Molecular Epidemiology of Hepatitis B Virus in Turkish Cypriot

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Abstract

There is an increased demand for molecular and epidemiological information regarding Hepatitis B Virus (HBV) infection as the disease severity depends on these specifications. We have aimed to analyze nucleos(t)ide analogues (NA) resistance and typical HBsAg escape mutations with the dispersion of HBV genotype/subgenotype/HBsAg serotypes on overlapping *pol/S* gene regions in the Turkish population. Samples were collected in Northern Cyprus. Reverse transcriptase (*rt*) region between 80–250 amino acids were amplified. Typical HBsAg escape mutations were determined as HBIg escape (6.48%), vaccine escape (8.34%), HBsAg misdiagnosis (9.25%), and immune escape mutations (8.34%). NAs resistances were determined as primary (2.78%), partial (2.78%), and compensatory mutations (26.85%) in overlapping *pol/S* gene region. The study patients were predominantly infected with HBV genotype D/D1 (98%). However, the predominant HBsAg serotype was *ayw*2 (99%). The most common NA resistance mutation was rtQ215H/P/S (16.67%), however, for *S* gene the misdiagnosis mutations were observed most frequently (9.25%). We can conclude that HBV D/D1 is the dominant strain and *ayw*2 is the dominant serotype in the Turkish Cypriot. Cyprus is an island located in the Eastern Mediterranean region, and it is, therefore, a key location for human trafficking and immigration; as a result of this reputation, it is necessary to analyze HBV phylogenetically for local dynamics, and our results indicate that treatment naïve population is prone to these *pol/S* gene mutations. However, if HBV strains were also analyzed among Greek Cypriots too, this would enable a complete island survey. With this work, we believe that we have enlightened this subject for further research.

K e y   w o r d s: hepatitis B Virus, genotype, drug resistance, hepatitis B surface antigens

Introduction

Hepatitis B virus (HBV), which belongs to the family of *Hepadnaviridae* and is one of the smallest enveloped DNA viruses, is a global health concern as more than 2 billion of people are affected and around 260 million of people are chronically infected. In 2015, according to WHO, 275 million people live with HBV, and as estimated, 887 000 deceased as a result of the infection (WHO 2019). The virus is very old, as it has been infecting humans for at least 28 centuries. Humans are the only reservoir for this pathogen, which is 50–100 times more contagious than the Human Immunodeficiency Virus (Cheah et al. 2018). Both morbidity and mortality rates are high, as there is an increased lifetime risk of hepatocellular carcinoma, cirrhosis and liver disease (Bissinger et al. 2015; Cheah et al. 2018; Kostaki et al.

2018). Due to error-prone reverse transcriptase activity, a high nucleotide mismatch rate (10^5 change/base/replication) and a high replicative capacity ($> 10^{12}$ virion/day) are observed, and HBV is characterized by a significant degree of genetic heterogeneity (Kostaki et al. 2018). The HBV genome encloses four partially overlapping open reading frames, which are PreS1/S2/S, PreC/C, P, and X encoding seven different proteins. Most significantly, Reverse Transcriptase (*RT*) and HBsAg frames overlap at *RT* amino acid 8–236, with HBsAg frameshift downstream by one nucleotide. Therefore, it indicates that mutations in these specific areas might result in drug resistance (Zehender et al. 2014; Zamor et al. 2017).

The high degree of genomic heterogeneity categorizes HBV into 10 genotypes (A-J), and an intergroup difference of around 7.5% is observed. All genotypes, except E and G, are classified further into 25 different

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subgenotypes, with a difference of around 4% being observed (Kramvis et al. 2005; Kostaki et al. 2018). HBV-A and HBV-D are present around the globe, whereas HBV-A is mainly seen in Europe and Africa, and HBV-D in the Middle East and Europe. HBV-B and HBV-C are generally found in Oceania and Eastern Asia, HBV-E in both Central and Western Africa. HBV-F and HBV-H are found in Alaska and Latin America only. HBV-D is considered to be pandemic. HBV-D1 is dominant in Australia, Europe, Indonesia, North Africa, and Western Asia, whereas HBV-D2 is seen in Albania, Japan, Malaysia, North-Eastern Europe, Russia, and United Kingdom (Tallo et al. 2008; Bissinger et al. 2015; Kostaki et al. 2018). A recent study in Brazil showed that due to Italian colonization, the dominance of genotype D/D3 is observed (Paoli et al. 2018). A similar study was performed before in the same region, and once again, genotype D/D3 was found to be the most relevant genotype (Chacha et al. 2017).

