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Article

Comparative diagnostic performance of a Cas13-based assay for detecting COVID-19 cases in Al-Dewaniyah province, Iraq.

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ABSTRACT

Effective, accurate, and rapid diagnostic tools for detecting SARS-Covid2 infection are urgently needed to prevent and control the pandemic. While RT-qPCR is a gold standard test for diagnosing SARS-Covid2 infections, this method is limited by the requirement for expensive equipment, experienced staff and specialized molecular laboratory. Recently, the CRISPR cas13 platform was used for the detection of COVID-19. This study evaluated the clinical performance of the CRISPR-based cas13a diagnostic assay for SARS-Covid2. In the current study, the diagnostic performance of the Cas13-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter Unlocking) assay was compared with RT-qPCR in terms of sensitivity, specificity and time consumption.

Keywords: Crispr-Cas13; Covid-19; Sherlock.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) [1]. SARS-CoV-2 is highly contagious, and the virus can transmit from human to human via droplet [1,2]. Thus, accurate, effective, and rapid diagnosis is crucial to monitor and control the infection. RT–WHO highly recommends the detection of the virus based on PCR and remains the standard diagnostic test for COVID-19 [3]. However, this test required well-trained personnel, specific equipment, and laboratory infrastructure[4]. In recent years, The CRISPR Cas system has been used for rapid, specific, and sensitive detection of nucleic acid [5]. The CRISPR systems represent the immune defense line of bacteria against foreign nucleic acid. Cas enzymes associated with CRISPR act on recognizing and eliminating the foreign genetic material through endonuclease activity [6]. Cas is accompanied by CRISPR RNA (crRNA), which guides Cas protein to recognize and degrade target DNA or RNA[7]. Cas13 is one of the CRISPR-associated proteins that specifically bind to RNA molecules, complementary to the target binding CRISPR RNA (crRNA) [8]. When combining target RNA

pre-amplification with RNA detection using custom crRNA and Cas13, this diagnostic platform is called SHERLOCK (specific high sensitivity enzymatic reporter unlocking) [5]. In this study, the clinical diagnostic performance of SHERLOCK basedCas13a for detecting COVID-19 was evaluated and compared with real-time reverse transcription assay(qPCR).

MATERIALS AND METHODS

Sample Collection

The infectious diseases department at Al-Dewanyiah Teaching Hospital, Al-Dewaniyah, Iraq, provided 157 RNA samples. The RNA sample was extracted from suspected COVID-19 patients.

RPA Primer & crRNA Design

RPA primers of two target genes (S gene and Orf1ab gene, Table 1) were designed to check the presence of COVID-19 RNA in nucleic acid extraction from patient samples. Primers were designed according to the instructions described in TwistAmp Assay Design (https://www.twistdx.co.uk/wp-content/uploads/2021/04/twistamp-assay-design-manual). Cas13a guide RNAs for detecting S & Orf1ab gene and reporter RNA for later follow read out (Table 1) were designed according to Zhang et al., 2020 [9]. Primer, Guide RNAs and reporter synthesized by Integrated DNA Technologies (IDT).

primer	Sequence	Ref.
S-RPA-Forward	GAA ATT AAT ACG ACT CAC TAT AGG GAG GTT TCA AAC TTT ACT TGC TTT ACA TAG A	9
S-RPA-Reverse	TCC TAG GTT GAA GAT AAC CCA CAT AAT AAG	9
Orf1ab-RPA-Forward	GAA ATT AAT ACG ACT CAC TAT AGG GCG AAG TTG TAG GAG ACA TTA TAC TTA AAC C	9
Orf1ab-RPA-Reverse	TAG TAA GAC TAG AAT TGT CTA CAT AAG CAG C	9
S-crRNA	rGrArU rUrUrA rGrArC rUrArC rCrCrC rArArA rArArC rGrArA rGrGrG rGrArC rUrArA rArArC rGrCrA rGrCrA rCrCrA rGrCrU rGrUrC rCrArA rCrCrU rGrArA rGrArA rG	9
Orf1ab-crRNA	: rGrArU rUrUrA rGrArC rUrArC rCrCrC rArArA rArArC rGrArA rGrGrG rGrArC rUrArA rArArC rCrCrA rArCrC rUrCrU rUrCrU rGrUrA rArUrU rUrUrU rArArA rCrUrA rU	9
Lateral-Flow-Reporter	mArA rUrGrG rCmAmA rArUrG rGrCmA	9

Table 1. Primers for SHERLOCK assay (RPA-CAS13-LFD)

Synthesis Positive Control of SARS-CoV-2 S Gene and Orf1ab Gene RNA Fragments by in Vitro Transcription

