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SARS COV 2 (COVID-19) DIAGNOSIS IN WILDLIFE ANIMALS USING RT-POLYMERASE CHAIN REACTION (PCR) IN DUBAI SAFARI PARK

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ABSTRACT

Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses. SARS-CoV-2 is a beta coronavirus, a genus that includes several coronaviruses (SARS-CoV, MERS-CoV, bat SARS-like CoV, and others) isolated from humans, bats, camels, civets, and other animals. Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) is the pathogenic agent that causes the disease COVID-19. SARS-CoV-2 is thought to have emerged from an animal source, most likely a bat, and subsequently spread to humans. While genetically closely related viruses have been isolated from Rhinolophus bats, the exact source of SARS-CoV-2 and its route of introduction into the human population have not been established. Monitoring animal infections is imperative to better understanding their epidemiological significance for animal health, biodiversity, and human health. According to evidence from risk assessments, epidemiological investigations, and experimental studies, animals play no significant role in the spread of SARS-CoV-2, which is sustained by human-to-human transmission. The possibility of Coronavirus testing by veterinary labs was considered after the Iowa State veterinary lab discovered the COVID-19 virus has a similar DNA testing process to porcine epidemic diarrhea virus (PED). PED is another form of coronavirus that killed many piglets in 2013 and was unresponsive to treatment. Several vet labs optimized their equipment and processes to test for PED, helping them determine that older pigs could recover and develop immunity against the virus. Those same labs are still set up for coronavirus testing. We in Dubai Safari Park, particularly within the laboratory Veterinary Hospital, are conducting RT-PCR analysis to test diverse animal species including non-human primates, Carnivores, small mammals, and Ungulates. Even though none of our animals show any respiratory signs, we have conducted this study to ensure that our animal collection is healthy and free of SARS-COV-2.

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INTRODUCTION

Although current evidence suggests that SARS-CoV-2 emerged from an animal source, likely a bat, the source has not yet been identified. The pandemic is being driven by human-to-human transmission, which happens through respiratory droplets from coughing, sneezing, and talking, that can remain in the air for some time as aerosols. The purpose of this study is to ensure that our animal collection is free from the novel corona virus, the Covid-19, and to proof that they are healthy and pose no risk to the staff and the public. Genetic sequence data reveal that SARS-CoV-2 is genetically closely related to other coronaviruses circulating in Rhinolophus bat (horseshoe bat) populations. To date, there is not enough scientific evidence to identify the source of SARS-CoV-2 or to explain the original route of transmission to humans

(which may involve an intermediate host) (World Organization for Animal Health, 2021). We are still learning about SARS-CoV-2 in animals. Based on the information available, the risk of animals spreading the virus to people is low (NVSL, 2021). Our facility has a high-throughput testing machine that can test 96 samples at a time, several times a day. It is also equipped to perform the real-time PCR diagnostic test that detects the presence of the SARS-Cov-2 virus.

MATERIALS AND METHODS

RT-qPCR assay for the Diagnosis of SARS CoV 2 (COVID-19)

Nucleic acid amplification and detection techniques are among the most valuable tools in biological research today. Polymerase chain reaction (PCR) is a relatively simple and widely used molecular biology technique to amplify and detect DNA and RNA sequences. PCR is highly sensitive and requires a minimal template for detection and amplification of specific sequences. Quantitative PCR (qPCR) is used to detect, characterize, and quantify nucleic acids. Commonly, in RT-qPCR, RNA transcripts are quantified by reverse transcribing them into cDNA first, as described above and then qPCR is subsequently carried out. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and elongation. However, in qPCR, fluorescent labeling enables the collection of data as PCR progresses. During each cycle, the fluorescence is measured. The fluorescence signal increases proportionally to the amount of replicated DNA and hence the DNA is quantified in "real time". The measured fluorescence is proportional to the total amount of amplicon; the change in fluorescence over time is used to calculate the amount of amplicon produced in each cycle.

