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Detection of coronaviruses, SARS-CoV-2 and MERS-CoV, in human and camel samples using different real-time RT-PCR kits

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ABSTRACT:
Coronaviruses are enveloped, large positive-sense, and single-stranded RNA viruses. Four coronavirus genera have been identified: alpha, beta, gamma, and delta. Non-structural proteins make up two-thirds of the coronavirus genome, and some of these (ORF1ab) are essential to create the replicase-transcriptase complex. The remaining coronavirus genome encodes four major structural proteins (S, E, M, and N). Coronaviruses infect both animals and humans, and the emergences of SARS-CoV, MERS-CoV, and SARS-CoV-2 highlight the viruses' importance as emerging pathogens. Accurate viral detection is crucial for treating cases and controlling diseases. MERS was first identified in Saudi Arabia in 2012 where as SARS-CoV-2, the causal agent of coronavirus disease 2019 (COVID-19), was first identified in Wuhan, China, in December 2019 and then spread globally, resulting in a pandemic. Many countries performed fast large-scale testing as a way to halt its spread. The National Veterinary Institute (NVI) of Ethiopia was among the organizations that took part in the COVID-19 testing campaign during the pandemic. The real-time reverse transcription polymerase chain reaction (real-time RT-PCR) is the gold standard method for diagnosing suspected cases of COVID-19 and MERS-CoV. These methods, which target viral ribonucleic acid (RNA), are currently the primary approach for directly identifying the virus. In this study, three real-time RT-PCR assays were employed to detect SARS-CoV-2 in human samples: two developed by CDC China for ORF1ab and the N genes, and one ORF1ab-based assay (BGI, China), and one RT-PCR assay developed by Charité laboratory (Berlin, Germany) for E gene targets. The other two RT-PCR assays developed by Corman *et al*., 2012 targeting MERS-CoV's upstream E (UpE) gene and ORF1a gene were used to screen and confirm MERS-CoV in camel swab samples. The uniplex, duplex, and triplex RT-PCR assays were assessed following the recommended protocols.

Keywords: Genome detection, SARS-CoV-2, MERS-CoV, real-time RT-PCR, human, camel

INTRODUCTION:

Coronaviruses are large, enveloped, non-segmented, single-stranded, positive-sense RNA viruses named for their crown (Latin "corona")-like surface projections observed under electron microscopy, which correspond to large surface spike proteins. Their genome varies in size from 28 to 32 kbp. Coronaviruses are classified into four groups based on genetic and antigenic characteristics: alpha coronavirus, beta coronavirus, gamma coronavirus, and delta coronavirus. All coronaviruses have four common structural proteins: three in the envelope and one associated with the genome [1, 2]. These major structural proteins are spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, all of which are encoded at the 3' end of the genome. The S protein mediates the virus's attachment to the host cell surface receptors, in which leads to fusion and subsequent viral entry. The M protein is the most abundant and determines the shape of the viral envelope. The E protein is the smallest of the major structural proteins and plays a role in viral assembly and budding. The N protein is the only one that binds to the RNA genome and is also involved in viral assembly and budding [3]. ORF1ab is the largest gene that codes for the replicase complex and is essential for viral replication. The replicase gene reading frame accounts for two-thirds of the 5′-end [4]. Coronaviruses cause respiratory and gastrointestinal infections in humans and

animals, making them important in both medical and veterinary practice [5, 6]. Currently, the main public health concerns are Middle East Respiratory Syndrome Coronavirus (MERS-CoV), which belongs to lineage C, and Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2), which belongs to lineage B of betacoronavirus [7, 8].

MERS is a viral respiratory infection in humans and dromedary camels caused by MERS-CoV [9]. The virus was first identified in 2012 by a man with pneumonia in Saudi Arabia [10]. Although its origins are not fully understood, it is believed that MERS-CoV, like many other coronaviruses, originated in bats and was transmitted to dromedary camels at some unknown time in the past. The dromedary camel is the only animal species that has been reported to transmit this virus to humans, and therefore is considered an intermediate reservoir [11, 12].

SARS-CoV-2 (2019-nCoV) is a novel coronavirus that has been discovered as the cause of COVID-19; it first began in Wuhan, China, in 2019 and has since spread worldwide [13]. Bats are thought to be the reservoir hosts for SARS-CoV-2. Since there is very limited close contact between humans and bats, viruses may have been transmitted to humans through an intermediate animal host, such as a domestic animal, a wild animal, or a domesticated wild animal, which has yet to be identified [14].

Molecular tests should be used to determine whether an active coronavirus infection exists. The accurate detection of SARS-CoV-2 and MERS-CoV is crucial for preventing virus spread. Real-time RT-PCR is the gold-standard test for amplification of viral RNA. To detect the SARS-CoV-2 RNA, several RT-PCR assays are available based on different protocols recommended by international institutions such as CDC China (target genes ORF1ab and N) Charité Germany CDC (target genes RdRP and E), and CDC United States (target genes N1-N3) [15, 16]. The human RNase P gene or human beta-actin genes could be used as internal controls to monitor viral RNA extraction efficiency and assess amplifiable RNA in the samples to be tested [17, 18]. Laboratory confirmation of MERS using a real-time RT-PCR assay requires positivity at two different MERS-CoV genomic target sites or detection at a single target site followed by sequence confirmation at a second site. Currently, an assay targeting the region upstream of the envelope gene (upE) is recommended for screening, and followed by an assay targeting ORF1a is used for confirmation [19].

