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Factors affecting SARS-CoV-2 variant distribution in military hospitals in Jordan

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ABSTRACT

The pandemic caused by SARS-CoV-2 has prompted a collaborative global effort to contain viral spread and improve health outcomes for those infected. The tracking of SARS-CoV-2 variants since the first sequence was published in January 2020 is an important part understanding the pandemic. There is limited data regarding SARS-CoV-2 circulation in Jordan. In this study we determined the prevalence of genetic variants of SARS-CoV-2 during June-September 2021 by sequencing the full genome of 213 viral samples from Jordanian Royal Medical Services military hospitals. Our analysis revealed the presence of 33 variants, with (B.1.617.2.AY.106) as the predominate strain. Six variants were present at a prevalence greater than 2% ((B.1.617.2.AY.106), 52.8%; Delta (B.1.617.2), 7.0%; (B.1.617.2.AY.34.1), 5.6%;(B.1.617.2.AY.44), 2.8%; (B.1.617.2.AY.121), 2.33%; (B.1.617.2.AY.102), 2.33%). Variant prevalence varied significantly by region and (B.1.617.2.AY.106) variant tended to be associated with mild to moderate symptoms, on the other hand other variants were asymptomatic. We did not find significant associations of variants with other factors such as age, gender or vaccination status. These data help us to understand the occurrence of new variants in Jordan, their geographic distribution, and associations with demographic variables, vaccination status, and symptom severity. The sustained circulation of SARS-CoV-2 continues to lead to novel variant emergence. These findings highlight the need to continue tracking new variants, monitor the dynamics of variant prevalence, and future efforts will guide prevention, vaccination, and control strategies.

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1. Introduction

Coronavirus disease 2019 (COVID-19), the highly contagious viral illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It had a catastrophic effect on the world's population resulting in more than 6 million deaths worldwide [1]. The cumulative number of COVID-19 cases was reported by the Ministry of Health to be 1,638,228 patients, with 13,849death [2].

Coronaviruses are large, enveloped, positive-stranded RNA viruses. Along with proteins necessary for viral replication, transcription, and host suppression, the genome of SARS-CoV-2 encodes 4 structural proteins—spike (S), membrane (M), envelope (E), and nucleocapsid (N) [3]. Of these, the S protein is an especially important molecular feature of SARS-CoV-2 and is crucial for viral entry. As a surface protein, S binds with receptors for angiotensin-converting enzyme 2 (ACE2) and this leads to cellular uptake [4,5]. The S protein

is also an important target for vaccine development, therapeutic antibodies, and diagnostic methods [6,7].

Genetic variation in S and other SARS-CoV-2 genes presents a major challenge to effective public health measures. In SARS-CoV-2, as in other RNA viruses, variation occurs largely due to the high error rate associated with viral RNA polymerases [8,9]. The resulting variation underlies, in part, differences in SARS-CoV-2 infection rates and COVID-19 pathogenesis [10]. Additionally, when new variants arise after the development of diagnostic tests, vaccines, and therapeutics, the effectiveness of these public health measures may be limited due to the differences in key viral proteins [11].

1.1. Viral surveillance and variants of concern

Whole genome sequencing has been used to continuously monitor changes to the SARS-CoV-2 genome and has identified multiple circulating subtypes. Variants may differ in transmissibility, virulence, disease phenotype, and clinical disease presentation. Continuous monitoring of variants helps to guide public health responses

[12]. Recently, several novel variants have been classified as variants of concern by the World Health Organization because of their potential for increasing COVID-19 morbidity and mortality around the world.

The dominant circulating SARS-CoV-2 variant in the Middle East (and globally) at the time of this study was Delta, and variants of concern included sub-lineages of alpha (B.1.1.7), beta (B.1.351, B.1.351.2, B.1.351.3), gamma (P.1, P.1.1, P.1.2), and Delta (B.1.617.2, AY.1, AY.2) [13]. Though some research has investigated regional variant diversity, and websites such as Global Initiative on Sharing Avian Influenza Data (GISAID) track the geographic distribution of variants, very little is known about viral diversity in Jordan and the risk factors associated with SARS-CoV-2 infection [14,15]. We hypothesize that there is a relationship between geography, vaccination status, and demographic characteristics associated with variant sub-lineages in Jordan. Here we report the distribution of SARS-CoV-2 variants, findings which provide information regarding variant distribution and factors contributing to variant circulation.

