Open Veterinary Journal, (2016), Vol. 6(1): 15-22
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Original Article

Submitted: 20/10/2015 Accepted: 05/01/2016 Published: 27/01/2016

The pulsed light inactivation of veterinary relevant microbial biofilms and the use of a RTPCR assay to detect parasite species within biofilm structures

M. Garvey[1,*], G. Coughlan[2,3], N. Murphy[2] and N. Rowan[3]
[1]*Department of Life Sciences, Institute of Technology Sligo, Sligo, Ireland*
[2]*Department of Parasitology, National University of Ireland Maynooth, Maynooth, Ireland*
[3]*Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland*

Abstract
The presence of pathogenic organisms namely parasite species and bacteria in biofilms in veterinary settings, is a public health concern in relation to human and animal exposure. Veterinary clinics represent a significant risk factor for the transfer of pathogens from housed animals to humans, especially in cases of wound infection and the shedding of faecal matter. This study aims to provide a means of detecting veterinary relevant parasite species in bacterial biofilms, and to provide a means of disinfecting these biofilms. A real time PCR assay was utilized to detect parasite DNA in *Bacillus cereus* biofilms on stainless steel and PVC surfaces. Results show that both *Cryptosporidium* and *Giardia* attach to biofilms in large numbers (100-1000 oo/cysts) in as little as 72 hours. Pulsed light successfully inactivated all test species (*Listeria, Salmonella, Bacillus, Escherichia*) in planktonic and biofilm form with an increase in inactivation for every increase in UV dose.
Keywords: Biofilms, *Cryptosporidium*, *Giardia*, PCR, Veterinary.

Introduction

The prevention and control of veterinary related infections is an important aspect of public health and safety due to the occurrence of zoonotic infections. The spread of pathogenic species within veterinary practices can lead to infection of both the housed animals and veterinary staff. Veterinary clinics are a focus for human and animal interaction, often in situations dealing with infected wounds or faecal matter. This is a significant concern for immunocompromised individuals who are animal owners. Animal associated pathogens of concern to immunocompromised persons include *Cryptosporidium, Salmonella, Listeria, Bacillus, Escherichia coli, Campylobacter* and *Giardia* (Grant and Olsen, 1999). Furthermore, many research studies have highlighted the connection between the spread of pathogenic organisms from surfaces to patients (Gebel *et al*., 2013). Consequently, the use of surface disinfectants for the control of pathogens in clinical and veterinary settings has become important due to the increase in antibiotic resistant microbial species and zoonotic infections. However, issues have arisen where some pathogens have shown resistance to commonly used chemical based disinfectants. Such pathogens include the parasites Cryptosporidium and Giardia, bacterial endospores and bacterial biofilm structures (Betancourt and Rose, 2004). Planktonic microbial cells are able to attach to and colonise environmental surfaces by producing an extracellular polymeric substance (EPS), these adherent (sessile) cells are referred to as biofilms. The descriptive terms sessile and planktonic are used to describe surface adherent and free floating bacterial cells respectively. Veterinary important species such as *Listeria, Escherichia, Bacillus* and *Salmonella* are capable of producing these biofilm structures allowing them to gain resistance to standard chemical disinfection methods. Biofilms communities spread largely by breaking of in clumps from the primary structure, these detached biofilm clumps may contain enough bacteria to give an infective dose to housed animals making them a potential health risk. Indeed, biofilms or sessile communities are believed to be the causative agent in diseases such as pneumonia, liver abscesses, enteritis, wound infections and mastitis infections in animals (Clutterbuck *et al*., 2007). Ingestion of a biofilm bacterial clump present in the surrounding environment could play an important role in the transmission of disease. In addition, in hosts with functioning innate and adaptive immune responses, biofilm-based infections are often very persistent and remain unresolved. In fact, surrounding tissues often undergo extensive damage by immune complexes and invading neutrophils when trying to eradicate the infection (Stewart and Costerton, 2001).

The prevention of biofilm formation on surfaces located in areas of animal housing would provide the best control measures for these robust structures; however, there is no agent available that will prevent cell adhesion and biofilm formation. Current methods rely on the use of disinfection agents and regular cleaning of surfaces exposed to possible pathogens. Research has indicated that sessile communities can be

