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Research article

Colorimetric isothermal nucleic acid detection of SARS-CoV-2 with dye combination

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ABSTRACT

RT-LAMP detection of SARS-CoV-2 has been demonstrated to be a valuable diagnostic method for the diagnosis of COVID-19[1,2], which can rapidly screen carriers of the virus to effectively control the spread of the SARS-CoV-2. Here, we present a combination of dyes for isothermal detection of SARS-CoV-2 as a commercial alternative, with expanded colorimetric spectrum. We compared them with commercial reagents and proved their suitability and sensitivity through clinical RNA samples. In addition, together with commercial single dye indicators, we believe the expanded color spectrum developed here as an indicator of rapid detection will promote the diagnosis of COVID-19.

Introduction

Nucleic acid amplification is essential for molecular diagnostic assays [1, 2, 3, 4]. The gold standard for RNA detection is reverse transcription quantitative PCR (RT-qPCR) which detected by fluorescence, and it requires access to expensive laboratory instruments, trained personnel or extended experimental workflows (e.g. electrophoresis, UV transilluminators, real-time thermocyclers). The limitations mentioned above are especially evident when the need for molecular diagnosis exceeds testing capacity [5, 6, 7]. A clear example was during the novel coronavirus SARS-CoV-2 pandemic outbreak worldwide. In those conditions, the use of simple approaches such as loop-mediated isothermal amplification (LAMP) [8, 9, 10] combined with reverse transcription (RT-LAMP) [11, 12, 13] and colorimetric detection [14, 15, 16] has the potential to offer a rapid, accurate and on-site diagnosis. We have recently established iLACO (isothermal LAMP based method for COVID-19) platform by isothermal amplifying the SARS-CoV-2 coupled with a single pH indicator present in the reaction mix causing a readout of the amplification [17, 18, 19]. In this method, six specific primers were designed using PrimerExplorer V5 to amplify ORF1ab gene fragment (conserved region of SARS-CoV-2) with DNA polymerase and reverse transcriptase with chain replacement activity in a short period of time. The accumulation of hydrogen ions during the amplification reaction results in a decrease in the pH of the reaction solution. Therefore, by using a visual pH indicator in the premix solution, the positive result can be determined by visually observing whether the reaction solution changes from pink to yellow. However, the unitary color change is inappropriate for the hundreds of millions of people effected by color weakness [20]. Also the commercial used color changing based kits limits the application in regions limited with delivery and budgets. In this study, we aimed to expand the colorimetric spectrum for a wider user application during COVID-19 pandemic, without affecting sensitivity and accuracy of iLACO.

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Materials and methods

RT-LAMP reaction

RT-LAMP assays were assembled in a total reaction volume of 20 μl, each 10 μl iLACO reactions contained: 2 μl of home-made 10X buffer (100 mM $(NH4)_2SO_4$, 500 mM KCl, 80 mM $MgSO_4$, and 1% v/v Tween-20), 2.8 μl of dNTP mix (10mM each, TianGen), l μl of Bst 2.0 WarmStart DNA Polymerase (New England Biolabs) or Bst V7.16, 0.5 μl of RTx reverse transcriptase (New England Biolabs) or home-made RT, 1 μl of a combination of dyes (both 0.5 μl of phenol red and azure II, 2mM of each dye), and above components were mixed in DEPC H_2O up to 10 μl, then adjusted pH to 7.5 with 1M KOH and measured by pH paper (Supelco). In addition to the above, 2 μl of 10X LAMP primer mix (2 μM F3, 2 μM B3, 16 μM FIP, 16 μM BIP, 4 μM LF and 4 μM LB), 1 μl sample and DEPC H_2O up to 20 μl were required.

iLACO primer design

During the test, ORF1ab gene was used as a target, as described before [17]. The concentration of primers required for the reaction were as followed: 0.2 μM of each outer primer (F3 5′-CCACTAGAGGAGCTACTGTA-3′ and B3 5′-TGACAAGCTACAACACGT-3′), 1.6 μM of each inner primer (FIP 5′-AGGTGAGGGTTTTCTACATCACTATATTGGAACAAGCAAATTCTATG-G-3′ and BIP 5′-ATGGGTTGGGATTATCCTAAATGTGTGCGAGCAAG AACAAGTG-3′), 0.4 μM of each loop primer (LF 5′- CAGTTTTTAA CATGTTGTGCCAACC-3′ and LB 5′-TAGAGCCATGCCTAACATGCT-3′).

Commercial reagents

All commercial reverse transcriptase (RT) and Bst enzyme or WarmStart Colorimetric master mix were obtained from New England Biolabs. Phenol red was obtained from Sigma-Aldrich, and bromothymol blue was obtained from BBI. Cresol red and neutral red and indigo were obtained from MBL. Alcian and methylene blue and azure II as well as toluidine blue were all purchased from Solarbio LIFE SCIENCES.

