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Évolution de l'endémicité du virus de l'hépatite B au Laos au XXI^e siècle : impact de l'immunisation universelle

Philavanh Sitbounlang

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THÈSE

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Présentée et soutenue par
Philavanh SITBOUNLANG

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ABBREVIATIONS

Anti-HBs = Anti-hepatitis B surface antigen antibodies

Anti-HBc = Anti-hepatitis B core antigen antibodies

ATP = Attapeu

anti-HBV IG = Hepatitis B immunoglobulin

BK = Borkeo

BLK = Bolikhamxay

CPS = Champasak

DTPw-HepB-Hib = Diphtheria, tetanus, pertussis, hepatitis B and Haemophilus influenzae 23 type b vaccine.

EPI = Expanded Program on Immunization

HBsAg = Hepatitis B surface antigen

HBV = Hepatitis B virus

HCW = Health care workers

HPN = Huaphan

HepB-3 = Hepatitis B third dose

HepB-BD = Hepatitis B birth dose

KHM = Khammouane

LNT = Luangnamtha

LPB = Luang Prabang

MTCT = Mother-to-child transmission

MOH = Ministry of Health

NBC = National Blood Centre

ODX = Oudonxay

OBI = Occult infection with HBV or occult B infection

PSL = Phongsaly

SVK = Savannakhet

SRV = Saravane

SK = Sekong

VTP = Vientiane province

VT = Vientiane Capital

XYB = Xayabury

XK = Xiengkhuang

XSB = Xaysomboun

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ABSTRACT

The health burden of chronic Hepatitis B infections is a major issue for Southeast Asian countries. In the region, the ethnically diverse and landlocked country of Laos belongs to the lower middle-income countries (LMICs) and ranks 99th in the Global Health Security Index. Historically, Laos has been a country highly (>8%) endemic for persistent infection with hepatitis B virus (HBV), notably in the adult population. As a consequence, the incidence of primary liver cancer in Laos is one of the highest in the world. To reduce the hepatitis B burden, beginning two decades ago, Laos gradually implemented the universal anti-hepatitis B immunization of newborns. It then later joined the WHO Regional Action Plan for Viral Hepatitis in the Western Pacific 2016-2020. This plan has the aim of eliminating hepatitis as a public health threat. A specific aim of the Sustainable Development Goal is a 90% reduction in new cases of chronic viral hepatitis by 2030. This necessitates a massive diagnostic effort to identify previously undetected virus carriers and correctly assess the disease burden in the country. Despite many seroprevalence surveys conducted in the last decade, the situation of chronic infection with HBV is still only partially characterized in Laos, and unexpected issues, such as significant geographical variation in prevalence, have been recently observed.

Using a rapid test for the qualitative detection of the HBV surface antigen (VIKIA® HBsAg), between Sep 1st, 2020 and Aug 31st, 2021 we conducted the largest-ever prospective survey of HBV prevalence in Laos. This survey included blood donors (n=42,277), patients attending care in capital and provincial hospitals (n=37,347) including attending mothers (n=20,548), HIV-infected patients (n=7,439, recruited from 2009 to 2020), students from the Health Sciences University (n=609), and outpatients (n=350) coming for diagnosis at the Center Infectiology Lao-Christophe Mérieux in Vientiane. In total, 88,022 persons were tested, representing approximately 1.22% of the national population. HBsAg prevalence estimates were further segmented by three variables: age (≤ 20 years as a cut-off), sex, and geographical origin. Blood donor values were used to estimate HBsAg prevalence in patients aged <20, while hospital survey prevalence was used to estimate the prevalence in those aged older than 20 years.

We observed an HBsAg seroprevalence ranging from 2.6% in blood donors to 8.0% in HIV-infected patients. In blood donors, men were significantly more at risk of being carriers than women (RR=1.2, P=0.00063). For blood donors, attending mothers, or HIV-infected patients, HBsAg was significantly more prevalent in northern Laos (5.1%- 8.4%) than in

central (2.0%-8.1%) or southern parts of the country (2.2%-6.9%), thereby delineating a North-to-South gradient.

Considering geographic and sex differences along with the relatively young age of the Laotian population (43% of the population is younger than 21 years), we estimate that the overall HBsAg prevalence in the country ranges between 5.0 and 6.0% of the total population. Our estimates suggest that Laos may no longer be a country highly endemic for chronic infection with HBV but rather a country with intermediate endemicity.

Finally, our work suggests that developing countries with predominantly rural populations can emerge from high endemicity for chronic hepatitis B virus infection within a single generation. The improving situation in Laos appears to have resulted from the determined application of public health measures benefitting populations whose many children are now protected from infection. It is, therefore, a message of hope for many other countries facing similar circumstances. Furthermore, we provide means for comparing baseline epidemiological situations and the results of public health policy implementation in Laos and other Southeast Asian countries. From these comparative assessments, we gain perspective and insight concerning the evolution of the hepatitis B burden in the region.

