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COMPARATIVE DIAGNOSTIC PERFORMANCE OF A CAS13-BASED ASSAY FOR DETECTION OF COVID-19 CASES IN AL-DEWANIYAH PROVINCE, IRAQ.

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Abstract: Effective, accurate and rapid diagnostic tools for detection SARS-covid2 infection are urgently needed to prevent and control the pandemic. While RT-qPCR is a gold standard test for diagnosis of SARS-Covid2 infections, this method limited by the requirement for expensive equipment, experienced staff and specialized molecular laboratory. Recently, CRISPR cas13 platform was used for the detection of COVID-19. This study conducted to evaluate the clinical performance of CRISPR based cas13a diagnostic assay for SARS-Covid2. In the current study, the diagnostic performance of Cas13-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter Unlocking) assay was compared with RT-qPCR in terms of Sensitivity, specificity and time consuming. The result shows that SHERLOCK assay is highly Sensitivity, specificity assay for detection of nucleic acid of SARS-Covid2. Furthermore , According to time for reading the results , it is one of the promising assay for rapid and accurate detection of this pathogen. Moreover , no instrument and lab. Infrastructure need to performed this diagnostic test.

Keywords: CRISPR-Cas13; covid-19; SHERLOCK

1. 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 constrain of the infection .Detection of the virus based on RT–PCR is highly recommended by WHO and remain the standard diagnostic test for COVID-19[3].However, this test required to well trained personnel , specific equipment and laboratory infrastructure[4]. In recent year, The CRISPR Cas system has been used for rapid, specific and sensitive detection of nucleic acid[5]. The CRISPR systems represent immune defense line of bacteria against foreign nucleic acid. Cas enzymes which associated with CRISPR acting on recognize and elimination of the foreign genetic material through endonuclease activity[6]. Cas accompanied with CRISPR RNA (crRNA), which guide Cas protein to recognize and degrade of target DNA or RNA[7]. Cas13 is one of CRISPR associated protein that specifically bind to RNA molecule ,complementary to the target binding CRISPR RNA (crRNA)[8]. When combine target RNA pre-amplification with RNA

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

2. Materials and Methods

Sample collection

A total 157 RNA samples were provided by infectious diseases department at Al-Dewanyiah teaching hospital, Al-Dewaniyah ,Iraq . the RNA sample were extracted from COVID-19 suspected patients.

RPA primer & crRNA design

RPA primers of two target genes (S gene and Orf1ab gene , Table1) were design to checking presence of cov-19 RNA in nucleic acid extraction from patient samples. primers were design according to instruction which 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 design according to Zhang et al., 2020 [9]. Primer , Guide RNAs and reporter synthesized by Integrated DNA Technologies (IDT).

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

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	GrArU rUrUrA rGrArC rUrArC rCrCrC rArArA rArArC GrArA rGrGrG rGrArC rUrArA rArArC rGrCrA rGrCrA CrCrA rGrCrU rGrUrC rCrArA rCrCrU rGrArA rGrArA rG	[9]
Orf1ab-crRNA	: rGrArU rUrUrA rGrArC rUrArC rCrCrC rArArA ArArC rGrArA rGrGrG rGrArC rUrArA rArArC rCrCrA ArCrC rUrCrU rUrCrU rGrUrA rArUrU rUrUrU rArArA rCrUrA rU	[9]

Lateral-Flow-Reporter	mArA rUrGrG rCmAmA rArUrG rGrCmA	[9]

Synthesis positive control of SARS-CoV-2 S gene and Orf1ab gene RNA fragments by in vitro transcription

SARS-CoV-2 RNA were provided by Al-Dewanyiah teaching hospital , RNA covert to cDNA using specific primer for s gene and orfab gene (Table1) 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 RPA product of each genes were clean up using Monarch® PCR & DNA Cleanup Kit(T1030S). Finally the constructed SARS-COV2 S gene & orfab gene were 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 were achieved by TwistAmp® Basic (TABAS03KIT) with addition of ProtoScript® II Reverse Transcriptase (M0368L). Each lyophilized RPA pellet were re-suspend with 29.5 rehydration buffer (supplied in the RPA kit.). The reaction was set according to the fallowing formula : 5.9 ul of Resuspended RPA solution ,0.5 S or Orfab RPA upstream primer (10 uM) , 0.5 S or Orfab RPA downstream primer (10 uM),0.2 Re-verse Transcriptase, 1.4ul of ddH2O. 1ul of RNA(sample) and 0.5 ul MgAc(280 mol/l). The reaction mix gently and incubated 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 fallowing: 2 ul cleavage buffer (400mM Tris pH 7.4), 9.6 ul ddH2O , 2 ul LwaCas13a protein (diluted), 1 ul of S or orfab crRNA (10 ng/ul), 1 ul Lateral-Flow-Reporter (20 uM),1ul RNase Inhibi-tor(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 mix with 80 ul of HybriDetect Assay Buffer then HybriDetect Dipstick was added to the reaction tube. Control positive and positive sample should show two line while negative control and negative sample show one line ,Figure1[3,9].

