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Phylogenetic analysis of isolates from new cases of HBV infection in Southern Italy

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ABSTRACT

The level of endemicity of hepatitis B virus (HBV) infections in Italy is low and genotype D infections predominant. New HBV strains may however be introduced as a result of movements of people from regions of high endemicity. The aim of the present study was to determine whether strains from new cases of acute hepatitis B detected in southern Italy were due to endemic or new HBV strains. We studied 34 isolates from patients with acute hepatitis B infection, and 35 from chronic hepatitis B patients. A phylogenetic analysis of preS/S region was done by comparing the sequences from the acute and chronic cases with references sequences. The study showed that 44% of strain from acute hepatitis B patients were of genotype A, 53% of genotype D, and 3% of genotype E. The molecular analysis of isolates from acute hepatitis B patients from Sicily showed a change in the local epidemiology of this infection, with an increase in HBV/A infections and a clustering effect for HBV D2, possibly correlated to immigration. The introduction of new genotypes , could have an effect on HBV-correlated diseases due to the different association between genotype, liver disease and response to antiviral therapy.

1. Introduction

Hepatitis B virus (HBV) strains display great genetic heterogeneity as a consequence of their unique replication cycle and are classified into eight genotypes (A–H) characterised by different geographic distribution (Norder et al., 2002; Schaefer, 2007). Genotypes A–D show such genetic diversity that they are divided into subtypes (Makuwa et al., 2006; Tatematsu et al., 2009; Utsumi et al., 2009). Genotypes A and D are distributed widely throughout the world, with the A1 subtype prevailing in Africa and India, the A2 subtype in northeast Europe and North America and genotype D predominating in the Mediterranean basin.

The highly conserved S gene is best suited for discriminating HBV isolates belonging to different genotypes and for study the phylogenetic correlations between different strains by comparison to sequences previously reported in Genbank (http://www.ncbi.nlm.nih.gov/genbank/).

In Italy, HBV genotype D is responsible for 90% of chronic HBsAg carriers, independent of whether they progress to liver disease or not (Dal Molin et al., 2006). On the contrary, there are limited studies regarding the circulation of strains in new cases of HBV infection (Biliotti et al., 2008). Over the last 20 years, the endemicity of HBV infections in Italy has shifted from an intermediate to low (<2%) as a result of improving socioeconomic standards and of the introduction of universal anti-HBV vaccination programs. However, 1.3 cases of acute hepatitis B (AHB) per 100,000 persons above the age of 25 were registered in 2005 (Mele et al., 2008). Displacement of people from regions where HBV vaccination has not been implemented universally, in combination with poor socioeconomic conditions, can result in pockets of high endemicity in countries where incidence rates are otherwise low (Palumbo et al., 2008). This scenario results in an increased risk of infection and the introduction of HBV strains other than those prevalent to a specific region (Williams, 2006).

The aim of this study was to characterise HBV isolates collected in Sicily, a region of southern Italy in the middle of Mediterranean basin, to determine whether new cases of HBV infection were due to endemic strains of HBV genotype D or to new HBV genotypes introduced into this population.

2. Materials and methods

2.1. Samples

Serum samples of 34 patients, enrolled consecutively with symptomatic acute hepatitis B (AHB) referred to the GI and Liver Unit at the University of Palermo and to the Infectious Disease Unit at the University of Catania between July 2006 and October 2009,

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were included in this study. Of the patients studied who presented with AHB (32 men, two women; mean age 44, range 26–81 years) 32/34 were Sicilian. Self-reported risk factors for HBV included promiscuous or unsafe sexual behaviours for 19 participants (56%), recent tattooing for three (9%), minor surgical procedures for five (15%), and intravenous drugs use for one (3%). Six patients (17%) did not describe any evident exposures to risk factors associated with infection.

HBV strains from 35 Sicilian patients (27 men, eight women, mean age 58, range 38–78 years) with untreated chronic hepatitis B (CHB) and diagnosed over the previous 15 years, also seen at the above clinics, were included as a control group.