RÉSUMÉ

Le fardeau sanitaire que représente l'hépatite B chronique est considéré comme un problème majeur pour les pays d'Asie du Sud-Est. Parmi eux, le Laos est un pays enclavé et très diversifié sur le plan ethnique. Il appartient aux pays à revenu faible ou intermédiaire de la tranche inférieure et occupe le 99^e rang de l'indice de sécurité sanitaire. En ce qui concerne la santé du foie, le Laos est toujours considéré comme un pays fortement endémique (>8%) pour l'infection persistante par le virus de l'hépatite B (VHB) notamment dans la population adulte. Par voie de conséquence, l'incidence du cancer primaire du foie au Laos est l'une des plus élevées au monde. Pour réduire le poids de l'hépatite B, le Laos a progressivement mis en œuvre, il y a deux décennies, la vaccination universelle des nouveau-nés contre l'hépatite B et a rejoint, par la suite, le Plan 2016-2020 d'action régional de l'OMS pour l'hépatite virale dans le Pacifique occidental, dans le but d'éliminer l'hépatite comme menace pour la santé publique. Un des objectifs de développement durable est la réduction de 90% des nouveaux cas d'hépatite virale chronique d'ici 2030. Cet objectif nécessite un effort de diagnostic massif pour identifier les porteurs de virus non détectés auparavant et évaluer correctement la charge de morbidité dans le pays. Malgré, de nombreuses enquêtes de séroprévalence menées au cours de la dernière décennie, la situation de l'infection chronique par le VHB n'est encore que partiellement caractérisée au Laos, et des problèmes inattendus, tels que des différences géographiques importantes, ont été observés récemment.

En utilisant le test rapide VIKIA® HBsAg pour la détection qualitative de l'antigène de surface du VHB, nous avons mené entre le 1^{er} septembre 2020 et le 31 août 2021 la plus grande enquête prospective de prévalence jamais réalisée au Laos. Cette enquête a inclus les donneurs de sang (n=42 277), les patients soignés dans les hôpitaux de la capitale et des provinces (n=37 347) dont les parturiantes (n=20 548), les patients infectés par le VIH (n=7 439, recrutés de 2009 à 2020), les étudiants de l'Université des Sciences de la Santé (n=609), et les patients externes (n=350) venant pour un diagnostic au Centre d'Infectiologie Lao-Christophe Mérieux à Vientiane. Au total, 88 022 personnes ont été testées, ce qui représente environ 1,22% de la population nationale. Pour parvenir à une estimation raisonnable de la prévalence de l'AgHBs au Laos, nous avons segmenté la population en fonction de trois variables, l'âge (≤ 20 ans comme seuil), le sexe et l'origine géographique. Les valeurs des donneurs de sang ont été utilisées pour estimer la prévalence de l'AgHBs

chez les patients de moins de 20 ans tandis que la prévalence de l'enquête hospitalière a été utilisée pour estimer la prévalence chez ceux âgés de plus de 20 ans

Nous avons observé une séroprévalence de l'AgHBs allant de 2,6% chez les donneurs de sang à 8,0% chez les patients infectés par le VIH. Chez les donneurs de sang, les hommes étaient significativement plus à risque d'être porteurs que les femmes (RR=1,2, P=0,00063). Chez les donneurs de sang, les parturiantes ou les patients infectés par le VIH, la prévalence de l'AgHBs était significativement plus élevée dans le nord du Laos (5,1%-8,4%) que dans le centre (2,0%-8,1%) ou le sud du pays (2,2%-6,9%), délimitant ainsi un gradient nord-sud.

Globalement, compte tenu de la différence géographique de la prévalence, de la différence entre les sexes et du jeune âge de la population laotienne (43% ayant moins de 21 ans), nous estimons que la prévalence de l'AgHBs dans le pays se situe entre 5,0 et 6,0% de la population totale. Ainsi, nous considérons que le Laos pourrait ne plus être un pays hautement endémique pour l'infection chronique par le VHB mais plutôt un pays d'endémicité intermédiaire. Enfin, nos travaux suggèrent qu'un pays en développement dont la population est essentiellement rurale peut sortir d'une forte endémicité pour l'infection chronique par le virus de l'hépatite B en l'espace d'une seule génération. Cette situation est avant tout le résultat du dynamisme démographique des populations dont les nombreux enfants sont protégés de l'infection par des mesures de santé publique appliquées avec détermination. Elle constitue donc un message d'espoir pour de nombreux autres pays confrontés à des circonstances similaires. Par ailleurs, dans le présent manuscrit, nous fournissons des éléments de comparaison des situations épidémiologiques de base et des politiques de santé publique mises en œuvre au Laos et dans les autres pays d'Asie du Sud-Est. Nous tirons de ces évaluations comparatives des perspectives concernant l'évolution de la charge de l'hépatite B dans la région.