The prevalence of HBV can be classified into three regions; low (< 2%), middle (2–7%), and high (> 8%) endemities. Turkey is categorized as middle endemicity with a prevalence rate of 0.8–5.7%, while the Turkish Republic of Northern Cyprus (TRNC) falls into a low category with a rate of 1.2% (Arikan et al. 2016; Ozguler and Sayan 2018). However, Cyprus is an island located in the Eastern Mediterranean, to the south of Turkey. Since 1974, there have been two communities living separately on the island: Turkish and Greek Cypriots. The exact population of North Cyprus is not known as the population number is dynamic, this is due to sex worker and immigrant trafficking that occurs along with constant international student and tourist travel. However, there is no data regarding HBV dynamics for South Cyprus. The South of the island is subject to more immigrations and human trafficking (Kaptanoglu et al. 2013; U.S. Department of State Publication 2018).

Cyprus had an estimated population of 1 193 635 in 2011. Around 352 000 were believed to live in North Cyprus, but the number has climbed up to half a million. Half of these are Cypriot-born children or Turkish settlers. Around 230 000 of those are classified as native-born TRNC citizens. The exact population remains unknown as North Cyprus has a dynamic society of students and tourists who regularly visit the island (Christou 2018; World Population Review 2018). Immunisation against HBV in the TRNC was rare in the late 80s, and the program was first introduced in the country in July 1998 (Kurugol et al. 2009). Between 2014 and 2018, 3149 HBsAg positive Turkish citizens were living in the TRNC, where 98.16% were of Turkish origin and 1.84% was Turkish Cypriots (KKTC Sağlık Bakanlığı 2019). Previous studies have concluded that the overall HBsAg positivity rate for the TRNC is 1.2% (Arikan et al. 2016).

There is a high demand for genotype information and investigation regarding HBV infected individuals. This importance rules to be informed regarding molecular and epidemiological specifications. As with the aim of this importance, we have aimed to analyze the dispersion of genotype/subgenotype/serotype together with *pol* gene mutations, which are related to NA therapy, and *S* gene mutations.

Experimental

Materials and Methods

Patients Samples. HBsAg reactive serum samples were collected and stored at –80°C in the Near East University Laboratory, Nicosia and Lancet Medical Diagnostic Laboratory, Famagusta between the dates of January 2015 and August 2018. This project obtained the Ethical Committee approval on 29 March 2018 from the Near East University Ethical Committee (Approval Number YDU/2018/56-539). As part of the Ethical approval, Helsinki Declaration principles were followed. Samples were taken from patients who presented to either of the health centers for residential permit screening, pre/post-operation screening, blood bank screening, and privately requested tests. The total number of samples in our study (n = 170) represented the total number of HBsAg reactive diagnoses made between the dates of January 2015 and August 2018. Unfortunately, information on the HBV infection status or phase of the patients is not available as they were not follow-up patients.

Serological Analysis. These samples were all screened for Anti-HCV, HBsAg, HIV Ag/Ab and Syphilis TP using Abbott Architect i1000SR/i2000SR automated analyzers. Out of the samples, only Turkish and Turkish Cypriot samples were selected (n = 170). Only HBsAg levels of the samples were measured primarily, and no other Hepatitis B serological markers were studied (such as AntiHBc).

