Complex HBV Quasispecies Affects Immunogenicity in Acute Hepatitis B Infection

Claudia Alteri[1], Valentina Svicher[1], Marianna Aragri[1], Nicola Coppola[2], Arianna Battisti[1], Caterina Sagnelli[3], Maria Antonietta Pisaturo[2], Maria Concetta Bellocchi[1], Evangelista Sagnelli[2], Carlo Federico Perno[1]

[1]Tor Vergata University, Department of Experimental Medicine and Surgery, Rome, Italy; [2]Department of Mental Health and Public Medicine, section of Infectious Diseases, Second University of Naples, Italy; [3]Department of Clinical and Experimental Medicine and Surgery, Second University of Naples, Naples, Italy

Dr. Claudia Alteri
University of Rome Tor Vergata
Via Montpellier 1, Rome 00133, Italy
Phone No: +39-06-72596553
e-mail: claudia.alteri@uniroma2.it

Università di Roma Tor Vergata

HIRMA PROJECT

Bandiera PB05 PROJECT

P-N11

Background & Objectives

- Even in the face of current vaccination programs, cases of acute HBV infection (AHB) are still registered and in some countries (such as Italy) the incidence of AHB remains higher than that observed for other hepatitis viruses (www.simi.iss.it/seieva.htm). This underlines the need to deeply characterize the virus circulating in these acutely infected patients.

- Limited information is so far available about the prevalence of drug resistance mutations in the setting of acute hepatitis B as well as of immune escape mutations and stop codons in HBsAg (associated with increased HBV oncogenic potential)

In this light, the goal of this study is to investigate HBV-RT and HBsAg quasispecies heterogeneity in patients with acute hepatitis B infection (AHB) in Italy and to define their clinical value.

Methods

- This study included 62 patients (44 infected by genotype D and 18 genotype A) positive for HBsAg and IgM/anti-HBc with a compatible clinical-history for AHB observed from 2000 to 2010.

- Plasma-samples obtained at the first-observation were analyzed by Ultra-deep pyrosequencing (UDPS). Shannon Entropy (weighted for the intra-patient prevalence of each mutated position) was used to measure the extent of amino acid variability in HBsAg.

Results

PATIENTS' CHARACTERISTICS

	Overall population	Genotype D	Genotype A	P value[a]
No. Cases	62	44	18	
Age, (years) median (range)	36 (29- 46)	34 (27- 46)	37 (30- 46)	ns
Male, n (%)	47 (75.8%)	30 (68.2%)	17 (94.4%)	0.03
Risk factor, n (%):				
Sexual intercourse	28 (45.2%)	19 (43.2%)	9 (50.0%)	ns
Drug addiction	18 (29.0%)	17 (38.6%)	1 (5.6%)	0.01
Tattoo/agopuncture, surgery, endoscopy, dentist intervetion	11 (17.7%)	4 (9.1%)	7 (38.9%)	0.01
Unknown	5 (8.0%)	4 (9.1%)	1 (5.6%)	ns
Italian nationality, n (%)	53 (85.5%)	36 (81.8%)	17 (94.4%)	ns
Years of observation (median, IQR)	2006 (2002- 2009)	2005 (2002-2008)	2006 (2002- 2010)	ns
AST, UI/dl (median, IQR)	1,653 (1,216- 2,253)	1,777 (1,334- 2,499)	1,528 (1,098- 2,006)	ns
ALT, UI/dl (median, IQR)	2,544 (1,938- 3,078)	2,716 (2,035- 3,319)	2,372 (1,841- 2,837)	ns
Log HBV-DNA, UI/mL (median, IQR)	5.88 (4.47- 7.37)	5.24 (4.42- 6.91)	6.53 (4.53- 7.84)	ns
Severe acute hepatitis B, n (%)	4 (6.5%)	4 (9.1%)	0 (0.0%)	ns
Co-infection, n (%):				
Anti-HDV	0 (0.0%)	0 (0.0%)	0 (0.0%)	ns
Anti-HCV	8 (12.9%)	8 (18.2%)	0 (0.0%)	ns
Anti-HIV	0 (0.0%)	0 (0.0%)	0 (0.0%)	ns
Clinical outcome, n (%):				
HBsAg- and Anti-HBs +	33 (53.2%)	24 (54.5%)	9 (50.0%)	ns
HBsAg - and Anti-HBs -	28 (45.7%)	19 (43.2%)	9 (50.0%)	ns
Chronic infection (HBsAg+)	1 (1.6%)	1 (2.3%)	0 (0.0%)	ns

[a]Statistically significant differences were assessed by Mann-Whitney Test.

