Outbreak of SARS-CoV-2 variants in Iraqi Kurdistan region

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Abstract

Introduction: Coronavirus disease 2019 (COVID-19) first appeared in Iraq, including the Iraqi Kurdistan region governorates, in March 2020.
Methodology: 48,494 samples were collected from public hospitals in the Kurdish governates from February 2021 to May 2022. Viral RNA was extracted, and real time quantitative polymerase chain reaction (RT-PCR) was used to detect the COVID-19 variants. Statistical analysis of patients' clinical data was performed.
Results: The RT-PCR results identified the Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) variants in the Kurdistan governorates. Young adults (20-39 years) had significantly higher rate of infection than children (1-11 months) and older adults (80-89 years). The Delta wave was more contiguous, spread more easily, and more fatal than the Alpha and Omicron waves. The highest number of COVID-19 cases was reported in Sulaymaniyah and Duhok; and the highest death rate was reported in Sulaymaniyah. The death rate in males was higher than in females, especially among older people. Fatigue, cough, and fever were common symptoms among the three variants. The phylogenetic tree revealed that the L-type of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was a descendant of the S-type at an early stage of evolution. The L-type could spread faster in Kurdistan. Multiple sequence alignment (MSA) confirmed that all L-type variants in different countries were 100% similar in sequence, and all were mutated in the regions 8782: ORF1ab and 28144: ORF8 703.
Conclusions: This study described the COVID-19 waves, pathogenesis, and evolution of the virus in the Iraqi Kurdistan region.

Key words: COVID-19 outbreaks; Alpha (B.1.1.7); Delta (B.1.617.2); Omicron (B.1.1.529); Iraqi Kurdistan Region; phylogenetic analysis.

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Introduction

Coronaviruses are a large group of pathogens known to target the human respiratory system. The single-stranded RNA viruses are positive-sense and possess envelopes. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent for coronavirus disease 2019 (COVID-19), is a member of the coronavirus group [1]. COVID-19 was first identified in late December 2019 in Wuhan, the capital of Hubei, China [2,3]. SARS-CoV-2 outbreaks spread globally within a few months, reaching pandemic proportions [4-6]. The ability of SARS-CoV-2 to spread has resulted in devastating human casualties and economic damage all over the world. Clinically, COVID-19 can be an asymptomatic disease or one with self-limiting fever, fatigue, rhinorrhea, sore throat, muscle and joint pain. The disease can also progress to coughing, hemoptysis, hypoxia, chest discomfort, or chest pain; possibly leading to respiratory failure or multiple organ failure [7,8]. Since its identification, SARS-CoV-2 has had numerous variants defined by lineages showing distinctive mutations in the spike protein [9]. A variant of concern (VOC) is associated with increased transmission rate, notable reduction of neutralization via antibodies produced during prior infections or vaccination, and evasiveness to treatment and diagnostic detection methods.

As of January 2022, the Kurdistan Regional Government (KRG) of Iraq has had four distinct waves of COVID-19 as reported by the local news channel (Rudaw). The first case of COVID-19 in Kurdistan was reported on March 1, 2020 [10,11]. The appearance of the first case coincided with the beginning of the first wave of COVID-19 [12]. Genomic sequences analyzed from patients during the first wave revealed that the variant was evolutionarily related to the Iranian Alpha variant (B.1.1.7) [13]. From an epidemiological point of view, the severity and mortality of the disease were lower when compared to other countries around the world. The first wave lasted till May 2020, after which

a subsequent wave followed where the Alpha variant was the causative agent [12,14]. In July 2021, the Delta variant (B.1.617.2) emerged in Kurdistan, thereby prompting a third wave, and it became the dominant variant of concern at that time. The earliest detection of the Omicron variant (B.1.1.529) in Duhok, KRG, was in late December 2021, which catalyzed the onset of the most recent wave in the region [15]. Preliminary data showed that the hospitalization rate was significantly higher during the dominance of the Delta variant, probably due to the high infectivity rate compared to its predecessors [16].