Genotyping, subgenotyping, serotyping and mutation analysis. Out of 170 samples, only 108 were sequenced, as rest of the samples did not yield any HBV DNA load. The clinical demographics of the samples are given in Table I. For HBV genotypes/subgenotypes, nucleos(t)ide analogue (NA) drug resistance analysis, and *S* gene analysis the overlapping the *Pol/S* gene region (*rt* region, between amino acids 80–250) were chosen. For HBV *pol* gene amplification (742 base pairs), forward (F: 5'-TCGTGGTGGAC TTCTCTCA ATT-3') and backward (R: 5'-CGTTGACAGAC TTTC CAATCAAT-3') primers were designed and used. HBV *Pol* gene amplification and Sanger dideoxy sequencing protocols were all performed as described previously by Sayan et al. (2010).

Characteristics	Patient group	Study group
Patients, n	170	108
Gender, M/F, n (%)	106 (63) / 64 (37)	68 (63) / 40 (37)
Age, years (mean ± SD)	49 ± 31	41.5 ± 23.5
Nationality Turkish	122 (71)	83 (77)
Turkish Cypriot	48 (29)	25 (23)
HBsAg value, S/Co* (mean ± SD)	3882.5 ± 3712.5	3882.5 ± 3712.5

Abbreviations: M – male; F – female; *S/Co: Sample/Cut-off.
HBsAg value was obtained using Abbott Architect i1000SR/i2000SR systems (Abbott, USA).

Sequences obtained were subsequently analyzed using a special online tool, the Geno2pheno (Centre of Advanced European Studies and Research, Bonn, Germany) drug resistance platform. The following target region and amino acid positions were analyzed for the determination of antiviral drug-associated potential vaccine-escape mutations (ADAPVEM) regions 161, 164, 172, 173, 175, 176, 182, and 193–196; HBIg selected escape mutation regions 118, 120, 123, 124, 129, 133, 134, 144, and 145; vaccine escape mutation regions 120, 126, 133, 143–145, and 193; Hepatitis B misdiagnosis mutation regions 120, 131, 133, and 143; immune-selected mutation regions 100, 101, 105, 109, 110, 114, 117, 119, 120, 123, 127, 128, 130–134, 140, and 143–145. The target region and amino acid position for the determination of HBV *pol* gene mutation were as follows: overlapping surface gene segments 100, 101, 105, 109, 110, 114, 117–121, 123, 124, 126, 127, 128–135, 137, 139, 140–142, 144–149, 151–153, 155–157, 161, 172, 173, 175, 176, and 193–196. The following target region and amino acid position for the determination of HBV *pol* gene mutation were analyzed: *rt* gene segments 74, 80, 82, 84, 85, 139, 149, 156, 169, 173, 180, 181, 184, 194, 200, 202, 204, 214, 215, 233, 236, 237, 238, and 250 (Sayan et al. 2012; Asan et al. 2018).

HBV genotype/subgenotypes were also phylogenetically analyzed using the Neighbour-Joining method. Primarily, the sample sequences and reference sequences were all aligned. A phylogenetic tree was created using CLC Sequence Viewer 8.0 (CLC bio A/S, Qiagen, Denmark). A bootstrap value of 1000 was chosen.

Three HBsAg glycoproteins share an *a* determinant epitope, which is located at the position 127–147. There are two other determinants namely, *d/y* at the position 122, lysine/arginine residue and *w/r* at the position 160; lysine/arginine residue represents each determinant, respectively. At the position 127, residue further differentiates *w* into four subtypes. The *adr* subtype only is further divided into q^-/q^+. With these combinations, nine different subtypes of HBV have been

identified (Yokosuka and Arai 2006). The analysis of HBsAg serotypes was also performed using the CLC sequence viewer, as the geno2pheno tool is not able to detect this information. After aligning the sequences to reference sequences, the phylogenetic parameters of UPGMA/Jukes-Cantor were used and the bootstrap value of 1000 was used.