We focused on the detection of SARS-CoV-2 and MERS-CoV RNA from human and camel samples, respectively. SARS-CoV-2 was detected using two RT-PCR assays developed by the China CDC and one real-time RT-PCR assay developed by Charité-Germany, whereas MERS-CoV was detected using Corman assays targeting the upE and ORF1a regions [20, 21].

MATERIALS AND METHODS:

Sources of samples:

During the COVID-19 pandemic, the National Veterinary Institute (NVI) of Ethiopia was one of the organizations that assisted public health services by testing human samples for the confirmation of SARS-CoV-2. Public health professionals collected around 8400 nasopharyngeal and oropharyngeal swab samples from COVID-19 suspected human cases and submitted to our molecular laboratory for SARS-CoV-2 detection. After SARS CoV-2 testing campaign completed, we continued MERS-CoV detection using about 400 swab samples collected from dromedary camels by NVI staff in close collaboration with FAO representatives in Ethiopia.

Table 1: Swab samples from humans and camels

Targeted virus	Sample source	Areas of sample collection	Years
SARS-CoV-2	human	Bishoftu, Mojo, Dukem, and Gelan	2020-2021
MERS-CoV	dromedary camels	Amibara, Awash, and Akaki	2021-2022

Extraction of RNA:

RNA was extracted from nasopharyngeal and oropharyngeal swabs collected from individuals with clinical signs related to SARS-CoV-2 infection using Da An Gene RNA purification kit (Da An Gene Co., Ltd. of Sun Yat-sen University, China), whereas MERS-CoV RNA was extracted from swab samples taken from turbinate nasal and turbinate swabs of dromedary camels using QIAamp® Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions [22, 23].

Detection kits:

SARS-CoV-2 was detected using the Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing) cat. #DA-930, manufactured by Daan Gene Co., Ltd. of Sun Yatsen University (Guangzhou, China), Real-Time Fluorescent RT-PCR kit for detecting 2019-nCoV (BGI Europe A/S, China), lyophilized 1-step RT-PCR polymerase mix, and Sarbecovirus E-gene plus EAV control (TIB Molbiol,

Germany) [24-26]. The commercial DAAN and BGI kits include primers and probes as premixes in the PCR reaction mix. The DAAN kit targets both the ORF1ab and N coding regions. This kit is used in conjunction with internal control, often a housekeeping human gene (RNase P gene), which is assumed to be present in all samples collected from human tissue, and is used to monitor the extraction, reverse transcription, and real-time PCR amplification processes [24,27]. The BGI kit targets only the ORF1ab region of the SARS-CoV-2, and an additional primer set targeting the human β-Actin as an internal control [25].

LightMix SarbecoV E-gene plus EAV control (cat. #:40-0776-96) was the kit used to detect only SARS-1 and SARS-CoV-2 pneumonia virus (bat-associated SARS-related Sarbecovirus), with no cross-reactivity with common human respiratory CoV. However, LightMix Modular SARS-CoV-2 (COVID-19) RdRP (cat. #:53-0777-96, TibMolBiol) was used as an alternative to confirm SARS-CoV-2 [28, 29]. The SuperScriptTM III One-Step RT-PCR System with PlatinumTM Taq High Fidelity DNA Polymerase (Invitrogen) was used to detect a conserved region upstream of the E gene (upE) and the open reading frame 1a (ORF 1a) genes of MERS-CoV RNA separately [30].

Specific primers and probes used for detection of SARS-CoV-2 and MERS- CoV:

Primers and TaqMan probes designed for conserved regions of SARS-CoV-2 and MERS-CoV genomes allow specific amplification and detection of viral RNA. Primer-probe sets targeting one or more of the SARS-CoV-2 and MERS-CoV genes and internal amplification controls such as human RNA targets (RNase P gene and β-Actin) are listed in **Table 2**. However, due to a lack of information, the primer and probe sequences of the extraction control target (Equine Arteritis Virus, EAV) did not include in **Table 2** for Charité (Sarbeco E gene) assay extraction control kit.

Table 2: Primers and probes used for real-time RT-PCR of SARS-CoV-2 and MERS-CoV

Assay developer	Target gene	Primer and Probe Sequences (5′-3′)	References
		For SARS-CoV-2	
China CDC	N gene	F: GGGGAACTTCTCCTGCTAGAAT	[31-36]
		R: CAGACATTTTGCTCTCAAGCTG	
		P: FAM-TTGCTGCTGCTTGACAGATT-TAMRA	
	ORF1ab gene	F: CCCTGTGGGTTTTACACTTAA	
		R: ACGATTGTGCATCAGCTGA	
		P: VIC-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1	
	human RNase P (US-CDC)	RP-F: AGATTTGGACCTGCGAGCG	
		RP-R: GAGCGGCTGTCTCCACAAGT	
		RP-P: Cy5-TTCTGACCTGAAGGCTCTGCGCG-BHQ2	
China CDC	ORF1ab	F: AGAAGATTGGTTAGATGATGATAGT	[33,34]
		R: TTCCATCTCTAATTGAGGTTGAACC	
		P: FAM-TCCTCACTGCCGTCTTGTTGACCA-BHQ1	
	human β-Actin	F: AATGAGCTGCGTGTGGCTC	
		R: GGCTGGGGTGTTGAAGGTC	
		P-VIC- TTCTCGCGGTTGGCCTTGGG-BHQ1	
Charité - Germany	E gene	E-Sarbeco-F1: ACAGGTACGTTAATAGTTAATAGCGT	[32- 34]
		E-Sarbeco-R2: ATATTGCAGCAGTACGCACACA	
		E-Sarbeco-P1:FAM-	