***Corresponding Author:** Dr. Mary Garvey. Department of Life Sciences, Institute of Technology Sligo, Ash Lane, Sligo, Ireland. Tel.: +353719305841. E-mail: *garvey.mary@itsligo.ie*

up to 1000 times more resistant to chemotherapeutics such as chlorhexidine than their planktonic counterparts (Garvey *et al*., 2014a). Furthermore, resistant bacteria originated in sessile communities can spread from animal to animal through veterinary staff, veterinary surfaces and equipment or farm equipment such as feeders and water dispensers (Aguilar-Romero *et al*., 2010) resulting in extended infection problems. Biofilm structures are also capable of trapping or incorporating other pathogenic species including enteric noroviruses (Wingender and Flemming, 2011) and parasites such as *Giardia* and *Cryptosporidium* (DiCesare *et al*., 2012). Harbouring of such species shields them from cleaning and disinfection techniques, increasing their already high resistance to such treatments. Studies have shown that aquatic biofilms represent a significant, long-term reservoir for pathogens such as *Cryptosporidium* and *Giardia*, which can be released back into water (Wingender and Flemming, 2011). Thus, explaining the presence of parasites in water networks long after disinfection protocols are completed following an outbreak. Ultraviolet (UV) light is well known for its antimicrobial activity, due to its bacteriostatic properties affecting the DNA of the organism, breaking DNA bonds, causing the formation of DNA adducts thus preventing bacterial cell replication (Ochoa-Velasco *et al*., 2014). Additionally, research focusing on the use of a pulsed light (PL) system for the inactivation of parasite species and bacterial endospores has shown this system to be highly efficient (Garvey *et al*., 2014a). PL technologies differ from standard UV lamps in their mode of delivery, penetration depth and wavelength range (Garvey *et al*., 2014a) making them a more potent disinfection system. Here we report on the use of a PL system for the disinfection of veterinary relevant biofilms on polyvinylchloride (PVC) and stainless steel surfaces. The use of polymerase chain reaction (PCR) methods provides a rapid species specific means of identifying species type and cell numbers present. Indeed, PCR methods have been used extensively to detect and quantify bacterial cells in food products and in biofilms (Pan and Breidt, 2007). Therefore, the present study also utilised a real time PCR assay to determine the extent at which *Bacillus* biofilm structures incorporated parasite species into their matrix, subsequently providing shelter from disinfection techniques.

Materials and Methods

Microbial test species

For this study a range of veterinary relevant biofilm forming microbial species *Listeria monocytogenes* (ATCC 11994), *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 13311) and *Escherichia coli* (ATCC 11775) were chosen for biofilm formation and PL inactivation studies. All strains were cultured and maintained in nutrient agar and nutrient broth (Cruinn Diagnostics Ltd, Ireland) at 37°C. *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts were purchased from Waterborne Inc USA. Oocysts and cysts were stored in sterile PBS (0.01 M phosphate buffer, containing 0.0027 M KCL and 0.137 M NaCl 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. Prior to use parasite identity was confirmed by a dye staining method comprising of propidium iodide (PI) 1 mg/ml in 0.1 M sterile PBS and 4', 6'-Diamidino-2-Phenylindole (DAPI) 2 mg/ml in methanol and a fluorescein-labelled mouse-derived monoclonal antibody Giardi-a-Glo™ or Crypt-a-Glo™ (Waterborne Inc, New Orleans, USA). Oo/cysts were counted using a haemocytometer and inverted microscope (Olympus, CKX41) with camera (Olympus, IX2-SLP) attached.

Growth of sessile communities using Centers for Disease Control (CDC) biofilm reactor

The CDC biofilm reactor (Biosurface Technologies Corp, Bozeman, Montana, USA) was used for the growth of biofilm structures as per the recommended procedure of the American Society for Testing and Materials (ASTM, 2012). Furthermore, the CDC reactor is a recognised method for the growth of biofilms under high shear and continuous flow (Coenye and Nelis, 2010) and is of sufficient capacity to provide numerous samples of biofilms for disinfection studies. In order to establish a dose response relationship for biofilm inactivation with UV light it is necessary to first obtain biofilm communities which were dense, reproducible and also treatable. For this study both PVC and stainless steel coupons were chosen as biofilm growth surfaces as both materials are commonly used in veterinary settings and are excellent matrixes for biofilm adhesion and proliferation.

For the growth of microbial biofilms methods were followed as per the recommended procedure for continuous fluid shear flow biofilm formation (ASTM E2562-12 2012) and Garvey *et al*., 2014b. The reactor was prepared containing 350 mL of tryptone soy broth (TSB) and 2% glucose as this concentration was previously found to promote biofilm adhesion and proliferation (Senevirantne *et al*., 2013). Once satisfied that the coupons were completely submerged the apparatus was sterilised by autoclaving. 1 mL of a 12 hour microbial culture was added to the reactor chamber to ensure that cells were in the log phase of reproduction. For each test strain the reactor was incubated at 37°C for 72 and 96 hours under rotatory conditions at 125 rpm. To allow for the enumeration of colony forming units (cfu) per microbial biofilm, all coupons were removed aseptically from each reactor rod and rinsed with sterile phosphate-buffered saline (PBS) to remove any planktonic cells. Biofilms were removed from each coupon by scraping the coupon using a sterile cell scraper into 10 mL of sterile PBS. The

standard plate count technique was used to determine the cfu/mL bacterial population in the biofilm as per the recommended procedure (ASTM E2562-12 2012). To allow for the entrapment of parasite test species within the biofilm matrix $1x10^6$ oo/cysts per mL was added to the reactor chamber and incubated for 72 hours. For biofilms containing parasite test species, 1 mL from the 10 mL PBS containing the scrapped biofilm was stained with parasite specific dyes as previously described to confirm identity and numbers present.