2. Materials and methods

2.1. Study population

Royal Jordanian Medical Services serve military personnel and their dependents through 12 military hospitals throughout Jordan. A total of 56,340 individuals from September 2021 through November 2021 sought testing for COVID-19 in military hospitals. Throat or nasopharyngeal swab samples for SARS-CoV-2 were obtained and sequenced as described below. Along with the samples, demographic information was recorded for each patient, including vaccination status, gender, age, geographic location, and clinical presentation (mild to moderate or severe). Mild to moderate patients were those with fever, joint pain, cough, loss of smell and taste, headache, back pain, and general weakness; severe patients were those who had chest pressure or pain, cyanosis, chills, dyspnea, intubation, low O_2 saturation, and patients that died.

2.2. Ethical approval

This study was approved by the Jordan Royal Medical Services Human Research Ethics Committee number (1/2022). Samples were collected as part of routine public health surveillance activities and data were de-identified prior to analysis by the research team.

2.3. Sample collection and storage

Specimens were collected at each military hospital, in accordance with the U.S. Centres for Disease Control and Prevention Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation for 2019 Novel Coronavirus [16]. Swab specimens were collected using iClean (BioMed Diagnostics; Singapore) nylon tipped swabs with plastic shaft and placed immediately into sterile tubes containing 3 ml of viral transport media with UTM-RT (W/O beads; Guangzhou, China). Specimens were packaged and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation [17], following shipping regulations for UN 3373 Biological Substance, Category B when sending potential SARS-CoV-2 specimens. Specimens were stored at 2 to 8°C and shipped overnight to the testing facility on ice packs. Specimens were stored at 2 to 8°C for up to 48 hours after collection prior to RNA extraction. If RNA extraction could not be performed within 48 hours, samples were stored at -70°C.

2.4. RNA extraction and virus detection at hospitals

The viral RNA was isolated and purified using 3DMed 2019-nCoV RT-qPCR Detection Kit (Shanghai, China). The 3DMed 2019-nCoV RT-qPCR Detection Kit is a 1-step RT-qPCR test for qualitative detection of SARS-CoV-2 specific RNA. Detection is achieved via fluorescence intensity measurement by an Applied Biosystems 7500 Real-Time PCR System. Briefly, this kit tests for 3 specific sequences in the SARS-CoV-2 genome found in the N, S, and E genes. A cycle threshold (C_T) value <40 was considered a positive result and a C_T value ≥40 was considered a negative result. If at least 1 of SARS-CoV-2 targets produced a positive result, the sample was interpreted to be positive. If all SARS-CoV-2 targets were negative, but the internal control was positive, the sample was considered to be negative.

2.5. Next generation sequencing (NGS)

Among 2000 qPCR-confirmed COVID-19-positive samples tested in Princess Iman Centre for Research and Laboratory Sciences, we selected 213 positive SARS-CoV-2 sample based on a low C_T (between 20 and 33), suggesting a high level of virus in the sample. Original clinical samples from this subset were transferred to Queen Alia Oncology Laboratory Department where whole genome sequencing of SARS-CoV-2 was performed as detailed below. The samples were transferred and stored according to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation for 2019 Novel Coronavirus [18].

2.6. Extraction and quality control of nucleic acids

QIAamp Viral RNA Mini Kits were used to purify viral RNA for amplification, per manufacturer's instructions (Qiagen, Germany). Following extraction, RNA quality assessment was performed with a Qubit fluorimeter (Qubit 4.0; ThermoFisher; Waltham, MA). The RNA was diluted to the final standard input concentration of 10 ng/μl according to the manufacturer's instructions (Illumina Inc., MiSeq, CA).

