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Covid -19 Virus Gene Distinction and Allelic Discrimination in Infected Patients in Babylon, Province

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ABSTRACT

The term coronavirus was first specified as a virus having spike-like projections, Coronaviruses (CoVs) are a group of related RNA viruses that cause diseases in both human and vertebrates. They can infect the respiratory, and gastrointestinal tract, hepatic, and central nervous system of human, livestock, birds, bat, mouse, and many other wild animals, which are enveloped viruses that possess large-sized single-strand RNA genomes. On December 29, 2019, the WHO officially named the novel disease as coronavirus disease 2019 (COVID-19). This virus can enter the human body by its Angiotensin-converting enzyme 2 (ACE2) receptors which are located in various organs such as the heart, lungs, kidneys, and digestive system, which is complementary in shape to the spike shape which allows successful attachment to occur and makes it easier for the virus to enter the target cells. From a group of probable cases in which that infected with SARS-COV-2 virus (Covid-19) taken the nasopharyngeal swab into viral transport Media (VTM) from 120 patients as studied group, all samples were chickened by using Direct Immunofluorescences antibody procedure provided by (Viro-Cell Company). The VTM containing Nasopharyngeal swab was taking to Real Time PCR, by Using Bioron– Real Time Kit, to evaluate the presence of gene differentiation of SARS -COV-2 as well as allelic discrimination through specific protocol provided with Bioron Kit on the Bio –Rad Thermo cycler system. The statistical analysis was done by using SPSS, ANOVA and Chi – Square.Gene distinction of SARS-CoV-2 with allelic discrimination by real time PCR. The presence of S gene mostly in first few days of infection while E gene present in the end time of infection over 1 -2 week of onset of infection as well as N gene but in less degree. The N gene has a higher level of stability and conservation, With high percent amino acid similarity and less mutations over time. The heterozygote of (S and / E gene) have higher percentage than other.

Keywords: COVID-19, allelic discrimination, N gene, E gene, S gene

Introduction

The term coronavirus was first used to describe a virus with spike-like projections, and the word "crown," which is derived from the Greek korn and means "crown" or "wreath." The term was originally used to describe certain characteristics of the infective type (virion) as seen through an electron microscope, which has a distinctive bulbous form projection (spike). These projections were later discovered to be proteins molecules anchored on the lipid bilayer membrane's surface [1]. Coronaviruses (CoVs) are RNA viruses that cause disease in humans and other vertebrates. They can infect humans, pets, birds, bats, mice, and a variety of other wild animal's respiratory, gastrointestinal, hepatic, and central nervous systems. Coronaviruses are enveloped viruses with large single-strand positive sense RNA genomes that belong to the Coronaviridae family [2]. At least seven forms of coronavirus have been identified as causing disease in humans; the 229E, OC43, NL63, and HKU1 viruses only cause moderate cold symptoms. The remaining three viruses, Extreme Acute Respiratory Syndrome (SARS-CoV), but caused the SARS outbreak in 2002 and 2003; Coronaviruses responsible for Middle East

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Respiratory Syndrome (MERS-CoV), which appeared in 2012 as circulating in camels; and SARS-CoV-2, which appeared in December 2019 in Wuhan, China, can cause serious illness, and a lot of work is being put in to stop them from spreading [3]. The COVID-19 outbreak was declared a Public Health Emergency of International Concern by the World Health Organization (WHO) on January, 2020. It has since grown into a global pandemic, affecting vast populations in Iran, S. Korea, and Italy, as well as causing a rise in worldwide cases in over 150 countries [4]. The coronavirus has spread rapidly through Europe, N. America, Asia, and the Middle East, with the first recorded cases in Latin American and African countries. According to the WHO, the number of countries, states, or territories affected by the coronavirus had risen dramatically outside of China by March, 2020, with 143 countries, states, or affected territories [5]. Coronaviruses are RNA viruses with a genome made up of a single stranded, positive–sense RNA with a size ranging from 26 to 31 kilobases. It is the largest of all RNA viruses in terms of morphology and genetics. The coronavirus genome is sequenced in the following order: 5′-leader–UTR–then replicate / transcriptase–spike (S)- Finally, the 3′UTR - poly (A) tail is attached to the envelope (E)-membrane (M)-nucleocapsid (N). It has multiple open reading frames (ORFs) 1a and 1b that overlap, and the other ORFs encode the main structural proteins spike, envelope, membrane, and nucleocapsid [6]. The Angiotensin-converting enzyme 2 (ACE2) receptors, which are present in various organs such as the heart, lungs, kidneys, and digestive system, are complementary in form to the spike shape, allowing effective attachment and facilitating the entry of viruses into the target cells. This binding occurs in the S protein domain of SARS-CoV-2 receptors, which is strongly linked to human ACE2. Following the entry and attachment processes, the viral membrane and the host cell fuse [7].

