Epidemiological study of patients with hepatitis E virus infection in Catalonia: a translational study of genotype 3 subtypes

Master in Zoonosis y One Health (Una Sola Salut)

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Content

Abstract

Hepatitis E virus (HEV) is a non-enveloped single stranded RNA virus member of the *Hepeviridae* family and genus *Orthohepevirus*, which is a common cause of acute hepatitis worldwide and, in high-risk groups, can develop into a chronic infection.

An epidemiological study of the from Hospital Universitari Vall d'Hebron (HUVH) HEV Data Base was carried out to study which is the most probable source of infections, risk factors, and diagnosis confidence test based on clinical data. Our results suggest that there HEV infection is associated with old age and/or immunocompromised status. A deeper analysis should be performed. Interestingly, our results show that HEV subtyping diagnosis classification should be improved, especially to classify HEV genotypes 3 infected patients. Sliding windows analysis was used to identify a short region of 400 nucleotides able to confidently classify genotype 3 genomes into subtypes. From the clinical analysis, we identified an HEV food-borne infected patient who got infected in 2012 and developed a long-lasting chronic infection having still HEV-RNA positive detection in 2017 samples. Surprisingly, this patient had HEV-RNA serum negativization periods (between 2014-2015). To study whether this patient had got multiple infections or whether it was the result of reactivation of the original infection, a sample from 2012 before starting Ribavirin treatment (pre-treatment) and a sample obtained in 2016 (post-treatment) were compared using Next Generation Sequencing (NGS).

Reactivation of the original infection was demonstrated by phylogenetic and genetic distance studies. This result has important clinical implications, since any patient that develop a chronic infection, despite negativization of circulating HEV-RNA in serum sample, should be followed up for several months or years with highly sensitive test before assuring that HEV infection has been completely resolved. Also, one described resistance-variant was observed and a list of variants were identificated in pre-ttm and post-ttm samples.

This study is a translational study which has a clinical relevance and which opens new lines of research for new future projects.

Introduction

Hepatitis, or inflammation of the liver, is a global health problem that results in injury and destruction of hepatocytes, leading to liver malfunction. Although hepatitis is usually caused by viral infections, other causes such as autoimmune diseases, drugs or toxins can also lead to hepatitis (WHO, 2016). Nowadays, five types of viral hepatitis are known: hepatitis A, B, C, D and finally hepatitis E, which will be discussed below.

Hepatitis E virus (HEV) is a non-enveloped single stranded RNA virus member of the *Hepeviridae* family and genus *Orthohepevirus*. HEV infection is a common cause of acute hepatitis worldwide (Holla et al., 2013; Wedemeyer et al., 2012) and, according to the Center of Disease Control and Prevention (CDC) and the World Health Organization (WHO), 20 million people get infected with HEV annually with 3 million of symptomatic infections (Wedemeyer et al., 2012).and 57 thousand estimated deaths (CDC, 2016; WHO, 2016).

Usually, numerous HEV outbreaks have been reported in regions where sanitation status is low, like developing countries, where it is probably more common than hepatitis A (Khuroo et al., 2004). However, few outbreaks have been recently documented in industrialized countries in Europe and other parts of the world like Japan (Cao and Meng, 2012), USA (Ditah et al., 2014; Faramawi et al., 2011), and recently UK (Cook et al., 2017; Meader et al., 2010).

Several studies have reported that HEV infection is mainly subclinical (Lenggenhager and Weber, 2017) showing a high prevalence of immunoglobulin (Ig)G anti-HEV among the general population. For instance, recent data from Europe shows IgG-HEV prevalences of 6.8% in German blood donors (Pischke et al., 2014), and 8% of Catalonian adults had a history of HEV infection (Buti et al., 2006; Buti et al., 2008). More recently, Sauleda et al reported a 10.7% /20% of seroprevalence, depending of the testing methodology, in 10.000 Catalonian donors (Sauleda et al., 2015). Therefore, HEV infection is usually self-limiting, but it has been considered a critical health concern especially for high-risk populations including pregnant women (Perez-Gracia et al., 2015; Pischke et al., 2014), patients with liver problems and immunocompromised patients (Wedemeyer et al., 2012). In these high-risk groups, an HEV infection could develop acute fulminant hepatitis that evolve into a chronic infection, which may progress to cirrhosis or even death (Fujiwara et al., 2014).

Virus structure

The HEV genome (~7,2 kb) is composed of a set of three overlapping open reading frames (ORFs), 5' and 3' untranslated regions (UTRs), and two essential cis-reactive elements (CRE), all of which are essential for HEV replication (Tong et al., 2016).

ORF1

This fragment is the most extensive (5kb) and heterogenetic one (Fry et al., 1992), which code for non-structural proteins involved in the replication and processing of viral proteins. These proteins are RNA-dependent RNA polymerase (RdRp), RNA Helicase (Hel), methyltransferase (MeT), papain-likecystein protease (PCP), and a hypervariable region that shows high levels of nucleotide variation between HEV isolates (Pérez-Gracia et al., 2014) **(Figure 1).**

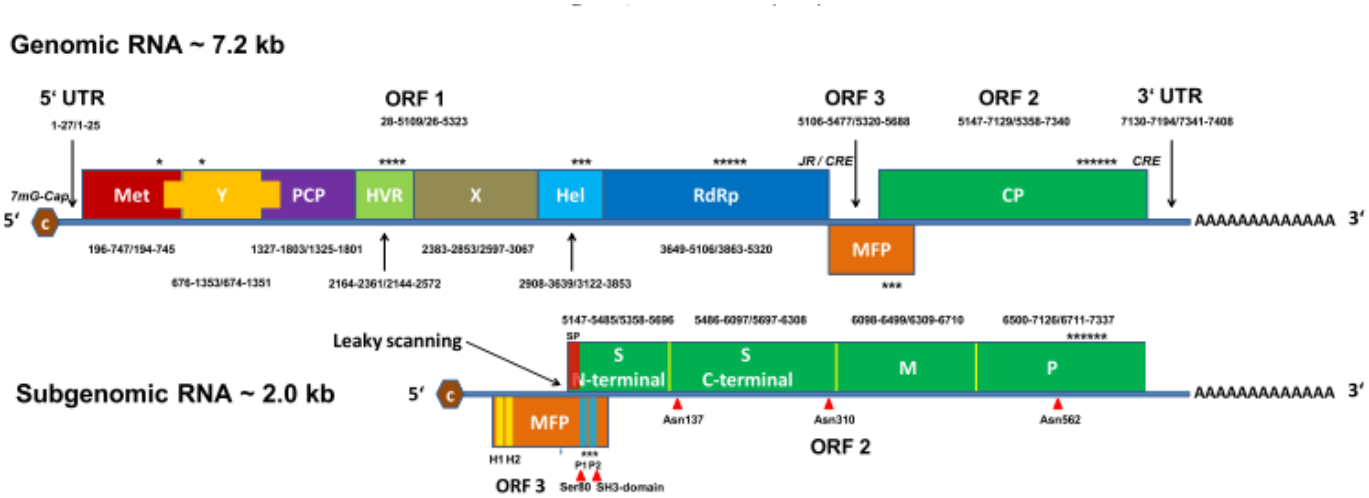


Figure 1: Description of the genome of hepatitis E virus and viral proteins. Nucleotide positions are relative to the HEV-1 Burmese strain (Acc. No. M73218) / HEV-3 47832 strain (Acc. No. KC618402). Figure extracted from Tong et al. (Tong et al., 2016).

ORF2

ORF2 extends almost 2kb and it is separated by 38 base pairs from ORF1 in 3' direction. ORF2 codes a structural protein that results in the virus's capsid. This structural protein consists in 3 domains: S domain, that forms de capsid, and P and M domains, that are involved in the virus-host cell interaction (Pérez-Gracia et al., 2014).

ORF3

This fragment has a length of 369 base pairs and it is partially overlapped with ORF1 and ORF2 **(Figure 1).** It codes for a structural phosphoprotein related to HEV pathogenicity (Chandra et al., 2008; Jameel et al., 2008).

Mutations

Some studies showed that the HEV genome sequence is quite stable (Arankalle et al., 1999) with high genomic homology among isolates from the same outbreak, and serial passages in animal models did not result in genetic drift (Worm et al., 2002).

However, the HEV mutation rates were estimated indirectly from clinical isolates as 1.40-1.72x10^{-3} base substitution per site per year and were similar to those reported for hepatitis C viruses (Takahashi et al., 2004). Sequence variability is not uniformly distributed along the HEV genome and some regions are highly polymorphic, for instance the hypervariable region located in ORF1. High variability and frequent selection of mutations in the HEV genome can be explained by transcription process, antiviral drugs selection pressure and host immune responses (Lhomme et al., 2014).

Molecular epidemiology of HEV genotypes

ORFs can have different arrangements that code for different proteins, resulting in different genotypes. At present, HEV has been classified into four main established and 3 newly discovered (Osterman et al., 2012). HEV genotypes 1 to 4 are globally distributed into different epidemiological patterns based on socioeconomic factors and ecology (Wang et al., 2013).

HEV-1 and HEV-2 infect only humans and are endemic in areas where fecal-oral transmission happens via contaminated water, and there are poor resources and low sanitation, such as Asia (in which HEV-1 is the most prevalent), Africa, India and Mexico (Colson et al., 2012). This results in repeated sporadic cases and large outbreaks.

By contrast, genotype 3 (HEV-3) and 4 (HEV-4) are zoonotically transmitted in developed countries such as USA (Ditah et al., 2014) and several European countries. Sporadic cases have been reported (Cook et al., 2017; Kamar et al., 2014). These genotypes infect humans and wild and domestic mammals such as pigs, rabbits, deers and wild boars and, while HEV-4 is mainly prevalent in Asia, HEV-3 is spread worldwide. Both genotypes are transmitted through contact with infected animals and by consumption of contaminated raw or undercooked meat or shellfish (Cook et al., 2017).

