Detection of viral aerosols by use of real-time quantitative PCR

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Received: 11 November 2008 / Accepted: 9 January 2009 / Published online: 4 February 2009
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Abstract PCR quantification is regarded as one of the most promising techniques for real-time identification of bio-aerosols. We have, therefore, validated a QPCR assay for quantification of a viral aerosol sample using the double-stranded DNA-binding dye SYBR green I, an economical alternative for quantification of target microorganisms. To achieve this objective we used mycobacteriophage D29 as model organism. Phage D29 aerosol was produced in an aerosol cabinet and then collected by use of an AGI liquid sampler. A standard curve was created by use of purified genomic DNA from the phage in liquid culture of known concentration measured by titration. To prevent false-positive results caused by formation of primer–dimers, an additional data-acquisition step was added to the three-step QPCR procedure; the new technique was called four-step QPCR. The standard curve was then used to quantify the total amount of phage D29 in liquid culture and aerosol samples. For liquid culture samples there was no significant difference ($P > 0.05$) between results from quantification of the virus using double-agar culture and QPCR. For aerosol samples, however, the result determined by the QPCR method was significantly

($P < 0.05$) higher than that from the double-agar culture method. The four-step SYBR green I QPCR method is a quick quantitative method for mycobacteriophage D29 aerosol. We believe that QPCR using SYBR green I dye will be an economical method for detection of airborne bio-aerosols.

Keywords QPCR · Viral aerosols · SYBR green I

1 Introduction

The sampling and analysis of airborne microorganisms has attracted much attention in recent years owing to concerns about mold contamination in indoor environments and the threat of bioterrorism. *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, and smallpox virus are causative agents of tularemia, plague, anthrax, and smallpox, respectively. These organisms and others have been listed as possible weapons of mass destruction (WMD), because of their potential to cause acute illness in the exposed public following aerosol release (Stetzenbach et al. 2004). The 2005 budget allocated to biological terrorism countermeasures by the US Department of Homeland Security (DHS) alone is over \$350 million. Any amount of biowarfare (BW) agents from a few milligrams to several kilograms may be released during a terrorist attack (Sabelnikov

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et al. 2006). Although the particles in bio-aerosols are generally 0.3–100 μm in diameter, the inhalable size fraction of 1–10 μm is of primary concern. Exposure to bio-aerosol particles, including airborne infectious agents, indoor allergens, fungal agents, and others, is usually measured by collecting such particles in solid, liquid, or agar media followed by qualitative and/or quantitative sample analysis using microscopic, biochemical, immunochemical, or molecular techniques (An et al. 2006). Microorganisms are capable of causing health effects whether they are in a culturable or non-culturable state. Culture also requires time for the organisms to grow into recognizable colonies, often taking days to weeks, depending on the microorganism. Light microscopy is tedious and lacks identification specificity, unless accompanied by specialized staining or immunological assay. Biochemical assays that measure (1,3)- γ -D-glucan and ergosterol indicate the presence of fungal biomass (Goto et al. 1994; Rylander 1999) which is useful in assessing the overall levels of fungal contamination, but these indicators do not reveal the presence of specific organisms. The inaccuracy of classical methods, lack of specificity, and lengthy analysis time required to characterize airborne bio-contaminant concentrations and populations emphasize the need to develop new sampling and analysis techniques that can provide rapid, reliable data for bio-aerosol exposure.

The use of molecular methods, for example quantitative polymerase chain reaction (QPCR) amplification, can enhance monitoring strategies by increasing sensitivity and specificity while reducing the time required for analysis. QPCR is evolving into a promising tool capable of reproducible and accurate measurement of total microorganism concentrations in environmental samples. The advantage of QPCR is its capacity for rapid sample quantification and species-specific identification. Furthermore, unlike conventional PCR, QPCR does not require post-PCR analysis, for example gel-electrophoresis. This detection system combines a thermal cycler and an optical module. At the end of the extension phase of each PCR cycle the optical module measures the fluorescence intensity of each cycle generated either by hybridization probes (TaqMan, molecular beacon, or fluorescence resonance energy transfer (FRET)) or by double-stranded DNA dyes such as Sybrgreen green (SYBR) (O'Mahony and Hill 2002; Stetzenbach et al.