Materials and Methods

From a group of probable cases in which those infected with SARS–Cov2 virus Covid -19) taken the nasopharyngeal swab into viral transport Media (VTM) from 120 persons as a patients group. All samples were chickened by using Direct Immunofluorescences antibody procedure provided by (Viro - Cell Company) with few modification . The test was changed by eliminating the first component (1milliter of VTM containing a Nasopharyngeal swab). Then centrifuged for 5 minutes at 2.500 RPM, took the precipitate and folded it onto a special modified Fluorescence slide, allowing it to ear dry for 60 mintes. Then, for 30 minutes, in a humid chamber to prevent evaporation, apply unique (Viro –Cell Fluorescence antibody Conjugates (FITC) – polyclonal forms, IgM and IgG) and wash with Phosphate Buffer Saline (PBS – pH -7.2). Allow it to dray before looking at it under a fluorescence microscope to see the virus-infected cells. The second part of the VTM, which included a Nasopharyngeal swab, was used to detect viral nucleic acid using Real Time PCR, using Bioron–Real Time Kit after extracting samples using Biocomma (Espan)–RNA/DNA nucleic acid extraction Kits, to assess the existence of Corona –Virus as well as gene differentiation and allelic discrimination using a particular protocol provided by the manufacturer test for the existence of Corona Virus, as well as gene differentiation and allelic discrimination, using Bioron Kit on Bio-Rad Thermo cycler device. SPSS, ANOVA, and Chi–Square were used in the statistical analysis. The study was done at September 2020 up to April 2021 in merjan medical city , Molecular Biology deparment laboratory , PCR unit .

Results and Discussion

Using direct immunofluorescence to identify positive patients that are likely to have the SARS-CoV-2, which will then be checked using real-time PCR on nasopharyngeal samples. The figure (1) shows different images of viral infected cells after staining with fluorescent stain , at magnification power , low and high , as well as a negative image.The findings were similar to

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those of another study by Marra M.A. *et al*.; 2003, which found that Through sequential titration, IFA was able to produce quantitative results. Depending on the time of serum collection, the serological response was linked to the confirmed date of COVID-19 symptom appear [8]. The tests approximate sensitivity was determined using the assumption that particular IgM and IgG antibodies should be detected 10 days after the onset of the disease.. Previous research on antibody kinetics showed that anti-SARS-CoV-2 antibodies were rarely detected before 10 days. Anti-SARS-CoV-2 antibodies were detected using an in-house indirect immunofluorescence assay (IFA) [9].

As shown in the following figures-2 , qualitative Real Time PCR was used to confirm the positive result of immunofluorescence and to detect the different viral gene types (S, N, and E) as well as allelic discrimination.The amplification curve began at cycle 32 above CT (cycle threshold line) and the concentration of RFU (relative fluorescent unit) up to 700 Unit, as shown in figure (2-A). This finding suggested that the patients were infected with SARS-CoV-2 at S-gene level; presence of S-gene may be linked to the presence of other SARS-CoV-2 genes such as N and E genes. Figure 2 -B shows that the amplification curve began at cycle 32 above CT and reached a maximum concentration of RFU of 2000 units. This meant that the patients had corona infection at E-gene stage. There is also an amplification curve that starts at cycle 32 above CT and goes up to 2100 Units of the N-gene stage, as shown in figure (2–C) . Figure (2 –D) shows the internal control level at cycle no.34 above CT with RFU up to 1000 Unit. Internal control was used to assess the content consistency, amplification, and dendity of viral nucleic acid at a semi quantitative level.

