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Detection of SARS-CoV-2 Reinfections by Rapid Inexpensive Methods

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***Abstract*—New SARS-CoV-2 infections are difficult to be verified, whether they are reinfections or persistent infections. The most prominent factors used for differentiating reinfections from persistent infections are whole-genome sequencing and phylogenetic analyses that require time and funds, which may not be feasible in most developing countries. This study explores reinfections with COVID-19 that harbors D614G and N501Y mutations by rapid inexpensive methods. It exploits the previously developed rapid economic methods that identified both D614G and N501Y mutations in clinical samples using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) probes and conventional PCR specific primers. In the present study, an immunocompetent patient has been found with a SARS-CoV-2 N501Y reinfection without comorbidities. According to the obtained results, this study suggests that the initial infection was due to a variant that contained only D614G mutation whereas the reinfection was potentially a result of alpha variant contained three mutations confirmed by DNA sequencing, including D614G, N501Y, and A570D mutations. These techniques will support rapid detection of SARS-CoV-2 reinfections through the identification of common spike mutations in the developing countries where sequencing tools are unavailable. Furthermore, seven cases of reinfections were also confirmed by these methods. These rapid methods can also be applied to large samples of reinfections that may increase our understanding epidemiology of the pandemic.**

***Index Terms*—Alpha variant, Iraq, Reinfections, SARS-CoV-2.**

I. Introduction

SARS-CoV-2 reinfections occur as a result of declining antibodies in convalescent people (Qureshi, *et al.*, 2021). However, a recent study found that reinfection occurs in an immunocompetent person who had neutralizing antibodies produced from the initial infections due to the patients moderate immune response (Brehm, *et al.*, 2021). The infections generally persist in immunosuppressed patients but the reinfections occur in immunocompetent individuals in regions where different variants are circulating (Choudhary, *et al.*, 2021). Immunosuppressed can be defined as the loss or deficiency in the quality of humoral or cellular immune components (CDC, 2022). These researchers concluded that in immunosuppressed individuals, accumulation of mutations is linked to viral evolution (Weigang, *et al.*, 2021). This is often perplexing to differentiate between reinfections and persistent infections. The reinfections can be differentiated from persistent infections by identifications of the viral variants in the clinical samples taken from both first and second infections. Viral variants are usually identified by phylogenetic analysis of the viral whole-genome sequences which are difficult to obtain as quickly as possible for both initial and second infections (Stokel-Walker, 2021). In other words, confirmations of reinfections have been under investigation. CDC recommends several criteria for suspecting SARS-CoV-2 reinfections including collecting respiratory samples daily for 7 days and serum samples in different times points on days 3, 7, 14, 21, and 60 (CDC, 2020). Therefore, alternative, rapid, molecular biological methods can also be exploited for variant identifications using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) probes, particularly when whole-genome sequencings are not available in poor or developing countries. Several methods have recently been developed (Al-Jaf and Niranji, 2021; Al-Jaf, *et al*., 2021; Banada, *et al.*, 2021; Durner *et al.*, 2021; Sandoval Torrientes, *et al.*, 2021). These methods, such as, specific primers, TaqMan probe-based method, and melting curve analysis, are more inexpensive and feasible than whole-genome sequencing.

Before emergence of SARS-CoV-2 variants of concerns (VOCs) such as B.1.1.7, B.1.351, P1, and B.1.617, reinfections were seldomly reported in the world. For example, reinfection with SARS-CoV-2 has first reported in a person from Hong Kong who migrated to Europe that was confirmed to be caused by a different lineage of the virus (Parry, 2020). Later on, several cases of reinfections have been reported worldwide (Choudhary, *et al.*, 2021). However, the variants of most of these reinfections were not confirmed by genomic sequencings; this makes reporting reinfections controversial and confusing with persistent

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infections (Costa, *et al.*, 2021). In other words, the majority of the global SARS-CoV-2 reinfections may have been a persistent infection not even reinfections (Simmonds, *et al*., 2021). This highlights the importance of reinfection confirmation and persistent infection exclusions using either whole-genome sequencings or rapid techniques to identify the virus's variants. Meanwhile, false-negative tests should be considered to avoid prolonged viral shedding from the patient (Falahi and Kenarkoohi, 2020) and emergence of new variants.