Pulsed Ultraviolet* (PUV) *light

The PUV machine used throughout this study was sourced through Samtech Ltd, Strathclyde, Scotland, UK. The bacteriostatic effects of PL are caused by the rich and broad-spectrum UV content, the short duration, and the high peak power of the pulse. The system was used as per Garvey *et al*. (2010) and is therefore not described in further detail herein.

Pulsed light inactivation of planktonic microbial species

*E. coli, S. t*yphimurium, *L. monocytogenes* and *B. cereus* cultures were grown and maintained as previously described. For PUV studies a single colony of the test strain was aseptically transferred to 100 mL of sterile nutrient broth followed by incubation at 37°C for 12 hours at 125 rpm. For surface treatment 100 µL of an appropriate dilution was spread onto agar surfaces. Test plates were then exposed to pulses of UV light at 16.2 J at varying doses (obtained by varying the pulse number) at a rate of 1 pulse per second as per Garvey *et al*. (2014). PUV studies were also conducted on samples diluted from the 12 hour broth in 20 mL final volumes of sterile PBS at 8 cm from the light source, after which 100 µL of treated liquid was transferred to suitable agar and incubated at 37°C for 24 hours.

Pulsed light inactivation of sessile communities

Coupons were aseptically removed from the reactor, rinsed with sterile PBS and transferred to a sterile petri dish. Samples were exposed to pulses of UV light at 16.2J at 8 cm from the light source at varying UV doses which were obtained by increasing the pulse number. Once treated, coupons were submerged in 10 mL of sterile PBS and surface scraped using a sterile cell scraper to remove the treated biofilms and to allow for the determination of inactivated rates. The liquid was then transferred to a sterile 20 mL container and centrifuged at 800 g for 10 minutes to pellet the cells. The sample was then re-suspended and agitated manually to ensure biofilm dispersion. Serial dilutions were made from the biofilms suspension and 100 µL spread on triplicate agar plates to determine the cfu/mL of treated samples. This process was repeated for coupons at varying UV doses (< 8µJ/cm^2) to determine the Log_{10} reduction obtained with increasing UV dose. Plates were incubated for 24 hours at 37°C to allow for the growth of bacterial colonies, which were subsequently gram stained and identify confirmed to ensure no contamination of the rector system had occurred.

Parasite entrapment and DNA extraction from biofilm structures

Biofilms of *B. cereus* were allowed to form while in the presence of $1x10^6$ oo/cysts per mL in the biofilm reactor, to allow for the entrapment of parasite species within the biofilm matrix. This species was chosen due to its enteric pathogenic nature and it greater resistance to PUV inactivation. Following 72 hours incubation, coupons were aseptically removed from the bioreactor to sterile petri dish. Coupons were then aseptically scrapped in to 10 mL volumes of PBS, which was subsequently centrifuged at 800g for 10 minutes to pellet the cells, followed by re-suspension in 200 µl of sterile PCR grade water. Target DNA extraction for *B. cereus, G. lamblia* and *C. parvum* was conducted as per kit instructions for *B. cereus* biofilm suspensions using a Roche DNA extraction kit and HP PCR template preparation kit (Roche Diagnostics, Roche, Ireland). All steps were performed as per manufacturer's instructions with treated and untreated microbial pellets which were suspended in 200 µL of sterile PBS.

Real time PCR

All primers and probes were sourced from Tib Molbiol, Berlin, Germany. For *B. cereus* the forward primer ACACACGTGCTACAATGGATG and reverse primer AGTTGCAGCCTACAATCCGAA with the taqman probe sequence F-ACAAAGGGCTGCAAGACCGCG—Q coding for the phaC gene was used as per Nayak *et al*. (2013). Primers coding for β-giardin of *G. lamblia* were used as per method of Bertrand *et al*. (2009) with the forward primer 5′-AAGCCCGACGACCTCACCCGCAGTGC-3′ and reverse primer 5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′. The Taqman probe with the following sequence: 5'-FAM TCACCCAGACGATGGA CAAGCCCTAMRA-3 was utilised for this study. For *Cryptosporidium parvum* the 18Si reverse primer 5'- CCTGCTTTAAGCACTTAATTTTC and 18Si forward primer 5'- ATGGACAAGAAATAACAATACAGG as first described by Morgan *et al*. (1997) were utilised as per Garvey *et al*. (2010). The Taqman probe had the following sequence: 5-'-(6-FAM) ACCAGACTTGCCCTCC (TAMRA) as per Keegan *et al*. (2003). Amplification reactions (20 µL) contained 5 µL of sample DNA (0.5 µM of each primer, 0.2 µM of probe) and 15 µL of reaction buffer (Roche Diagnostic, West Sussex, England). Both positive and negative controls were included in RT-PCR to validate the results. DNase–RNase free water was used as negative control throughout. Cycling parameters were initial denaturation for 10 min at 95°C followed by 65 cycles of denaturation for 10 s at 95°C,

