Title
Real-time quantitative PCR for enteric adenovirus serotype 40 in environmental waters

Permalink
https://escholarship.org/uc/item/41q7p2gx

Journal
Canadian Journal of Microbiology, 51(5)

ISSN
0008-4166

Authors
Jiang, Sunny C
Dezfulian, H
Chu, W P

Publication Date
2005-05-01

Peer reviewed
Real-time quantitative PCR for enteric adenovirus serotype 40 in environmental waters

Sunny Jiang, Hojabr Dezfulian, and Weiping Chu

Abstract: Adenoviruses 40 and 41 have been recognized as important etiological agents of gastroenteritis in children. A real-time PCR method (TaqMan® assay) was developed for rapid quantification of adenovirus 40 (Ad40) by amplifying an 88 bp sequence from the hexon gene. To establish a quantification standard curve, a 1090 bp hexon region of Ad40 was amplified and cloned into the pGEM®-T Vector. A direct correlation was observed between the fluorescence threshold cycle number (Ct) and the starting quantity of Ad40 hexon gene. The quantification was linear over 6-log units and the amplification efficiency averaged greater than 95%. Seeding studies using various environmental matrices (including sterile water, creek water, brackish estuarine water, ocean water, and secondary sewage effluent) suggest that this method is applicable to environmental samples. However, real-time PCR was sensitive to inhibitors present in the environmental samples. Lower efficiency of PCR amplification was found in secondary sewage effluent and creek waters. Application of the method to fecal contaminated waters successfully quantified the presence of Ad40. The sensitivity of the real-time PCR is comparable to the traditional nested PCR assay for environmental samples. In addition, the real-time PCR assay offers the advantage of speed and insensitivity to contamination during PCR set up. The real-time PCR assay developed in this study is suitable for quantitative determination of Ad40 in environmental samples and represents a considerable advancement in pathogen quantification in aquatic environments.

Key words: adenovirus, real-time PCR, environmental waters, serotype 40.

Introduction

Human enteric viruses represent a diverse group. The term enteric viruses reflect the fact that most of the species inhabit the alimentary (enteric) tract. Most enteric viruses are non-enveloped RNA viruses with the adenoviridae family being the only enteric viruses containing double stranded DNA genome. Human adenoviruses have been recovered from virtually every human organ and have been associated with a wide spectrum of clinical diseases (Hierholzer 1995). They are also frequently found in urban rivers as well as polluted coastal waters (Puig et al. 1994; Pina et al. 1998; Jiang et al. 2001). There are 51 known serogroups of adenoviruses. Adenovirus 40 (Ad40) and 41 (Ad41) together account for approximately 50% of all types of adenoviruses found in stool specimens (Wigand et al. 1995).
Construction of the real-time PCR standard human adenovirus including Ad 40. The probe is specific for 17 different serotypes of Ad40 and its application in environmental samples. Development and validation of a real-time PCR method for the simultaneous detection of both serotypes and the high stringency requirement of real-time PCR, a single assay for the simultaneous detection of both serotypes has not been successful. This report focuses on the objective of this study is to develop a real-time PCR method for the quantification of human enteric adenovirus for application in complex environmental matrices. However, there has not been a method for rapid, sensitive, and accurate detection of the load of adenoviruses in environmental waters. The development and validation of a real-time PCR method for Ad40 and its application in environmental samples.

Materials and methods

Primers and probe selection

All PCR primers and probes were designed from the human Ad40 hexon gene sequence using Primer Express® Software v. 2.0 (Applied Biosystems, Foster City, Calif.) and are listed in Table 1. Primers and probes were manually selected based on estimated annealing temperature (Tm), the desire for small amplicon size, and the location of the probe and were tested empirically as described below. The specificity of the primer and probe combinations was evaluated using BLAST web software (Altschul et al. 1990). The forward primer has homology with human adenovirus serotypes 40 and 41 and the reverse primer is specific for human adenovirus serotypes 40, 3, 7, and 16. Therefore, the combination of forward and reverse primers is only specific to Ad40. The probe is specific for 17 different serotypes of human adenovirus including Ad 40.