At present, 3 new genotypes have been described. HEV-5 and HEV-6 are genotypes exclusive for animals, and cannot infect humans. They are described in rabbits, wild boars, and swines.

(Geng et al., 2011; Takahashi et al., 2014; Zhao et al., 2009). Furthermore, a new HEV genotype was discovered in dromedary camels in Arabia and in the United Arab Emirates, and was identified as HEV-7 (Khuroo et al., 2016). Recently, a new HEV genotype HEV-8 was proposed to be the one identified in Bactrian camels in China (Woo et al., 2016).

Mode of transmission

As regards the form of transmission, HEV can be transmitted through different modes. HEV is mainly transmitted by fecal-oral route, which is associated with contaminated water due to the usage of untreated river water in developing countries and industrialized ones (Ahmad et al., 2010; Ceylan et al., 2003). In addition to this form of transmission, there are other emmerging ones such as vertical transmission (pregnant woman to her fetus) and from transfusion of infected blood products (horizontal transmission), being the first one very common in developing countries, and the second one spread worldwide (Riveiro-Barciela et al., 2017; Sauleda et al., 2015).

Likewise, food-borne transmission (or zoonotic transmission) has also been reported after consumption of meat products obtained from infected HEV pigs and deers (Minuk et al., 2007). In fact, some articles present a phylogenetic demonstration of hepatitis E infection transmitted by pork meat ingestion in Catalonia (Riveiro-Barciela et al., 2015) or relate infection with consumption of Figatellu sausage in France (Colson et al., 2010). In Japan, consumption of undercooked or raw pork in a restaurant was associated with three cases of HEV infection (Miyashita et al., 2012) and recently in UK, hepatitis E virus is known as Brexit Virus, because of HEV infection cases which are emerging by consumption of sausages with infected meat from Europe and its time coincidence with politics situation of the country (Cook et al., 2017).

Laboratory diagnosis of HEV and treatment

There are some serological and molecular techniques for HEV detection. These techniques include detection of antibodies, proteins and viral genome. Detection of anti-HEV antibodies such as anti-HEV IgM or anti-HEV IgG, provides a good understanding of the infection, but a positive test does not reflect the presence or absence of HEV RNA in blood. In fact, it may cause a misdiagnosis of HEV in many cases due to the affectation of the seroprevalence of HEV by the sample size, type of assay, etc.

Because of this, HEV RNA detection is the best way to uncover viremia (Gupta et al., 2013; Hewitt, 2014; Vollmer et al., 2014). This is performed using a real-time PCR, so value of cycle threshold (CT) is used to make a conversion to know samples viral load using a standard sample with viral load and CTs known provided from WHO (Baylis et al., 2011) to make possible samples quantification.

Therefore, a good diagnosis of infection will be based on serology, blood viral load (RNA) and biochemical data. The most important biochemical data to consider are the values of aspartate-aminotransferases (AST) and alanine aminotransferases (ALT), which will have high levels if they exceed 35 IU/L. Bilirubin levels (0.30-1.20 mg/dl), hemoglobin (12.0-15.0 g/dl) and platelets (140-400 $x10^9$/L) may also be taken into account.

Nevertheless, there is not a FDA-approved serological assay available to accurate clinical diagnosis of HEV, so seroprevalence and detection of HEV-RNA are the best available measures to know more about HEV infection (Kodani et al., 2016; Rossi-tamisier et al., 2013; Wenzel et al., 2013).

With regard to the treatment of HEV infection, it is limited to two different drugs: Sofosbuvir (SOF) and Ribavirin (RBV). SOF is a medication used in combination with other drugs, such as RBV (Dao Thi et al., 2016), simeprevir, ledipasvir, etc., for the treatment of hepatitis C chronic infection (Gallego et al., 2016). SOF inhibits RNA-dependent RNA-polymerase activity (NS5B protein) and has been recently used to treat HEV chronically infected patients.

The most extensively used treatment for chronic HEV infection has been RBV, which has been also applied to treat other viral infections such as RSV infection, hepatitis C, and viral hemorrhagic fever. This drug can be used in monotherapy (Gouttenoire et al., 2016) or

combined with other drugs such as SOF (Dao Thi et al., 2016). Some studies have shown that RBV is a mutagenic drug (Perales et al., 2015). Recently, it has been reported that mutations in the RNA polymerase which affect its replication capacity (Debing et al., 2014; Debing et al., 2016; Lhomme et al., 2016). may cause treatment failure in Organ Transplant Recipients (Debing et al., 2014; Debing et al., 2016). This mutagenic characteristic has been seen In Vivo (Todt et al., 2016). Some of these "Ribavirin-resistance" mutations are Y1320H, K1383N, D1384G, K1398R, V1479I, Y1587F and G1634R, which are associated with viral replication (fitness), RBV sensitivity and virus infectivity (Debing et al., 2014; Debing et al., 2016; Todt et al., 2016).

Objectives

1. To identify possible risk factors associated with acute HEV infection.
2. To determinate whether a patient HEV food-borne infected in 2012 which is still positive in 2017 after periods of HEV-RNA negativization is the result of multiple infections or a reactivation of the same virus becoming a long-lasting chronic infection.
3. To improve HEV subtyping diagnosis classification of HEV genotypes 3.
4. To study by NGS, the presence of RBV HEV-resistance mutations in the chronically infected patients that had failed to RBV treatment.

Patients and Methods

1.Epidemiological study:

A Deep analysis of the Clinical HEV Data Base from Liver Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH) has been performed. 2050 patients, who had HEV serology tests, were selected. Starting from these data, 95 patients with HEV infection have been analyzed to study the putative HEV source of infection and risk factors, and also to assure that they have been properly characterized as HEV real infected patients. Clinical histories and laboratory data were analyzed to obtain information of interest.

All HEV-RNA positive patients were treated with RBV. In all HEV acute patients, RBV treatment caused a decrease in viral load until undetectable levels. However, one patient foodborne infected, despite initial treatment in 2012 with RBV developed a not clear chronic infection since HEV-RNA was undetectable in some samples but reappears in others. HEV-RNA has been detected in some samples from 2016. it is not clear whether the patient has followed a continuous chronic infection with fluctuating increases and decreases of viral load or whether this fluctuating pattern is the product of multiple reinfections.

All samples previously reported as indetectable were retested by an HEV-RNA ultrasensitive assay (COBAS 6800 Analyzer-Roche diagnostics, sensitivity 10 IU/mL) currently available in the Liver Pathology Laboratory (Biochemistry and Microbiolgy, confirming that HEV-RNA was indetectable

2. Molecular study:

2.1.HEV Genotyping and subtyping

2.1.1Selection of patient and samples

Selected study patient was a man of 67 years old from Granada who lives in Catalonia since he was 17 years old, and who was diagnosed with hepatitis E virus in 2012 with a possible food-borne origin of infection for consumption of non-bottled water and raw vegetables in Istanbul, Turkey.

After been diagnosed with HEV in 2012, this patient was treated with RBV in doses of 200 mg/d for 90 days. On May 2014 his viral levels in blood were undetectable and it was diagnosed as a

chronic E hepatitis healed. On January 2015 viral RNA presence in blood was positive and on January 2016 was included in a clinical study. On January 19, 2016 the patient started a second treatment with RBV in doses of 200 mg/d for 24 weeks. At present, HEV-RNA still detected in his blood.

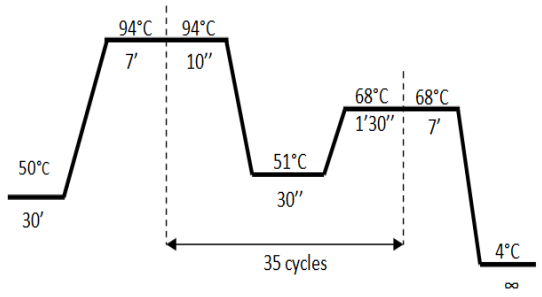
Two different samples of this patient obtained from Liver Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH) were coded according to confidentiality rules and analyzed. First sample correspond to 2012 basal sample, obtained the same day in which the first RBV treatment started (pre treatment sample). The second sample corresponds to a sample obtained on January 2016, before starting the second RBV treatment (post treatment sample). Both samples had a viral load of $1x10^6$ and $5x10^6$ respectively. In addition, three more HEV-RNA positive samples from the same patient were analyzed. One corresponded to a sample from November 2012, which was first one with RNA-HEV positive; the second one was from February 2014 and third one from 12 weeks Follow-up of 2016.

2.1.2RNA extraction

For the extraction and manual collection of viral RNA, 140 µl of serum was used using QIAmp Viral RNA Mini Kit, following instructions of the manufacturers (Qiagen, Hilden, Germany) and eluting the RNA in a final volume of 30 µl. Standard measures to avoid contamination were strictly enforced (Kwok and Higuchi, 1989).

2.1.3RT-PCR

Reverse transcription was performed using OneStep RT-PCR Transcriptor kit (Roche Applied Science, Basel, Switzerland). In this reaction, complementary DNA or cDNA is obtained from previous RNA, since the kit contains an enzyme which first acts as reverse transcriptase. This first enzyme is then inhibited at 94 °C and a second polymerase is activated, resulting in the amplification of a specific region of this RNA according to primers that have been used (**Figure 2**).



MIX	1x (µL)
H_2O	28.5
Buffer	10
DMSO	2.5
P_{FW}	1.5
P_{RV}	1.5
Polymerase	1
RNA	5

Figure 2: RT-PCR program and components of Mix to amplify a target of 1.1 kb of RNA from hepatitis E virus samples.

The following primers were used to amplify a target of 1.1 kb, in ORF-2 region: forward primer HE1Chf6547 (5'- CCGACAGAATTGATTTCGTCGGC -3') and reverse primer HE1Chr7675 (5'- ACTCCCGRGTYTTACCYACCTT -3'). RT-PCR reaction was carried out with DMSO and other components named in **Figure 2.** DMSO prevent primers bind together to form dimers, increasing their affinity for target cDNA.

