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Development of a combined *in vitro* cell culture – Quantitative PCR assay for evaluating the disinfection performance of pulsed light for treating the waterborne enteroparasite *Giardia lamblia*

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HIGHLIGHTS

- Pulsed UV light successfully inactivated *Giardia lamblia* cysts as determined by real time PCR.
- *G. lamblia* appears more UV resistant than *Cryptosporidium parvum*.
- The HCT-8 human intestinal cell line acts as a suitable host for *Giardia* infection.
- A reduced level of infectivity was observed with the Caco-2 colon cell line.
- Real Time PCR provided a suitable tool for measuring *Giardia* infectivity *in vitro*.

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ABSTRACT

Giardia lamblia is a flagellated protozoan parasite that is recognised as a frequent cause of water-borne disease in humans and animals. We report for the first time on the use of a combined *in vitro* HCT-8 cell culture-quantitative PCR assay for evaluating the efficacy of using pulsed UV light for treating *G. lamblia* parasites. Findings showed that current methods that are limited to using vital stains before and after cyst excystation are not appropriate for monitoring or evaluating cyst destruction post PUV-treatments. Use of the human ileocecal HCT-8 cell line was superior to that of the human colon Caco-2 cell line for *in vitro* culture and determining PUV sensitivity of treated cysts. *G. lamblia* cysts were also shown to be more resistant to PUV irradiation compared to treating similar numbers of *Cryptosporidium parvum* oocysts. These observations also show that the use of this HCT-8 cell culture assay may replace use of animal models for determining disinfection performances of PUV for treating both *C. parvum* and *G. lamblia*.

1. Introduction

Giardia lamblia is a flagellated protozoan parasite that is frequently associated with gastrointestinal infection in both developed and developing countries where the main routes of transmission including consumption of contaminated food and water, person-to-person and animal-to-person zoonosis through the faecal-oral route. Waterborne transmission of *Giardia* is the most common cause of outbreaks as *G. lamblia* cysts are abundant in surface and source waters (Shin et al., 2010). Notably, 20 of the 45 European countries have reported the prevalence of *Giardia* in human and water samples with 10 countries reporting its presence in recreational waters. Furthermore, giardiasis has recently been categorised as a "neglected disease" by the World Health Organisation (WHO) (Plutzer et al., 2010). The excretion of extensive numbers of *Giardia* cysts by high numbers of cattle and sheep around water supplies make infected animals an important route of water contamination. Indeed occurrence data from developed and developing countries suggest that *Cryptosporidium* and *Giardia* are commonly found in raw sewage with the latter present in higher numbers more frequently. As with parasites such as *Cryptosporidium parvum* the removal of *Giardia* from water supplies has proven problematic due to its resistance to current water disinfection methods and the low cyst number required for infection to occur.

Indeed it is due to the emergence of such recalcitrant chlorine-resistant pathogens that the need for alternative water disinfection methods has arisen. The use of UV light technology for the treatment of water has proven effective for numerous water borne microorganisms including parasitic protozoan (Craik et al., 2000;

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Garvey et al., 2010). The inactivation of microbial species by UV light involves the alteration of DNA following the absorbance of UV energy by the treated species which in turn inhibits the reproductive abilities of the organism. Standard UV disinfection methods involve the usage of lamps which emit UV in at set wavelength and are termed low press UV. Recent research including that carried out by this research group has focused on the potential of a novel pulsed UV light system as a suitable method of water and waste water disinfection. Pulsed UV operates by storing energy in a capacitor and releasing it as short bursts of energy into the treatment area. Compared to conventional UV light, pulsed light treatment has the advantage of being fast, effective and more efficient at converting electrical energy into photon energy.

Studies on the UV inactivation of organisms such as *Cryptosporidium* and *Giardia* are problematic due to the infective nature of the parasites which require a live host to initiate its reproductive cycle. This coupled with the mode of action of UV light (inducing genetic damage as opposed to cell membrane damage) raises difficulties with accurately determining if *Giardia* has lost its infective abilities following UV exposure.

Research to date on the UV inactivation of *Giardia* has been based on the use of vital dyes, *in vitro* excystation and *in vivo* infection of live rodents with the former consistently proven to overestimate inactivation (Maux et al., 2002) and the latter raising ethical issues as well as the difficulties and time demands associated with animal testing. The use of an *in vitro* cell culture model as an alternative to *in vivo* testing has proven successful for other parasites such as *C. parvum* (Garvey et al., 2010). By providing an *in vitro* environment similar to that of the host intestines, the parasite can be stimulated to infect cells growing in culture and to initiate its life cycle. *Giardia* trophozoites strongly adhere to the epithelial surface of the intestine via a ventral adhesive disc. A number of parasitic surface molecules are engaged in this tight interaction, including giardins (primarily alpha, beta, delta and gamma giardins), as well as a complex network of contractile proteins which play key roles in trophozoite attachment. The extraction and amplification of parasitic DNA via real time PCR may then provide a rapid measurement of infective parasite numbers allowing for the measurement of live or dead parasites. This represents the first study on the use of a combined cell culture - real time PCR *in vitro* assay for the viability assessment of low-pressure and pulsed UV light treated *G. lamblia* cysts using human intestinal derived cell lines. It is envisioned that such an assay may provide an alternative approach to that of *in vivo* testing by allowing for a rapid method of determining parasitic inactivation following UV and other enabling processes for water treatment. Working towards such methods will aid in the treatment and elimination of this pathogenic organism from water supplies by allowing for reproducible studies on the inactivation of *Giardia* cysts for effective water treatment and control.