The study protocol was approved by hospital's Ethics Committee, and written consent for collection of clinical data and sequence analysis of HBV–DNA was obtained from all patients.

2.2. HBV–DNA testing

Viral DNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Indianapolis, USA). Full-length HBV DNA (3.2 kb) was amplified (Günther et al., 1995), using the Expand High Fidelity PCR System (Roche Diagnostics). The first-round PCR product was amplified to obtain three overlapping fragments covering the entire preS/S region in three different reaction mixtures, with 800 nM of each primer (HBV1 5′ GGTCACCATATTCTTGGGAA 3′ and HBV10 5′ CCTGATGTGATGTTCTCCATG 3′; DON2 5′ TCCACAACCTTCCACCAAACT3′ and HBV2 5′ AATGGCACTAGTAAACTGAG 3′; 377 5′ GGATGTGCTTGCGGCGTTT 3′ and OS2 5′ TCTCTGACATACTTTCCAAT 3′).

2.3. Sequencing

The nucleotide sequence of the preS/S region was determined by sequencing three overlapping fragments amplified by nested-PCR. Approximately 15 ng of purified DNA were labelled by Big Dye Terminator v 1.1 and analysed by a sequencer ABI Prism 3100 instrument (Applied Biosystems, Foster City, USA).

2.4. Sequence analysis

A consensus sequence of 1.3 kb corresponding to the preS/S region was generated by assembling the three partially overlapping fragments with BioEdit software (Tom Hall, USA). Ambiguities were resolved by sequencing both sense and antisense strands. The preS/S sequences obtained were compared to sequences of genotypes A (A1, A2,A4, A5, A6 subtype) and genotype D (D1, D2 , D4 and D6 subtype), published in GenBank (http://www.ncbi.nlm.nih.gov/genbank).

All sequences were aligned based on viral genotype, using the Clustal W algorithm and confirmed by visual inspection. Phylogenetic trees and nucleotide difference matrixes were constructed using the Mega 4.1 program using the Kimura two-parameter system and the neighbour-joining method (NJ). Phylogenies were also reconstructed using the maximum-likelihood method (ML), and topologies confirmed by both methods.

The mean synonymous (d_S) and nonsynonymous (d_N) substitution rates were estimated using the model of Nei and Gojobori with the Jukes–Cantor correction with MEGA 4.1. The d_N/d_S ratios were used to measure immune pressure selection.

2.5. Statistical analysis

Differences in the means between groups were compared using an unpaired Student's-*t* test. A *p*-value of <0.001 was considered statistically significant.

3. Results and discussion

The study showed a high rate of HBV strains genotype non-D, isolated from new cases of infection in Sicilian patients infected mainly through unsafe sex. Indeed, HBV genotyping and subtyping of the new strains, assessed by phylogenetic tree analysis (data not shown), demonstrated that 15 (44%) were HBV genotype A (HBV/A), 18 (53%) genotype D (HBV/D), and one genotype E (3%) (HBV/E). The HBV/A strains identified were of subtype A1 (HBV/A1, $n = 1$) and subtype A2 (HBV/A2, $n = 14$). The HBV/D strains identified were subtype D1 (HBV/D1, $n = 13$), and subtype D3 (HBV/D3, $n = 5$). The single genotype E strain identified was obtained from a patient born in Ghana where the prevalence of HBsAg chronic carriage is the highest in the world and genotype E strains are dominant (95%) (Garmiri et al., 2009). Risk factors for HBV subtypes are described in Table 1.

Most strains of subtype HBV/A2 was observed among patients living in western Sicily.

Of the 35 strains from CHB patients enrolled, 33 (94%) were HBV/D, of which 26 (78%) were HBV/D1, one (3%) HBV/ D2, six (18%) HBV/D3 and two (5.7%) HBV/A2. This high prevalence of the HBV/D genotype in CHB in Southern Italy has already been reported (Mangia et al., 2008).