The frequency and percentage of each studied gene are shown in the graphs below.In contrast to nuclear (N –gene) marked as (circles) in which that have (43 : 96 = 44.8%), heterozygote of (S and N genes) marked as (triangle) have (4: 96= 4.2%), while spike (s) gene (marked as square) have (33: 96= 34.4%). The low heterogeneity of (SN genes) can mean that the low S gene in relation to N genes may indicate infection chronicity or that the patients have had the infection for at least two weeks. The N gene is more preserved and stable, with 90 percent amino acid homology and fewer mutations across time. (8,9). Many coronavirus N proteins are highly immunogenic and are abundantly expressed during infection [10]. In a vaccine setting, the N protein serves as a model antigen for the T-cell response, inducing SARS-specific T-cell multiplication and cytotoxicity. [11,12]. High levels of IgG antibodies against virus have been found in sera from SARS patients [13]. When compared to the genetic sequence of the original SARS-CoV viral strain from Wuhan, early findings by Cong Y. *et al*.; 2020 from genetic sequence analysis revealed the existence of many mutations in SARS-CoV-2 [14]. In addition, new strains with higher virulence than the Chinese SARS-CoV-2 isolates have been reported. A point mutation in D614G induces changes in viral spike glycoprotein, making it more resistant to cleavage during viral replication, resulting in these newly described strains [15].

Figure (4) shows that the heterozygote of (S and E genes) marked as (triangle) has (46: 96= 47.9%), while spike (s) gene (marked as circles) has (15: 96= 15.6%), in contrast to Envelope (E –gene) marked as (square) which has (33: 96= 34.4%). The high heterogeneity of (SE genes) may indicate that the high S gene in comparison to E genes may indicate an acute infection or that the patients are infected in the first week. The ongoing rapid dissemination and global spread of SARS-CoV-2 has raised serious concerns about the viral population's evolution and adaptation as it encounters diverse host immune systems and counter-measures as it spreads across the world, powered by mutations, deletions and/or recombination [16]. Several studies by Okada M. *et al*.; 2005 and Korber B. *et al*.; 2020 indicate that viral proteins, especially the S protein, can adapt at the nucleotide, amino acid (aa), and structural levels [17,18]. Intriguingly, Shen *et al*. discovered intra-host viral evolution in patients after infection, which may be linked

