RESEARCH PAPER

Human Vaccines & Immunotherapeutics 11:1, 53–57; January 2015; © 2015 Landes Bioscience

Hepatitis B vaccination

Are escape mutant viruses a matter of concern?

Luisa Romanò[1], Sara Paladini[1], Cristina Galli[1], Giovanni Raimondo[2], Teresa Pollicino[3], and Alessandro R Zanetti[1,*]

[1]Dipartimento di Scienze Biomediche per la Salute; Università degli Studi di Milano; Milano, Italy; [2]Dipartimento di Medicina Clinica e Sperimentale; Università degli Studi di Messina; Messina, Italy; [3]Dipartimento di Scienze Pediatriche, Ginecologiche; Microbiologiche e Biomediche, Università degli Studi di Messina; Messina, Italy

Keywords: Hepatitis B, HBV, Hepatitis B vaccination, HBV escape mutants, drug resistant mutants

Abbreviations: aa, amino acid; ADV, Adefovir; ALT, alanine aminotransferase; anti-HBs, antibody to hepatitis B surface antigen; cccDNA, covalently closed circular DNA; ETV, Entecavir; HB, hepatitis B; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LdT, Telbivudine; LMV, Lamivudine; MHR, major hydrophilic region; NA, Nuclos(t)ide analogue; ORF, open reading frame; RT, reverse transcriptase; TDF, Tenofovir; VEMs, vaccine escape mutants; WHO, World Health Organization

Hepatitis B virus is a worldwide leading cause of acute and chronic liver disease including cirrhosis and hepatocellular carcinoma. Effective vaccines have been available since the early '80s and vaccination has proved highly successful in reducing the disease burden, the development of the carrier state and the HB-related morbidity and mortality in the countries where vaccination has been implemented.

Neutralizing (protective) antibodies (anti-HBs) induced by vaccination are targeted largely towards the amino acid hydrophilic region, referred to as the common a determinant which is present on the outer protein coat or surface antigen (HBsAg), spanning amino acids 124–149. This provides protection against all HBV genotypes (from A to H) and is responsible for the broad immunity afforded by hepatitis B vaccination. Thus, alterations of residues within this region of the surface antigen may determine conformational changes that can allow replication of the mutated HBV in vaccinated people.

An important mutation in the surface antigen region was identified in Italy some 25 years ago in infants born to HBsAg carrier mothers who developed breakthrough infections despite having received HBIG and vaccine at birth. This virus had a point mutation from guanosine to adenosine at nucleotide position 587, resulting in aa substitution from glycine (G) to arginine (R) at position 145 in the a determinant. Since the G145R substitution alters the projecting loop (aa 139–147) of the a determinant, the neutralizing antibodies induced by vaccination are no longer able to recognize the mutated epitope. Beside G145R, other S-gene mutations potentially able to evade neutralizing anti-HBs and infect vaccinated people have been described worldwide.

In addition, the emergence of Pol mutants associated with resistance to treatment with nucleos(t)ide analogues can select viruses with crucial changes in the overlapping S-gene, potentially able to alter the S protein immunoreactivity. Thus such mutants have the potential to infect both naïve and immunized people, negatively affecting the efficacy of both the antiviral treatment and the vaccination programs.

Despite concern, at present the overall impact of vaccine escapes mutants seems to be low and they do not pose a public health threat or a need to modify the established hepatitis B vaccination programs. The development of novel NAs with a high barrier to resistance is warranted.

Introduction

Hepatitis B virus (HBV) is a leading cause of acute and chronic liver disease including cirrhosis and liver cancer, which ranks as the third cause of cancer deaths worldwide. WHO estimates that at least 2 billion people have been globally infected with HBV. Over 240 million (14 million living in Europe) are chronically infected. An estimate 500 000–700 000 (36 000 in Europe) people die each year for HBV-related diseases, and 4.5 million new cases of acute hepatitis B occur each year, and a quarter of these may progress to chronic liver disease.[1,2]

Despite this impressive burden, hepatitis B is now considered a largely treatable and preventable disease, thanks to the availability of effective antiviral drugs and the adoption of several public health measures, including vaccination.