2.7. Library generation, sequencing, and validation

Libraries for RNA samples were prepared for sequencing following the Illumina Covidseq workflow (Illumina Inc.) in accordance with the manufacturer's specifications. The extracted RNA was reverse-transcribed to generate cDNA using Amplise cDNA synthesis for Illumina (Illumina Inc.). Each reverse transcription reaction requires 1 to 100 ng per pool of DNase-treated total RNA. The recommended input is 10ng of RNA per pool. After total RNA was generated, cDNA was amplified in 2 pools according to the designed primers in AmpliSeq cDNA kit (Illumina Inc.). The 2 pools were then recombined to be simultaneously fragmented and tagmented and partially digested by using FuPa Reagent. The tagmented amplicons were amplified once more with the addition of indexes to each sample using AmpliSeq CD Indexes for Illumina, DNA ligase, and switch solution. The indexed libraries were subjected to a post-tagmentation clean-up step using AM Pure XP beads using Beckhman Coulter Life Sciences kit and 70% ethanol. Then the libraries were amplified using 50 μl Amplification Mix following the Equalizer workflow protocol (Illumina Inc.). A second clean-up was performed using Equalizer beads. From each amplicon an equal volume was transferred into a new microcentrifuge tube to be validated, denatured, and then diluted as specified by the manufacturer workflow (Illumina Inc.). The libraries were quantified using Qubit 4.0 fluorometer by dsDNA HS Kit of Qubit High-Sensitivity Assay (Thermo Fisher Scientific). High Sensitivity DNA electrophoresis with Agilent 2100 bioanalyzer system assessed the size and quality of DNA over a range of sizes and concentrations using HS dsDNA Assay Kit. qPCR for Illumina by Biosystems 7500 using

KAPA qPCR kit was employed to calculate the molarity of libraries then denatured by 0.1 fresh NaOH, which were then diluted to final loading concentration (10pM), as specified by the manufacturer. The resulting DNA libraries were sequenced on a MiSeq using reagent Kit v2 300-cycle.

2.8. Phylogenetic and lineage analysis

The sequencing quality was analyzed with FASTQ files raw data processed by EDGE COVID-19 [19,20]. EDGE provides web platform tools for both read-based and assembly-based analyses that enables rapid, automated, and standardized process available for GISAID submission.

2.9. General workflow for processing SARS-CoV-2 genome

Quality control for FASTQ or FAST5 files was performed to remove and trim bad quality reads. Then, the reads were mapped to a reference genome (Wuhan-Hu-1 genome for SARS-CoV-2). The consensus genomes were assembled based on read mapping (21).

2.10. Statistical analysis

Sample numbers, date of collection, demographic information, symptoms, vaccination status, and variants detected were entered into Microsoft Excel (Microsoft, Redmond, WA), which was then imported into SPSS 20 (SPSS Corp., IBM, Armonk, NY) for analysis. Univariate analyses were conducted to analyze SARS-CoV-2 variants according to the number of variables including demographic information, vaccination status, severity of symptoms, and geographical distribution. A final logistic regression was run that included variables found to be statistically significant (P value <0.05) in the multivariate analysis and using variables reported as significant factors affecting SARS-CoV-2 variant distribution in the literature. The model fitness was evaluated with the Hosmer-Lemeshow goodness-of-fit-test.

3. Results

A total of 213 COVID-19 cases (derived from 56,340 cases collected from military hospitals across the country) were analyzed to evaluate the genetic diversity of the SARS-CoV-2 virus between June and October 2021. Our data identified Delta sub-lineage AY.106 as the dominant variant in Jordan during this period. Of the samples analyzed, 48.3% (103) were female and 51.7% (110) were male. The majority of samples were collected from middle-aged adults (42.7%), followed in decreasing order from young adults, elderly, and children. 5.1% were asymptomatic, 87% had mild to moderate symptoms, and 7.9% had severe disease (Fig. 1). Our sample set included individuals with a range of vaccination status; 45.5% were unvaccinated, 19.8% were vaccinated with the Pfizer-BioNTech vaccine (1.5% had 1 dose and 18.3% had both doses), 31% had the Sinopharm vaccine (1% had 1 dose and 30% had both doses), 3.7% had the AstraZeneca vaccine (all had both doses).