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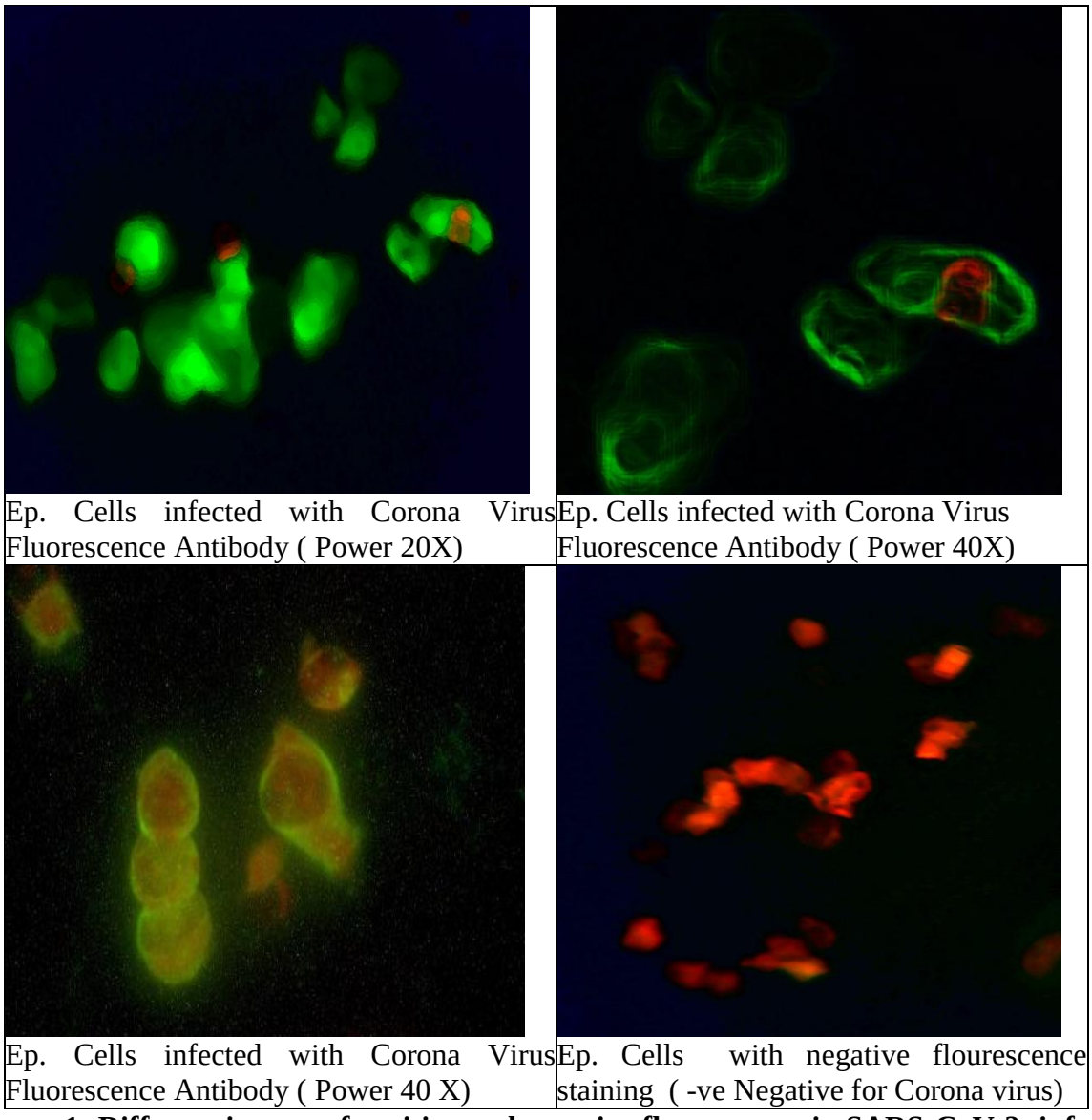
to the viral virulence, transmissibility and / or evolution as a result of the immune response [19]. Sardar R. *et al*.; 2020 discovered a rare mutation in the S protein (A930V) in Indian SARS-CoV-2 strains that was not seen in other similar strains from different geographical regions [20].

Figure (5) shows that the heterozygote of (N and E genes) marked as (triangle) has (46: 96= 47.9%), while nuclear (N) gene (marked as circles) has (26: 96= 27.1%), while Envelope (E – gene) marked as (square) has (22: 96= 22.9%). The high heterogeneity of (NE genes) could mean that the patients have been infected for longer than 2 weeks. According to one study by Armijos-Jaramillo V. *et al*.; 2020, a combined model for disease susceptibility with a number of common specific genes that reflect the preferred context in which additional private mutations may decide disease progression has been created [21]. Some of the genes were linked to TLR pathways, while others were linked to C-lectin pathways and inflammasome activation (cytokine storm). Also indicative of the help in explain of severe COVID-19 that should be included in gene panels and during meta-analysis of various variants in COVID-19 host genetics studies [22,23].



Ep. Cells infected with Corona Virus Fluorescence Antibody (Power 20X)

Ep. Cells infected with Corona Virus Fluorescence Antibody (Power 40X)

Ep. Cells infected with Corona Virus Fluorescence Antibody (Power 40 X)

Ep. Cells with negative flourescence staining (-ve Negative for Corona virus)

Figure - 1: Different images of positive and negative fluorescence in SARS-CoV-2 -infected cells.