annealing for 40 s at 40 °C, extension for 1 s at 70°C and cooling for 30 s at 40°C on a Nanocycler® device (Roche Diagnostics). These cycling parameters were the same for all samples. Additionally, large numbers of cycles were used to ensure detection of low levels of infection. On completion of each RT-PCR run amplification curves were analysed by Nanocycler software (Roche Diagnostics) and a standard curve (Fig. 1) of cell DNA concentration determined. DNA standards were prepared from fresh cells or oo/cysts ranging in concentration from 10 to 10^8 oocysts or cysts/mL by dilution in PBS following standard viable count determinations.

Statistics

All experimental data is an average of 3 experimental replicates with 3 internal replicates. Bacterial inactivation is expressed as $\log_{10}$ reduction of the untreated control. Student's t-tests and ANOVA one-way model (MINITAB software release 16; Mintab Inc., State College, PA) were used to compare the relationship between UV treatments and bacterial inactivation at 95% level of confidence. Student t-tests were used to determine the relationship between the sensitivity of biofilms from different strains to PL treatment.

Results

Sessile communities and parasite detection

All bacterial strains under study formed densely populated sessile communities on both PVC and stainless steel surfaces after 72 hours. Findings also demonstrate (data not shown) that with longer incubation times, exceeding 72 hours (96 hours), there was no increase in cell number of the biofilms as detected by plate counts. Following 72 hours, a ca. 5 and 6.6 $\log_{10}$ biofilm formed for *B. cereus* and *S. typhimurium* respectively, and a 6 $\log_{10}$ for *E. coli* and 6.5 $\log_{10}$ for *L. monocytogenes* on PVC surfaces. A similar level of cell density was detected on stainless steel surfaces, where a ca. 5 $\log_{10}$ to 6.6 $\log_{10}$ biofilm formed for *B. cereus, L. monocytogenes*, *S. typhimurium* and *E. coli*. The determination of cell number for *B. cereus* biofilms via PCR was slightly higher than the standard cell count method. A Ct value of 18.39, corresponding to a cell count of ca. 7 $\log_{10}$ cfu/ml (Fig. 1) for both materials was determined by analysis of the standard curve. An important fact to note is that PCR detects the presence of target DNA, but cannot differentiate between live and dead cells. In contrast, the standard cell count technique reports viable cell numbers only via the enumeration of colonies grown on nutrient agar. The lack of an increase in biofilm cell density after a 72 hours period suggests the presence of a stationary phase or steady state of biofilm growth. PCR analyses showed the presence of total cells (non-viable and viable) at 72 hours, when viable cell counts as determined by the spread plate technique are subtracted from this, a value for non-viable cells can be determined. In this case a biofilm viable cell density of 5 $\log_{10}$ was formed, indicating that approximately 2 $\log_{10}$ of non-viable cells were also present in the biofilm matrix as detected by PCR. The presence of these non-viable cells further confirms that incubation for 72 hours provided an optimal period of time for biofilm formation, after which cell death occurs to some extent. These findings correspond to that of Senevirantne *et al*. (2013), who concluded that 72 hours was also the optimal incubation time for the growth of *Enterococcus faecalis* biofilms. Therefore, the findings of this study suggest that 72 hour duration of incubation is sufficient to reproducibly produce a robust, densely populated biofilm of *B. cereus, E. coli, L. monocytogenes* and *S. typhimurium* using a CDC reactor. Consequently, 72 hour biofilms were used for inactivation studies for all test species.

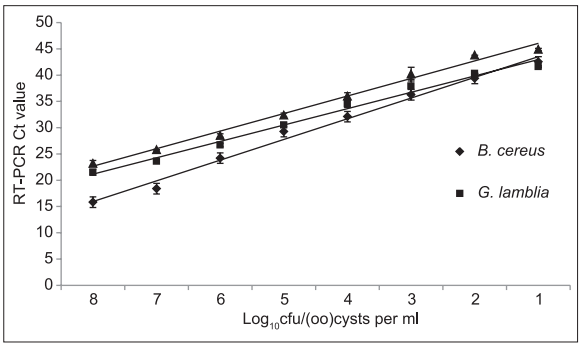

Fig. 1. Linear regression analysis of DNA standard curve as determined by real time PCR analysis for planktonic *Bacillus cereus* ($\log_{10}$ cfu/ml) and the parasite species *Cryptosporidium parvum* ($\log_{10}$ oocysts/ml) and *Giardia lamblia* ($\log_{10}$ cfu/(oo) cysts per ml) (+/-S.D) using species specific primers.