Of the identified variants, the most frequent was AY.106 (53.1%), followed by B.1.617.2 (7.0%), B.1.617.2.AY.34.1 (5.60%), B.1.617.2. AY.44 (2.80%), B.1.617.2.AY.121 (2.33%), B.1.617.2.AY.102 (2.33%), B.1.617.2.AY.39 (1.86%), B.1.617.2.AY.43 (1.40%), B.1.617.2.AY.45 (1.40%), B.1.617.2.AY.59 (1.40%) and Jordan lineage B.1.1.312 (0.9%) (Fig. 2).

We evaluated the relative prevalence of the dominant variant Delta AY.106 based on age, gender, geographical region, vaccine status, vaccine brand, and symptoms. Only the geographical occurrence of SARS-CoV-2 AY.106 variant cases varied significantly ($P < 0.005$). There was no association by age, gender, vaccination status, vaccine brand, or symptom status.

Table 1
Factors associated with AY.106 incidences in military hospitals in Jordan compared to other variants, June-October 2021.

Variable	% lineage Ay. 106 (# Ay.106 / # sequenced)	χ^2 test	P-value
COVID positive	53.1% (113/213)		
Age in years		3.023	0.388
0-16	68.8% (11/16)		
17-30	49.2% (31/63)		
31-45	56.0% (51/91)		
Above 45	46.5% (20/43)		
Gender		0.204	0.652
Male	54.5% (60/110)		
Female	51.5 % (53/103)		
Region		10.837	0.004
North	64.4% (38/59)		
Middle	55.7% (59/106)		
South	33.3% (16/48)		
Vaccination status		2.669	0.849
Pfizer (1 dose)	66.7% (2/3)		
Pfizer (2 doses)	51.3% (20/39)		
Sinopharm (1 dose)	50.0% (1/2)		
Sinopharm (2 doses)	53.2% (33/62)		
AstraZeneca (2 doses)	50.0% (4/8)		
unvaccinated	54.6% (53/97)		
Vaccinated (1 or 2 doses) vs unvaccinated		0.013	0.909
Unvaccinated	53.5% (54/101)		
Vaccinated	52.7% (59/112)		
Symptom presentation		1.305	0.521
Asymptomatic	36.4% (4/11)		
Mild To Moderate	54.1% (100/185)		
Severe	52.9% (9/17)		

A similar percentage of females (51.5%) and males (54.5%) were positive for AY.106 ($P > 0.05$). Children aged 0 to 16 years displayed the highest incidence of AY.106 (68.8%), followed by middle aged adults (56%), young adults (49.2%), and the elderly (46.5%) ($P > 0.05$). 54.1% of patients with mild to moderate symptoms were infected by the AY.106 variant, followed by 52.9% with severe clinical manifestation, and 36.4% of asymptomatic patients were infected with the AY.106 variant. The cases with AY.106 differed in the vaccination status, type of vaccination, and number of doses. The incidence of the AY.106 variant had minimal variations ($P > 0.05$) by vaccine status (Table 1). The AY.106 variant is widespread over the territory of The Hashemite Kingdom of Jordan, with the highest incidence in the north, where it accounts for 64.4% of all infections tested, although it constitutes the dominant variant in the middle (55.7%) and southern regions (33.3%) as well ($P < 0.005$) (Fig. 3).

4. Discussion

In this study, conducted within a large integrated health care system in Jordan (Royal Medical Services), the weekly percentage of all infections attributed to the Delta AY.106 variant rapidly increased to 53% between June and September 2021. Infection with the Delta AY.106 variant tended to be more common among middle aged group persons (aged 31-45 years) and among unvaccinated personnel. According to GISAID database guidelines, our analysis revealed the presence of 33 sub-strains. The lineages of Delta most commonly detected among sequenced samples were AY.106, AY.34.1, and AY.44. In February 2021 the AY.106 variant dominated in India (19%), Germany (18%), the United States (11%), Israel (8%), and Jordan (8%). Soon after a rapid change in the distribution of SARS-CoV-2 variants was observed, with alpha becoming the dominant variant between approximately mid-April and late-May 2021, and Delta quickly became the dominant variant thereafter [22−24].