		ACACTAGCCATCCTTACTGCGCTTCG-BBQ	
	RdRp gene	RdRP_SARSr-F2: GTGARATGGTCATGTGTGGCGG	[32-34]
		RdRP_SARSr-R1: CARATGTTAAASACACTATTAGCATA	
		RdRP_SARSr-P2:FAM-CAGGTGGAACCTCATCAGGAGATGCBBQ (Specific for 2019-nCoV,)	
		RdRP_SARSr-P1:FAM-CCAGGTGGWACRTCATCMGGTGATGCBBQ (Pan sarbeco) (W=A/T, R=A/G,M=A/C,S=G/C)	
		For MERS-CoV	
Corman *et al*., 2012	UpE gene (Assay 1)	EMC-upE F: GCAACGCGCGATTCAGTT	[20,42]
		EMC-upE R: GCCTCTACACGGGACCCATA	
		EMC-upE-P: FAM-CTCTTCACATAATCGCCCCGAGCTCG-TAMRA	
	ORF1a gene (Assay 2)	EMC-Orf1a-F: CCACTACTCCCATTTCGTCAG	
		EMC-Orf1a-R: CAGTATGTGTAGTGCGCATATAAGCA	
		EMC-Orf1a-P: FAM-TTGCAAATTGGCTTGCCCCCACT-TAMRA	

Detection of SARS-CoV-2 and MERS-CoV using different real-time RT-PCR assays:

The current standard test for both SARS-CoV-2 and MERS-CoV is detection of viral RNA using the highly sensitive reverse transcription and quantitative PCR (RT-qPCR) method. As mentioned in **Table 2** above, the detection of the SARS-CoV-2 virus was conducted using two real-time RT-PCR assays developed by the CDC China and one developed by the Charité Institute. MERS-CoV was detected using a two-step RT-PCR assay developed by Corman and his colleagues, which suggests screening using an assay that detects the upper stream envelop (UpE) gene, followed by confirmatory testing with the open reading frame1a (ORF1a) gene [42].

Master Mix preparation:

RT-PCR Master Mix was prepared for all assays as per the recommendation provided along with the RT-PCR kit to be used and dispensed into a 96-well plate or strips, depending on the number of samples. SARS CoV-2 detection kits (DAAN and BGI) are mixtures of amplification primers and fluorescently labeled probes and provided with SARS CoV-2 positive and negative controls; however primers and probes for MERS-CoV detection kits were reconstituted to recommended concentration from the original stock before the master mix preparations. A detection kit used for end point RT-PCR was modified and used for a real time RT-PCR assay for MERS CoV detection. External positive and negative controls were used only in real-time RT-PCR assays of MERS-CoV.

Table 3: Master Mix preparation protocols to detect SARS-CoV-2 and MERS-CoV

kits	Master mix components	Vol/reaction (µL)	references
	Detection of SARSCoV-2 (N, ORF1ab or		

	E-gene)		
2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing) (DAAN)	NC (ORF1ab/N) PCR reaction solution A (Specific primers, probes, Tris-HC)	17	[24]
	NC (ORF1ab/N) PCR reaction solution B (Hot start Taq DNA polymerase, c-MMLV enzyme)	3	
	Extracted RNA	5	
	Total volume per reaction	25	
Real-Time Fluorescent RT-PCR kit for detecting 2019- nCoV (BGI)	2019-nCoV Reaction Mix (reagents, probes and primers of the target gene and internal reference)	18.5	[25]
	2019-nCoV Enzyme Mix (Taq polymerase, Reverse transcriptase and UDG)	1.5	
	Extracted RNA	10	
	Total volume per reaction	30	
Modular Diagnosis Kit SARS-CoV-2 E-gene (Tib Molbiol) + Light Cycler Multiplex RNA Virus Master (Roche)	Pathogen-specific reagents (primers and probes)	0.5	[26]
	EAV extraction target control	0.5	
	qPCR Master mix	10	
	Extracted RNA	9	
	Total volume per reaction	20	
	Detection of MERS-CoV (E-gene and ORF1a gene,uniplex RT-PCR)		
SuperScript™ III One-Step RT-QPCR System with Platinum™ Taq High Fidelity DNA Polymerase (Thermo Fischer/Invitrogen)	RNase-free water	3.6	[30,37]
	2X Reaction Mix	12.5	
	5mM magnesium Sulfate (0.8 mM)	0.4	
	SuperScript III RT/Platinum Taq High Fidelity Enzyme Mix	1	
	EMC UpE/ ORF1a-F (10 μM)	1	
	EMC UpE/ ORF1a-R (10 μM)	1	
	EMC upE-P/Orf1a-P (10 μM)	0.5	
	Extracted RNA	5	
	Total volume per reaction	25	

Thermal cycling of real-time RT-PCR:
All assays were performed using a real-time PCR machine (Bio-Rad CFX96™ fluorescent quantitative PCR system) as per the RT-PCR Kit. The thermal profile is different for each kit as indicated in **Table 4**.
The confirmation assay for MERS- CoV's ORF1a gene used the same conditions (master mix preparation and thermal cycling profile) as the upE gene RT-PCR assay, with the exception that the primer and probe sequences were different [9].

