Noroviruses Surrogate Detection Using Loop-Mediated Isothermal Amplification (LAMP), Conventional RT-PCR and Quantitative RT-PCR in Sg. Tekala and Sg. Gabai Stream Water

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Abstract. A preliminary study was carried out in order to evaluate a reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay for detecting Noroviruses (NoV) RNA surrogates as external standard for NoV.The detection limit of the NoV RT-LAMP assay was observed to be 22 copies/μL. This RT-LAMP assay sensitivity was comparablewith the quantitative reverse transcriptase-Polymerase Chain Reaction (PCR) and shown to be 10-fold more sensitive than end-point conventional reverse transcriptase-PCR (RT-PCR). The NoV RT-LAMP assay showed high specificity to NoV targeted gene when specificity test was completed with no cross-reactivity with other 17 environmental strains. The assay also was performed with 11 spiked recreational stream water randomly picked from two recreational areas in Hulu Langat Malaysia, Sg. Tekala and Sg. Gabai. The RT-LAMP assay is simpler compared to the conventional PCR and real-time PCR, which in optimum isothermal temperature of 63°C, the amplification can be completed in 40 minutes. Results of spiked recreational stream water samples suggested that the NoV RT-LAMP assay can be used as monitoring tool for NoV surveillance in recreational stream water.

1 Introduction

There are not many surveillance of NoV in Malaysia have been carried out in water and environment area except very few studies had been performed in food application[1,2]. The lack of information could be contributed to misdiagnose of severe diarrhea symptoms of NoV to other gastroenteritis bacterial such as *Shigella*sp. and *Escherichia coli* and other enteric viruses like Rotavirus and Adenovirus [3].It is suggested that the infection of NoV occurred from the activities of swimming, canoeing or other recreational activities relating

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to the fecal contaminated water [4–6]. For decades, fecal indicator bacteria (FIB) such as *E.coli* and enterococci has been widely used as standard procedure to evaluate fecal borne pathogen contamination in water. Although this technique is being used for water quality criteria [7], it has limitation in protecting the public from recreational waterborne disease especially viruses infection. The requirement of 18 to 24 hours of virus incubation post-exposure prior to issue advisory on infectivity may increased the greater risk of infection[8,9]. Another drawback of using FIB as indicator is that these bacteria has been reported to continuously grow in the environment, leads to a bias in assessment especially in tropical weather like Malaysia[10–12]. Human enteric viruses such as enteroviruses, NoV and adenoviruses which directly associated with human diseases found to be more reliable indicators for fecal contaminated water [13,14].These enteric viruses have the ability to survive for longer period of time and reflect their existence in real number since they cannot replicates without permissive host organism in water [13,15].

Although, the public health risks assessment of NoV in recreational water is very important, the need on the advanced diagnostic tool and high analytical skill in determining the availability of NoV in water samples has concealed the perceptive on the real existence, concentration and behaviours of NoV in aqueous environment[16]. PCR and RT-PCR method is time saving and able to detect non cultivable viruses or slow-growing viruses. However, the downside of these methods is the need of bulky, expensive thermo cycler which usually inaccessible in a remote area associated with waterfalls and streams.These lacks of accessible leads the development of loop-mediated isothermal amplification (LAMP) which employs simpler method for DNA amplification; isothermal condition around 63 to 65^{o}C and directly visualized after 30to 60 minutes incubation.Since no cultivation activity involved like FIB enumeration method [7], this molecular methodhas the advantage to serve as a faster and safer platform for monitoring NoV contamination in recreational water with simplified risk consideration for recreational water contamination management.In this study, recreational stream waters were spiked with NoV to mimic the real detection of NoVusing PCR, real-time RT-PCR and RT-LAMP, and the performances of these methods for NoV detection in environmental sample were compared.

2 Materials and methods

2.1 Laboratory strains and specificity test

Specificity test for the primers used in this study were validated using 15environmental DNA strains as described in our previous study [17]: *Burkholderia cepacia, B. thailandensis, B. pseudomallei, Bacillus subtilis, B. macerans, B. circulans, B. megaterium, Staphylococcus* sp*., S. epiderminis, S. heamolyticus, E. coli* BL21, *E. coli* Nova Blue, *Shigella* sp., *Salmonella* sp. and *Shinella granuli*. All strains, positive control (DNA plasmid of NoV) and negative control (steril dH_2O) were assessed simultaneously in optimized LAMP condition.