2.1.4 Nested PCR

A second internal or Nested PCR amplifying a shorter fragment was performed in order to obtain enough DNA for Sanger sequencing. This Nested PCR was performed using FastStart High Fidelity PCR system, dNTPack (Roche Applied Science, Basel, Switzerland) with same conditions, although in some cases it has been necessary to increase number of cycles up to 40 (**Figure 3**).

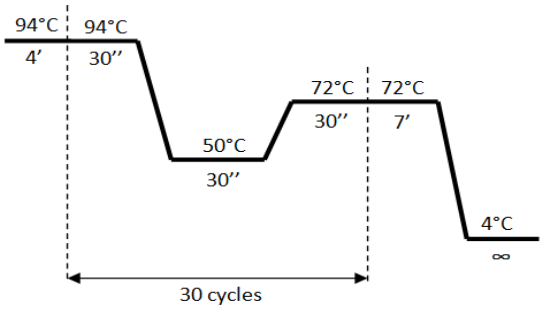


Figure 3: Nested PCR program to amplify the resulting DNA from RT-PCR to obtain shorter fragments to be read by Sanger sequencing.

The following primers were used to amplify a target of 400 pb: forward primer ECf6598 (5'- GTCGTCTCAGCCAATGGCGAGCC -3') and reverse primer ECr7009 (5'- CASARAANGTCTTNGARTACTGCT -3').

2.1.5 DNA purification

Amplification products were analyzed by electrophoresis on a 2% agarose gel. Negative controls (amplification in the absence of RNA) have been included in parallel to ensure absence of contamination (Kwok and Higuchi, 1989). With the aim of recovering DNA without residues from agarose gel, bands obtained on gel were cut out and the DNA purified using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), eluted in a final volume of 30 µl and finally stored at -20 ° C.

2.1.6 Nanodrop quantification

Once the DNA was recovered from agarose gel, it was quantified using Nanodrop (Desjardins and Conklin, 2010), a total spectrum spectrophotometer that measures the concentration of dsDNA in 1.5 µl of sample.

2.1.7 Sanger sequencing

Purified DNA fragments with a concentration of 100 ng/µl recovered from PCR amplifications were Sanger sequenced. We performed a bidirectional sequencing process, obtaining for each sample two sequences with forward and reverse primers, respectively. Primers used were the same as those used in the Nested PCR but in a concentration of 5 µM.

2.1.8 Alignment and analysis

Pre treatment and post treatment sequences obtained from Sanger sequencing were analyzed in order to correct possible errors, and were aligned, using GeneDoc Software (Nicholas and Nicholas, 1997). To classify the subtype of pre-treatment sample (2012) and post-ttm (2016) 40 reference GenBank sequences with well HEV subtype classification and two previously classified local samples (Hepatic Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH)) were included in the study.

2.2.Primers design for HEV Genotype 3 sybtyping as a diagnosis tool.

2.2.1 Sliding window

Genotype 3 is the most common HEV genotype in Europe, but a good diagnosis tool for HEV correct subtyping classification has not been developed. To develop a translational assay 76 complete well classified sequences from different genotypes, with a highest prevalence of genotype 3 sequences, published by Smith et al in two reference manuscripts (Smith et al., 2013; Smith et al., 2015) were downloaded. The alignment of the whole genome from selected

genotype 3 isolates was subjected to a sliding window analysis to find out which is the best genomic region, along the whole genome that better discriminate with the highest quality between genotypes, in case they are different from genotype 3, and between subtypes, in case they are genotype 3.

To calculate the distances between pairs of sequences, a model of substitutions like Kimura's, which describes the probabilities of substitutions between pairs of nucleotides, was performed. This model was used to determine matrix of genetic distances between set of sequences in order to make a multidimensional scaling (MDS) which project a cloud of points on 3 main components to obtain in reduced dimensions a map of distance between objects (Borg and Groenen, 2005). This method represents an alternative to phylogenetical trees and allows a better display of distances between pairs of sequences. Finally, substitutions number was determined between pairs of sequences in order to know in which part of the sequence are more differences with other sequences.

2.2.2Primer design

In order to improve diagnosis of HEV patients, and due to the lack of reliable specific primers to genotyping and subtyping of HEV samples, specific virus subtype primers have been designed. To perform a manual design of primers spanning all genotype 3 subtypes and all HEV genotypes, a selection of 51 sequences from the first sequence selection named before (**see 2.2.1 Sliding windows**) was used and aligned using GeneDoc (Nicholas and Nicholas, 1997), to make a manual nucleotide position count in each sequence but more thoroughly in neighboring regions of the sliding window selected as the most differentiating one.

A own made count colour code was used (**Figure 4**): red positions are those conserved in all 51 sequences, green positions those ones that have more than one nucleotide possible, being this position a degenerated one in primers, and yellow positions. This last category is referred to those positions where the most sequences have a specific nucleotide but 1-4 sequences have a different one. In this case, an analysis of which sequences had a different nucleotide was made in order to know if all were the same genotype, supposing a degenerated position on primers at the end. In the case that these positions aren't representative, they would be discarded and considered as conserved ones.

Pos	1870	1871	1872	1873	1874	1875
A	0	48	0	2	0	0
C	25	0	47	17	0	51
G	0	3	0	4	51	0
T	26	0	4	28	0	0

Figure4: Extraction of an analysis area for the design of primers. **Pos**: position

Once neighboring regions were selected, primers designed should must had characteristics numbered below:

- Primers length is between 18 and 25 pb.
- Number of degenerated bases is equal or under 4 bases per primer.
- Annealing Temperature (Ta) is equal or superior to 45ºC.
- Forward and Reverse primers have a similar percentage of C-Gs and less than a 60%.
- All primers have at least one Thymine (T) or Adenine (A) at the 5' extreme and 66% (8/12) have at least one Cytosine (C) or Guanine (G) at the 3' extreme. This situation improves primers joining to DNA, since T-A union is weaker than C-G union, facilitating joining even when there is a mutation into target.
- Low self-complementarity to avoid formation of dimers of primers.

2.3. Phylogenetic assay

2.3.1 Primer design

Due to the knowledge about mutagenic characteristic of RBV treatment (Debing et al., 2016), one of the aims of the present study consisted in investigating viral genome changes comparing pre-treatment (2012) versus post treatment to RBV 2016 samples. Thus, specific virus subtype primers have been designed to the Open Reading Frame 1 region (ORF1) of the genome, which corresponds to polymerase protein of the virus. This region, which is the most hypervariable one, has a length of 1671 pb (Tong et al., 2016). Since Next Generation Sequencing (NGS) (Hardwick et al., 2017) using MiSeq platform technology just allows a sequence length of approximately 400 nts to obtain high quality reads (sequences), six overlapping pairs of primers were designed to obtain fragments of 380-450 pb each one covering the polymerase gene placed into ORF1 region.

Chronic HEV samples were classified as subtype 3f, then, specific primers were manually desgined by aligning eight sequences extracted from our previous selection (Smith et al., 2013; Smith et al., 2015) using GeneDoc Software (Nicholas and Nicholas, 1997). In a neighboring zone of the region of interest it was necessary to look for conserved regions to design primers with the maximum probability of join the DNA of samples. After selecting a possible zone candidate for designing each primer, Oligocalculator Software (Kibbe, 2007) was used to know primers characteristics and confirm they were into criteria numbered before **(2.2.2 Primer design).**

Resulting primers were subtype 3f specific and are numbered in **Table 1.** Designed primers were ordered to TibMolBiol (Berlin, Germany).

Table 1: subtype G3f specific primers of HEV manually designed. Degenerations are: Y=T/C, R=G/A, S=G/C, W=A/T, M=A/C, B=G/T/C, D=G/A/T, H=A/C/T.

Region	Primer name	Sequence	Position	Length (pb)
1	HEVFW3346	TTY GAY GCY TGG GAR CGB AA	3346 - 3796	450
	HEVRV3796	ATC AAC ATY CCY CTG CTG TAT		
2	HEVFW3711	TYA RCC CYG GGA TTG TSC AYT A	3711 - 4120	409
	HEVRV4120	TAT GAG CTC RCA MAC ATC RGC		
3	HEVFW3989	TGA YCC HAA CCA GAT YCC TGC	3989 - 4418	438
	HEVRV4418	AAA TRC CRA CYT CRC GSA RYA GGC C		
4	HEVFW4317	TTA TYC ART CYT CCC GBG CMC A	4317 - 4706	389
	HEVRV4706	TAT CRG ACA CCG TRA GCT CC		
5	HEVFW4571	ATY AGT GCC TAY CAY CAR YTR GC	4571 - 4993	422
	HEVRV4993	TRC ARA GGT CRA GCT CRA GYA C		
6	HEVFW4934	TWG AGG CYA TGG TGG AGA A	4934 - 5376	442
	HEVRV5376	ATD AGC CAC TGR GGC ATG		

2.3.2 RNA extraction and RT-PCR

Once primers were designed, RNA extraction of both pre- and post treatment samples was performed following the same protocol explained before in section **2.1.2 RNA Extraction**. Reverse transcription was performed using OneStep RT-PCR Transcriptor kit (Roche Applied Science, Basel, Switzerland) but with a different program which add 5 seconds in each cycle of the process. (**Figure 5**). In this step a DNA target of 2 kb was amplified with two of 12 designed primers. These two primers were HEVFW3346 and HEVRV5376.

