Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of SARS-CoV2

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Background. Highly sensitive real-time reverse transcription polymerase chain reaction (qRT-PCR) methods have been developed for the detection of SARS-CoV2 . However, they are costly. Loop-mediated isothermal amplification (LAMP) assay has emerged as a novel alternative isothermal amplification method for the detection of nucleic acid.

Methods. A rapid, sensitive, and specific real-time reverse transcription LAMP (RT-LAMP) assay was developed for SARS-CoV2 detection.

Results. This assay detected 1 copy of SARS-CoV2 RNA in 30 mins. Both the clinical sensitivity and specificity of this assay were 100%. The RT-LAMP showed comparable performance with real-time qRT-PCR. Combining simplicity and cost-effectiveness, this assay is therefore recommended for use in resource limiting settings.

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2 **Isothermal Amplification for Rapid Detection of SARS-**

3 **CoV2**

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Abstract

Background. Highly sensitive real-time reverse transcription polymerase chain reaction (qRT-PCR) methods have been developed for the detection of SARS-CoV2. However, they are costly. Loop-mediated isothermal amplification (LAMP) assay has emerged as a novel alternative isothermal amplification method for the detection of nucleic acid.

Methods. A rapid, sensitive, and specific real-time reverse transcription LAMP (RT-LAMP) assay was developed for SARS-CoV2 detection. Forty-seven qRT-PCR positive and 42 qRT-PCR negative nasopharyngeal swabs samples were tested.

Results. This assay detected one copy of SARS-CoV2 RNA in 30 mins. Both the clinical sensitivity and specificity of this assay were 100%. The RT-LAMP showed comparable performance with real-time qRT-PCR. Combining simplicity and cost-effectiveness, this assay is therefore recommended for use in resource limiting settings.

Introduction

A novel coronavirus, SARS-CoV2, was recently identified causing pneumonia in humans, termed coronavirus disease 2019 (COVID-19). Cases of this new infection were first reported in Wuhan, China in December 2019, and the outbreak spread to more than 155 other countries in a short time [1]. The World Health Organization (WHO) declared the COVID-19 outbreak as a pandemic on 11 March 2020. Several real-time reverse transcription polymerase chain reaction (qRT-PCR) methods have been developed and recommended by Centers for Disease Control (CDC) of the United States [2] and WHO [3,4] for detection of SARS-like coronaviruses and specific detection of SARS-CoV2. These methods are highly sensitive and specific but are too expensive to be widely used in many developing countries. Real-time RT-PCR also requires experienced personnel,

46 maintenance of reagents in cold storage facility, and use of a high-precision thermal cycler. Loop-

47 mediated isothermal amplification (LAMP) has emerged as a novel alternative isothermal

48 amplification method for the detection of nucleic acid [5]. The LAMP assay was reported to take

49 less than one hour to perform at a constant temperature. Since LAMP does not require any major

50 equipment and is simple to perform, it represents an ideal diagnostic tool for use in areas with

51 limited resources. LAMP assays have been described for the detection of various other infectious

52 agents including dengue viruses [6,7] and malaria parasites [8]. In order to further reduce costs

53 and enable detection by the naked eye, we used hydroxynaphthol blue (HNB) dye for the

54 colorimetric detection of the amplification reaction. To our knowledge, this is the first report of

55 the detection of SARS-CoV2 by real-time reverse transcription LAMP (RT-LAMP) assay with

56 HNB.

57

58 **Materials & Methods**

59 **RT-LAMP assay**

60 Primers were designed using the Primer-Explorer V4 software (Eiken Chemical Co., Ltd., Tokyo,

61 Japan) based on SARS-CoV2 nucleoprotein (GenBank accession no MN988713.1, LC528233.1

62 and MT123293.1). The sequences of the selected primers were conserved among the SARS-CoV2

63 sequences (Supplementary Figure 1) that do not share homology with other SARS-like coronavirus

64 and Middle East respiratory syndrome coronavirus (MERS) sequences (Supplementary Figure 2).

65 Reverse transcription LAMP (RT-LAMP) was carried out using Loopamp RNA amplification kit

66 (Eiken Chemical Co., Ltd., Japan). The amplification was carried out in a Loopamp real-time

67 turbidimeter (LA-320; Teramecs, Co., Ltd., Tochigi, Japan) at 65 °C for 30 min with 2X reaction

68 mixture, 5 μL RNA template, and species-specific primers. The primer sequences are listed in

69   Supplementary Table 1. Endpoint assessment was done by visual inspection following the addition

70   of 1 µl HNB (Sigma, USA) in the master mix; a positive amplification was indicated by a colour

71   change from violet to sky blue (Figure 1).