INTRODUCTION

I. General introduction

Chronic hepatitis B is considered a major problem in Southeast Asian countries. Historical estimates of hepatitis B prevalence in Laos came from infectious disease screening surveys conducted among migrants in the United States of America about four decades ago. During these campaigns, which frequently mixed participants from different countries in the region (Laos, Cambodia, Vietnam), the hepatitis B virus (HBV) surface antigen (HBsAg) positivity rate was very high (e.g., 12.6-20.0% in the 1980s) [1]. In the following decade, however, it was recognized that Laotians in the United States were often members of specific ethnic groups (Hmong, Mien) who could be considered underserved compared to the rest of the population in their country of origin [2]. This worrying situation of hepatitis B among Lao refugees in their countries of migration has persisted until recently [3]. Taken together, these data indicate that chronic hepatitis B was and still is a serious health problem in many segments of Lao society and perhaps for the population as a whole. Laos is, therefore, generally considered to be a highly endemic country (>8%) for persistent hepatitis B virus (HBV) infection, particularly in the adult population [4], [5].

In the absence of appropriate antiviral treatment, HBV is directly responsible for the death of 40.0% of chronically-infected patients. All-cause mortality is increased in these patients. However, the two most important complications associated with fatality are hepatocellular carcinoma (HCC), followed by liver cirrhosis (LC) [6]. In Laos, the age-standardised incidence rate of liver cancer is currently one of the highest in the world for both women and men (14.2 and 35.7 cases per 100,000, respectively, in 2021) [7]. In parallel, the situation for children appears to have improved recently due to the routine vaccination of newborns against hepatitis B, which began to be implemented two decades ago (2001). Under this scheme, newborns must receive a first injection of the hepatitis B vaccine at birth. Three additional injections follow this at weeks 6, 10, and 14 [8]. It is important to note that there is no difference in procedure for HBsAg-carrier and non-carrier newborns. Past immunisation surveys indicate that while there has been considerable improvement in the percentage of newborns covered by the birth dose, rising from 34% in 2011 to between 58% and 66% in 2020 [9], there is still much work to be done. Despite many Lao children remaining unvaccinated, researchers found that they had a much lower HBsAg prevalence than mothers (1.0% vs 7.0%), and within a few years the number of HBsAg-positive toddlers more than halved (0.75% vs 1.82%) [10]. In addition, serological evidence of better protection against hepatitis B is already detectable among older schoolchildren (11-18

years), both in the capital and the provinces. [11]. Despite the numerous seroprevalence surveys conducted over the past decade, chronic HBV infection is still only partially characterised in Laos, and unexpected results, such as significant geographical differences in HBV surface antigen (HBsAg) prevalence, are observed [12].

Obtaining and providing to the Lao government representative information on HBV epidemiology is important for determining whether the hepatitis B control strategy is sufficient or needs strengthening. To this end, we have conducted the largest-ever serological study of HBsAg status in the Lao population. In total, we surveyed 88,022 individual blood samples using the VIKIA rapid test, representing 1.22% of the total population of Laos. The survey included five categories of participants: blood donors, hospitalized patients, HIV(+) patients at the Center Infectiology Lao-Christophe Merieux (CILM), students from the University of Health Sciences in Vientiane, and persons from the general population coming to the CILM for diagnostic purposes.

To gain an in-depth understanding of the changing burden of hepatitis B in the region, we extended our work to include a review of surveys from 11 Southeast Asian countries that make up this vast region, home to 681 million people. This review facilitates comparisons of baseline epidemiological situations and the impact of public health policies in Laos and the other Southeast Asian countries.

Chronic HBV infection may lead to liver disease, and this risk is affected by HBV genotype. In Southeast Asian countries, genotype C is the most common, followed by genotype B and the recently identified genotype I [13]. In Laos, the distribution of HBV genotypes appears similar to that observed in neighbouring countries. However, there is still a lack of knowledge regarding the association between HBV genotype and liver disease progression in Laos. Therefore, we conducted a cross-sectional study to describe the characteristics of liver disease progression in 125 HBsAg carriers aged more than 40 years who were followed up for viral load testing at the Center Infectiology Lao-Christophe Mérieux (CILM). In addition, we provide the near-complete (n=48, only segment 1790-1855 is lacking) and partial sequences (n=31) of HBV genomes and describe molecular features of the virus associated with disease progression.

II. A brief presentation of Laos

Laos is a landlocked country located in the north-eastern central part of Southeast Asia, sharing borders to the north with China, to the northeast and east with Vietnam, to the south with Cambodia, to the west with Thailand, and to the northwest with Myanmar (Burma) (Figure 1A) [14].



