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Next-Generation Sequencing to better characterise viral infections: Hepatitis as an example

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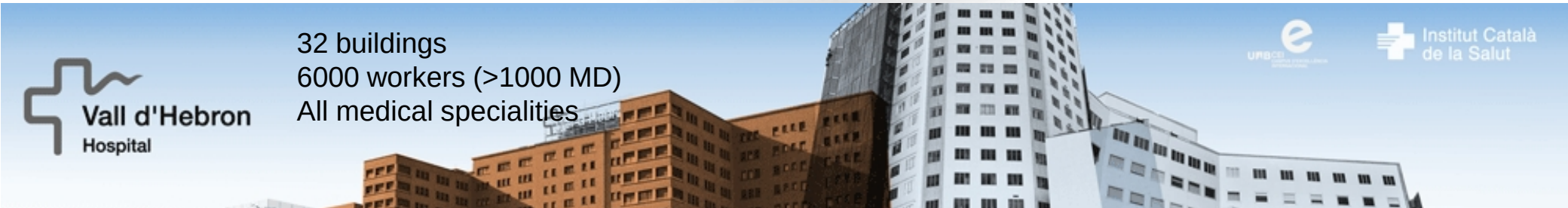


NEXT GENERATION SEQUENCING TECHNIQUES (NGS)

In this talk:

-The use of NGS in viral infections: Molecular biology methods with considerable potential in the study of viral populations

-Brief outline of how these systems work (the most widely used)

-Some relevant characteristics of viral populations:nature of quasispecies and importance of some mutations

-Examples of the use of these techniques in viral hepatitis including a potential future application: Phylogenesis of haplotype populations in the study of transmission

-Overview of HEV infection and the preliminary application of NGS

BACKGROUND

Viral hepatitis: More than 500 million people affected worldwide. Five different viruses (A-E). B,C,D,E transmitted through blood and blood products.

Viral infection: heterogeneous population of viral particles (quasispecies), a fact with clinical consequences, such as the response to antiviral treatment. For example, the different HCV genotypes (G1-G7) and subtypes (Ex G1a/G1b) are associated with differing response rates to standard and to new therapies. However, current lab techniques (reverse hybridization, real-time PCR) cannot correctly differentiate between these subtypes (15% of G1 cannot be subtyped into G1a/G1b).

Furthermore, mutations in certain viral therapeutic target regions are associated with treatment failure (HBV, HCV), as you will see later.

Therefore, one approach to attack these infections would be to know the viral genotype and resistant variants. Classic sequencing approach (direct sequencing, molecular cloning), are laborious, time consuming, and can only characterize limited portions of the viral population.

This is where NGS come in, which enable relatively fast, correct phylogenetic classification of many components of the viral population and quantification of variants that may be resistant to treatment. However, each platform has advantages and limitations, so we have to choose the best one for our purposes.

NEXT-GENERATION SEQUENCING (NGS)

- Next-generation, ultra-deep, massive-parallel, etc. refers to new high-throughput technologies that can analyse large numbers of different DNA sequences in a single reaction: thousand of sequences from a specific sample and a specific genome region can be analysed in a short time.

NORWEGIAN SEQUENCING CENTRE
http://www.sequencing.uio.no/

	Illumina HiSeq	Illumina MiSeq	454	PacBio	Ion Torrent (PGM)
PCR / Single Molecule	Bridge PCR	Bridge PCR	Emulsion PCR	Single molecule	Emulsion PCR
Read length	50/100 bp	50/150 bp	Mode 500 bp	Mean 2500 bp, 95 percentile is at 5500 bp	100/200 bp
Single read (SR) / Paired end (PE)	SR and PE	SR and PE	SR and PE	Circular	SR
Multiplexing in one run	8 lanes and	1 lane with	2, 4 or 8 lanes, up to	Probably possible, not	32 samples with
Problems caused by GC content	Possible bi bridge PCI				?
Homopolymer indel problems	minor prob				problem
Typical applications	Re-sequen genome ar expression				Bacterial genome sequencing, amplicon sequencing
Status at NSC	In product				In testing (4 test runs performed)

Note that the technology is in constant development, these specifications are those that were available in March 2012

No platform is perfect for all tasks. Each has advantages and disadvantages relative to the others. For some purposes, especially for phylogenetic classification, the read length is very important

HOW THEY WORK

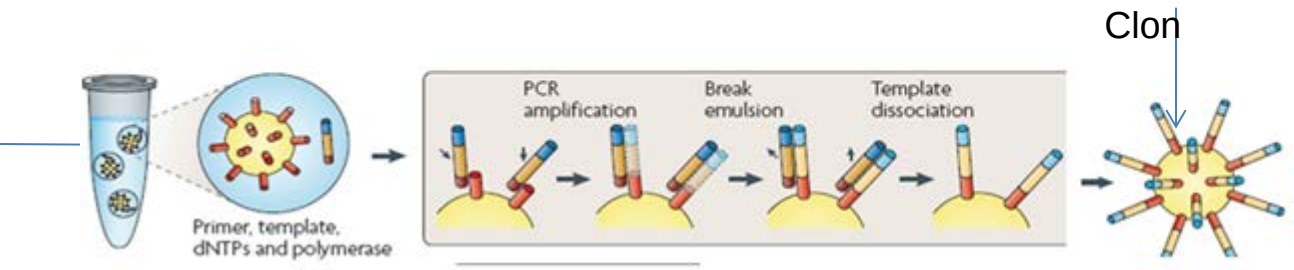
1-DNA template preparation: amplicon "library" generation

DNA molecules are labelled with specific sequences in both ends, mainly by the addition of such sequences in the PCR primers. Same complexity for both platforms. Manual processing, but we have developed an automatic processing for 454 platform on an standard robotic station, useful for studying all viral genomes by adapting only the first PCR step to make it genome specific

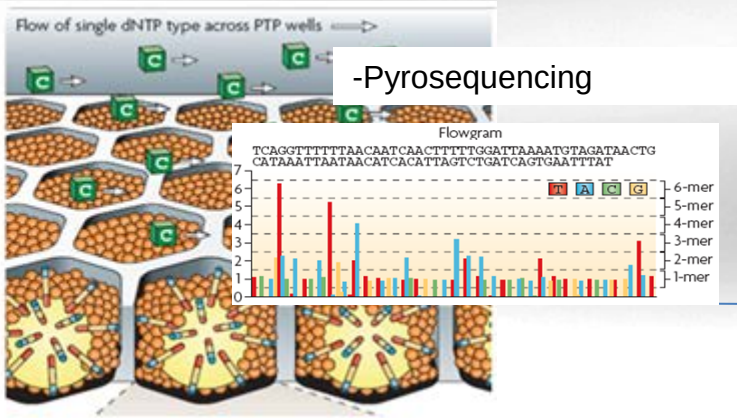
2-Clonally amplified templates

-Emulsion PCR (*emPCR*)

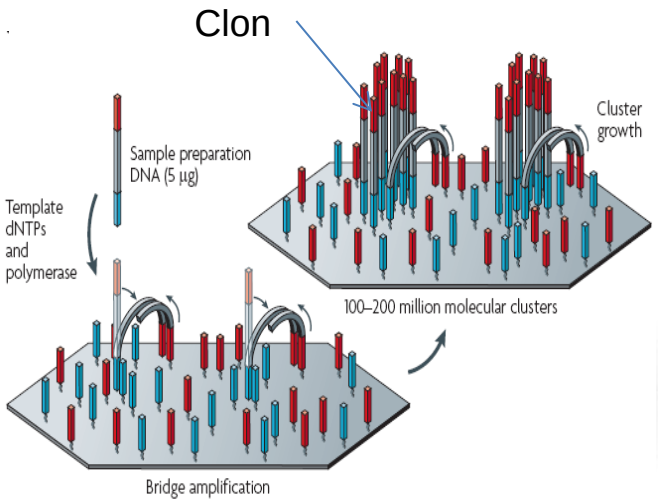
Roche 454 (Two manual steps: Clonal amplification and Sequencing)

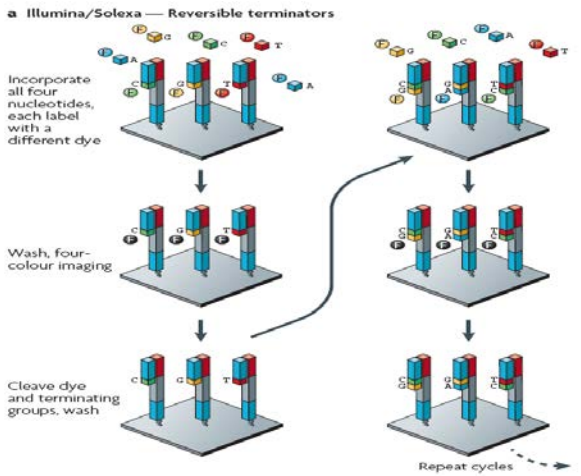


-Pyrosequencing



-Solid surface (*bridge PCR*) Illumina/Solexa (One automatic step: clonal amplification/sequencing)





Both based on PCR for clonal sequencing: but the different approaches condition their advantages and limitations

454 ROCHE

Viral Detection and Research
A review of publications featuring Illumina® Technology

from the Illumina website:
http://res.illumina.com/documents/products/research_reviews/viral_detection_research_review.pdf

Han Y., Zhang Y., Mei Y., Wang Y., Liu T., et al. (2013) Analysis of hepatitis B virus genotyping and drug resistance gene mutations based on massively parallel sequencing. J Virol Methods 193: 341-347

Although the major limitations of HiSeq sequencing is that it provides short sequences, which more difficult to accurately align if the exploring domain is heavily mutated, and it is impossible to link mutations> 200 bp apart,

Complex library preparation

Not as high throughput

Long read length (800 nt). High capacity for multiplexing

High sensitivity and reproducibility for detecting minority variants (<0.5%) Own experience
HBV minor variants 0.03 % (Hepatology 2014)
HBV haplotypes 0.25 % (Ramirez 2013)
HCV haplotypes 0.5 % (Gregori 2014)

Short read length (150-300 bp), may be too short for phylogenesis study in complex genomic populations such as viruses

High sensitivity and reproducibility for detecting minority variants (<0.1%)

Insertion/deleti
homopolymeric

ing/fluorescence.
licated: (Kircher BMC,

Because the main applications in virology are: phylogenesis for viral genotyping, and minor variants detection , the 454 is more suitable as it allows analysis of longer fragments (500-800 nt) and has similar sensitivity for detecting minority variants.

VIRAL QUASISPECIES

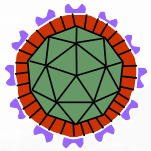
Viral populations are comprised of a complex mixture of different, but closely related, genomes, known as a quasispecies.

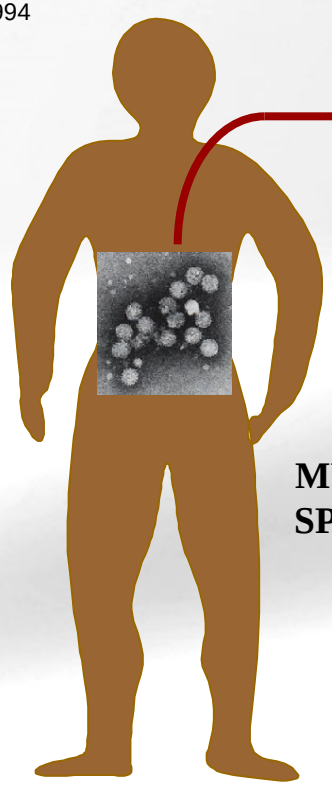
RNA viruses (eg, HCV and influenza virus) and reverse transcriptase-dependent viruses (eg, HBV and HIV), show high variability within each host because of high replication rates and low fidelity of the replication enzyme.