Both parasites species were detected in the *B. cereus* biofilms at a concentration of between 2 and 3 $\log_{10}$ for PVC and stainless steel surfaces by PCR (Fig. 2). Additionally, PCR proved a more efficient reliable method of detecting *Cryptosporidium* and *Giardia* than the use of specific dyes. Fluorescent dye staining of biofilms containing oo/cysts greatly underestimated the number of organisms present. A maximal oo/cyst count of 10 (+/-2) was measured for *C. parvum* and 14 for *G. lamblia* (+/-4) via fluorescent staining. Issues arose in relation to non-specific binding of dyes to biofilm constitutes believed to be EPS components resulting in unreliable counting of parasite numbers.

The impact of PL on microbial species was assessed for surface treated organisms, organisms in suspension and sessile communities. All test strains proved to be susceptible to the pulsed light treatment, albeit with varying levels of sensitivity as shown in (Figs. 3 and 4) *E. coli* showed the greatest level of inactivation on agar surfaces (Fig. 3a) with complete inactivation of an initial concentration of ca. 9 $\log_{10}$ with as little as 5 $\mu J/cm^2$ of pulsed light. The order of decreasing sensitivity for test

strains was *E. coli, L. monocytogenes, B. cereus* and *S. typhimurium* on surfaces. When treated in suspension this sensitivity changed with *L. monocytogenes* showing the highest resistance to PL treatment and *S. typhimurium* showing the greatest sensitivity to pulsed light (Fig. 3b) for all treatment doses ($p<0.05$). Indeed a maximal 9.6 $\log_{10}$ inactivation of *S. typhimurium* was achieved with 5.39 µJ/cm^2 compared to a 2.73 $\log_{10}$ for *L. monocytogenes*. This same dose resulted in a 3.45 and 5.38 $\log_{10}$ inactivation of *B. cereus* and *E. coli* respectively, highlighting the significant difference in susceptibility to pulsed light. These findings are in conjunction with Cheigh *et al*. (2012) where *E. coli* also proved more sensitive to PL than *L. monocytogenes* when treated in suspension. High levels of biofilm inactivation were also achieved for all test strains present on both surface materials (Fig. 4). For the Gram negative species *E. coli* and *S. typhimurium* a 4.04 and 5.11 $\log_{10}$ reduction in viable cell counts was obtained on PVC surfaces with 5.39 µJ/cm^2 (Fig. 4a). This same dose resulted in a significantly ($p<0.05$) greater level of inactivation of the same species on stainless steel surfaces, with a maximal 4.2 and 6.6 $\log_{10}$ reduction obtained for *E. coli* and *S. typhimurium* respectively (Fig. 4b). Both Gram positive species tested showed increased sensitivity on stainless steel surfaces compared to PVC. A dose of 5.39 µJ/cm^2 resulted in a 3.23 and a 4.34 $\log_{10}$ inactivation on PVC and 5.95 and 4.6 $\log_{10}$ inactivation on stainless steel for *B. cereus* and *L. monocytogenes* respectively. A PL dose of 7.56 µJ/cm^2 resulted in complete inactivation of *L. monocytogenes* and *S. typhimurium* of ca. 6.51 $\log_{10}$ (Fig. 4a) on PVC surfaces.

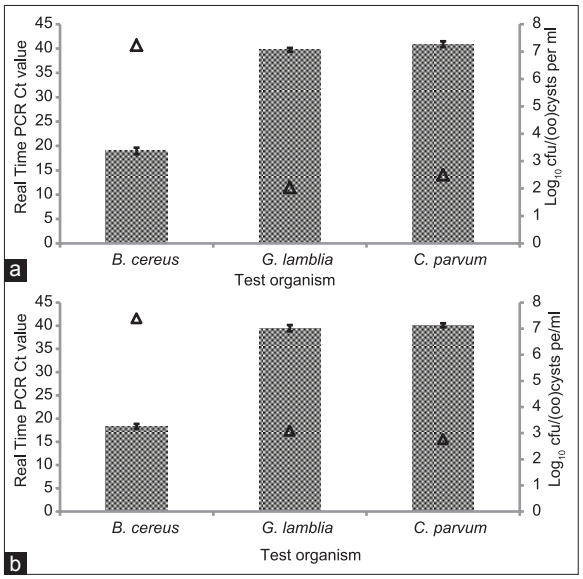

Fig. 2. Real time PCR Ct value (column graph) for microbial test species and corresponding cell count in $\log_{10}$ cfu/(oo)cysts per ml (**Δ**) as determined by using the equation of the line of the standard curves, results show both parasite test species as detected in *B. cereus* biofilms on (a) PVC and (b) stainless steel surfaces (+/- S.D).