Table 4: Real-time thermal profile setup for detection of SARS-CoV-2 and MERS-CoV

Detection kit	Cycle	Temperature (°C)	Running time	References
DAAN (ORF1ab and N)	1	50	00:15:00	[24]
	1	95	00:15:00	
	45	94	00:00:15	
		55	00:00:45	
BGI (ORF1ab)	1	50	00:20:00	[25]
	1	95	00:10:00	
	40	95	00:00:15	
		60	00:00:30	
Tib-Molbiol (Sarbeco E gene)	1	55	00:03:00	[26]
	1	95	00:00:30	
	40	95	00:00:03	
		60	00:00:12	
SuperScript™ III One-Step RT-PCR System (upE or ORF1a gene of MERS-CoV)	1	55	00:20:00	[37]
	1	95	00:03:00	
	45	95	00:00:15	
		58	00:00:30	

RESULT AND DISCUSSION:
One-step real-time RT-PCR was used for all assays, which without the need for a separate reaction for cDNA synthesis, making it quick and simple. After the reaction was completed, the results were automatically saved, and the amplification curves of the detected targets and the internal control were analyzed separately. Before the interpretation of the sample results, each assay's test controls (internal or extraction control, negative control, and positive control) were examined. All data were analyzed based on the genes that were detected; the Ct values and amplification curves were compared to the instructions supplied in the respective kits used for the genes tested. The Ct values and sigmoid amplification curve were used to decide if the sample was positive, negative, inconclusive, or invalid. In cases of inconclusive results, the test was repeated; whereas in cases of invalid test, either RNA was re-extracted from the original specimen, or in the case of SARS-CoV-2 suspected samples, re-sample was ordered where insufficient human cellular material was suspected. Even though we performed more tests and analyzed a large data for both SARS-CoV-2 and MERS-CoV, only a few representative results are included in this study to each RT-PCR assay.

SARS-CoV-2 RNA detection using real time RT-PCR:

The DAAN kit: The primer-probe mix in the DAAN kit contains two pairs of primers to detect N-gene and ORF1ab genes of SARS-CoV-2 RNA and one pair of primers to detect RNase P gene of human RNA. The N-gene and ORF1ab genes of SARS-CoV-2 were monitored simultaneously, and an endogenous internal control (Human RNase-P) transcript was used to track the occurrence of false negative results. The N-gene, ORF1ab, and RNase P signals were detected using the FAM, VIC, and Cy5 channels, respectively, as shown in **Figure 1**. The sample tested was considered as positive for SARS-CoV-2 based on S-shaped amplification curve with Ct ≤40 for the N and ORF1ab genes. A positive control contains an in vitro RNA transcript for the two SARS-CoV-2 target genes (N gene and ORF1ab gene) as well as the human RP gene (internal control) was included in each RT-PCR reaction.

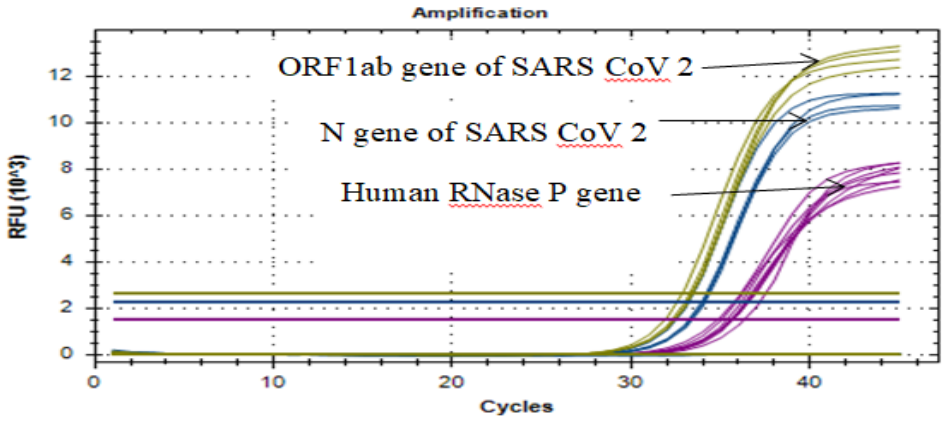


Figure 1: Amplification curves for the N gene (FAM), ORF1ab gene (VIC), and Human RNase P gene (Cy5) (Triplex PCR)

BGI kit: RT-PCR assay using the BGI kit was one of the most rapid, accurate, and specific for detecting SARS-CoV-2 RNA. Prior to interpreting the sample results, all test controls were examined to determine whether the test was valid or not. Each run included one positive and one negative control. The SARS-CoV-2 ORF1ab gene and the internal control (human β-actin) were amplified concurrently. All samples which have amplification curves with Ct ≤38 for ORF1ab gene were considered positive for SARS-CoV-2.