SARS-CoV-2 has several major VOC, which have been known to influence on the transmissibility, infectivity, and fatality of the virus, including B.1.1.7 (alpha variant) (Challen, *et al.*, 2021; Davies, *et al.*, 2021), B.1.351 (beta variant), P.1 (gamma variant), and B.1.617 (delta variant). Up to our best knowledge, there are few case reports of reinfections in Iraq (Hussein, *et al.*, 2020, 2021). Nonetheless, no studies have confirmed reinfection using DNA sequencings, particularly with the alpha variant and few reports in the world. This was possibly due to difficulties in comparing the whole-genome sequencings between the initial infections and reinfections. In this study, we aim to investigate reinfections in eight individuals reported in Kalar town, Sulaymaniyah Province, Kurdistan regional government of Iraq, where sequencing facilities are hardly obtained. We also aim to apply the previous rapid methods to confirm reinfections with variants carrying N501Y mutations that have occurred in the region.

II. Materials and Methods

Seventy-eight out of 255 individuals who visited Coronavirus Research and Identification Lab, University of Garmian, Kurdistan Region, Iraq, were positive for COVID-19 rRT-PCR test ((MutaPLEX® Immundiagnostik, Germany). Eleven persons revisited the laboratory, after 5–10 months, having COVID-19 symptoms, but only eight of them were tested positive. Only the patients were included in the current study that has their tests negative after 2 weeks of first infection. All positive samples were tested for D614G and N501Y mutations using the methods that described previously by Al-Jaf and Niranji, 2021; Al-Jaf, *et al*., 2021.

Nasopharyngeal samples were tested by rRT-PCR kits (MutaPLEX® Immundiagnostik, Germany). The positive samples were tested for mutations from both initial infections and reinfections. Specific primers method was applied for identification of both N501Y and D614G that can detect both mutations utilizing conventional PCR and electrophoresis. rRT PCR method was applied for the identification of both N501Y and D614G mutations (TaqMan probes designed to detect both N501Y and D614G mutations). Spike 748 primers were used to amplify a 748 nucleotides region of the spike protein. The PCR products from this region were sequenced to detect the common mutations that occurred in the VOCs including (K417N, L452R, T478K, E484K, N501Y, A570D, and D614G). These methods were previously described by Al-Jaf and Niranji, 2021; Al-Jaf, *et al*., 2021.

Initially, a person was presented to the Coronavirus Research and Identification Lab, University of Garmian, Kurdistan Region, Iraq. He agreed to fill a consent form as a participant of this study, which was approved by an ethical committee at the Department of Biology, University of Garmian (Ethical approval code: 00087, October 01, 2020) that follows the rules adhered to the Declaration of Helsinki for human and animal research. He was 42 years old, his body weight was 70, height = 170 cm. A nasopharyngeal swab was taken in viral transport medium (VTM) on October 7, 2020. The initial infection was tested for diagnosis as SARS-CoV-2 using a coronavirus real-time RT-PCR kit (MutaPLEX® Immundiagnostik, Germany). Blood tests were performed to observe complete blood counts (CBC), ferritin, D-dimer, LDH, CRP, and ESR. The patient had no comorbidities without receiving vaccination. The reinfection was diagnosed on March 23, 2021 (after 5.5 months), using the same protocol as the initial infection.

