ARTICLE

Development of a closed-tube QUASR-LAMP assay for the detection of human adenoviruses

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Human adenoviruses (HAdVs) are double-stranded DNA viruses capable of causing a wide array of diseases in humans. They are ubiquitous in nature and may cause life-threatening opportunistic infections in immunocompromised individuals. Diagnosis is traditionally by cell culture or antigen detection methods. Development of rapid diagnostic kits for the detection of enteric adenoviruses can help to rule out other underlying causes of gastroenteritis in patients. Also, these kits may be used to detect HAdVs in environmental samples. This present study presented the development of a quenching of unincorporated amplification signal reporters–loop-mediated isothermal amplification (QUASR–LAMP) assay to detect HAdVs for clinical and environmental applications. The optimized assay can be completed in 50 min at 63-64 ºC. The minimum detection limit of the QUASR–LAMP is 50 copies/µL while the polymerase chain reaction (PCR) assay was 100 copies/µL. Thirteen (13) human fecal samples and 6 water samples were used to determine the measures of accuracy. The sensitivity of the assay was 85.71%, while the specificity was 92.86%. The positive predictive value (PPV) was 82.76% while the negative predictive value (NPV) was 94.20% and an accuracy of 90.82%. The assay is also specific to HAdV DNA. The QUASR–LAMP assay can potentially be modified and applied as a simple diagnostic tool to detect infections in humans and to determine water quality for risk assessment especially in resource-limited areas.

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INTRODUCTION

Human adenoviruses (HAdVs) can cause an array of clinical diseases which include conjunctivitis, hepatitis, gastroenteritis, myocarditis and pneumonia. There are 7 known HAdV species, HAdV-A to HAdV-G, where HAdV-A has been associated with gastrointestinal tract infections (Ghebremedhin, 2014). Adenovirus infections are relatively common and may be caused by consumption of contaminated water or inhalation of aerosolized droplets during water recreation. Eighty percent (80%) of adenovirus infections result in prolonged periods of viral excretion in the feces or urine of infected individuals. Enteric viruses including HAdVs have been recognized as potentially hazardous waterborne pathogens. They have shown to be more persistent in the environment, resistant to water treatment than bacteria and can infect individuals even at low doses. (Cooksey et al. 2019). In the Philippines, cases of waterborne diseases caused by viruses are not always detected and viral indicators for measurement of microbial quality are not being done routinely, thus, the immediate need for a test that can do both. Detection methods for enteric HAdVs include polymerase chain reaction (PCR) (Ko et al. 2005; Ahmed et al. 2010; Lu and Erdman 2016; Marti and Barardi 2016), immunofluorescence (Calgua et al. 2011), immune real-time PCR (which enabled detection of lower virus concentrations in stool samples) (Bonot et al. 2014), immunochromatographic (Levent et al. 2009), and cell culture assays (Polston et al. 2014).