SARS-CoV-2 RNA was provided by Al-Dewanyiah Teaching Hospital, RNA covert to cDNA using specific primer for s gene and orfab gene (Table 1) and according to manufacturer instruction(ProtoScript® II Reverse Transcriptase (M0368L), New England Bi-oLabs). cDNA of the two genes were amplified using TwistAmp® Basic kit (TABAS03KIT, TwistDx Limited). Then, the RPA product of each gene was cleaned up using Monarch® PCR & DNA Cleanup Kit(T1030S). Finally, the constructed SARS-COV2 S gene orfab gene was Transcripted in vitro

using HiScribe™ T7 Quick High Yield RNA Synthesis Kit (E2050S) according to the manufacturer's protocol.

Reverse Transcriptase Recombines Polymerase Amplification (RT-RPA)

The basic RT-RPA was achieved by TwistAmp® Basic (TABAS03KIT) with the addition of ProtoScript® II Reverse Transcriptase (M0368L). Each lyophilized RPA pellet was re-suspended with 29.5 rehydration buffer (supplied in the RPA kit.). The reaction was set according to the following formula: 5.9 ul of Re-suspended RPA solution,0.5 S or Orfab RPA upstream primer (10 uM), 0.5 S or Orfab RPA downstream primer (10 uM),0.2 Reverse Transcriptase, 1.4ul of ddH2O. 1ul of RNA(sample) and 0.5 ul MgAc(280 mol/l). The reaction was mixed gently and incubated at 42°C for 25 minutes[9].

Detection of The Target RNA Using Cas13

LwaCas13a protein(GenCRISPR™ Cas13a) diluted in storage buffer(1M Tris pH7.4, 5M NaCl, 2.5 mL of glycerol, and 1M DTT). The RPA-CAS13 reaction was as follows: 2 ul cleavage buffer (400mM Tris pH 7.4), 9.6 ul ddH2O, 2 ul LwaCas13a protein (diluted), 1 ul of S or fab crRNA (10 ng/ul), 1 ul Lateral-Flow-Reporter (20 uM),1ul RNase Inhibitor (M0314S), 0.6 ul T7 RNA Polymerase (New England biolab, M0251S), 1 ul MgCl2 (120mM) and 1 ul S or orfab RT-RPA reaction. The reaction was incubated at 37°C for 40 minutes.

Lateral Flow Readout

Readout of the Cas13 detection reaction is achieved by Lateral-Flow-Reporter: (5'-/56-FAM/mArArUrGrGrCmAmArArUrGrGrCmA/3Bio/-3)[9]andHybriDetect Dip-stick((Milenia HybriDetect , TwistDx). The 20µl-LwaCas13a reactions were mixed with 80 ul of HybriDetect Assay Buffer, and then HybriDetect Dipstick was added to the reaction tube. Control positive and positive samples should show two lines, while negative control and negative samples show one line, Figure 1[3,9].