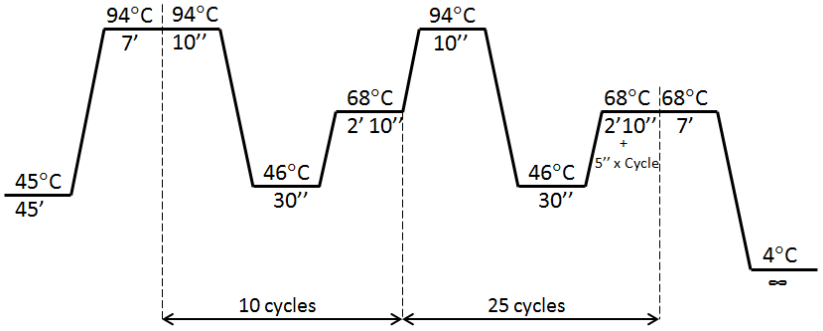
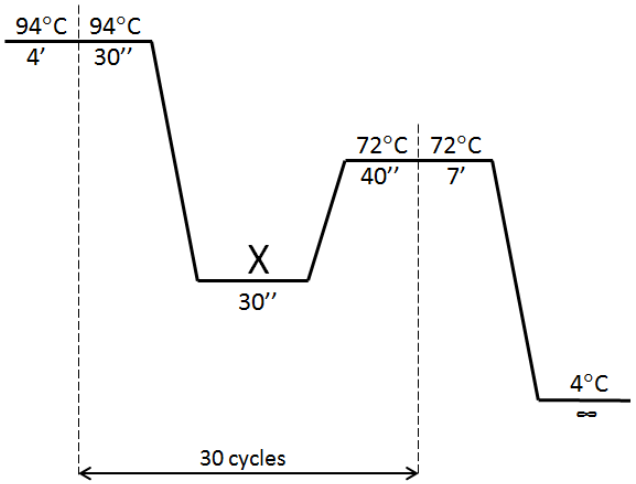


Figure 5: RT-PCR program which add 5 seconds in each cycle to amplify a target of 2 kb of RNA from hepatitis E virus basal samples.

2.3.3 Hemi-Nested PCR

A Hemi-Nested PCR was performed in order to obtain fragments of 380-450 pb using FastStart High Fidelity PCR system, dNTPack (Roche Applied Science, Basel, Switzerland). See **Figure 6**.



X	
T (°C)	Fragment
47	6
48	1,5
49	2,4
52	3

Figure 6: description of Hemi Nested PCR program with different temperatures of annealing due to different primers used. Primers Forward (FW) and Reverse (RV) of the same region had a similar temperature of annealing. Elongation time is adjusted to the length of resulting fragment, 380 - 450 pb.

2.3.4 DNA purification, quantification and Sanger Sequencing

Amplification products obtained from step before were analyzed by electrophoresis on a 2% agarose gel and purified using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), Nanodrop quantification and Sanger sequencing was performed as commented in **2.1.5 DNA purification, 2.1.6 Nanodrop quantification and 2.1.7 Sanger sequencing**. Sequences resulted from Sanger sequencing corresponding to each fragment were analyzed and aligned to each other.

2.3.5 Next Generation Sequencing (NGS)

To study the dynamics of HEV viral populations isolated in 2012 compared with the one after RBV treatment in 2016, and also to study the presence of already described resistance-associated mutations in the polymerase region, we performed NGS analysis of the polymerase gene.

DNA quality and quantification

To start library preparation for NGS (Hardwick et al., 2017), 2µL of both pre-and post treatment DNA samples were sent to Bioanalyzer (Agilent Genomics) to find out DNA quality in order to get the maximum efficiency in NGS (Hardwick et al., 2017). Later, samples were quantified using Qubit dsDNA BR Assay kit, following manufacturer's instructions.

Pool preparation and purification

With Qubit quantification results, two pools corresponding to six different fragments of each sample were made. These pools were later purified using Ampure Beads Agentcourt AMPure XP Beckman Coulter (ref#A63880), a technique based in the use of magnetic beads to the recovery and purification of amplicons obtained in a PCR, and remove primers excess, nucleotides, salts and enzymes with a wash process, obtaining the PCR final product without residues. Later, final product was newly quantified with Qubit as did before to know if pools had the optimal amount of sDNA (between 100ng and 250 ng) to End repair and A-tailing reactions.

End repair and A-tailing

Resulting fragments cut-out by primers may have cohesive extremes making adapters, short known and identifiable sequences useful to identify samples once NGS is over, not join well. In order to make a successful adapter joining, an End repair and A-tailing process of the fragment was performed in both pools, preparing reaction and following the program is shown in Kapa Hyper Prep for Illumina libraries protocol.

Adapter ligation a, post-ligation cleanup and library amplification

Two different adapters were selected to difference between pools. Product obtained from Ligation was newly purified using Kapa Pure Beads (Roche ref#KK8001) and following manufacturer's instructions. Amplification of the library was made following instructions explained in Kapa Hyper Prep for Illumina libraries protocol.

Post-amplification cleanup and Bioanalyzer

Once library is amplified; a second wash was performed in order to remove all not incorporated adapters and amplified short fragments. This second wash was made using Kapa Pure Beads following the same protocol as the first wash, and after that, pools were sent to Bioanalyzer (Agilent Genomics) to know their quality and newly quantify using Qubit dsDNA HS Assay Kit. Once it was quantified, nanomolar concentration (nM) was calculated to prepare a library with a concentration of 4 nM in an adequate volume not to take less than 1 µL.

Real-time qPCR quantification and library preparation

Pools also were quantified by real-time qPCR to, according to viral loads obtained, adjust concentrations to 4nM (in case they are too different from 4 nM) to know exactly which volume of it would be at final pool, depending on the desired coverage.

Library preparation was made following protocol "Denature and Dilute Libraries Guide" (Document No.15039740 vo1) and "MiSeq Reagent Kit v3 Reagent Preparation Guide" (Document No.15044983 Rev. B.) guide.

NGS Results analysis

Results extracted from NGS were analyzed with some pipelines to obtain haplotypes without errors and with population frequencies. Flash process was performed in order to join reads (Forward and Reverse) allowing at least three differences between sequences, and discarding those which have more than three. Resulting "Fasta" file was submitted to multiplex process to separate reads from samples and chain (Forward and Reverse). Later, some filters were use in order to take only reliable sequences. Some of these quality filters removed from the analysis those reads which did not cover the entire fragment to amplify, those ones which had bad quality because indeterminations or gaps, ones that were in very low frequencies and also ones that were unique sequences.

In addition, to determinate nucleotide alignments per sample, another filter was done to "Fasta" file to shape sequencing errors resulted from joining haplotypes Forward and Reverse chains. Consensus haplotypes were those ones that had a minimal abundance of 0,1% in both chains, and they were common between chains. Later, a second filter was done, which consisted in a filter of abundance, on those haplotypes present in less than a 0,5% were excluded. Final haplotypes included in the analysis were later translated to protein.

Resulting haplotypes alignments were analyzed to study the presence of resistance-associated mutations to RBV at a level of sensitivity of 1% (Tong et al., 2016). Haplotypes that passed all filters imposed, were aligned using GenBank LC055973 as a reference one. Once positions were fixed, described mutations (Tong et al., 2016) were searched in pre- and post samples and these ones were compared, due to the lack of a consensus sequence, to the master sequence (sequence which is in major proportion of population).

Results and discussion

1.Epidemiological study

A total of 95 patients included into the Data Base from Liver Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH), which had anti-HEV IgM serology positive, were analyzed. Clinical histories from these patients, firstly diagnosed as HEV infected ones, were investigated in order to know whether they were immunocompromised patients, had other viral infections, had possible foodborne origin infection and/or if they were correctly diagnosed.

Results show that although 95 patients were serologically diagnosed as acute HEV infection, eleven were incorrectly diagnosed since acute hepatitis infection was caused by Hepatitis B, Hepatitis C or Epstein-Barr viral infections, and not by HEV, resulting in 84 HEV real infections. From these, nine HEV infected patients were immunocompromised due to organ transplantation, being bi-pulmonary transplant the most frequent one (**Figure 7**). In addition, four patients proceeding from countries where Hepatitis E is endemic indicated that they ate undercooked pork meat, and/or raw vegetables or other type of meat. This result suggest that food-borne transmission could be the route of transmission similarly as what it was previously reported by the Vall d'Hebron group (Riveiro-Barciela et al., 2015).

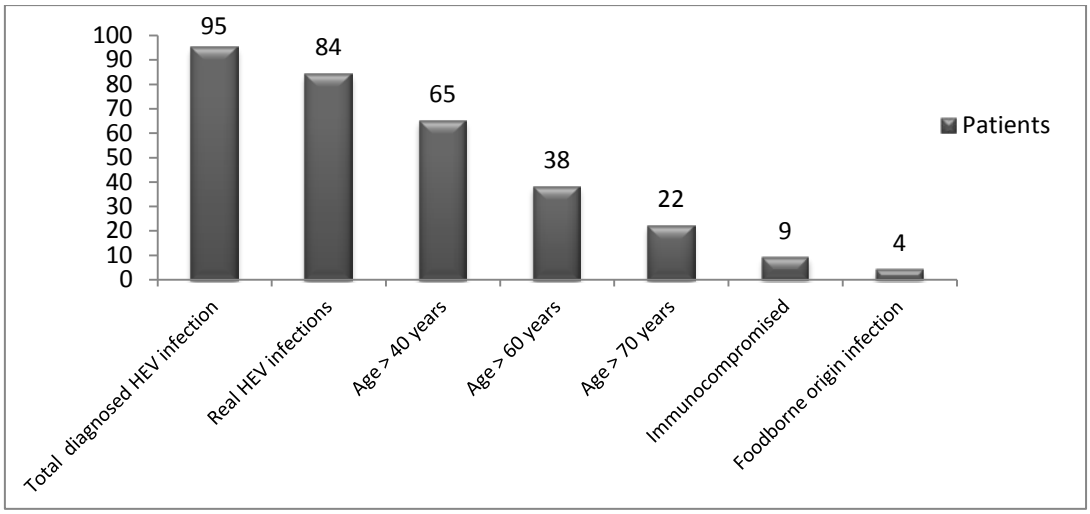


Figure 7: graphic representation of epidemiology data recollected from patients who were diagnosed with HEV infection. From 95 patients diagnosed, 11 were incorrectly diagnosed, 9 were immunocompromised, and 4 could have a foodborne origin. As regards age, 65 patients were older than 40, 38 patients from these 65 were older than 60 and 22 older than 70 years old.