72

73   **Analytical sensitivity and specificity**

74   To determine the analytical sensitivity of the SARS-CoV2 RT-LAMP assay, *in vitro* transcript

75   RNAs were prepared using a previously published method [9]. Briefly, target gene fragments were

76   cloned to a pGEM-T vector as described in the manufacturer's protocol (Promega, USA).

77   Following digestion with *Sal*I restriction endonuclease (NEB, USA), the plasmids were purified.

78   *In vitro* transcribed RNAs were prepared with RiboMAX™ System (Promega, USA) and digested

79   by deoxyribonuclease (DNase) I as described in the manufacturer's protocol. The product was then

80   purified by phenol-chloroform extraction method. Finally, *in vitro* transcribed RNAs were

81   quantified by UV spectrophotometry. The limit of detection (LOD) was determined using 10-fold

82   serially diluted *in vitro* transcript RNA with known numbers of nucleic acid copies (10 cp/µL, 5

83   cp/µL, 2 cp/µL, 1 cp/µL and 0.1 cp/µL) and comparing the assay with qRT-PCR. The reactions

84   were carried out in duplicates. One µL of each diluted *in vitro* transcript RNA was used for qRT-

85   PCR and RT-LAMP assay.

86   The specificity of the RT-LAMP assay was determined by using genomic RNA of coronaviruses

87   (HCoV-OC43 and SARS-CoV), adenovirus, human metapneumovirus, influenza A

88   (A/H1pdm2009 and A/H3) viruses, influenza B virus, parainfluenza virus 3, rhinovirus A,

89   respiratory syncytial virus B and enterovirus D68.

90

91   **Clinical sensitivity and specificity**

92   Forty-seven qRT-PCR positive and 42 qRT-PCR negative nasopharyngeal swabs samples were

93   randomly chosen, regardless of qRT-PCR threshold cycle value (Ct-value) from a recent COVID-

94   19 outbreak in Malaysia (2020) were collected by Hospital Sungai Buloh, Malaysia.

95   Nasopharyngeal swabs were collected using Dacron swabs made of polyester fiber. The swab was

96   inserted into the nostril and back to the nasopharynx and left in place for a few seconds then slowly

97   withdrawn with a rotating motion. The swab was immediately placed into a sterile vial containing

98   2 ml of viral transport media. Total RNA was extracted using QIAamp viral RNA Mini kit (Qiagen,

99   Germany) according to the manufacturer's instructions. In brief, 140 μL of cell lysates were

100   transferred into 1.5 mL tubes containing 560 μL of Buffer AVL followed by vortex for 15 s. After

101   standing at room temperature for 10 minutes, the collection tube was briefly centrifugated. Then

102   560 μL of ethanol (96–100%) was added to the sample, and mixed by vortex for 15 s. The mixture

103   was then transferred to QIAamp Mini column and washed with Buffer AW1 and AW2. A 50 μL-

104   elution was obtained for each sample. The RNA samples were analysed by qRT-PCR (Figure 2),

105   as previously described [3,4]. Briefly, 5 μL of extracted RNA were added as template into 20 μL

106   of the reaction mixture containing 12.5 μL of 2 × reaction buffer provided with the Superscript III

107   one step RT-PCR system with Platinum Taq Polymerase (Invitrogen, USA), 1 μL of reverse

108   transcriptase/Taq mixture from the kit, 0.4 μL of a 50 mM magnesium sulphate solution and 1 μg

109   of nonacetylated bovine serum albumin. Primer and probe sequences targeted at RdRP and E genes

110   were used. Thermal cycling was performed at 55 °C for 10 min for reverse transcription, followed

111   by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s, 58 °C for 30 s.

112   Extracted RNA for qRT-PCR were kept in -80°C until further analysis by RT-LAMP. SARS-

113   CoV2 RT-LAMP reactions were run at 65°C for 30 min. Clinical sensitivity was calculated as

114   (number of true positives)/(number of true positives + number of false negatives) and clinical

115   specificity was calculated as (number of true negatives)/(number of true negatives + number of

116   false positives) comparing to qRT-PCR. Ethical approval for this study was obtained from Medical

117   Research Ethics Committee (MREC) Ministry of Health Malaysia (NMRR-20-535-53855).

118

Results

120   The SARS-CoV2 RT-LAMP assay was able to detect one copy per reaction of SARS-CoV2 RNA

121   in 30 mins while the LOD for qRT-PCR was five copies per reaction (Supplementary Table 2).

122   The time taken for amplification did not change with or without the addition of HNB in the master

123   mix (Supplementary Table 2). No cross-reactivity with other respiratory viruses (coronaviruses,

124   adenovirus, human metapneumovirus, influenza A viruses, influenza B virus, parainfluenza virus

125   3, rhinovirus A, respiratory syncytial virus B and enterovirus D68) was found in either assay

126   (Supplementary Table 2).

