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Long amplicon (LA)-qPCR for the discrimination of infectious and noninfectious phix174 bacteriophages after UV inactivation

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ABSTRACT

Waterborne viruses are increasingly being considered in risk assessment schemes. In general, virus detection by culture methods is time consuming. In contrast, detection by quantitative polymerase chain reaction (qPCR) is more rapid and therefore, more suitable for monitoring. At present, qPCR lacks the essential ability for discriminating between infectious and non-infectious viruses, thus limiting its applicability for monitoring disinfection processes. In this study, a method was developed to quantify UV inactivation by long amplicon (LA)-qPCR. Bacteriophage phiX174 was used as a surrogate for human pathogenic viruses. A qPCR protocol was developed with new sets of primers, resulting in amplicon lengths of 108, 250, 456, 568, 955, 1063, 1544, and 1764 nucleotides. The log reduction of gene copies increased with increasing amplicon length. Additional treatment with the intercalating dye, PMA, had no effect, indicating that the bacteriophage capsids were not damaged by low pressure UV irradiation. A qPCR of nearly the complete genome (approx. 5000 nucleotides) showed similar results to the plaque assay. The log reduction in qPCR correlates with [specific amplicon length x UV dose]. The normalized DNA effect constant can be applied to calculate phiX174 inactivation based on qPCR detection.

1. Introduction

In recent years, polymerase chain reaction (PCR) detection methods have been developed for many health-related waterborne microorganisms (Brettar and Höfle, 2008). The application of molecular methods, such as PCR, enables more rapid detection of pathogenic bacteria and viruses compared to standard culture methods. Highly specific PCR can be created for each microbe of interest by in silico primer design. Quantitative PCR (qPCR) uses fluorescent dyes such as SYBR Green or probes to determine the DNA concentration of each amplification cycle, and quantitative results can be obtained within few hours. qPCR has become a powerful and widespread tool for the detection of pathogens and microbial indicators (Ahmed et al., 2014; Girones et al., 2010; Kunze et al., 2015), microbial source tracking (Gomez-Donate et al., 2012; Raith et al., 2013) and the direct detection of viruses in wastewater treatment plants (Ottoson et al., 2006), infected patients (Mesquita et al., 2011) and shellfish (Costafreda et al., 2006). The quantification of viruses in drinking water is of increasing interest because viruses may react differently to water treatment processes when compared to individual fecal indicator bacteria (WHO, 2011). Bacteriophages serve as model organisms as they are more easily manageable in water laboratories than viruses that are pathogenic to humans (Pei et al., 2012). Furthermore, standardized cultivation assays and qPCR method exist for comparison studies.

To date, qPCR detection lacks the ability to differentiate between infectious and non-infectious organisms (Szewzyk et al., 2000). The correlation of results from cultivation and qPCR is essential for the monitoring of disinfection and quantitative microbial risk assessment. To provide safe water, several methods for disinfection are used in water treatment. In Germany, UV is used in 42% of disinfecting waterworks (Niehues, 2009). UV radiation is also applied in widespread areas, such as water treatment plants (Simmons and Xagoraraki, 2011), swimming pools (WHO, 2006) or for the reuse of wastewater for crop irrigation (Nasser et al., 2006; Verbyla and Mihelcic, 2015).

The treatment with UV causes direct DNA damage, as photons are absorbed by the DNA nucleotides thymine and cytosine. During UV irradiation, adjacent nucleotides can form dimers and induce

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strand breaks preventing the action of polymerases (Rastogi et al., 2010). Proteins can also absorb UV light, resulting in damage of viral surface structures. However, this effect is only observed for medium-pressure UV lamps, which emit at a broader wavelength (Eischeid and Linden, 2011). In contrast, low-pressure UV lamps emit at 254 nm, which is close to the adsorption maximum of DNA (260 nm). UV light has been found to be effective against all waterborne pathogens, specifically, viruses, bacteria and parasites (Hijnen et al., 2006). The dose of UV disinfection in Germany must exceed 400 J/m^2 (§ 11 TrinkwV, 2001), which is similar to the requirements in other European countries or the USA.

To date, disinfection studies have shown that the results obtained by conventional qPCR often do not correspond to the results obtained in cultivation tests (Calgua et al., 2014; Duizer et al., 2004; Leifels et al., 2015; Pecson et al., 2009). Therefore, several advanced methods have been suggested for the discrimination between living and dead organisms by PCR detection.

The treatment of samples with fluorescent dyes, such as propidium monoazide (PMA) or ethidium monoazide (EMA), has been studied in recent years (Nocker et al., 2006). These photoactive fluorescent dyes can only enter cells with damaged membranes and intercalate with nucleic acids. When exposed to blue light, the azido group of these dyes bind covalently to single or double stranded DNA or RNA (Prevost et al., 2016). Labelled nucleic acids cannot be amplified in PCR and consequently, only nucleic acids of intact organisms are available for PCR. While this method was mainly used for bacteria, some studies with viruses are also available (Fittipaldi et al., 2010).

