Diyala Journal for Veterinary sciences
Open Access Journal Published by College of Veterinary Medicine
University of Diyala, Iraq
P-ISSN: 2410-8863
Vol. 1, NO. 2, June 2021
Proceedings of 2nd National & 1st International Scientific Conference
Of Veterinary Medicine & Science , (NISCVMS-2021)

Molecular Diagnostic Approaches For SARS-COV2

Ali Ibrahim Ali AL-Ezzy

Department of pathology, College of Veterinary Medicine, University of Diyala, Iraq
Corresponding author : alizziibrahim@gmail.com

Received: 10-4-2021 **Accepted: 20-5-2021** **Published: 1-7-2021**

Abstract

Coronaviruses make up a massive household of viruses that can infect birds and mammals, which includes humans .These viruses have been accountable for quite a few outbreaks round the world, inclusive of the Sever acute respiratory syndrome (SARS) ; the Middle East respiratory syndrome (MERS) ; a novel coronavirus (SARS-CoV-2, additionally acknowledged as COVID-19) . Several molecular methods have been used for analysis such as the reverse transcription-polymerase chain reaction (RT-PCR) assay ; multiplex-tandem PCR (MT-PCR) Assay ; real-time nanopore target sequencing (NTS) and amplification methods; isothermal amplification reaction for nucleic acid ; nucleic acid hybridization using microarray ; amplicon-based meta genomic sequencing . In conclusion , The SARS-CoV-2 pandemic, which is developing throughout the borders, is a horrifying international situation and is presently the most serious emergency around the world . The ongoing lookup depends on speedy and correct diagnostic techniques, vaccine improvement and identification of new remedy out of the current drugs. Early diagnosis of infected people is the most vital step , and appropriate diagnostic approach that can precisely notice the virus in the early tiers of infection is sought after.

Key words : covid-19,diagnosis,molecular techniques

How to cite the article:
AL-Ezzy, A. I. A. (2021). Molecular Diagnostic Approaches For SARS-COV2..Diyala Journal For Veterinary Sciences 1(2): 10-20.

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Introduction : Coronaviruses belong to the subfamily Coronavirinae in the family Coronaviridae. Coronaviruses make up a large family of viruses that can infect birds and mammals, including humans, according to WHO [1] . WHO 2020stated that These viruses have been responsible for several outbreaks around the world, including the severe acute respiratory syndrome (SARS) pandemic of 2002-2003 and the Middle East respiratory syndrome (MERS) outbreak in South Korea in 2015 . Recently, WHO Stated that a novel coronavirus (SARS-CoV-2, also known as COVID-19) triggered an outbreak in China in December 2019, sparking international concern . While some corona viruses have caused devastating epidemics, others cause mild to moderate respiratory infections, like the common cold. WHO has declared COVID-19 to be a global pandemic. While the vast majority of cases in Iraq are attributed to people who traveled to or recently returned from Iran, WHO has acknowledged that the spread of COVID-19 in Iraq has now reached community transmission levels, and robust preventative measures continue to the best defense against a rising caseload[2]. WHO has stated that the relative youth of Iraq's population (60 % of the population is under 25 years old) could contribute to relatively milder symptoms and quicker recovery times for those who are infected, as the virus does not affect young people as severely as older people .The declaration of pandemic status for COVID-19 will enable increased production of medications which can successfully treat the symptoms of the virus in Iraq and elsewhere.