This characteristic enables the viral quasispecies to quickly adapt to dynamic environments through mutations, and gives rise to viral resistance to vaccines and antiviral drugs.

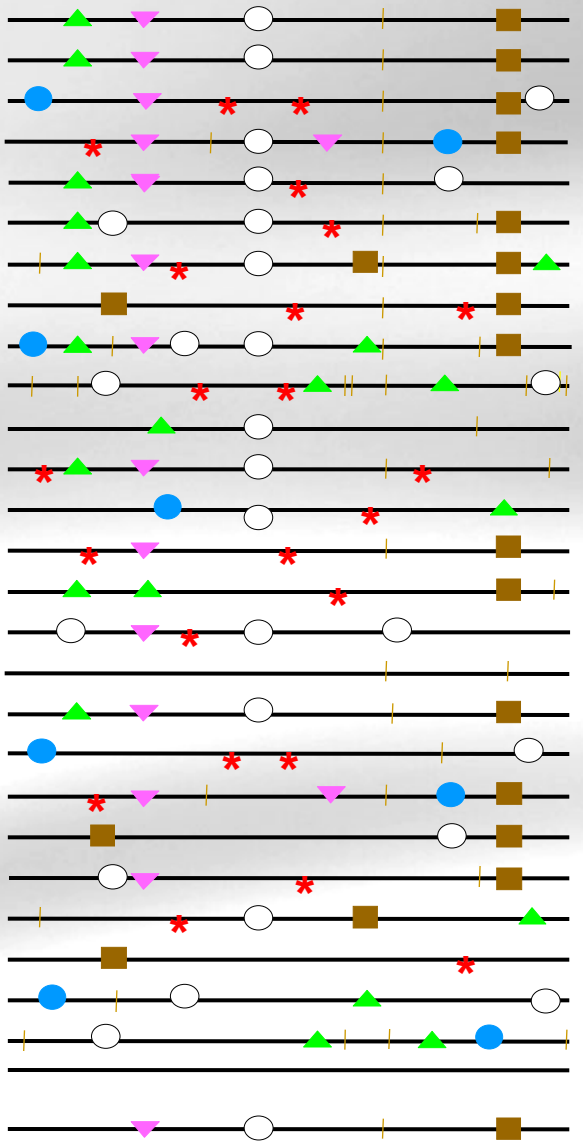
QUASISPECIES STRUCTURE

Holland JJ et al. Science 1982; 215(4540):1577-1585
Domingo E & Holland JJ. Evolutionary biology of viruses. 1994
Martell et al. J.Virol. 1992; 66(5):3225-3229
Vignuzzi Nature 2006; 439:344-348
Vignuzzi M, et al. Nature 2005.





QUASISPECIES



MUTANT SPECTRA

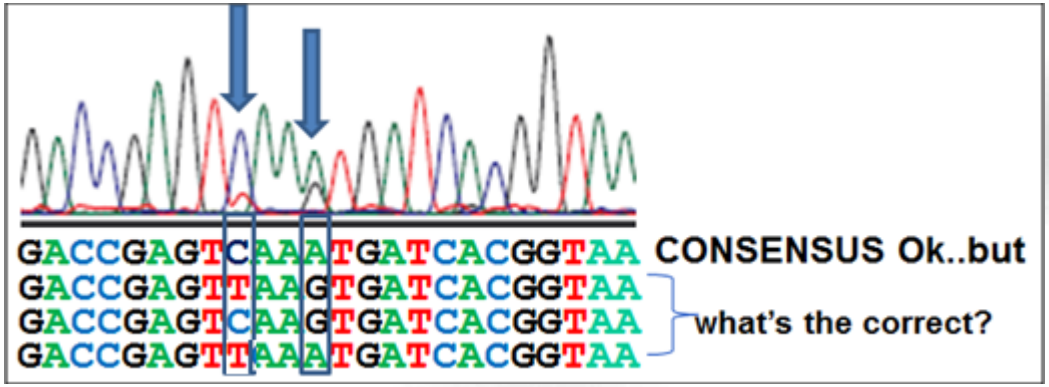
CONSENSUS (CS)

Direct sequencing

-Show only the consensus sequence, which is the one containing an average of all the nucleotides present.

-The consensus sequence itself may not be present in the sample or be present in low numbers.

-Only variants comprising at least 20% of the total can be detected and it is not possible to know if these variants are located in the same sequence



HOW CAN WE STUDY THESE COMPLEX POPULATIONS?

By the classic techniques (Sanger or indirect methods) ..viral particles seem identical: consensus sequence

We need a kind of magnifying glass to get detailed knowledge of this population.

Here is a peculiar “quasispecies”…from a distance men in uniform look alike ¡¡¡

A closer look reveals considerable differences.

Up to now molecular cloning has been used to better characterise viral populations. However, it is highly time consuming and expensive, and it is extremely dificult to obtain more than 100 clones…when a viral population contains billions of particles

NGS can help to solve this problem... let's see what it can do in hepatitis virus

APPLICATIONS OF NGS IN VIROLOGY

Some examples from our lab (454 technology)

NGS allows high-resolution study of viral infection, useful for:

-Prediction of therapy effectiveness by genotyping and sequencing to detect mutants

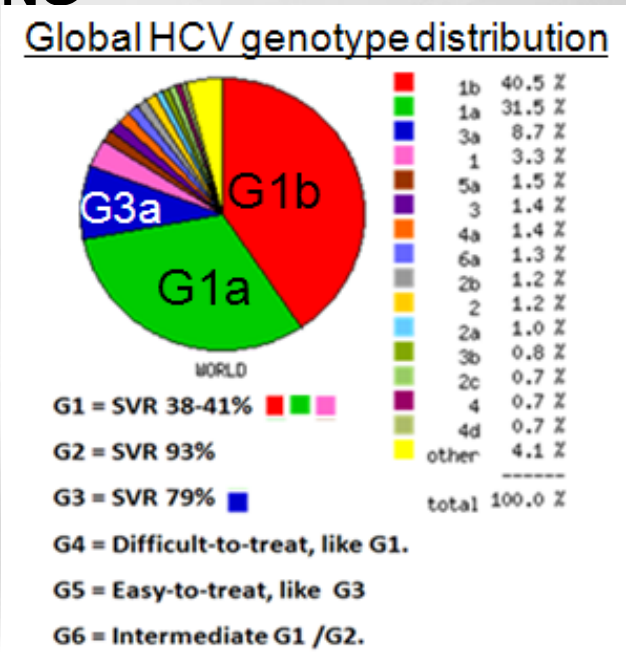
-Determining relevance of minor variants

-Determining origin of transmission

Additional applications …not covered in this talk ¡¡

Complete viral genome resequencing and new virus discovery

THERAPY EFFECTIVENESS: GENOTYPING



HCV genotype (G1-G7) and subtype (>60) important viral factors for predicting treatment response:

Eg: In major HCV genotype G1, new treatments have better response in G1b than G1a. Even the most expensive one (Sofosbuvir , 1,000 $/tablet), which has the same activity for G1a/G1b, is low active in G3.

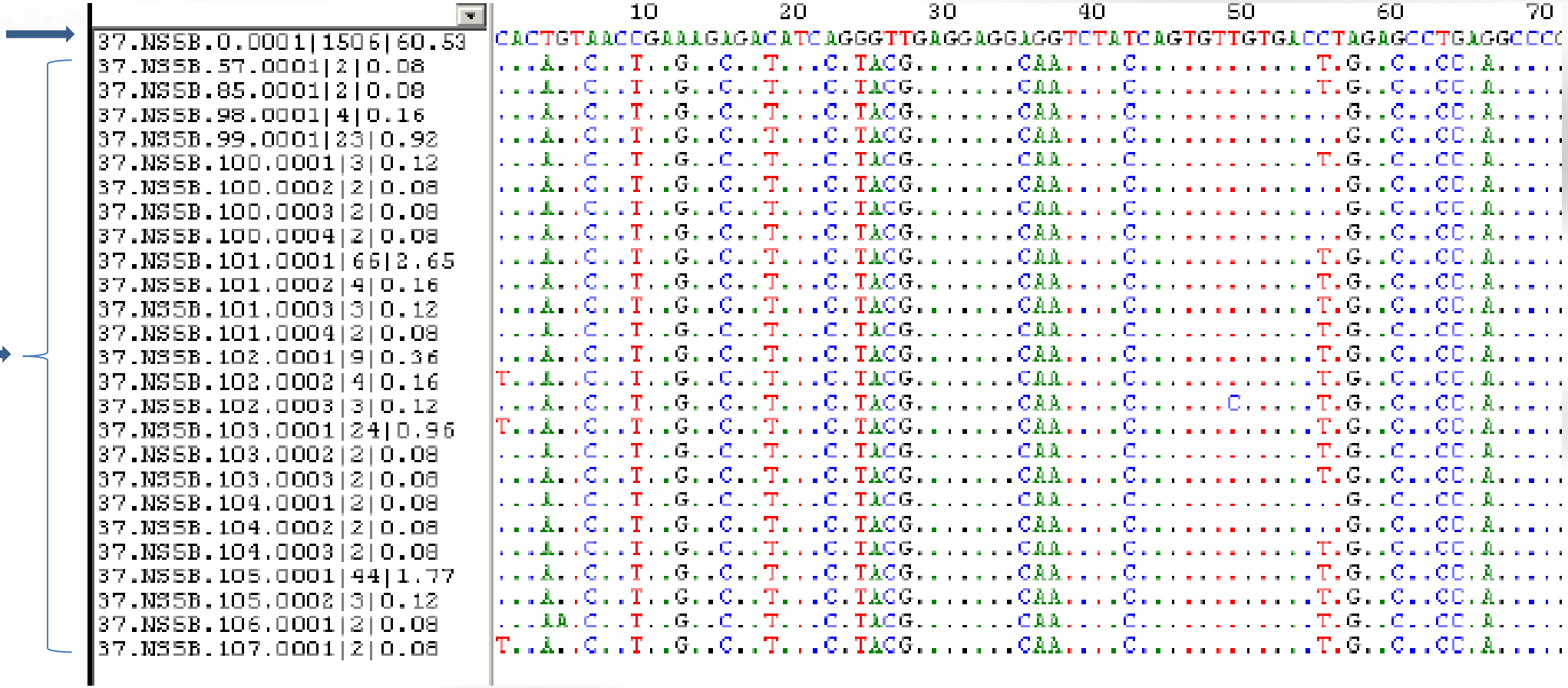
HBV genotype associated with differences in the natural history of chronic HBV infection and response to antiviral therapy. (Pourkarin 2014, Kranvis 2014). Emerging data have shown that HBV genotype recombination (30% Shy 2012) and mixtures are common (22% Jardi 2008) and may be clinically relevant.

HCV and HBV genotyping can be done with classic techniques, but they are limited for detecting genotype mixtures: (the predominant one masks the others)
Detection of recombinant genotypes is not possible with classical methods.

NGS (454) is more reliable and has provided the following preliminary data.