Designation | Function | Sequences | Amplicon size (bp)
--- | --- | --- | ---
f-AD_{157} | Real-time PCR forward primer | 5‘-ACCCACGATGTAACCACAGACA-3‘ | 88
r-AD_{245} | Real-time PCR reverse primer | 5‘-ACTTTTGAAGATAGCGGGTTTCC-3‘ | 
\(p-AD_{106}\) | Real-time PCR probe | 6-FA-MCTGGGCTCTGTTCCGGTGC-TAMRA | 
f-AD_{9} | Standard control forward primer | 5‘-CCCCTCGATGATGCAGCAA-3‘ | 
r-AD_{1097} | Standard control reverse primer | 5‘-CTGTCCTGCTGCTGTTCCA-3‘ | 1090

Real-time PCR for the Ad40 hexon gene

To evaluate the efficiency and specificity of the primers, real-time PCR was first conducted using SYBR green nucleic acid dye in an ABI Prism 7000 sequence detection system (Applied Biosystems). The reactions were carried out in a 96-well plate in a 25-µL reaction volume containing 12.5 µL of 2x SYBR green master mix (Applied Biosystems), a 900 nmol/L concentration of each forward and reverse primer, and pAD40 DNA. The following thermocycling profile was used for each reaction: 50 °C for 2 min to activate uracil N-glycosylase in the master mix, 95 °C for 10 min to activate AmpliTaq Gold® followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation analysis was run at the end of each SBYR green real-time PCR reaction to verify a single PCR amplicon. The size and purity of the amplicons were also examined using conventional agarose gel electrophoresis in some cases.

Real-time PCR with a TaqMan® probe was performed in a 25-µL reaction mixture with a TaqMan® PCR core reagent (Applied Biosystems). The reaction mixture contained TaqMan® core buffer, 5 mmol/L MgCl₂, 200 µmol/L each of dATP, dCTP, and dGTP, 400 µmol/L dUTP, 300 nmol/L of forward primer, 900 nmol/L of reverse primer and 250 nmol/L probe, 0.25 U of AmpErase uracil N-glycosylase, 1 U of Taq Gold polymerase, and titrated pAD40. The thermocycling profile was identical to that used for the real-time PCR with SYBR green dye. Each run contained at least 3 no-template controls to establish the baseline emission intensity of the quenched reporter dye.

To achieve optimal real-time PCR settings, primer and probe concentrations were optimized using a concentration matrix spanning a concentration range from 50 to 900 nmol/L for each primer and a concentration range from 50 to 250 nmol/L for the TaqMan® probe. The amounts of target DNA used per reaction varied from 10 to 10⁵ copies to determine the real-time PCR detection limits. Each sample was run in replicate or triplicate. The sensitivity and reproducibility of the reaction were also tested using serially diluted titrated Ad40 viral particles, expanding the range from 10⁻⁴ to 10⁷ plaque forming units (PFU) per reaction.

A quantification standard curve was generated using serial dilutions of a known copy of pAD40 under optimal PCR conditions determined as described above. A triplicate of

Table 1. Primers and probe for adenovirus 40 hexon gene.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Function</th>
<th>Sequences</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-AD_{157}</td>
<td>Real-time PCR forward primer</td>
<td>5’-ACCCACGATGTAACCACAGACA-3‘</td>
<td>88</td>
</tr>
<tr>
<td>r-AD_{245}</td>
<td>Real-time PCR reverse primer</td>
<td>5’-ACTTTTGAAGATAGCGGGTTTCC-3‘</td>
<td></td>
</tr>
<tr>
<td>(p-AD_{106})</td>
<td>Real-time PCR probe</td>
<td>6-FA-MCTGGGCTCTGTTCCGGTGC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>f-AD_{9}</td>
<td>Standard control forward primer</td>
<td>5’-CCCCTCGATGATGCAGCAA-3‘</td>
<td></td>
</tr>
<tr>
<td>r-AD_{1097}</td>
<td>Standard control reverse primer</td>
<td>5’-CTGTCCTGCTGCTGTTCCA-3‘</td>
<td>1090</td>
</tr>
</tbody>
</table>
at 37 °C, 5% CO₂ before plaques were counted under an Olympus Microscope using a 10x magnification lens.