Figure 1A: Laos and its ten Southeast Asian neighbors

Figure 1B: the 18 provinces of Laos



Laos is relatively underpopulated compared neighbouring countries, with 7.2 million people inhabiting a total land area of 236,800 km² (30 habitants/km²). There are 18 provinces (including Vientiane Capital): 8 in the north, 6 in the middle, and 4 in the south of the country (Figure 1B) (<https://laosis.lsb.gov.la>). The current population of Laos came mainly from Chinese territory during vast population movements in the last 2000 years. More than 200 ethnolinguistic groups have been identified in Laos, making it the most ethnically diverse country in Southeast Asia [15]. However, Laotian tribes can be classified into three main groups based on their ethnolinguistic family, customary habitat, and type of agricultural production. These groups are the Lao Lum ("lowlanders", 62.4% of the population) speaking Lao-Tai (also named Tai-Kadai), Lao Thoeng/Teung/Kang ("mid- or uplanders") speaking Mon-Khmer (23.7%), and Lao Sung/Song ("highlanders") speaking either Hmong-lu Mien (9.7%) or Sino-Tibetan languages (2.9%) (Figure 2B) [16]. The country is most densely populated in and around the capital, Vientiane. Much of the rural population is scattered in

often hard-to-reach communities (11 236 villages in Laos), isolated by distance, mountainous terrain, and poor or non-existent road infrastructure[17]. In 2015, the proportion of the population living in urban or rural areas was 32.9% and 67.1%, respectively, and among the rural group, 8% lived in locations without roads. Concerning sanitation, about 61.4% of all households have access to clean water. About 2.4% of households have water resources more than 200 meters from the home, and 2.1% where water is more than 1000 meters away [16].

According to data from the 2022 world economic and financial survey, Laos has the third lowest Gross Domestic Product *per capita* (2,757 USD) among ASEAN countries, followed by Myanmar and Cambodia [18].

In 2020, the average life expectancy in Laos was 62 years for men and 65 years for women[16], with a birthrate of 21.2 per 1,000 people (<https://laosis.lsb.gov.la>).

Laos remains quite traditional, and its culture is still primarily influenced by Buddhism (Theravada Buddhism) [19] which shapes Laotian daily life. Some traditions and cultural habits have been shown to promote viral transmission. Examples include ear piercing of newborns, sometimes using surgical needles and sutures already used for their mother after birth, and ritual tattoos (called *Sak Yant*) [20] linked to the Buddhist belief that they confer protection against bad luck and provide energy, success, and fortune [21].

Traditional ways of drinking at a party or ceremony (e.g., *Baci*, Wedding party, village festivals etc.)—where there is generally a single vessel shared by everyone around the table, and then once filled, the glass is then passed to each participant—may also facilitate disease transmission. There is also the tradition of sharing the Lao-Lao or Laoi Hai (a traditional drink of fermented rice alcohol) using the same straws ([Figure 2A](#))



Buddhist tattoos (Sak Yant).
photo by BT tatouage Thailand



share the Lao-lao or Lao-hai (traditional drink, fermented rice alcohol)

Figure 2A: Laos culture



Lao Sung/Song (Hmong), photo by Paul Edward Evans



Lao Thoeng/Teung/Kang, photo by Ania Blazejewska



Lao Lum, Photo by "www.seeasiadifferently"

Figure 2B: The three main ethnic groups of Laotian tribes

CHAPTERS

CHAPTER I: STATE OF ART

PART I – HEPATITIS B VIRUS

1. Descriptive virology

1.1. Classification

HBV is a member of the *Hepadnaviridae* family, the hepatotropic DNA viruses. Historically *hepadnaviruses* were divided into two genera, depending on their host. The *Orthohepadnaviruses* infect mammals such as squirrels, woodchucks, bats and primates, including humans. The *Avihepadnaviruses* infect birds, including ducks, geese, herons, storks, cranes and parrots (Table 1) [22], [23]. *Hepadnaviruses* from fish (bluegills) and amphibians were recently discovered and found to be closely related to mammalian *hepadnaviruses* [24], [25] suggesting that the viral family is much larger than believed even a decade ago (Figure 3). The most recent phylogenetic analysis proposed adding three new genera to *Hepadnaviridae*: the *Herpetohepadnavirus* (for viruses infecting amphibians and reptiles), *Metahepadnavirus* (for those infecting Osteichthyes), and *Parahepadnavirus* (for those infecting Chondrichthyes) [26].

Table 1: Comparison of the different members of the *Hepadnaviridae* family

	<i>Orthohepadnaviridae</i>			<i>Avihepadnaviridae</i>
	HBV	WHV	BtHV	DHBV
Physical characteristics				
Genome size	3182-3284	3308-3320	3149-3377	3021-3027
nt Homology with HBV (in %)	100	70	48	40
Number of ORFs	4	4	4	4
ORF—amino acid lengths				
Pre-S	163	204-205	159-224	161-163
S	226	222	223-224	167
Pre-C	29	30	28-33	43
C	183	187	188-189	262
P	838	879	827-902	786-788
X	145	141	135-144	114

HBV (hepatitis B virus); WHV (woodchuck hepatitis virus); BtHV (bat hepatitis virus); DHBV (duck hepatitis B virus); nt (nucleos(t)ides); ORFs (open reading frames); Pre-S (presurface); S (surface); pre-C (precore); C (core); P (polymerase).