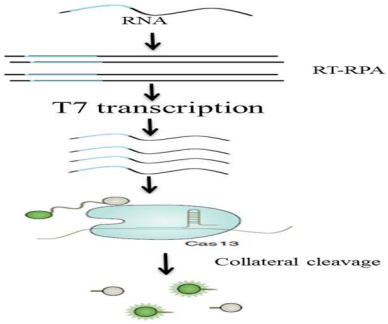


Figure (1) : Schematic illustration of SHERLOCK platform using Cas13a

3. Results

Optimization of RPA-Cas13a-LFD assay (SHERLOCK)

In this study ,Clinical performance of SHERLOCK assay to detecting Covid-19 cases was estimated using RNA extracted from patients with Covid-19 . Two target genes (S & orfab) were used to detect presence of the virus by using two set of primer as described in table 1 .T7 promoter sequence were synthetically added to the forward primer of the both genes in pre-amplification stage and using of T7 RNA polymerase in detection stage to allow of t7transcription 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 for successfully amplified of the target genes using RT-RPA assay. Incubation of Cas13 reaction which previously described at method section at 37℃ for 30 minutes show successfully detection of Viral RNA by S or orfab crRNA guided Cas13a protein and generate of signal RNA reporter . The detection reaction were Dipstick buffer (supplied by manufacturer). then the stick dip in the mixture. Three minutes were need to show and interrupt the result. According to time for reading the results , SHERLOCK assay is one of the promising assay for rapid and accurate detection of pathogens including SARS2-COVID19 virus.