PRIMARY DRUG RESISTANCE MUTATIONS OCCUR WITH A LOW INTRA-PATIENT PREVALANCE WHILE COMPENSATORY MUTATION AS MAJOR SPECIES

Patient ID	HBV genotype	HBV RT position[a]	Intra-patient prevalence[b] (Number of reads)	Mutational Load[c] (UI/ml)
7	D	$_{rt}$A181T	0.11% (4)	105,756
7	D	$_{rt}$A194T	0.23% (4)	210,841
7	D	$_{rt}$M204I	0.23% (4)	206,904
16	D	$_{rt}$A181T	47.50% (3587)	14,769
47	A	$_{rt}$V173L	10.50% (423)	1,516
51	A	$_{rt}$A194T	1.13% (44)	52,083
53	A	$_{rt}$V173L	63.48% (2051)	4,532,386
53	A	$_{rt}$L180M	99.98% (4223)	7,138,310

[a]The table reports drug resistance mutations that were evaluated by using RT sequences obtained by UDPS. Only mutations detected in ≥ 4 reads in both forward and reverse primers were considered for the analysis. The list of drug resistance mutations is available at the following website http://hbv.bioinf.mpi-inf.mpg.de/index.php updated on October 2013.
[b]Intra-patient prevalence was expressed as % of reads with the specific mutation.
[c]Mutational load was calculated by the following formula: n° of reads with the specific mutation x patient's viremia / tot n° of reads obtained for each patient.

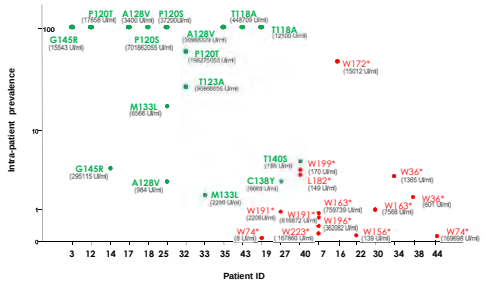
THE MAJOR HYDROPHYLIC LOOP IN HBsAg IS CHARACTERIZED BY A TREND OF HIGHER DEGREE OF GENETIC DIVERSITY IN GENOTYPE D

	Genotype D	Genotype A
(A)Genetic Distance (mean±SE):	0.027±0.011	0.003±0.002
(B)Amino acidic Complexity:		
H (mean±SE)	0.184±0.053	0.123±0.054
H_d (mean±SE)	0.084±0.024	0.059±0.027
S_n (mean±SE)	0.141±0.034	0.109±0.038
Mf (mean±SE)	0.008±0.003	0.004±0.002
K (mean±SE)	0.120±0.048	0.065±0.030

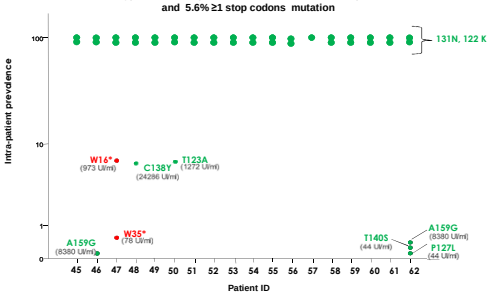
Genetic distance and Shannon entropy parameters were shown as mean and Standard Error (SE).
H: Haplotype. **H_d:** Haplotype diversity. **Sn:** Normalized quasispecies Shannon entropy. **Mf:** Mutation frequency.**K:** Mean number of pairwise differences.
Genetic distances were estimated by using the software MEGA v5.2. H, HD, Sn, Mf, K were calculated according to the formulas described by Ramirez et al., 2013. Genetic distance and Shannon entropy parameters were shown by mean and Standard Error (SE). A trend of increase of parameters that estimate genetic variability in HBsAg was observed in genotype D compared with genotype A. Among these, only the genetic distance shows a significant difference between the two genotypes (P-value <0.001, by Mann-Whitney).