Human infection : Coronaviruses vary significantly in risk factor, Some Coronaviruses can kill more than 30% of those infected, such as MERS-CoV, and some are relatively harmless, such as the common cold[3] . Coronaviruses can cause colds with major symptoms, such as fever and a sore throat from swollen adenoids [4]; pneumonia and bronchitis as a direct cause or as a complication when bacterial coinfection happened. Six species of human coronaviruses are known, with one species subdivided into two different strains , making seven strains of human coronaviruses altogether.(HCoV 229E , HCoV NL63),HCoV OC43),HCoV HKU1) human coronaviruses produce symptoms that are generally mild and continually circulate in the human population and produce the generally mild symptoms of the common cold in adults and children worldwide . These coronaviruses cause about 15% of common colds, while 40 to 50% of colds are caused by rhinoviruses [5] . The four mild coronaviruses have a seasonal incidence occurring in the winter months in temperate climates [6] . There is no preponderance in any season in tropical climates [7] .Three human coronaviruses produce symptoms that are potentially severe : MERS-CoV, SARS-CoV, SARS-CoV2.SARS-CoV first appeared in southern China and quickly spread around the world between 2002 and 2003 . This virus was identified as the causative agent of the global pandemic SARS, which led to substantial morbidity and mortality. A decade after SARS, an outbreak of MERS-CoV emerged in 2012 [8].Most people with MERS had no previous contact with bats, leading to the identification of camels as an intermediate host [9]. Chan *et al.* 2015;stated that Patients with SARS or MERS present with a variety of clinical features, ranging from

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asymptomatic or mild respiratory illness to fulminant severe ARDS with extra-pulmonary complications. SARS-CoV-2 from evolutionary point of view has 84% homology to SARS-like coronavirus re-covered from the Chinese horseshoe bat [10],78% with SARS-CoV and 50% with MERS-CoV [10].snakes, mink, and pangolins could be intermediate hosts, based on codon preference and viral infection patterns[11].At the onset of the COVID-19 pandemic, the main symptoms were fever (98%),cough (76%), and myalgia (44%) [12].About half of the patients developed breathing difficulty in one week and the severely ill patients soon developed ARDS, acute cardiac injury, sec-ondary infections, or a combination thereof[12].The diagnosis of the disease main-ly depends on SARS-CoV-2 RNA detection in nasopharyngeal swab by real-time PCR , epidemiological history, clinical manifesta-tions, and lung imaging[13] .

Diagnostic Approaches :

Espejo *et al.* 2020 stated that SARS-CoV-2 is a large positive-sense single-stranded ri-bonucleic acid (RNA) virus that comprises of four structural proteins , i.e., nucleocap-sid protein (NP) that holds the viral RNA, spike protein (SP), envelope protein (EP), and membrane protein (MP), that create the viral envelope . It has a diameter of 50–200 nm and possesses spikes on its surface (up to 20 nm in length) that provide it the crown-like appearance, a characteristic of corona-viruses (CoVs).Cheng *et al.* 2007 and Tai *et al.* 2020 reported that the structure of SARS-CoV-2 comprises of spikes, which are formed by the SP (Figure 1). SP is a ma-jor glycoprotein (Mol. Wt. ~180 kDa) that consists of two subunits, i.e., S1 and S2 [14]. S1 contains a receptor binding domain (RBD), which is responsible for recognizing and binding with the host cell receptor, i.e., angiotensin converting enzyme 2 receptor (ACE2) found in the lower respiratory tract [15, 16] . On the other hand, S2 contains other basic elements needed for the membrane fusion. SP is the common target for neutral-izing antibodies and vaccines, while the amino-terminal S1 subunit of SP is the most variable immunogenic antigen. Additionally, SARS-CoV-2 has NP (Mol. Wt. ~40 kDa), the most abundant viral phosphoprotein pro-duced and shed during infection[17] . The template mRNA of NP is the most abundant subgenomic RNA. NP exhibits high immu-nogenicity and can be detected in either se-rum or urine samples during the first two weeks of infection with peak viral shedding around ten days after infection [18] . Vashist 2020 ,recorded that SARS-CoV-2 contains MP, which is the most abundant protein on the complete virion particle. The EP is the smallest major structural protein of SARS-CoV-2, which is involved in viral assembly, release of virions, and pathogenesis[19] .