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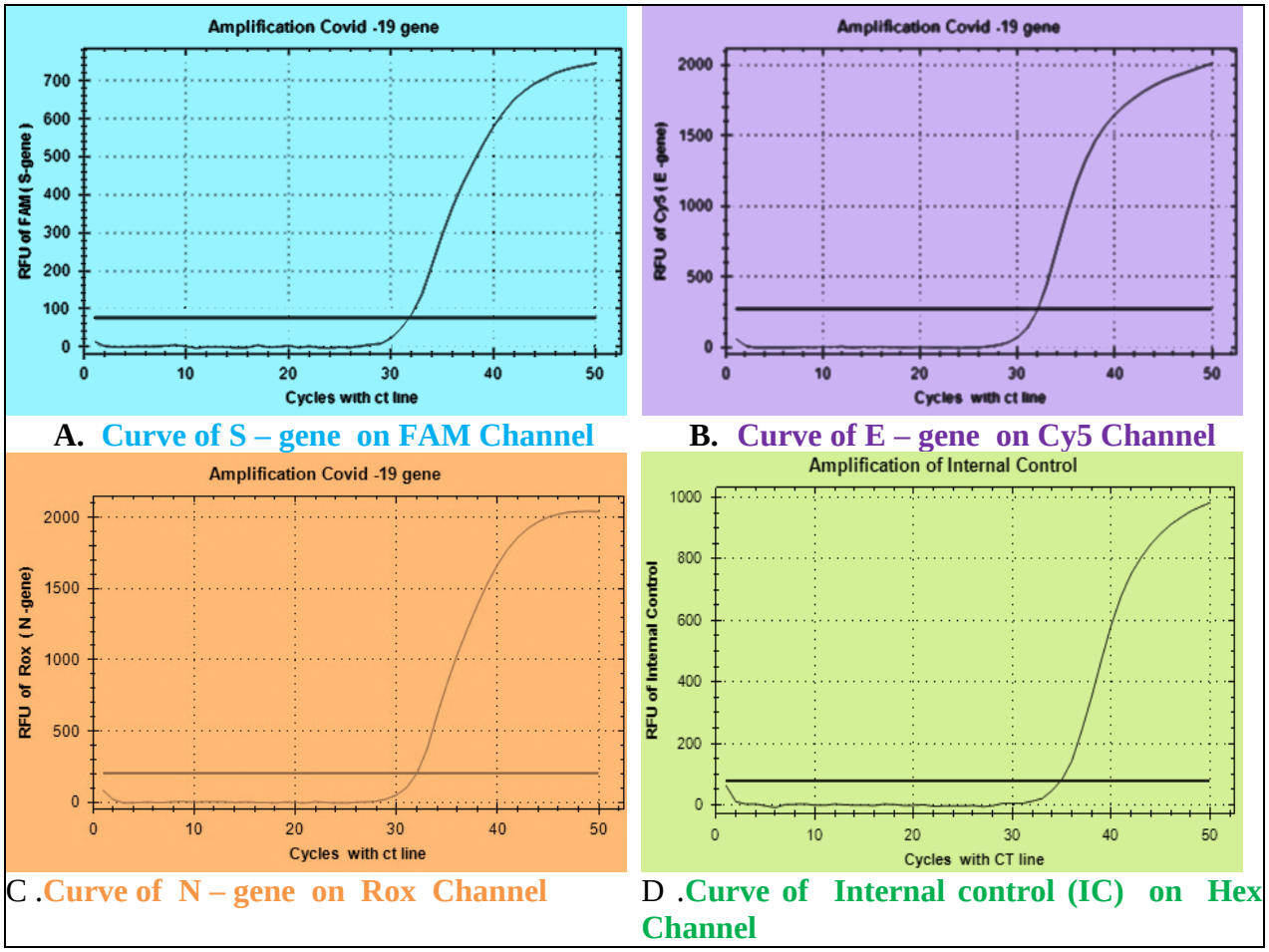

Figure 2: Real-time PCR amplification of Covid-19 genes with an internal monitor.