2. Methods

2.1. Pulsed UV light system

A bench-top pulsed power source (PUV-1, Samtech Ltd., Glasgow) was used to power a low-pressure (60 kPa) xenon-filled flashlamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV transparent quartz tube), that produced a high-intensity diverging beam of polychromatic pulsed light, was used in this study as per Garvey et al. (2010, 2012). This delivery system kills microorganisms by using ultra-short duration pulses of an intense broadband emission spectrum that is rich in the UV-C germicidal wavelength. PUV is produced by storing electricity in a capacitor over relatively long times and releasing it as a short duration pulse using sophisticated pulse compression techniques. The pulsed light has a broadband emission spectrum extending from the UV to the infrared region with a rich UV content and its intensity also depends on the level of the voltage applied. The light source has an automatic frequency control function which allows it to operate at 1 pulse per second (pps); this setting was used throughout the study. Light exposure was homogenous as the xenon lamp measuring 9×0.75 cm was longer than the 8.5 cm standard diameter.

2.2. Mammalian cell culture and maintenance of cell lines

Monolayers of the human ileocecal adenocarcinoma cell line HCT-8 (ATCC CCL-244: American Type Culture Collection, Rockville, Md.) were grown with regular sub-culturing in RPMI 1640 growth media with L-glutamine and supplemented antibiotics (penicillin G, 100,000 U/L, streptomycin, 0.5 g/L and amphotericin B, 0.5 g/L), sodium bicarbonate, 2 g/L, and 10% foetal calf serum adjusted to pH7.4. Caco-2 cells (ATCC HTB-37), established from a human colon adenocarcinoma Caco-2 cells were maintained at 37 °C in Dulbecco modified Eagle's medium/Ham's F-12 medium, supplemented with 20% (v/v) foetal bovine serum, 1% 200 mM L-glutamine, 1% (v/v) non-essential amino acids, 0.5% (v/v) penicillin–streptomycin and 0.5% (v/v) amphotericin B (Sigma–Aldrich). Maintenance media was stored at 4 °C and heated to 37 °C prior to use. HCT-8 and Caco-2 cells were cultured and maintained in T75 cm^2 cell culture flasks in a humidified incubator at 37 °C in an atmosphere containing 5% (vol/vol) CO_2 for circa. 24 h until 80 to 90% confluent monolayers had formed. Once confluent, cells were trypsinised to remove the cell monolayer from the flask and seeded into 6 well plates for 24 h at 37 °C at a seeding density of 1×10^6 cells/well for use in real time pcr studies and at a density of 1×10^5 cells/well for chamber slides for infectivity studies using fluorescent stains.

2.3. Viability and infectivity determination of G. lamblia

G. lamblia cysts (derived from experimental infected gerbils) were purchased from Waterborne Inc USA. Cysts were stored in sterile PBS (0.01 M phosphate buffer, containing 0.0027 M KCL and 0.137 MNaCl at a pH of 7.4) with 100 U of penicillin/ml, 100 μg of streptomycin/ml and 100 μg of gentamicin/ml at 4 °C until they were used for UV treatment studies. A combined surrogate dye staining method comprising propidium iodide (PI) 1 mg/ml of 0.1 M sterile PBS, 4′,6′-Diamidino-2-Phenylindole (DAPI) 2 mg/ml in methanol and a fluorescein-labelled mouse-derived monoclonal antibody Giardi-a-Glo™ (having corresponding epitope on cyst cell wall; Waterborne Inc, New Orleans, USA) was used to confirm the viability of cysts. The excystation rate was determined for each batch of cysts by microscopic observation following sequential incubation at 37 °C in acidified hanks balanced salt solution (HBSS) for 1 h as per method of Garvey et al., 2010. All experiments were carried out using cysts with greater than 90% viability, as determined by *in vitro* excystation and the uptake or exclusion of vital dyes. Cysts were counted using a haemocytometer and inverted microscope (Olympus, CKX41) with camera (Olympus, IX2-SLP) attached.

Cell culture infectivity was confirmed by immunofluorescent (IF) staining of treated HCT-8 and Caco-2 cell monolayers following exposure to viable cysts. Cell monolayers were seeded into each of 8 well chambered slides (Lab Tec II, Nunc) at a concentration circa.1×10^5 cells per well. Cysts were stimulated to excyst by re-suspension in acidified HBSS pH 2.7 for 1 h at 37 °C. After one washing step with sterile PBS, cysts were re-suspended in cell culture media containing varying concentrations of proteose and thereafter 350 μl aliquots were then added to each well. Samples were incubated for up to 48 h at 37 °C in 5% (vol/vol) CO_2 atmosphere, to determine optimal conditions for cell infectivity.