2004). The data analysis software provided with a QPCR system calculates a threshold based on background fluorescence, and determines the cycle number (CT) at which the fluorescence in the sample crosses this threshold. The CT is inversely correlated with the concentration of the microorganism in the sample. By using known microorganism concentrations as templates, standard curves can be produced to quantify total target microorganism concentrations in an unknown sample. This method has been successfully used for detection of airborne microorganisms in environmental samples (Meklin et al. 2007; Vesper et al. 2005; Zeng et al. 2004, 2006; Pyankov et al. 2007; Hietala et al. 2005; Haugland et al. 1999, 2002). Sequence-specific fluorescence-labeled probes (e.g., TaqMan) have been regarded as a conventional detection method in many diagnostic and research applications but are not suitable for quantification of a large number of different sequences, because a new and relatively expensive probe is usually required for each amplicon under investigation. We have therefore optimized and validated a QPCR assay for accurate quantification of aerosol samples using the double-stranded DNA-binding dye SYBR green I, a much more economical alternative for quantification of target microorganisms. The main objective of the work discussed in this paper was to develop a QPCR detection system for quantification of a viral aerosol sample using SYBR green I. For this purpose, we used mycobacteriophage D29 as model organism and developed standard curves by using purified genomic DNA from known liquid phage culture titrated to a certain CT value determined by QPCR. The standard curves were then used to quantify the amount of total phage D29 in liquid culture and aerosol samples.

2 Methods

2.1 Microorganism

As test organism for this study, we used mycobacteriophage D29 as simulating virus. *M. smegmatis* strain (ATCC 607) was the host bacterium of phage D29. Phage and mycobacteria were obtained from National Institute for the Control of Pharmaceutical and Biological Products of China. Phage Φ X174, phage f2, and phage SM701 were culture collections

in our laboratory. Phage D29 was prepared in *M. smegmatis* by the standard method and stored, as described by Eltringham et al. (1999).

2.2 Titer of phage D29 determined by plaque-forming units (pfu) count

The titer of phage D29 was determined by the double-agar cultivation method. *M. smegmatis* strain (ATCC 607) was inoculated in 7H9 liquid medium and cultured at 160 r/min and 37°C for 48 h as phage D29 host. 7H9 solid plates were pre-warmed in an incubator then overlaid with 5 ml melted 7H9 semi-solid medium which contained 1 ml host *M. smegmatis* and 100 µl of different dilutions of phage D29. The second layer was left to harden. The double-agar plates were incubated at 37°C for 48 h and the number of plaque-forming units (pfu) was counted. The same procedure, except for the dilution factor used, was used to enumerate pfu from air samples.

2.3 Preparation of DNA samples

The genomic DNA of phage D29 was extracted by use of a QIAamp DNA mini kit (Qiagen) in accordance with the manufacturer's recommended procedure. The resulting genomic DNA was then stored at –20°C.

2.4 Standard PCR

Oligonucleotide primers for PCR were designed by Primer Prime 5.0 and synthesized by Sunbiotech, as follows: the forward primer, 5'-GCCACCAGGAGC-CACGAAC-3' and the reverse primer, 5'-AATAGG GAAGGAGTCTGCGTTTG-3' were used at final concentrations of 0.25 µM for each primer to produce a 139-bp amplicon. PCR was performed in a reaction mixture (25 µl) including 11.5 µl Taq MasterMix (Tiangen Biotech; all reagents were provided in MasterMix), 10 µl phage genomic DNA, 0.5 µl of each primer, and 2.5 µl double-distilled water (ddH₂O), using a Biometra thermocycler. The PCR conditions used were: 95°C for 5 min; 35 cycles of (94°C for 30 s, 55°C for 30 s, 72°C for 30 s); 72°C for 10 min; cool to 4°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel to confirm that the molecular weight of the amplicon was correct. The PCR product was purified using a

Doupson gel-extraction kit in accordance with the manufacturer's recommended procedure. The purified PCR product was sequenced by Sunbiotech to confirm correct sequence of the amplicon.

2.5 Development of QPCR standard curves

The resulting genomic DNA from phage D29 of known titer was then used to prepare a series of ten dilutions. QPCR was performed on a Bioer line-gene detection real-time PCR system using SYBR green I. QPCR was performed in a reaction mixture (25 µl) containing 11.5 µl RealMasterMix (SYBR green I) (Tiangen Biotech; all reagents were provided in the MasterMix and prepared in accordance with the manufacturer's recommended procedure), 10 µl phage genomic DNA, 0.5 µl of each primer, and 2.5 µl ddH₂O. The QPCR conditions used were: 95°C for 5 min; 45 cycles of (94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and detection of the fluorescence). To prevent all possible false-positive results, including primer-dimer-induced positive results, all QPCR procedures were followed by melting curve analysis.