Sample collection

This study used excess RNA samples from patients with suspected SARS-CoV-2 infection based on routine clinical diagnosis, chest imaging and epidemiological evidence. No patient identifiable information was collected. The only data collected from the samples were types of specimens (53 nasopharyngeal swabs). We obtained positive patient samples from Shenyang Center for Disease Control And Prevention (Shenyang CDC). Samples were collected between Mar 16th and Apr 1st 2020. We conducted the study on April 22nd. All samples were tested with Taqman RT-qPCR, which Ct values under 37 were called as positive, while Ct values were not determined or above Ct 37 were called as negative. For RT-LAMP, each sample was heat inactivated at 95 °C for 15 min and stored at -80 °C prior to experiments.

Ethics statement

Sample collection and analysis of samples were approved in the P2 laboratory by the local CDC of Shenyang city. The internal use of samples was agreed under the medial and ethical rules for each participating individual and the written informed consent was waived.

Results and discussion

Optimized combination of dyes have been developed that can be used as a complementary spectrum to expanded the application of colorimetric isothermal nucleic acid detection. In addition, we aim to research and development a new none-commercially viable product as a substitute for currently existing products. Thus, we combined pH-sensitive dyes with pH-insensitive dyes, to create combined mixed color by following some basic color matching principles, and WarmStart® Colorimetric

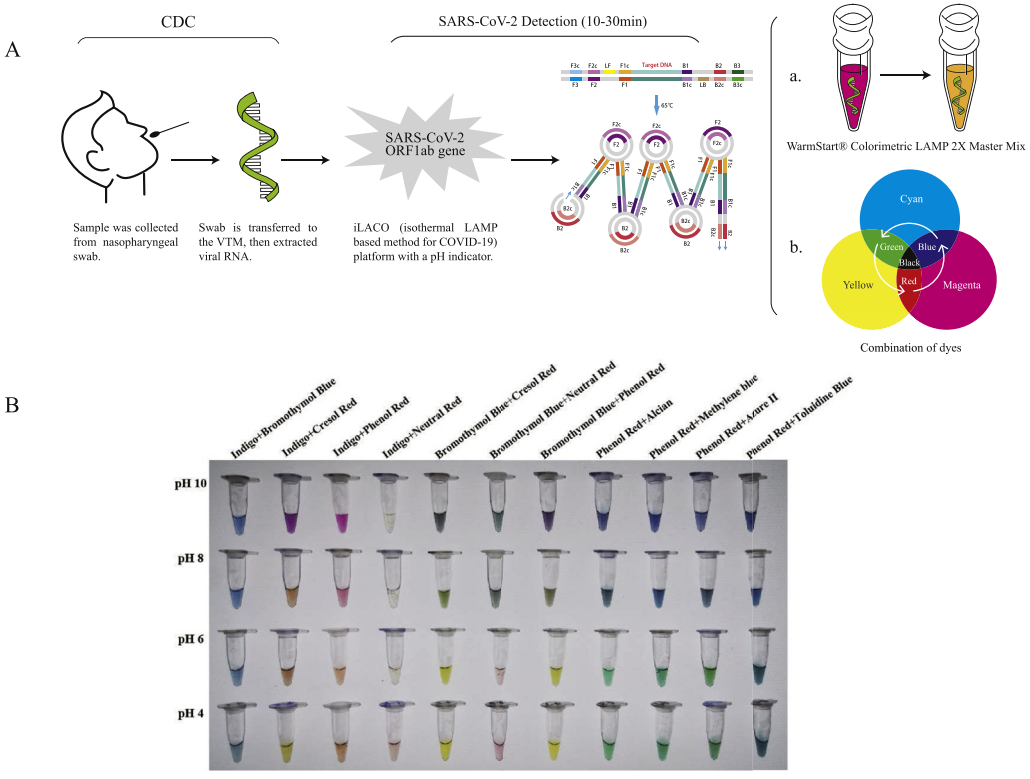

Figure 1. (A) Process for dye combination based on iLACO is shown. (B) Color-array of combined mixed color changed with pH range at 10, 8, 6, and 4 in iLACO reaction buffer condition.

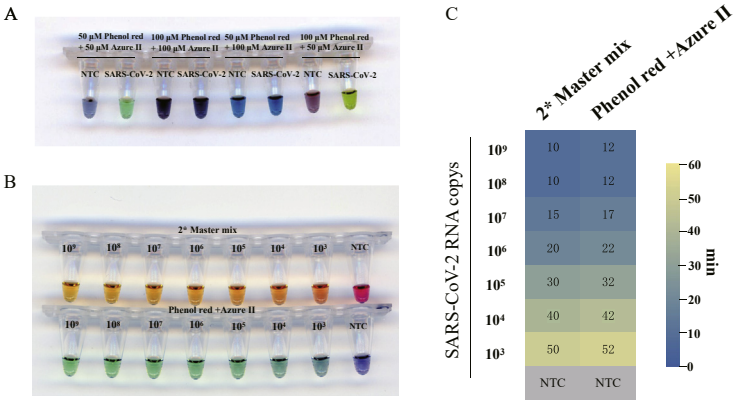

Figure 2. Colorimetric isothermal nucleic acid detection of SARS-CoV-2 with dye combination. (A) Detection of SARS-CoV-2 with different concentrations of azure II-pheno red combined dye. (B) Sensitivity and accuracy of SARS-CoV-2 detection, WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs) as a reference with diluted patient samples (duplicates of 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies of RNA as well as non template control (NTC)). (C) Summary of SARS-CoV-2 detection for diluted patient samples, WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs) as a reference, color indicates time of detection of amplifying, gray indicates no amplification of no template control.