As a means of studying the phylogenetic correlations of the AHB isolates, viral sequences obtained from the enrolled AHB patients were compared to sequences from CHB patients and to reference HBV sequences available in GenBank.

The single HBV/A1 isolate, found in the AHB group, segregated with reference sequences from the Philippines and from India (phylogenetic tree not shown) and was isolated from an intravenous drug user patient who had a travel history to the Indian subcontinent 2 months before the onset of hepatitis.

Eleven of the 14 HBV/A2 strains examined were isolated from patients referred to Palermo (western Sicily) and all the HBV/A2 strains identified were grouped to the top of the phylogenetic tree (Fig. 1). Within this group was observed one cluster, supported by a 94% bootstrap value, consisting of one sequence from one of two Sicilian CHB/A2 patient and five sequences from AHB patients. The results of phylogenetic analysis comparing to sequences from a limited number of Sicilian CHB/A2 patient and sequences available in GenBank does not allow to trace a origin of these new isolates. According to the promiscuous and unsafe sexual behaviour in the most cases and the high endemicity of HBV/A2 in northeast Europe allows to hypothesise that the HBV/A2 isolates could come from these region.

HBV/D1 was the second most prevalent genotype detected among AHB patients and the most prevalent among CHB cases. Unlike HBV/A2, 11/13 HBV/D1 strains were obtained from patients referred to Catania (Eastern Sicily). Among them, the main risk factor for HBV infection was also promiscuous and unsafe sexual practices.

The phylogenetic tree of this subtype (Fig. 2) showed that the sequences HBV/D1 from Sicilian CHB patients and from AHB

Table 1
Risk factors associated with acute hepatitis B infections by subtype.

HBV Subtype	Risk factors				
	Unsafe sex	Tattoo	Minor surgery	IDU[a]	Unknown
A1	–	–	–	1	–
A2	8	1	2	–	3
D1	10	2	1	–	–
D3	–	–	2	–	3
E	1	–	–	–	–
Total	19	3	5	1	6

[a] IDU: intravenous drugs use.