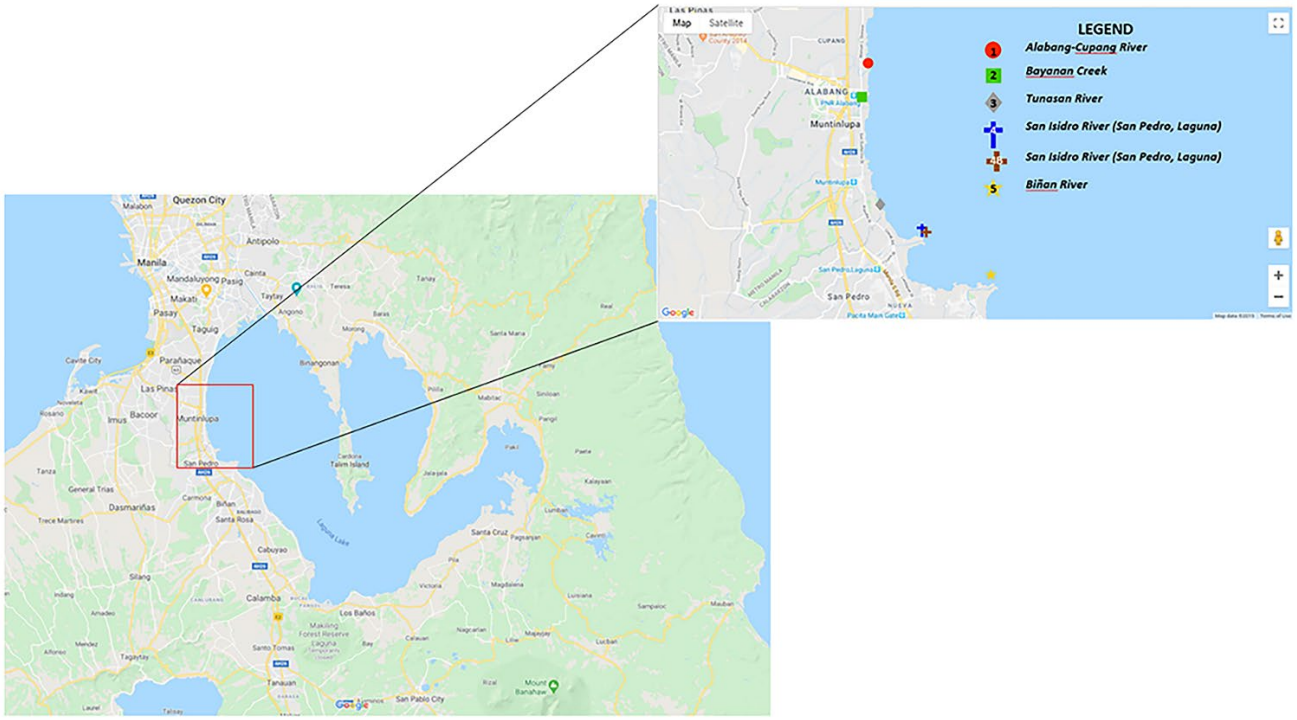

Figure 1: Map of Laguna Lake, Philippines showing the 5 tributaries where water samples were collected.

A novel gene amplification method that does not require specialized equipment was developed by Notomi et al. (2000) which is called the loop-mediated isothermal amplification (LAMP) assay. The DNA synthesis involves auto-cycling and strand displacement by the *Bst* DNA polymerase large fragment and is performed under isothermal conditions, around 65 °C for 15-60 min using 4 to 6 primer pairs. This can be used as an alternative to PCR for low-cost or point-of-care diagnosis for infectious diseases (Notomi et al. 2000). A LAMP assay has been used to detect HAdVs in nasopharyngeal aspirates from pediatric patients with acute respiratory infections (Sun et al. 2014) while another LAMP coupled with a lateral flow dipstick assay was developed to detect fowl adenovirus serotype-4 (Zhai et al. 2019).

Detection in LAMP assays is usually accomplished by observing turbidity, running the product on an agarose gel to observe the ladder-like formation, adding an intercalating dye like SYBR Green to observe color change, adding manganese-quenched calcein to generate fluorescence upon amplification, or adding a colorimetric indicator such as hydroxynaphthol blue or pH-sensitive dyes to generate a color change upon amplification. However, these have their own drawbacks including poor discrimination capability, inability to multiplex targets, high rates of false positives, and in some cases, the requirement of opening reaction tubes post-amplification (Ball et al. 2016). Another novel approach for endpoint determination developed by Ball et al. was based upon quenching of unincorporated amplification signal reporters (QUASR) that can show higher discriminatory power, can be used for multiplexing and does not require opening the tube after the reaction (Ball et al. 2016). Therefore, this present study aimed to develop a QUASR–LAMP assay to detect HAdVs for clinical and environmental applications.

MATERIALS AND METHODS

DNA Controls and Samples

Human adenovirus 41 DNA control was purchased from Vircell Microbiologists (Spain). Water samples were collected from five tributaries of Laguna Lake, Philippines (Figure 1) and filtered using 0.2 µm nitrocellulose membranes before nucleic acid extraction. Thirteen (13) human fecal samples were also collected from individuals working in farms near Laguna Lake. The protocol for handling human samples was reviewed and approved by the University of the Philippines Manila Research Ethics Board (UPMREB-2018-356-01). DNA were extracted using ZymBiomics DNA/RNA Miniprep (Zymo Research, USA) following the manufacturer's protocol.