Four-step QPCR cycles were used: 95°C for 5 min; 45 cycles of (94°C for 30 s, 55°C for 30 s, 72°C for 30 s, then detection of the fluorescence at 85°C for 5 s). The reaction mixture was the same as that used for three-step QPCR. To prevent all possible false-positive results, including primer-dimer-induced positive results, all QPCR procedures were followed by melting curve analysis. On the basis of the result of the four-step QPCR program, a standard curve was obtained by use of the QPCR software.

2.6 Application of standard curves for quantification of liquid culture samples

Liquid phage D29, phage ΦX174, phage f2, and phage SM701 were mixed together. The phage mixture was detected by double-agar culture and QPCR. The genomic DNA was extracted by use of the QIAamp DNA mini kit in accordance with the manufacturer's recommended procedure. The isolated genomic DNA was then detected by four-step QPCR and the titer of phage D29 was obtained by use of the QPCR software and the developed standard curve. The results from the two methods were analyzed by SPSS 11.0. *P* < 0.05 was considered statistically significant.

2.7 Aerosolization and sampling of phage D29 aerosol

Phage D29 suspension was aerosolized using a DV40 glass nebulizer operated with filtered air at 10 psi and a flow rate of 10 l/min. The test aerosol was generated in a 2 m³ test chamber. The mass median diameter (MMD) of phage D29 aerosol was determined by use of a multigrade Anderson sampler (Kangjie Instrument). After nebulization for 5 min, the phage D29 aerosol was collected by use of a liquid microbial sampler AGI-10 (Kangjie Instrument). The BioSampler was operated at a flow rate of 7 l/min. During each test, the biosampler collected phage D29 aerosol in 10 ml sterilized PBS for 10 min. Collection of phage D29 was repeated five times. The entire test system was placed in a BSL-2⁺ laboratory so that any aerosol particles not collected by the sampler were properly eliminated. The chamber was disinfected by aerosolizing 70% ethanol followed by the aerosolization of sterilized water.

2.8 Application of standard curves for quantification of air samples

After collection, each air sample was processed as follows:

- 1 Sample suspension (1 ml) was transferred to a sterilized microcentrifuge tube and 0.1 ml of the sample was used to obtain 10⁰–10^{−7} serial water-based dilutions. Serial dilutions were used to obtain pfu counts by the double-agar cultivation method as described above.
- 2 Sample suspension (1 ml) was transferred to a sterilized microcentrifuge tube and 200 µl of the sample was used for DNA extraction. The genomic DNA was extracted by use of the QIAamp DNA mini kit in accordance with the manufacturer's recommended procedure. The isolated genomic DNA was then detected by four-step QPCR and the titer of phage D29 was obtained by use of the QPCR software using the developed standard curve.

The results from the culture and QPCR methods were analyzed by use of SPSS 11.0. *P* < 0.05 was considered statistically significant.

3 Results

3.1 Result of standard PCR

Results from gel electrophoresis confirmed that the molecular weight of the amplification was correct (139 bp; Fig. 1). The oligonucleotide sequence was identical with that of the target fragment, which indicates the primers were specific.

3.2 Result from standard curves

Figure 2 shows the three-step QPCR melting curve analysis. A signal was obtained from the negative control, and its melting curve had two peaks. The *T_m* of the target product was approximately 88°C and the *T_m* of the primer-dimer was approximately 82°C. This showed that primer-dimers had appeared when templates were at a low concentration. To eliminate fluorescence signals caused by generation of primer-dimers, the assay was optimized by lowering the primer concentrations and redesigning the primers, but none of the specific amplifications still appeared. An additional data-acquisition step was then added to conventional three-step cycling procedure. For this step, the temperature was set above the *T_m* of the

Fig. 1 Gel electrophoresis of PCR products. *M*, DNA marker; 1, 10⁴ pfu/ml phage D29; 2, 10⁶ pfu/ml phage D29

Fig. 2 Melting curve of three-step QPCR program

primer–dimers but approximately 3°C below the T_m of the specific PCR product. This additional step thus increased the dynamic range and reliability of quantification by several orders of magnitude if primer–dimers were co-amplified (Zhang et al. 2004).