Identifications of SARS-CoV-2 N501Y and D614G mutations were performed for both initial infection and reinfection, using rapid molecular biological methods (TaqMan probes, Macrogen, South Korea) as previously developed (Al-Jaf and Niranji, 2021; Al-Jaf, *et al*., 2021) and illustrated in Fig. 1. In addition, a pair of primers (F-AGAGGTGATGAAGTCAGACAAAT) and (R- CTATTAAACAGCCTGCACGT) amplifying a region of 748 nucleotides (22768-23516) that cover common mutations of VOC (including K417N, L452R, T478K, E484K, N501Y, A570D, and D614G) in the spike protein gene of SARS-CoV-2 were used (Al-Jaf and Niranji, 2021) and the PCR products were confirmed by DNA sequencings (Sanger sequencing, Macrogen Co., Seoul, KR). ELISA test for SARS-CoV-2 IgG and IgM (Ideal Tashkhis Atieh Co., Iran) was performed a day post reinfection to reveal antibody state of the patient against the virus. The rapid molecular methods were also applied to seven persons suspected of either reinfections or persistent infections. They agreed to fill consent forms and were approved by the ethical committee as previously mentioned.

III. Results

A. Initial Infection

The clinical features were as follows: Severe sore throat with excessive cough for 2 weeks that were initially dry but wet and purulent at the end. Sneezing, malaise, fatigue, and diarrhea were also present. Oxygenation saturation was normal as indicated by normal SPO_2 more than 96%. The results of the rapid methods showed that the initial infection resulted from a SARS-CoV-2 variant, which has had N501 wild type and D614G mutant. The Ct value of 17.14 was obtained. The sequencing result also confirmed amino acids N501 wild type, A570 wild type, and D614G mutant in the

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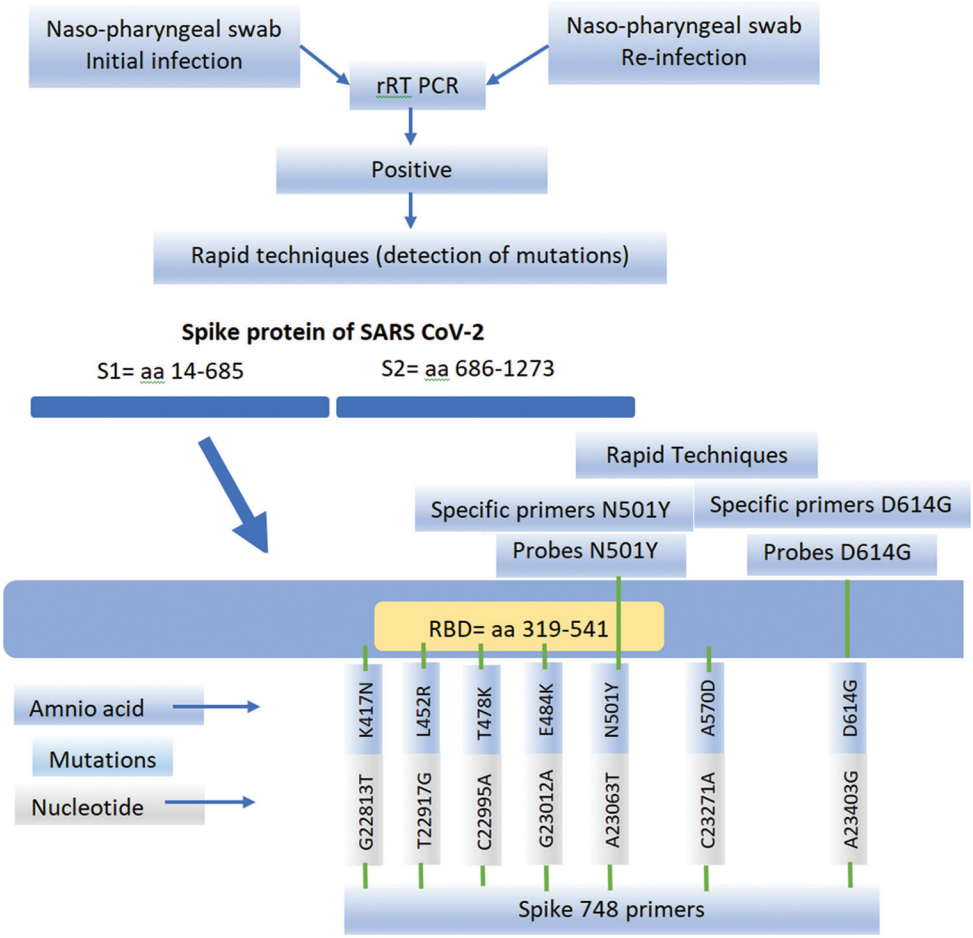