The European Centre for Disease Prevention and Control (ECDC) has classified SARS-CoV-2 variants into three categories: variant under monitoring (VUM), variant of interest (VOI), and variant of concern (VOC); based on the importance and/or threat of a new or emerging variant. The classification depends on criteria such as genetic change, transmissibility, immunity, and infection severity which are provided by the ECDC. Compared to the previous variants, Omicron, which is still listed as a VOI, had a higher infectivity rate according to ECDC; however, it produced less severe symptoms, and the vaccines were less effective against the variant, thereby necessitating the use of boosters [17]. Our study aimed to show the effects of the SARS-CoV-2 variants outbreaks in the four governates that comprise Iraqi Kurdistan: Erbil, Sulaymaniyah, Duhok, and Halabja; using clinical data, and to demonstrate the detailed evolution of SARS-CoV-2 in the region.

Methodology

Sample collection

Oro-nasopharyngeal swabs were collected from 48,494 patients in multiple public hospitals in the Kurdish governates as follows: Erbil (Hospitals of Rizgari, Peshmarga, Central Emergency, and Attaya Emirati), Sulaymaniyah (Hospitals of Sarchnar, and Ali Naji), Dohuk (Burn and Plastic Surgery Hospital), and Halabja (hospitals of Wafa and COVID-19). The samples were collected in the period between February 2021 to May 2022. Aside from the swabs, a questionnaire was used by the assisting healthcare professionals to collect further data about the clinical features of the patients.

Viral RNA extraction

The diagnostic laboratories at the hospitals mentioned above were used to diagnose SARS-CoV-2, and the test was standardized by the Ministry of Health of the KRG. The test consisted of extracting viral RNA from the samples using a QIAampViral RNA Mini kit (Cat. No. 52904 or 52906; QIAGEN, Hilden, Germany). Then, the RNA was reverse-transcribed to complementary DNA (cDNA), as described in the cDNA synthesis kits (QIAGEN, Hilden, Germany). The expression level was then measured via the real-time quantitative polymerase chain reaction (RT-PCR) machines (QIAGEN, Hilden, Germany). The region's health authorities confirmed that the COVID-19 testing kits were mainly provided by the World Health Organization (WHO), Germany, the People's Republic of China, and the U.S.A.

SARS-CoV-2 detection by RT-PCR

In Iraq, WHO provided primer and probe sequences for RT-PCR as shown in Supplementary Table 1 [18]. The manufacturer's instructions were followed using the AgPath-ID one-step RT-PCR reagents (Cat. No. AM1005, Applied Biosystems, Foster City, CA, USA). The viral RNA sample (5 µL) was added to the RT-PCR reagents, and the *RdRp* or *E* gene primers (1 µL, 10 pmol) and probe (0.5 µL, 10 pmol). Carboxyrhodamine (ROX) was employed as a passive reference dye. The temperature and duration of each cycle of RT-PCR are described in Supplementary Table 2.