Results

Baseline data. Out of 170 samples included in the study, only 108 (63.5%) were sequenced. Demographic characteristics of the patients from whom these samples were collected are listed in Table I. Genotypes/subgenotypes/HBsAg serotype analysis of 108 samples were performed following aligning for phylogenetic analysis (Fig. 1 and 2).

RT mutations. NA resistance mutations were detected in 35/108 (32.4%) of the samples, and as a novel data 2/108 (1.85%) of these samples confer ADAPVEMs (Table II). The compensatory resistance *pol* gene mutations were the most frequent as 29/108 (26.85%) of the samples comprise this category, followed by primary resistance mutations in 3/108 (2.78%), and lastly, partial resistance mutations in 3/108 (2.78%) samples analyzed. The most prevalent *pol* gene mutation was rtQ215H/P/S and it falls in the compensatory mutation category.

The *S* gene mutations. A total of 17/108 (15.74%) the *S* gene mutations were detected, together with 3/108 (2.78%) combined escape mutations in the same region (Table III). The highest *S* gene escape mutations were detected for HBsAg misdiagnosis 10/108 (9.25%), both vaccine and immune escape mutations in 9/108 (8.34%), and the lowest number mutations were observed for HBIg escape with a frequency of 6.48% (7/108).

Genotyping, subgenotyping and serotyping. According to our results, HBV-D/D1 was observed to be the major genotype/subgenotype with a prevalence of 106/108 (98%), and *ayw2* was the major serotype that accomplishes 99% (96/108) in Turkish Cypriots (Table IV). Also, it is important to mention that only 1/108 (1%) Turkish Cypriot were infected with HBV-D/D2, and 1/108 (1%) Turkish citizen were infected with HBV-E. A significant finding is that HBV-D/D2 was the only *ayw3* serotype 1/108 (1%).