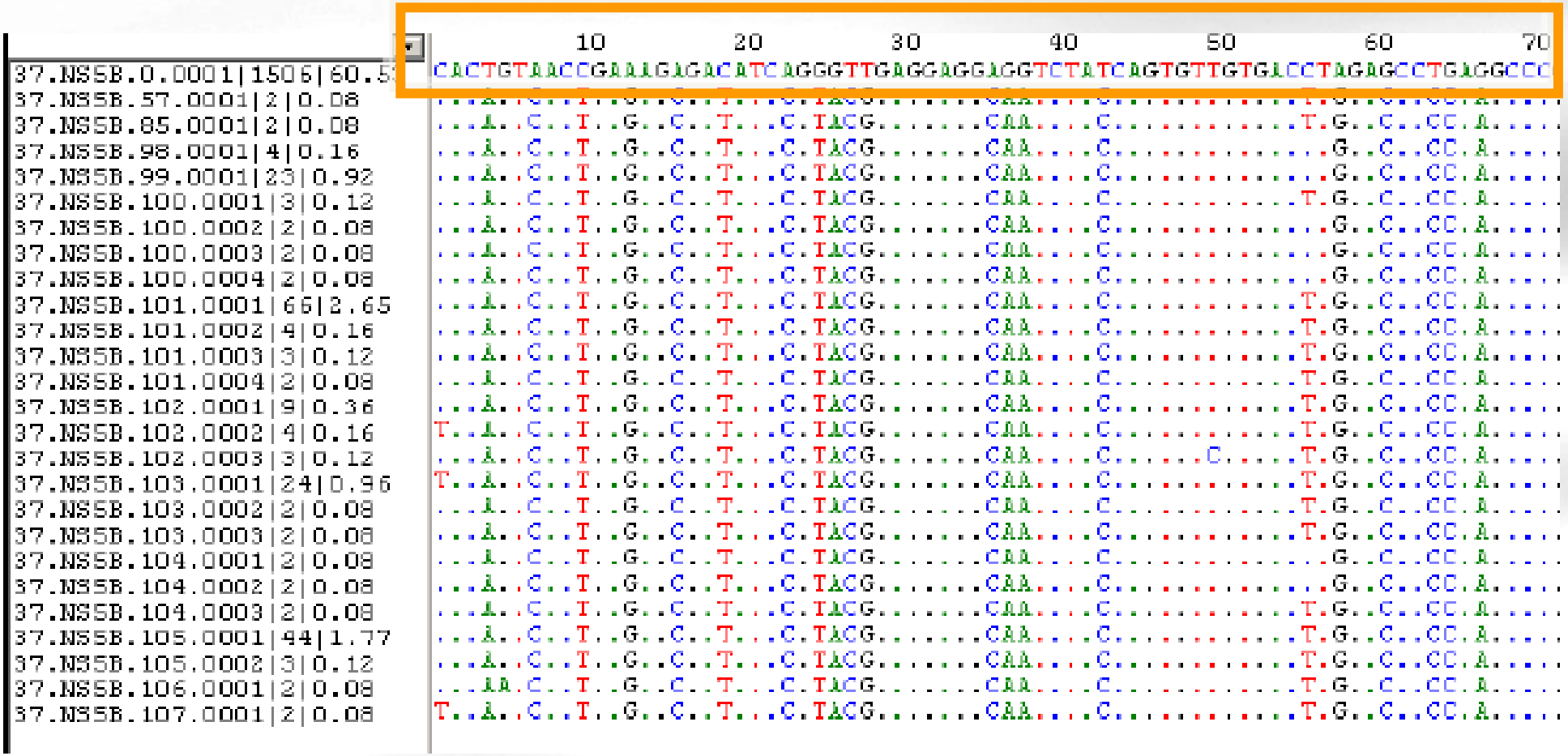
HIGH-RESOLUTION HCV SUBTYPING 454/GS-Junior

Detection of genotype/subtype mixes (infection by more than one subtype at the same time): such this case, in which simple alignment clearly suggests the presence of two different populations



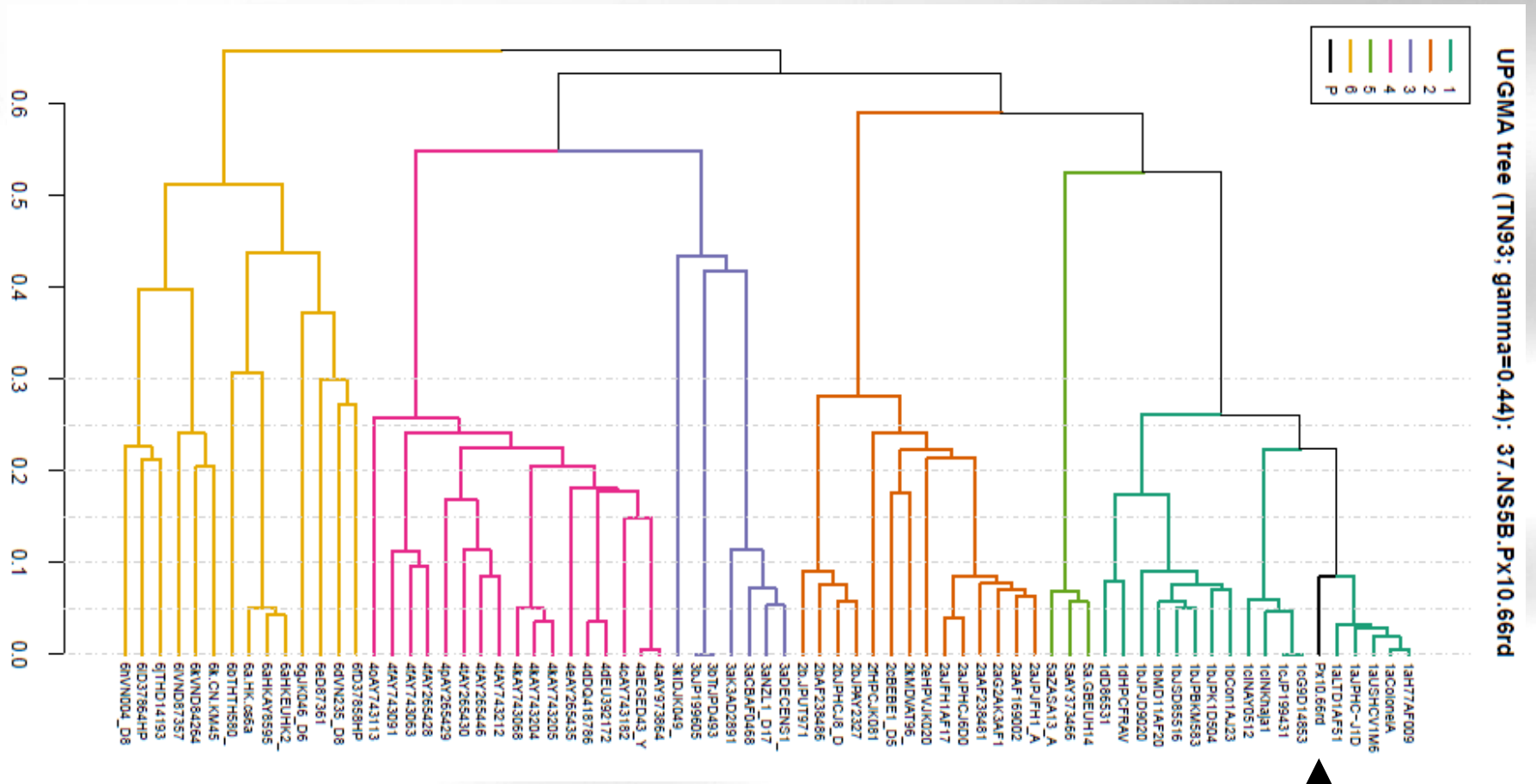
HIGH-RESOLUTION HCV SUBTYPING 454/GS-Junior

First the major or master sequence (60.53%)



HIGH-RESOLUTION HCV SUBTYPING 454/GS-Junior

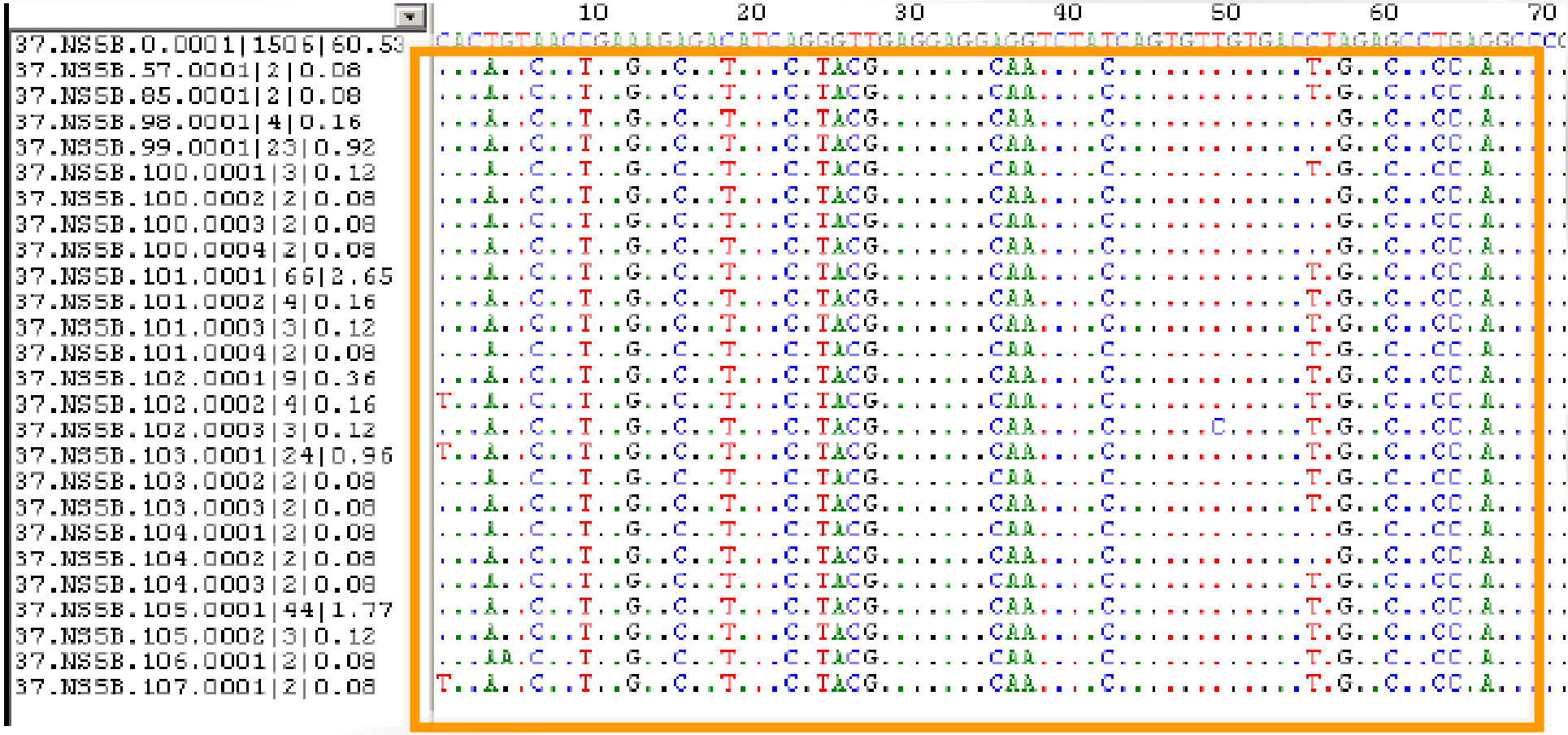
First the major or master sequence (60.53%): G1a