A final concentration of 10⁶ PFU/mL Ad40 was seeded into each of the following environmental matrices: creek water, estuarine water, ocean water, and secondary sewage effluent. Sterilized DI water was used as the reference matrix to determine PCR efficiencies in each type of environmental matrix. All environmental samples were collected in June 2004 using sterilized containers. Creek water was taken from San Diego Creek, a tributary of Newport Bay, Calif. Estuarine water was taken from the middle of Newport Bay, ocean water was taken from Huntington Beach, Calif., and the secondary sewage effluent was obtained from Orange Country Sanitation District, Calif.

Viral nucleic acid was extracted from seeded samples using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif.) following manufacturer’s recommendation. According to the manufacturer, QIAamp Viral RNA Kit is also efficient at recovery of viral DNA. In comparison, it is more efficient than other blood DNA extraction kits for viral DNA recovery from sewage and other environmental samples where PCR inhibitors may be of concern. Four microlitres of each nucleic acid extract was used in the TaqMan® real-time PCR reaction using optimized condition described above.

**Assay of environmental samples**

To test the feasibility of the real-time PCR method for the detection and quantification of human Ad40 in environmental waters, twelve water samples artificially contaminated with sewage, human feces, dog feces, gull feces, or cattle feces were used as test subjects. These samples were provided to researchers in the lab as “blind samples”. Each sample was prepared by mixing 1 to 3 sources of fecal material in sterile water. Ten litres of each water sample was concentrated using a Centramate Tangential Flow recirculation ultrafiltration system (Pall Life Science, East Hills, New York) with a 30 kDa molecular mass cutoff Omega filtration cassette following the manufacturer’s instructions. The concentrates were frozen immediately in aliquots. Viral nucleic acid was purified from concentrates using a QIAamp Viral RNA Mini Kit (Qiagen) and used for adenovirus assay by TaqMan® real-time PCR as described above or nested PCR as previously described by Jiang et al. (2001).

**Sequence analysis of adenoviral amplicons**

Amplicons from nested-PCR were cloned into the pGEM-T vector (Promega) following manufacturer’s instructions. Recombinant plasmid with insert was purified and submitted for cycle sequencing using a Dye-Terminator Cycle Sequencing Ready Reaction FS Kit (PE Applied Biosystems) following manufacturer’s instructions. Primers targeted at the T7 and Sp6 promoters were used for bi-directional sequencing. The fragments were trimmed using AssemblyLIGN (Oxford Molecular Group, Campbell, Calif). The final sequences were submitted to a BLAST search of the NCBI GenBank database for identification. ClustalX (Thompson et al. 1997) was used to align sequences. Multiple alignments were performed using default parameters and the final aligned sequences were refined visually. A phylogenetic tree was created using the neighbor-joining method (Saitou and Nei 1987). The branching confidence was estimated by bootstrap.
analysis using the following parameters: random number generator seed: 111; number of bootstrap trials: 1000. The final tree was viewed in TreeView software. All sequences were submitted to NCBI database under the accession numbers AY747670 to AY747675.

