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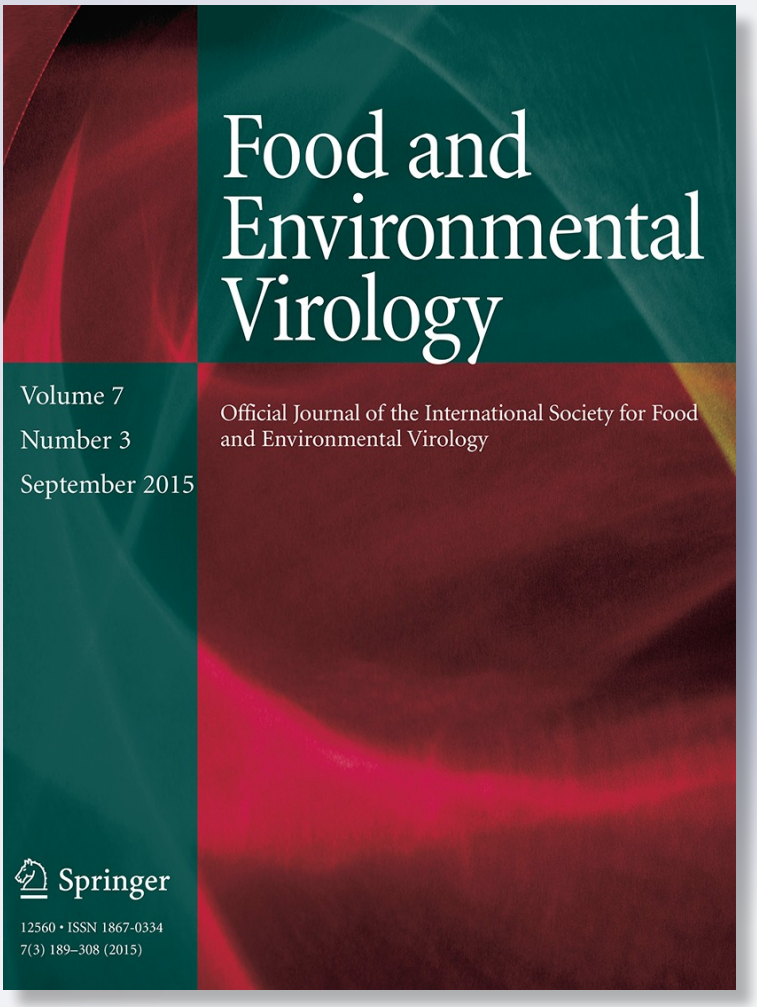

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ORIGINAL PAPER

Development and Evaluation of a Loop-Mediated Isothermal Amplification Assay for the Detection of Adenovirus 40 and 41

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Abstract Human adenoviruses (hAdVs) of subgroup F (enteric serotypes 40 and 41) display characteristic gut tropism, in vivo, fastidious growth characteristics in cell culture, and are estimated to be associated with 5–20 % worldwide of acute gastroenteritis cases among infants and young children. Adequate hAdV gastroenteritis case management requires laboratory-based diagnosis. The present study aimed to the development and evaluation of a simple and cost-effective, one-step, single-tube adenovirus type 40/41 specific loop-mediated isothermal amplification (LAMP) assay for the detection of hAdV40/41 DNA in environmental and/or clinical samples, since no LAMP assay has previously been reported for the detection of these virus types. The assay targeted the hexon gene and had the advantages of being rapid, simple, specific, and sensitive. Results could be obtained within 60 min, under isothermal conditions at 69 °C. The detection limits for hAdV genomes were between 50 and 100 copies/reaction for hAdV40 and hAdV41, and no cross-reactions with other selected viruses, were found. The assay was evaluated with clinical as well as environmental samples. The developed assay is expected to provide a potential molecular tool in obtaining greater knowledge of the hAdV40/41 importance in the epidemiology and clinical manifestations of gastroenteritis.

Keywords Loop-mediated isothermal amplification · Adenovirus 40 and 41 · Molecular detection

Introduction

Human adenoviruses (hAdVs) are ubiquitous double-stranded DNA viruses, classified in the family *Adenoviridae*, genus *Mastadenovirus*, which contains seven known hAdV species (A–G). Historically, hAdVs were classified by hemagglutination and serum neutralization reactions into 51 serotypes, but new adenovirus types have since been identified based on genomic data for a total of 54 types being officially recognized (Harrach et al. 2011). New AdV types have recently been identified based on genomic data, including several emerging and recombinant viruses (Buckwalter et al. 2012; Robinson et al. 2013). The disease spectrum of hAdVs include, but is not limited to, respiratory, ocular and urinary tract infections, and gastroenteritis (Bányai et al. 2009). It is estimated that more than 90 % of the human population is seropositive for one or more types of adenoviruses (Fong et al. 2010). HAdVs are present at a higher frequency in sewage than are other enteric viruses and are excreted in high concentrations from infected patients (up to 10^{11} viral particles per gram of feces) (Fong et al. 2010). HAdVs species F types 40 and 41 are enteric pathogens which are estimated to be associated with 5–20 % of acute gastroenteritis cases among infants and young children (Haramoto et al. 2007, 2010; Bányai et al. 2009; Sdiri-Loulizi et al. 2009; Lee et al. 2012; Rodríguez-Lázaro et al. 2012). Although they are