Discussion

In our former research the following HBV genotypes were found, namely: D/D1; 70.6%, D/D2; 5.9%, D/D3; 1.5%, A/A1; 7.4%, A/A2; 2.9%, and E; 11.8% (Sayıner and Abacıoglu 2010; Arikan et al. 2016). However, in this study, D/D1 was found in 98% of the
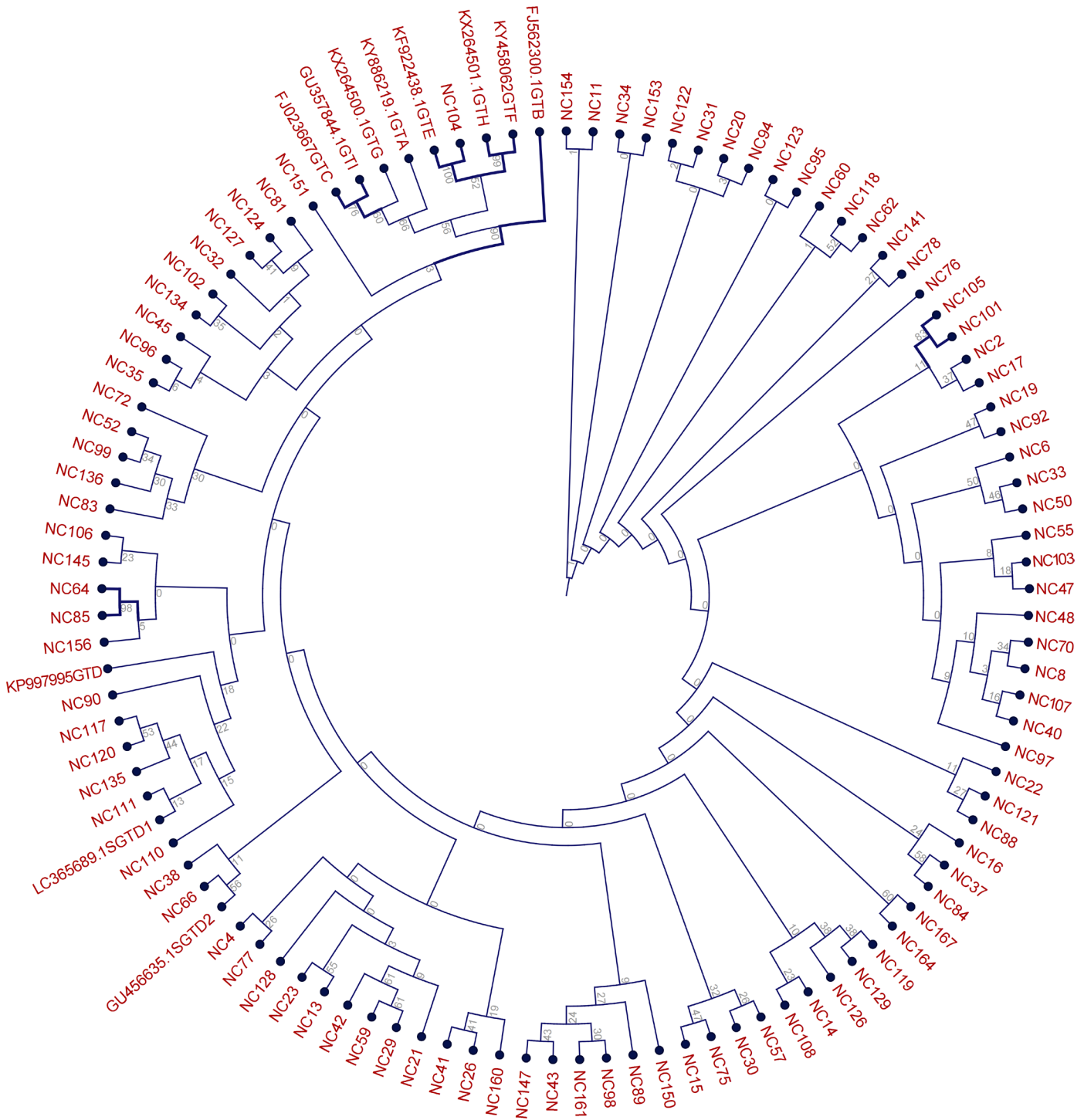
Fig. 1. Circular cladogram of HBV genotypes and subgenotypes. The phylogenetic tree was constructed using the CLC sequence viewer (CLC bio A/S, Qiagen, Denmark). The HBV reverse transcriptase region length was 495 base pairs in the alignment. The Neighbour-Joining and Jukes-Cantor methods were used. The Bootstrap value was chosen as 1000. HBV genotype A: KY886219.1, B: FJ562300.1, C: FJ023667 D: KP997995, D/D1: LC365689.1, D/D2:GU456635.1, E: KF922438.1, F: KY458062, G: KX264500.1, H: KX264501.1, I: GU357844.1 reference sequences were obtained from GenBank.

samples, and D/D2 and E only in 1% of the samples examined, respectively (Table IV). The most often detected genotype was D/D1 for both patient groups. This is the only similarity with our previous works and the main dissimilarity we observed in this study was that D/D2 was found in a Turkish Cypriot, and

a Turkish person was found to have genotype E, which has not been previously observed (Arikan et al. 2016).