Testing for the presence of coronavirus is an essential diagnostic tool for monitoring and managing the current COVID-19 pandemic. The genetic material in SARS-CoV-2 virus is stored in RNA. In the case of COVID-19 testing, RNA from a SARS-CoV-2 sample is first converted to its complementary DNA sequence by reverse transcription (RT). The detection kit (3DMed ANDiS Fast SARS-CoV-2 RT-qPCR Detection Kit) mainly targets the three specific genes of SARS-CoV-2 virus that are ORF Lab, E gene and N gene. The collection of amplified data enabled by the application of FAM, VIC, and ROX fluorophores targeting ORF Lab, gene and N gene respectively. Moreover, an additional fluorophore, Cy5 is used to measure the internal control.

Here at Dubai safari park, we are equipped with BIORAD CFX96 Optical reaction module with C1000 thermal cycler. The CFX96 Optical Reaction Module converts a C1000 Touch Thermal Cycler into a real-time PCR system that is operated by Bio-Rad CFX Manager 3.1 software. It is a six-channel (five colors and one FRET channel) real-time PCR instrument combines advanced optical technology with precise temperature control to deliver sensitive, reliable detection for singleplex or multiplex reactions.

Figure 1. RT-qPCR test kit.

Animals

Samples from different animal species were collected, including ten Blackbucks, one Impala, four Arabian gazelle, one Pygmy goat, two Axis deer, three Tigers, one Egyptian fruit bat, three Lions, one Marmoset, one Vervet monkey, three Hamadryas baboons, one Racoon, one Chimpanzee and one White handed gibbon.

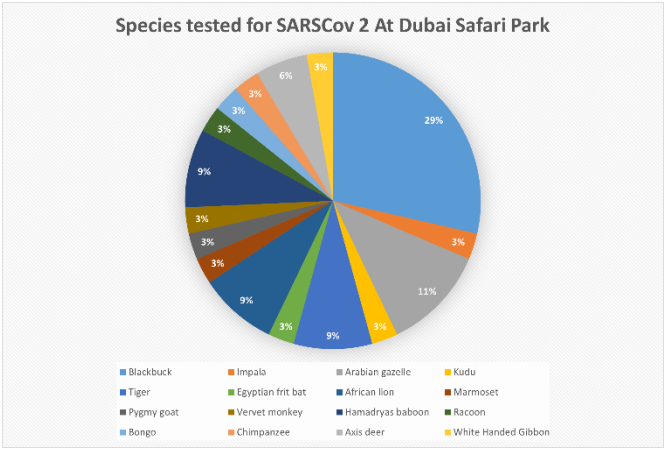

Figure 2. Percentage of the different species of animals tested for SARS Cov-2 Virus.

Sample Collection

Samples were collected (Figures 3 and 4) using swabs with synthetic nylon tips (Jiangsu Mole Bioscience Co., Ltd) from the animal's Nasopharyngeal /Oropharyngeal area and immediately stored in viral transport media (Mole Bioscience) for storage and transportation. In order to ensure a good quality specimen for the identification of respiratory viruses, it is important to collect epithelial cells from the nasal septum and the pharynx (NSW Health, 2021).

Figure 3. a) collection of nasopharyngeal swab sample from an African lion, b) collection of nasopharyngeal swab sample collection from an African lion. Samples were immediately transferred to a commercially available Viral transport media.

Nasopharyngeal /Oropharyngeal area and immediately stored in viral transport media (Mole Bioscience) for storage and transportation. In order to ensure a good quality specimen for the identification of respiratory viruses, it is important to collect epithelial cells from the nasal septum and the pharynx (NSW Health, 2021). In details, we measured the distance from the corner of the nose to the front of the ear and insert the shaft only half this length, the swab should be inserted along the medial part of the septum, along the base of the nose, until it reaches the posterior nares – gentle rotation of the swab may be helpful (PROVLAB, 2022). Sample tubes are labeled according to a patient's unique identification, species, sex, location, date and time of collection. Samples are then stored in the laboratory refrigerator. Regarding animal species Blackbucks, Impala, Arabian gazelle, Pygmy goat, Axis deer were restrained physically with the help of trained animal keepers during Dubai safari preventive medicine program. However, for the Tigers, Egyptian fruit bat, Lions, Marmoset, Vervet monkey, Hamadryas baboons, Racoon, Chimpanzee, White handed gibbon were chemically immobilized during their sampling procedure. Opportunistically we sampled one Bongo during postmortem examination. The total number of samples obtained was 35.