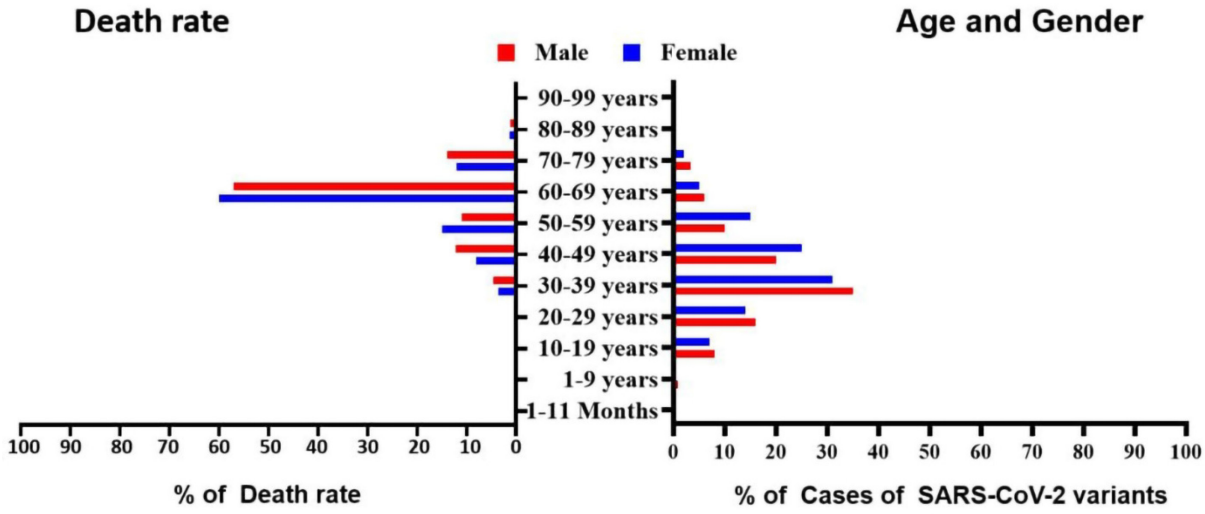
Statistical analysis

The clinical data were analyzed and visualized using GraphPad Prism (Version 8.0.1). This software also analyzed the results of RT-PCR to determine whether the sample was positive or negative for COVID-19. In this research, 48,494 individuals were followed up; 1495 were not hospitalized, and 47,000 were hospitalized patients. For the RT-PCR, the cycle threshold (Ct) value of the SARS-CoV-2 target gene was determined using the Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA).

Phylogenetic analysis for SARS-CoV-2 variants

A complete genome of SARS-CoV-2 was sequenced by Sangar sequencing at the Immunogene Center in Erbil. The whole genome sequence is available in the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis was then performed to understand the evolutionary relationship between the SARS-CoV-2 variants found in different countries. The primary local alignment sequence (BLAST) tool was applied to identify 41 SARS-CoV-2 nucleotide sequences from other countries. The sequences for phylogenetic analysis were collected from BLAST NCBI, and the sequence was submitted to the GISAID database, accession no. MT447177 as a query sequence to identify 41 similar

Figure 1. Percentage of coronavirus disease 2019 (COVID-19) infected cases and death rate in Iraqi Kurdistan governates by age and gender.



SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

sequences. MUSCLE alignment and maximum likelihood tools of the Molecular Evolutionary Genetic Analysis (MEGA) software, version 7, were used to create the maximum likelihood tree (ML tree) and multiple sequence alignment (MSA) [19,20]. The sequence analysis was performed to identify mutations in *Open Reading Frame ab* (*ORF1ab*) and *ORF8* sequences.

Phylogenetic analysis for SARS-CoV-2 and other coronaviruses

Phylogenetic analysis was performed to identify the evolutionary relationship between the SARS-CoV-2 and other viral species. Basic local alignment search tool (BLAST) was used to collect 41 sequences to reconstruct an evolutionary phylogenetic tree. Then, MUSCLE alignment and maximum likelihood tools of MEGA7 software were used to create the MSA and maximum likelihood tree (ML tree), respectively [19,20].

Ethical approval

Ethics approval was obtained from the Ethics Committee of Salahalddin University-Erbil (4e/198) since it involved human participants. The authors ensured that this study had been carried out following the ethics code of the World Medical Association (Declaration of Helsinki of 1964) for human experiments. All participants provided informed consent before participating in the present study.