For many years, qPCR detection was based on the amplification of short DNA fragments, i.e., 100 to 200 base pairs (bp). However, in recent years the detection of larger amplicons, i.e., exceeding 200 bp, has been considered for practical application due to the availability of affordable polymerases. Studies on *E. coli* (Rudi et al., 2010), hepatitis B virus (Allain et al., 2006) and caliciviruses (Xu et al., 2015) have demonstrated that longer amplicons in qPCR increase the probability of detecting DNA damage caused by UV treatment. A recent study (Beck et al., 2014) also successfully demonstrated the use of a two-step PCR with long amplicons to capture UV damage in adenovirus. A long amplicon RT-PCR was also reported for RNA viruses (Tellier et al., 1996; Zhang et al., 2001). Furthermore, the detection of DNA damage in UV treated adenovirus by integrated cell culture PCR (ICC-qPCR) has been described (Ryu et al., 2015).

In our study, we investigated the effect of amplicon size and PMA on qPCR results. The bacteriophage phiX174 was used as model virus for the experiments. Phages are often used as process controls and surrogates for pathogenic viruses, in applications such as testing UV disinfection (Li et al., 2011), removal of viruses by flocculation (Kreißel et al., 2014), inactivation of viruses in food industry (Davis et al., 2015) or for material integrity tests (Standard ASTM F1671).

The aim of this study was to develop a qPCR method allowing for the rapid monitoring of UV disinfection. The model viruses in tap water were treated with different doses of low pressure UV, and the samples were analyzed via qPCR with different amplicon sizes. The results were also compared to the standardized plaque assay.

2. Material and methods

2.1. Plaque assay and phage stock solutions

Phage plaque assays were performed according to DIN EN ISO 10705–2:2001. phiX174 (DSM-4497) and its host *E. coli* (DSM-13127) were supplied by DSMZ (German Collection of Microorganisms and Cell Cultures). For the treatment experiments, a phage stock solution was produced. The phages were concentrated using *Vivaspin*® 15R 30 kDa centrifugation units (Sartorius, Göttingen, Germany) and washed three times using tap water to remove the culture media. The washed phages were added to one liter of tap water to final concentrations of 10^8 - 10^9 PFU per mL.

2.2. qPCR analysis

2.2.1. Primer design

Primers were taken from previously published literature as stated below or were designed using Primer Blast (Ye et al., 2012). For primer design, the complete genome sequence (NC_001422) of phiX174 was used. Using different primer combinations, qPCR amplicons of 110–1764 bp were obtained. The sequences of all of the primers used in this study are listed in Table 1.

2.2.2. qPCR

All qPCRs were performed using a Rotor-Gene 6000 cycler (Corbett, Mortlake, Australia) with *SensiMix SYBR No-Rox Kit* (Bioline, Luckenwalde, Germany) for amplicons up to 568 bp or *SsoFast EvaGreen* (BIO-RAD, München, Germany) for larger amplicons. qPCR efficiency for long amplicons was very low using SensiMix. Better efficiencies were achieved with SsoFast EvaGreen, most likely due to a different enzyme and a shorter initial activation phase not damaging the template DNA. The PCR program used was as follows: 15 min at 95 °C (initial phase), 45 cycles of 20 s at 94 °C (denaturation), 20 s at 60 °C (annealing) and 20 s at 72 °C (elongation), followed by melting curve analysis for *SensiMix*. The initial incubation step was set to 3 min for the reaction kit *SsoFast EvaGreen*. The elongation time was increased for larger amplicons, as shown in Table 2. For complete genome amplification with the 648FW and 369RW primers, the following PCR program was used: 1 min at 95 °C for initial denaturation, followed by 35 cycles of from 10^1 to 10^7 genomic copies per µL was used.

For qPCR calibration, an extract of phiX174 ssDNA was used. The

Table 1
Primer sequences used in this study.

No.	Orientation	Position (NC_001422)	Sequence (5′-3′)	Source
1	FW	4758	GGCCGTCTTCATTTCCATGC	This Study
2	FW	4916	GCAGGACGCTTTTTCACGTT	This Study
3	FW	11	GCTTCCATGACGCAGAAGTT	(Crews et al., 2007)
4	FW	119	ACTGCTGGCGGAAAATGAGA	This study
5	FW	261	GGTTCGTCAAGGACTGGTTT	(Crews et al., 2007)
6	RW	369	TTGAACAGCATCGGACTCAG	(Crews et al., 2007)
7	RW	575	CGTACCATAAACGCAAGCC	This Study
8	FW	648	CATCCCGTCAACATTCAAACG	(Myers et al., 2009)
9	RW	687	CTTCCATGATGAGACAGGC	(Weaver and Malling, 2003)
10	RW	1074	ATCTGACCAGCAAGGAAGCC	This Study
11	RW	1136	CGTCCATCTCGAAGGAGTCG	This Study