Sample Processing; Nucleic Acid Extraction

RNA extraction was done via obtaining 200 ul of the vortexed sample (Swab within the viral media) and inserted in the dedicated sample well (1st and 7th well) of the 96 well plate (alphaPrep Viral DNA/RNA Extraction Kit Model: VDR-B0967). Filter tips were used to avoid any contamination and the whole process was performed in a class 2 biosafety cabinet. The plate is then inserted in the instrument, an automated nucleic acid extraction machine (Hanwool TPC NC-15 Plus) along with the adequate number of strip tip combs based on the number of blocks placed in the machine (2 strip tips combs per each 96 well plate). The extraction process usually takes around thirty minutes to be completed. During the automated extraction procedure, the electromagnetic bead within the plate mixes the sample (DNA/RNA) followed by three steps of lysing treatment. After the lysing process, the extracted sample obtained from the elution buffer and transferred to disposable micro centrifuge tubes. Finally, samples were labelled according to their unique identification number and prepared for the amplification process. At this stage, extracted RNA

could be placed in the freezer for one month at -20 or

RT-qPCR COVID-19 Programming

RT-PCR COVID-19 programming was done as per 3DMed ANDiS Fast SARS-CoV-2 RT-qPCR Detection Kit protocol (Table 1).

Table 1. RT-qPCR COVID-19 test protocol set up,

Stage	Temperature	Time	Cycle number
1	50° Celsius	2 Minutes	1
2	95° Celsius	2 Seconds	1
3	95° Celsius 60° Celsius	1 Second 13 Seconds	41

processed immediately.

As per the reference, Stage 1 amplification temperature is set to 50° Celsius for 2 minutes in cycle no.1, Stage 2 amplification temperature set to 95° Celsius for 2 seconds. Stage 3 programmed in to two substages the first one is set to 95° Celsius for 1 second and the second one is set to 60° Celsius for 13 seconds. Additionally, 40 more repeated cycles programmed at stage 3, which will result in a total of 41 cycles (1 initial cycle followed by 40 repeated cycles). Total sample volume is set to 20 µL (10 µL sample plus 10 µL super mix). After verifying all these parameters, the program was saved in the system.