Results

Evaluation and optimization of real-time PCR

Empirical testing of PCR with primers f-AD\textsubscript{157} and r-AD\textsubscript{245} using SYBR Green I detector showed a linear increase of SYBR Green I fluorescence intensity with cycle number. Dissociation analysis following PCR yielded a single melting peak at 90.8 °C. This dissociation peak was within 2 °C of the expected Tm, suggesting a specific amplification product (Ririe et al. 1997). A single 88-bp amplicon was also confirmed when the final reaction mixture was visualized on a 2% agarose gel. Therefore, these results suggest that primers f-AD\textsubscript{157} and r-AD\textsubscript{245} are capable of specifically amplifying the target with high efficiency. Addition of the probe (without fluorescent dye) did not reduce the amplification efficiency (data not shown), suggesting that there is no interference between the probe and the 2 primers.

Real-time PCR with TaqMan\textsuperscript{®} probe (p-AD\textsubscript{196}) and serially diluted pAD40 DNA yielded a log linear relationship between initial pAD40 copy and threshold cycle number (Ct), spanning a 6-log dilution series (Fig. 1a). Correlation coefficients were always greater than 99%, indicative of accuracy within each experimental run. Slope values varied between –3.25 to –3.57 from run-to-run and averaged –3.40. This translates to an amplification efficiency (E = (10^{-1/slope} – 1) \times 100) of 90% to 103% (average 97%). Optimization of primer and probe concentrations using a concentration matrix indicated that primer concentrations of 300 nmol/L forward and 900 nmol/L reverse, and a probe concentration of 250 nmol/L yielded the lowest threshold cycle (Ct) value. Therefore, this optimized condition was used for all further studies and analyses of environmental samples described below.

The sensitivity of this assay, using pAD40 DNA as targets, yielded a lower detection limit of less than 10 genome copies per reaction (Fig. 1a). The slope value for the amplification of standard DNA insert (pAD40) and titrated Ad40 viral particles was within a margin of less than 0.1, indicating that the efficiencies of the amplification for both types of target were equal (Fig. 1a). The sensitivity of this assay, using titrated Ad40 viral particles as targets, yielded a lower detection limit of 10^{-4} PFU per reaction, equivalent to 2 copies of the hexon gene target (Fig. 1b).

Application in environmental matrices

Table 2 shows the application of this method to various environmental matrices seeded with Ad40. The results indicated that the method is applicable to quantify Ad40...
viral concentration in all environmental matrices tested. The viral concentration varied within 1 log unit (3-fold difference between lowest and highest value) in different matrices. However, the efficiency of PCR reduced with the increase of concentration of complex organic material in the sample. Secondary sewage effluent had the lowest PCR efficiency of 32%, whereas ocean water had relatively higher PCR efficiency because of the lower concentration of PCR inhibitors in the sample.

Application of the method to fecal contaminated waters indicated that this method is specific to human fecal source (Table 3). There was no cross reaction with fecal contamination from non-human sources (i.e., dog and gull feces). A positive signal for adenovirus was detected by real-time PCR in samples 1, 2, 6, 7, 8, 9, and 10, which were contaminated with either sewage or human feces. However, the Ct value for samples 2, 6, 7, 8, and 9 fell outside the range of the standard curve (less than 10 copies per reaction), which prevented an accurate quantification of genome copy in the original water samples. Only samples 1 and 10, which were contaminated with 0.69% and 0.14% of sewage, respectively, yielded Ct values within the range of the standard curve. Calculation of the viral genome number in the original water samples after correcting for concentration and extraction factors indicated the concentration of Ad40 detected was proportional to the level of sewage contamination in samples 1 and 10. However, only a weak signal (1 of 2 replicates was above threshold Ct) was detected in sample 8, which was also contaminated with 0.69% of the sewage. Later sequence analysis of adenovirus amplicons from nested PCR indicated that Ad41 was the dominant serotype presented in sample 8, explaining the low amplification efficiency using Ad40 real-time PCR. Using nested PCR, adenoviruses were detected in samples 1, 4, 8, and 10 (Table 3). All PCR amplicons from the nested-PCR were used for cloning. However, no positive clone was obtained from PCR amplicons of sample 1.