Although SARS-CoV-2 shares genetic features attuned with the other members of the CoV family, Wang *et al.* 2020 recorded that it possesses considerably varied genetic sequence compared with that of earlier se-quenced CoVs . Ludwig and Zarbock 2020 ,reported that SARS-CoV-2 shares around 79.5% identical genetic sequence with SARS-CoV and 96.2% genetic sequence similarity with RaTG13, a short RNA-dependent RNA polymerase (RdRp) region present in the CoV that originated from bats. SARSCoV-2 belongs to the genus Beta coronavirus and subgenus Sarbeco virus and is different from SARS-CoV .SARS-CoV-2 first originated in bats with pangolins as an

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intermediate mammalian host [20]. Zhang *et al.* 2020 recored that A closely related virus obtained from the lung samples of Malayan pangolin showed similarity with the SARS-CoV-2 given that SARS-CoV- 2 and Pangolin-CoV share five key amino-acid substitutions in the receptor binding domain (RBD) and are 91.02% identical. Pangolin-CoV is the second closest to SARS-CoV-2 after RaTG13 .

Hoffmann *et al.* 2020 and Huang *et al.* 2020, reported that The envelope (E) S protein of SARS-CoV-2 is used by the CoV to attach to the host cell [21].The S protein is responsible for binding to the receptor and host membrane (M) fusion and is vital for the determination of transmission capacity and tropism of hosts . The two functional domains of S protein are regarded as S1 (liable for binding to receptor) and S2 (assists in cell M fusion) [22] . Wang *et al.* 2020 illustrated that three-dimensional structural analysis of the virions revealed the presence of RBD (Figure 1C), which consists of an external subdomain and a core and can bind to angiotensin-converting enzyme II (ACE2) receptors in a manner similar to that of SARSCoV [23]. The crystal structure of SARS-CoV-2 S protein C-terminal domain in complex with human ACE- 2 was developed, revealing the strong affinity of C-terminal domain with ACE-2 with high number of atomic contact points . Two additional crystal structures of SARS-CoV-2 RBD bound to ACE-2 were reported . The residues of SARS CoV- 2 RBD, which are critical in binding to ACE-2, were identified. Surface plasmon resonance was employed to show that SARS-CoV-2 RBD binds more strongly to ACE-2 than SARS CoV [24]

Ahmed *et al.* 2020; reported that The S protein is the primary target for vaccines and neutralizing antibodies, whereas the S1 subunit acts as the most vulnerable antigen causing immunogenicity. In addition to S protein, SARS-CoV-2 has a nucleocapsid (N) protein containing viral RNA, which is commonly detected by the immunoassay of blood and serum samples of infected patients during the early days of infection .Abd El-Aziz and Stockand 2020 recorded that M protein is the most abundant protein in the virus , whereas the pathogenesis is attributed to the E protein. SARS-CoV-2 enters the host cells in the respiratory system by binding to the ACE2 receptor and multiplies rapidly to form new virions . The signs and symptoms of the disease generally appear after 2–14 days of the infection, that is, the viral incubation period .

Types of specimen

According to (CDC) recommendations the upper respiratory specimens should be collected for RT-PCR based testing of COVID-19 and especially the nasopharyngeal specimen is a preferred choice [25] . When the nasopharyngeal swab is not possible the other specimen such as; an oropharyngeal specimen, nasal mid-turbinate swab, an anterior nares (nasal swab) specimen and nasopharyngeal wash/aspirate or nasal aspirate (NA) specimen can be collected alternatively . According to WHO 2020; All specimens should be placed in a tube containing viral transport medium and transported to the laboratory on time. Similarly for serological assay the blood sample can be collected. The healthcare professional should adhere to infection prevention and control guidelines of WHO and use the personal protective equipment such as, gown,