2.2 NoV surrogates construction and transcription *in-vitro*

The external standard for NoV was constructed synthetically in plasmid (Integrated DNA Technology, USA) by inserting specific target genes sequences for NoVd etection.The molecular weights of nucleotide base pairs of the products recombinant plasmid are 207bp and the sequences are:

5′GTGGTATGGATTTTTACGTGCCCAGGCAAGAGCCAATGTTCAGATGGATGAGATTC
TCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATG

GCGTCGAATGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTCAACA ATGAGGTTATGGCTTTGGAGCCCGTTGTC-3′. The target gene was previously being used as NoV specific marker [18]. Briefly, a total of 400 complete and partial RNA-dependent RNA polymerase and capsid protein gene sequences of NoV from Genbank database were aligned using Vector NTI Advance 11 (Invitrogen, Auckland, New Zealand). The most conserved region among these genes was inserted into pIDT-SMART-NoV vector and sequencing-verified (Integrated DNA technology, USA). This template was further used as template for *in-vitro* transcription using the Ribo-MAX^{TM} Large Scale RNA production System-T7 (Promega, Madison, USA)as described by the manufacturer's protocol. The transcribed RNA was then purified with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and measured using Nanodrop 1000 (Thermo Scientific, USA). The reading of RNA absorbencies were then converted to molecular copies according to Krieg formula [19] and serially 10-fold diluted from 10^{-1} to 10^{-8} for further use.

2.3 Primers design for RT-LAMP of NoV

The oligonucleotides were designed using LAMP primer designing software Primer Explorer 4.0 (http://primerexplorer.jp/e/) according to the conserved region of target gene inserted to the pIDT-Smart-NoV vector. List of primers suggested by the software were evaluated as described by Notomi *et al*.[20]. The primers set consist of six primers; two outer primers (B3 and F3), two inner primers (BIP and FIP) and two loop primers (LF and LB) that recognizing eight target sequence. The B3 and F3 oligonucleotide primers used for the RT-LAMP of NoV were also used for quantitative RT-PCR and conventional RT-PCR. The details of the selected primers sequences are shown in Table 1.

Table 1 Sequences of primer set used for Noroviruses surrogate RT-LAMP, RT-PCR and qRT-PCR

Assays	Primer	Sequence (5'-3')	Reference
RT-LAMP/ RT-PCR/ qRT-PCR	F3	GTGGTATGGATTTTTACGTGCC	[18]
RT-LAMP/ RT-PCR/ qRT-PCR	B3	GACAACGGGCTCCAAAGC	
RT-LAMP	FIP	CAGATTGCGATCGCCCTCCCGAG CCAATGTTCAGATGGATGA	
RT-LAMP	BIP	TGAAGATGGCGTCGAATGACGCA ACCTCATTGTTGACCTCTGG	
RT-LAMP	LF	CGTGCTCAGATCTGAGAATC	
RT-LAMP	LB	TCCGCAGCCAACCTCGT	

2.4 RT-LAMP reaction and detection

The RT-LAMP reaction mixture consist of 1.0 µL of 10x ThermoPol Reaction Buffer, 0.4 µL of 8U/µL*Bst* DNA polymerase (New England Biolabs), 0.4 µL of 200U/µL Superscript III Reverse Transcriptase (Invitrogen, USA), 1.0 µL of 10mM dNTP mix (Invitrogen, USA), 1.6 µL of 5M Betaine (Sigma, USA), 1.2 µL of 50mM $MgCl_2$ (Invitrogen, USA), 0.4 µLof 5 µM each primers F3 and B3 (Integrated DNA Technology, USA), 0.4 µL of 40 µM each primers FIP and BIP, 0.4µL of 20µM each LF and LB primers and 0.8 µL of target RNA/DNA. The reaction mixture was incubated at 63^{o}C for 45 min and heated at 80^{o}C for 5 min to terminate the reaction.

The RT-LAMP reaction were observed visually for colour changing from soft orange to fluorescent green in the presence of LAMP amplified products. Alternatively, the RT-LAMP also been observed by electrophoresis in 2.5% agarose gel (Fermentas , USA) added

with 1:10000 SYBR® safe (Bio Rad, California, USA) staining dye and viewed in UV-transilluminator (Gelcompany, San Francisco, USA) for the present of ladder-like pattern.