Safe and effective vaccines have been available since the early '80 when the so called plasma-derived vaccines were first introduced and then replaced by DNA-recombinant vaccines around the mid-'80s.

*Correspondence: Alessandro R Zanetti; Email: alessandro.zanetti@unimi.it
Submitted: 07/03/2014; Accepted: 07/10/2014; Published Online: 08/06/2014
http://dx.doi.org/10.4161/hv.34306

WHO recommends to introduce hepatitis B vaccination into national childhood immunization programs and catch up programmes focused to people at increased risk of HBV exposure.[3] At present, 181 countries have implemented this recommendation, and Italy was one of the first countries to do so, starting in 1991.

Several hundred million vaccinations have been administered worldwide with an outstanding record of safety and efficacy. Vaccination has proved highly successful in reducing the disease burden, the development of carrier state and the hepatitis B-related morbidity and mortality in the countries where vaccination has been implemented.[4]

Thus thanks to the use of antiviral drugs such as the last generation nucleos(t)ide analogues (NAs) for the treatment of chronically infected patients as well as the implementation of extensive programs of vaccination may lead in the end to the elimination of hepatitis B and hepatitis B-related diseases.

However, against this view, there are some concerns due to the peculiar biology of HBV, in particular its propensity to develop—through mutation—drug resistant and vaccine-escape mutant viruses which may potentially challenge the therapeutic and prevention programs currently in place.

Hepatitis B Virus (HBV)

Briefly, HBV is a 42 nm DNA virus (the so-called Dane particle), composed by an outer glycoprotein envelope containing HBsAg (hepatitis B surface antigen), an inner icosahedral core (HBcAg) surrounding a circular, partially double-stranded DNA molecule (composed by a complete minus strand and a partial plus strand) of approx 3.2 kb in length, and a large polymerase that functions as both a reverse transcriptase for synthesis of the negative DNA strand from pregenomic RNA and an endogenous DNA polymerase for synthesis of the positive DNA strand using the negative strand.[5-7]

The super compact HBV genome contains four overlapping genes. The preS/S gene has 3 ORFs that encode 3 forms of HBsAg: the large (pre-S1), medium (pre-S2) and small (S) structural proteins of the viral envelope. The C gene has two ORFs (C and pre-C) encoding the HBcAg (hepatitis B core antigen) and the e protein, which is processed to produce soluble HBeAg (hepatitis e antigen). The X gene encodes a small protein with transactivator activity, while Pol (polymerase) gene encodes a large polymerase protein.[8]

On the basis of a minimal divergence of 8% of the complete genome sequences, eight HBV genotypes (from A to H) and a number of sub-genotypes—with a minimal genetic divergence of 4%—have been identified so far. In addition, there are 4 major subtypes: *adw*, *adr*, *ayw*, *ayr*, and 9 minor subtypes. HBV serotypes and genotypes have distinct geographical and ethnic distribution worldwide and may play a role in determining the severity of liver disease and treatment outcome of HBV infection.[9-15]

The viral life cycle is complex since HBV adopts, at least in part, a retrovirus-like strategy involving reverse transcription of a pre-genome RNA to the negative strand DNA. After the DNA minus strand is formed the DNA polymerase starts to synthesize the positive strand. Virions are then formed via budding into the endoplasmic reticulum. Virus particles can either exit the cell or re-enter into the nucleus to initiate another round of replication which involves covalently closed circular DNA (cccDNA) formation followed by its transcription of mRNA, including pregenome RNA.

Because of this strategy of replication, which involves a RT lacking proof-reading capacity, HBV shows greater mutability than other DNA viruses. Mutations can occur in all four genes through spontaneous errors of the viral polymerase or as a consequence of pressure by the host immune system or by exogenous factors including immunization or treatment with antivirals.

The HBsAg a Determinant

All HBV genotypes and serotypes share the common determinant a which spans aa 124–149 within the major hydrophilic region (MHR) and is in a form of two major and one minor loops with cysteine-disulphide bonds, protruding from the outer surface of the virus; the second hydrophilic loop (aa 139 to 147 or 149) is the major target for neutralizing anti-HBs produced following natural infection or vaccination.