P. G. Ziros and P. A. Kokkinos have contributed equally to this work.

P. G. Ziros · P. A. Kokkinos (✉) · A. Vantarakis
Environmental Microbiology Unit, Department of Public Health, School of Medicine, University of Patras, 26504 Rion, Greece
e-mail: pkokkin@med.upatras.gr

A. Vantarakis
e-mail: avanta@upatras.gr

A. Allard
Department of Clinical Virology, Umeå University Hospital, 901 85 Umeå, Sweden

sensitive to chemical disinfection, they are more resistant to the effects of UV light than other enteric viruses (Rodríguez-Lázaro et al. 2012). HAdV40/41 have been detected in different aquatic environments (Chapron et al. 2000; Lee and Kim 2002; He and Jiang 2005; Van Heerden et al. 2005; Haramoto et al. 2007; Albinana-Gimenez et al. 2009a, b; Haramoto et al. 2010; Kokkinos et al. 2011; Wyn-Jones et al. 2011) and shellfish (Myrmel et al. 2004), and are of sufficient concern to public health that they have been placed by the U.S. Environmental Protection Agency in its Contaminant Candidate List for drinking water (Jothikumar et al. 2005). A recent Europe-wide surveillance study carried out to determine the frequency of occurrence of two human enteric viruses in recreational waters showed that about 20 % of freshwater samples and about 47 % of marine water samples contained infectious adenovirus, supporting laboratory observations that these agents are environmentally robust (Wyn-Jones et al. 2011).

Despite the replication of hAdV40/41 to high titre in the intestine, these viruses display fastidious growth characteristics in cell culture (De Jong et al. 1983; Kidd et al. 1983; Jothikumar et al. 2005). Indeed, cultivation of hAdV41 results in a 20-fold lower yield than cultivation of AdV2 (Siqueira-Silva et al. 2009).

Differential diagnosis for hAdV gastroenteritis is impossible based on the clinical picture alone; therefore, adequate case management requires laboratory-based diagnosis (Bányai et al. 2009).

Various molecular techniques have been exploited for the development of highly sensitive and rapid assays for the detection of causative agents of viral gastroenteritis, including hAdV40/41. It has been reported that the detection of adenovirus was increased by more than 200 % using PCR compared to results with electron microscopy (EM) (Logan et al. 2006). In recent years, real-time PCR assays for the detection of viruses associated with gastroenteritis have been described as demonstrating greater sensitivity and specificity than antigen-detection assays (Van Maarseveen et al. 2010). Conventional PCR assays have been described for AdVs based on hexon and fiber gene sequences but are labor intensive due to the need for post-PCR product analysis by gel electrophoresis and confirmatory hybridization assays or sequencing (Tiemessen and Nel 1996; Jothikumar et al. 2005; Bányai et al. 2009). In addition, diagnostic use of conventional multiplex PCR, real-time PCR, and multiplex real-time PCR assays for the detection of various agents of viral gastroenteritis, including AdVs, has been described (Jothikumar et al. 2005; Logan et al. 2006; Albinana-Gimenez et al. 2009a, b; Fong et al. 2010; Van Maarseveen et al. 2010; Feeney et al. 2011; Higgins et al. 2011; Buckwalter et al. 2012; Jex et al. 2012; Kim et al. 2012; Wessels et al. 2013). A simple protocol combining a one-step multiplex reverse transcription polymerase chain reaction (RT-PCR) with microsphere-based fluorescence detection was developed for different viruses including AdVs (Liu et al. 2011). In some cases, real-time PCR has been shown to be less sensitive than conventional RT-PCR (Noble et al. 2003; Fong and Lipp 2005). Conflicting results between direct qPCR and immunomagnetic separation technique combined with PCR (IMS–qPCR) has been attributed to the different extraction efficiency of viral DNA from concentrated water samples pretreated with/without the IMS procedure (Haramoto et al. 2010). The sensitivity of the Seeplex DV assay, a commercial multiplex RT-PCR assay that detects five diarrheal pathogens, including adenovirus, was not higher than that of EM for detection of adenovirus, and the required time was longer than that of rRT-PCR (6 h) (Higgins et al. 2011).

There is a need to provide rapid, sensitive, and often high throughput detection of pathogens in diagnostic virology (Feeney et al. 2011). In 2000, Notomi et al. developed a novel nucleic acid amplification method, designated loop-mediated isothermal amplification (LAMP). Compared with other rapid detection methods, LAMP has many advantages, such as high specificity and sensitivity, simple operation, and low cost, which constitutes a potentially valuable tool for rapid diagnosis. Both simple and real-time detection (http://loopamp.eiken.co.jp/e/index.html) has been applied for specific detection of pathogens (Notomi et al. 2000).