Fig. 1. Rapid detection of reinfections by identifying spike mutations of variants of concerns. S1: Spike 1, S2: Spike 2, RBD: Receptor-binding domain of the spike protein, WT: Wild type, MT: Mutant, CT: Threshold cycle for rRT-PCR.

initial variant (MW897351). Treatment was Vitamin D, zinc, Vitamin C, paracetamol, and azithromycin for 10 days. PCR result was negative after 2 weeks. Another PCR test was performed 4 months later.

B. Reinfection

The clinical features were as follows: Moderate sore throat with mild coughs for 10 days. Dry mouth was much more than the initial infection. Malaise and fatigue were less prominent than the previous infection, and the patient had no diarrhea. He had normal SPO_2. The rapid methods and sequencing result (MW897356) found two amino acid changes N501Y and A570D, in addition to the D614G which was also present in the initial infection. The presence of Y501, D570, and G614 mutations in the reinfection suggested alpha variant. Other parameters are as follows: Ct value = 17.9, normal CBC, ferritin, D-dimer, LDH, and ESR, but CRP was 15 mg/dl (normal CRP titer <5 mg/dl). Treatment was Vitamin D, zinc, Vitamin C, paracetamol, and azithromycin for 7 days. The patient recovered with negative PCR after 2 weeks with no clinical signs. Both IgG and IgM were considered as negative results with very low titers, 0.28 and 0.182, respectively (antibody titer above 1 is considered as positive).

C. Application of the Rapid Molecular Biological Methods on Other Reinfections

Seven persons, who were previously positive for SARS-CoV-2 from June to September 2020 where no N501Y mutations were present in the region, were tested for reinfections by the same molecular methods as previously mentioned. Results of the rapid methods showed that all the eight persons were reinfected with a variant having both D614G and N501Y mutations, as shown in Table I. The patients' demographic and clinical information are shown in Table II. The results revealed that one out of eight reinfections carried the wild-type N501 variant (Case No. 3); these were confirmed by DNA sequencings. Whereas other 7 persons were re-infected with N501Y mutated variants. This mutation is present in the Alpha, Beta and Gamma variants of COVID-19.

IV. Discussion

This study has reported SARS-CoV-2 reinfections using rapid inexpensive techniques that can be used to discriminate between reinfections and persistent infections. The reinfections occurred in different ages ranging between 26 and 55 years, with either having comorbidities or not. In

addition, the manifestation and the severity of the symptoms were variable among different individuals and between the initial and second infections in the same patient. The first case reported in this study was confirmed by the rapid tests and DNA sequencings in the Kurdistan Region of Iraq. In addition, these rapid methods, which can detect both D614G and N501Y mutations, were applied to eight clinical samples. Out of eight persons, one (Case No. 3, Table II) was suspected to be either reinfected or suffered from persistent infections because she carried only D614G but not N510Y mutations in both initial and second infections as confirmed by DNA sequencings too. However, she seemed to be reinfected rather than persistent infection as the time between both infections was more than 6 months (Stokel-Walker, 2021). The other seven individuals carried both D614G and N501Y mutations; the latter was not present in the first infection that suggested reinfections. The reinfected patients include different ages with or without comorbidities suggested that reinfections with alpha variant may occur in various ages with or without comorbidities. The comorbidities were obesity, hypertension, diabetes, and asthma.