Regarding other than Mediterranean region, high rates of genotype D have also been observed in the Middle East, South Asia, and North-East Europe (Sunbul et al. 2014; Zehender et al. 2014). Our results support
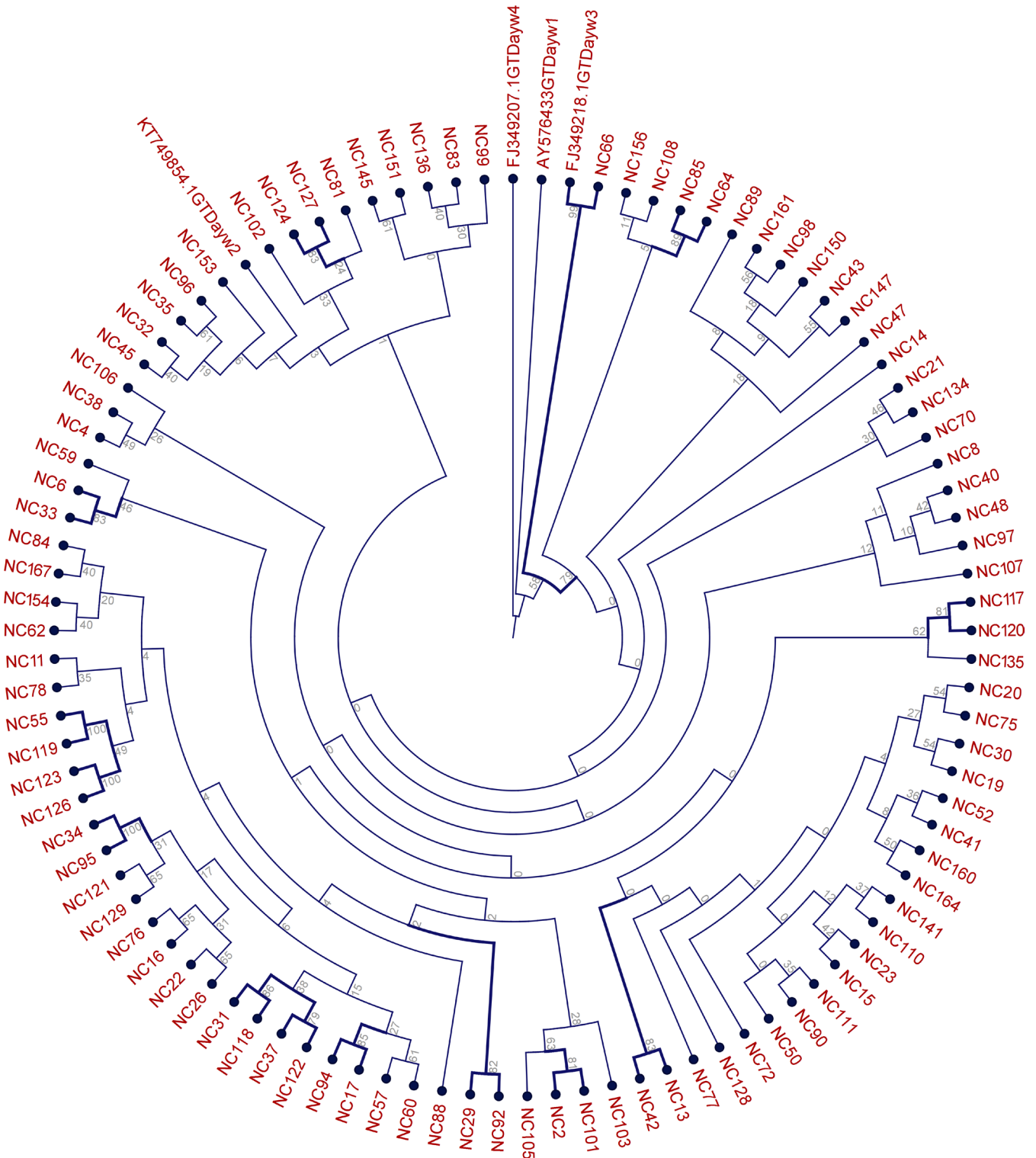
Fig. 2. Circular cladogram of HBsAg serotypes. The phylogenetic tree was constructed using the CLC sequence viewer (CLC bio A/S, Qiagen, Denmark). The HBV reverse transcriptase region length was 495 base pairs in the alignment. The UPGMA and Jukes-Cantor methods were used. The Bootstrap value was chosen as 1000. HBV D Serotype *ayw1*: AY576433, *ayw2*: KT749854.1, *ayw3*: FJ349218.1, *ayw4*: FJ349207.1 reference sequences were obtained from GenBank.

these findings as the majority of the students, sex workers, and labor workers mostly travel and immigrate to the TRNC from Europe, Turkey, and Africa. Therefore, we can state that genotype D was introduced into the society via migrations in the past several decades from

these regions, while other genotypes such as the serotype E could also be observed in the future (Zehender et al. 2014). In 2018, the article by Velkov et al. (2018) has been published that presents the global genotype distribution of HBV, assessing 125 countries and over

Table II
HBV *pol* gene mutation pattern and frequency in the study patients.