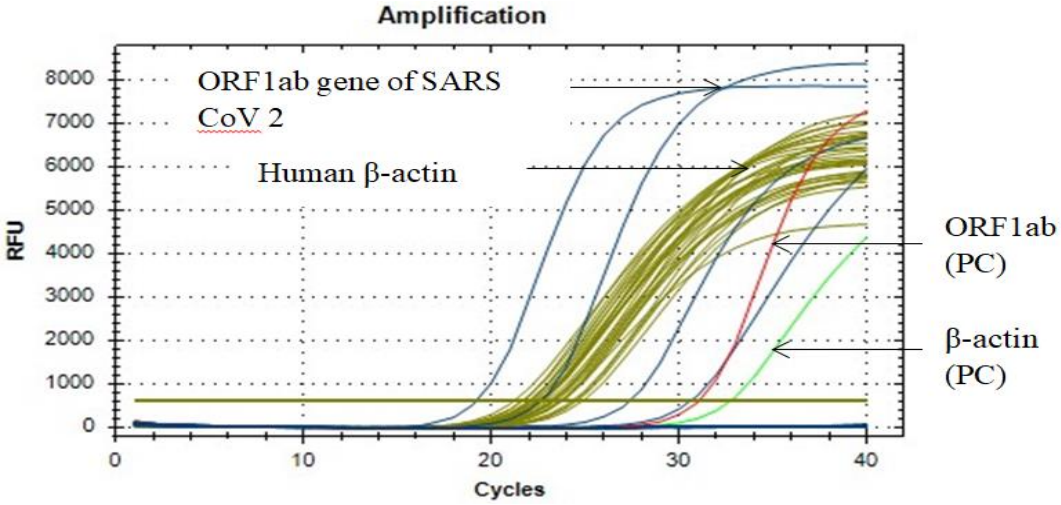


Figure 2: Amplification plots for the ORF1ab gene (FAM) of SARS-CoV-2 RNA as domain target and Human β–actin (VIC) as an internal control (Duplex PCR)

LightMix SarbecoV E-gene plus EAV control kit: This kit was the third kit used to detect SARS-CoV-2 RNA in our study. The assay included an EAV extraction control, which could monitor the presence of amplification inhibitors in the samples to avoid false negative results. The EAV extraction control was added to each sample during the RT-PCR step of the test. It consists of artificial RNA sequences from the Equine Arteritis Virus (EAV) used to monitor the reverse transcription and PCR amplification processes in each

sample. Amplification of the E gene and EAV with a Ct value less than 36 indicated that the sample tested was positive for SARS-CoV-1 and SARS-CoV-2. Because SARS-CoV-1 is currently not assumed to be circulating, a positive result from the E-gene could be interpreted as SARS-CoV-2 [26]. However, even though the Sarbeco E-gene protocol developed by Charité laboratory (Berlin) needs additional time, all E-gene-positive samples were further confirmed for SARS-CoV-2 using RdRP-SARSr primers and 2019-nCoV-specific probes (P2) as shown in Table 2. Nevertheless, the amplification graphs for RdRp gene (confirmatory tests) are not presented here.

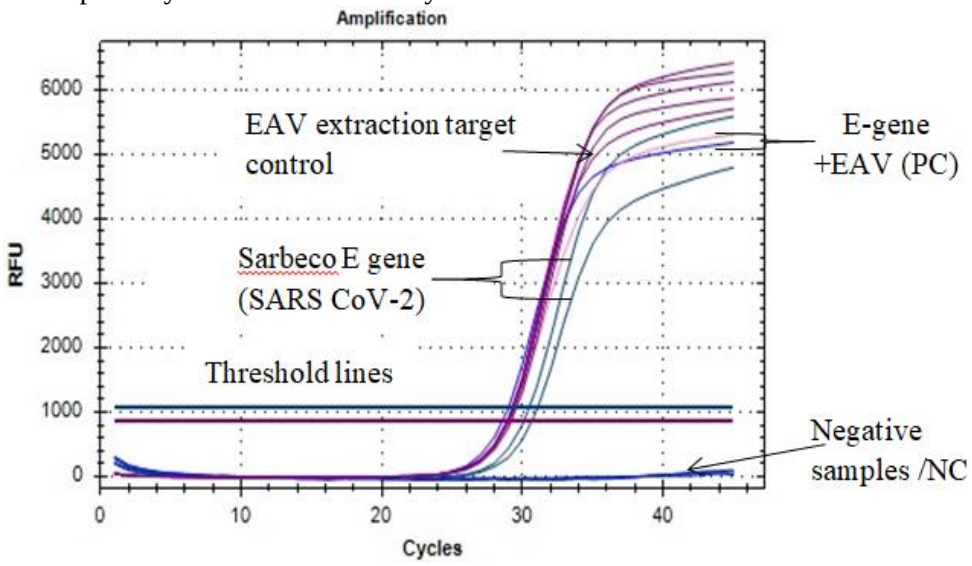
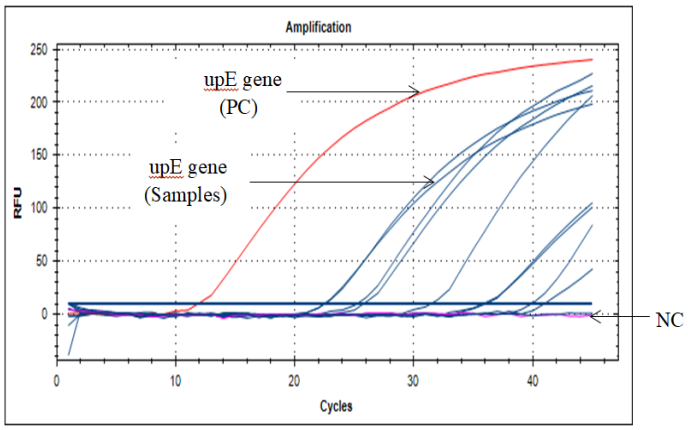


Figure 3: The duplex RT-PCR amplification of conserved E-gene (Sarbecovirus) in the FAM channel along with extraction control EAV in the Cy5 channel.