127   The RT-LAMP assay demonstrated a 100% sensitivity as all the 47 RNA samples that were

128   positive by qRT-PCR were tested positive with RT-LAMP. None of the 42 qRT-PCR negative

129   samples were positive for SARS-CoV2 using this assay (Supplementary Figure 3). No false-

130   positive reactions were noted (Supplementary Table 2, Supplementary Figure 4).

131

Discussion

133   The COVID-19 RT-LAMP reaction was sensitive enough to detect one copy of RNA per reaction,

134   5-fold better than real-time PCR. Addition of HNB to the LAMP reaction solution did not affect

135   the time taken for amplification. Several studies have found that LAMP out-performs qRT-PCR

136   for other viral infections [6,10,11], which is consistent with our results.

137   The analysis showed that the RT-LAMP developed is 100% specific and sensitive for the detection

138   of SARS-CoV2 with no false positives detected. The specificity and sensitivity levels of RT-

139   LAMP are comparable to real-time qRT-PCR methods as reported in other studies [12,13].

140   Encouragingly, the time required for confirmation of results by the RT-LAMP assay was less than

141   30 mins, 2.5-fold faster than the time required by real-time PCR. Even including the RNA isolation

142   step, RT-LAMP assay can be completed in less than one hour, which is very short when compared

143   to that for real time qRT-PCR (two to two and a half hours).

144   Due to its high sensitivity, RT-LAMP is prone to aerosol contamination. LAMP assays can be

145   analysed by running an agarose gel or adding SYBR Green I. As it has an inhibitory effect, SYBR

146   Green I has to be added after completion of the LAMP reaction. Tubes used for RT-LAMP

147   reactions have to be opened for gel electrophoresis for addition of SYBR Green I, which can

148   contaminate the air and subsequent reactions. Therefore, in our study, to avoid contamination,

149   HNB dye was used to enable interpretation of the results by the naked eye, without requiring the

150   tubes to be opened. Addition of HNB to the LAMP reaction solution did not affect amplification

151   efficiency. This approach has been shown to be sensitive and simple for visual detection of turkey

152   coronavirus RNA in tissues and faeces [14].

153

Conclusion

155   There are several limitations in this study. First, the LOD of the SARS-CoV2 RT-LAMP assay

156   was not determined using serial dilutions of purified cell culture supernatant of SARS-CoV2 due

157   to the unavailability of BSL3 laboratory. Secondly, although we showed specificity when testing

158   against the most closely-related human coronavirus SARS-CoV, we were unable to obtain RNA

159   from other coronaviruses HCoV-229E, HCoV-NL63, and MERS-CoV for specificity analysis.

160   These experiments should be included in the future to enhance the stringency of the RT-LAMP

161   assay.

162   In order to improve the efficiency of the RT-LAMP reaction, parameters such as heating

163   temperature, dNTP concentration, and reaction time can be further optimized. There is a high risk

164   of contamination due to the large amount of LAMP products, which often leads to false-positive

165   results. A typical molecular laboratory should be divided into at least three areas for sample

166   preparation, master mix preparation and product detection. However, this may not be available in

167   resource-limited countries. To avoid contamination, general cleaning practices such as

168   decontaminating all surfaces with 10–15 % sodium hypochlorite solution, followed by 70 %

169   ethanol should be routinely carried out. It is highly recommended to adopt a closed end-point

170   detection method in order to avoid carry-over contamination. On top of that, additional mineral oil

171   inside the reaction tubes can reduce the risk of contamination. Sample processing time and cost

172   can be reduced by using direct pathogen detections without upstream RNA extraction by

173   commercial kits. Nie et al., (2012) [15] demonstrated that direct RT-LAMP assay can detect EV71

174   in heat-treated nasopharyngeal swab specimens.

175   Lastly, the sensitivity and specificity of the RT-LAMP assay can only be compared with qRT-

176   PCR as there is no true gold standard for SARS-CoV2 detection. It is possible that either test may

177   have misclassified the true result of some of the samples. It is also important to note that the LAMP

178   primers are highly specific, different primers may be needed for detection of different types of

179   mutations of the target gene in the future.

180   SARS-CoV-2 can also be detected in other biological samples such as sputum, bronchoalveolar

181   lavage fluid, feces and blood [16]. Future studies are expected to assess the feasibility of RT-

182   LAMP in detection of SARS-CoV2 RNA extracted from these biological samples.

183   To conclude, an inexpensive, rapid, sensitive, and specific RT-LAMP assay was successfully

184   designed for SARS-CoV2 detection. The simplicity of RT-LAMP combined with rapid turnaround

185   time has shown it to be a valuable and applicable tool for the diagnosis of infectious diseases,

186   particularly in resource-limited countries. In addition, RT-LAMP can be easily adapted to point-

187   of-care diagnosis of COVID-19 as supported by a review written by Nguyen et al., (2020) [17]

188   and a non-peer-reviewed preprint by Zhang et. al., (2020) using seven clinical samples [18]. Early

189   and accurate diagnosis is crucial to identify patients with COVID-19 for prompt

190   institutionalization of infection control and public health measures, and when available, treatment.