G1a

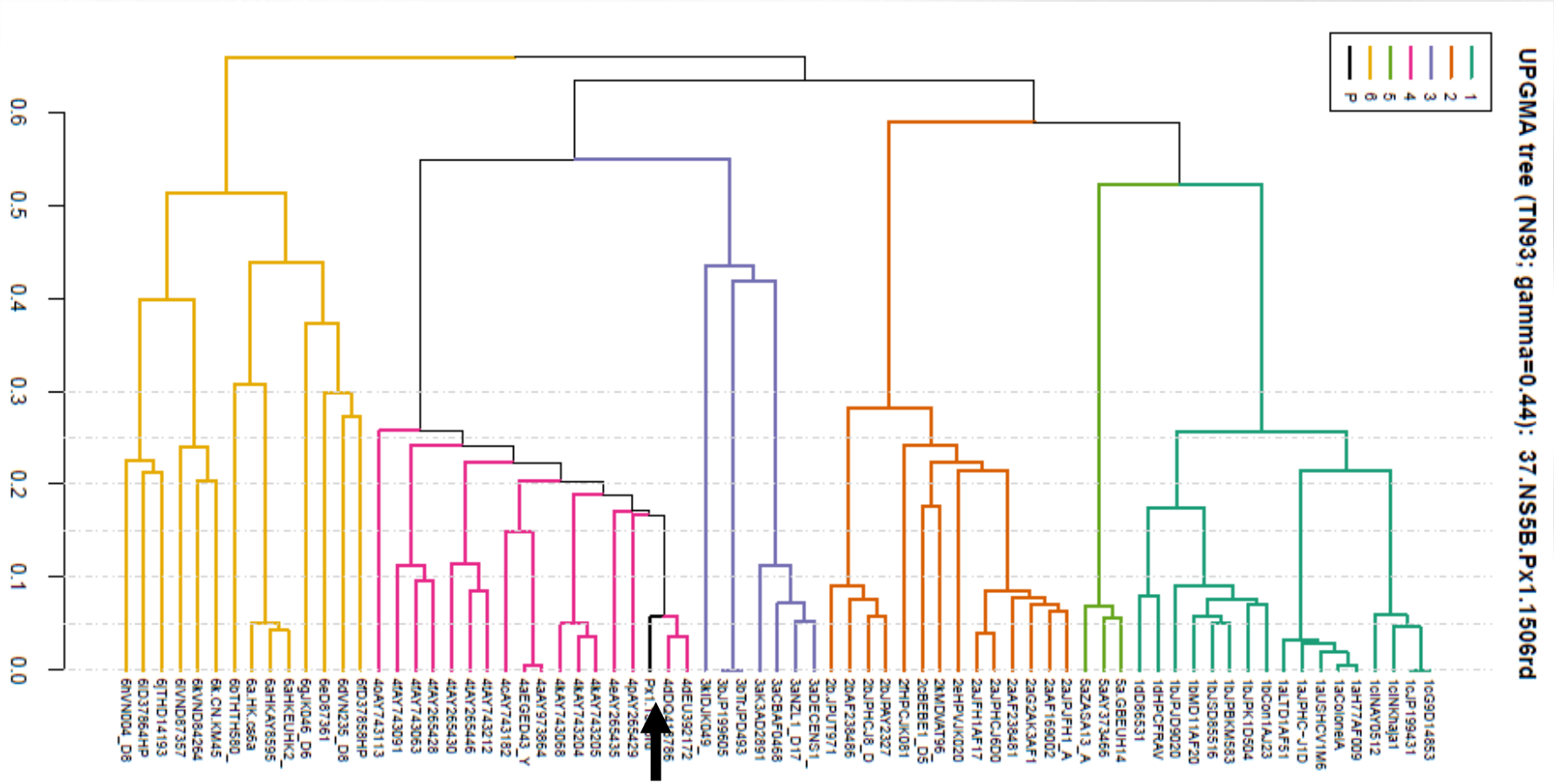
HIGH-RESOLUTION HCV SUBTYPING 454/GS-Junior

Then the second more heterogeneous group of haplotypes (39.47%):



HIGH-RESOLUTION HCV SUBTYPING 454/GS-Junior

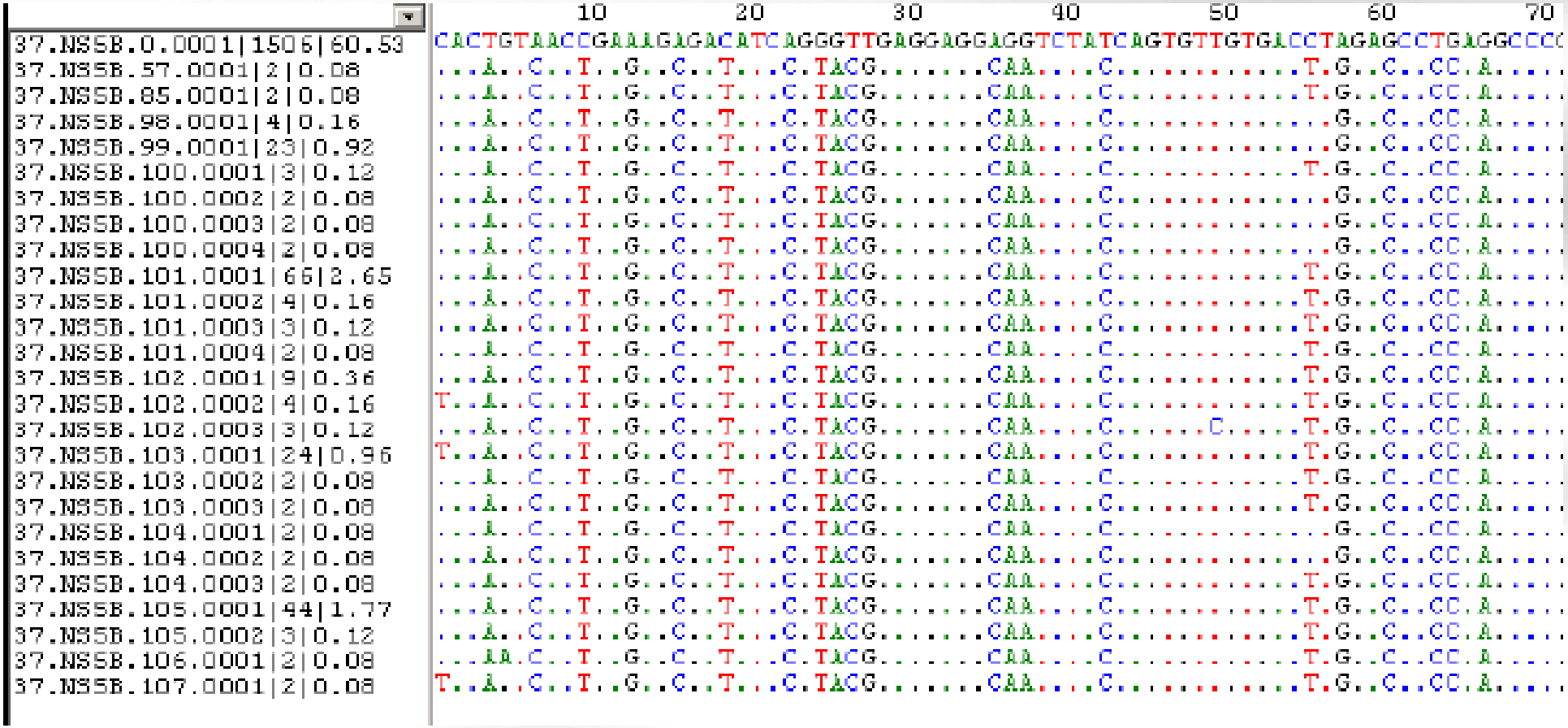
Then the second most heterogeneous group of haplotypes (39.47%): G4d



G4d

HIGH-RESOLUTION HCV SUBTYPING 454/GS-Junior

Therefore, this patient has a mixture of HCV subtypes (coinfection):
60.53% G1a, and 39.47% G4d: which have different SVR rates in some treatments
Eg: Abbie cocktail : G1a possible good response, but G4d probably not so effective



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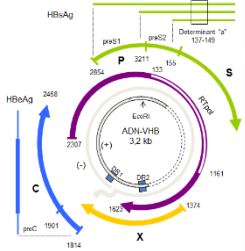


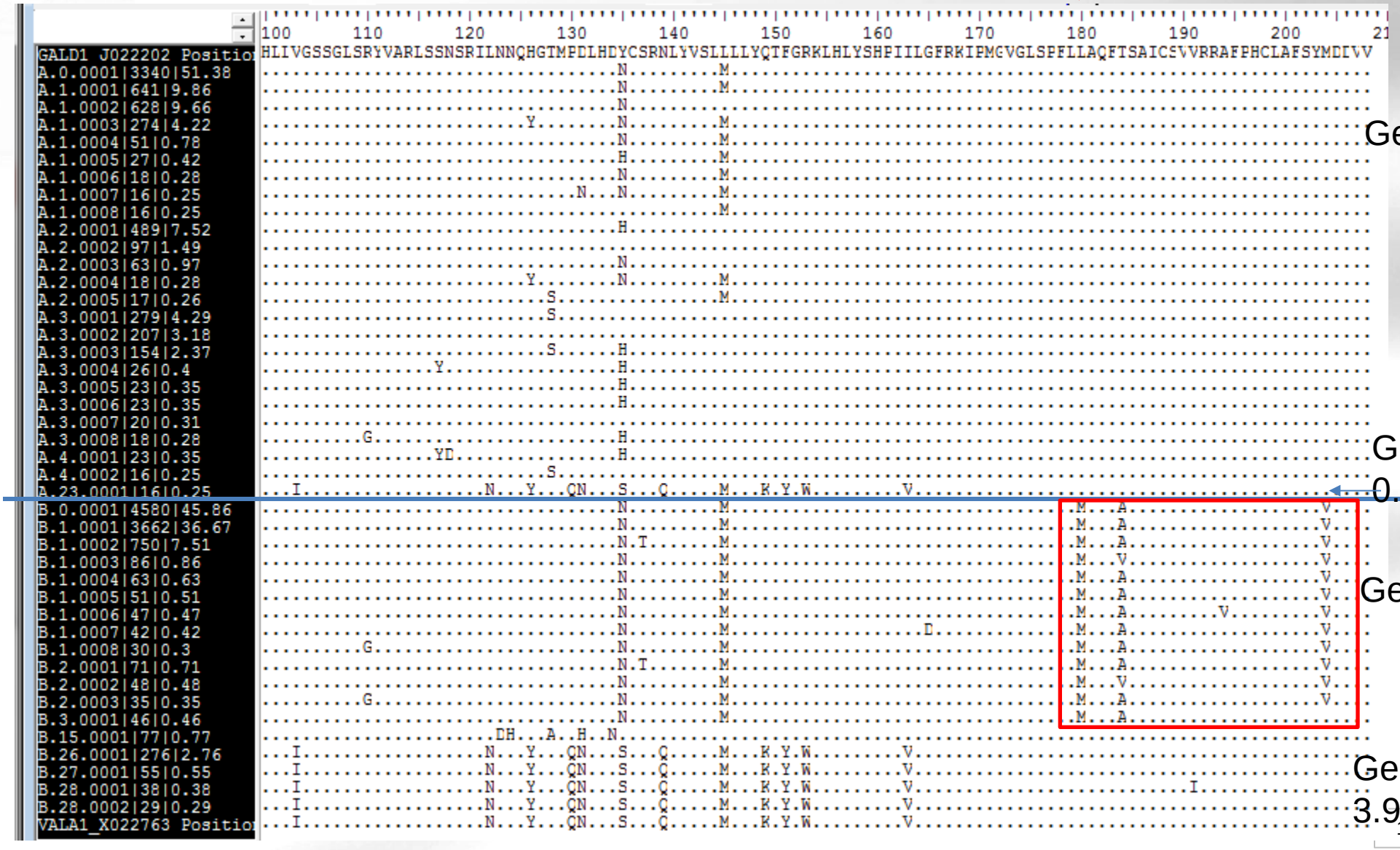
HBV GENOTYPING, RESISTANT VARIANTS, POLYMERASE REGION

Lamivudine failure: Pretreatment HBV genotype pattern D 99,75% /A 0,25% changes after treatment failure D 96.02%/A 3.98% in a percentage undetectable by LIPA.