Neutralizing (protective) antibodies induced by vaccination are targeted largely towards the conformational epitope of the a determinant. This provides protection against all HBV genotypes and subtypes and is responsible for the broad immunity afforded by HBV vaccination. Thus, alterations of residues within this region of the surface antigen can determine conformational changes that can allow replication of mutated viruses in vaccinated people (vaccine escape mutants or VEMs). In addition, such mutated viruses can be undetectable by the current diagnostic assays, posing a potential threat to the safety of blood supply.[16-19]

S-Gene Mutants

An important mutation in the surface antigen region was first identified in Italy some 25 years ago in infants born to HBsAg carrier mothers who developed breakthrough infections despite the presence of protective levels of antibody produced in response to the administration of HBIG (hepatitis B iperimmune gamma globulins) plus vaccine received at birth.[20,21] This virus has a point mutation from guanosine to adenosine at nt position 587, resulting in aa substitution from glycine (G) to arginine (R) at position 145 (G145R) of the a determinant of the surface antigen. Since the G145R substitution alters the projecting second loop of the a determinant, the neutralizing antibodies induced by vaccination are no longer able to recognize the mutated epitope. G145R was shown to be viable infectious and pathogenic in chimpanzees.[22]

Beside the prototype G145R, other S gene mutations (alone or in combination) across the entire a determinant potentially able to evade neutralizing anti-HBs have been identified worldwide rising concern that these mutant viruses may take advantage on the wild type in escaping the immunity of vaccinated people.[23-26] HBV infection with S-gene mutant viruses has been reported to

occur in presence of protective levels of anti-HBs in infants born to HBV-infected mothers who received HBIG plus vaccine (suggesting that HBIG, when used, can be the major driving force for the selection of vaccine escape mutants), in liver transplanted patients who received HBIG for prophylaxis, and in HBsAg-negative chronic carriers of HBV. Globally speaking the emergence of G145R is an uncommon event more generally associated with the use of HBIG rather than vaccination.[27,28]

In 2010 a survey conducted in Taiwan failed to detect an increased prevalence of vaccine escape mutants in a population of children and adolescents fully covered by universal infant immunization over a period of 20 years.[29]

Of concern, a recent study carried out in China reported that HBV mutants capable of infecting people are emerging 13 years after the implementation of a successful universal vaccination program.[30,31] However, a more careful analysis of data from such study showed that following vaccination both the HBsAg carrier rate and prevalence of variants indeed decreased, even though the variant prevalence decreased at lower rate (71% versus 33%). In addition in this study a clear differentiation between proven- and not-proven VEMs among the HBsAg variants is lacking.[32,33]

Nucleos(t)ide Analogues Resistant Mutants

The availability of nucleos(t)ide analogues able to suppress HBV replication by inhibiting the reverse transcriptase (rt) has provided effective treatments to patients with chronic hepatitis B, significantly reducing morbidity and mortality. However, the selection and emergence of drug-resistant HBV mutants can lead to treatment failure and progression to liver disease. The development of resistance due to mutations in the Pol gene is usually followed by a virological breakthrough (rise in HBV DNA levels of at least 1 log compared with the nadir value), a biochemical breakthrough (ALT elevation) and worsening of liver disease.[34,35]

Mutations of the Pol gene located within the catalytic domain of the RT region are particularly common after treatment with Lamivudine (LMV). Evidence indicates that LMV resistance increases progressively over the time of treatment (up to 80% after 48 m of the treatment).[36]

The rate of emergence of resistant HBV following treatment with other NAs, particularly Adefovir (ADV) and Telbivudine (LdT), is lower than that of LMV but still substantial in many cases.[36]