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gloves, eye protection and N95 mask while collecting the specimen .WHO 2020 stated that If the shipping of specimen to the reference diagnostic laboratories required, the sample must be transported in triple packaging system; the sample vial must be properly labeled and sealed and kept in outer covering of absorbent material and then placed in secondary container. After that the secondary container should be placed with frozen gel packs in the thermocol box . All the processes; including packaging, labeling and shipping must be done as per the WHO guideline . Peng *et al.* 2020 reported that the SARS-CoV-2 can invade digestives system and haematological system along with respiratory system . Peng *et al.* 2020 reported that Different types of specimen for detection of SARS-CoV-2 RNA were collected in china ,the highest positivity rate was reported in pharyngeal swab (78%), after that equally in blood and anal swab (22% each) and 11% in urine . Huang *et al.* 2020 and Holshue *et al.* 2020 reported that In USA they found that stool, oropharyngeal swab and nasopaharyngeal swab were positive for SARS-CoV-2, while serum and urine were found negative . Blood samples show minimal sensitivity for SARS-CoV2 detection (6 out of 41 , 14.6%) ,In study from china compared with 100% respiratory specimens were found positive . The virus can be detected in feces and blood along with respiratory specimens , however the percentage is not very promising[26] . Hase *et al.* 2020 stated that when the clinical suspicion is high the negative PCR test on throat swabs is not sufficient to rule out the COVID-19,but the bronchoalveolar lavage (BAL) of the lower respiratory tract specimen should be preferred for diagnosis .Thus it is very important to conclude the final result of the highly suspected SARS-CoV-2 infected individuals; we should consider collecting various types of specimen. This will improve the detection rate and can reduce the false negative results .

Molecular techniques

[1] The Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay

Mathuria and Yadav 2020 reported that In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions in nucleic acid testing assay. The real time reverse transcription–polymerase chain reaction (real time RT-PCR) is one of the best and accurate laboratory methods for detecting, tracking, and studying the coronavirus. Real time RT-PCR is a method by which we can detect the presence of specific target genetic material . Sethuraman *et al.* 2020 recorded that various fluorescent dyes are used as marker to detect the specific genetic target; earlier radioactive isotopes were used as marker. The most important aspect of using real-time RT-PCR assays is that the amplification and analysis will be carried out in a closed system; therefore the chances of false positive results will be minimized

The molecular testing is still a gold standard for relevant case diagnosis [27] . Yi-Wei Tang 2020 stated that Most of the molecular diagnostics being developed are based on real-time RT-PCR assays for COVID-19 infection. loop-mediated isothermal amplification, clustered regularly interspaced short palindromic repeats and multiplex isothermal amplification followed by microarray detection are the some other

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methods which were developed and evaluated worldwide

Vashist 2020 and Corman *et al.* 2020 reported that RT-PCR is the most widely employed IVD test for the confirmatory detection of COVID-19. A highly specific, novel, and robust RT-PCR assay was developed by Tib-Molbiol, Germany; this assay can specifically detect SARS-CoV-2 but not other CoVs . It was highly specific for SARS-CoV-2 RNA and did not cross-react with other coronaviruses. The test detects the SARS-CoV-2 RNA via envelope (E) and RNA-dependent RNA polymerase (RdRp) gene assays. The E-gene assay was used for first line screening, while the RdRp gene assay was employed for confirmatory testing . Jiang *et al.* 2020 and Chu *et al.* 2020 stated that another relatively quick RT-PCR method was developed to target the Orf1b and N regions of the virus; this method provides results in 75 Minutes [28] . The N-gene assay gives the initial results, and the Orf1b assay confirms the diagnosis. However, this assay can also detect other closely related sarbecoviruses, such as SARS-CoV, due to the presence of Orf1b and N regions . This problem can be overcome by using sequence analysis of positive amplicons once the RT-PCR test is positive . Chan *et al.* 2020 and Wolters *et al.* 2020 reported that another RT-PCR assay was developed targeting the RdRp and helicase genes of the virus with an added advantage of specificity for SARS-CoV-2 . This assay is highly sensitive and can be used specifically for COVID-19 detection despite the low viral loads. In the continuation of the development of RT-PCR assays that can yield results rapidly, a real-time rapid test (XpertR Xpress SARS-CoV-2 test) was recently developed by Cepheid, USA.This test gives confirmatory results within 45min and can qualitatively detect the virus in different specimens, such as oropharyngeal/nasopharyngeal swabs, nasal wash, or aspirates .