The four-step QPCR employed an additional step for detecting fluorescence at 85°C for 5 s after the extension step. Melting curve analysis showed a single melting point of the phage D29 amplicon (88°C). As shown in Fig. 3, no fluorescence signal was detected in the no-template control during amplification. An example of the graphical output of QPCR for serial dilution of diluted–isolated DNA

obtained from phage D29 cells is shown in Fig. 4. On this graph the CT values for tenfold DNA dilutions ranging from 10^3 to 10^8 are evenly distributed, which indicates the linearity of the reaction within the concentration range investigated. The fluorescence of the samples of 10^2 , 10^1 , 10^0 pfu/ml, and the negative control did not increase. The standard curve of phage D29 calculated by use of the software of the QPCR machine was $Y = -3.08X + 37.46$, where Y is the CT value and X the logarithm of the titer of phage D29. The error was 0.026 and the correlation coefficient was 0.996 (Fig. 5). The sensitivity of four-step QPCR was 10^1 pfu/reaction.

Fig. 3 Amplification curve of positive and negative samples by four-step QPCR **Fig. 4** Amplification curve of the phage D29 by four-step QPCR

Fig. 5 Standard curve of phage D29 by four-step QPCR

3.3 Result from quantification of liquid culture samples

The titer of phage D29 of the phage mixture determined by use of the four-step SYBR green I QPCR was $2.92 \pm 0.62 \times 10^7$ pfu/ml. The titer of phage D29 determined by the double-agar culture method was $2.25 \pm 0.39 \times 10^7$ pfu/ml. There was no significant difference ($P > 0.05$) between results from the QPCR and culture methods, which showed the very good specificity of the four-step QPCR method.

3.4 Result from quantification of air samples

The mass median diameter (MMD) of phage D29 aerosol was $2.21 \mu\text{m}$ (Table 1). The results from detection of five aerosol samples by the QPCR and double-agar cultivation methods are shown in Table 2. The results for the aerosol samples determined by the QPCR method were significantly ($P < 0.05$) higher than those obtained by the double-agar cultivation method. The titer of phage D29 in aerosol samples determined by QPCR was approximately 78.19 ± 16.22 times that obtained by the cultivation method.

Table 1 Biological particle mass median diameter of phage D29 aerosol

Sampler grade	Distribution of particle diameter (μm)	Mean pfu (pfu/plate)	Accumulative frequency
First grade	>7	21 ± 2.5	21
Second grade	4.7–7	23 ± 1.5	44
Third grade	3.3–4.7	55 ± 2.6	99
Fourth grade	2.1–3.3	580 ± 30.2	679
Fifth grade	1.1–2.1	486 ± 51.1	1,165
Sixth grade	0.65–1.1	91 ± 12.6	1,256

Mass median diameter = $L + (i/f)(50\%n - C) = 2.1 + (1.2/580)(679 - 628) = 2.1 + 0.11 = 2.21 \mu\text{m}$

Table 2 Results from quantification of the aerosol sample

Test	Titer of phage D29 aerosol determined by QPCR (pfu/m ³)	Titer of phage D29 aerosol determined by cultivation (pfu/m ³)
First	218,637	2,458
Second	85,740	1,315
Third	71,879	1,172
Fourth	237,214	2,372
Fifth	133,754	1,772

4 Discussion

SYBR green I real-time PCR assays are the least expensive option for performing QPCR assays, because they do not require an amplification-specific probe. Unfortunately, these dyes detect all double-stranded DNA (dsDNA), including primer–dimers and other undesired products. The specificity of the method is determined entirely by its primers, because the presence of any dsDNA can generate fluorescence. We observed accumulation of primer–dimers (PD) in low-template samples and even in no-template-control (NTC) tubes when using the three-step program. This might obscure the true result in quantitative assays using genetic DNA dyes, especially for samples in which the gene of D29 is of low abundance and PD are readily formed. Extensive PD formation during quantitative three-step QPCR in NTCs has not been intensively considered yet, probably because most assays use a TaqMan probe that does not detect this kind of nonspecific amplification (Meklin et al. 2007; Vesper et al. 2005; Zeng et al. 2004, 2006; Pyankov et al. 2007; Hietala et al. 2005). In the four-step NTC reactions, no amplification of PD was observed compared with the presence of PDs in the three-step NTCs. Four-step SYBR green I QPCR thus eliminated fluorescence readings caused by generation of primer–dimers and quantified phage D29 in both liquid culture and aerosol samples. SYBR green I

is, perhaps, a suitable choice for accurate and reproducible real-time measurements of aerosol samples. Obscuring PD observed in three-step QPCR reactions were eliminated by use of the four-step procedure; this was shown to be a prerequisite for accurate QPCR and facilitated primer design.