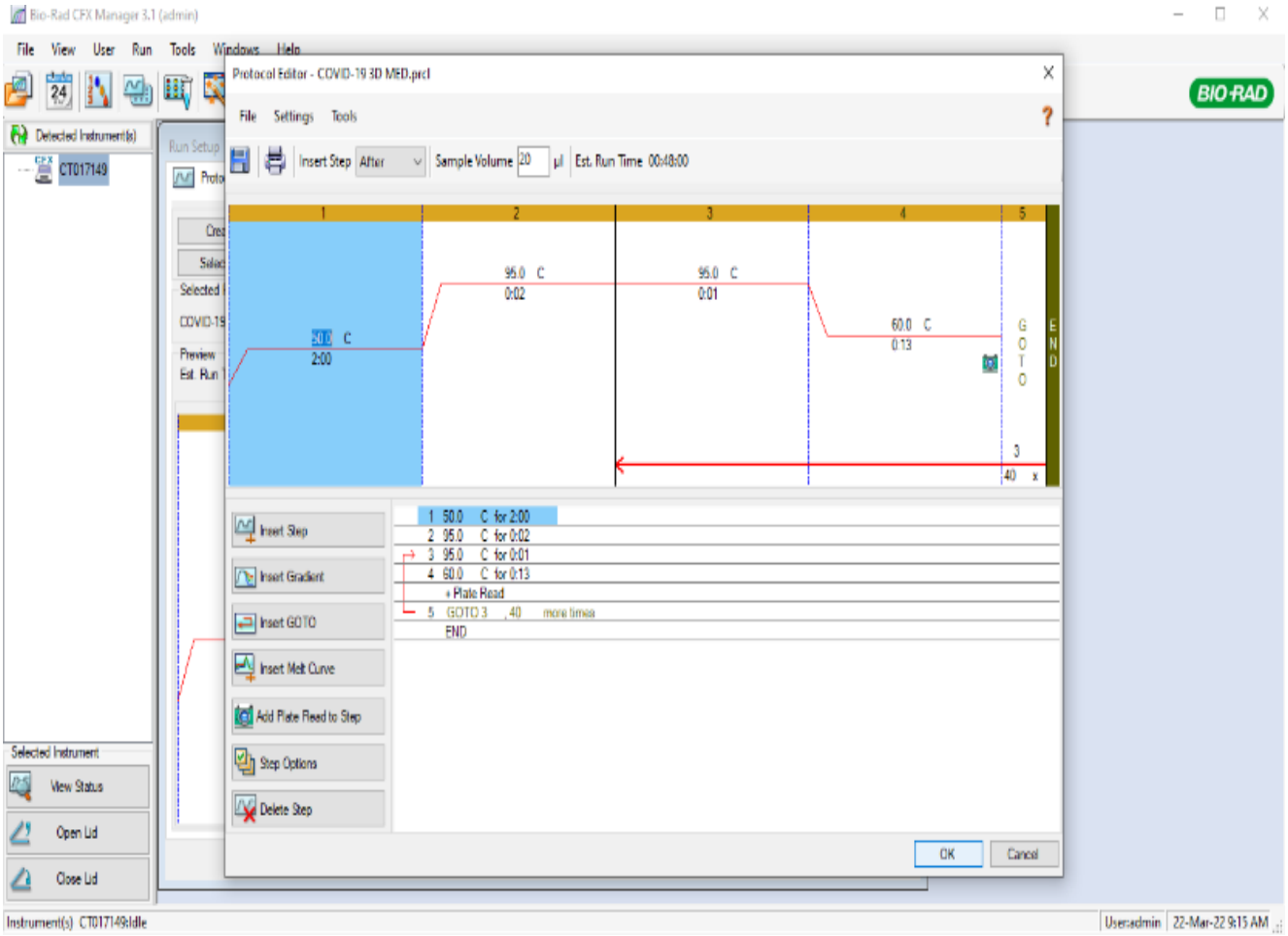

Figure 4. RT-qPCR COVID-19 test protocol set up page in Bio-Rad CFX Manager 3.1 software.

RT-PCR COVID-19 Plate Setup

Referring to 3DMed ANDiS Fast SARS-CoV-2 RT-qPCR Detection Kit protocol, initiated a new plate set up for SARS-CoV-2 RT-qPCR by clicking new plate setup. Once the set-up window is open, all wells were selected and activated by assigning sample type as unknown. Then by clicking 'Select fluorophores' icon the fluorophores window will appear. Selected FAM, VIC, ROX and Cy5 fluorophores as per the 3DMed ANDiS Fast SARS-CoV-2 RT-qPCR Detection Kit protocol (Pic.02) and assigned each fluorophore towards ORF Lab, E gene, N gene and internal control respectively as the target (Figure 5).