The Delta AY.34.1 percentage reached 26% in the United Kingdom in April 2021, while it was 6% in Jordan between June and September 2021[25]. At the time of our study, during September 2021, more
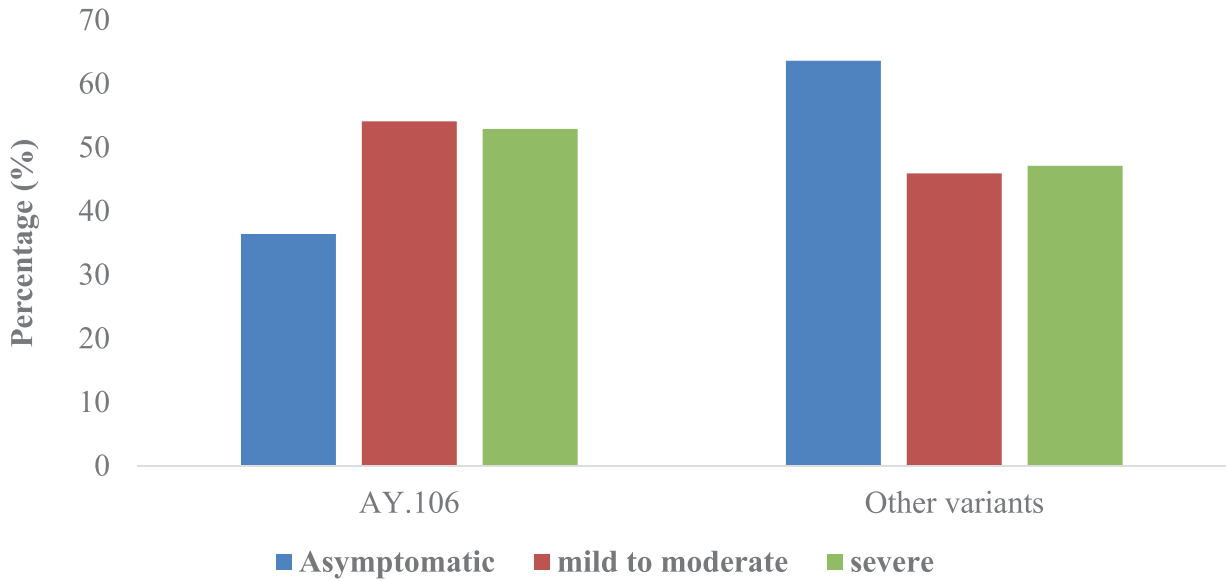
Fig. 1. Symptoms severity according to SARS-CoV-2 Delta variants in Jordan, June-October 2021, whereas AY.106 Delta variant tended to be associated with mild to moderate symptoms. SARS-CoV-2 = Severe Acute Respiratory Syndrome Coronavirus 2.

than 99% of cases in the United Arab Emirates, Saudi Arabia, and Egypt were related to the Delta variant [22]. Worldwide in June 2021, 90.2% of cases were Delta and about 9% were the alpha variant [22].

There is not yet enough evidence to suggest whether these variants are resistant to vaccines and therapeutics or are associated with differences in morbidity and mortality. It is still not possible to determine the precise effect of viral mutation on infectivity; it is possible that these mutations are associated with differences in viral

pathogenesis, but additional studies will be required to identify such a connection.