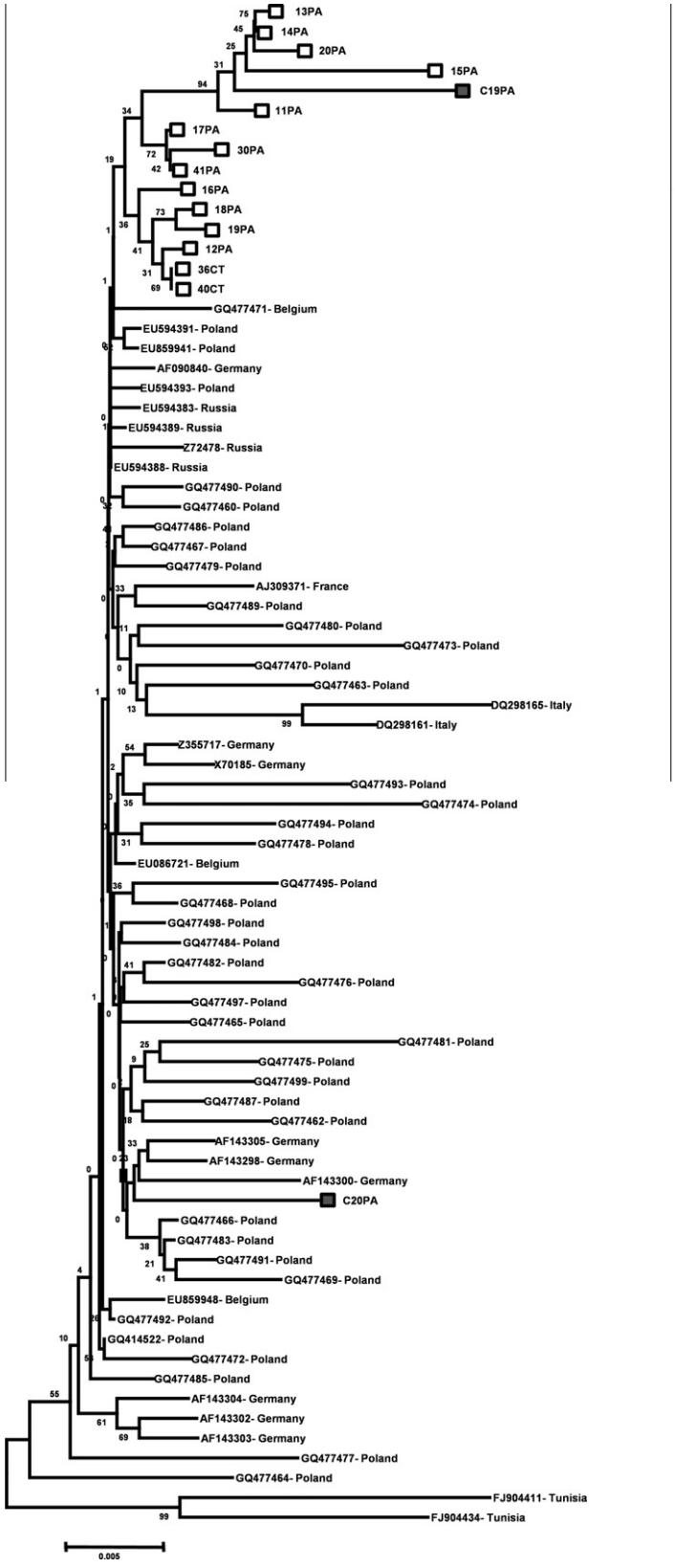

Fig. 1. Neighbor-joining tree of the preS/S region of HBV/A2 sequences using boostrap values of 1000 replicates. The sequences from Sicilian patients presenting with acute hepatitis B are prefixed by "□" and those with chronic hepatitis B by "■". The geographic location from where the reference sequences were obtained is indicated to the right of the tree.