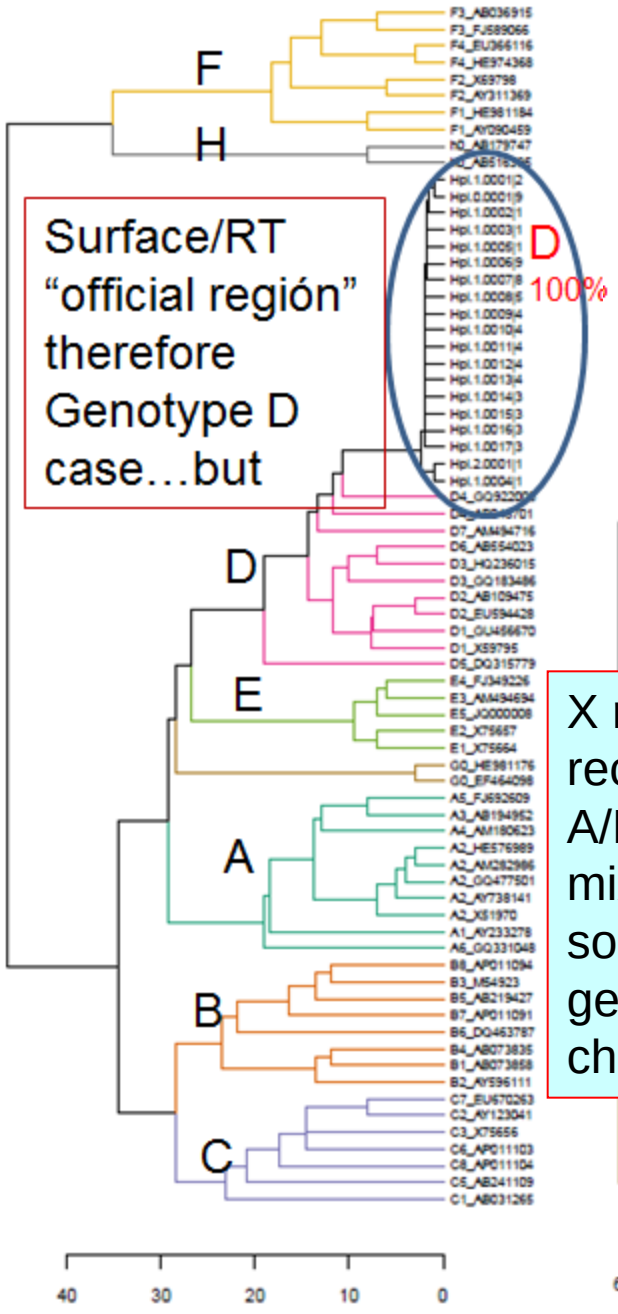
Genotype “A” Haplotypes do not show any known resistant variant¡¡

Haplotypes above the blue line are pretreatment

Resistant Variants

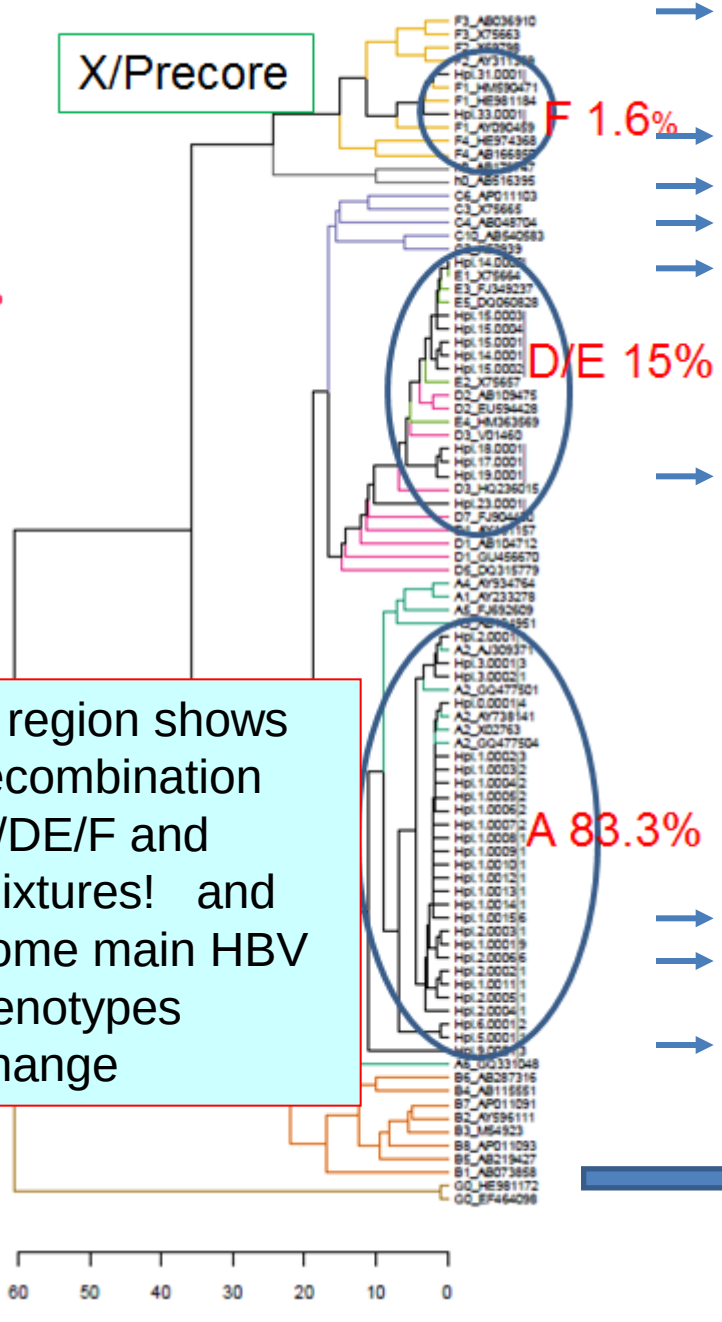


HBV GENOTYPING: MIXTURES AND RECOMBINATION?



Surface/RT "official región" therefore Genotype D case…but

D 100%



X region shows recombination A/DE/F and mixtures! and some main HBV genotypes change

	ID	GEN_FLX RT			GEN_FLX X		
		A	D	F	A	D/E	F
1	AGC BA	93	3,6	3,4	92,2	5,3	2,5
	UT	100	0	0	98,9	1,1	0
	LA	100	0	0	84,5	14	1,1
2	AVL BA	**6**	**16**	**78**	**88,5**	**12**	**0**
	UT	99	1	0	86,9	8,4	4,6
	LA	96	3,6	0	81,3	12	6,7
3	EAA BA	99	1,5	0	92,5	7,5	0
	UT	100	0	0	93,5	4,3	2,2
	LA	100	0	0	83,6	15	1,3
4	JFCE BA	100	0	0	87,8	11	1,5
	UT	**0**	**100**	**0**	**81,4**	**12**	**7,1**
	LA	**9,4**	**90**	**0,7**	**97,6**	**2,1**	**0,3**
5	JSR BA	100	0	0	100	0	0
	UT	100	0	0	87,5	12	0,3
	LA	100	0	0	90	7,4	2,6
6	MMD BA	100	0	0	98,9	1,1	0
	UT	100	0	0	97,1	2,1	0,9
	LA	100	0	0	82,9	9,9	7,2
7	PRT BA	100	0	0	93,2	2,6	4,3
	UT	100	0	0	95,9	2,2	1,8
	LA	100	0	0	71,4	0	29
8	RCP BA	77	3	20	89,4	9,7	0,9
	UT	93	7	0	86,5	10	3,2
	LA	100	0	0	90,4	8,3	1,3
9	RLZ BA	87	13	0	86,8	7,9	5,4
	UT	0	100	0	*	*	*
	LA	100	0	0	76,5	19	4,2
10	RRD BA	**0**	**100**	**0**	**83,3**	**15**	**1,6**
	UT	**0**	**100**	**0**	**94,5**	**1,1**	**4,4**
	LA	100	0	0	89,2	4	6,8

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HCV THERAPY EFFECTIVENESS: MUTANT DETECTION:

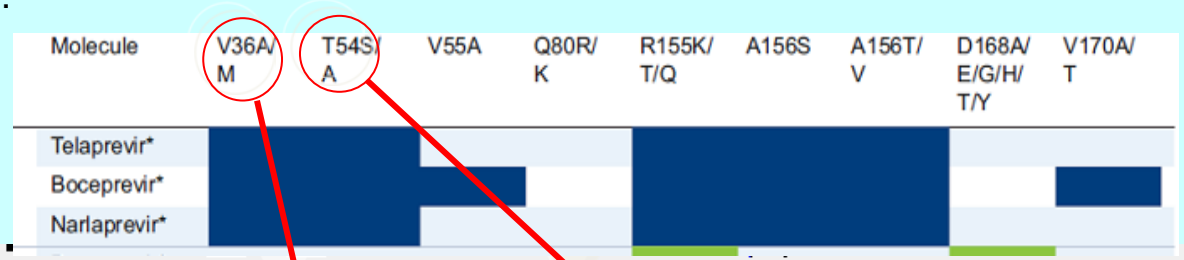
Resistant variant have been described in all HCV therapeutic targets regions (NS3, NS5A, NS5B) and for all treatments.

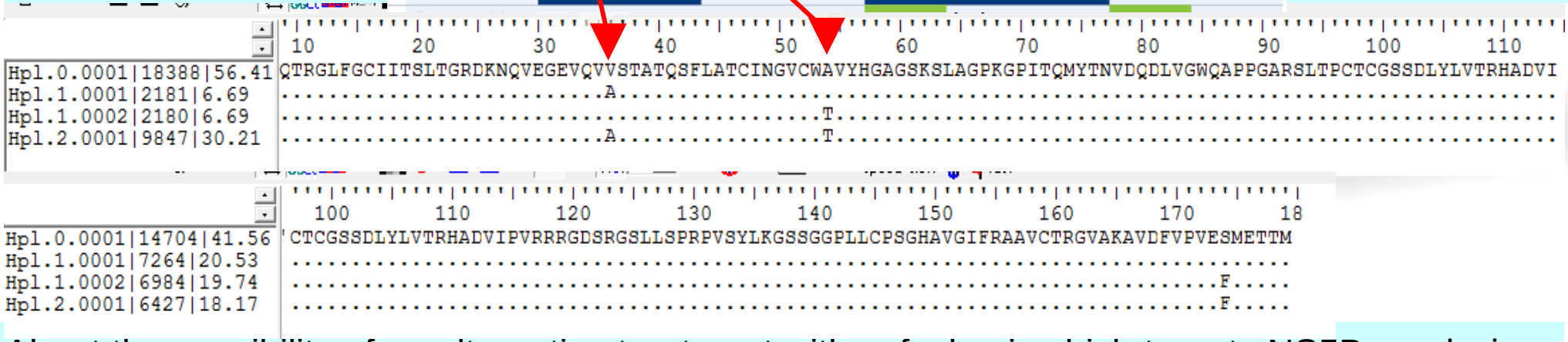
FAILURE TRIPLE HCV THERAPY: HCV G1b Liver transplant recipient treated with triple antiviral therapy: telaprevir+pegIF+RBV with persistence of HCV viremia and severe anemia after one month of treatment. Attending physicians asked us if they should stop treatment. We studied the treatment targeted NS3 region searching for resistant variants

NGS analysis detected two resistant variants to telaprevir: V36A (37%), T54A (63%)

Haplotype study showed:

- Wt V36-T54 (7%)
- A36/A54 (7%)
- V36/A54 (56%)
- A36/T54 (30%)