Yi-Wei Tang 2020 reported that this test has received US FDA EUA approval and targets multiple regions of the SARS-CoV-2 genome .However, mounting evidence shows that RT-PCR methods cannot detect the virus especially in the early stages of infection, giving false negative results .Sheikhzadeh *et al.* 2020 stated that The false negative results can be attributed to the insufficient and improper extraction of nucleic acid for the test. Therefore, in these cases,a computerized tomography scan of the chest is suggested as a complementary tool and a suitable diagnostic assay which can accurately detect the specific biomarkers of SARS-CoV-2 in the initial stages of infection is still required .

[2] Multiplex-tandem PCR (MT-PCR) assay

Recently,[29] stated that Aus Diagnostics Multiplex-tandem PCR (MT-PCR) assay which includes two tandem amplification steps were also applied for the detection of SARS-CoV-2. The first amplification step (enrichment) utilized a specific outer primer with fewer numbers of PCR cycles. In the second amplification step, the target region within the product from the first step was amplified by inner primers. Comparative analysis with State Reference Laboratory showed 118/127 (92.9%) consistency. After investigation of discrepancies, 125/127 (98.4%) positive results were obtained and this method has demonstrated reliable diagnosis for SARS-CoV-2

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[3] Real-time nanopore target sequencing (NTS) and amplification methods

Butler *et al.* 2020 recorded that these methods were employed for the simultaneous detection of SARS-CoV-2 and 10 other respiratory viruses in 6–10 h with LOD of 10 copies $mL^{-1}$with at least 1 h sequencing data . Oxford nanopore sequencer is a small device that can be coupled with a personal computer for data processing. In this method, 11 virulence-related and specific gene fragments of ***ORF1ab*** of SARS-CoV-2 were amplified with an in-house primer panel. Then, the amplified fragments were sequenced on a nanopore platform . Wang *et al.* 2020 recorded that A comparative study of approved qPCR kits and NTS method with samples from patients have shown that NTS provided more positive results. The system has shown two orders of magnitudes more sensitivity than qPCR as well as specificity against other mutated nucleic acid sequences or various respiratory virus infections in the samples . Butler *et al.* 2020 cecorded that Total RNA sequencing was carried out by the Shotgun metatranscriptomics method. The obtained data were utilized for phylogenetic analysis and were assigned to subclade in New York subway samples

[4] Isothermal Amplification Reaction For Nucleic Acid

Nguyen *et al.* 2019 recorded that isothermal amplification reaction, used to amplify nucleic acids at constant temperature avoiding the complex requirement of the regular PCR that needs changing multiple temperatures in each cycle . isothermal amplification reaction could serve as an alternative method to the RT-qPCR to detect COVID-19. The isothermal amplification reaction amplifies the DNA in isothermal condition with rapidity and high specificity. This method can be utilized for the diagnosis of COVId-19 without the need of specialized equipment and trained analysts. In near future the point-of-care device based on isothermal amplification reaction can be a potential diagnostic tool for the diagnosis of COVID-19 infected individuals

Four types of isothermal amplification reaction for nucleic acid were developed :

[i] Transcription-Mediated Amplification (TMA) :

Priyadarshi *et al.* 2020 reported that TMA **i**s a patented single tube, isothermal amplification technology modeled after retroviral replication which can be used to amplify specific regions of either RNA or DNA much more efficiently than RT-PCR . Shah *et al.* 2020 recorded that It uses a retroviral reverse transcriptase and T7 RNA polymerase and has been used for detection of nucleic acids from multiple pathogens

[ii] Loop-Mediated Isothermal Amplification (LAMP):

Park *et al.* 2020 reported that the amplification product in the LAMP method can be detected by measuring the turbidity of the solution or the fluorescence of an intercalating dye. unpurified samples can be applied in LAMP directly .This method is a rapid and cost-effective way for virus detection but is limited only to one sample per run .