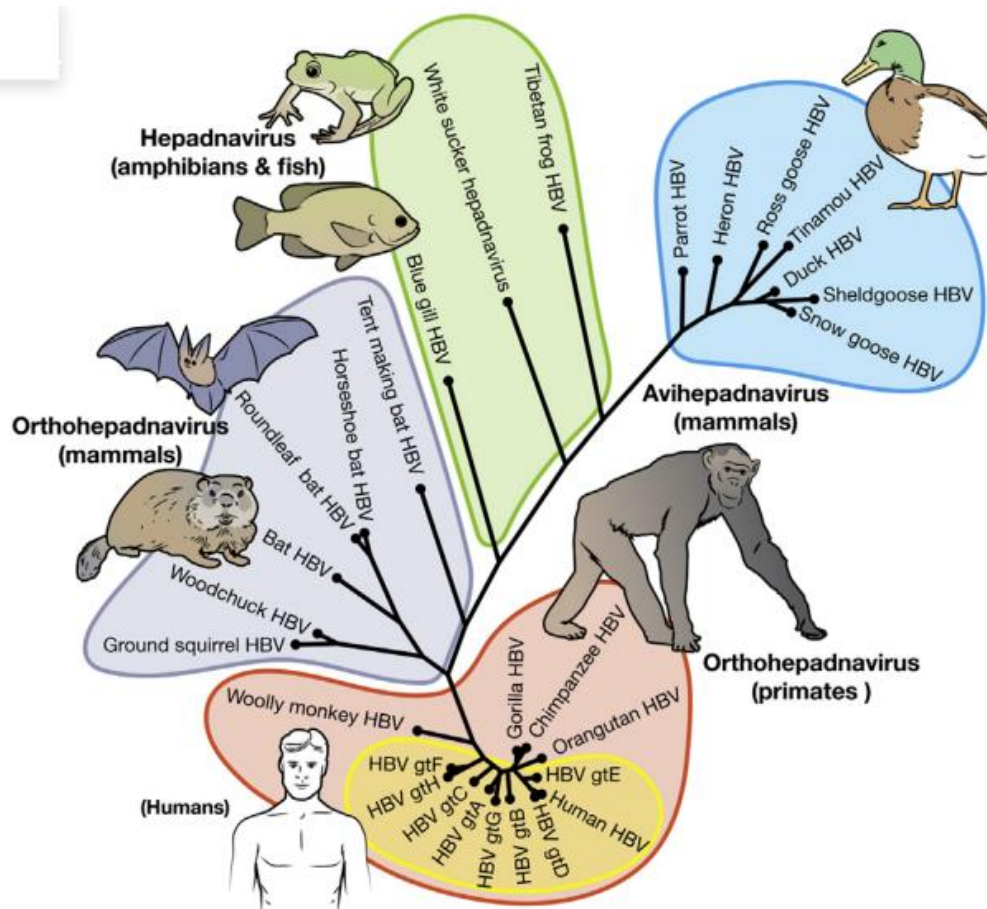


Figure 3: Phylogenetic relationships among avian, mammalian, and other *hepadnaviruses* [27].

1.2. Genomic structure, organization

HBV is an enveloped DNA virus; its surface antigen was initially described from virus isolated from an Australian patient in 1965 and named Australia antigen. In 1970, virologist David Dane and colleagues made the first observation of HBV viral particles by electron microscopy. They observed at least three types of HBV particles in the serum of infected patients. These included so-called Dane's particles (42 nm spherical particles now known to harbour HBV DNA), smaller spherical particles with a diameter of 22 nm, and filamentous particles of variable length with a cross-sectional diameter of 22 nm. The smaller particles do not contain nucleic acids and are thus non-infectious. These empty particles are thousands to tens of thousands times more abundant in the plasma than infectious Dane's particles. The Dane particles are infectious virions consisting of a lipid bilayer membrane

containing three viral surface antigens (HBs): large (L-HBs), middle (M-HBs), and small (S-HBs). The largest parts of these three proteins are domains embedded within the viral membrane, with surface domains exposed to the outside environment (Figure 4) [28], [29]. The capsid core inside the viral envelope is comprised of small protein subunits called capsomers. The single capsomer of hepatitis B virus is called hepatitis B core antigen (HBcAg). E antigen, or hepatitis B e antigen (HBeAg), is found between the capsid core and the envelope. Circular partially double-stranded DNA, also called relaxed-circular DNA (rcDNA), is found in the capsid core along with its own polymerase enzyme (Figure 4) [28].