Out of 8400 human samples tested for SARS-CoV-2 RNA detection, around 540 were SARS-CoV-2 positive. Even though the three commercial kits were used to detect different SARS-CoV-2 RNA target genes with a particular real time RT-PCR assay, all the kits were sensitive, specific and accurate with slight variations. The results observed in this study were agreed with previous research works [38, 41,42].

MERS-CoV RNA detection using real time RT-PCR:

Corman assays targeting the UpE and ORF1a regions were used to detect MERS-CoV using the primers and probes listed in Table 2. These primers and probes were designed by Erasmus Medical Center (Netherlands), which FAO purchased and provided to the National Veterinary Institute. Laboratory confirmation of MERS-CoV infection requires either a positive real-time RT-PCR result for at least two specific genomic targets or a single positive target followed by sequencing of a second target [39]. Because of both probes (upE-P and Orf1a-P) were labeled with the same channel (FAM), only one target could be detected at a time. For multiplex detection, the two target genes (upE and ORF1a) and internal controls should be labeled with the different fluorescent dyes [40]. Another limitation of this assay was the lack of internal controls to detect false negatives. MERS-CoV was detected in dromedary camel samples using a screening assay (Assay 1) that targeted the sensitive RNA upstream of the E gene (upE) and a confirmation assay (Assay 2) targeting open reading 1a (ORF1a). Amplification of the upE gene (screen-positive) with a Ct≤ cutoff value was further validated using MERS-CoV-specific ORF1a gene primers and fluorescent-labeled probes. Swab samples from dromedary camels were tested for MERS-CoV with screening, and confirmatory, or specific assays. Out of the 400 total camels that were sampled and tested, 54 were positive for MERS-CoV. A high rate of MERS-CoV positive was observed compared to the previous research conducted in Ethiopia from 2017–2020 [43].



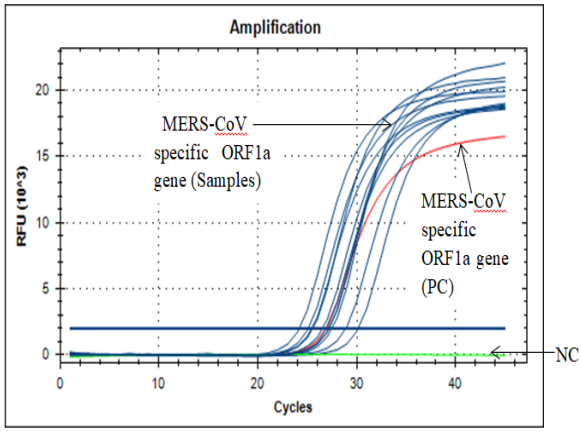


Figure 4: A) Real-time RT-PCR results for UpE-gene (screening test) B) Real-time RT-PCR test results for ORF1a gene (confirmatory test)

CONCLUSION:

Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses. SARS-CoV-2 and MERS-CoV are highly pathogenic coronaviruses which have a global public health concern. Real time RT-PCR is the gold standard and most reliable method for SARS-CoV-2 and MERS-CoV detection. In this study, three different commercial kits (DAAN, BGI, and Sarbeco E-gene) were used to detect SARS-CoV-2 and one detection kit (SuperScript™ III One-Step RT-QPCR System with Platinum™ Taq High Fidelity DNA Polymerase) was used to detect MERS-CoV. SARS-CoV-2 RNA detection was done by one step real-time RT-PCR using primers and Probes which were developed at different institutes like China CDC and Charite (Germany). MERS-CoV RNA detection was performed using two consecutive assays (E gene detection as screening assay and ORF1a gene detection as Confirmatory assay) developed by Corman and his colleagues in 2012.

REFERENCES:

[1] Britton P. Coronaviruses: General Features (*Coronaviridae*). Encyclopedia of Virology. 2019:193–7. doi: 10.1016/B978-0-12-814515-9.00139-9. Epub 2021 Mar 1. PMCID: PMC7831903.

[2] Gorkhali R, Koirala P, Rijal S, Mainali A, Baral A, Bhattarai HK. Structure and Function of Major SARS-CoV-2 and SARS-CoV Proteins. Bioinform Biol Insights. 2021 Jun 22; 15:11779322211025876. doi: 10.1177/11779322211025876. PMID: 34220199; PMCID: PMC8221690.

[3] Malik YA. Properties of Coronavirus and SARS-CoV-2. Malays J Pathol. 2020 Apr; 42(1):311. PMID: 32342926.

[4] Mingaleeva RN, Nigmatulina NA, Sharafetdinova LM, Romozanova AM, Gabdoulkhakova AG, Filina YV, Shavaliyev RF, Rizvanov AA, Miftakhova RR. Biology of the SARS- CoV-2 Coronavirus. Biochemistry (Mosc). 2022 Dec; 87(12):1662-1678. doi: 10.1134/S0006297922120215. PMID: 36717455; PMCID: PMC9839213.