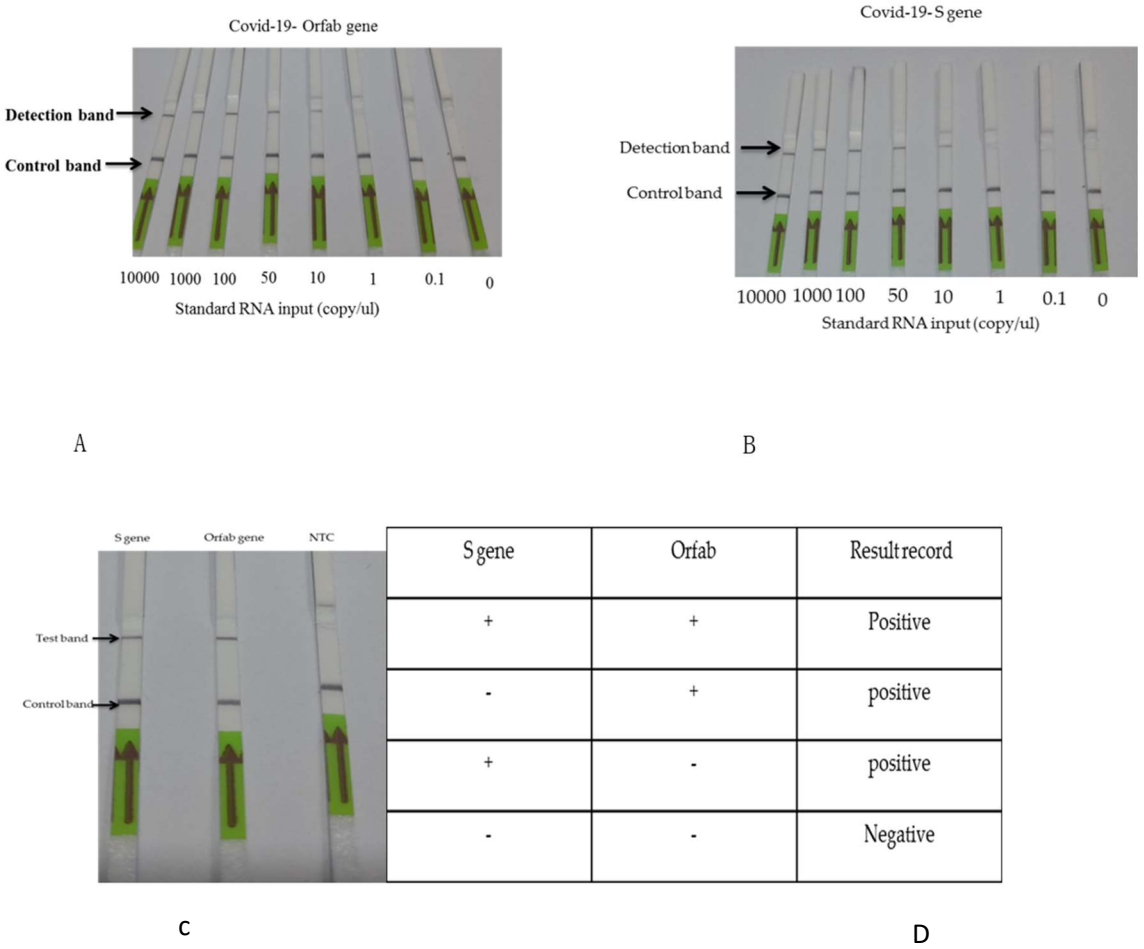
Sensitivity of the RPA-Cas13a-LFD assay

The sensitivity of SHERLOCK assay was estimated by using synthetic control positive RNA of SARS-Covid2 S gene & orfab gene. Serial dilution of the S & orfab synthetic RNA fragment were made using ddH2O and including negative control. The dilution con-taining RNA ranging from 0 to 1000 copy per microliter. As shown in Figure 1 , we demonstrate that Sherlock assay can detect of 10 cp/ul of target RNA input (S & orfab gene)(Figure2,A&B). As a compare with golden assay for detection covid-19 (qPCR) the SHERLOCK assay is highly sensitive promising assay for detection of nucleic acid of viral pathogens including SARS-Covid2.

Clinical performance of RPA-Cas13a-LFD assay in comparison with qPCR.

A total of 157 clinical sample were used to evaluate clinical performance of the RPA-Cas13a-LFD assay in comparison with qPCR. Of the 157 sample , 100 sample were positive and

57 were negative for Covid-19 by qPCR. All the Covid-19 positive sample were positive by SHERLOCK test and no false negative results were recorded. While 55 sample of the 57 Covid-19 negative sample, were negative by SHERLOCK assay. Only 2 Covid-19 negative sample show false positive result by SHERLOCK assay when read it out using lateral flow and RNA reporter. Over all specificity of Sherlock assay for de-tecting of Covid-19 in 157 clinical sample was 98.8% relative to qPCR assay(Figure 2 C&D). As a compare with qPCR ,SHERLOCK is a rapid test where just 30 minutes were need to record results . Furthermore no instrument and lab. Infrastructure need to performed 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 detection of at least one of the two SARS-CoV-2 viral gene targets(S gene or Orfab gene).

4. Discussion

RT-qPCR is a gold standard test for diagnosis of many viral infections including Covid-19. However, The test comes with limitation of requirement for expensive equipment, experienced staff

and specialized molecular laboratory[10]. During Covid-19 pandemic ,CRISPR based diagnostic covert from experimental tool to clinical clinically relevant diagnostic technology.

SHERLOCK is one of the CRISPR based assay in which nucleic acid pre-amplification combined with CRISPR-Cas enzyme(Cas13a) in order to recognition of DNA or RNA sequence[5]. the common approaches for pre-amplification of nucleic acid is RPA ,which amplified target gene at room temperature and short time[11]. CRISPR Cas13a specifically bind to RNA molecule (crRNA) that include complement to the target[6]. Binding of crRNA to the target lead to activate of RNase activity of Ca13 enzyme. Activated Cas13 act on cleavage of the specific target RNA then cleavage of neighbor non-target sequence and this called collateral activity , Reporter RNA molecule were used in SHERLOCK reaction to report cleavage of the target RNA through generate detachable signal[12]. Theses fluorescence signal can be direct visualize using LFD. A variety of pathogen including SARS-Cov-2 can be efficiently detected by CRISPR-Cas13a system[13].

As a compare with qPCR, the SHERLOCK is highly sensitive assay for detection of nucleic acid of viral pathogens including SARS-Covid2. According to time for reading the results , it is one of the promising assay for rapid and accurate detection of pathogens[14]. Furthermore no instrument and lab. Infrastructure need to performed this diagnostic test. Therefore SHERLOCK assay must be valid for clinical uses. However this assay has several of limit include need to RNA extraction from sample , cleavage activity of Cas13a is highly depend on the specific target sequence for instance T rich PAM & G rich PAM , Random mutation in the target sequence may prevent pathogen detection[15].

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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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