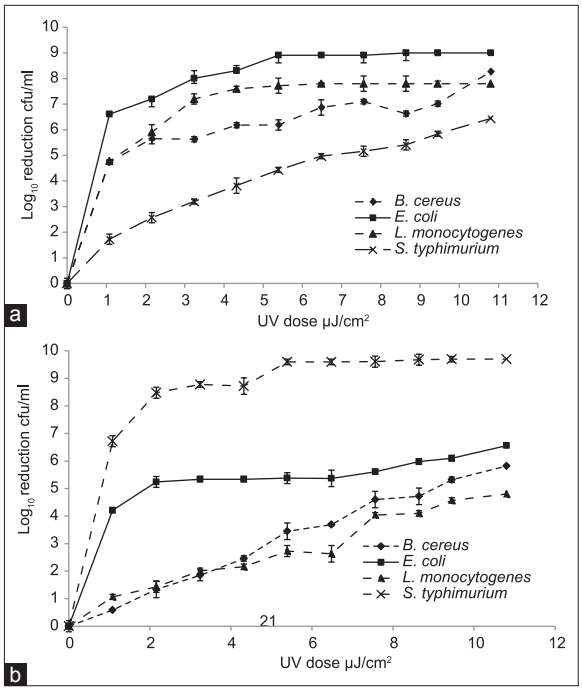

Fig. 3. Pulsed light inactivation of a range of Gram negative and Gram positive test species on (a) agar surfaces and (b) in suspension (+/- S.D).

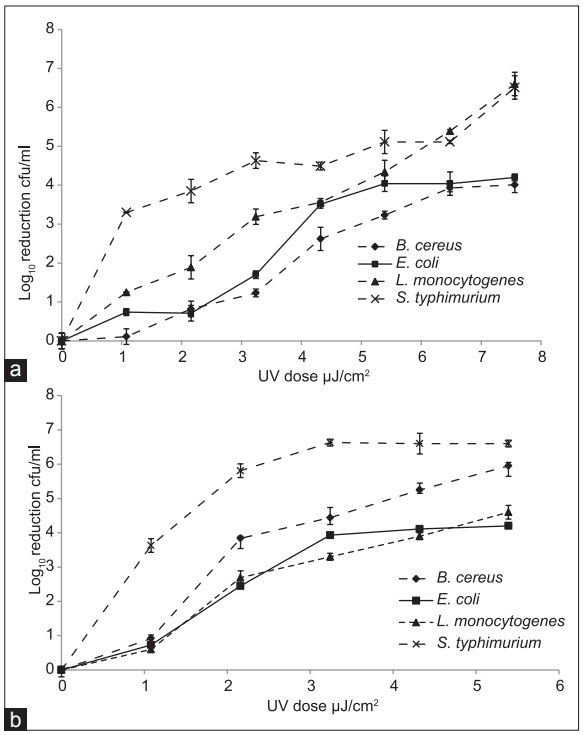

Fig. 4. Pulsed light inactivation of bacterial biofilms of varying test species on (a) PVC surfaces and (b) stainless steel surfaces (+/- S.D).

Discussion

The change from a planktonic free floating cell to that of a biofilm sessile cell induces physiological changes in bacteria, occurring via a series of gene expression alterations including gene repression and induction (Donlan and Costerton, 2002). It is the induction of genes, relating to antibiotic resistance that leads to the increased pathogenicity of sessile bacteria over their planktonic counterparts (O'Leary *et al*., 2015). Consequently, this causes the increased resistance to antibiotics and disinfectants such as chlorine commonly observed with these complex structures (Aguilar-Romero *et al*., 2010). For this reason, it is of the upmost importance to establish alternative ways of eradicating these problematic often pathogenic structures from veterinary surfaces. The pulsed light system used in this study proved successful at disinfecting densely populated biofilms of veterinary relevant microorganisms. Indeed, complete inactivation of a 6.5 $\log_{10}$ biofilm of *L. monocytogenes* and *S. typhimurium* was achieved with 7.56 μJ/cm^2. However, there was a significant difference ($p<0.05$) in the susceptibility of biofilm communities on PVC and stainless steel surfaces. *S. typhimurium* and *B. cereus* proved more sensitive to pulsed light inactivation on stainless steel surfaces compared to PVC. Stainless steel is the predominant material used in veterinary practices as clinical surfaces and animal housing due to their easy to clean nature. Consequently, the higher susceptibility of microbial biofilms on stainless steel surfaces further establishes this materials benefit for use in such environments.

Traditionally, the sensitivity of planktonic cells to disinfection has been used as an indication of biofilm sensitivity and resistance (Buckingham-Meyer *et al*., 2007). However, disinfection studies such as those described herein based on actual biofilm communities is much more representative of the environmental situation. Additionally, high levels of planktonic cell inactivation (4 – 9 $\log_{10}$ cfu/ml) were also achieved following pulsed UV exposure for both surface treated and microbial suspensions. Therefore, pulsed light as a disinfectant has the ability to reduce biofilm formation at the planktonic stage of attachment, which can be assisted by choosing surface materials that are more readily disinfected by this approach such as stainless steel.