2.5 Quantitative RT-PCR and RT-PCR

The cDNA of RNA transcript was produced using Superscripts III first-strand synthesis system (Invitrogen, USA). The reaction was done in one reaction tube with two steps. The first step was done with mixture of 1.0µL of 50ng/µL random hexamers, 1.0 µL of 10mM dNTP mix and 8.0 µL of total RNA and incubated at 65^{o}C for 5 min, and cold down in ice for 1 min. Next, 2.0 µL of 10x RT buffer, 4.0µL of 25 mM $MgCl_2$, 2.0 µL of 0.1 MDTT, 1 µL of 40 U/µL RNAse OUT and 1 µL of 200U/ µL Superscript III reverse transcriptase were added. The cDNA were synthesized at 25^{o}C for 10 min, 50^{o}C for 50 min and terminated at 85^{o}C for 5 min. The product was stored in -20^{o}C for further usage.

RT-PCR reaction mixture was performed with the NoV specific primers targeting the same region as RT-LAMP using the B3 and F3 primers (Table 1). The reaction mixture was carried out in a total volume 10 µL containing 5 µL of 2X Gotaq® Green Mastermix (Promega, USA), 1 µL of 10 µM F3 and B3 primers, 1 µL of cDNA template and 2 µL of dH_2O. The tube were incubated in thermal cycler (MJ Research,MA, USA) using programme; 94^{o}C for 5 min, 30 cycles of 94^{o}C for 45 s, 55^{o}C for 30 s and 72 ^{o}C for 30s and a final extension cycles at 72^{o}C for 5 min. The expected PCR product was 207bp.

The quantitative RT-PCR was performed in Mastercycler®quantitative PCR system (Eppendorf,USA) using SYBR Green I based real-time PCR amplification system. The amplification was carried out in a 25 µL reaction using SsoFast^{TM}EvaGreen® Supermix (BioRad, California, USA). The reaction mixture consist of 12.5 µL of SsoFast$^{™}$ EvaGreen® Supermix, 1.0 µL of each F3 and B3 primers (10µM), 2.0 µL of cDNA template and 8.5 µL of dH_2O. The cycler was programmed to95^{o}C for 30 s, followed by 40 cycles of 95^{o}C for 5 s, 55^{o}C for 10 s and 72^{o}C for 10s. The melting curve analysis was performed after the amplification cycles finished verifying the targeted products.

2.6 Application of the RT-LAMP and RT-PCR in spiked water samples

Aliquots from 11water samplesrandomly collected from two recreational streams located in the Hulu Langat province (Sg. Tekala and Sg. Gabai) were used as spiked water samples.NoV surrogates (RNAs)from the stock solution were measured based on theabsorbance at 260 nm and 10 µL of 55 ng/µL of NoV surrogates were added in 10% aliquots of the pelleted water samples continue with the RNA extraction. All extracted RNA from 11 spiked water samples were used for RT-LAMP and RT-PCR assays.

3 Results

3.1 RT-LAMP amplification and specificity test

The reaction mixture of NoV RNA surrogates RT-LAMP was incubated at 63^{o}C for 45 min. Although a positive reaction could be detected after 20 to 25 min incubation, the incubation time was prolonged to ensure the optimum reaction occurs during incubation.Positive RT-LAMP amplification can be detected due to the presence of fluorescent detection reagent. The observation made by colour changes from light orange to fluorescent green and presence of a ladder-like pattern on 2.5% agarose gel electrophoresis under UV-transilluminator. No positive reactions were found in all other environmental species *Burkholderia cepacia*, *B. thailandensis*, *B. pseudomallei, Bacillus subtilis, B.*

macerans, B. circulans, B. megaterium, Staphylococcus sp., *S .epiderminis, S. heamolyticus, E. coli* BL21, *E. coli* Nova Blue, *Salmonella* sp., *Shigella* sp. and *Shinella granuli*(data not shown).

3.2 Detection Limit of the RT-LAMP assay

The detection limit of the assay was determined by copy number of the 10-fold dilution of RNA transcripts. RNA concentration measured based on the absorbance at 260 nm was 55ng/μL and the transcribed length of RNA is 448 bp. The NoV RNA initial concentration was calculated and converted to copy numbers to get 2.17x 10^8copies μL^{-1} based on the formula by Krieg [25]. The lowest of detection (LOD) of the RT-LAMP assay was estimated to be ~22 copies μL^{-1} (Fig. 1B). The last positive results from the copy number dilution was defined as the detection limit (only samples with three replicates tested positives was considered positive (3/3))

3.3 Evaluation of the RT-LAMP, RT-PCR and quantitative RT-PCR

Standard curve of the 10-fold serially diluted RNA transcripts of NoV were tested in parallel with RT-PCR and quantitative RT-PCR. The detection limits of both assays were 220 copies and 22 copies, respectively (Fig. 1). The amplification of quantitative RT-PCR, conventional RT-PCR and RT-LAMP assays showed a linear plots against range of input RNA transcripts concentration generating coefficient of correlation for NoV surrogates detection R^2= 0.995 (Fig. 2A). Amplification and dissociation plots showed in melting curve analysis proved the primers were specific to the target gene sequence where all the amplified products have same melting temperature at 85.9°C (Fig. 2B).