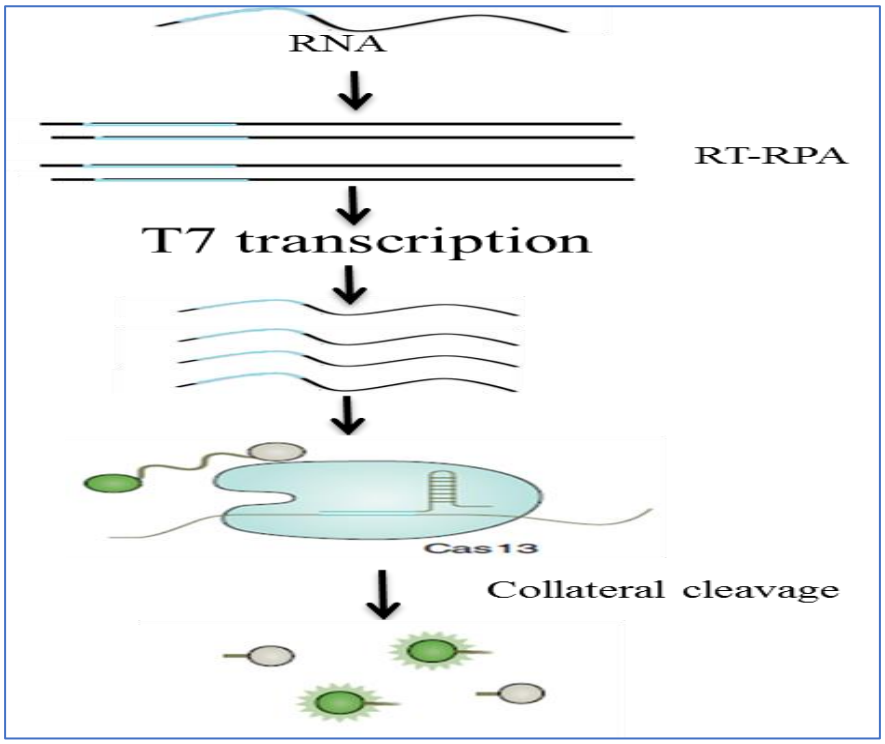


Figure 1. Schematic illustration of the SHERLOCK platform using Cas13a

RESULTS

Optimization of RPA-Cas13a-LFD Assay (SHERLOCK)

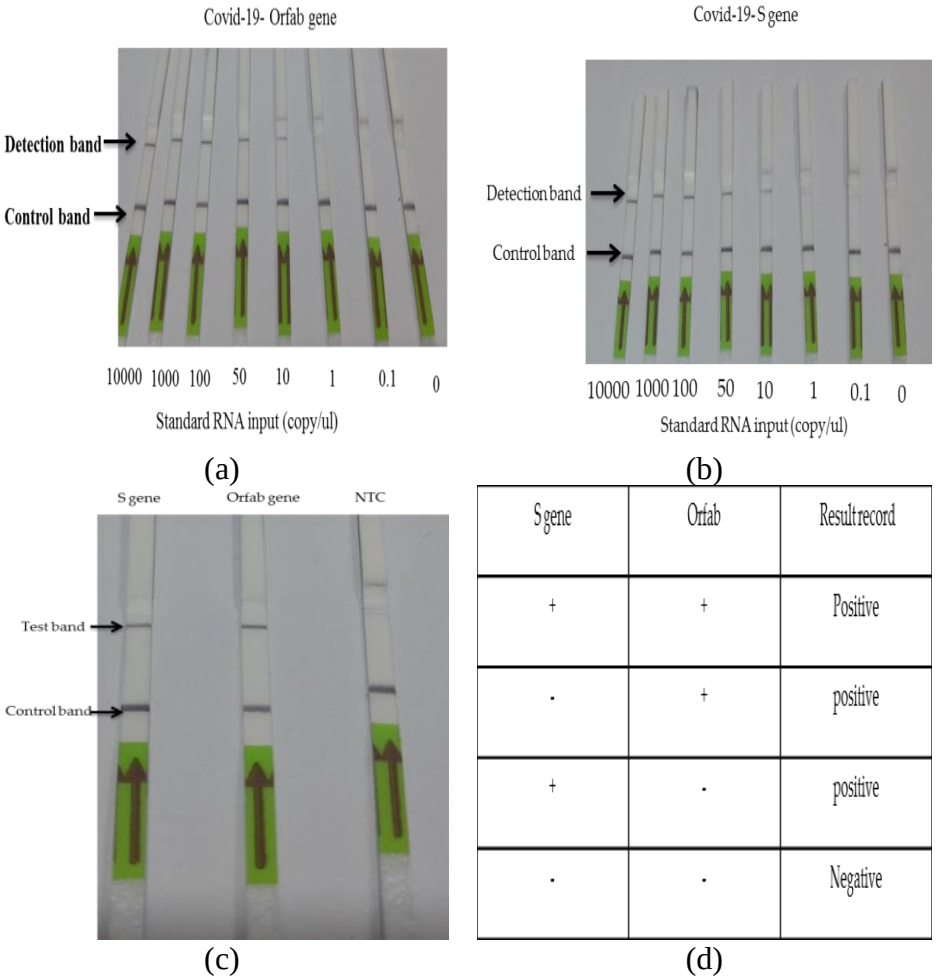
In this study, the Clinical performance of SHERLOCK assay to detect COVID-19 cases was estimated using RNA extracted from patients with COVID-19. Two target genes (S & orfab) were used to detect the presence of the virus by using two sets of primer as described in Table 1 .T7 promoter sequence was synthetically added to the forward primer of both genes in the pre-amplification stage and using T7 RNA polymerase in detection stage to allow of T7 transcription then detect of the target RNA by Cas13a collateral activation, according to Zhang Lab. Method 9, we find that 42℃ for 25 minutes is enough to amplify the target genes using RT-RPA assay successfully. Incubation of the Cas13 reaction, previously described in the method section at 37℃ for 30 minutes, shows successful detection of Viral RNA by S or fab crRNA-guided Cas13a protein and generated signal RNA reporter. The detection reaction was Dipstick buffer (supplied by the manufacturer). Then, the stick is dipped in the mixture. Three minutes were needed to show and interrupt the result. According to the time for reading the results, the SHERLOCK assay is a promising assay for rapid and accurate detection of pathogens, including the SARS-COVID-19 virus.