Impingement collection enables processing of the sample by dilution or concentration to maximize accuracy in quantification. A liquid sample can also be used with a variety of analytical methods, including culture, microscopy, immunoassay, flow cytometry, and molecular methods. In our study an AGI sampler was used to collect the phage D29 aerosol. The sensitivity of the QPCR method was much higher than that of the cultivation method. The reason may be that during the collection period the impingement sampling stress destroyed the structure and the decrease of phage D29 in the aerosol state thereby reduced the viability of collected phage D29. The QPCR method detected all of the phage D29 whether it was dead or alive whereas the double-agar cultivation method only detected the live phage D29. The QPCR method does not underestimate the total quantity of the target virus in the same way as the cultivation method when performing risk assessment. Unlike bacteria, viruses are sensitive and fragile and needed the optimized sample method to reduce impingement sampling stress and increase virus viability when collecting the virus aerosol.

The TaqMan assay is based on measuring the fluorescence released during PCR as the 5'-nuclease activity of Taq DNA polymerase cleaves a dual-labeled fluorescent hybridization probe, designed to bind inside the amplified region, during primer extension. SYBR green I is an intercalating dye that gives a fluorescence signal when bound to double-stranded DNA, while being otherwise virtually non-fluorescent. SYBR green I lacks the specificity of the TaqMan assay, in which the fluorescent signal is derived from a specific probe. However, in several published studies comparisons have been made between the two methods, and it has been shown that the specificity of SYBR green assays can approach that of Taqman probe assays if the primers are carefully selected and the assays thoroughly optimized (Ravva and Stanker 2005; Maeda et al. 2003; Malinen et al. 2003; Ponchel et al. 2003; Arikawa et al. 2008). SYBR green I can be applied to fluorescence monitoring of any amplification reaction,

which gives more flexibility to the real-time PCR. It is widely used because of the ease in designing the assays and its relatively low setup and running costs. Unlike TaqMan fluorescent probes, SYBR green dye intercalates into double-stranded DNA to monitor the amplification of target gene specifically initiated by gene-specific primers. One drawback of SYBR green assays, however, is that the dye is non-specific and can generate false positive signals if non-specific products or primer-dimers are present in the assay. Those problems can be addressed by carefully designing the primers and validating the PCR products with dissociation curve analysis immediately after PCR. In addition, other approaches have been practiced to further increase the specificity of the SYBR green detection, for example a “hot start” strategy using a DNA polymerase that requires heat activation, or acquisition of fluorescence signals at a temperature slightly below the melting temperature of the desired amplicon but above which nonspecific primer-dimer related products will denature and produce minimal signals (Arikawa et al. 2008).

The real-time PCR quantification method has many advantages compared with other conventional quantification methods in terms of accuracy, sensitivity, dynamic range, high-throughput capacity, and absence of post-PCR manipulations. The sensitivity of the QPCR-based method is considered one of the highest. It is considered one of the most promising techniques for real-time identification of airborne microorganisms. In contrast with airborne fungi, there are only a few papers describing detection of viral aerosols by use of QPCR (Weesendorp et al. 2008; Pyankov et al. 2007; Hietala et al. 2005). Recent bioterrorism events, emerging infectious diseases, for example SARS-COV, and concern about fungal contaminants in indoor environments have increased the need for enhanced detection of airborne microorganisms. This study established a preliminary procedure for detecting virus aerosols by four-step SYBR green I real-time PCR. This method is a much more economical alternative to Taqman probe QPCR for quantification of target microorganisms. This study was performed in a laboratory experimental environment and had no field background and little genomic complexity. The methods developed in this work need further study in an actual field environment and could then serve as rapid, specific, and sensitive means of detection of virus aerosols and could thus facilitate investigations

of their distribution, ecology, and clinical diagnosis, and assessment of risk from exposure.

Acknowledgments Wang Guozhi, Sun Zhenhai, and Zheng Jinlin are thanked for their excellent technical assistance. Zhai Junhui is thanked for his revising of this manuscript.

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