Mutation characteristic	Mutation pattern	Nucleos(t)ide analogue	Patient, n (%)
Primary resistance mutation	rtM204I	LAM, LDT, L-FMAU, FTC	2 (1.85)
	rtI233V	ADV	1 (0.92)
Total*	–	–	3 (2.78)
Partial resistance mutation	rtL80I	LAM, LDT	1 (0.92)
	rtL180M	LAM, LDT, L–FMAU, FTC	2 (1.85)
Total*	–	–	3 (2.78)
Compensatory mutation	rtL91I	LDT	7 (6.48)
	rtQ149K	ADV	7 (6.48)
	rtV214A	LAM, L-FMAU, FTC, TDF	2 (1.85)
	rtQ215H/P/S	LAM, L-FMAU, FTC, TDF	18 (16.67)
	rtN238D	ADV	6 (5.56)
Total*	–	–	29 (26.85)
ADAPVEM	rtM204I/sW196L	LAM, LDT	2 (1.85)
Total*	–	–	2 (1.85)

Abbreviations: LAM – lamivudine; LDT – telbivudine; L-FMAU – clevudine; FTC – emtricitabine;
TDF – tenofovir; ADV – adefovir; ETV – entecavir;
ADAPVEM – antiviral drug-associated potential vaccine escape mutant.
*Total: the number of total included *rt* gene mutations in 108 sequenced samples. The aa position 250 is where
we expect a specific mutation to occur, two unknown mutations rtM250G/H detected were ETV related
amino acid substitutions; these are not mutations which cause nucleostide resistance.

Table III
HBsAg escape mutations in the study patients.

HBsAg escape mutation category	Mutation pattern	Patient, n (%)	Combined pattern	Patient, n (%)
HBIg escape	sP120T, sQ129H, sM133I, sY134N, sD144E, sC147S	7 (6.48)	sM133I + sD144E	1 (0.92)
Vaccine escape	sP120S, sQ129H, sS143L, sD144E, sC147S, sS193L	9 (8.34)	sT126S + sS193L	1 (0.92)
HBsAg misdiagnosis	sP120T, sP120S, sR122K, sT131I, sM133I, sC147S	10 (9.25)	–	–
Immune escape	sQ101H, sG119R, sP120T, sT123N, sT131N, sY134F, sD144E	9 (8.34)	sG119R + sT123N	1 (0.92)
Total*	–	17 (15.74)	–	3 (2.78)

* The total number of patients which HBsAg mutation was detected.

Table IV
HBV genotypes, subgenotypes, and HBsAg serotypes of the samples.

HBV genotype	HBV subgenotype	Patients, n (%)	HBsAg serotype, n (%)*	Nationality
D	D1	106 (98)	*ayw2*, 96 (99)	TR, TRNC
	D2	1 (1)	*ayw3*, 1 (1)	TRNC
E	–	1 (1)	–	TR
Total	–	108 (100)	97 (100)	–

Abbreviations: TR – Turkey, TRNC – Turkish Republic of Northern Cyprus.
*Genotype E strain and short sequences (n = 11) were not included in serotype analysis.

900 publications. Their findings indicated that genotype D was dominant in Eastern Europe, the majority of Asia and North Africa. HBV genotype distribution shows a similar pattern among the countries in the same region but varies amongst different parts of the world.

Large population migrations can modify public health dynamics. High frequency of genotypes A-D was observed in North America following migrations from Asia and Europe. A similar situation was observed in the Caribbean where genotypes A and D were found as a result of migrations from the African continent (Velkov et al. 2018; Al-Sadeq et al. 2019). We can see that migrations mainly from Turkey for working and living, and other parts of the Middle East for other purposes such

as studying have caused genotype D to be dominant and new genotypes such as E introduced to the TRNC.