LAMP 2X Master Mix (New England Biolabs) as a reference (Figure 1A). On that basis, we chose four pH-sensitive dyes (phenol red, bromothymol blue, cresol red, neutral red) and five pH-insensitive dyes (indigo, alcian, methylene blue, azure II, toluidine blue). Using those dyes, we created a color-array to select the best combinations by mixing 100 μM dyes at 1:1 ratio. We tested the combined color change with pH range at 10, 8, 6, and 4 in iLACO reaction buffer condition respectively (Figure 1B). This combination gave us the opportunity to pick new indicator colors. For example, the mixed dyes for phenol red-azure II and phenol red-methylene blue gave the color change from blue-purple to green, when pH changed from 7.5 to approximately 6.5 (Figure 1B). And the mixed dyes for bromothymol blue-cresol red and bromothymol blue-phenol red gave the color change from green to yellow (Figure 1B). We chose the dye combinations that were cheap and non-toxic for visual detection and selected two sets as the optimal combination that facilitated the visual identification of DNA amplification. The apparent color switch would help people in diagnostics field to interpret the COVID-19 data properly without additional assistance.

Once established this optimized colorimetric dye combination, we decided to apply it to the detection of SARS-CoV-2 with the iLACO platform. In addition to the commercial reverse transcriptase (RT) and Bst enzyme or WarmStart® Colorimetric LAMP 2X Master Mix, we used the home-made RT and Bst (Bst V7.16) to perform iLACO [21, 22].

iLACO reactions were performed using the home-made solution without Tris-HCl, containing only a small amount of Tris-HCl carried over from the DNA polymerase storage buffer. Initially, we used both Bst 2.0 WarmStart DNA Polymerase and Bst 3.0 to select the optimal enzyme. Thereafter, we found that Bst 2.0 WarmStart DNA Polymerase was more efficient with a concentration of 0.32 U/μL compared to Bst 3.0 (data not shown). And we used Bst 2.0 WarmStart to proceed the following experiments together with home-made Bst V7.16 to compare the performance. In addition, 0.3 U/μL RTx reverse transcriptase (New England Biolabs or home-made) should be added to a direct detection of SARS-CoV-2 for iLACO. The combination of dyes was added to the reaction mixture at final concentration of 100 μM with ratio of 1:1. And the reaction was performed at 65 °C for 40 min. The color of the reaction mixture was checked after the reaction and removed from metal bath or water bath. Before and after the reaction, pictures were recorded with a mobile phone or scanner.

We first tested the initial optimal pH in the reaction solution for colorimetric iLACO using only one dye. We checked reaction solutions with pH gradient from 8.8 to 7.5 with COVID-19 patient samples and 100 μM phenol red indicator. And we found that the optimal initial solution pH was 7.5 and did not observe any color change with reaction solution at pH 8.8, which was previously recommended [18] (data not shown). Then we evaluated the novel combination dyes with SARS-CoV-2 RNA extracted from COVID-19 patients with optimized buffer solution. For bromothymol blue and neutral red combination, we observed that the color changed from green to pink (bromothymol blue 200 μM and neutral red 100 μM). Similarly, we conducted the concentration gradient experiment of the dye indicator combination of azure II and phenol red. The result elucidated that when the ratio of azure II to phenol red was 1:1 at concentrations 50 μM, the color changed from blue-purple to green with best contrast (Figure 2A). To evaluate the sensitivity of the new dye combinations, we used commercial WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs) as a reference, and conducted iLACO with azure II-phenol red combined dye and home-made RT and Bst V7.16. Fifty-three RNA samples from COVID-19 patients were tested and series dilution was made to check the sensitivity. Notably, it was observed that the new combination of pH dyes showed the same sensitivity and accuracy but with different color spectrum compared with commercial colorimetric kit in 53 patient samples (Figures 2B and 2C and data not shown).

Conclusion

Here, we developed a combination of dyes for isothermal detection of SARS-CoV-2 with iLACO platform. This new colorimetric combination could be used for the detection of SARS-CoV-2 without compromising sensitivity and accuracy. Together with single dye indicators, we believe the expanded color spectrum developed here as on-site rapid detection indicator would facilitate the COVID-19 diagnosis.

Declarations

Author contribution statement

Shanshan Wu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xiyang Liu: Performed the experiments; Analyzed and interpreted the data.

Shenglong Ye: Performed the experiments.

Jianmin Liu, Wei Zheng, Xue Dong: Contributed reagents, materials, analysis tools or data.

Xiushan Yin: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare the following conflict of interests: Combination of pH-sensitive dyes for visual detection of SARS-CoV-2 or other viruses, which authors have filed a patent application describing this method.

Additional information

No additional information is available for this paper.

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