The aim of the present study was the development and evaluation of a simple and cost-effective, one-step, single-tube adenovirus types 40/41 specific LAMP assay for the detection of hAdV40/41 DNA in environmental and/or clinical samples, since no LAMP assay has previously been reported for the detection of these virus types.

Materials and Methods

LAMP Primer Design and Reaction Conditions

The primers used for hAdV40/41 LAMP were designed against the sequence of the hexon gene by using Primer Explorer V software (Fujitsu, Tokyo, Japan) after a ClustalW2 analysis of known AdV sequences (http://www.vmri.hu/~harrach/AdVtaxlong.htm) (Harrach et al. 2011).

The LAMP assay was conducted with a 20 μL reaction mixture consisting of the set of six primers (outer, inner, and loop primers), *Bst*2.0 DNA polymerase reaction buffer (New England Biolabs, MA), $MgSO_4$ (7 mM), betaine (0.6 M) (Sigma Aldrich, St. Louis, MO), dNTPs (1.2 mM), *Bst*2.0 DNA polymerase (8 U) (New England Biolabs), and target DNA. All the primers were high-performance liquid chromatography-purified. The sequences of the oligonucleotide primers are shown in Table 1. The reaction components were mixed in a tube and were tested at 60–70 °C

for 30–60 min using a thermal cycler (MJ Mini^{TM} Personal Thermal Cycler, BIO-RAD, USA) and then heated to 80 °C for 20 min to terminate the reaction. Positive and negative controls were included in each run. The amplified product was detected by adding 1 μL of 1,000× SYBR green dye to each reaction tube. After incubation for 15 min in the dark at room temperature, a yellowish green color indicated a positive reaction, while a reddish orange (the color of the unbound dye) indicated a negative reaction. The color change in the reaction tubes was also examined under natural light conditions. In addition, the LAMP products were detected by agarose gel (2 %) electrophoresis with UV light transillumination. The hAdV genomes quantitation by QPCR was performed as described previously (Ziros et al. 2011) using a plasmid with the hAdV41 hexon gene sequence (Viroclime FP7 EU project).

Specificity and Sensitivity of the LAMP Assay

The specificity of the LAMP assay was evaluated by using a panel of different AdV strains from the Department of Clinical Virology of the Umeå University Hospital (Sweden) (Table 2) (Allard et al. 2001). Moreover, the specificity was evaluated by using porcine adenovirus (pAdV), bovine polyomavirus (bPyV), human, and *Salmonella* spp. DNAs. Viral nucleic acids were extracted using QIAamp Viral RNA mini kit (Qiagen), either manually or using QIAcube (Qiagen, Hilden, Germany) fully automated platform. The same number of genome copies (GCs) was used for all samples in the specificity tests. To determine the sensitivity of the assay serially diluted plasmids containing the hexon gene sequence of AdV40 or AdV41 DNA which was previously quantified by real-time PCR were used. For measuring the detection limit of the LAMP assay, samples were tested in triplicate and the lowest concentration of GCs was taken as the limit when all of the triplicate samples were positive.

Environmental Samples Collection and Processing

Sewage samples (500 mL) were collected from the inlet of the municipal wastewater treatment plant (WWTP) of Patras (Greece), during a 2-year period (2010–2012). Twenty-eight (28) samples were collected in total. Samples (50 mL) were processed by the skimmed milk (SM) flocculation procedure for virus concentration (Calgua et al. 2008). Nucleic acids (100 μL) were extracted with the QIAamp Viral RNA Mini kit (Qiagen). HAdV molecular detection was based on a previously described *Taq*Man assay (Hernroth et al. 2002). The analysis method is accredited according to ISO 17025 (ESYD 550-2). Positive samples for hAdV were further confirmed by nested PCR and sequencing (Kokkinos et al. 2011). LAMP assay robustness was also tested by analyzing sewage samples (500 μL) which were heated at 100 °C for 5 min, without prior nucleic acids extraction (2 μL).

Table 2 Adenovirus strains tested during the specificity analyses for the development of hAdV40/41 LAMP assay of the present study

Adenovirus	Species	Strain identity
Ad 31	A	na
Ad 3	B1	GB
Ad 7	B1	Gomen
Ad 21	B1	na
Ad 11	B2	Slobitsky
Ad 35	B2	Holden
Ad 1	C	na
Ad 5	C	Ad75
Ad 19	D	na
Ad 37	D	35S
Ad 50	D	WAN
Ad 4	E	Prototype
Ad 40	F	HoviX
Ad 41	F	TAK

na not available

Clinical Samples Collection and Processing

Seven (7) fecal specimens, obtained from confirmed gastroenteritis outbreaks (Mellou et al. 2013a, b) and from hospitalized children admitted with symptoms of acute diarrhea to the University Hospital of Patras, Greece

Table 1 Sequences of oligonucleotide primers used for hAdV40/41 LAMP assay of the present study

Label	Primer sequence 5′–3′	Working concentration (μM)
F3_ADE	CCGCAATGGTCTTACATGCA	0.2
B3_ADE	GAATAAGCGGTGTCCTCGC	0.2
FIP_ADE	CTGAAGTACGTATCGGTGGCGCGACGCCTCGGAGTATCTGAG	1.6
BIP_ADE	TCAGAAATCCCACTGTGGCTCCCGAATCGCAGCGTCAGTC	1.6
LF_ADE	AATTGCACCAGGCCCGG	0.8
LB_ADE	CACGATGTAACCACAGACAGGTC	0.8