Results

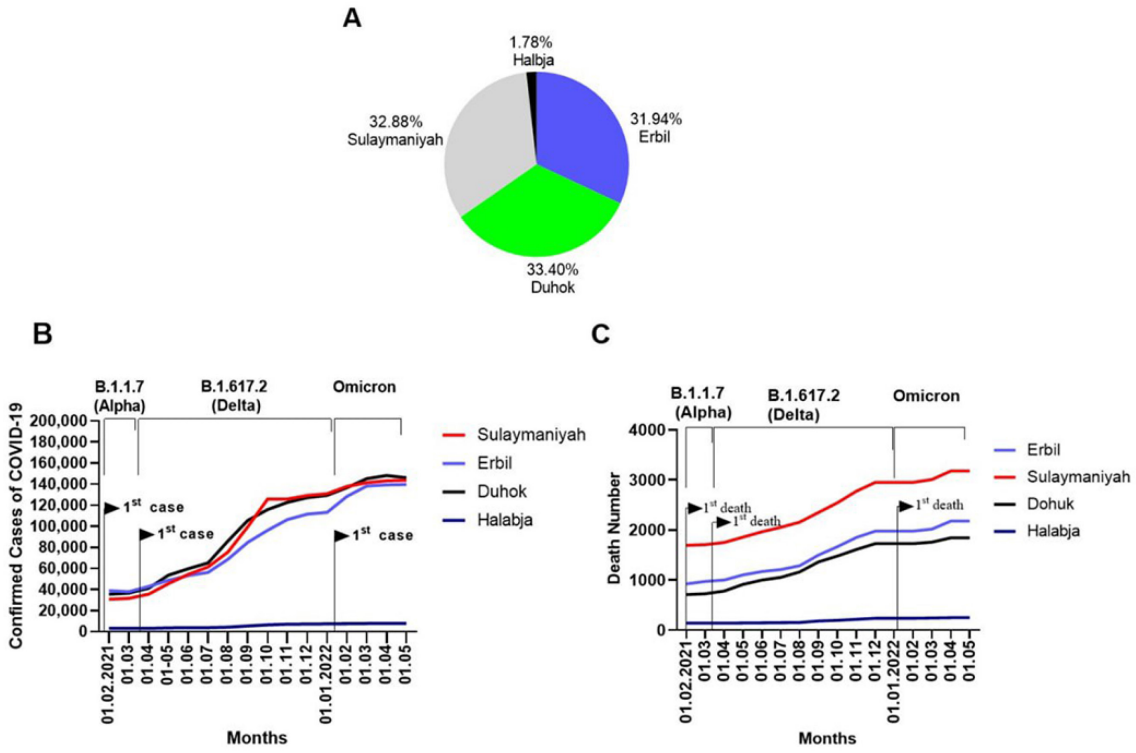
Age and gender

SARS-CoV-2 can infect men and women of all ages. In our study, males of ages 10-39 and 60-79 years were infected at a higher rate than females in all four Kurdistan governorates. However, the infection rate in females between 40-59 years of age was higher than in males. People between the ages of 30-49 years had the highest infection rate. The percentage of COVID-19 infected and death rates by age and gender are presented in (Figure 1). The age group of 60-69 years had the highest death rates compared to the other age groups. The age groups 30-39 years and 40-49 years had the highest rates of COVID-19 infection. Children aged 10-19 years were infected at a higher rate than adults aged 60-79 years. From February 2021 to May 2022, children < 1 year of age and older people aged 90-99 years had the lowest COVID-19 infection rates. Furthermore, our study showed that males are more likely to die from the disease than females.

COVID-19 outbreaks in KRG, Iraq

From February 2021 to May 2022, the total number of confirmed cases in our study was 48,494, as shown in the pie chart (Figure 2A). Duhok and Sulaymaniyah had the most infected cases, 33%, and 32%, respectively; while 31% of cases were in Erbil; and Halabja had the lowest number at 1.7% of the infected cases. We found that the Alpha variant was dominant until mid-March 2021, after which the Delta variant became more prominent among the population. Most recently, in early 2022, the Omicron variant became

Figure 2. Cities of the Kurdistan Region in Iraq with coronavirus disease 2019 (COVID-19) cases.



