'spontaneous excystation' of *C. parvum* and therefore on the viability and infectivity of the oocysts. Thus a high percentage of oocysts undergo excystation in distilled water exposed for different lengths of time to temperatures of between 40 and 48 °C, with the process being favoured at 46 °C. However, in a recent study on the adaptation of *Cryptosporidium* oocysts to different conditions, temperature was found to be an important trigger for excystation in *Cryptosporidium muris* but not in *C. parvum*, and so the authors suggest that high temperature by itself does not induce efficient excystation of *C. parvum* oocysts, as such a step would not be essential for intestinal species (Widmer *et al.* 2007).

Staining with vital dyes is a widely used method for evaluating oocyst viability (Campbell *et al.* 1992; Belosevic *et al.* 1997; Dowd and Pillai, 1997; Jenkins *et al.* 1997; Neumann *et al.* 2000), although it is known to overestimate viability in comparison with that measured in animal models and cell cultures, and the values obtained are conservative estimates. However, Robertson and Gjerde (2007) consider this method as a simple assay technique that can provide useful preliminary data for studies on the effects of environmental pressures such as temperature. Application of this technique in the present study revealed that when the oocyst suspension is exposed to temperatures higher than 44 °C for longer than 4 h, the percentage of oocysts that incorporated the PI dye (indicative of the integrity of the oocyst cell wall) increased to between 25 and 50% compared with 8·74% for the initial isolate. Increased temperatures do not only increase the permeability of the cell wall (Jenkins *et al.* 1997) but may also denature proteins that are essential for the survival of *Cryptosporidium* and affect the energetic metabolism of the oocysts. Fayer *et al.* (1998) showed that the ability of *Cryptosporidium* oocysts to initiate infection has been linked to finite carbohydrate energy reserves in the form of amylopectin, which is consumed in direct response to environmental temperatures. It has also been concluded that amylopectin constitutes the energy reserve required for excystation and invasion of host cells by coccidian sporozoites, and that when the amylopectin content falls below a critical level, sporozoites lack sufficient energy to invade cells (Vetterling and Doran, 1969). Moreover, King *et al.* (2005) identified a close relationship between oocyst infectivity and ATP content, and demonstrated that temperature inactivation at higher temperatures is a function of increased oocyst metabolic activity. This may explain why the percentages of spontaneous excystation obtained at 50 °C were slightly lower or similar to those obtained at 37 °C. However, the percentage of oocysts that incorporate the PI dye after 4 h of exposure at 50 °C was considerably higher than at 37 °C (41·65% compared with 10·45%). In addition, at the maximum temperature and as the time of exposure was increased, the oocysts were observed to collapse and adhere to one another to form accumulations.

Jenkins *et al.* (2003) showed that *C. parvum* oocysts stored in water at 15 °C for 7 months are capable of infecting mice and cell cultures, even though the concentration of amylopectin is extremely low. In the present study, infection was achieved in neonatal

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mice at low levels of intensity of infection after the oocysts were maintained at 48 °C for 8 h. However, exposure for 12 h at the same temperature completely destroyed the oocyst infectivity.

The global viability (calculated with the previously described formula) decreased as the temperature and time of exposure increased, and the lowest value reached was that obtained after exposure of the oocysts at 46 °C for 12 h (24·71%), due to the higher percentage of excystation detected at this temperature (53·81%). Uptake of the vital dye PI was tested in the remaining intact oocysts and it was found that 46·50% incorporated the dye and were therefore unviable. At higher temperatures there was a slight increase in the number of oocysts permeable to PI, but the levels of excystation decreased significantly, possibly because of damage to the oocysts caused by the high temperature. However, the results were contradictory in that infection was achieved in 19·38% of the neonatal mice inoculated with oocysts exposed at 46 °C for 12 h, whereas the infection was null at 48 °C and 50 °C. This may be due to the conservative values obtained with the method used to assess the viability or to damage caused by the high temperatures and not detected with the methodology applied.

As the objective of the present study was to evaluate the effect of a range of temperatures that can be reached in water exposed to solar radiation during SODIS procedures, the results demonstrate the important effect that temperature has on the excystation of *C. parvum* and therefore on its viability. Under natural solar water disinfection conditions, the excystation and consequently inactivation of *Cryptosporidium* oocysts can be better, because of the synergistic effect of temperature and ultraviolet light (McGuigan *et al*. 1998). The results also suggest that the techniques for measuring excystation are not suitable for evaluating the potential viability of oocysts in water subjected to SODIS, given the high levels of excystation detected. These findings, in addition to the observed alteration in the oocyst morphology and in the cell wall integrity, and the irreversible effect that ultraviolet light has been shown to have on oocysts (Rochelle *et al*. 2005) support the use of solar water disinfection to guarantee the safety of drinking water contaminated with *C. parvum* oocysts.

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