Primers were designed within the sequence of hexon gene, using Primer Explorer V software (Fujitsu, Tokyo, Japan)

(Kokkinos et al. 2013), were used for the specificity and evaluation tests of the LAMP assay. Nucleic acids were extracted with the QIAamp Viral RNA mini kit (Qiagen) from 140 µL of the supernatant of approximately 10 % fecal specimens. HAdVs were detected by real-time PCR, as described above. Positive samples for hAdV were further confirmed by nested PCR and sequencing.

Prevention of PCR Carryover Contamination

To prevent any LAMP carryover contamination, all standard precautions were followed by adhering to strict laboratory practices. The pre-LAMP manipulations (DNA isolation and LAMP set-up) were performed in a clean room that was physically isolated from the LAMP PCR machine and the post-LAMP processing area. Dedicated pipettes and reagents were used for each location. Negative controls were run with all assays, and no indications of contamination were detected. Plasmids were prepared in a separate room and special attention was paid when opening the caps of the used reaction tubes for addition of SYBR green dye or subsequent electrophoresis.

Results

LAMP Specificity

The specific amplification of the DNA of hAdV40/41 strains generated ladder-like pattern bands on agarose gel which are indicative of a mixture of stem-loop DNA of varying sizes and multiple loops of DNA formed by annealing between alternately inverted repeats of the target in the same strand (cauliflower-like structures). No amplification was observed in LAMP reactions without template DNA (negative control) and in the control reactions with non hAdV40/41 DNA (adenovirus 35, AdV35; porcine adenovirus, pAdV; bovine polyomavirus, bPyV; human DNA; *Salmonella* spp.). Similarly, no cross-reaction was observed in LAMP reactions with AdV strains other than hAdV40/41 (adenovirus 1, AdV1; adenovirus 3, AdV3; adenovirus 4, AdV4; adenovirus 5, AdV5; adenovirus 7, AdV7; adenovirus 11, AdV11; adenovirus 19, AdV19; adenovirus 21, AdV21; adenovirus 31, AdV31; adenovirus 35, AdV35, adenovirus 37, AdV37) (Fig. 1). The diagnostic accuracy of the assay was 100 %.

LAMP assay detected AdV40/41 within 60 min. There was no difference between the LAMP results detected by agarose gel electrophoresis of LAMP products or visual detection of LAMP products under UV light or day light (Fig. 2).

The hAdV40/41 specific LAMP assay was evaluated at different temperatures of 70, 69, 68, 66, 64, 62, 61, and 60 °C. The temperature of 69 °C was finally selected as an optimum temperature to minimize the possibility of potential non-specific amplifications (Fig. 3), even though the assay worked well at lower temperatures, with the used panel of control samples.

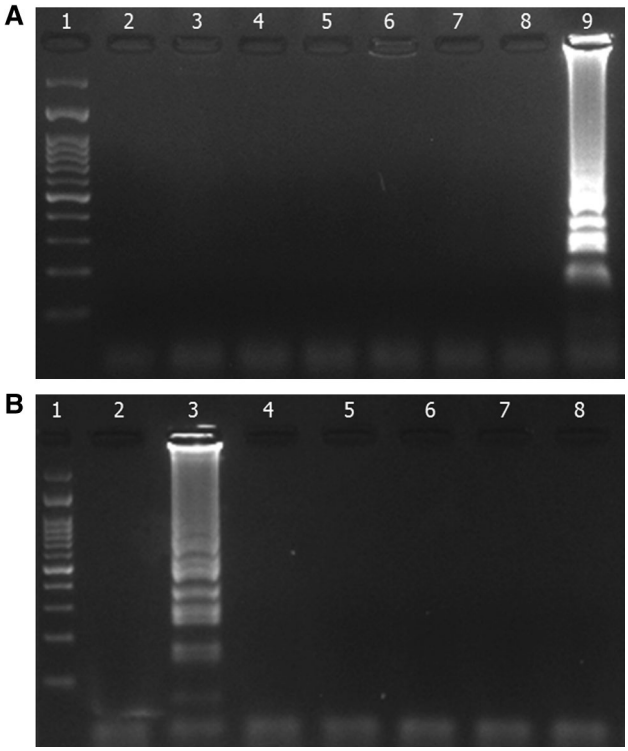

Fig. 1 Specific detection of hAdV40/41 DNA compared to other AdV strains. **a** *lane 1* 100-bp DNA ladder; *lane 2* negative control; *lane 3* adenovirus 1 (AdV1); *lane 4* adenovirus 3 (AdV3); *lane 5* adenovirus 4 (AdV4); *lane 6* adenovirus 5 (AdV5); *lane 7* adenovirus 7 (AdV7); *lane 8* adenovirus 11 (AdV11); *lane 9* AdV 41 (AdV41). **b** *lane 1* 100-bp DNA ladder; *lane 2* negative control; *lane 3* adenovirus 40 (AdV40); *lane 4* adenovirus 19 (AdV19); *lane 5* adenovirus 21 (AdV21); *lane 6* adenovirus 31 (AdV31); *lane 7* adenovirus 35 (AdV35); *lane 8* adenovirus 37 (AdV37)

LAMP Sensitivity

Serial dilutions of 10^0–10^5 GCs of AdV40/41 were used for the sensitivity assays. The analytical sensitivity of the LAMP assay was estimated at 50–100 copies/reaction (Fig. 4).