The sensitivity results of NoV RT-LAMP and quantitative RT-PCR were both equal and 10-fold more sensitive than conventional RT-PCR on agarose gel electrophoresis.However, the RT-LAMP has the advantage over quantitative RT-PCR for its visibility by naked eye. The total end result of the RT-LAMP amplification was completed in 45 min faster than that quantitative RT-PCR (2.0 h) and conventional RT-PCR (2.5h).

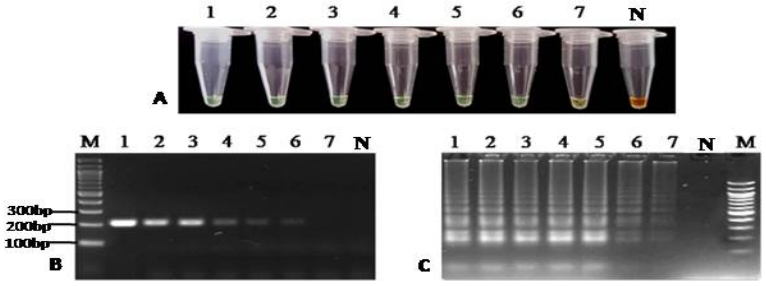

Fig. 1 Limit of detection of NoV RT-LAMP assay (positive results will show ladder-like amplicons, while negative results shows no amplification) was examined against the concentration of NoV surrogates ranging from 2.17 x 10^7 to ~22 copies/μL in 10-fold serial dilutions (lane M; 100bp marker, lane 1; 2.17 x 10^7, lane 2; 2.17x 10^6, lane 3; 2.17x 10^5, lane 4; 2.17x 10^4, lane 5; 2.17x 10^3, lane 6; 2.17x 10^2, lane 7; 2.17x 10^1, lane N; non-template control).(**A**) Color changes of the RT-LAMP reaction stained with fluorescent detection reagent showed the detection limit is 22 copies/μL.(**B**) Gel electrophoresis of PCR product (207bp) from conventional RT-PCR showed a 10-fold less sensitive then (**C**) the RT-LAMP method electrophoresis results in 2.5% agarose gels.

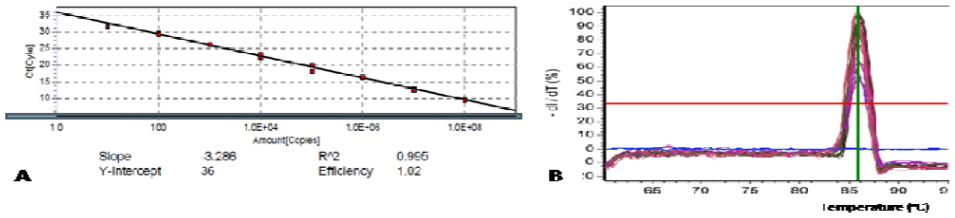

Fig.2 The amplification by quantitative RT-PCR (**A**) A graph plotted by real-time PCR system showed the coefficient of correlation for range of cDNA concentration input was R^2= 0.995. (**B**) Melting curves analysis showed amplification and dissociation of all PCR products happened at the same temperature of 85.9°C. A single target gene was amplified from the NoV surrogates.

3.4 Application of NoV RT-LAMP in spiked sample

The RT-LAMP assay was further evaluated with spiked water samples randomly picked from Hulu Langat province. Using the RT-LAMP and conventional RT-PCR the samples were examined for the presence of Noroviruses surrogates spiked in it. The results found that the RT-LAMP assays showed all positive (100%) for the presence of Noroviruses surrogates while the conventional RT-PCR showed only 8 (72.3%) out of 11 spiked of positive samples (Table 3).The gel electrophoresis analysis for RT-PCR assays and eyes observation of the RT-LAMP assays are shown in Fig. 3.