Target Sequence and Primer Design

QUASR–LAMP primers were designed from the alignment of human adenovirus 40 and 41 hexon gene of 5 reference strains (GenBank accession numbers AB330122, HQ005289, AB610527, X51782 and AB330121) to screen a conserved sequence for use as target sequence. The selected fragment was used as template for designing LAMP primers via PrimerExplorer V4 (Eiken Chemical Co., Ltd). Primer sets generated from the software were also adjusted manually to meet the requirements for LAMP reaction conditions. Complementary quencher probes were designed manually avoiding primers that will likely form stable hairpins.

PCR Assays

Samples were subjected to conventional PCR assays prior to LAMP assays using previously designed primers (Biscaro et al. 2018) targeting the hexon gene. The PCR protocol used was as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s, and final extension of 72 °C for 5 min. PCR products were run in 1.5% agarose gel. The expected product for positive result is ~300 bp when viewed on an LED transilluminator (Hercuvan, UK).

QUASR–LAMP Assays

The QUASR–LAMP reaction mix contained 8U *Bst* DNA polymerase, Exonuclease Minus (Lucigen, USA), 1X DNA polymerase Buffer B containing 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1 % Triton X-100, 0.2 µM of outer and loop primers (F3/B3, LoopF/LoopB), 1.6 µM of inner primers (FIP/BIP), 2.4 µM of the quencher probe, and 5 µL of the DNA template. The mixture was incubated at 63-64 °C using the Biotek-M™ LAMP Heater (Manila Healthtek Inc., PH) for 50 min. Results were viewed using the EASYView™ LED Transilluminator (Manila Healthtek Inc., PH). LAMP products were also viewed on agarose gels to confirm results of positive LAMP with ladder-like formations.

Data Analyses

The limit of detection (LOD) was determined by performing the assays using different viral copy numbers (10, 50, 100, and 500 copies/µL). The lowest copy number in which a positive result is observed is the LOD. On the other hand, specificity and sensitivity were used as measures of accuracy. The positive predictive value (PPV) and the negative predictive value (NPV) were also obtained. Measures were obtained at $\alpha = 0.05$, confidence interval of 95% and power of 80% with a margin of error of ± 2. Based on Table 1, the sensitivity, specificity, PPV and NPV of the test were computed using the following formula (MedCalc Statistical Software, 2020):

a. Specificity = D/ B+D x 100%
b. Sensitivity = A/A+C x 100%
c. PPV = A/ A+B
d. NPV = D/ C+D

Table 1: 2 x 2 cross-tabulation of comparative sensitivity between QUASR-LAMP and PCR.

QUASR LAMP	PCR	
	POSITIVE	NEGATIVE
POSITIVE	A	B
NEGATIVE	C	D
TOTAL	A+C	B+D

RESULTS AND DISCUSSION

Optimization of the temperature for the QUASR-LAMP assay showed that the optimal temperature was at 63-65 °C, making it easier to perform the assay even at varying temperatures within this range (Figure 2).

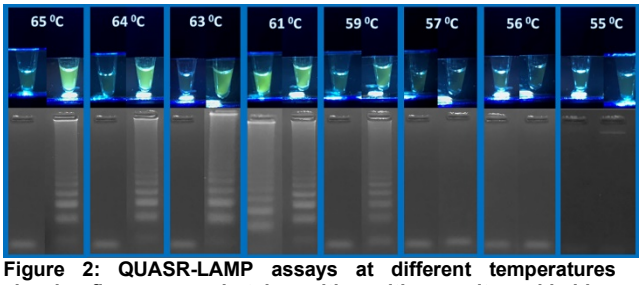

Figure 2: QUASR-LAMP assays at different temperatures showing fluorescence in tubes with positive results and ladder-like formations on agarose gel.

Using the assays described earlier, the LOD of the QUASR–LAMP was determined to be at 10 copies/µL (Figure 3 A, B) while the PCR assay was at 100 copies/µL (Figure 3 C).