Rapid and Specific Detection of hAdV40/41 in Environmental Samples

Sixteen (16) out of twenty-eight (28) sewage samples of urban sewage analyzed in total, were found positive for hAdVs, by initial screening with real-time PCR (Hernroth et al. 2002). LAMP assay detected fifteen (15) hAdV41 positive urban sewage samples, out of the 16 samples found positive for hAdVs by real-time PCR, within 60 min. One sample which was found positive for AdV2 by initial screening with real-

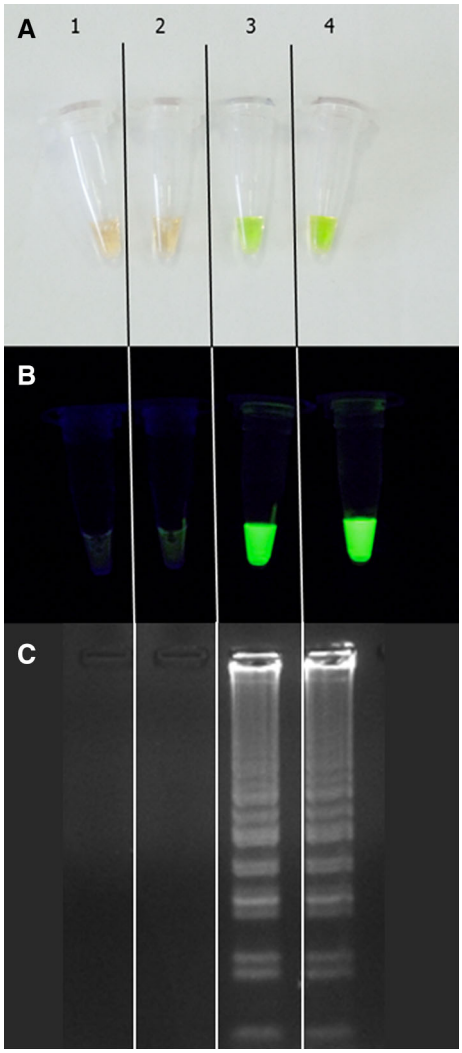

Fig. 2 Specific detection of LAMP products, using different methods. **a** Visual detection of LAMP products under day light. The tubes with a positive reaction show a *color* change to *yellowish green*, which can be distinguished from the *reddish orange color* of the negative reactions. **b** Visual detection of LAMP products under UV light, after the addition of SYBR Green I dye. **c** Agarose gel electrophoresis of the same samples. *Lane 1* negative control, *lane 2* hAdV35, *lane 3* hAdV40, *lane 4* hAdV41 (Color figure online)

time PCR and subsequent typing by nested PCR and sequencing was not detected by hAdV40/41 specific LAMP assay, as expected (Fig. 5). Moreover, the robustness of the LAMP assay for the analysis of environmental samples was shown by the detection of the target hAdV41 DNA in sewage samples without prior nucleic acids extraction.

Rapid and Specific Detection of hAdV40/41 in Clinical Samples

Fecal specimens, including samples obtained from gastroenteritis outbreaks and from hospitalized children admitted with symptoms of acute diarrhea to the University Hospital of Patras, Greece, were analyzed (Fig. 6). All hAdV40 and hAdV41 isolates were consistently detected within 60 min, whereas all other adenovirus types were not, including AdV1, AdV2, and AdV6.

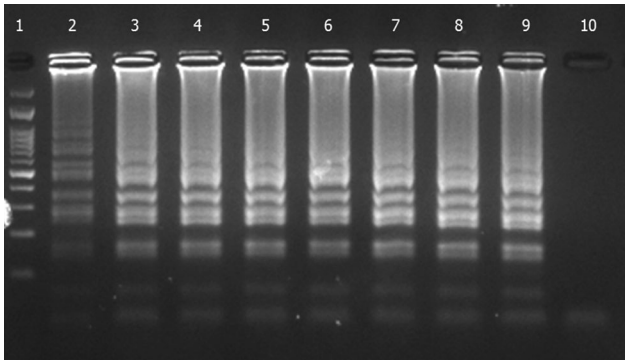

Fig. 3 HAdV40/41 specific LAMP assay at different temperatures. *Lane 1* 100-bp DNA ladder; *lane 2* 70 °C; *lane 3* 69 °C; *lane 4* 68 °C; *lane 5* 66 °C; *lane 6* 64 °C; *lane 7* 62 °C; *lane 8* 61 °C; *lane 9* 60 °C; *lane 10* negative control