Indeed, current chronic hepatitis B treatment guidelines recommend that Entecavir (ETV) or Tenofovir (TDF), drugs with high potency and the lowest rates of resistance, should be chosen as first-line treatment options to minimize the likelihood of resistance development and to increase the chances of achieving treatment goals.[34] Since the Pol-gene encoding the polymerase completely overlaps the S gene encoding the surface protein (**Fig. 1**), each mutation occurring in the Pol-gene implies possible changes in the envelope protein and viceversa. Thus, drug-resistant HBV mutants emerging under NAs therapy may also show mutations in the S protein (**Table 1**) causing alteration in the antigenicity of the protein.[35-37]

HBV S escape variants are viable and pathogenic and may infect properly vaccinated people Thus, "NAs-resistant, possible vaccine escape mutants" have the potential to infect both naïve and immunized people, negatively affecting the efficacy of both the antiviral treatment and the vaccination programs.

What are the Public Health Implications of S-Gene and Pol-Gene Mutants?

The implementation of universal vaccination had a tremendous impact in terms of reduction in morbidity and mortality of hepatitis B and HB-related diseases.

In Taiwan, for example, the HBsAg prevalence in children under 15 of years of age decreased from 9.8% in 1984 (the year of introduction of mass vaccination) to less than 0.5% in 2009. The annual average incidence of HCC in children declined from 0.7 per 100 000 to less than 0.2 after 20 years of vaccination.[38,39]

Similar successful results were reported in other hyperendemic countries such as the Gambia, China, Singapore, and Alaska where the impact of vaccination in terms of reduction in the burden of hepatitis B, the rate of carrier state, and hepatitis B-related mortality has been impressive.

In Italy, where approx 20 million children have been vaccinated since implementation of vaccination in 1991, the overall

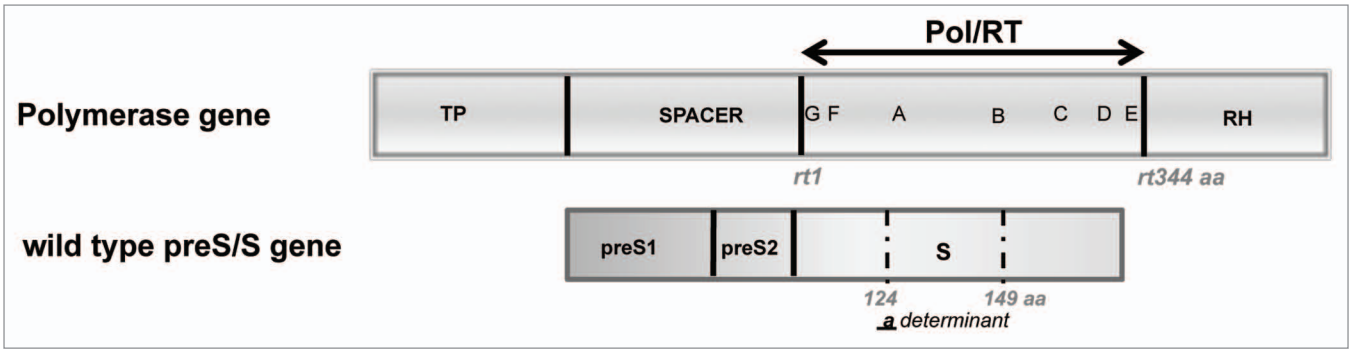

Figure 1. Schematic representation of the overlap between the HBV polymerase and envelope open reading frames. The numbers indicate amino acid (aa) sites. Numbering is according to genotype D. The a determinant of HBsAg that is located between aa 124 and 149, and which includes the major antibody neutralization domain of HBV, is indicated.

Table 1. Major drug-resistant mutations in the HBV reverse-transcriptase (rt) resulting in structural changes in the HBsAg protein

Nucleos(t)ide	Pol-gene mutations	S gene mutations
S gene mutations	(Primary or compensatory)	
LMV and LdT	rtM204V	sI195M
	rtM204I	sW196S/L/*
	rtV173L	sE164D
ADV	rtA181T[§,#]	sW172L/*
([#]LMV, [§]LdT)	rtA181V[§]	sL173F
ETV	rtI169T	sF161H/L
	rtT184C	sL175F+sL176V
	rtT184L/S	sL175F
	rtT184G	sL176V
	rtT184M	sL176stop
	rtS202C/G	sS193F/L
	rtS202I	sV194F/S