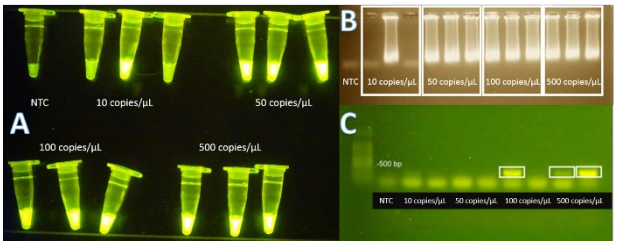

Figure 3: Limit of detection (LOD). (A) QUASR–LAMP assay showing the limit of detection at 10 copies/µL under the transilluminator; (B) agarose gel of the QUASR–LAMP products with ladder-like formations, confirming positive results; and (C) PCR products in agarose gel.

To determine the specificity, QUASR-LAMP assays were carried out using other bacterial and viral DNAs. Results showed that primers and probes only amplified the HAdV positive control as indicated by the fluorescence in the tube and ladder-like formation in agarose gel (Figure 4).

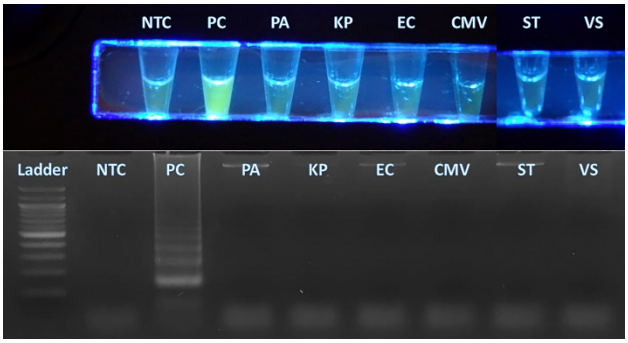

Figure 4: Specificity of the QUASR LAMP assay. Results as seen in the LED transilluminator using other organisms to demonstrate specificity of the assay in detecting HAdV only. NTC- no template control; PC- positive control; PA- *Pseudomonas aeruginosa*; KP- *Klebsiella pneumoniae;* EC- *Escherichia coli*; CMV- Cytomegalovirus; ST- *Salmonella* Typhimurium; VS- *Vibrio* spp.

Thirteen (13) human fecal samples and 6 water samples were used to test the ability of the QUASR–LAMP assays to detect enteric adenoviruses in these samples. Results showed that only one sample was positive in PCR and one in QUASR–LAMP (data not shown) while 5/6 of the water samples were positive for both assays (Figure 5).

Several studies have reported the use of LAMP assays for the detection of different pathogens but used expensive real-time turbidimeter or a real-time PCR system (Goto et al. 2009). The optimized QUASR–LAMP assay in this study was optimized at 63-65 °C in a simple device, the Biotek-M™ LAMP Heater (Manila Healthtek Inc., PH), a heating apparatus that has a dual temperature set-up with temperature range of 25-100 °C. This unit is used for LAMP reactions (first setting at 63-65 °C) and stop reactions (second setting at 80 °C).

LAMP has advantages compared to PCR which include having low-cost instrumental requirements, reactions that can be run as quickly as 30 to 60 min, being specific because of the use of 4 to 6 primers that corresponds to 6 to 8 target sites, sensitive enough to detect as low as one copy (10 times lower than PCR), efficient amplification that can reach up to 10^9 fold in 30 to 60 min, high impurity tolerance and easier judgment of result (Nakauchi et al. 2014; Shang et al. 2020). The present QUASAR–LAMP was optimized at 50 min incubation which