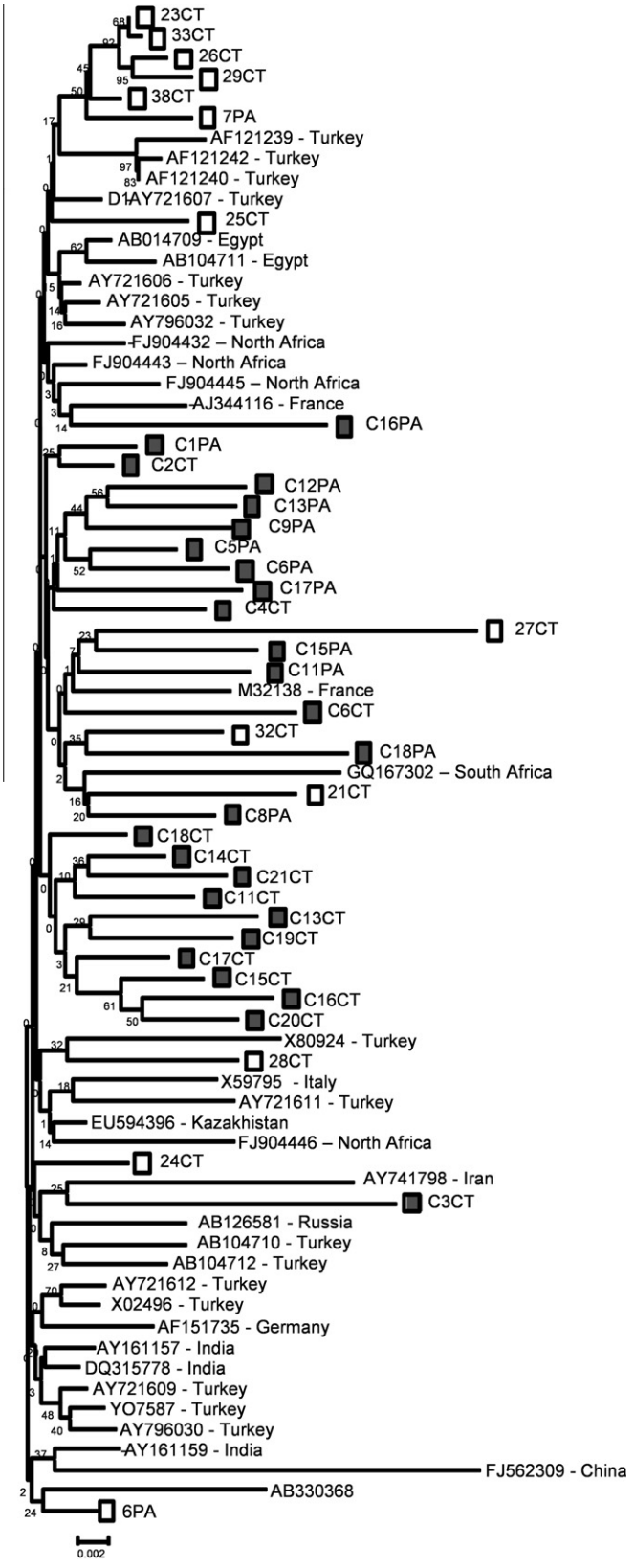

Fig. 2. Neighbor-joining tree of the preS/S region of HBV/D1 sequences using boostrap values of 1000 replicates. The sequences from Sicilian patients with acute hepatitis B infections are prefixed by "□" and those with chronic hepatitis B infections by "■". The geographic location from where the reference sequences were obtained is indicated to the right of the tree.