5. Conclusions

Within our study population, infections attributed to the Delta AY.106 variant tended to be middle-aged relative to all people with positive sequenced specimens. This could be due to multiple factors,
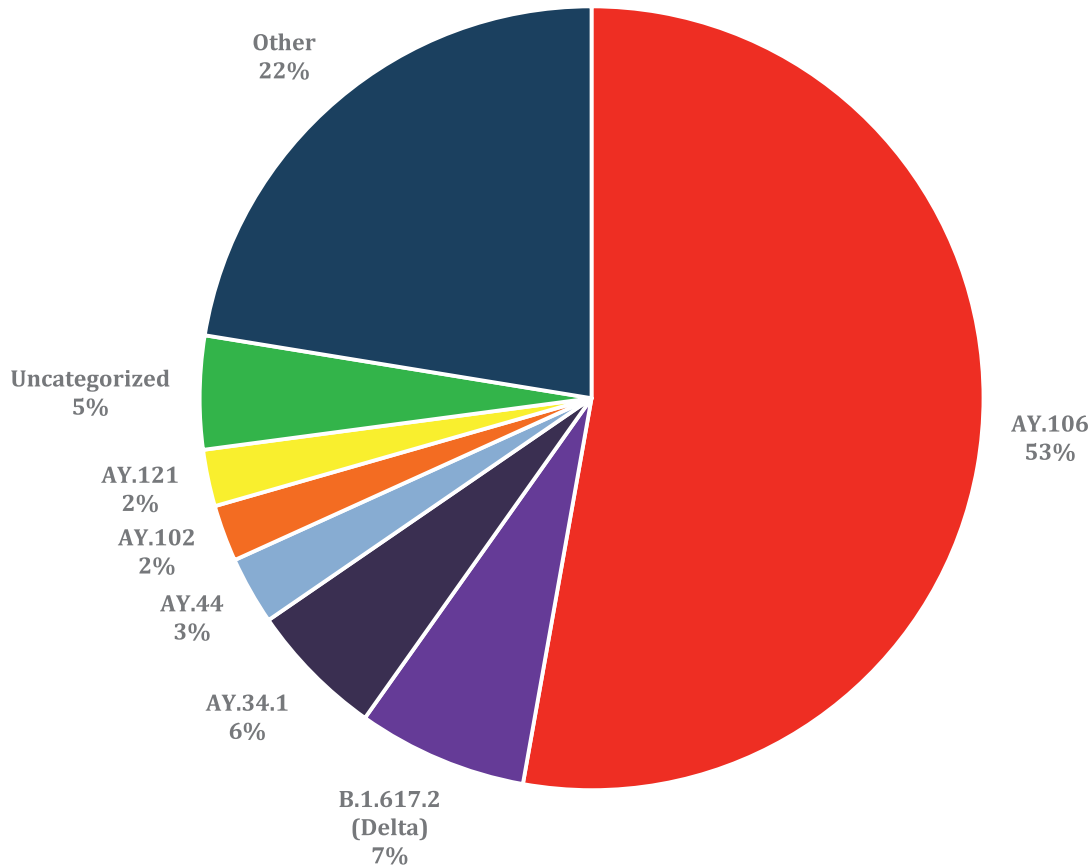
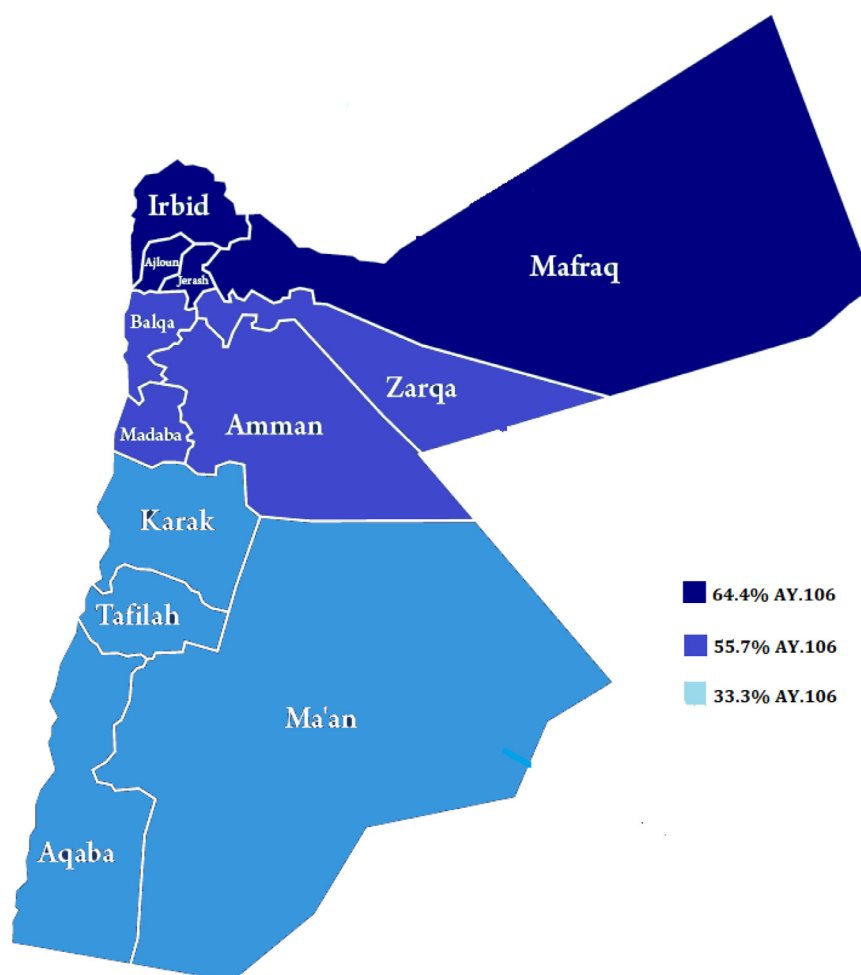**Fig. 2.** Percentages of sequenced SARS-COV-2 variants in Jordan, June-October 2021.