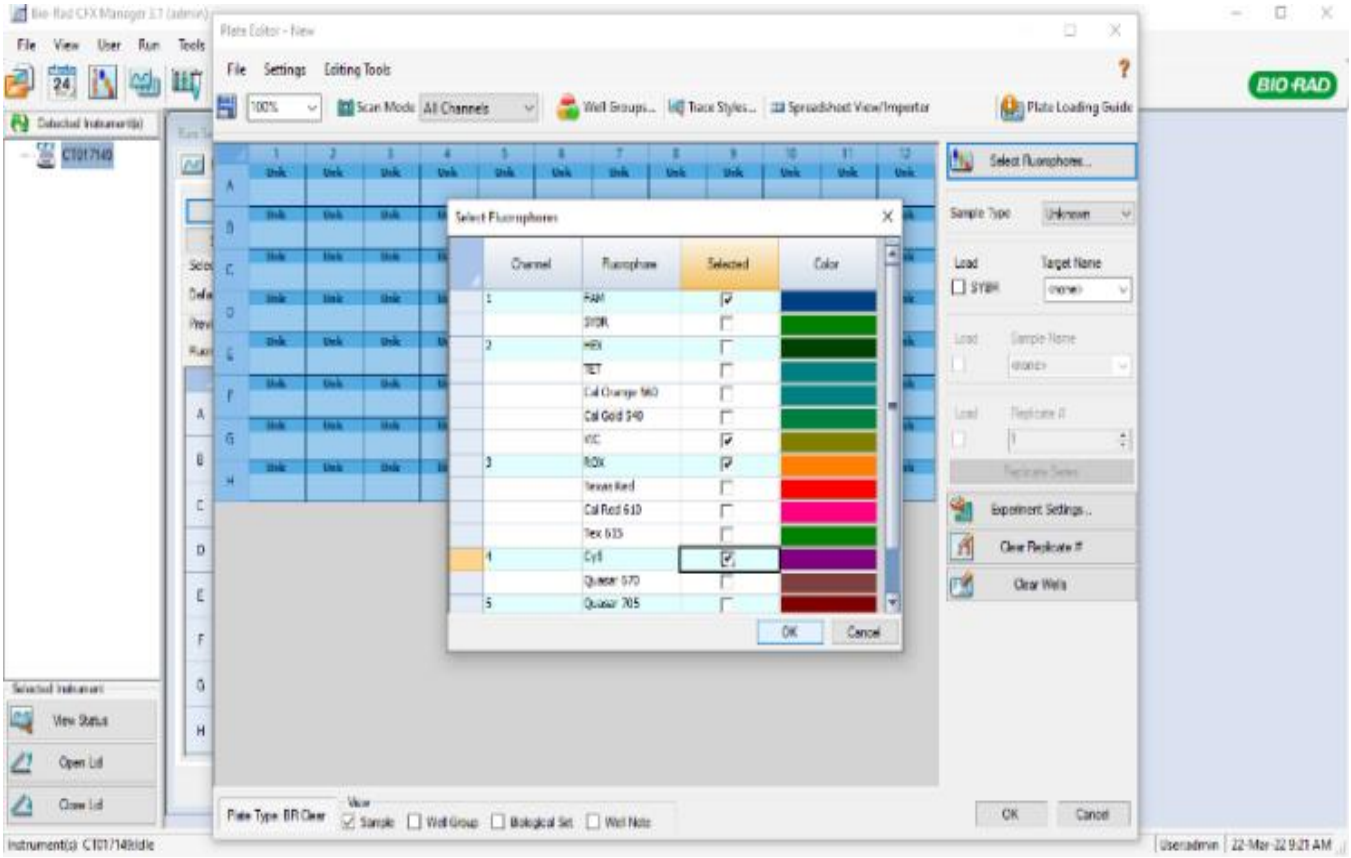

Figure 5. Selection of fluorophores in Bio-Rad CFX Manager 3.1 software.

Table 2. RT-qPCR results interpretation criteria.

ORF LAB (FAM)	N gene (ROX)	E gene (VIC)	Internal control (CY5)	Results	Interpretation
+	+	+	+/-	SARS-CoV-2 Positive	SARS-CoV-2 RNA is detected
+	-	+/-	+/-	SARS-CoV-2 Positive	SARS-CoV-2 RNA is detected
+	+	+/-	+/-		
+	+	+	+/-		
-	-	+	+/-	Presumptive positive for SARS-CoV-2	SARS-CoV-2 RNA is detected. But SARS-CoV-2 specific RNA targets are not detected
-	-	-	+	SARS-CoV-2 Negative	SARS-CoV-2 RNA is not detected
-	-	-	-	Invalid	Results are invalid. Repeat test. If result is still invalid, a new specimen should be collected

Then by selecting, the last three wells assigned as negative control, positive control and NTC (No Template Control) (Figure 6). Finally, all the information was reviewed and saved the plate setup in the system.