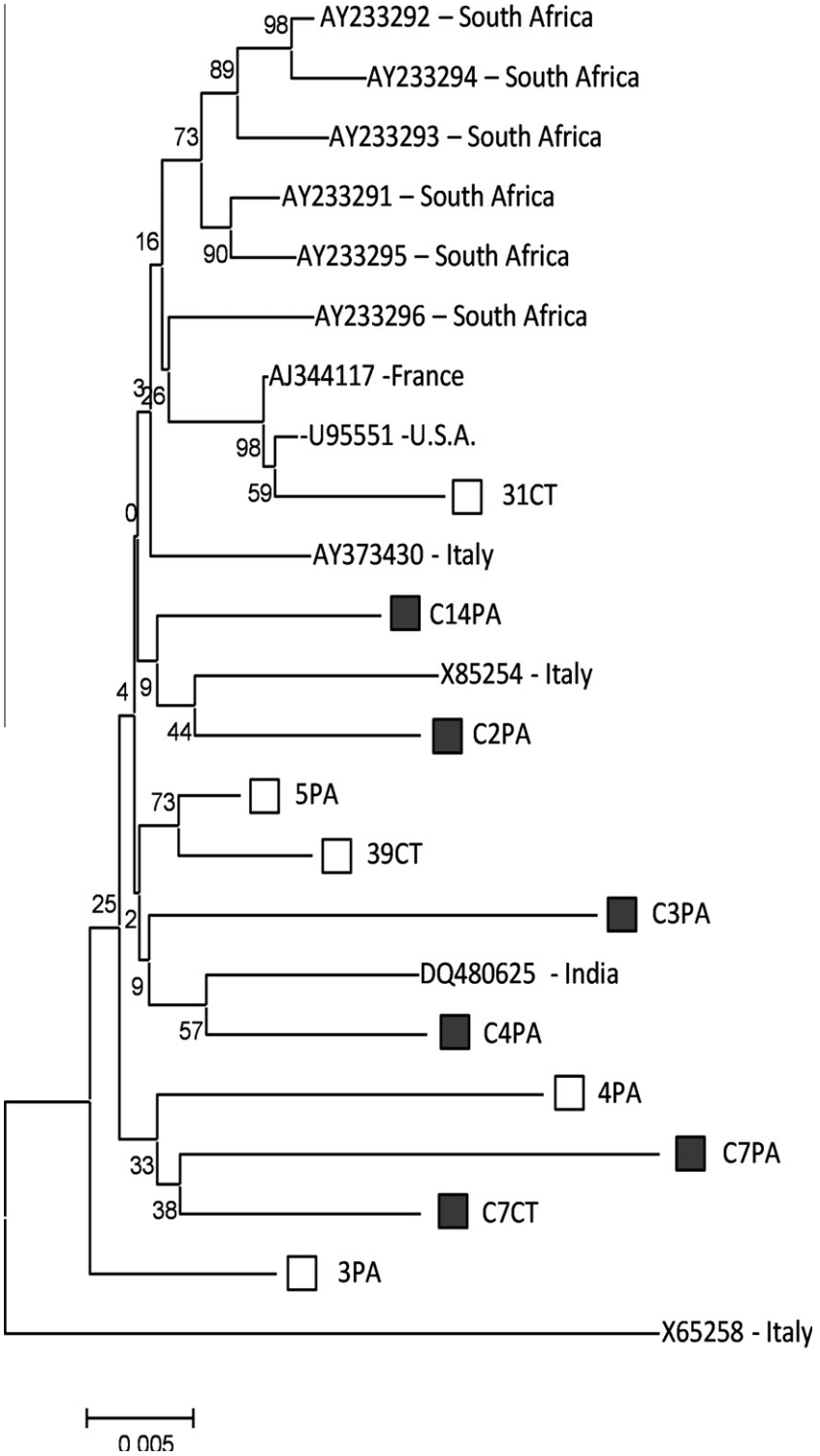

Fig. 3. Neighbor-joining tree of the preS/S region of HBV/D3 sequences using Boostrap values of 1000 replicates. The sequences from Sicilian patients with acute hepatitis B are prefixed by "□" and those with chronic hepatitis B are prefixed by "■". The geographic location from where the reference sequences were obtained is indicated to the right of the tree.

patients are present heterogeneously in the tree as a result of the simultaneous circulation of endemic strains in our geographical area.

Six HBV/D1 sequences from AHB patients segregated on the top of the tree. The bootstrap value (<60%) of this group not support a phylogenetic correlation of the strains. However, the nucleotide sequences analysis showed a similarity into the group with four substitutions (G71A, G235A, G453A, A1190G) present only in these isolates. Moreover, four of these six sequences result phylogenetically related, as supported by 92% bootstrap value. This sequences derived from patients from Catania infected in the same year because of unsafe sex (23 CT e 33CT, bootstrap 68%) and tattoo (26CT and 29 CT, bootstrap 95%). These date showed that four sequences might have an common source of infection.

Table 2
Nucleotide and amino acid matrix distances of PreS/S, "a" determinant and RT-domain according to genotype and subtype from acute hepatitis B infected patients.

Region	Genotype	Subtype		Genotype	Subtype
	D	D1	D3	A	A2
Nucleotide matrix distances					
PreS/S	2.5	2.2	2.2	1.3	0.8
"a" determinant	2.6	1.7	4.0	0.6	0.5
RT-domain	2.4	1.9	2.4	0.8	0.5
Amino acid matrix distances					
PreS/S	3.2	2.8	4.1	1.4	1.4
"a" determinant	3.7	3.5	8.1	0.0	0.0
RT-domain	3.2	2.5	3.7	0.86	0.8

Five HBV/D3 strains derived from three patients from Palermo and two patients from Catania. The risk factor for three patients was not identified, but minor surgery was established for two individuals. These HBV/D3 isolates formed a heterogeneous group with six sequences from Sicilian CHB patients (Fig. 3). All five of the AHB cases resulting from infections with HBV/D3 originated seemingly from local viral isolates.