[§,#]Also induced by [#]LMV or [§]LdT; *stop codon.

morbidity rate per 100 000 inhabitants fell from 7 in 1990 to 1 in 2012. This decline was even more striking in 15–24-year-old individuals where incidence per 100 000 dropped from 17 to less than 0.4 in the same period of time.[40] Moreover several seroprevalence studies show a dramatic drop of HBV markers in vaccinated people, particularly those under 30 years of age.[41] According to our national surveillance system (SEIEVA) breakthrough infections due to vaccine escape mutants are rarely reported.

On the whole, data collected from Italy and elsewhere show the success of vaccination as the most effective measure to control and prevent hepatitis B and its severe sequelae.

Conclusions

Vaccination has clearly proved to be very successful resulting in remarkable progress towards the prevention and control of hepatitis B and HBV-related diseases on global scale. Cases of hepatitis B in fully vaccinated people are generally rare. Breakthrough infections caused by S-gene mutants are occasionally reported but at present they do not pose a serious threat to the established vaccination programs. The emergence of drugs resistant mutants with alteration in the a determinant of the S protein is of some concern. The development of novel NAs with a high barrier to resistance is warranted.

Global surveillance networks should be set up to monitor the epidemiological dynamics and public health impact of vaccine escape and treatment escape mutants.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. World Health Organization. Hepatitis B. 2008. Available at: http://www.who.int/mediacentre/factsheets /fs204/en/ (last access 18 june 2014).
2. World Health Organization, Regional Office for Europe. Hepatitis B and C infect one in fifty adults in European Region. Available at: http://www.euro.who.int/en/health-topics/communicable-diseases/hepatitis/news/news/2013/06/hepatitis-b-and-c-infect-one-in-fifty-adults-in-european-region# (last access 18 june 2014).
3. World Health Organization. WHO expanded programme on immunisation. Global Advisory Group. Wkly Epidemiol Rec 1992; 3:11-6
4. Zanetti AR, Van Damme P, Shouval D. The global impact of vaccination against hepatitis B: a historical overview. Vaccine 2008; 26:6266-73; PMID:18848855; http://dx.doi.org/10.1016/j.vaccine.2008.09.056
5. Ganem D, Schneider RJ. Hepadnaviridae: the viruses and their replication. In: Knipe DM, et al., eds. Fields Virology, 4th ed. Philadelphia, Lippincott Williams & Wilkins, 2001:2923-69
6. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev 2000; 64:51-68; PMID:10704474; http://dx.doi.org/10.1128/MMBR.64.1.51-68.2000
7. Dienstag JL. Hepatitis B virus infection. N Engl J Med 2008; 359:1486-500; PMID:18832247; http://dx.doi.org/10.1056/NEJMra0801644
8. Rossner MT. Review: hepatitis B virus X-gene product: a promiscuous transcriptional activator. J Med Virol 1992; 36:101-17; PMID:1583465; http://dx.doi.org/10.1002/jmv.1890360207
9. Prince AM, Ikram H, Hopp TP. Hepatitis B virus vaccine: identification of HBsAg/a and HBsAg/d but not HBsAg/y subtype antigenic determinants on a synthetic immunogenic peptide. Proc Natl Acad Sci U S A 1982; 79:579-82; PMID:6176997; http://dx.doi.org/10.1073/pnas.79.2.579
10. Norder H, Couroucé AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004; 47:289-309; PMID:15564741; http://dx.doi.org/10.1159/000080872
11. Erhardt A, Blondin D, Hauck K, Sagir A, Kohnle T, Heintges T, Häussinger D. Response to interferon alfa is hepatitis B virus genotype dependent: genotype A is more sensitive to interferon than genotype D. Gut 2005; 54:1009-13; PMID:15951551; http://dx.doi.org/10.1136/gut.2004.060327
12. Schaefer S. Hepatitis B virus: significance of genotypes. J Viral Hepat 2005; 12:111-24; PMID:15720525; http://dx.doi.org/10.1111/j.1365-2893.2005.00584.x
13. Liaw YF, Chu CM. Hepatitis B virus infection. Lancet 2009; 373:582-92; PMID:19217993; http://dx.doi.org/10.1016/S0140-6736(09)60207-5
14. Pujol FH, Navas MC, Hainaut P, Chemin I. Worldwide genetic diversity of HBV genotypes and risk of hepatocellular carcinoma. Cancer Lett 2009; 286:80-8; PMID:19683385; http://dx.doi.org/10.1016/j.canlet.2009.07.013
15. Guirgis BS, Abbas RO, Azzazy HM. Hepatitis B virus genotyping: current methods and clinical implications. Int J Infect Dis 2010; 14:e941-53; PMID:20674432; http://dx.doi.org/10.1016/j.ijid.2010.03.020

16. Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, Decker R. Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. Lancet 1995; 345:1406-7; PMID:7539089; http://dx.doi.org/10.1016/S0140-6736(95)92599-6
17. Mimms L. Hepatitis B virus escape mutants: "pushing the envelope" of chronic hepatitis B virus infection. Hepatology 1995; 21:884-7; PMID:7875688
18. Jongerius JM, Wester M, Cuypers HT, van Oostendorp WR, Lelie PN, van der Poel CL, van Leeuwen EF. New hepatitis B virus mutant form in a blood donor that is undetectable in several hepatitis B surface antigen screening assays. Transfusion 1998; 38:56-9; PMID:9482395; http://dx.doi.org/10.1046/j.1537-2995.1998.38198141499.x
19. Coleman PF, Chen YC, Mushahwar IK. Immunoassay detection of hepatitis B surface antigen mutants. J Med Virol 1999; 59:19-24; PMID:10440803; http://dx.doi.org/10.1002/(SICI)1096-9071(199909)59:1<19::AID-JMV4>3.0.CO;2-B
20. Zanetti AR, Tanzi E, Manzillo G, Maio G, Sbreglia C, Caporaso N, Thomas H, Zuckerman AJ. Hepatitis B variant in Europe. Lancet 1988; 2:1132-3; PMID:2460710; http://dx.doi.org/10.1016/S0140-6736(88)90541-7
21. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC. Vaccine-induced escape mutant of hepatitis B virus. Lancet 1990; 336:325-9; PMID:1697396; http://dx.doi.org/10.1016/0140-6736(90)91874-A
22. Ogata N, Zanetti AR, Yu M, Miller RH, Purcell RH. Infectivity and pathogenicity in chimpanzees of a surface gene mutant of hepatitis B virus that emerged in a vaccinated infant. J Infect Dis 1997; 175:511-23; PMID:9041321; http://dx.doi.org/10.1093/infdis/175.3.511
23. Hsu HY, Chang MH, Ni YH, Lin HH, Wang SM, Chen DS. Surface gene mutants of hepatitis B virus in infants who develop acute or chronic infections despite immunoprophylaxis. Hepatology 1997; 26:786-91; PMID:9303514; http://dx.doi.org/10.1002/hep.510260336
24. Carman W, Thomas HC, Zuckerman AJ, et al. Molecular variants of hepatitis B virus. In: Zuckerman A, Thomas HC ed. Viral Hepatitis. 2nd ed. London, Churchill Livingstone, 1998: 141-72.
25. Zuckerman AJ. Effect of hepatitis B virus mutants on efficacy of vaccination. Lancet 2000; 355:1382-4; PMID:10791517; http://dx.doi.org/10.1016/S0140-6736(00)02132-2
26. Hsu HY, Chang MH, Ni YH, Chen HL. Survey of hepatitis B surface variant infection in children 15 years after a nationwide vaccination programme in Taiwan. Gut 2004; 53:1499-503; PMID:15361503; http://dx.doi.org/10.1136/gut.2003.034223
27. Protzer-Knolle U, Naumann U, Bartenschlager R, Berg T, Hopf U, Meyer zum Büschenfelde KH, Neuhaus P, Gerken G. Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. Hepatology 1998; 27:254-63; PMID:9425945; http://dx.doi.org/10.1002/hep.510270138
28. Nainan OV, Khristova ML, Byun K, Xia G, Taylor PE, Stevens CE, Margolis HS. Genetic variation of hepatitis B surface antigen coding region among infants with chronic hepatitis B virus infection. J Med Virol 2002; 68:319-27; PMID:12226817; http://dx.doi.org/10.1002/jmv.10206