Sheikhzadeh *et al.* 2020 ,stated that RT-LAMP was carried out in one step at 63 °C within 30 min to detect SARS-CoV-2. The optical density at 400 nm and color change from orange to green were used to detect amplification. The assay was capable to

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identify ORF1ab gene, E gene and N gene simultaneously with accuracy rates of 99%, 98.5%, and 92.3%, respectively. ORF1ab and N genes showed higher specificity and sensitivity

Zhang *et al.* 2020 recored that the technique was specific because of using six to eight primers to distinguish eight different regions on the target DNA .SARS-CoV-2 virus from purified RNA or cell lysis was visually detected with the LAMP method. The LAMP method was performed with 5 full primers sets targeting SARS-CoV-2 RNA with amplicon regions designed to the 50 region of the ORF1a gene and N gene. The test showed identical results with the RT-qPCR test for RNA samples of respiratory swabs .

Yu *et al.* 2020 reported that ORF1ab region and online software Primer Explorer V5 were applied to design RT-LAMP primers. By specificity analysis, one primer set with several pairs of loop primers was selected. These six primers were distinguished by 8 distinct regions of the ORF1ab gene. The primer sequence was checked against similar 11 related viruses by the BLAST method and there was no similarity with selected viruses. Amplification was detected by the change of the color from pink to yellow. The method illustrated a similar sensitivity with the RT-qPCR method and was capable to detect 10 copies of SARS-CoV-2

Lamb *et al.* 2020 reported that different spiked samples like serum, saliva, urine, oropharyngeal swabs and nasopharyngeal swabs were utilized to determine the interference of the RT-LAMP method with other viruses and no cross-reactivity was observed . Urine and plasma samples were used without any treatment. A comparison between urine and serum samples with an equal amount of virus illustrated that urine samples could show greater signals . Tran *et al.* 2020 recorded that three different colorimetric isothermal amplification methods including LAMP , cross-priming amplification (CPA), and polymerase spiral reaction (PSR) were investigated and compared. Phenol red was used to detect the amplification products by color change. Among them, LAMP showed better results for the genomic RNA of SARS-CoV-2.The lyophilized LAMP kit has shown advantages such as fast detection of SARS-CoV-2 in spiked nasopharyngeal and oropharyngeal samples, high sensitivity (43 copies), early detection of virus, portability and capability of being used by untrained staff

[iii] **Rolling Circle Amplification (RCA)**

Tian *et al.* 2020 stated that Circle-to-circle amplification which was a homogeneous and isothermal nucleic acid quantification method coupled with optomagnetic chip for sensitive (0.4 f M) detection of SARS-CoV-2 was reported . The method utilized the conserved region of the SARS-CoV-2 *RdRp* gene. It provided a wide linear range of 3 orders of magnitude and was able to distinguish SARS-CoV RdRp cDNA from SARS-CoV-2 RdRp cDNA .

[iv] Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) .

Broughton *et al.* 2020 reported that CRISPR is also another promising isothermal amplification method for the detection of viruses. In this method, some bacterial enzymes such as Cas12 and Cas13 are pro-

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grammed to cut certain viral RNA sequences followed by isothermal amplification and a visual readout with a fluorophore on paper strips. Broughton et al. 2020 have reported a CRISPR– Cas12-based detection method for SARS-CoV-2 in respiratory swabs .