As regards to age and gender, there was not a significant difference between male and female, being 42 (50%) female infected versus 42 (50%) males. However, age resulted to be a significant variant. 84 patients were subclassified in 3 groups depending they were or not older than 40, older than 60 and older than 70. A chi-square statistical analysis was performed comparing Data Base patients with Catalonian population (data extracted from Idescat). Results for age were significant in three groups with a significance level of 0,05; getting values of p-value 0,000861, 0,000032 and 0,000009 to groups of patients older than 40, older than 60 and older than 70 respectively. These results suggest that older age could be related with an increase in the probability to get HEV infected, compared to the rest of Catalonian population. Nevertheless, it can be dismissed that age is linked with other pathologies and disabilities.

Nevertheless, this study had a few limitations. In first place, due to the lack of a data analysis system capable of synthesizing such a complex cross-database, it was not possible to carry out the analysis of the 2050 patients who had negative serology or who were not asked for serology. It is necessary to transform data with the aim of making its analysis available.

Last but not least, this study has been carried out considering only as patients with infection those patients with anti-HEV IgM positive serological tests, not crossing these data with positive blood RNA tests. To perform a more accurate study, it is necessary to take into account both serology and viral load quantification in all patients from Liver Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH), as well as to have a control group corresponding to healthy patients without any pathology that may be linked to HEV infection.

Summarizing, this analysis shows a possible relationship between HEV infection and older age, immunocompromise status and foodborne origin. However, a deeper analysis should be performed. In addition, present analysis highlights the need to improve HEV diagnosis in order to correctly diagnose hepatitis viral infections. Public health measures must be pushed through to warn the population, especially at risk groups, of how to prevent hepatitis E infection.

2.Molecular study

2.1 HEV sequencing analysis

2.1.a Genotyping and Subtyping of the chronic samples

Pre-ttm and post-ttm HEV isolates were Sanger sequenced and phylogenetically classified (**Figure 8**) using 40 GenBank downloaded sequences with known genotype and subtype, and 2 local-control sequences from patients from Hospital Universitari Vall d'Hebron (HUVH) that had no relationship with the chronic patient.

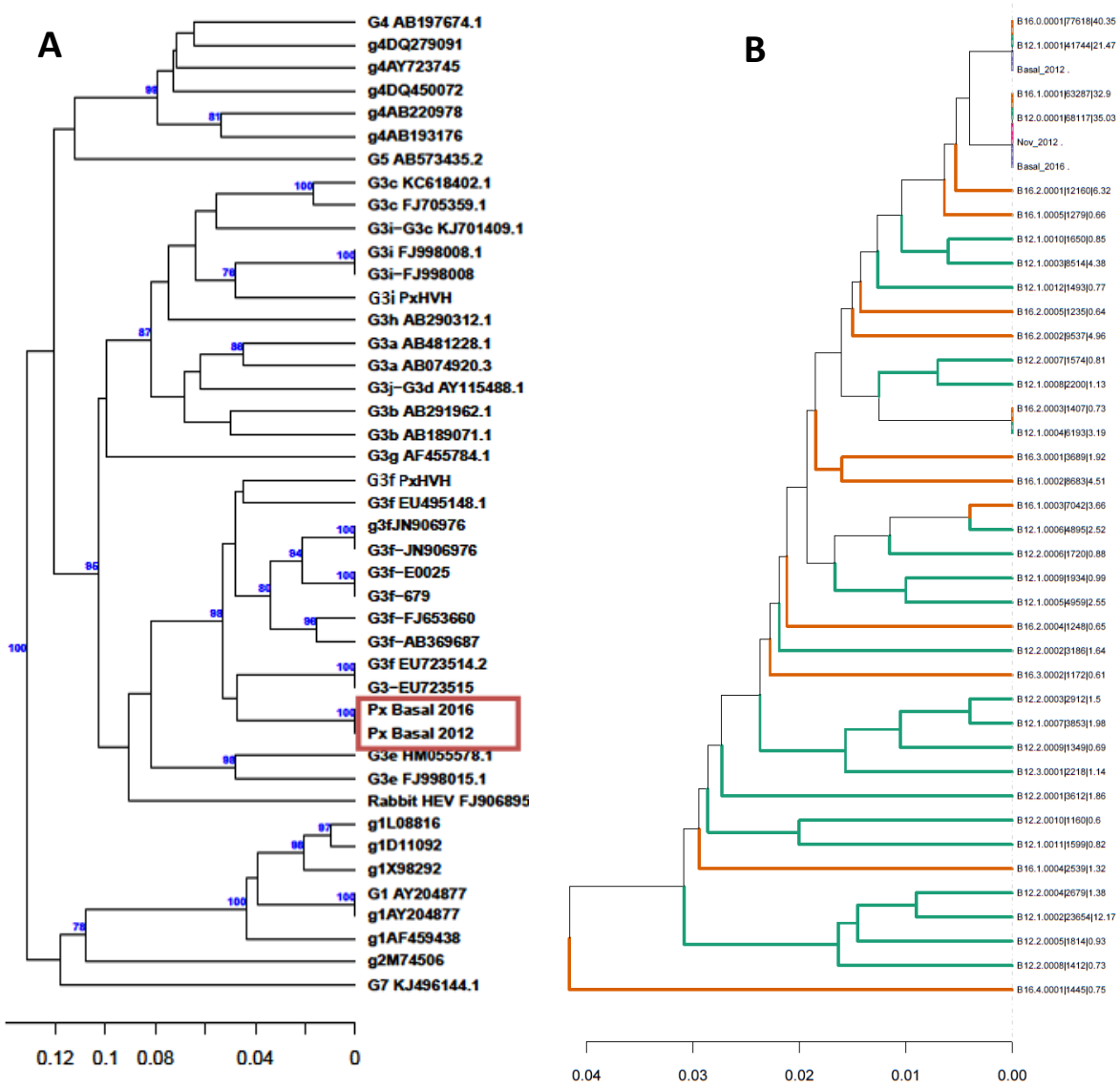


Figure 8: **A**: phylogenetical tree of K80 distances where pre- and post treatment samples are joined up in 100% of replicas of bootstrap and known genotype sequences are clustered in groups of the same genotype and subtype, even both sequences from Hospital database. **Px Basal 2012**: pre treatment sample. **Px basal 2016**: post treatment sample. **G3i/G3f PxHVH:** Hospital patients database samples. **B**: Phylogenetical tree for first fragment from polymerase region, where consensus sequences from November 2012 and post-ttm samples are clustered with

two haplotypes from 2012 and 2016, and also consensus sequence from pre-ttm sample. Pre-ttm (green) and Post-ttm (orange) haplotypes are clustered together, not showing a Bottlenecking effect. Genetic distances have a maximum value of 0,04.

In phylogenetical tree performed with consensus sequences, Pre-ttm(2012) and post-ttm(2016) patient sequences were clustered together in 100% of replicas performed between them, and both samples also form a cluster in 96% of replicas with known genotype 3f known sequences, meaning that study patient genotype was infected by HEV genotype 3 subtype f, which is one of the most common in Europe.

In addition, the fact that in 100% of replicas both samples (pre- and post-) have been linked suggest that HEV infection in 2016 was a reactivation of the same virus that started infection in 2012, despite having HEV-RNA negative result on May 2014. However, this should be confirmed using a population approach (NGS) and including local control subtype 3f samples.

A possible explanation for the undetectability of HEV-RNA on May 2014 was the limit of detection of HEV-RNA in blood samples using Cobas 6800 system (Analyzer-Roche diagnostics), which is an integrated qPCR system used in Liver Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH). System's sensitivity is 15 IU/mL what means that if sample has a viral load under that limit it will be diagnosed as non-infected. It is possible that in this case, sample from May 2014 has a viral load lower than 15 IU/mL. This result opens the discussion on the protocol that should be followed to declared curation on a treated chronic hepatitis. However, it must be kept in mind that this sensitivity is the highest in the market, being used in blood banks for testing donation useful for transfusions or blood products processing.

The first conclusion from this study, is that the patient was infected by subtype 3f. Besides, it has been observed that there is a lack to correctly classify genotype 3 viruses into subtypes, despite being the most common genotype in Europe (Perez-Gracia et al., 2015). To solve the problem, we have downloaded and aligned well classified reference sequences (see 2.2 point), have designed new specific primers and improved the protocol.

2.1.bNGS STUDY. New infection or Reactivation.

Dynamics of viral populations before and after RBV treatment were studied to confirm it was a reactivation. Results from NGS (Hardwick et al., 2017) were analyzed and are described in **Figure 9**, where it is shown that from 1500000 original reads, after Flash and multiplex process, and quality filters, there are almost 300000 left (±20%), which were aligned and newly analyzed.