SARS-CoV-2 reinfections were previously rare in the world as in a study conducted in approximately 9 thousands of positive samples in the USA from December 2019 to November 2020, only 63 samples (0.7%) were reported as reinfections, which were linked with low antibody responses in the initial infection (Qureshi, *et al.*, 2021). However, recent research has reported 58 out of 1300 suspected reinfections among about 700,000 positive individuals in India (Mukherjee, *et al.*, 2021). Likewise, 138 out of 28,875 positive cases were reported as reinfections in Denmark (Hansen, *et al.*, 2021). An ecological research conducted in the UK concluded no evidence of increasing rate of reinfections with the alpha variant (B.1.1.17) which were confirmed by whole-genome sequencings (Graham, *et al.*, 2021). During the writing of this manuscript, several cases of SARS-CoV-2 reinfections were reported in the USA, Italy, Columbia, Brazil, and Luxemburg, in which their second infections were due to B.1.1.7, B.1.1.7, B.1.1.269, P1, and B.1.351 variants (Marquez, *et al.*, 2021; Novazzi, *et al.*, 2021; Ramírez, *et al.*, 2021; Staub, *et al.*, 2021).

For how long antibodies persisted in previously infected persons is uncertain. However, studies suggested that antibodies may remain for approximately 6 months (Stokel-Walker, 2021). The persistence of antibodies varies from an individual to another or it depends on the severity of the disease or the type of the variants. The antibody status of the first case was performed a day of the reinfection indicated that his adaptive immunity had no response yet and results showed that both IgG and IgM were negative. This suggested that the reinfected person has had no protective antibodies to prevent the reinfection. Limitations of the present study were lack of checking antibodies in all cases. Despite the small number of samples, this study reported reinfections with SARS-CoV-2 N501Y mutant.

Table I
SARS-CoV-2 Mutations in Initial and Reinfection Confirmed by the Rapid Methods and Sequencing

Cases	Initial infections		Reinfections	
	Mutations	Date	Mutations	Date
1	G614, A570 and N501 MW897351	07/10/2020	G614, D570, and Y501 MW897356	23/03/2021
2	G614 and N501	10/09/2020	G614 and Y501	22/04/2021
3	G614 and N501 sequencings: MW897353.1	10/07/2020.	G614 and N501 sequencings: MW897354	10/02/2021
4	G614 and N501	10/07/2020	G614 and Y501	24/04/2021
5	G614 and N501	10/07/2020	G614 and Y501	24/04/2021
6	G614 and N501	25/06/2020	G614 and Y501	26/04/2021
7	G614 and N501	10/07/2020	G614 and Y501	26/04/2021
8	G614 and N501	10/07/2020	G614 and Y501	26/04/2021

Table 2
Demographics and Clinical Information of the Patients in Both Initial Infection and Reinfections

Cases	Demography		Comorbidities	Clinical manifestations	
	Age	Sex		Initial infections	Reinfections
1	42	M	None	Severe sore throat, excessive cough, sneezing, malaise, fatigue, diarrhea, normal SPO_2 more than 96%	Moderate sore throat, mild coughs, dry mouth, malaise, fatigue. Normal CBC, ferritin, D-dimer, LDH and ESR, CRP (15 mg/dl). Negative IgG and IgM
2	43	M	None	Asymptomatic with normal CBC and acute phase parameters	Severe dry cough, malaise for 3 weeks. Loss of taste and smell
3	35	F	Obese	Tiredness, fever, headache, and loss of taste and smell	Tiredness, fever, and headache for 4 days
4	55	M	Hypertension, diabetes, obesity	Malaise, fever	Malaise, hypoglycemia, and diarrhea
5	26	F	None	Fever, tiredness, sore throat, headache, loss of taste and smell	Fever, tiredness, sore throat, headache, loss of taste and smell
6	41	M	Obese	Myalgia, fever, headache, loss of appetite. High platelet and WBC count	Tiredness and headache
7	54	F	None	Dry cough, myalgia, fever, headache, loss of appetite	Dry cough, tiredness, and fever
8	34	F	Asthma	Malaise, sore throat, headache	Malaise, sore throat, headache

V. Conclusion

This research explores SARS-CoV-2 reinfections using rapid low-cost methods and reported first reinfections with a SARS-CoV-2 N501Y mutant variant in the Kurdistan region of Iraq. Further study is required to apply these methods in a large number of samples. This will open our understandings of the epidemiology and reinfections of the virus.

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