A: The total number of reported COVID-19 cases in Erbil, Sulaymaniyah, Duhok, and Halabja; B: COVID-19 prevalence from February 2021 to May 2022; C: Number of deaths from February 2021 to May 2022.

apparent in the Kurdistan region. Statistical analysis identified the Alpha variant was the most dominant until mid-March 2021, after which the Delta variant became more prominent among the population. Most recently, the Omicron variant became apparent in early 2022 in the Kurdistan region (Figure 2B); thus, different variants of COVID-19 were prevalent from February 2021 to May 2022. Figure 2C presents the numbers of deaths due to COVID-19. We found that Sulaymaniyah had the highest number of deaths compared to the other governorates, followed by Erbil and Duhok. Lastly, Halabja had the lowest number of deaths.

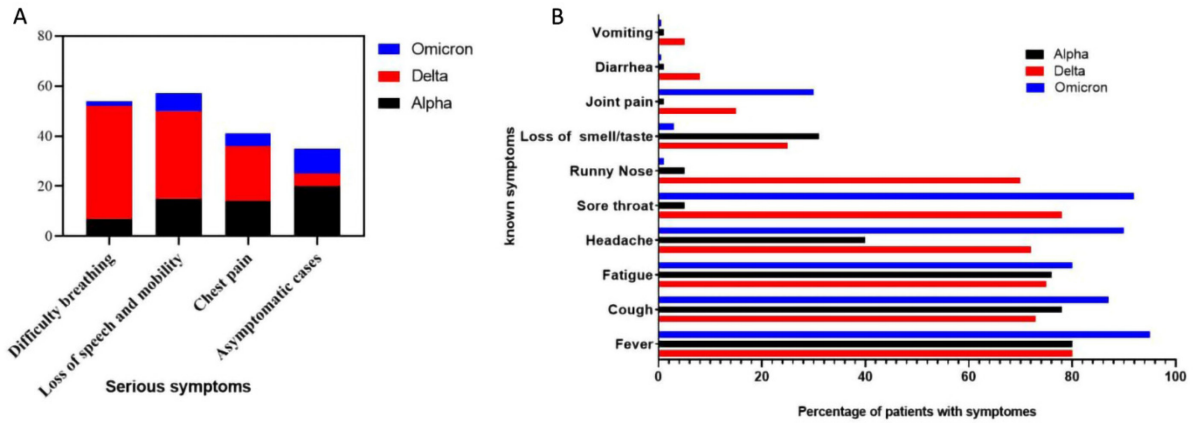
Serious and typical symptoms in the COVID-19 cases

COVID-19 affected patients in a variety of ways. Most infected people experienced mild to severe symptoms and recovered without medication or hospitalization. However, some COVID-19 patients became seriously ill and required medical attention. When comparing the three variants, asymptomatic cases were primarily found in patients infected with the Alpha variant. Patients infected with the Delta variant had the highest reports of chest pain, reduced speech and mobility, and difficulty breathing (Figure 3A), which comprises cases with serious symptoms. Patients infected with the Omicron variant reported the lowest rates of severe symptoms compared to the Alpha and Delta variants (Figure 3A). COVID-19 disease affects a variety of patients in various ways. When reporting mild to moderate symptoms, fatigue, cough, and fever appeared to be shared among all three variants. However, when comparing the variants, patients infected with the Omicron variant had more reports of joint pain, sore throat, headache, fatigue, fever, and coughing (Figure 3B), which present the most and least common symptoms of COVID-19. At the same time, patients infected with the Delta variant reported more vomiting, diarrhea, and runny nose. Patients infected with the Alpha variant had more reports of loss of smell and taste when compared to the Delta and Omicron variants (Figure 3B).

Molecular diagnosis for COVID-19 by RT-PCR

All coronavirus tests from the start of the COVID-19 outbreak were carried out at public hospitals in Kurdistan's governorates. The most commonly used technique for COVID-19 diagnosis was quantitative polymerase chain reaction (qPCR) which is widely used in infectious disease diagnosis. Both COVID-19 test

Figure 3. Coronavirus disease 2019 (COVID-19) patients with serious and typical symptoms in Kurdistan governates.