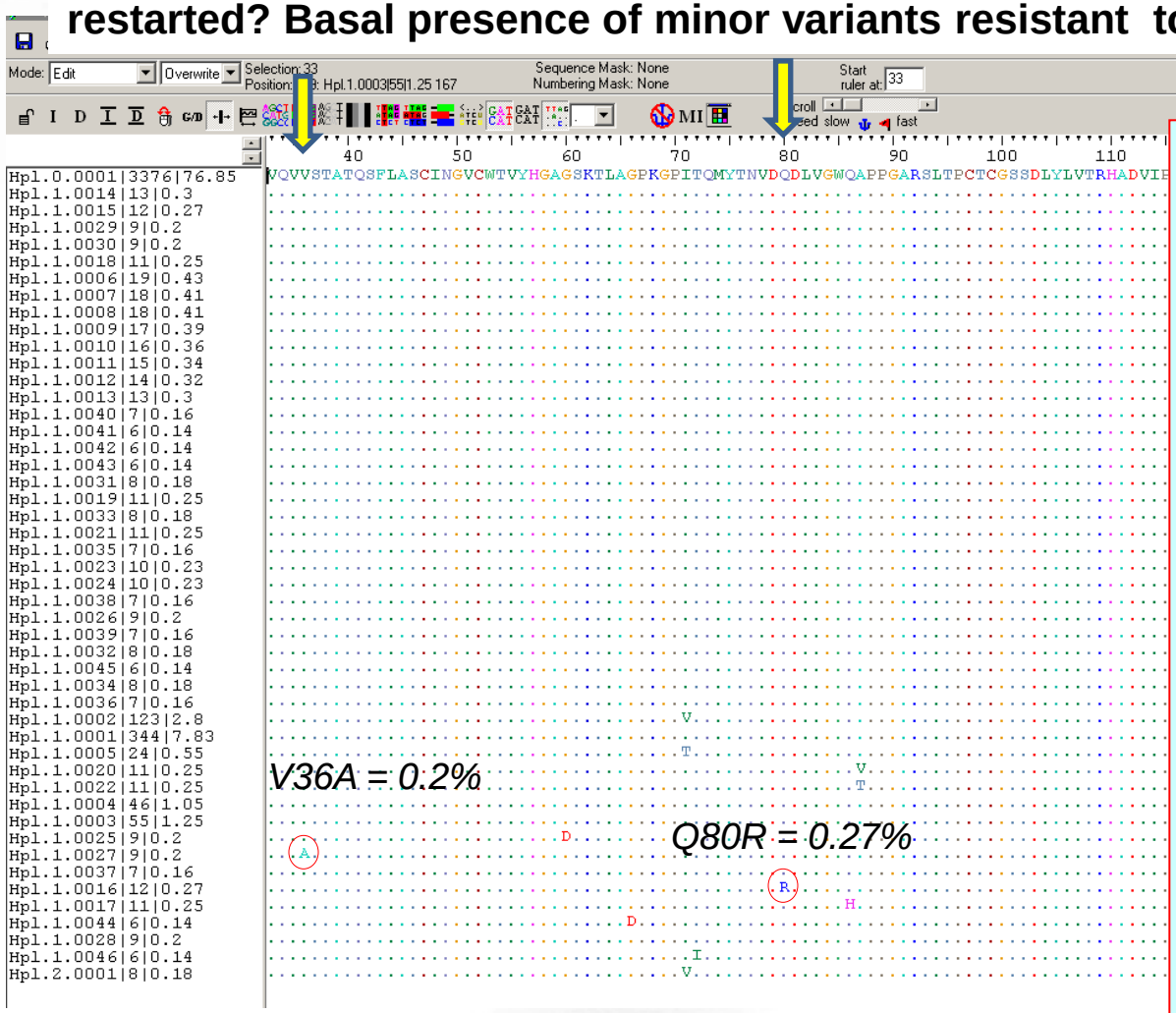


About the possibility of an alternative treatment with sofosbuvir which targets NS5B, analysis of this region showed no resistant variants, so we recommended discontinuation of telaprevir treatment and a switch to sofosbuvir, which achieved a sustained virologic response

HCV THERAPY EFFECTIVENESS: MUTANT DETECTION:

Resistant variant have been described in all HCV therapeutic targets regions (NS3, NS5A, NS5B) and for all treatments.

Telaprevir treatment in 2012, stop at 8w due to skin adverse effects. 2014 can therapy be restarted? Basal presence of minor variants resistant to TVR/BOC or SMV



These variants can remain in the quasispecies “memory” years after treatment discontinuation, questioning the adequacy of starting treatments with similar resistant profiles,:

”Eg:Vermehren 2012: 20-52% even four years after treatment failures”

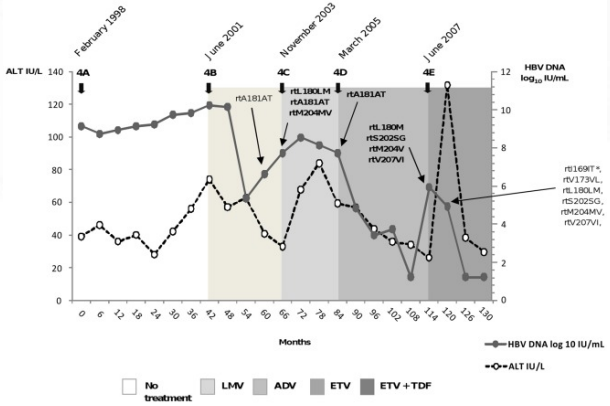
However, to date there is little evidence that a minor variant present in the basal quasispecies can cause treatment failure

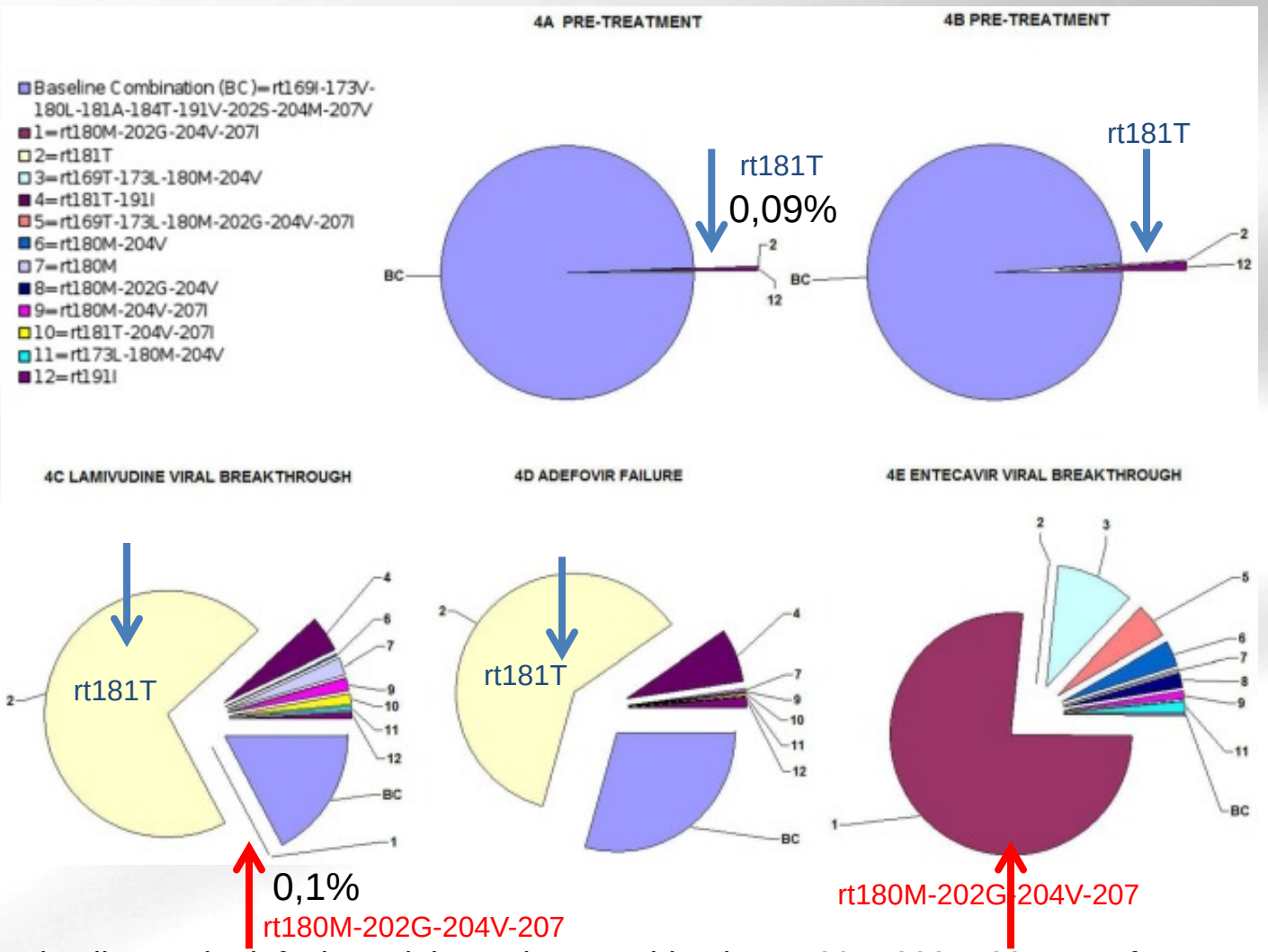
Eg, 3/10 telaprevir non-responders had resistant variant associated with prior treatment at baseline

De Meyer J Hepatol 2012.

DETERMINING RELEVANCE OF MINOR VARIANTS: HBV

HBV polymerase region dynamics: natural and under antiviral treatment:
Minor haplotype selection. **Resistant variants in very low proportions (0,1%) in pretreatment samples may be selected by the pressure of antiviral treatment and be responsible for treatment failure** (Rodriguez-Frias F PLoS 2012;19(12):867-71)





-Minor basal variant **rt181T** (0.09%, blue arrows) was selected by lamivudine treatment and minor variant rt180M-202G-204V-207I (0.1%, red arrows) was selected by lamivudine and ultimately was responsible for entecavir failure

NOTE: rt181T confers resistance to lamivudine and adefovir, and the variant combination rt180M-202M-204V confers resistance to entecavir

DETERMINING ORIGIN OF TRANSMISSION

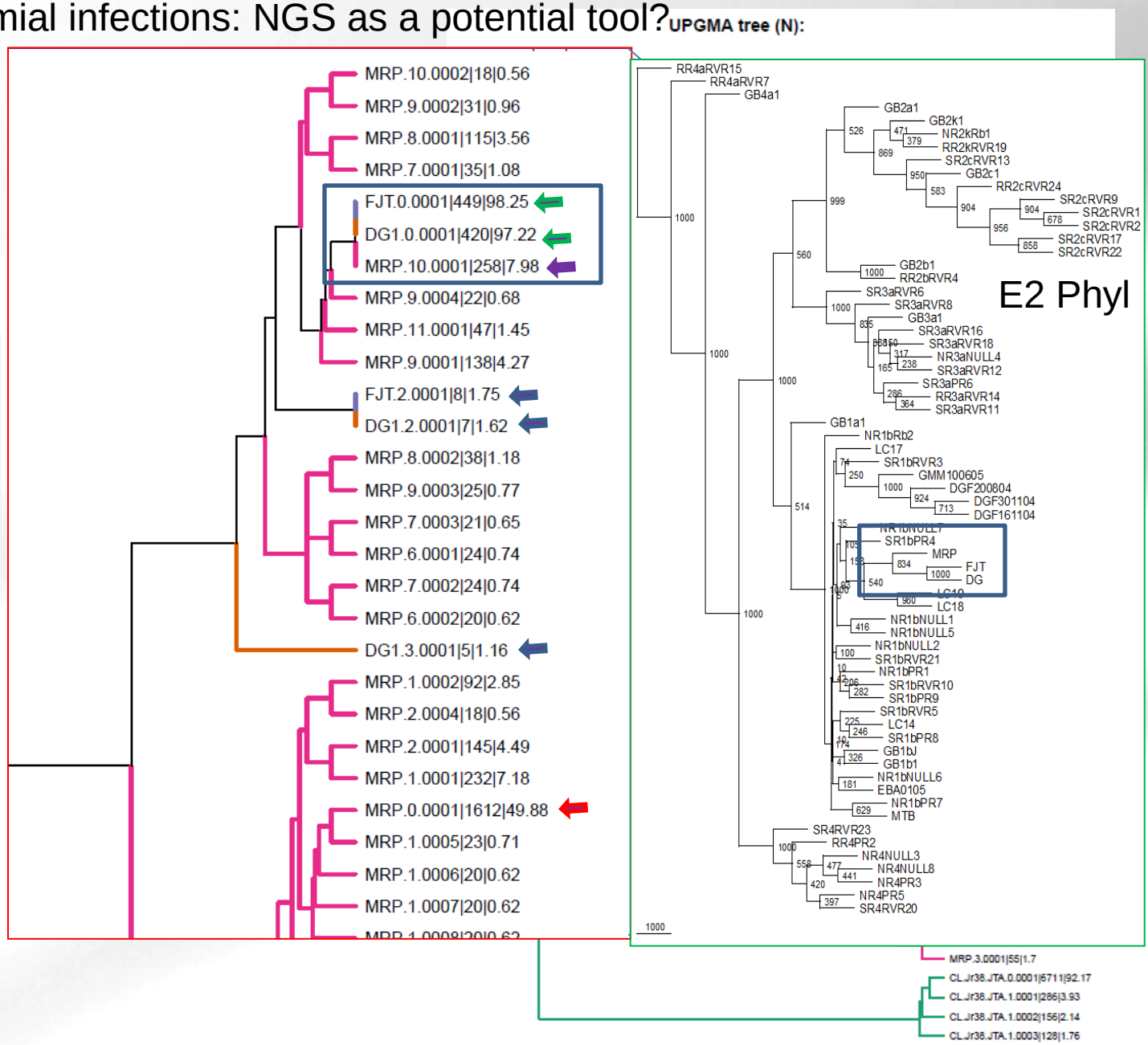
HCV acute nosocomial infections: NGS as a potential tool?