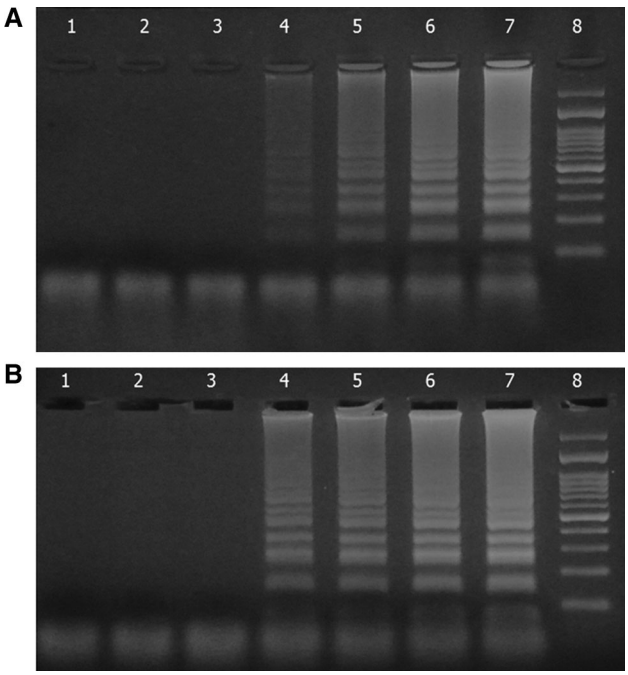

Fig. 4 Sensitivity assay for hAdV40/41 specific LAMP. **a** Sensitivity assay for hAdV40; *lane 1* negative control; *lane 2* 10^0; *lane 3* 10^1; *lane 4* 10^2; *lane 5* 10^3; *lane 6* 10^4; *lane 7* 10^5; *lane 8* 100-bp DNA ladder. **b** Sensitivity assay for hAdV41; *lane 1* negative control; *lane 2* 10^0; *lane 3* 10^1; *lane 4* 10^2; *lane 5* 10^3; *lane 6* 10^4; *lane 7* 10^5; *lane 8* 100-bp DNA ladder

Discussion

F species adenoviruses types 40 and 41 are the most recognized adenoviruses in suspected fecal–oral transmission

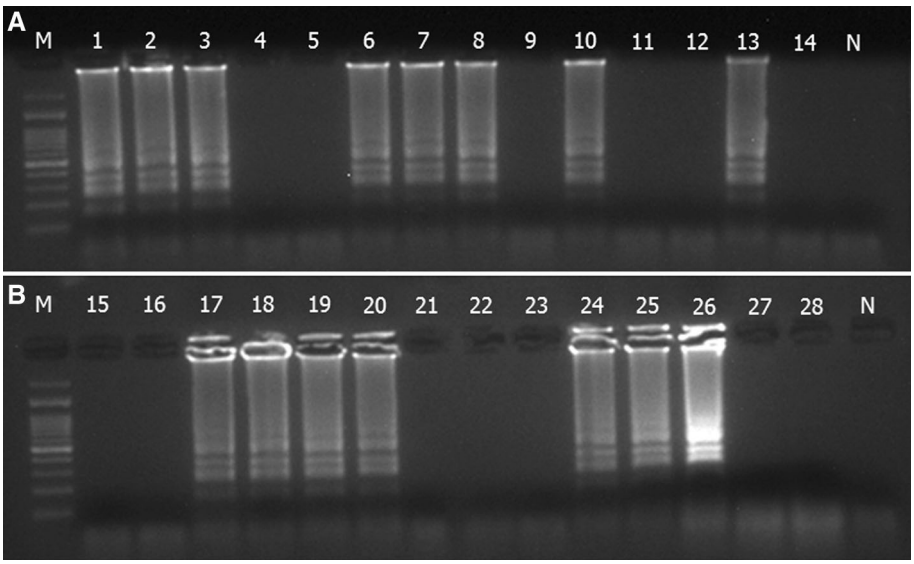

Fig. 5 LAMP assay detected fifteen (15) hAdV41 positive urban sewage samples, out of the sixteen (16) samples found positive for hAdVs by real-time PCR (28 samples were analyzed in total). One sample was found positive for AdV2 (**a** 14), as was confirmed by nested PCR and sequencing. Where *M*, in **a**, **b**: 100-bp DNA ladder; where *N*, in **a**, **b**: negative controls; hAdV41 positives, **a** 1, 2, 3, 6, 7, 8, 10, 13; **b** 17, 18, 19, 20, 24, 25, 26

through water and have been the prototypes for studies of gastroenteritis caused by adenoviruses, displaying characteristic gut tropism, in vivo (Leung and Brown 2011). Types 40 and 41 are particularly difficult to isolate, in contrast to other adenovirus species, because they grow slowly in cell culture. Identification of adenovirus isolates can be accomplished by neutralization with type-specific antisera or by DNA restriction analysis. However, these methods are time-consuming and results are often difficult to interpret, and therefore, these methods are impractical for routine testing of clinical or environmental samples (Jothikumar et al. 2005).