Sensitivity of The RPA-Cas13a-LFD Assay

The sensitivity of the SHERLOCK assay was estimated by using synthetic control positive RNA of the SARS-Covid2 S gene & orfab gene. Serial dilution of the S & orfab synthetic RNA fragment was made using ddH2O and including negative control. The dilution containing RNA ranges from 0 to 1000 copies per microliter. As shown in Figure 1, we demonstrate that the Sherlock assay can detect 10 cp/ul of target RNA input (S & orfab gene)(Figure 2, A&B). As a comparison with a golden assay for the detection of COVID-19 (qPCR), the SHERLOCK assay is a highly sensitive and promising assay for the detection of the nucleic acid of viral pathogens, including SARS-Covid2.

Clinical Performance of RPA-Cas13a-LFD Assay in Comparison with qPCR.

A total of 157 clinical samples were used to evaluate the clinical performance of the RPA-Cas13a-LFD assay in comparison with qPCR. Of the 157 samples, 100 were positive, and 57 were negative for Covid-19 by qPCR. All the COVID-19-positive samples were positive by the SHERLOCK test, and no false negative results were recorded. At the same time, 55 samples of the 57 COVID-19 negative samples were negative by SHERLOCK assay. Only 2 COVID-19 negative samples showed false positive results by SHERLOCK assay when read out using lateral flow and RNA reporter. The overall specificity of the Sherlock assay for detecting COVID-19 in 157 clinical samples was 98.8% relative to the qPCR assay(Figure 2 C&D). Compared with qPCR, SHERLOCK is a rapid test where just 30 minutes were needed to record results. Furthermore, there is no instrument or lab. Infrastructure needs to perform this diagnostic test.



S gene	Orfab	Result record
+	+	Positive
-	+	positive
+	-	positive
-	-	Negative

Figure 2: A Serial dilution of synthetic Covid-19 S gene, B Serial dilution of synthetic Covid-19 Orfab gene, C& D Lateral flow strip assay readout. A positive result requires detecting at least one of the two SARS-CoV-2 viral gene targets(S gene or Orfab gene).

DISCUSSION

RT-qPCR is a gold standard test for diagnosing viral infections, including Covid-19. However, The test comes with the limitation of the requirement for expensive equipment, experienced staff and specialized molecular laboratory[10]. During the COVID-19 pandemic, CRISPR-based diagnostic covert from experimental tool to clinical clinically relevant diagnostic technology. SHERLOCK is one of the CRISPR-based assays in which nucleic acid pre-amplification is combined with CRISPR-Cas enzyme(Cas13a) to recognize DNA or RNA sequence [5]. The standard approach for pre-amplification of nucleic acid is RPA, which amplifies the target gene at room temperature and short time [11]. CRISPR Cas13a specifically binds to RNA molecules (crRNA) that include a complement to the target[6]. The binding of crRNA to the target leads to activation of the RNase activity of the Ca13 enzyme. Activated Cas13 acts on the cleavage of the specific target RNA and then cleavage of neighbor non-target sequence, and this is called collateral activity; reporter RNA molecules were used in the SHERLOCK reaction to report cleavage of the target RNA through generating detachable signal [12]. These fluorescence signals can be directly visualized using LFD. A variety of pathogens, including SARS-Cov-2, can be efficiently detected by CRISPR-Cas13a system [13]. As a comparison with qPCR, the SHERLOCK is a highly sensitive assay for the detection of the nucleic acid of viral pathogens, including SARS-Covid2. According to the

time for reading the results, it is one of the promising assays for rapid and accurate detection of pathogens [14].
Furthermore, there is no instrument or lab. Infrastructure needs to perform this diagnostic test. Therefore, the SHERLOCK assay must be valid for clinical uses. However, this assay has several limits, including the need for RNA extraction from the sample, the cleavage activity of Cas13a is highly dependent on the specific target sequence, for instance, T, rich PAM and rich PAM, and Random mutation in the target sequence may prevent pathogen detection [15].

CONCLUSIONS

The result shows that the SHERLOCK assay is a highly sensitivity, specificity assay for the detection of nucleic acid of SARS-Covid2. Furthermore, According to the time for reading the results, it is one of the promising assays for rapid and accurate detection of this pathogen. Moreover, no instrument or lab. Infrastructure needs to perform this diagnostic test.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are available upon request to the authors.

Conflicts of Interest: The authors declare no conflict of interest.

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