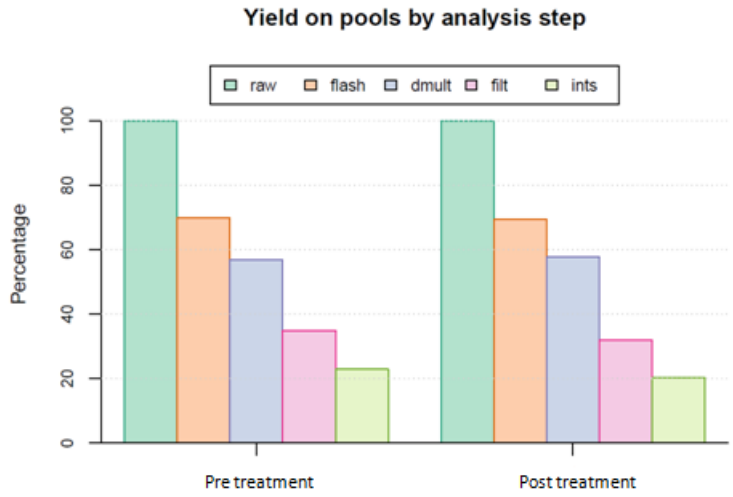


Figure 9: graphic where original reads of pools from pre- and post treatment are reduced ¼ after steps from analysis. **Raw**: original reads, **Flash**: flash process, **dmult**: multiplex process, **Filt**: quality filters (indeterminations, gaps…), **ints**: intersection of common haplotypes.

Phylogenetical trees of the 6 overlapping regions covering the polymerase gene of HEV were performed by including all haplotypes that passed the quality filters after MiSeq sequencing of pre-treatment sample (2012) and post-treatment sample (2016) together with consensus sequences of November 2012, February 2014, and 12 weeks follow-up 2016 (**Figure 8**). Interestingly, the number of haplotypes obtained in each overlapping regions were different, suggesting that even inside the HEV polymerase there are subregions subjected to different selective pressure.

Secondly, different haplotypes isolated from 2016 are clustered together with haplotypes from 2012, suggesting that despite HEV-RNA was not detected in a sample on May 2014, a basal replication was maintained and virus reactivates later on, causing a positive RNA detection in serum on January 2015, making patient be included in a clinical assay and started to be treated on January 2016 (**Figure 10**). These results confirm the hypothesis of a reactivation of the same virus of 2012. However, despite second RBV treatment, twelve weeks after ending it RNA

detection stills positive. Patient is still under follow-up according to his positive RNA detection in blood. A new clinical approach is needed in order to heal this patient.

In addition to this assay, consensus sequences from November 2012, Pre-ttm, Post-ttm and 12 weeks follow-up samples were used to perform a genetic distances matrix (see **2.2.1 Sliding window** and **Figure 10**). With results, fixation mutation rate was calculated as published in Quer, J et al (Quer et al., 2005) and was $5{,}1x10^{-3}$ base substitutions/site/year. This result can be explained by the immunocompromised status of the patient, due to which there is no selection pressure that affects substitution fixation.

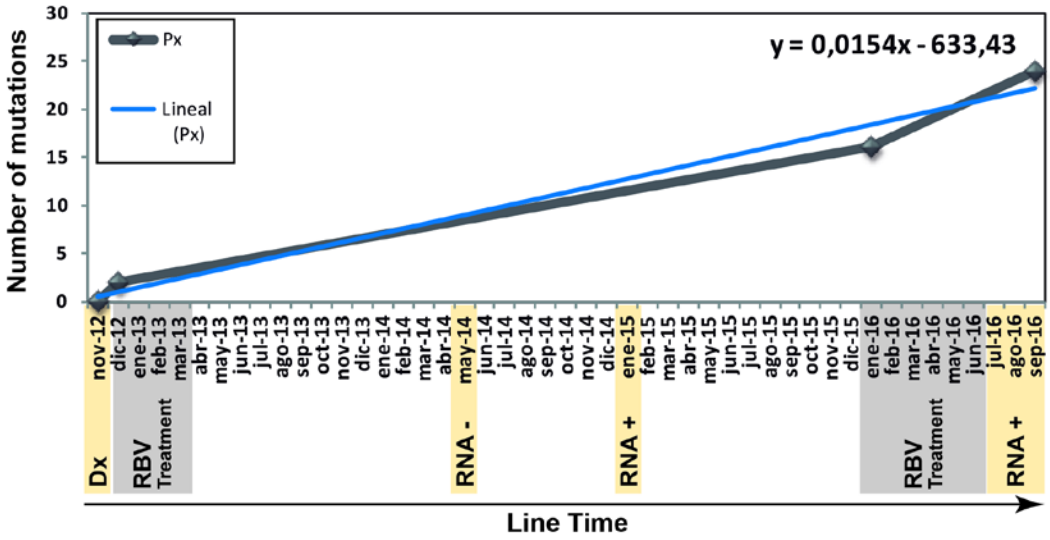


Figure 10: Patient line time since first HEV infection diagnosis on 2012 till sample of 12 weeks follow-up on 2016. In the middle time, he had a first RBV treatment and a negative RNA test on May 2014, and received a second RBV treatment on 2016.

Summarizing, NGS (Hardwick et al., 2017) results suggest that due to a virus can maintain a basal replication for years, as it has been demonstrated, after getting a negative HEV-RNA result, the patient should be followed up during several months and/or years to discard reactivation of HEV infection, having this result an important clinical implication in chronic patient follow up. In addition, phylogenetical trees analyses comparing the number of haplotypes in each region, suggest that HEV polymerase can be subdivided into regions subjected to different selective pressure.

2.2 Identification of the highest discriminating HEV region for genotyping and subtyping

Before performing sliding windows analysis of the whole genome to identify the highest discriminating genomic region to subtype genotype 3 isolates, we performed a phylogenetic analysis (**Figure 11)** using 76 sequences published by Simth et al (Smith et al., 2013; Smith et al., 2015), to assure the correct clustering by genotype and subtype. Six of the 76 sequences previously classified as "unknown" were classified, two (?.AY535004 and ?.JQ001749) as genotype 4, one (?.AY575857) as genotype 3 subtype a, and the other three have not a clear classification. In addition, eleven sequences of genotype 3 but with unknown subtype have been correctly subtyped.

Nevertheless, this assay has a few limitations. First of all, there are three sequences which could not be classified into any of the already known genotypes, ?.HQ731075, ?.JN998606 and ?.GU345042. There are two putative explanations. First one is that the three sequences have too many differences with other known genotypes used to build the tree, so more sequences of each genotype are needed in order to build a more reliable tree. Also, the papers used as a reference to download sequences do not provide any genotype 2 reference to be downloaded (Smith et al., 2013; Smith et al., 2015). Therefore, it is possible that, at least sequences ?.JN998606 and ?.GU345042 belong to genotype 2.

Second limitation of this tree is that there is only one reference sequence for genotype 1, one for genotype 7, two for genotype 5, one for 3h, one for 3i and none for genotype 2. More sequences of each genotypes should be included in the reference tree for HEV classification.

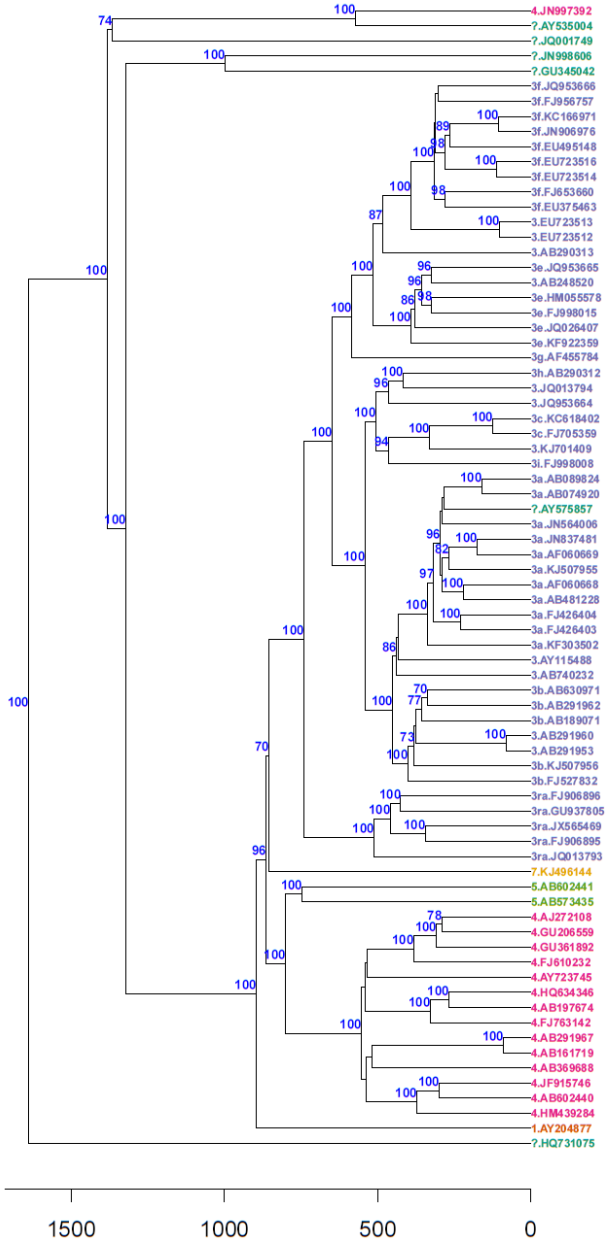


Figure 11: phylogenetical tree obtained with 76 sequences where sequences with unknown genotype and subtype are classified and known genotype sequences are joined in clusters.

Once reference sequences were clearly grouped in the phylogenetical tree, we selected sequences from different subtypes of genotype 3 to perform a sliding window analysis to find out most discriminative region along the whole genome to design primers for subtype genotype 3 samples, and to transfer into a diagnosis laboratory. Results comparing all subtypes with genotype 3 subtype f are represented in **Figure 12**, results for other subtypes are represented in **Annex 1 (Figure 13)**. Substitution model of Kimura was used to obtain a map of distance where a sequence is compared with other sequences from different subtypes, in order to see

which is the most differentiating region. In this case, a region of 400 nucleotides was selected. This "window" is between 1890 and 2290 pb.