Heterogeneity of S and N Gene		Mark shape	Frequency	Percent	Chi-Square P. Value
Genes	Homozygote of S Allele	Square	33	34.4	37.750
	Homozygote of N Allele	Circle	43	44.8	0.000
	Heterozygote of (SN)	Triangle	4	4.2	
	Undetermined	X-shape	16	16.7	
	Total		96	100.0	

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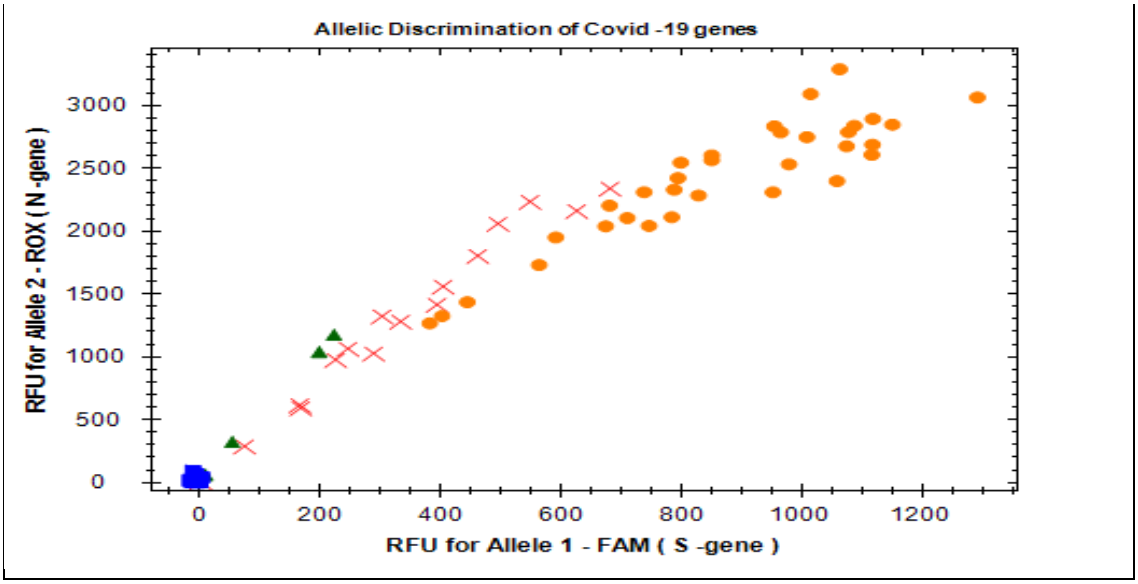

Figure - 3: Allelic Discrimination of Covid -19 - S and N genes Heterogeneity.

SE -Gene		Mark shape	Frequency	Percent	Chi-Square P. Value
Valid	Homozygote of S Allele	Circle	15	15.6	47.083
	Homozygote of E Allele	Square	33	34.4	0.000
	Heterozygote of (SE)	Triangle	46	47.9	
	Undetermined	X-shape	2	2.1	
	Total		96	100.0	