Sample Processing; Sample Amplification

After the nucleic acid extraction, the samples were placed in a separate biosafety cabinet. Simultaneously a SARS-CoV-2 RT-qPCR detection kit (3DMed ANDiS Fast SARS-CoV-2 RT-qPCR Detection Kit) taken out from -20 ULT freezer and kept in the refrigerator for gradually thaw. Once all required components like the enzyme mix, RT PCR reaction, negative control and positive control thawed then placed in the biosafety cabinet. All the components were brought to room temperature and each components undergone vortexing in order to make sure they are mixed properly, then the components undergone

a short centrifugation in order to re suspend any micro droplet of the components sticks to we inner surface of the tubes. Once all the components are ready for mixing a sterile RNAse/DNAse free micro centrifuge tube placed on a rack to prepare a super mix. Based on the number of samples 1.5 µL of enzyme mix/per each sample and 8.5 µL SARS-CoV-2 RT PCR reaction/ per each sample added to the micro centrifuge tube. This super mix then undergone vortexing and quick centrifugation. Finally, a disposable PCR well set prepared. 10 µL of super mix added to all the required wells. 10 µL sample added in respective wells. As part of the quality control a negative control, Positive control (SARS-CoV-2 synthetic positive control) and NTC (No Template Control) placed. Sample wells were closed with disposable well caps placed in the PCR amplifier machine and start the amplification by selecting SARS-CoV-2 plate setup and SARS-CoV-2 RT PCR protocol. Later on, sample wells are labelled in the system according to their unique identification numbers and saved.