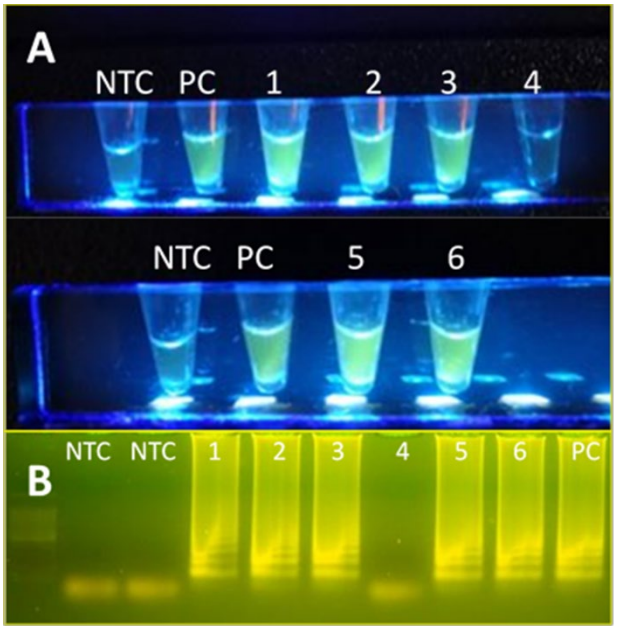

Figure 5: QUASR–LAMP result of water samples from 6 different sites. (A) Tubes inside the LED Transilluminator and (B) QUASR–LAMP products in an agarose gel showing ladder-like formation of positive samples.

makes it faster compared to the PCR assay targeting the same gene in 2 h.

QUASR enables non-inhibitory, bright, single-step, closed-tube detection of DNA, as well as RNA targets with LAMP and RT–LAMP. It can even be used for multiplexing, enabling the detection of two targets in one reaction tube (Ball et al. 2016). LAMP has a detection limit of few copies (<10) in the reaction mixture compared to PCR and time to positivity is also lesser (Notomi et al. 2000; Datta, 2017). In the present study, it can detect at least 10 copies/µL compared to the PCR assay which was 100 copies/µL (Figure 3). The assay was also able to detect HAdVs both in water samples (Figure 5) and in human fecal samples (data not shown). These results further illustrate that the assay is sensitive enough to detect HAdVs in a wide range of samples.

Using the four-cell decision matrix shown in Table 2, the measures of accuracy of the QUASR–LAMP assay were calculated and are reflected in Table 3. Based on the matrix, 6 samples (human stool and water samples) were positive for both PCR and QUASR–LAMP, while one was positive for QUASR–LAMP but negative in PCR. Additionally, 1 sample was negative for QUASR–LAMP but positive in PCR and 13 were negative in both assays.

Table 2: Four-cell decision matrix for the computation of the measures of accuracy.

QUASR LAMP	PCR	
	POSITIVE	NEGATIVE
POSITIVE	A (6)	B (1)
NEGATIVE	C (1)	D (12)
TOTAL	A+C (7)	B+D (13)

Table 3: Summary statistics of the measures of accuracy of the HAdV QUASR–LAMP assay (MedCalc Statistical Software, 2020).

Statistics	Value	95% CI
Sensitivity	85.71%	42.13 to 99.64%
Specificity	92.86 %	66.13% to 99.82%
Positive Predictive Value	82.76%	41.48% to 97.02%
Negative Predictive Value	94.20%	72.47% to 99.01%
Accuracy	90.82%	70.07% to 98.93%

The HAdV QUASR–LAMP assay developed in this study had a laboratory sensitivity of 85.71% (95% CI 42.13 to 99.64%) and specificity of 92.86% (95% CI 66.13% to 99.82%) in a four-cell decision matrix. The assay has an accuracy of 90.82% (95% CI 70.07% to 98.93%).

QUASR–LAMP could lower testing costs and increase access to diagnostics and biosurveillance tools in setting where resources are limited. The ability of QUASR–LAMP to discriminate positive from negative at a defined end-point (in this case 50 min) and provide a positive or negative result is very helpful especially for laboratory technicians with limited molecular biology training and/or experience.

CONCLUSION

The QUASR-LAMP technique described in this study is rapid, sensitive and specific for the detection of enteric adenoviruses making it very useful diagnostic method for point-of-care settings. However, it is recommended to test the protocol using more samples to improve the sensitivity of the assay.

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CONFLICT OF INTEREST

The authors declare that there are no competing financial conflicts of interest and ethical issues exist.

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