A: COVID-19 cases with serious symptoms; B: COVID-19 patients with mild to moderate common symptoms.

kits and qPCR machines were urgently provided by the Ministry of Health of Iraq's Kurdistan region to public hospital laboratories in Erbil, Sulaymaniyah, Duhok, and Halabja. The COVID-19 test kits, techniques, equipment, and RT-PCR machines were provided by Iraq, the People's Republic of China, Germany, and WHO. The COVID-19 test was carried out by COVID-19 care workers trained by the Kurdistan region's Ministry of Health. The critical steps of the COVID-19 molecular test are summarized in Supplementary Figure S1.

Briefly, oro-nasopharyngeal swabs were taken from patients; RNA was isolated from the specimen using RNA isolation kits and reverse-transcribed to cDNA; the cDNA was amplified using qPCR; and finally, the presence or absence of the virus in the specimen was determined based on the results.

Phylogenetic analysis of SARS CoV-2 from different species

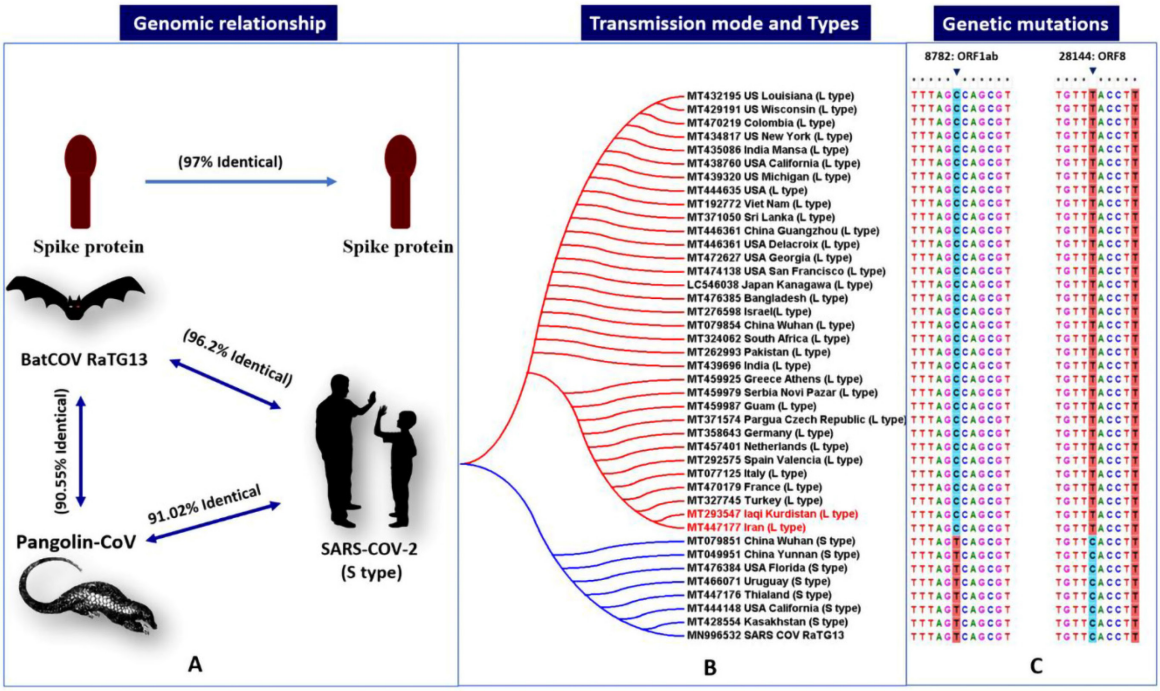
SARS-CoV-2, BatCoV-RaTG13, and Pangolin-CoV have a close genomic relationship. According to the whole genome analysis, the percentage of genomic identity between BatCoV RaTG13 and human SARS-CoV-2 was higher than that between Pangolin-CoV and SARS-CoV-2. BatCoV RaTG13 was found to be 96.02% similar to SARS-CoV-2 and 90.55% identical to the pangolin virus. Figure 4A shows the relationship between BatCoV-RaTG13, Pangolin-CoV and SARS-CoV-2, in a phylogenetic tree. Furthermore, the glycoprotein structures of spike protein in SARS-CoV-2 and BatCoV RaTG13 were very similar, and 97% of their spikes were similar.