CL.	Local Control
DG1	Acute nosocomial infections
FJT	
MRP	Source of infection?

1-Acute cases quasispecies simpler than chronic (just blue and green arrows: 5 vs 27 haplotypes from chronic source MRP)

2-The study indicates that the infectious source was a minor haplotype (violet arrow) rather than the master (most frequent) haplotype: Bottle neck phenomenon.

3- In this case, classic analysis by direct sequencing Sanger was not as conclusive as NGS approach.



THE EMERGING PROBLEM OF HEV

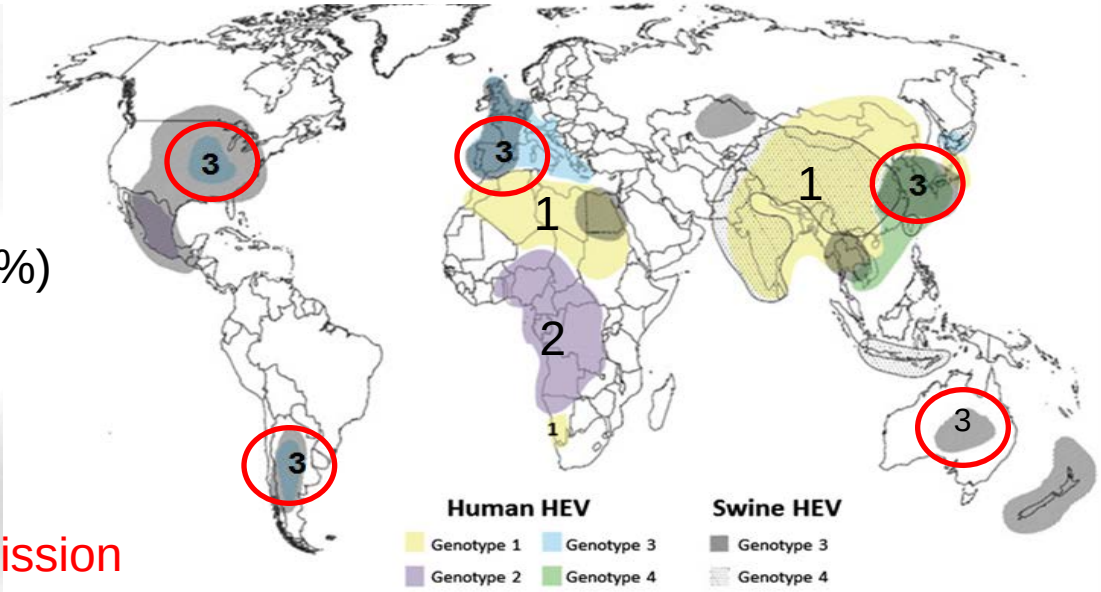
3 M symptomatic cases per year.70,000 deaths per year

Rein et al. Hepatology 2011.

-Four genotypes: two (G3/G4) also affect animals (zoonotic transmission)

-Not a third world disease: **G3** prevalent in industrialized countries (seroprevalence >20%) therefore native infection , with pigs as main reservoirs.

Fecal oral and by pork meat to human transmission **(G3),** but also parenteral transmission: Potential for transfusion transmission



Dual behavior:

-Acute infections: usually asymptomatic and self-limited in healthy subjects, but fulminant in specific high-risk groups (e.g. pregnant women >20% deaths, up to now only **G1/G2).**

-Sometimes progresses to chronic: Potential morbidity/mortality in immunosuppressed subjects: transplant recipients, HIV coinfected, hematological patients on chemotherapy, all **G3**. Fast progression of liver fibrosis in transplantation with chronic HEV.

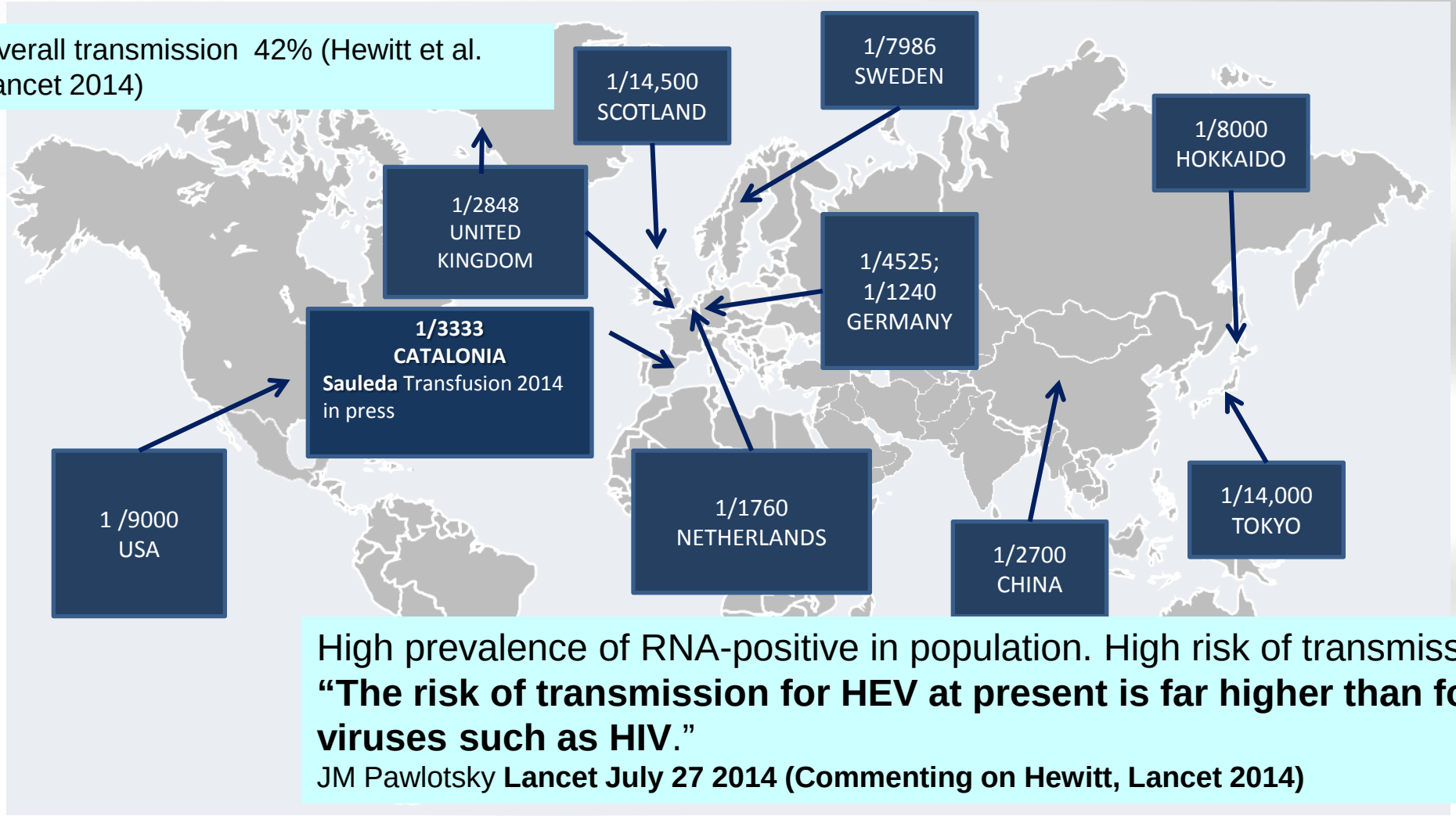
NGS potential utility: genotypìng and determining the origin of transmission

THE EMERGING PROBLEM OF HEV

HEV-RNA PREVALENCE IN BLOOD DONORS

Dr. S. Sauleda

Overall transmission 42% (Hewitt et al. Lancet 2014)



High prevalence of RNA-positive in population. High risk of transmission: **"The risk of transmission for HEV at present is far higher than for viruses such as HIV."**

JM Pawlotsky **Lancet July 27 2014 (Commenting on Hewitt, Lancet 2014)**

Ong ISBT 2014; Satake ISBT 2014; Sakata ISBT 2014; Cleland Vox Sang 2013; Slot Eur Sur 2013; Ren Transfusion 2014 Ushiro-Lumb IPFA 2014; Linnen IPFA 2014; Baylis Vox Sang 2012, Hewitt Lancet 2014, Hagema Transfusion 2014,, Vollner J Clin Microbiol 2012, Sauleda Transfusion 2014

THE EMERGING PROBLEM OF HEV

BLOOD TRANSFUSION HEV HEPATITIS: documented in a number of countries

AUTHOR	COUNTRY	BLOOD COMPONENT	RECEPTOR	CLINICS
Colson et al 2004	France	conc. erythrocytes	immunosupressed	acute hepatitis
Matsubayashi 2004	Japan	plasma	heart surgery	acute hepatitis
Boxall et al 2006	UK	conc. erythrocytes platelets	immunosupressed PBC	acute hepatitis no seroconversion
Tamura et al 2007	Japón	conc. erythrocytes	?	acute hepatitis
			5 receptos	no seroconverssion

Therefore, as in other potentialy transmissible infections such as HBV, HCV or HIV optimal phylogenesis is needed to confirm the origin of infection, and this can be done with NGS strategies like the one we used to investigate HCV transmission.

-Feray (Lancet 2014;383:218) France: 5/367 liver transplant recipients infected by blood transfusion developed liver graft damage

-HEV in blood products: 0.7% plasma minipools in England with HEV RNA , (Ijaz S Vox Sanguinis 2012, Cleland A Vox Sanguinis 2013).

-Since the first HEV blood transmission in the UK in 2006 (Boxall E Transfus Med 2006) just 8 cases (2 in 2013, 5 in 2012, and 1 in 2011)..

-Disease mainly in immunosuppressed

-Up of 60% of HEV infections in immunocompromised solid organ transplantation recipients may lead to chronic infection.