Broughton et al. 2020 this method showed high sensitivity and selectivity in RNA extracts compared to RT-PCR. In this method, CRISPR-Cas12 and lateral flow technology were coupled for the rapid detection of SARS-CoV-2 from nasopharyngeal or oropharyngeal swabs . This assay is low-cost ,relatively rapid and thus, has great potential for POC diagnosis of COVID 19 . The procedure of this assay is illustrated in Fig. 1. All in one Dual CRISPR-Cas12 assay was also developed for SARS-CoV-2 and HIV with a high sensitivity of few copies . Ding *et al.* 2020 stated that the one-pot reaction system was applied in this method and all materials which were needed for amplification and CRISPR detection were mixed in a single step and incubated at 37 °C. This fast and robust method can be further developed to form a POC test.

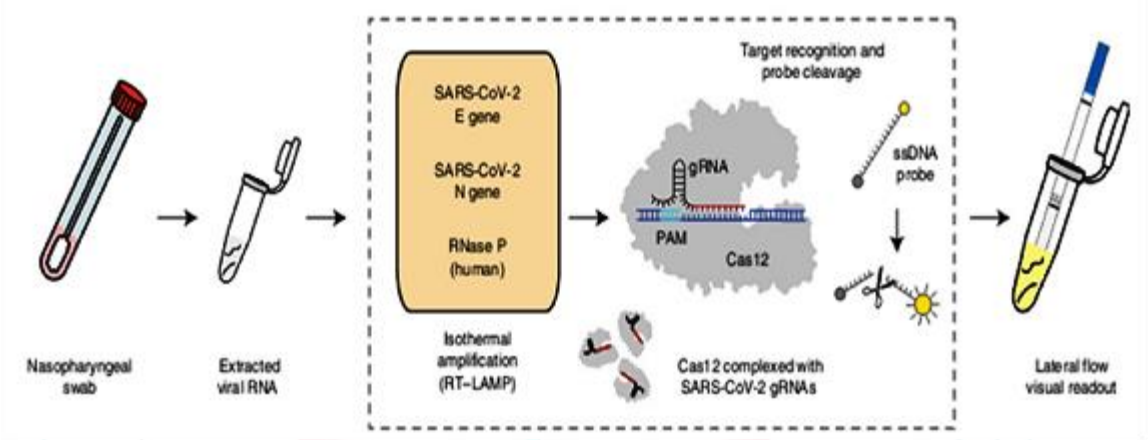

Fig. 1.CRISPR-Cas12DETECTR lateral flow assay for SARS-CoV-2 with permission ref [30]

[5] Nucleic Acid Hybridization Using Microarray

Chen *et al.* 2010 reported that microarray assays have been used for rapid high-throughput detection of SARS-CoV nucleic acids . They rely on the generation of cDNA from viral RNA using reverse transcription and subsequent labeling of cDNA with specific probes. The labeled cDNAs are loaded into the wells of microarray trays containing solid-phase oligonucleotides fixed onto their surfaces. If they hybridize, they will remain bound after washing away the unbound DNA, thus signaling the presence of virus-specific nucleic acid

[6] Amplicon-Based Meta genomic Sequencing.

Moore *et al.* 2020 reported that this dual technique is particularly relevant to SARS-CoV-2 in assessment of its rate of mutation and to detect its possible recombination with other human coronaviruses, both of which have implications for vaccine development and antiviral efficacy. Amplicon

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and metagenomics MinION based sequencing were used by Moore et al. (2020) to rapidly (within 8h) sequence the genome of SARS-CoV-2 and the other microbiome in nasopharyngeal swabs obtained from patients with COVID-19 by the ISARIC 4C consortium.

Conclusions

The SARS-CoV-2 pandemic, which is growing rampant across the borders, is a frightening global concern and is currently the most important health emergency around the world . The lack of vaccine and a suitable treatment for the disease further worsens the issue. The ongoing research relies on rapid and accurate diagnostic techniques, vaccine development and identification of effective therapy out of the existing drugs. Early diagnosis of infected individuals is the most important step, and a suitable diagnostic technique that can accurately detect the virus in the early stages of infection is sought after.

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