SARS-CoV-2 has been classified into two types: S and L. A phylogenetic tree was reconstructed using 41 homologous SARS-CoV-2 genomes from various countries worldwide to show the original lineage of SARS-CoV-2 types. During the early stages of evolution, the tree split into two major groups (S- and L-type) (Figure 4B). The ML tree shows that the S lineage (blue) evolved from an animal, Bat SARS CoV RaTG13; and that the L lineage (red) evolved from the S lineage. The L-type variant is more widely distributed worldwide than the S-type, but the S lineage has been linked to animal origin. Since they were clustered, the L-type of SARS-CoV-2 is predicted to travel from Iran (red) to Iraqi Kurdistan (red). Furthermore, MSA revealed that substitutions were the most critical mechanisms in viral evolution, particularly during SARS-CoV-2 outbreaks. The SARS-CoV-2 genomes differ at two single nucleotide polymorphisms (SNPs) at positions 8782 in *ORF1ab* and 28144 in *ORF8*, implying that L-type variants have C instead of T at site 8782: *ORF1ab*, and T instead of C at site 28144: *ORF8* (Figure 4C).

Discussion

The COVID-19 pandemic was caused by the rapid global spread of the SARS-CoV-2 in early 2020. Multiple unique SARS-CoV-2 variants evolved in various populations as the virus diversified and propagated, causing both local and worldwide waves of illness. Based on data showing that specific variants can worsen the disease, enhance transmissibility, or elude immunity from previous infection or vaccination, they have been classified as VOIs or VOCs [21]. Thus, it is critical to accurately estimate the epidemiological features and implications to inform public health responses, such as tracking the efficacy of vaccinations and therapeutic antibodies. These results may also shed light on the long-term course of SARS-CoV-2 after the pandemic.

Figure 4. Whole genomic analysis and potential coronavirus recognition.



A: Genomic relationship between Pangolin-CoV, BatCoV RaTG13, and SARS-CoV-2; B: A phylogenetic tree of 41 sequences of coronaviruses in different countries in the world was constructed by collecting sequences from BLAST NCBI, and the sequence was submitted to the GISAID database, with the accession no. MT447177 as a query seq to collect 41 similar sequences. Accession numbers and countries in parentheses. The tree was created to show the evolutionary relationship of S and L types of SARS-CoV-2 using MEGA's maximum likelihood (ML) tool, version 7. Accession numbers of some sequences were unavailable, so complete genome accession numbers were used; C: Multiple sequence alignment (MSA) of the 41 homologous sequences from different countries. The sequences were aligned using the MUSCLE tool of MEGA7. Using the NCBI BLAST tool, SARS-CoV-2 (MT447177) of Iran was used as a query sequence to harvest the 41 homologous sequences. The asterisks on the top of the alignment correspond to conserved nucleotides, and dark blue arrows represent mutated nucleotides at 8782n: ORF1ab and 28144n: ORF8.

The findings showed that the Delta wave was more contiguous, easily dispersed, and continuous throughout the Kurdistan governorates than the Alpha and Omicron variants waves. Sulaymaniyah and Duhok had the highest death rates overall, whereas Sulaymaniyah had the greatest number of cases; this could be due to the adherence of the residents to the COVID-19 restrictions placed by the regional government. Erbil and Halabja had the lowest number of cases. Halabja has the lowest population when compared to the other governates, which possibly explains the low number of cases. However, Erbil had the highest population but represented the second lowest percentage in the total number of cases. Thus, although there was a slight similarity between governates, the number of cases were different. Moreover, the study revealed the percentage of males infected was higher than females, and the groups between 30-49 years old were the most infected. In addition, this study found that men died at a higher rate than women, particularly among the elderly. Our results were similar to those of Pijls *et al.* who reported that in a meta-analysis of 59 studies, men aged above 70 years have the most risk of COVID-19 infection, severe disease, and death [22], due to infectious diseases of the respiratory tract being more severe in males. As a result, the mortality rate in men was higher than in females [23].