29. Hsu HY, Chang MH, Ni YH, Chiang CL, Chen HL, Wu JF, Chen PJ. No increase in prevalence of hepatitis B surface antigen mutant in a population of children and adolescents who were fully covered by universal infant immunization. J Infect Dis 2010; 201:1192-200; PMID:20210630; http://dx.doi.org/10.1086/651378
30. Bian T, Yan H, Shen L, Wang F, Zhang S, Cao Y, Zhang S, Zhang Y, Bi S. Change in hepatitis B virus large surface antigen variant prevalence 13 years after implementation of a universal vaccination program in China. J Virol 2013; 87:12196-206; PMID:24006443; http://dx.doi.org/10.1128/JVI.02127-13
31. Bian T, Yan H, Shen L, Wang F, Zhang S, Cao Y, et al. Universal hepatitis B vaccination in China boosts breakthrough mutant viruses. Microbe 2013; 8:393-4
32. Jilg W, Norder H, Kane M, Van Damme P, Vorsters A. Viral Hepatitis Prevention Board. Reduced prevalence of HBsAg variants following a successful immunization program in China. J Virol 2014; 88:4605-6; PMID:24672049; http://dx.doi.org/10.1128/JVI.03654-13
33. Locarnini S, Shouval D. Commonly found variations/mutations in the HBsAg of hepatitis B virus in the context of effective immunization programs: questionable clinical and public health significance. J Virol 2014; 88:6532; PMID:24803487; http://dx.doi.org/10.1128/JVI.00234-14
34. European Association For The Study Of The Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 2012; 57:167-85; PMID:22436845
35. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology 2009; 137:1593-608, e1-2; PMID:19737565; http://dx.doi.org/10.1053/j.gastro.2009.08.063
36. Gish R, Jia JD, Locarnini S, Zoulim F. Selection of chronic hepatitis B therapy with high barrier to resistance. Lancet Infect Dis 2012; 12:341-53; PMID:22326017; http://dx.doi.org/10.1016/S1473-3099(11)70314-0
37. Pollicino T, Cacciola I, Saffioti F, Raimondo G. Hepatitis B Virus PreS/S Gene Variants: Pathobiology and Clinical Implications. J Hepatol 2014; 61:408-17; PMID:24801416; http://dx.doi.org/10.1016/j.jhep.2014.04.041
38. Ni YH, Chang MH, Wu JF, Hsu HY, Chen HL, Chen DS. Minimization of hepatitis B infection by a 25-year universal vaccination program. J Hepatol 2012; 57:730-5; PMID:22668640; http://dx.doi.org/10.1016/j.jhep.2012.05.021
39. Chang MH, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM, Chu HC, Wu TC, Yang SS, Kuo HS, et al.; Taiwan Hepatoma Study Group. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. J Natl Cancer Inst 2009; 101:1348-55; PMID:19759364; http://dx.doi.org/10.1093/jnci/djp288
40. SEIEVA. Sistema Epidemiolgico Integrato dell'Epatite Virale Acuta. Istituto Superiore di Sanità, Roma. Available at http://www.iss.it/binary/seie/cont/Tassi_EpatiteB_2012.pdf (last access: 18 june 2014).
41. Da Villa G, Romanò L, Sepe A, Iorio R, Paribello N, Zappa A, Zanetti AR. Impact of hepatitis B vaccination in a highly endemic area of south Italy and long-term duration of anti-HBs antibody in two cohorts of vaccinated individuals. Vaccine 2007; 25:3133-6; PMID:17280750; http://dx.doi.org/10.1016/j.vaccine.2007.01.044