-HEV non-enveloped virus, behaviour similar to other non-enveloped viruses (HAV, B19), resistent to plasma inactivation strategies. Hauser et al. Blood 2014,Andonov et al. Vox Sang 2014)

THE EMERGING PROBLEM OF HEV

BLOOD TRANSFUSION HEV HEPATITIS

www.thelancet.com Published online July 28, 2014 http://dx.doi.org/10.1016/S0140-6736(14)61034-5

Hepatitis E virus in blood components: a prevalence and transmission study in southeast England

Patricia E Hewitt, Samreen Ijaz, Su R Brailsford, Rachel Brett, Steven Dicks, Becky Haywood, Iain T R Kennedy, Alan Kitchen, Poorvi Patel, John Poh, Katherine Russell, Kate I Tettmar, Joanne Tossell, Ines Ushiro-Lumb, Richard S Tedder

Phylogenesis is needed to confirm the source of infection.

NGS can help in this task.

225,000 individual donations screened and 79 donations contained HEV RNA. Prevalence 1:2848 donations: about 80,000–100,000 acute HEV infections may have occurred in England during the year of the study.

129 components derived from the 79 HEV RNA-positive donations and 62 (48%) components given as transfusions to 60 recipients. 43 patients were followed and 18 (42%) had evidence of infection. **Transmission confirmed by phylogenesis**.

THE EMERGING PROBLEM OF HEV

HEV INFECTION AND NGS: A SINGLE ARTICLE, EXPERIMENTAL HUMAN TO SWINE TRANSMISSION

High-throughput sequencing. Illumina GAII sequencing was subcontracted to GATC (Constance, Germany). High-molecular-weight DNA (5 g), resulting from genomic RNAs as described above, was fragmented into 200- to 350-nt fragments, to which adapters were ligated. Adapters included a nucleotide tag allowing for multiplexing of the three samples in one channel.

Phylogenesis was not performed

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UMR 1161 Virology, ANSES, Laboratoire de Santé Animale, Maisons-Alfort, France[a]; UMR 1161 Virology, INRA, Maisons-Alfort, France[b]; UMR 1161 Virology, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France[c]; Pathoquest, Paris, France[d]; and Department of Virology, Institut Pasteur, Paris, France[e]

High-throughput sequencing of bile and feces from two pigs experimentally infected with human hepatitis E virus (HEV) of genotype 3f revealed the same full-length consensus sequence as in the human sample. Twenty-nine percent of polymorphic sites found in HEV from the human sample were conserved throughout the infection of the heterologous host. The interspecies transmission of HEV quasispecies is the result of a genomic negative-selection pressure on random mutations which can be deleterious to the viral population. HEV intrahost nucleotide diversity was found to be in the lower range of other human RNA viruses but correlated with values found for zoonotic viruses. HEV transmission between humans and pigs does not seem to be modulated by host-specific mutations, suggesting that adaptation is mainly regulated by ecological drivers.

THE EMERGING PROBLEM OF HEV

HEV INFECTION AND NGS

Pork meat to human transmission and mutagenic effect of ribavirin treatment

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Volume 60, Issue 1, Supplement, Page S287, April 2014 | Next Article »

P657 *IN VIVO* MUTAGENIC EFFECT OF RIBAVIRIN ON SUBTYPE 3F HEV QUASISPECIES ISOLATED FROM A PATIENT INFECTED FROM PORK MEAT

R. Casillas, F. Rodríguez-Frias, B. Mínguez, M. Riveiro, J. Gregori, D. Garcia-Cehic, D. Tabernero, M. Homs, M. Blasi, A. Caballero, L. Nieto, A. Hundesa, R. Girones, M. Buti, J. Quer

DOI: http://dx.doi.org/10.1016/S0168-8278(14)60819-9

PATIENTS and METHODS

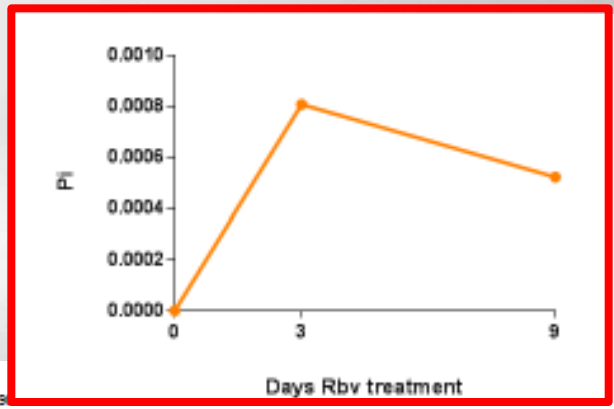
An immune compromised patient with Acute Hepatitis E was treated with RBV for 14 days. The epidemiological survey revealed that one month before admission the patient ate undercooked pork meat from a local family farm. Thus, a possibility of zoonotic transmission emerged.

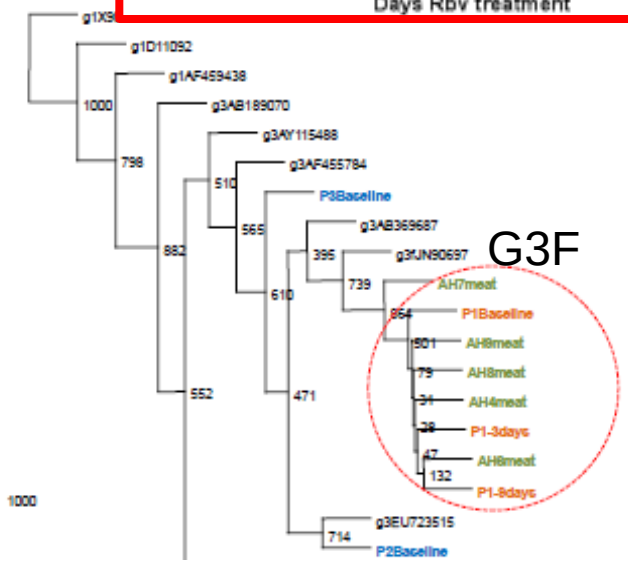
We phylogenetically studied an RT-PCR fragment of the ORF2 core gene (Figure 1) isolated from patient's serum samples and from a frozen pork meat. Serum HEV population at day 0 (t0), 3 (t3), 9 (t9), 15 (t15) and 79 (t79) of Rbv therapy, were studied by ultra-deep pyrosequencing (UDPS, 454/GS-Junior, Roche).

RESULTS

Sequence obtained from the patient was identical to pork meat isolates (consensus sequence and six clones, Figure 2) suggesting possible foodborne zoonotic transmission. Phylogenetic tree confirms this connection, since the sequences are in the same cluster, and show that the patient was infected with genotype 3f (Figure 3).

A total of 26239 sequences were obtained by UDPS from patient samples. HEV RNA declined from $4,87x10^5$ at t0, to $1,21x10^3$ at t3 and $1,11x10^2$ genome-copies/ml at t9. It was negative at t15 and t79. HEV variability increased from t0 (PI [Nucleotide Diversity]= 0.0000, 1 haplotype) to t3 (PI=0.00081, 15 haplotypes) (35% of substitutions associated to Rbv treatment) and declined at t9 (PI= 0.00052, 6 haplotypes) (Figure 4, Table 1).





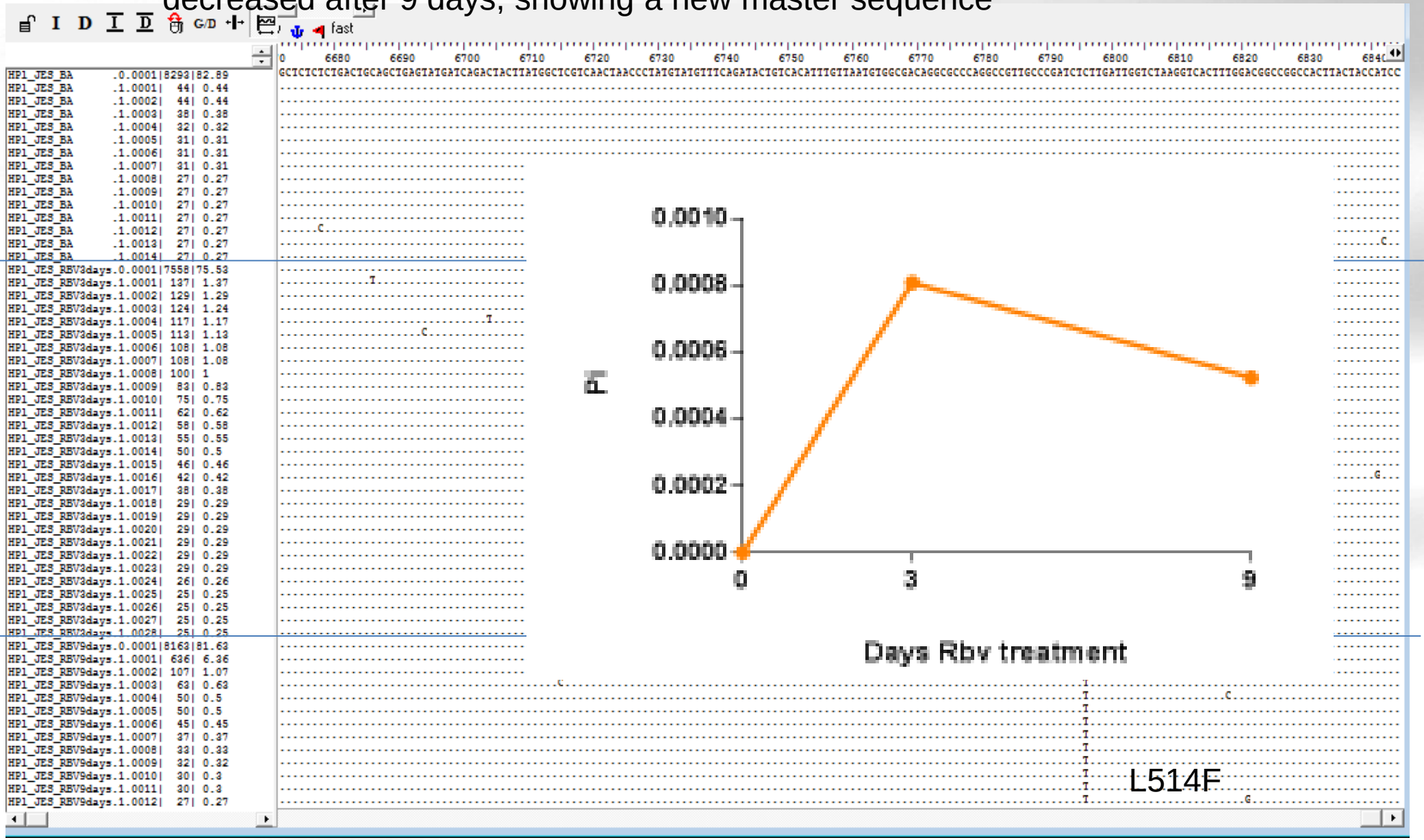
CONCLUSION

These results suggest that HEV was transmitted by ingestion of pork meat. The Ribavirin was mutagenic in vivo causing a temporal increase in variability at day 3. At t9, the mutagenic effect of Ribavirin was not detected, probably due to viral load decay observed just before resolution of infection.

THE EMERGING PROBLEM OF HEV

HEV INFECTION AND NGS: Mutagenic effect of RBV?

After only 3 days of RBV therapy, complexity of HEV quasispecies increased, then decreased after 9 days, showing a new master sequence



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CONCLUSIONS

NGS techniques are useful to deeply study the viral quasispecies

When equipping your laboratory, choose the one best suited to your needs

Knowledge gained with the use of these techniques will help us to better understand and better treat viral infections

Thanks for your attention

And thanks to my team, here in one of our routine **N**ormal **G**eneral **S**miling experiments

"Choose a job you love and you will not have to work a day in your life" Confucius

"*The difference between a politician and a statesman is that a politician thinks about next elections, while the statesman thinks about the **Next Generations***."

Winston Churchill…

And they are the "**N**ext **G**enerations"

Dr. Josep Gregori Dr. Josep Quer