We discovered that the common symptoms associated with the three variants were fever, coughing, and fatigue. However, the patients infected with the Delta variant had the highest incidence of chest pain, reduced speech, reduced mobility, and difficulty breathing. This could explain the higher rates of hospitalization in the region during the prevalence of the Delta variant. Our data agreed with Menni *et al*. who reported higher severity of the Delta variant than the Alpha and Omicron variants [24]. Patients infected with the Omicron variant reported the lowest rates of severe symptoms compared to the Alpha and Delta variants. The disease severity and differences in symptoms in the three variant waves may be due to factors, such as vaccine approval and the steps taken to control the pandemic which led to decline in the mortality rates [25].

The phylogenetic tree indicated that the L-type of SARS-CoV-2 had descended from the S-type at an early evolutionary stage. The L-type spread faster in Kurdistan than the S-type. The 100% sequence similarity among all L-type sequences across national borders was verified by MSA. They were all mutated in regions 8782: ORF1ab and 28144: ORF8 703. Whole genome analysis detected that the percentage of genomic identity of BatCoV RaTG13 and human SARS-CoV-2 was higher in comparison to that between Pangolin-CoV and SARS-CoV-2. The SARS-CoV-2 and BatCoV RaTG13 glycoprotein structures of spike protein were 97% similar; despite that, Wrobel *et al.* found that there were also differences between the S proteins, the main difference being that the SAR-CoV-2 spike protein can bind 1000 times tighter to human receptor [26]. The phylogenetic tree analysis of 41 SARS-CoV-2 genomes predicted that the L-type of SARS-CoV-2 travelled from Iran to Iraqi Kurdistan.

The pandemic started in Wuhan in December 2019. Chinese researchers reported that SARS-CoV-2 can be classified into two variants, L-type and S-type; and S-type is less prevalent and phylogenetically older [27]. Despite the international effort to identify different elements of SARS-CoV-2, such as clinical symptoms, epidemiology, mortality and morbidity, and diagnosis, there are still numerous unanswered questions and significant gaps in our understanding of the disease.

Conclusions

Based on our findings, the Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529), variants were distributed in the Kurdistan governates from February 2021 to May 2022, with the Delta variant spreading faster and more fatal than the Alpha and Omicron variants. Furthermore, people infected with the Delta variant produced the most serious symptoms, while people infected with the Omicron variant mostly presented with mild to moderate symptoms. Lastly, phylogenetic analysis implies that the COVID-19 L-type identified from Iraqi Kurdistan probably originated in Iran.

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Authors' contributions

SAM, RD, writing original draft; SOM, methodology and data collection, data analysis; RKY, review and editing.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Primers and probes used to identify SARS-CoV-2.

Gene target	Primer/probe	Primer/probe sequence 5'→3'
RdRp gene	RdRp_SARSr-F2	5'-GTGARATGGTCATGTGTGGCGG-3'
	RdRp_SARSr-R1	5'-CARATGTTAAASACACTATTAGCATA-3'
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BHQ
E gene	E_Sarbeco_F1	5'-ACAGGTACGTTAATAGTTAATAGCGT-3'
	E_Sarbeco_R2	5'-ATATTGCAGCAGTACGCACACA-3'
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ

R is G/A; FAM: 6-carboxyfluorescein; BHQ: black hole quencher; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Supplementary Table 2. Steps of RT-qPCR.

Cycles	1×	40×		
Step	Initial denaturation	Denaturation	Annealing	Extension
Temperature	95°C	95 °C	55 °C	72 °C
Time	120 sec	30 sec	30 sec	60 sec

RT-qPCR: reverse transcriptase quantitative polymerase chain reaction.

Supplementary Figure S1. Workflow of COVID-19 molecular test.