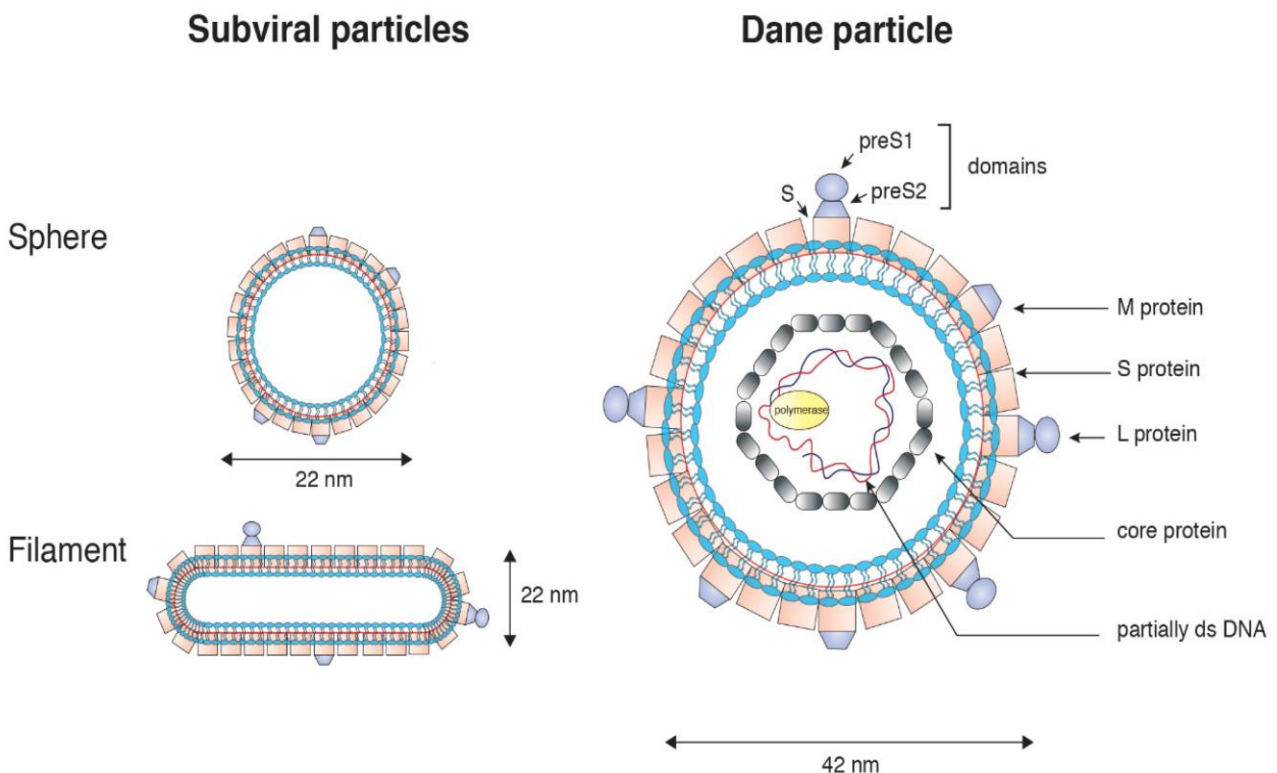


Figure 4: three types of HBV particles[29]

The circular HBV genome is approximately 3,2 Kb long, and consists in four overlapping open reading frames (ORFs) with the same orientation (Figure 5): 1) polymerase (P), the largest ORF encoding the viral polymerase and its 4 different domains; 2) Surface (S; pre-S1 and pre-S2) located out-of-frame of the Polymerase ORF; 3) Precore/core(C) with two in-phase initiation codons that code for secreted HBeAg and HBcAg (capsid or core protein); and 4) HBx, the smallest ORF, encoding X protein, a regulatory protein required for HBV replication [27], [30]. (Figure 5).

The HBV genome can be found in different forms. First, the genome can stay as rcDNA, a partially double-stranded DNA with the polymerase protein covalently attached to the 5' end of the minus-strand DNA. The negative DNA strand is completed while the synthesis of the positive strand is stopped and left unfinished by the polymerase when capsid assembly is complete. The size of the gap created is quite variable, sometimes large or small [25], [31].

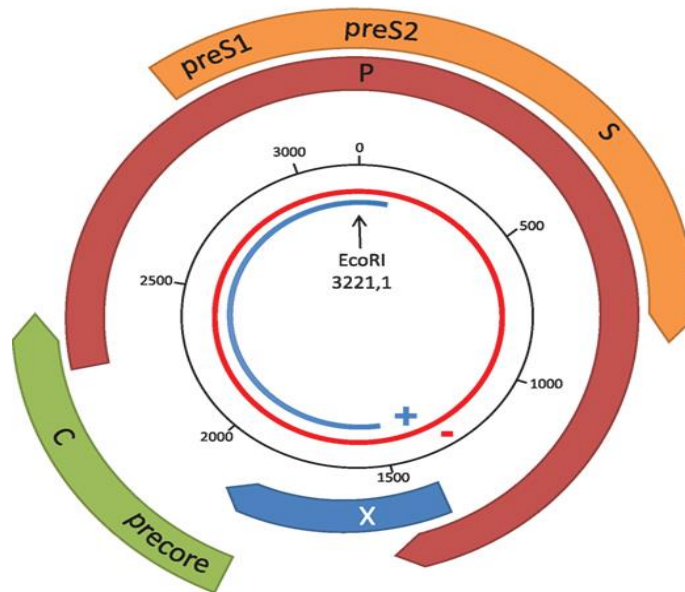


Figure 5: Hepatitis B virus genome. The HBV genome organization with nucleotide numbering starting at the EcoRI site of serotype ayw.