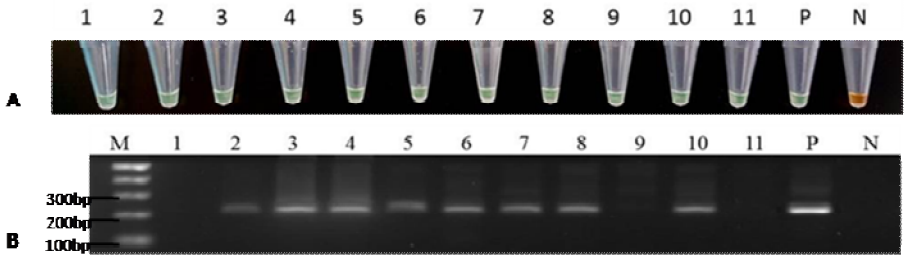

Fig. 3 RT-PCR and RT-LAMP assaysfor NoV surrogates detection in spiked recreational stream water samples. Results in lane 1 to 11 (stream water samples from lane M; 100bp marker, lane 1;Tekala 1, lane 2;Tekala 2, lane 3; Tekala 3, lane 4; Tekala 4, lane 5; Tekala 5, lane 6; Gabai 1, lane 7; Gabai 2, lane 8; Gabai 3, lane 9; Gabai 4, lane 10; Gabai 5, lane 11; Gabai 6, lane P; positive control, lane N; non-template control) showed that (**A**) RT-LAMP assay was able to detect NoV*in-vitro* transcribed surrogates spiked in all recreational stream water samples. (**B**) Conventional RT-PCR assay was unable to detect the NoV surrogates from sample 1, sample 9 and sample 11 and showed amplified products (207 bp) only in other 8 samples.

Table 2 Results of spiked water samples tested with RT-LAMP and RT-PCR

Water type	RT-LAMP assay	RT-PCR assay
Stream water tested	11	11
Positive/tested	11/11	8/11
(%)	(100%)	(72.3%)

4 Discussion

Noroviruses are shed in the faces of infected individuals and have high possibility to exist in very low concentration in water environment [21]. In recreational water, the risk for contamination may associate with overt fecal accidents or direct contact of contaminated bodies in the water [5].As a major causative agents of nonbacterial gastroenteritis in many countries, a rapid detection on the occurrence of NoV in recreational water is very much needed[1,3,5]. A study based on human volunteer showed the NoV infectious dose may be as low as 10to 100 viruses particles [6]emphasizes the need on highly sensitive method to detect their prevalence in water. NoV is usually detected in 3to 4h by RT-PCR method[22]. In latest innovation in DNA detection by amplification techniques, loop-mediated isothermal amplification (LAMP) has gain attention for it versatility in shorten the DNA amplification time to around one hour with results visible by naked eye using fluorescent dye. In this study, the RT-LAMP assay was demonstrated for the detection of NoV in comparison with the quantitative RT-PCR and conventional RT-PCR to evaluate the performance of each assay. The RT-LAMP has shown higher sensitivity with detection limit as low as 22 copies of NoV surrogates in 10µL reaction mixture with fluorescent dye. Detection of target NoV surrogates by RT-LAMP when compared to detection by quantitative RT-PCR was equivalent or more sensitive. The specificity test of the RT-LAMP, quantitative RT-PCR and conventional RT-PCR assays showed no-cross reactivity towards other 15 water and environmental bacteria strains available in our laboratories. Previously, the primers have been tested to be specific to NoV GII amongst other viruses including rotavirus, astrovirus, adenovirus, and other calicivirus and sapovirus[18].

Although DNA plasmid has been used to replace the RNA templates for assay optimization purpose in some study [23] (for the reason of RNA molecules are instable *in-vitro*) however, the DNA plasmid is not fully representative the RNA viruses such as NoV.In this study, the DNA plasmid was transcribed *in-vitro* which would served as better NoV surrogates. The sensitivity test and spiked recreational stream water sample was done using the *in-vitro* transcribed RNA. This form of surrogates would provide comparative reference to other laboratories doing the similar research.The use of fluorescent detection reagent for visualizing f RT-LAMP results simplifies the need of agarose gel electrophoresis. The step for opening the reaction tube also would introduce risk of contamination to the handler. Despite higher sensitivity thequantitative RT-PCR assay demands higher cost commitment which off benefit to the unequipped laboratoriesand therefore provide advantage toRT-LAMP assay for routine detection of NoV isolated from recreational stream water even on-site application.

5 Conclusion

The feasibility of RT-LAMP assay as method for detection of NoV surrogates in stream water should have a great potential for surveillance of NoV in Malaysia to improve the capacity for early prevention management.

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