Fig. 3. Geographic distribution of sequenced SARS-CoV-2 variants in Jordan, June-October 2021.

including increasing vaccination coverage among older adults or increased social interactions among the middle-aged groups during periods when the Delta AY.106 predominated. In general, the weekly percentages of isolated variants in this population and their dependents were similar by vaccination status and was not affected by gender, but differed according to geographical distribution. On the other hand, these results help inform the efficient deployment of public health resources and vaccination programs in Jordan and reduce the spread of COVID-19. Sequencing COVID-19 positive samples in hospitals will help identify the most contagious and/or the most virulent lineages, and thus guide effective vaccine use, tracking of virus evolution, and aid in developing efficient public health measures and vaccination programs to control COVID-19 spread.

The findings in this report are subject to multiple limitations. First, only a subset of the total COVID-19-positive population in Jordan were included in this study, and of those included some specimens were not successfully sequenced. Therefore, the study population was not representative of all positive specimens in the population. The lack of financial resources limited the numbers of samples that could be analyzed. Second, sequence success rate depends on the amount of viral genetic material in the specimen. Thus, the sequences analyzed may be skewed towards specimens with high virus levels which can be influenced by many factors such as age, vaccination status, sample collection quality, time of collection, or virus sub-strain. Nonetheless, the size of the subset analyzed was sufficient to provide a snapshot of the variants circulating during the study period. Success in managing the pandemic depends on how we develop social strategies to stop the spread of the virus informed by knowledge generated through surveillance and high-quality research. We believe it is important to continue to monitor SARS-CoV-2. To stay abreast of the current situation and inform the scientific community it is necessary to continuously monitor the evolution of the virus.

Author Contributions

Rame Hamdi Khasawneh: collecting data and statistical analysis

Shirin Shaher Almharat: writing methods and material and results.

Ruba Abedelmajeed Al-Smadi: writing abstract, introduction, and references.

Lamees Abasi: writing conclusion

Maha Al-Amr: performing tables and figures

Ali Saleh Alhuniti: samples sequencing

Abdullah Mohammad Almuhasen: samples sequencing

Mohammad Ahmad Dojan: samples sequencing

Osama Ismail Alshdifat: samples sequencing

ALanood Faheem: samples sequencing

Declaration of Competing Interest

The authors confirm that the research was conducted in the absence of any commercial or financial relationship that would present a conflict of interest.

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