Sequence analysis of a 130-bp Ad40 hexon region recovered from nested PCR indicated that Ad41 was the dominant serotype present in sample 8, explaining the low amplification efficiency using Ad40 real-time PCR. Using nested PCR, adenoviruses were detected in samples 1, 4, 8, and 10 (Table 3). All PCR amplicons from the nested-PCR were used for cloning. However, no positive clone was obtained from PCR amplicons of sample 1.

**Discussion**

The results of this study demonstrated that the developed primers and probe are capable of efficiently amplifying the Ad40 hexon gene using a real-time fluorescence detector. The real-time PCR is also applicable to environmental samples. The sensitivity of the method is 1000 to 10 000 times greater than that of the plaque assay since the lower detection limit by this method equals $10^{-4}$ PFU. The assay is rapid. The entire procedure can be completed within 4 h. However, the efficiency of amplification is reduced in presence of PCR inhibitors. Further improvement of nucleic acid purification could improve the efficiency of quantification and detection. Internal seeding of control targets may help to understand the variability of PCR inhibition during field application of this method.
Application of the real-time PCR method to fecal contamin-
ated water samples has demonstrated the specificity of the
method. There is no cross-reaction with fecal source of non-
human origin. Real-time PCR yields positive signals for 7 of
the 8 samples contaminated with sewage and human feces.
The negative results may be because of the low concentra-
tion of Ad40 in sample 4. Only low signals were detected in
samples contaminated with human feces (sample 6). This is
likely because of the low level of viruses in the individual
human feces used for this study. Sewage from a population
of individuals is more likely to contain enteric human patho-
gen. Compared with nested PCR assay, which detected
adenoviruses in 50% of the samples contaminated with
sewage and human feces, this single-step real-time PCR is
perhaps more sensitive. However, the quantitative ability of
this assay is limited only to the samples with relatively
higher concentration of viruses. This is in agreement with
previous clinical studies comparing single-step real-time
PCR with nested PCR (van Elden et al. 2001). Development
of nested real-time PCR protocol may further improve the
sensitivity of the assay. Nevertheless, the real-time PCR
method developed here represents a significant advancement
for the rapid quantification of viral load in environmental
waters.

Acknowledgements

The funding for this project was provided by Water
Environmental Research Foundation award 01-HHE-2a.
Partial support for this project was also provided by the
Southern California Coastal Water Research Project Author-
ity for Comparative Evaluation of Microbial Source
Tracking Techniques. Fecal contaminated water samples
were provided by the research team of the microbial source
tracking study. We thank Dr. Yu-Li Tsai from Orange
County Sanitation District and Dr. Jian-Wen He from
Beckman Coulter Inc. for technical advice, and Mandy Han
for technical support in the Ad40 plaque assay. We also
thank Dr. Steve Weisberg for coordinating the microbial
source tracking study effort.

References

Altschul, S.F., Gish, W., Miller, W.W., Myers, E., and Lipman, D.J.
410.
inactivation of enteroviruses and adenovirus 2 by UV light.
Trans E1 component requirements for maximal replication of E1-
viral, rickettsial and chlamydial infections. Edited by E.H. Lennette,
D.A. Lennette, and E.T. Lennette. American Public Health
Association, Inc., Wash., DC. pp. 169–188.
Jiang, S., Noble, R., and Chu, W. 2001. Human adenoviruses and
coliphages in urban runoff-impacted coastal waters of Southern
pollution in the environment and in shellfish: Human adenovirus
detection by PCR as an index of human viruses. Appl. Environ.
Microbiol. 64: 3376–3382.
Puig, M., Jofre, J., Lucena, F., Allard, A., Wedell, G., and Girones,
R. 1994. Detection of adenoviruses and enteroviruses in polluted
60: 2963–2970.
differentiation by analysis of DNA melting curves during the
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and
strategies for multiple sequence alignment aided by quality
van Elden, L.J., Nijhuis, M., Schipper, P., Schuurman, R., and van
Wigand, R., Baumeister, H.G., Maass, G., Kuhn, J., and Hammer,

© 2005 NRC Canada