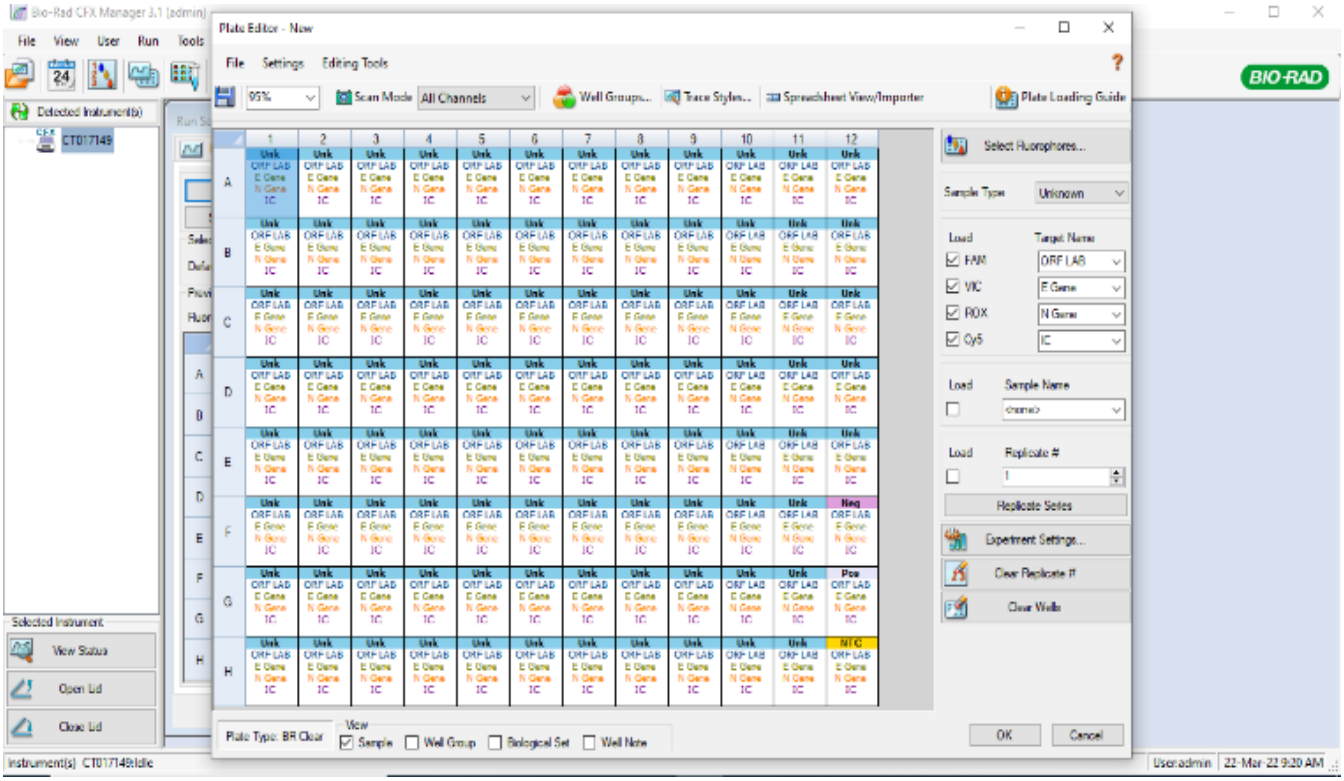

Figure 6. RT-qPCR COVID-19 test plate set up page in Bio-Rad CFX Manager 3.1 software.

RESULTS AND DISCUSSION

Based on the above data and graph the assay was completed and the threshold value was set. The controls including positive, negative, and non-reactive template were validated through the results. Upon the validation, the samples' Cq values were interpreted and if any of the controls do not exhibit the expected performance, the assay should be considered as invalid. With reference to the above analysis report all the negative controls indicate negative and positive controls indicate positive with Cq values. Moreover, a non-reactive well was assigned as blank which indicates no reaction as well. Then the Cq values of the samples were analyzed and none of them exhibited any reaction. Hence the analyzed samples are declared as non-reactive or negative, in other words SARS-CoV-2 virus RNAs were absent in the given samples (Table 3).

Table 3. Superior clinical performance.

Test Reagent	Comparative Reagent		
	Positive (+)	Negative (-)	Total
Positive (+)	121	0	121
Negative (-)	1	114	115
Total	122	114	236

Clinical study using 121 positive samples and 115 negative samples demonstrated that the positive percent agreement (PPA) is 99.18%, and the negative percent agreement (NPA) 100%.

Quantification Data:

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
A01	Cy5	IC	Unkn	Tiger	35.1	35.09	0
B01	Cy5	IC	Unkn	Bat	32.7	32.7	0
C01	Cy5	IC	Unkn	Lion Raja	32.5	32.52	0
D01	Cy5	IC	Unkn	Lion-Ahmed	36.5	36.54	0
E01	Cy5	IC	Unkn	Marmoset	31.6	31.62	0
F01	Cy5	IC	Unkn	Pygmy goat	34.6	34.6	0
G01	Cy5	IC	Unkn	Vervet monkey	31.8	31.8	0
H01	Cy5	IC	Neg Ctrl	NEG CTRL	N/A	0	0
A02	Cy5	IC	Pos Ctrl	POS CTRL	N/A	0	0
A03	Cy5	IC	NTC	NRT	N/A	0	0
A01	FAM	ORF Lab	Unkn	Tiger	N/A	0	0
B01	FAM	ORF Lab	Unkn	Bat	N/A	0	0
C01	FAM	ORF Lab	Unkn	Lion Raja	N/A	0	0
D01	FAM	ORF Lab	Unkn	Lion-Ahmed	N/A	0	0
E01	FAM	ORF Lab	Unkn	Marmoset	N/A	0	0
F01	FAM	ORF Lab	Unkn	Pygmy goat	N/A	0	0
G01	FAM	ORF Lab	Unkn	Vervet monkey	N/A	0	0
H01	FAM	ORF Lab	Neg Ctrl	NEG CTRL	N/A	0	0
A02	FAM	ORF Lab	Pos Ctrl	POS CTRL	37.21	37.21	0
A03	FAM	ORF Lab	NTC	NRT	N/A	0	0
A01	ROX	N gene	Unkn	Tiger	N/A	0	0
B01	ROX	N gene	Unkn	Bat	N/A	0	0
C01	ROX	N gene	Unkn	Lion Raja	N/A	0	0
D01	ROX	N gene	Unkn	Lion-Ahmed	N/A	0	0
E01	ROX	N gene	Unkn	Marmoset	N/A	0	0
F01	ROX	N gene	Unkn	Pygmy goat	N/A	0	0
G01	ROX	N gene	Unkn	Vervet monkey	N/A	0	0
H01	ROX	N gene	Neg Ctrl	NEG CTRL	N/A	0	0
A02	ROX	N gene	Pos Ctrl	POS CTRL	34.8	34.8	0
A03	ROX	N gene	NTC	NRT	N/A	0	0
A01	VIC	E gene	Unkn	Tiger	N/A	0	0
B01	VIC	E gene	Unkn	Bat	N/A	0	0
C01	VIC	E gene	Unkn	Lion Raja	N/A	0	0
D01	VIC	E gene	Unkn	Lion-Ahmed	N/A	0	0
E01	VIC	E gene	Unkn	Marmoset	N/A	0	0
F01	VIC	E gene	Unkn	Pygmy goat	N/A	0	0
G01	VIC	E gene	Unkn	Vervet monkey	N/A	0	0
H01	VIC	E gene	Neg Ctrl	NEG CTRL	N/A	0	0
A02	VIC	E gene	Pos Ctrl	POS CTRL	31.48	31.48	0
A03	VIC	E gene	NTC	NRT	N/A	0	0