Second, found only in the nucleus of infected hepatocytes, the HBV genome is present as covalently closed circular DNA (cccDNA), a completed circular form of HBV DNA. cccDNA molecules adopt a chromatin-like structure, considered a viral minichromosome [32], and associate with viral and host proteins such as histones and other cellular proteins [28]. It serves as the template for the transcription of viral RNAs that are subsequently translated into viral proteins in the cytoplasm [27]. cccDNA is maintained in the nucleus according to a poorly characterized mechanism that includes recycling neosynthesized genomes or *de novo* infections of hitherto uninfected hepatocytes [28]. Elimination of cccDNA is observable during acute infections. It is due to either cytopathic or non-cytopathic mechanisms requiring a combination of immune system responses and an imbalance between cell death and cell division [33].

1.3. Viral proteins

HBV produces seven viral proteins: polymerase, three surface proteins (Small, Middle and Large), the capsid/core, hepatitis B early antigen (HBeAg) encoded by the precore region, and the X protein [34], [35]. Below I discuss the structure and function of the first six. The X protein has been discussed above.

- **Capsid protein (HBcAg)** consists of 183-185 amino acid (aa) residues [36] with a molecular weight of 21 kDa. The protein self-assembles to form a protective shell where the reverse transcription from pgRNA to rcDNA and viral replication occurs [37], [38]. Antibodies against the HBcAg, called anti-HBc, appear in both acute and chronic hepatitis HBV infections. Hence, this marker could not distinguish whether the infection was acute and resolved, or persistent. Anti-HBc IgM antibodies are the first markers of HBV infection but decrease rapidly with time below the detection threshold. Anti-HBc IgM antibodies may reappear during viral reactivation [39].
- **Hepatitis B-e-Antigen (HBeAg)** is a secreted protein translated from a pgRNA that also acts as the messenger for polymerase and core protein [40]. HBeAg is a 15.5-kDa polypeptide in serum [41]. While not required for viral replication, due to its tolerogenic properties HBeAg is important for the persistence of infections and has been used clinically as an index of viral replication, infectivity, disease severity, and response to treatment [42]. In addition, maternal HBeAg is thought to induce T-cell tolerance to HBV and facilitate HBV persistence during vertical (mother-to-child) transmission [43]. It may function as an immune tolerogen *in utero* as the secreted, soluble HBeAg can cross the placenta [44].
- **The polymerase (pol)** is a large protein containing about 800 amino acids encoded by the P ORF. Three domains play important roles in genome synthesis and replication: the terminal protein domain is involved in encapsidation and initiation of minus-strand synthesis; the reverse transcriptase (RT) domain catalyzes genome synthesis; and the ribonuclease H domain degrades pre-genomic RNA and facilitates replication [45], [46].

- **Surface proteins (S-, M- and L-HBsAg):** Three viral envelope proteins (S, M, and L) are translated by the surface ORF encoding two different mRNAs: S- and M-HBsAg are translated from PreS2/S (2.1kb) and L-HBsA from PreS1/preS2/S (2.4 kb) mRNAs [47]. The small envelope protein (S-HBsAg) comprises 226 aa coded by the S region in the C-terminal of the ORF. The middle envelope protein (M-HBsAg) is also coded for by the S region, along with a 55 aa N-terminal extension encoded by the preS2 region. Lastly, the large envelope protein (L-HBsAg) sequence is coded for by both preS2 and S segments with an additional 108 or 119 additional aa, depending on the genotype, coded by the preS1 domain [36], [48]–[50]. The main functions of the S-, M- and L-HBsAg are 1) assembly and secretion of HBV, 2) induction of immune tolerance in the infected host, and 3) attachment and penetration to host hepatocytes. The L-HBsAg and S-HBsAg are believed to be responsible for viral entry of HBV [51]. For the host, surface proteins are responsible for the development of protective immunity in naturally infected or vaccinated individuals.

1.4. HBV infectious cycle

The HBV life cycle involves the attachment and penetration of host hepatocytes and the uncoating of the capsid core, followed by replication and the assembly and release of infectious Dane particles and non-infectious SVPs. Details of each of these steps in the cycle are discussed below.