[5] Zeidler A, Karpinski T M. SARS-CoV, MERS-CoV, SARS-CoV-2 Comparison of Three Emerging Coronaviruses. Jundishapur J Microbiol. 2020; 13(6):e103744. https://doi.org/10.5812/jjm.103744.

[6] Masters PS. The molecular biology of coronaviruses. Adv Virus Res. 2006; 66:193-292. doi: 10.1016/S0065-3527(06)66005-3. PMID: 16877062; PMCID: PMC7112330.

[7] Abdelrahman Z, Li M, Wang X. Comparative Review of SARS-CoV-2, SARS-CoV, MERS-CoV, and Influenza A Respiratory Viruses. Front Immunol. 2020 Sep 11; 11:552909. doi: 10.3389/fimmu.2020.552909. PMID: 33013925; PMCID: PMC7516028.

[8] David W. Kimberlin, M., FAAP; Elizabeth D. Barnett, MD, FAAP; Ruth Lynfield, MD, FAAP; Mark H. Sawyer, MD, FAAP, Coronaviruses, Including SARS-CoV-2 and MERS-CoV. 32nd Edition ed. January 2021: merican Academy of Pediatrics. http://publications.aap.org/redbook/book/chapter-pdf/1568397/rbo2021_sec3_036_en.pdf

[9] WOAH, Middle East Respiratory Syndrome (Infection of dromedary camels with Middle East Respiratory Syndrome Coronavirus). WOAH Terrestrial Manual 2021.

[10] Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012 Nov 8; 367(19):1814-20. doi: 10.1056/NEJMoa1211721. Epub 2012 Oct 17. Erratum in: N Engl J Med. 2013 Jul 25; 369(4):394. PMID: 23075143.

[11] Mohd, H.A., Al-Tawfiq, J.A. & Memish, Z.A. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) origin and animal reservoir. *Virol J* 13, 87 (2016). https://doi.org/10.1186/s12985-016-0544-0

[12] NaTHNaC: Middle East respiratory syndrome coronavirus (MERS- CoV) reminder Jun 2022

[13] Kumar A, Singh R, Kaur J, Pandey S, Sharma V, Thakur L, Sati S, Mani S, Asthana S, Sharma TK, Chaudhuri S, Bhattacharyya S, Kumar N. Wuhan to World: The COVID-19 Pandemic. Front Cell Infect Microbiol. 2021 Mar 30; 11:596201. doi: 10.3389/fcimb.2021.596201. PMID: 33859951; PMCID: PMC8042280.

[14] Chathappady House NN, Palissery S, Sebastian H. Corona Viruses: A Review on SARS, MERS and COVID-19. Microbiol Insights. 2021 Mar 19; 14:11786361211002481. doi: 10.1177/11786361211002481. PMID: 33795938; PMCID: PMC7983408.

[15] CerTestBiotec, S.L., VIASURE SARS-CoV-2 Real Time PCR Detection Kit. 2020.

[16] Afzal A. Molecular diagnostic technologies for COVID-19: Limitations and challenges. J Adv Res. 2020 Nov; 26:149-159. doi: 10.1016/j.jare.2020.08.002. Epub 2020 Aug 6. PMID: 32837738; PMCID: PMC7406419.

[17] Feng H, Wu H, Wang H, Yuan J, Chen L, Jin H, et al. (2022) Detection of a Novel Coronavirus (SARS-CoV-2) by Real-Time Reverse Transcription-Polymerase Chain Reaction, China, 2020. J Antivir Antiretrovir. S23:003.

[18] Zhen W, Berry GJ. Development of a New Multiplex Real-Time RT-PCR Assay for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV- 2) Detection. J Mol Diagn. 2020 Dec; 22(12):1367-1372. doi: 10.1016/j.jmoldx.2020.09.004. Epub 2020 Sep 19. PMID: 32961315; PMCID: PMC7501774.

[19] Arabi YM, Balkhy HH, Hayden FG, Bouchama A, Luke T, Baillie JK, Al-Omari A, Hajeer AH, Senga M, Denison MR, Nguyen-Van-Tam JS, Shindo N, Bermingham A, Chappell JD, Van Kerkhove MD, Fowler RA. Middle East Respiratory Syndrome. N Engl J Med. 2017 Feb 9; 376(6):584-594. doi: 10.1056/NEJMsr1408795. PMID: 28177862; PMCID: PMC5362064.

[20] Corman VM, Müller MA, Costabel U, Timm J, Binger T, Meyer B, Kreher P, Lattwein E, Eschbach-Bludau M, Nitsche A, Bleicker T, Landt O, Schweiger B, Drexler JF, Osterhaus AD, Haagmans BL, Dittmer U, Bonin F, Wolff T, Drosten C. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 2012 Dec 6;17(49):20334. doi: 10.2807/ese.17.49.20334-en. PMID: 23231891

[21] Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer TM, Muth D, Müller MA, Drexler JF, Zambon M, Osterhaus AD, Fouchier RM, Drosten C. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012 Sep 27; 17(39):20285. doi: 10.2807/ese.17.39.20285-en. Erratum in: Euro Surveill. 2012; 17(40): pii/20288. PMID: 23041020.