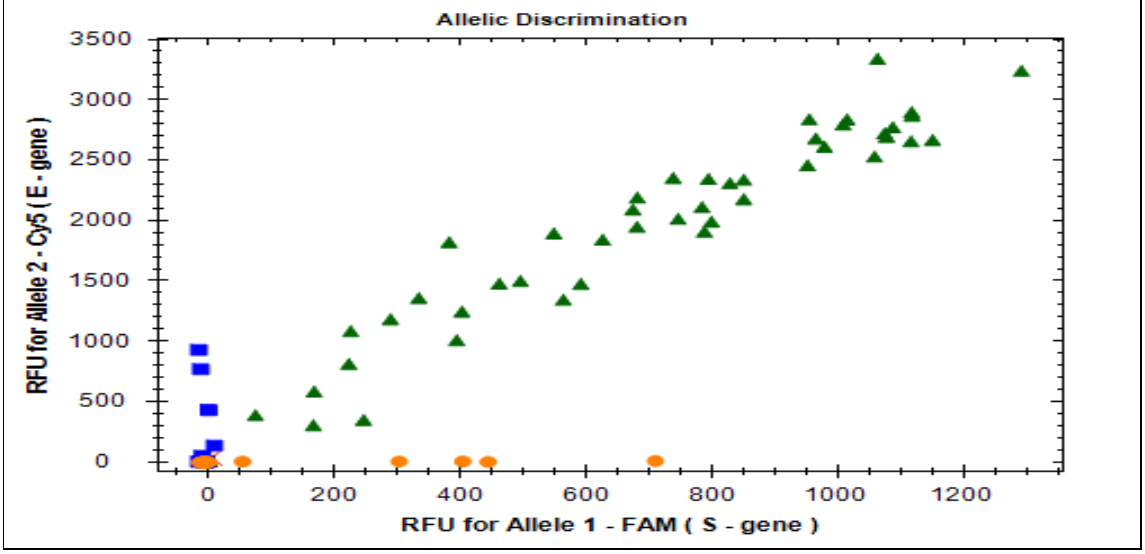

Figure 4: Allelic Discrimination of Covid -19 - S and E genes Heterogeneity.

NE-Gene	Mark shape	Frequency	Percent	Chi-Square P. Value

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Valid	Homozygote of N Allele	Circle	26	27.1	40.667
	Homozygote of E Allele	Square	22	22.9	0.000
	Heterozygote of (NE)	Triangle	46	47.9	
	Undetermined	X-shape	2	2.1	
	Total		96	100.0	

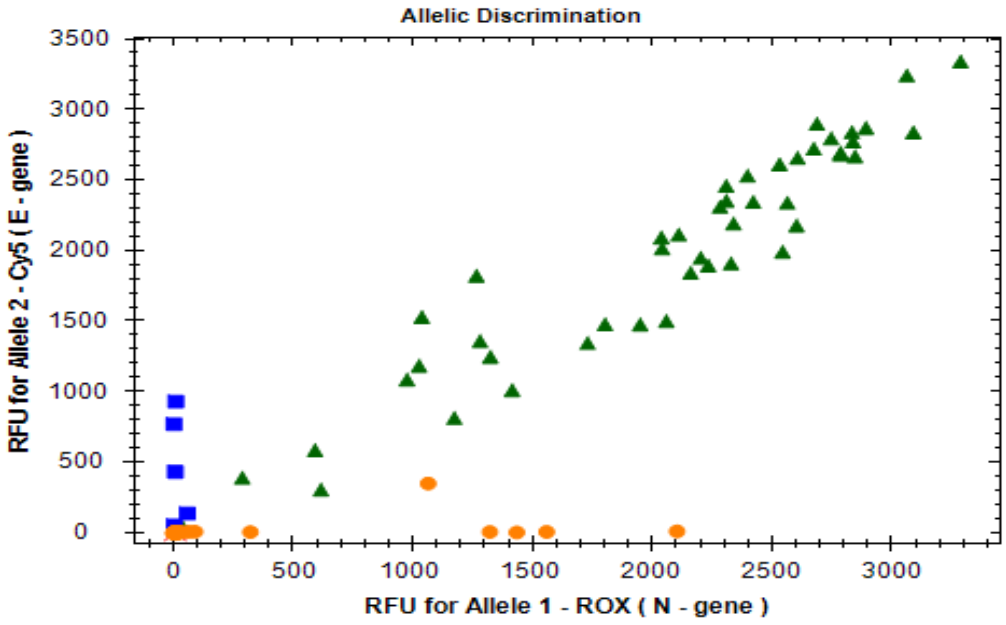

Figure 5: Allelic Discrimination of Covid -19 - N and E genes Heterogeneity.

Conclusion

The development of an in-house direct immunofluorescence assay (DIFA) to diagnoses SARS-CoV-2 antigen on nasopharyngeal swab and confirm positive patients with corona virus using real-time PCR to reduce the cost of PCR materials, which are expensive and supplement difficulties . SARS-CoV-2 gene differentiation with allelic discrimination by real-time PCR, with the presence of S gene mainly in the first day of infection, E gene present in the end of infection over 1 -2 weeks after appear of infection, with lesser extent of N gene .

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