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

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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 pathogen 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...
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 1 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 (Seneviratne 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
E. coli, S. typhimurium, 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 16.2 J 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/cm²) 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 identified confirmed to ensure no contamination of the reactor system had occurred.

**Parasite entrapment and DNA extraction from biofilm structures**

Biofilms of B. cereus were allowed to form while in the presence of 1x10⁶ 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 its greater resistance to PUV inactivation. Following 72 hours incubation, coupons were aseptically removed from the bioreactor and the high peak power of the pulse. The system was rinsed with sterile PBS and transferred to 10 mL of sterile 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 described in further detail herein.

**Real time PCR**

All primers and probes were sourced from Tib Molbiol, Berlin, Germany. For B. cereus the forward primer ACACACGTGCTACAATGGGAT and reverse primer AGTTGCAGCCTACAATCCGAA with the taqman probe sequence F-ACAAGGGCTGCAAGACCGCQ-G—Q coding for thephaC 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′-AAGCCCCAGCAGCCTCACC CGCACGTGC-3′ and reverse primer 5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′. The Taqman probe with the following sequence: 5′-FAM TCACCAGACGATGGA CAAGCCTAMRA-3′ was utilised for this study. For Cryptosporidium parvum the 18Si reverse primer 5′- CCTGCTTTAAGCACTTAATTTTTC and 18Si forward primer 5′- ATGGACAAGAAATAAACATGAG 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) ACCAGACTTGGCCCTCC (TMRA) as per Keegan et al. (2003). Amplification reactions (20 μL) contained 5μL 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 90 °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 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 Senevirante et al. (2013), who concluded that 72 hours was also the optimal incubation time for 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.

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. lambia (+/-4) via fluorescent staining. Issues arose in relation to non-specific binding of dyes to biofilm constituents 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 μJ/cm² 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 log₁₀ inactivation of *S. typhimurium* was achieved with 5.39 μJ/cm² compared to a 2.73 log₁₀ for *L. monocytogenes*. This same dose resulted in a 3.45 and 5.38 log₁₀ 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 log₁₀ reduction in viable cell counts was obtained on PVC surfaces with 5.39 μJ/cm² (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 log₁₀ 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/cm² resulted in a 3.23 and a 4.34 log₁₀ inactivation on PVC and 5.95 and 4.6 log₁₀ inactivation on stainless steel for *B. cereus* and *L. monocytogenes* respectively. A PL dose of 7.56 μJ/cm² resulted in complete inactivation of *L. monocytogenes* and *S. typhimurium* of ca. 6.51 log₁₀ (Fig. 4a) on PVC surfaces.
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 utmost 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 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/cm²) which have been previously shown to inactivate both parasite species (Cryptosporidium and Giardia), have also provided complete inactivation of all biofilms tested.

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Conflict of interest
The authors declare that there is no conflict of interest.
References


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