[22] Daan Gene, Instructions for Use ofRNA/DNA Purification Kit (Spin Column),China (2020) https://en.daangene.com/products/spin-column-dna-rna-extraction-kit/

[23]QIAGEN, QIAamp Viral RNA Mini Kit,2020. https://www.studocu.com/cl/document/universidad-mayor/biologia-celular/hb-0354-007-hb-qa-viral-rna-mini-0720-ww/28387372

[24] Daan Gene, Instructions for use for Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing), DA0930~DA0932,China,2020. https://viaglobalhealth.com/wp-content/uploads/2020/05/DaAn-Gene-nCoV-RNA-COVID-19-test-Instruction-Manual.pdf

[25] BGI, G., Real-Time Fluorescent RT-PCR Kit for Detecting SARS-CoV-2,China,2020. https://www.fda.gov/media/136472/download

[26] TIB,M., LightMix® SarbecoV E-gene plus EAV control, 2020.

[27] Pasqualotto, A.C. and A.L. Seus, *COVID-19 PCR:* frequency of internal control inhibition in clinical practice. Access Microbiology, 2023. 5(5).

[28] Corman, V.M., et al., Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Eurosurveillance, 2020. 25(3).
[29] Corman, V.M., et al., Diagnostic detection of 2019-nCoV by real-time RT-PCR. Unpublished work,2020.
https://www.who.int/docs/defaultsource/coronaviruse/protocol-v2-1.pdf
[30] Thermofisher(invitrogen), SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase. 2016.
[31] He Y, Luo J, Yang J, Song J, Wei L, Ma W. Value of Viral Nucleic Acid in Sputum and Feces and Specific IgM/IgG in Serum for the Diagnosis of Coronavirus Disease 2019. Front Cell Infect Microbiol. 2020 Aug 6; 10:445. doi: 10.3389/fcimb.2020.00445. PMID: 32850506; PMCID: PMC7423831.
[32] Zhou Y, Zhang L, Xie YH, Wu J. Advancements in detection of SARS-CoV-2 infection for confronting COVID-19 pandemics. Laboratory Investigation; a Journal of Technical Methods and Pathology. 2022 Jan; 102(1):4-13. DOI: 10.1038/s41374-021-00663-w. PMID: 34497366; PMCID: PMC8424153
[33] Zhongtian, L.Y.R.H.Q., Isolation and identification of pathogens of novel coronavirus pneumonia,China,2020.
https://rs.yiigle.com/cmaid/1230124
[34] Li D, Zhang J, Li J. Primer design for quantitative real-time PCR for the emerging Coronavirus SARS-CoV-2. Theranostics. 2020 Jun 1;10(16):7150-7162. doi: 10.7150/thno.47649. PMID: 32641984; PMCID: PMC7330846.
[35] Kilic T, Weissleder R, Lee H. Molecular and Immunological Diagnostic Tests of COVID-19: Current Status and Challenges. iScience. 2020 Aug 21; 23(8):101406. doi: 10.1016/j.isci.2020.101406. Epub 2020 Jul 25. PMID: 32771976; PMCID: PMC7381402.
[36] Zhen W, Berry GJ. Development of a New Multiplex Real-Time RT-PCR Assay for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Detection. J Mol Diagn. 2020 Dec; 22(12):1367-1372. doi: 10.1016/j.jmoldx.2020.09.004. Epub 2020 Sep 19. PMID: 32961315; PMCID: PMC7501774.
[37] FAO, Veterinary laboratory testing protocols for priority zoonotic diseases in Africa. FAO Animal Production and Health Guidelines No. 34. 2023Rome. https://doi.org/10.4060/cc3956en
[38] Sisay, A., *et al*., Diagnostic accuracy of three commercially available one step RT-PCR assays for the detection of SARS-CoV-2 in resource limited settings. PLoS One, 2022. **17**(1) https://www.ncbi.nlm.nih.gov/pubmed/35051204
[39] WHO, Laboratory Testing for Middle East Respiratory Syndrome Coronavirus,2018. https://iris.who.int/bitstream/handle/10665/259952/WHO-MERS-LAB-15.1- Rev1-2018-eng.pdf? Sequence=1
[40] Kazuya, S., *et al*., Ultra-Rapid Real-Time RT-PCR Method for Detecting Middle East Respiratory Syndrome Coronavirus Using a Mobile PCR Device, PCR1100. Jpn J Infect Dis, 2020. **73**(3): p. 181-186.
https://www.ncbi.nlm.nih.gov/pubmed/31875608
[41] Jiankang Zhao, Y.Z., Bin Cao, Comparison of the Performance of Six SARS-CoV-2 Nucleic Acid Detection Kit in Positive Samples Using RT-PCR. Journal of Clinical Chemistry and Laboratory Medicine, 2021. Vol.4 (Iss.3 No:1000170).
[42] Peihua Niu, R.L., Li Zhao,Huijuan Wang,Baoying Huang,Fei Ye,Wenling Wang,Wenjie Tan, Three Novel Real-Time RT-PCR Assays for Detection of COVID-19 Virus. 2020.
https://weekly.chinacdc.cn/fileCCDCW/journal/article/ccdcw/2020/25/PDF/PCR.pdf
[43] Zhou, Z., et al., Genetic diversity and molecular epidemiology of Middle East Respiratory Syndrome Coronavirus in dromedaries in Ethiopia, 2017-2020. Emerg Microbes Infect, 2023. 12(1): p. e2164218.