LAMP (Notomi et al. 2000) is potentially applicable as a bedside diagnostic method, because it does not require specific instruments and rapidly provides results (Nakamura et al. 2012). LAMP assays have extremely high specificity and selectivity because of the use (generally) of six primers, with two loop primers recognizing eight distinct regions on the target sequence. The specificity and selectivity of LAMP assays have been documented in different studies, the number of which is exponentially increasing. LAMP assays have been developed for hAdV-D53 and hAdV-D54. Nakamura et al. reported the detection of HAdV-Ds from patients with epidemic keratoconjunctivitis in the Fikai Prefecture during 1995–2010 by different methods including LAMP (Nakamura et al. 2012).

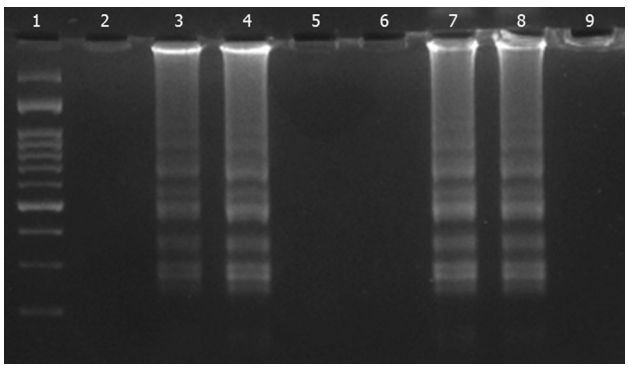

Fig. 6 Application of LAMP assay to clinical samples. *Lane 1* 100-bp DNA ladder; *lane 2* negative control; *lane 3* AdV40; *lane 4* AdV41; *lane 5* AdV1; *lane 6* AdV2; *lane 7* AdV41; *lane 8* AdV40; *lane 9* AdV6. Tested clinical strains derived from diarrheal fecal specimens obtained from gastroenteritis outbreaks and from hospitalized children admitted with symptoms of acute diarrhea to the University Hospital of Patras, Greece. All strains were confirmed by nested PCR and sequencing

In the present study, we achieved rapid detection of hAdV40/41 genomes by using a one-step, single-tube F species adenovirus types 40/41 specific LAMP assay. This is to the best of our knowledge the first report of the development of a LAMP assay for the detection of hAdV40/41 genomes. The specificity of the LAMP assay for hAdV40/41 genomes was established by examining the cross-reaction with standard AdV40/41 DNAs, DNA templates of different AdV genotypes, and of other viral, as well as human and bacterial DNA samples. In the present study, the LAMP assay specifically amplified only AdV40/41; and no cross-reactivity with other targets was observed.

A real-time PCR assay for the detection of all 57 known hAdV types demonstrated 99 % specificity (Buckwalter et al. 2012). When a nested multiplex assay was replaced by two *Taq*Man single round real-time multiplex assays, a higher specificity for adenoviruses was achieved (95.2 vs. 99 %) (Feeney et al. 2011). Similarly, different *Taq*Man assays have been developed for the specific detection of group F AdVs in singleplex (Jothikumar et al. 2005; Jiang et al. 2005), or multiplex formats, which showed comparable specificity to the individual assays for the detection of

AdV group F (Van Maarseveen et al. 2010). Two different QPCR procedures when evaluated for their suitability in the analysis and quantification of hAdV present in different wastewater matrices showed significant differences of specificity (Bofill-Mas et al. 2006). Luminex xTAG® Gastrointestinal Pathogen Panel (xTAG GPP) showed a diagnostic specificity of 100 % for AdV40/41 (Navidad et al. 2013), similarly to Seeplex Diarrhea-V ACE (Seeplex DV) (Higgins et al. 2011). The first assay is performed in an 8 h shift, while the second within 5 h.

The LAMP assay of the present study was able to detect hAdV40/41 genomes with sensitivity levels of approximately 50–100 copies/tube, in serial tenfold dilution tests of DNA templates. The assay was robust to detect target viruses in environmental (sewage) samples, with or without prior nucleic acids extraction, and clinical (fecal) samples, after a nucleic acids extraction step. According to previous studies, hAdVs are almost always present in sewage samples from different geographical areas and show a mean concentration of 10^3 genomic copies (GC)/mL, respectively (Calgua et al. 2013). Similarly, hAdVs were present in the sewage specimens obtained from Patras' WWTP at concentrations which were higher than the levels of sensitivity of the hAdV40/41 specific LAMP assay of the study.