The importance of this study was fulfilled through (COVID-19) RT-qPCR Screenings in Dubai safari park for various animals including non-human primates, Carnivores, small mammals and Ungulates and test results indicated negative (note detected) for all the animals undergone the screening. Continues RT-qPCR screenings will be conducted against SARS-CoV-2 (COVID-19) as part of preventive medicine programs and quarantine health screenings to ensure our animal collection is healthy and free of SARS-COV-2 (COVID-19) at Dubai safari park. This provides crucial evidence that provides data with negative results from the new emerging COVID-19 virus, which gives a sense of satisfaction to the visitors and staff while dealing with animals and break the cycle of the disease to be spread. Monitoring animals in a wildlife park like Dubai Safari Park is of great importance, particularly in terms of disease control. Early detection of illnesses, safeguarding endangered species, preventing zoonotic diseases, and contributing to research are some key benefits. This practice ensures the health of animals, protects biodiversity, and maintains visitor safety, making it a cornerstone of responsible wildlife management. Multifaceted endeavor with profound implications for disease control, conservation, and public safety, which gives the advantage for the safari park to attract more visitors with confidence of a disease-free collection. It is a commitment to the health of our animals, the preservation of biodiversity, and the safety of all who visit our park, which is central to our mission of responsible wildlife management." Many studies of animal models have been reviewed in connection with SARS and MERS infection, these models usually failed to replicate the conditions which are similar to the human environment for trail and testing of vaccines. In addition, the results of this systematic review are also consistent with the above findings when COVID-19 infection is considered. Unfortunately, the common complications like acute respiratory distress syndrome (ARDS) and coagulopathy, which is common in human COVID patients, were not replicated in any of these animal models. Primates have similar binding affinity to COVID-19 virus as when related to humans (TIB Diagnostics, 2021) which differs in stark contrast to other animals such as hamsters and mice, which have low-to-medium affinity.

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REFERENCES

NSW Health. 2021. Respiratory Swab Collection. NSW Health, Australia.

NVSL. 2021. Confirmation of COVID-19 in a Canada Lynx at a Pennsylvania Zoo. National Veterinary Services Laboratories, United States Department of Agriculture (USDA), Washington, DC 20250, United States.

PROVLAB. 2022. Collection of A Nasopharyngeal and Throat Swab for Detection of Respiratory Infection. PROVLAB, Alberta Health Services, Edmonton, Alberta, Canada.

TIB Diagnostics. 2021. How veterinary diagnostic labs support human COVID-19 testing. Medium, San Francisco, USA.

WHO (World Organization for Animal Health). 2021. Infection with Sars-Cov-2 in Animals: Aetiology Epidemiology Diagnosis Prevention and Control References, in: Sheet, O.T.F. (Ed.). World Organization for Animal Health, Paris, France.