In order to study the genetic heterogeneity of viral isolates we calculated the nucleotide and amino acid matrix distances of the preS/S, the "a" determinant, and overlapping-RT domain, according to genotype and subtype (Tables 2). D genotypes had a mean nucleotide divergence significantly greater than A genotypes ($p = 0.001$). In particular, the "a" determinant showed a null amino acid divergence in genotype A. This same condition was found, even though the analysis was performed on the major subtypes. Moreover, the D3 subtype had a higher value of divergence in the S portion, and in the "a" determinant, compared with the D1 subtype, as previously described (De Maddalena et al., 2007; Zehender et al., 2008).The study of amino acid sequences, evaluated for each subtype, for the preS/S and RT regions, using the model of Nei and Gojobori, showed that the mean synonymous (d_S) was always higher than nonsynonymous substitution (d_N), with a rate $d_N/d_S < 1$.

Amino acid characterization showed that the isolates come from new infections did not have a common mutational pattern. Antibody-escape mutants in the "a" determinant of the S gene, and mutations in the overlapping P gene, associated with resistance to antiviral drugs, were not observed. Similar findings were reported in another study examining chronic HBV patients who did not receive nucleos(t)ide analogues (Pollicino et al., 2009). This absence may reflect the rarity of these viral mutants in reservoir of chronic HBV carriers, their poor transmissibility rates or a weak replicative potential which prevented these isolates from becoming more highly prevalent.

4. Conclusions

In Italy, HBV genotype D is responsible for 90% of chronic HBV infection (Dal Molin et al., 2006). However, there are limited reports regarding the epidemiology of new cases of acute hepatitis B (Biliotti et al., 2008; Coppola et al., 2010) and characterization by molecular means has yet to be carried out.

Studies in countries that have low incidence rates of HBV infections have demonstrated that immigration of individuals from countries with high HBV endemicity resulted in the introduction of HBV genotypes different from those endemic to the respective host nations. For example, in Japan, where HBV/B and HBV/C are the endemic genotypes, and in the Netherlands new AHB infections with HBV/A genotype were shown to be on the rise (Matsuura et al., 2009; Takeda et al., 2006; van Houdt et al., 2007).

The high incidence of HBV/D genotype in Sicilian patients with chronic infection, infected more than 15 years, it was associated with the circulation of endemic strains transmitted mostly by intrafamilial spread, as previously demonstrated (Craxì et al., 1991). Instead, phylogenetic analysis of viral isolates from new cases of HBV infection showed the presence of A genotypes not endemic in Sicily introduced mainly through sexual promiscuity and unsafe. These data are in keeping with the findings of Coppola et al., who recently described in Campania, another region of southern Italy, an increase of genotype non-D HBV strains, isolated from new cases of infection in the last 10 years, spread predominantly through sexual transmission (Coppola et al., 2010).

HBV mutants able to elude vaccine-elicited protective immunity were not found among Sicilian acute hepatitis B patients. Hence in our area, where universal HBV immunisation programs started since the late 80s, most acute cases of HBV infection are attributable to infection with non-endemic HBV genotypes because of a increase of unsafe practices, mostly promiscuous sex, among non-immune individuals.

The introduction of non-D genotypes could affect in the long term the pattern of expression of HBV-related diseases due to the variable association between genotype, liver disease and response to antiviral therapy (Fung and Look, 2004).

Our study has identified HBV variants without immune escape and drug resistant mutations, which could spread and prevail in the population. This has been reported in a large outbreak of HBV infection in the United Kingdom where two new variants of A and D genotypes have spread in large population groups through injecting drug use and heterosexual sex (Hallett et al., 2004; Andersson et al., 2012).

It is worth noting that, since half of our patients with AHB were infected by HBV genotype A and the reduction of viral load which would theoretically be obtained by treating chronic HBV carriers (most of them being infected by HBV genotype D) would change only partially the circulation of HBV in our area. This is likely to be due to the fact that the pool of chronic HBV carriers infected by genotype A belong to a social-demographic setting (commercial sex workers) which is not reached by screening for HBV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.07.006. These data include Google maps of the most important areas described in this article.

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