The sensitivities of QPCR assays for the detection of hAdVs were estimated to be of less than five GCs per reaction for hAdVs 40/41 (Haramoto et al. 2007), 1–10 GCs for hAdVs (Bofill-Mas et al. 2006), fewer than 10 GCs per reaction (He et al. 2005), 10 GCs/μL for all 57 known hAdV types (Buckwalter et al. 2012), 10 GCs per reaction (Logan et al. 2006; Fong et al. 2010), and ≤15 copies/run for 51 hAdVs (Heim et al. 2003). A broadly reactive *Taq*Man assay was able to detect five copies of AdV40 and eight copies of AdV41, while a hAdV 40/41 specific assay had a detection limit of 3–5 copies of AdV40 and AdV41 standard DNA (Jothikumar et al. 2005). The analytical sensitivity for hAdV41 was determined at 10^{-1} 50 % tissue culture infective dose (TCID50)/mL (Buckwalter et al. 2012), while the lower limit of detection was 100 PFU/reaction, using serial dilutions of purified adenoviral particles of serotype 40 (He and Jiang 2005). Jiang et al. reported a LDL of 10^{-4} PFU per reaction for AdV40, equivalent to two copies of the hexon gene target (Jiang et al. 2005). The sensitivity of a conventional PCR was 10–100 GCs/reaction (Fong et al. 2010), while multiplex, probe-based PCR assays showed improved sensitivity for AdV detection (97.3 vs. 86.1 %), in comparison to nested multiplex PCR assays (Feeney et al. 2011). Commercial multiplex PCR assays, Seeplex Diarrhea-V ACE (Seeplex DV) (Higgins et al. 2011), and Luminex xTAG Gastrointestinal Pathogen Panel, showed 100 % of sensitivity for AdV40/41 detection (Navidad et al. 2013). The analytical sensitivity of the ProAdeno+ assay for AdV41 was determined at $10^{-1.5}$ $TCID_{50}$/mL. Luminex xTAG can amplify and detect viruses in 96 specimens in an 8 h shift, while FilmArray gastrointestinal panel can test only a single specimen in 1 h. Real-time PCR assays provide the most sensitive diagnosis for AdV40/41, but the developed LAMP assay is a much more robust technique (Francois et al. 2011) and has also a significant potential as an alternative tool for detection in the field.

We expect that the F species adenovirus types 40/41 specific LAMP assay will be routinely used in laboratories because of its simplicity and specificity. The greatest advantage of the LAMP assay is the substantial reduction in required time compared to that required for PCR. Our simple-to-use LAMP assay shortens the reaction time to maximum 60 min.

Visual detection of a color change under day light (Fig. 2a), or UV light (Fig. 2b), by using SYBR Green I is a simple and effective method of detecting LAMP amplification products. Moreover, this detection approach eliminates the need for gel electrophoresis and staining with ethidium bromide, and thus shortens the overall assay time by approximately 50 min. Gel electrophoresis is unnecessary for routine diagnosis purposes, because a large amount of DNA is synthesized by LAMP and the products can be detected simply by the presence of turbidity or fluorescence (Yang et al. 2010). The entire process of the LAMP reaction occurs in only one reaction tube. To minimize the possibility of post-amplified cross-contamination the reaction tube does not need to be opened, and thus the selection of the appropriate detection method is of a pivotal role for regular screening purposes in diagnostic laboratories or for deployment in field-testing. By incorporation of a fluorescent dye and fluorometer or turbidimeter, the assay time can be further shortened, as well as be converted to a real-time format (Techathuvanan and D'Souza 2012). The possibility of the elimination of nucleic acids (NA) extraction step, which adds time, cost, and complexity to the analysis, has also to be evaluated in base of the required application (Kokkinos et al. 2014).

Thus, the overall assay time depends on the reaction detection method and the type of application. Reactions set-up and isothermal amplification, may be completed in approximately 1 h and 20 min. If a nucleic acids extraction step is required, an additional approximate 30-min time period is required, dependently on the number of analyzed samples. Ideally, the use of a fluorometer or turbidimeter, for real-time detection, without opening the reaction tube, as well as the elimination of the NA extraction step, could provide the most rapid, simple, and cheap diagnostic solution for hAdV40/41 in environmental and/or clinical samples.

The European Standardization Committee (CEN), in collaboration with the International Organization for

Standardization (ISO) has proposed a general guideline for PCR testing that requires the presence of IAC in the reaction mixture; and only IAC-containing PCRs may undergo multicenter collaborative trials, which is a prerequisite for standardization (Hoorfar et al. 2004). Although LAMP is a much more robust technique compared to other nucleic acid amplification methods (Francois et al. 2011), different environmental and clinical samples may still inhibit LAMP reactions. Thus, incorporation of an IAC into the reaction is significant for more reliable results, to ensure that the amplification results contain no false negatives. At present, only the external positive and negative controls have been used, and the developed hAdV40/41 LAMP assay contains no IAC; therefore, the presence of inhibitors in the samples cannot be monitored. The lack of IAC would pose a problem for its use in routine diagnosis and thus, the construction and implementation of an appropriate internal amplification control is a future aspect of priority for the developed molecular assay.

The developed hAdV40/41 specific LAMP assay is expected to provide a very robust, innovative, and powerful molecular diagnostic method for environment safety testing services and Public Health authorities, and will be used for environmental monitoring, epidemiological investigations of disease outbreaks, and molecular epidemiological investigations of adenovirus infections and illnesses associated with waterborne exposures. Importantly, LAMP has the potential to support the development of new on-site diagnostics. Incorporation of LAMP assays in simple, low power, handheld devices for real-time detection of the LAMP reaction will provide promising solutions for real-time on-site screening for hAdV40/41 contamination.

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