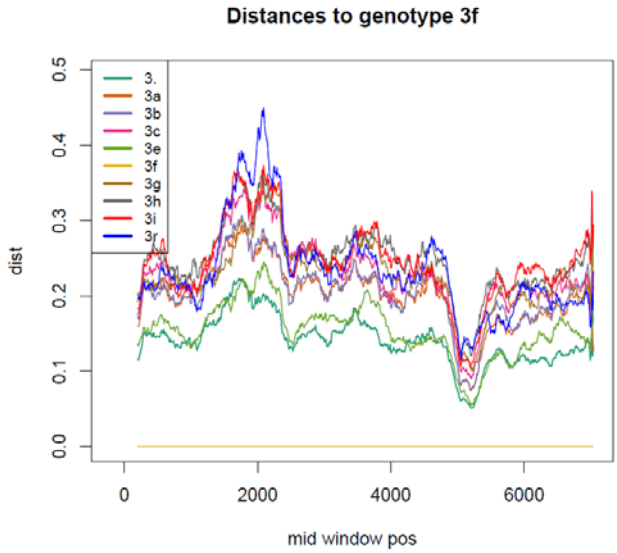


Figure 12: model of sliding windows with Kimura's model. Genotype 3 subtypes are represented in different colours. The line behind is the subtype compared with others. There is a region of 400 nucleotids from 1890 and 2290 where there are more differences between subtypes (more distance).

Once a region of interest was selected, a primers design was performed.

2.3 Development of a diagnosis tool for correct genotype 3 subtype classification

Due to the absence of a good reliable protocol based in specific primers, a primer design was performed (see section **2.2.2 Primer design)**. Nevertheless, although some primers have been proposed, it has not been possible to design primers that fulfill all the necessary characteristics to be specific and to improve the diagnosis of HEV. This project will continue in order to achieve a battery of effective specific subtype HEV primers.

2.4 Identification of resistance associated mutations to RBV antiviral treatment

According to resistance-associated mutations to RBV treatment, we have identified resistance substitution D1384G (Aspartic acid that changes to Glycine at position 1348), which have also been associated with HEV replication fitness decay in in vitro experiments (Debing et al., 2014; Debing et al., 2016; Todt et al., 2016). This substitution was present in pre treatment sample in only one haplotype representing 1,06% (727 reads) of total haplotypes. When analyzing the haplotypes sequenced in post-ttm sample 4 years later (2016) this mutation was not present nor any of the resistance mutations described in literature (Tong et al., 2016) (**Table 5 Annex 2**.

See comments on **Table 2**). However, by comparing the pre-ttm sample with the post-ttm sample several changes have been observed.

Table 2: selection of variants seen in Pre- and/or Post treatment samples with nº haplotypes where they were seen, nº of reads of every variant, percentage they represent and in which amplified fragment (amplicon) have been seen.

	Pre treatment				Post treatment			
Variant	nº Haplotype	nº reads	%	Amplicon	nº Haplotype	nº reads	%	Amplicon
I948V	5	53672	27,61	1	-	-	-	-
V948I	-	-	-	-	9	102322	49,51	1
P1035S	1	277	1,01	2	-	-	-	-
S1035P	-	-	-	-	4	9562	20,05	2
V1195A	1	557	0,89	5	1	395	0,82	5
V1195I	-	-	-	-	2	2485	5,18	5
I1195V	-	-	-	-	2	9092	16,04	4
Y1439C	2	1114	1,63	6	-	-	-	-
Y1439H	2	1745	2,55	6	-	-	-	-
Y1439N	1	443	0,65	6	-	-	-	-
H1439Y	-	-	-	-	2	14608	37,63	6

Position 948 in pre treatment sample, where there is an Isoleucine (I) in 72,48% of the reads and a Valine (V) in 27,61%. However, in the post treatment sample Valine had increased to 50.49% of the reads. Similarly, at position 1035, Serine (S) increases to 79,95% of the reads from 1,01% represented before starting treatment.

Besides, at position 1439, Tyrosine (Y) is present in 95,17% of pre treatment reads with Histidine (H) representing 2,55%. After treatment its abundance decays to 37,63%, while Histidin (H) increased to 62,37%.

In the same way, there were six variants which were in both pre- and post treatment samples. These variants are numbered in **Table 3.** Three of these variants (E936K, I1072V and V1195A) decreased their abundance after RBV treatment, while other three (A931V, A1164T and V1378A) increased their abundance in post treatment sample. The most selected variant is A1164T, which in pre treatment sample is 0,68% of reads while in post treatment is 6,08%.

Table 3: selection of variants those are present in Pre- and Post treatment samples.

	Pre treatment	Post treatment
Variant	%	%
A931V	0,88	4,44
E936K	4,09	3,63
I1072V	4,19/6,92	1,13
A1164T	0,68	6,08
V1195A	0,89	0,82
V1378A	7,56	7,57

In sum up according to resistance-associated mutations to RBV treatment, a putative explanation to the absence of D1384G mutation in post-ttm sample is that resistance mutations such as D1384G causes a fitness lost, therefore, once RBV treatment was stopped, it is possible than wild type virus (D1348) displaced resistance mutation 1348G because of its highest competitive value. To demonstrate such possibility we should have access to a sample during or just stopping RBV treatment and sequential samples afterwards. In addition, to identify observed variants resistance-association, an in vitro assay should be done.

Conclusions

1. Analysis of Data Base from Liver Pathology Unit of Biochemistry and Microbiology services from Hospital Universitari Vall d'Hebron (HUVH) shown a possible relationship between HEV infection and older age, immunocompromise status and foodborne origin. Nevertheless, a deep analysis must be carried out due to the need of HEV diagnosis improvement.
2. Phylogenetical tree performed with pre-ttm and post-ttm consensus sequences suggested HEV infection in 2016 was a reactivation of the same virus as in 2012. This result was confirmed with a population approach (NGS) where it was shown that a basal replication was maintained since 2012. Results from both assays suggest that after getting a negative HEV-RNA result, the patient should be followed up during several months and/or years to discard reactivation of HEV infection.
3. Improvement of HEV subtyping diagnosis was done with sliding window assay, where a region of 400 nucleotids was selected as the most differentiating one. However, it has not been possible to design a battery of specific primers to improve HEV diagnosis in available time.
4. As regards to presence of resistance-associated mutations to RBV investigation, just one described variant was identified in pre-ttm sample. In addition, other non described variants were detected, opening a new line of research for new future projects.
5. Finally, the present study has demonstrated the need to perform a follow-up of patients who have been correctly diagnosed with chronic hepatitis E once they have a negative RNA viral load, in order to rule out reactivation of the virus.

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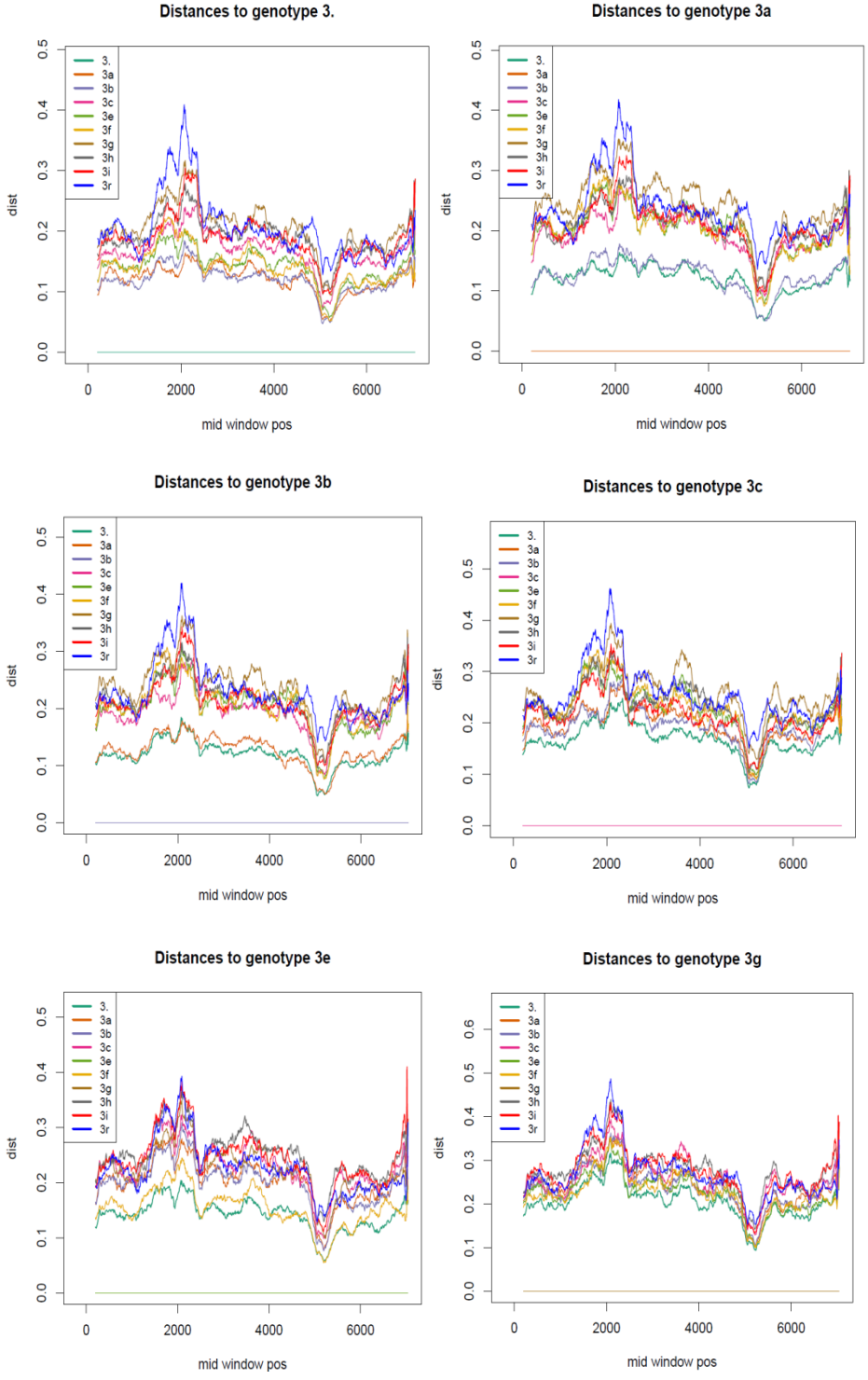
Annex 1: Sliding window results