The findings of this study confirm that both parasite species studied can quickly attach or become entrapped in bacterial biofilms. This is in keeping with the findings of recently published literate outlining the presence of parasite species in biofilm structures (DiCesare *et al*., 2012; Koh *et al*., 2013). The findings of Koh *et al.* (2013) conclude that biofilm communities accumulate *Cryptosporidium* species over time as determined by qPCR detection. The detection of these pathogens within biofilm structures has important public health implications in relation to animal and human exposure. The infectious dose for *Cryptosporidium* has been established to be less than 20 oocysts (Zambriski *et al*., 2013) with prolonged infection occurring with little success following medical intervention. The robust, chemical disinfection resistant nature of biofilms and these parasites increases the probability that the survival and detachment of biofilm-associated viable parasites may occur at concentrations exceeding that required for infection. This possibility needs to be considered in risk assessments relating to the cleaning of veterinary environments particularly where animals are housed.

Previous studies by this research group reported a ca. 5 $\log_{10}$ inactivation of *Cryptosporidium parvum* (Garvey *et al*., 2013) and ca. 1 $\log_{10}$ inactivation of *Giardia lamblia* (Garvey *et al*., 2014b) with a PL dose of 7.38 μJ/cm^2. Nevertheless, further studies are warranted to determine the exact dose required to inactivate parasites within biofilm matrixes, which will undoubtedly shield parasites to some extent. However, issues are expected to arise in relation to viability determination post treatment and cell culture infectivity. Specifically, issues relating to the sterility of the parasites following extraction from biofilms and subsequent exposure to mammalian cell lines. Nonetheless, PL shows potential for use as a disinfectant for veterinary environments given its highly effective bacteriostatic properties towards bacterial biofilms and parasite species. Regardless of microbial exposure to PL in suspension or on surfaces findings demonstrate that cell inactivation increased significantly ($p<0.05$) with increasing UV dose or treatment time.

In conclusion, the findings reported here contribute to existing literature in many ways:

Firstly, all veterinary relevant strains produced densely populated biofilms structures on both surface materials used.

Secondly, PL repeatedly inactivated the range of test species on surfaces and in suspension. Additionally, it provided high levels of biofilm inactivation on PVC and stainless steel surfaces.

Thirdly, a real time PCR assay proved successful for determining the level of *C. parvum* and *G. lamblia* present in the biofilms of *B. cereus* where fluorescent staining greatly underestimated the numbers present.

Finally, pulsed light doses (7.38 μJ/cm^2) which have been previously shown to inactivate both parasite species (*Cryptosporidium* and *Giardia)*, have also provided complete inactivation of all biofilms tested.

Acknowledgments

All research studies were conducted in the Bioscience Research Institute, Athlone Institute of Technology, Ireland.

Conflict of interest

The authors declare that there is no conflict of interest.

References

Aguilar-Romero, F., Perez-Romero, A.N., Diaz-Aparicio, E. and Hernandez-Castro, R. 2010. Bacterial biofilms: Importance in animal diseases. Current Research, Technology and Education topics in Applied Microbiology and Microbial Biotechnology. A Mendez Vilas Edition, pp: 700-703.

ASTM. 2012. ASTM E2562 - 12: Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with High Shear and Continuous Flow Using CDC Biofilm Reactor. In Annual Book of ASTM Standards; ASTM International: West Conshohocken, PA, USA.

Bertrand, I., Maux, M., Helmi, K., Hoffmann, L., Schwartzbrod, J. and Cauchie, H.M. 2009. Quantification of *Giardia* transcripts during *in vitro* excystation: Interest for the estimation of cyst viability. Water Res. 43, 2728-2738.

Betancourt, W.Q. and Rose, J.B. 2004. Drinking water treatment processes for removal of *Cryptosporidium* and *Giardia.* Vet. Parasitol. 126(1-2), 219-234.

Buckingham-Meyer, K., Goeres, D.M. and Hamilton, M.A. 2007. Comparative evaluation of biofilm disinfectant efficacy tests. J. Microbiol. Methods 70, 236-244.

Cheigh, C.I., Park, M.H., Chung, M.S., Shin, J.K. and Park, Y.S. 2012. Comparison of intense pulsed light- and ultraviolet (UVC)-induced cell damage in *Listeria monocytogenes* and *Escherichia coli* O157:H7. Food Control 25(2), 654-659.

Clutterbuck, A.L., Woods, E.J., Knottenbelt, D.C., Clegg, P.D., Cochrane, C.A. and Percival, S.L. 2007. Biofilms and their relevance to veterinary medicine. Vet. Microbiol. 121, 1-17.

Coenye, T. and Nelis J.J. 2010. *In vitro* and *in vivo* model systems to study microbial biofilm formation. J. Microbiol. Methods 83, 89-105.