191

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Figure 1

Asessment of RT-LAMP results based on hydroxynaphthol blue visualization of color change.

A positive reaction indicated by sky blue colour is seen in tubes 1–2 and the positive control (PC), while a violet colour indicates a negative reaction in tubes 4-5 and the non-template negative control (NC).
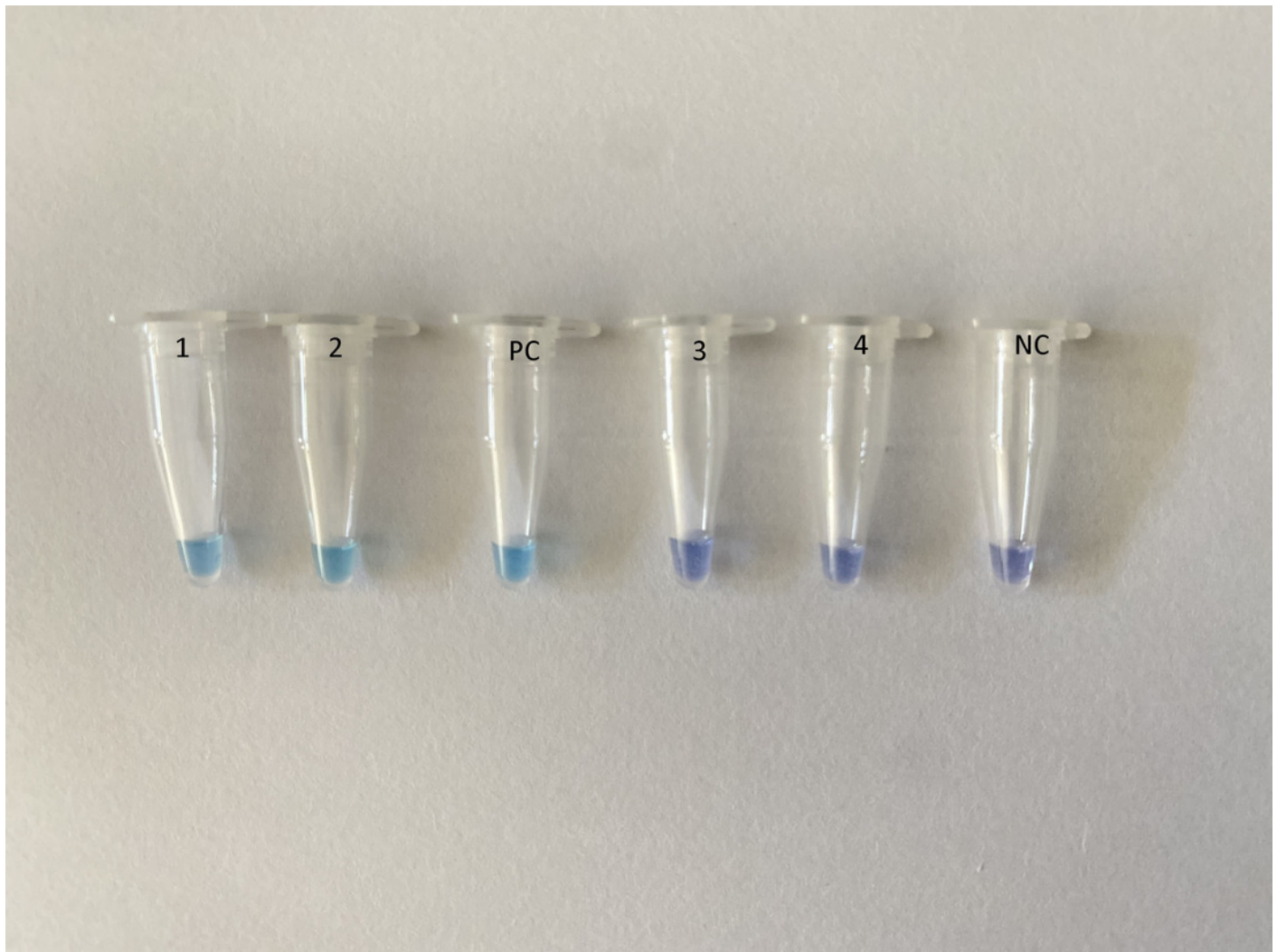
Figure 2

qRT-PCR results for SARS-CoV2 detection.

The chart was generated by plotting relative fluorescence (RFU) vs. cycle number, with each colored line representing one sample. Seven positive samples and a positive control (PC) are shown with cycle threshold levels between 18 to 40 cycles.