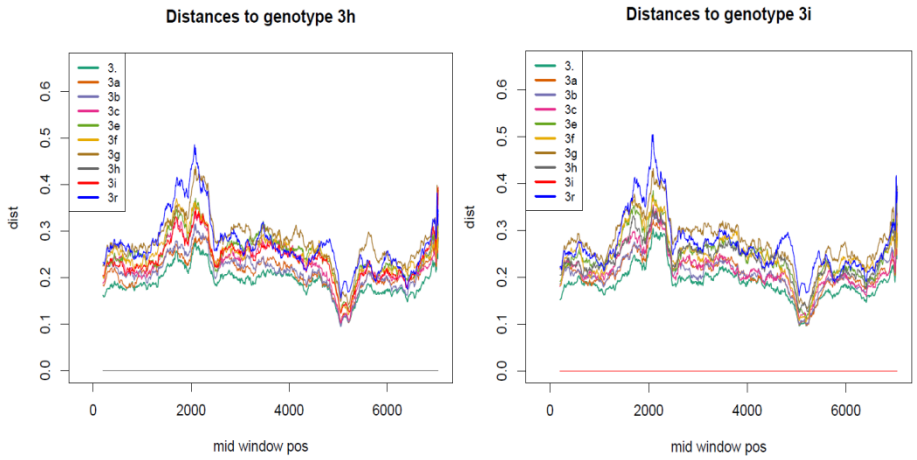


Figure 13: model of sliding windows with Kimura's model. Genotype 3 subtypes are represented in different colours. The line behind is the subtype compared with others. There is a region of 400 nucleotids from 1890 and 2290 where there are more differences between subtypes (more distance).

Annex 2: Table 5

Table 5: Compilation of variants seen in Pre- and/or Post treatment samples with nº haplotypes where they were seen, nº of reads of every variant, percentage they represent and in which amplified fragment (amplicon) have been seen.

	Pre treatment				Post treatment			
Variant	nº Haplotype	nº reads	%	Amplicon	nº Haplotype	nº reads	%	Amplicon
Y913H	1	2218	1,14	1	-	-	-	-
T915P	1	3612	1,86	1	-	-	-	-
A920T	-	-	-	-	1	1637	0,79	1
N925I	-	-	-	-	1	2682	1,3	1
T928K	-	-	-	-	1	1231	0,6	1
Q929R	6	12904	6,64	1	-	-	-	-
A931T	-	-	-	-	1	3861	1,81	1
A371V	1	1720	0,88	1	1	9181	4,44	1
T933I	1	3186	1,64	1	-	-	-	-
T933A	-	-	-	-	1	1673	0,79	1
T935S	1	1574	0,81	1	-	-	-	-
E936A	1	4959	2,55	1	-	-	-	-
E936G	4	29559	15,21	1	-	-	-	-
E936K	3	7964	4,09	1	1	7507	3,63	1
E936V	1	1934	0,99	1	-	-	-	-
D937A	1	1599	0,82	1	-	-	-	-
D937G	1	1160	0,6	1	-	-	-	-
T938A	1	1650	0,85	1	-	-	-	-
T938S	1	8514	4,38	1	-	-	-	-
T938I	-	-	-	-	1	10071	4,87	1
A942T	2	3774	1,94	1	-	-	-	-
A942V	1	6193	3,19	1	1	1505	0,73	1
N943T	-	-	-	-	1	1323	0,64	1
I948V	5	53672	27,61	1	-	-	-	-
V948I	-	-	-	-	9	102322	49,51	1
V954A	1	1814	0,93	1	-	-	-	-
T959A	1	2679	1,38	1	-	-	-	-

T962A	1	1493	0,77	1	-	-	-	-
T962I	-	-	-	-	1	1637	0,79	1
V967I	-	-	-	-	3	18870	9,14	1
V968I	-	-	-	-	1	1340	0,65	1
H969R	-	-	-	-	1	1306	0,63	1
S982P	-	-	-	-	1	404	0,85	2
L1000P	-	-	-	-	1	404	0,85	2
A1018V	-	-	-	-	1	333	0,7	2
V1026I	-	-	-	-	1	819	1,72	2
S1033A	1	223	0,81	2	-	-	-	-
P1035S	1	277	1,01	2	-	-	-	-
S1035P	-	-	-	-	4	9562	20,05	2
L1041P	1	268	0,98	2	-	-	-	-
Q1044R	1	226	0,82	2	-	-	-	-
N1056Y	1	223	0,81	2	-	-	-	-
G1067S	1	318	0,84	3	-	-	-	-
A1071V	1	383/500	1,4/1,32	2,3	-	-	-	-
I1072V	1	1148/2626	4,19/6,92	2,3	1	365	1,13	3
E1075G	1	2542/1417	9,27/3,73	2,3	-	-	-	-
A1077T	-	-	-	-	1,2	374/3973	0,78/12,26	2,3
H1086Y	-	-	-	-	1	253	0,78	3
C1088Y	-	-	-	-	1	780	1,64	2
A1119V	-	-	-	-	1	240	0,4	3
R1123K	-	-	-	-	1	386	1,19	3
A1130V	-	-	-	-	1	672	2,07	3
K1131T	1	341	0,9	3	-	-	-	-
A1164T	1	426	0,68	5	2	2917	6,08	5
A1167V	-	-	-	-	1	10374	21,63	5
A1168T	1	625	1	5	-	-	-	-
L1170V	1	1133	1,81	5	-	-	-	-
P1171S	1	3103	4,95	5	-	-	-	-
L1174P	-	-	-	-	1	356	0,63	4

T1175A	-	-	-	-	1	224	0,69	3
K1180R	-	-	-	-	1	351/689	1,08/1,22	3,4
I1183V	1	820/17272	2,16/21,88	3,4	-	-	-	-
L1184P	1	331/465	0,87/0,59	3,4	-	-	-	-
E1186G	2	1869	2,98	5	-	-	-	-
S1190P	-	-	-	-	1	417	0,87	5
D1191A	-	-	-	-	1	1413	2,95	5
S1192G	1	743	1,18	5	-	-	-	-
L1194Q	-	-	-	-	1	8768	18,28	5
V1195A	1	557	0,89	5	1	395	0,82	5
V1195I	-	-	-	-	2	2485	5,18	5
I1195V	-	-	-	-	2	9092	16,04	4
F1196L	1	704	1,12	5	-	-	-	-
E1197K	-	-	-	-	1	346	0,72	5
F1203L	-	-	-	-	1	548	0,97	4
C1204Y	-	-	-	-	1	815	1,7	5
L1205P	1	1248	1,58	4	-	-	-	-
P1209S	-	-	-	-	1	387	0,81	5
V1210A	-	-	-	-	1	355	0,63	4
R1222G	1	732	1,17	5	-	-	-	-
P1223L	1	504	0,64	4	-	-	-	-
R1225C	1	946	1,2	4	-	-	-	-
T1227A	1	1265	2,02	5	-	-	-	-
T1227S	-	-	-	-	2	5238	10,92	5
K1228R	2	1583	2,53	5	-	-	-	-
Q1231R	-	-	-	-	4	15949	28,15	4
A1233T	-	-	-	-	1	8768	18,28	5
A1233V	1	851	1,36	5	-	-	-	-
V1237I	-	-	-	-	1	458	0,95	5
Q1238H	-	-	-	-	1	8462	14,93	4
Q1238R	-	-	-	-	1	630	1,11	4
S1240G	-	-	-	-	1	795	1,4	4

G1242R	-	-	-	-	1	387	0,81	5
F1244L	1	1594	2,54	5	-	-	-	-
A1246V	-	-	-	-	1	511	0,9	4
P1250S	1	443	0,71	5	-	-	-	-
V1251A	1	667	1,06	5	-	-	-	-
A1254T	-	-	-	-	2	2548	4,5	4
A1257V	-	-	-	-	1	8542	15,07	4
L1260V	1	596	0,76	4	-	-	-	-
P1261S	1	1081	1,37	4	-	-	-	-
E1263A	1	725	1,16	5	-	-	-	-
G1269D	-	-	-	-	1	716	1,49	5
G1359D	-	-	-	-	1	445	1,15	6
D1384G	1	727	1,06	6	-	-	-	-
T1393A	-	-	-	-	1	269	0,69	6
W1406R	1	2220	3,25	6	-	-	-	-
F1418L	1	1141	1,67	6	-	-	-	-
E1424G	8	18518	27,07	6	-	-	-	-
E1424K	3	2754	4,03	6	-	-	-	-
L1426R	-	-	-	-	1	11669	30,06	6
I1433T	1	1596	2,33	6	-	-	-	-
Y1439C	2	1114	1,63	6	-	-	-	-
Y1439H	2	1745	2,55	6	-	-	-	-
Y1439N	1	443	0,65	6	-	-	-	-
H1439Y	-	-	-	-	2	14608	37,63	6
E1440D	2	2123	3,1	6	-	-	-	-
E1440G	1	1038	1,52	6	-	-	-	-
E1441G	-	-	-	-	1	910	2,34	6
G1452S	-	-	-	-	2	1923	4,95	6
F1464Y	1	525	0,77	6	-	-	-	-
T1467A	3	11146	16,29	6	-	-	-	-
G1474A	1	930	1,36	6	-	-	-	-
G1474S	3	4095	5,99	6	-	-	-	-

L1475F	1	479	0,7	6	-	-	-	-
V1478A	2	5172	7,56	6	1	2939	7,57	6