DiCesare, E.A., Hargreaves, B.R. and Jellison, K.L. 2012. Biofilm roughness determines *Cryptosporidium parvum* retention in environmental biofilms. Appl. Environ. Microbiol. 78(12), 4187-4193.

Donlan, R.M. and Costerton, J.W. 2002. Biofilms: survival Mechanisms of Clinically Relevant Microorganisms. Clin. Microbiol. Rev. 15(2), 167-193.

Garvey, M., Rabbit, D., Stocca, A. and Rowan, N. 2014a. Pulsed Ultraviolet light inactivation of Pseudomonas aeruginosa biofilms. Water Environ. J. 29, 36-42. DOI: 10.1111/wej.12088.

Garvey, M., Stocca, A. and Rowan, N. 2014b. 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*. Exp. Parasitol. 144, 6-13.

Garvey, M., Clifford, E., O'Reilly, E. and Rowan, N. 2013. Efficacy of using harmless Bacillus endospores to estimate the inactivation of Cryptosporidium parvum oocysts in water. J. Parasitol. 99(3), 448-452.

Garvey, M., Farrell, H., Cormican, M. and Rowan, N. 2010. Investigations of the relationship between use of *in vitro* cell culture-quantitative PCR and a mouse-based bioassay for evaluating critical factors affecting the disinfection performance of pulsed UV light for treating *Cryptosporidium parvum* oocysts in saline. J. Microbiol. Methods 80(3), 267-273.

Gebel, J., Exner, M., French, G., Chartier, Y., Christiansen, B., Gemein, S., Goroncy-Bermes, P., Harteman, P., Heudorf, U., Kramer, A., Maillard, J.Y., Oltmanns, P., Rotter, M. and Sonntag, G.H. 2013. The role of surface disinfection in infection prevention. GMS Hyg. Infect. Control 8(1): Doc10. doi:10.3205/dgkh000210.

Grant, S. and Olsen, C.W. 1999. Preventing Zoonotic Diseases in Immunocompromised Persons: The Role of Physicians and Veterinarians. Emerg. Infect. Dis. 5(1), 159-163.

Keegan, A.R., Fanok, S., Monis, P.T. and Saint, C.P. 2003. Cell culture-Taqman PCR assay for evaluation of Cryptosporidium parvum disinfection. Appl. Environ. Microbiol. 69, 2505-2511.

Koh, W., Clode, P.L., Monis, P. and Thompson, R.C. 2013. Multiplication of the waterborne pathogen Cryptosporidium parvum in an aquatic biofilm system. Parasit. Vectors. doi: 10.1186/1756-3305-6-270.

Morgan, U.M., Constantine, C.C., Forbes, D.A. and Thompson, R.C. 1997. Differentiation between human and animal isolates of Cryptosporidium parvum using rDNA sequencing and direct PCR analysis. J. Parasitol. 83(5), 825-830.

Nayak, P.K., KumarMohanty, A., Gaonkar, T., Kumar, A., Bhosle, S.N. and Garg, S. 2013. Rapid Identification of Polyhydroxyalkanoate Accumulating Members of Bacillales Using Internal Primers for phaC Gene of Bacillus megaterium. ISRN Bacteriol. 1-12: doi:10.1155/2013/562014.

Ochoa-Velasco, C.E., Cruz-Gonzalez, M. and Guerrero-Beltran, J.A. 2014. Ultraviolet-C light inactivation of Escherichia coli And Salmonella typhimuriumin coconut (Cocos nucifera L.) milk. Innovative Food Sci. Emerg. Technol. 26, 199-204.

O'Leary, D., McCabe, E.M., McCusker, M.P., Martins, M., Fanning, S. and Duffy, G. 2015. Acid environments affect biofilm formation and gene expression in isolates of Salmonella enterica Typhimurium DT104. Int. J. Food Microbiol. 206, 7-16.

Pan, Y. and Breidt, F. 2007. Enumeration of Viable Listeria monocytogenes Cells by Real-Time PCR with Propidium Monoazide and Ethidium

Monoazide in the Presence of Dead Cells. Appl. Environ. Microbiol. 73(24), 8028-8031.

Senevirantne, C.J., Yip, J.W., Chang, J.W., Zhang, C.F. and Samaranayake, L.P. 2013. Effect of culture media and nutrients on biofilm growth kinetics of laboratory and clinical strains of Enterococcus faecalis. Arch. Oral Biol. 58, 1327-1334.

Stewart, P.S. and Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358(9276), 135-138.

Wingender, J. and Flemming, H.C. 2011. Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Environ. Health 214(6), 417-423.

Zambriski, J.A., Nydam, D.V., Wilcox, Z.J., Bowman, D.D., Mohammed, H.O. and Littoa, J.L. 2013. *Cryptosporidium parvum*: Determination of ID50 and the dose–response relationship in experimentally challenged dairy calves. Vet. Parasitol. 197, 104-112.