CHARACTERIZATION OF IMMUNE ESCAPE MUTATIONS AND STOP CODONS IN HBsAg

In Genotype D, 29.5% of patients carried ≥1 immune escape mutation and 22.7% ≥1 stop codons mutation



In Genotype A, 100% of patients carried ≥1 immune escape mutation and 5.6% ≥1 stop codons mutation



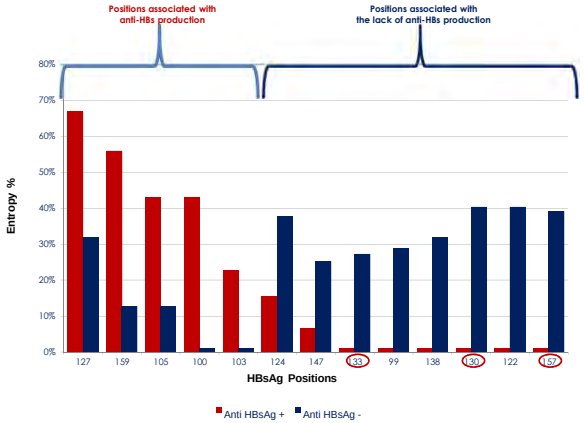
Both graphs report the intra-patient prevalence of immune escape mutations (in green color) and the inta-patient prevalence of stop codons (in red color), detected in the 62 patients infected by HBV genotype D (upper graph) and A (lower graph). Intra-patient prevalence was expressed as % of reads with the specific mutation. Values in parentheses correspond the mutational load defined according to the following formula: n° of reads with the specific mutation x patient's viremia / tot n° of reads obtained for each patient. Immune escape mutations were retrieved from literature (Hou et al., 2001; Torresi et al., 2002; Sheldon et al., 2008; Olyaee et al., 2010)

- Although no patients received HBV-vaccination, vaccine-escape mutations (sG145R, sM133L, and sP120S) were found in 11.4% of patients, all genotype D-infected. Among them, sG145R (known to affect not only recognition but also production of anti-HBs) was detected in 2 patients with an intra-patient prevalence of 3.9% (mutational-load:295,000IU/ml) and 99.9% (mutational-load:15,543IU/ml).

- In **genotype A**, the amino acid K and N (known to hamper humoral HBsAg recognition) are the wild-type aa at HBsAg positions 122 and 131. These positions may be responsible for a higher intrinsic propensity of HBV genotype A to an easier escape from the immune pressure. This aspect needs to be further investigated.

- Stop-codons were found in 19.3% patients with a intra-patient prevalence ranging from 1.6% to 47.5% (mutational-load from 149 to 15012U/ml) . They occurred at 11 HBsAg positions including 172 and 182, known to increase HBV oncogenic potential.

THE EXTENT OF GENETIC VARIABILITY AT SPECIFIC HBSAG POSITIONS CAN IMPACT ANTI-HBS TITER PRODUCTION



The graph reports HBsAg positions with a statistically significant difference in the values of Shannon entropy (expressed as %) between the group of patients developing anti-HBs (red bars) and the group of patients not developing anti-HBs (blue bars). Shannon Entropy measures the extent of genetic variability at each HBsAg amino acid positions.
To define the amino acidic complexities of viral population for each patient, we estimated the Shannon entropy value, by using the tool available in Los Alamos database (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy.html).

IC *:	Anti-HBs+	Anti-HBs-	P value
sAA_130 (mean±SE)	0±0.000	1.98±0.011	0.05
sAA_133 (mean±SE)	0±0.000	1.95±0.033	0.02
sAA_157 (mean±SE)	0±0.000	1.98±0.010	0.05

*IC, (Information contents at a site): Given the relative frequency of each nucleotide in the population, at a specified site.
Refining the analysis by using a quantitative description of HBV viral quasispecies, an higher variability at 3 HBsAg positions (AA position 130, 133, 157) was reconfirmed to be associated with the Anti-HBc +/ Anti-HBs – status.

- The extent of genetic variability at some specific HBsAg positions (99, 122, 124, 130, 138 and 147) correlates with the lack of anti-HBs production.

- Refining the analysis by using a quantitative description of HBV viral quasispecies, an higher variability at 3 HBsAg positions (AA position 130, 133, 157) was reconfirmed to be associated with the Anti-HBc positivity and Anti-HBs negativity status.

Conclusions

A substantial fraction of AHB-patients is characterized by a complex viral quasispecies with reduced antigenicity/immunogenicity, enhanced oncogenic-potential and/or altered drug-susceptibility. These viral variants may potentially expose patients to severe and/or difficult-to-treat forms of HBV-infection (also in the setting of HBV reactivation), and may affect the efficacy of current HBV vaccination strategy.