In this study, a total of 3/108 (2.78%) primary, 3/108 (2.78%) partial and 29/108 (26.85%) compensatory mutations were observed in the *rt* gene (Table II). However, previously, primary/partial resistance mutations occurred with a frequency of 1% and compensatory mutations were of 37% (Arikan 2015). When analyzed in greater details, rtM204I, rtI233V, rtL80I, and rtL180M mutations were not detected before, and these mutations, particularly rtL80I and rtL180M, restore the activity of viral polymerase to near wild type levels, which helps to promote the replication of mutants (Lazarevic 2014). This indicates that treatment naïve population is prone to such mutations, and has a significant impact on the treatment procedures and costs. Also, primary/compensatory mutations alone may increase HBV DNA levels and cause failure in future treatment (Sayan 2010; Sayan et al. 2010; Sayan et al. 2011).

The *S* gene mutations; however, indicate different structure when compared to earlier work. In our study, the total number of the *S* gene mutations was 17/108 (15.74%), and combined *S* gene mutations were 3/108 (2.78%) (Table III). The previous work revealed the frequency of 29% and 9%, respectively (Arikan et al. 2016). HBIg selected escape mutations in former work was 6%; however, in this study, different mutation pattern is observed with a similar percentage of 7/108 (6.48%); (Table III) (Arikan et al. 2016). sQ129H, sM133I, and sY134N mutations are associated with occult infection with D genotype; also they impair *S* protein secretion (Lazarevic 2014). HBV vaccine escape mutations in the prior work were observed with the frequency of 10% (Arikan et al. 2016). In this study, it was observed in 9/108 (8.34%) of the samples analyzed (Table III). Hepatitis B misdiagnosis mutation patterns in earlier work were only 4%, whereas in this study it was higher, and accounted for 9.25% (10/108) (Table III). Lastly, immune escape mutations in previous work were as often as in 24% of the samples. On the other hand, in this study, only 9/108 (8.34%) samples carried these mutations (Table III) (Arikan et al. 2016). Combined HBsAg mutations in this research were dissimilar to earlier study as 9% of the samples had such mutations, but in this study only 3/108 (2.78%) samples carried them (Arikan 2015). In summary, *S* gene mutations may lead to misdiagnoses (false-negative results) and cause insufficient protection using HBIg.

In the study by Al-Sadeq et al. (2019) performed in the Middle East and North Africa region, *S* gene mutations were detected in Egyptian, Saudi, Palestinian and Tunisian patients, in whom the genotypes B, D/D1, D/D3, and D/D7 were identified. We have detected only five common mutations and all the *S* gene mutations were observed only in D/D1 patients (Table III). This indicates that different geographical regions may have different *S* gene mutation profiles, even though the genotypes of the patients are the same. The ADAPVEM analysis revealed 2/108 (1.85%) of the samples carried such mutations. These were rtM204I/sW196L mutations (Table II). In Turkey, the same mutation pattern was observed in 8.7% of the patients together with other ADAPVEMs. The ADAPVEM status has not yet been known for the TRNC, these results are initial data for monitoring of such mutations in the future. (Sayan et al. 2013; Asan et al. 2018; Ozguler and Sayan 2018).

In conclusion, HBV-D/D1 was the dominant strain, and *ayw2* is the serotype most often detected among Turkish Cypriots. Cyprus is an island located in the Eastern Mediterranean region, a strategic location for human trafficking and immigration, and as a result of this reputation, it is necessary to analyze HBV phylogenetically for international and local importance. However, data from Greek Cypriot is necessary, as it would enable a complete island survey to be performed. With this work, we believe that we have set the ground for further research of this topic.

One of the limitations of this study is a sample size, as larger samples will generate more significant results. The lack of prior work is another limitation as there is only one previous study, and additional work will uncover significant results in the future. Another limitation is that information is not available about the HBV infection status or phase of the patients as they were not follow-up patients.

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