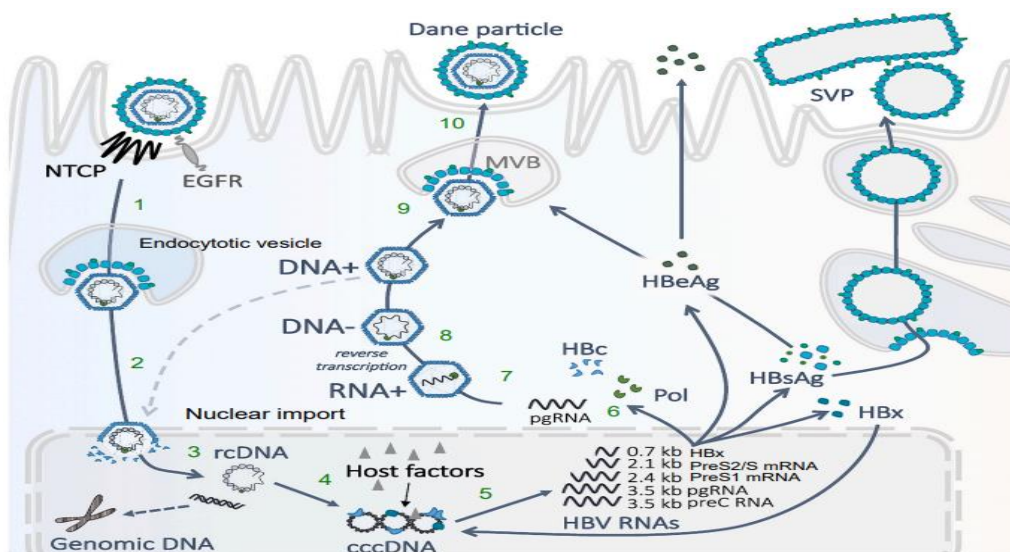


Figure 6: overview of the HBV life cycle

Attachment and entrance: The receptor for HBV entry into host hepatocytes is the Na⁺ taurocholate cotransporting polypeptide (NTCP) (gene name *SLC10A1*, for Solute Carrier Family 10A1). This peptide is localized at the basolateral membrane of hepatocytes and was recently identified as the main effector of hepatic bile acid transport [52]. Receptor binding occurs through contact with the PreS1 region (myristoylate peptide spanning amino acids 2-48) of viral envelope proteins. HBV entry begins with low-affinity binding with heparan sulfate proteoglycans (HSPGs) [53]. This is followed by high-affinity binding with NTCP and subsequent penetration into the cell [28], [50], [54].

Steps of viral uncoating, replication, assembly and release:

1) Inside the host cell, the envelope of virus fuses with the membrane of endocytic vesicles and nucleocapsid core is released into the cytoplasm.

2) The nucleocapsid core comes close to the nuclear pore. Then, the capsid core disintegrates and the DNA and DNA polymerase enters the cell nucleus.

3-4) Inside the nucleus, rcDNA is then repaired by the host cell DNA repair machinery and converted into covalently closed circular DNA (cccDNA) [55].

5) HBV genes located on cccDNA are then transcribed into their respective mRNA molecules by human RNA polymerase 2. There are five viral mRNA species: HBx (0.8kb), PreS2/S (2.1kb), PreS1/preS2/S (2.4 kb), PreCore and pgRNA (3.5kb). In practice, PreCore mRNA and pgRNA are very similar in size and differ only by their 5' end (preC is a bit longer). All these mRNAs are exported into the cytoplasm after transcription.

6) pgRNA is the template strand of the negative DNA strand of HBV genome and was previously the template for polymerase and core protein translation.

7) Once translated, the viral polymerase quickly attaches to the pgRNA via eight to ten nucleotides complementary to pgRNA extremity. The pgRNA encodes also for the capsid. Once a sufficient pool of core proteins is synthesized, capsid assembly begins in close association with a pgRNA-polymerase complex.

8) The polymerase then synthesizes the negative DNA strand by reverse transcription of the pgRNA. This is why hepadnaviruses are considered para-retroviruses. After the first DNA strand is synthesized, the RNA is destroyed by the RNase domain of the Pol. The second (positive) DNA strand is then only partially synthesized due to the premature closure of the capsid around the polymerase-HBV genome complex.

9) Assembly of the envelope protecting the capsid begins when surface protein mRNAs (PreS2/S and preS1) are translated inside the lumen of ribosome. The HBV surface proteins are then embedded in the membrane of endoplasmic reticulum. The nucleocapsid interacts thereafter with areas of endoplasmic reticulum where the HBV surface proteins are embedded and enters inside the lumen eventually, once envelope assembly is completed, to be pinched outward and become a virus particle.

10) Virions are then packed into special vesicles and the so-called Dane particle is then exported outside the cell by the process of exocytosis. Concomitant to the production of Dane particles, empty, non-infectious sub-viral particles (SVP) and HBeAg are also produced in excess and exported outside the cell. Interestingly, SVPs and HBeAg are produced in much greater quantity compared to Dane particles.

1.5. HBV genotypes and subgenotypes

The first analysis of HBV genomic variation identified four groups (genotypes) A, B, C and D [56]. Since that time, six additional genotypes (E-J) have been distinguished [47]. The frequency of the different genotypes varies geographically (see figure 7). Genotypes A and D more prevalent in Europe and Africa [57]. Genotypes B and C are more prevalent in Asia with genotype C particularly abundant in the Asia Pacific region [58]. Genotype E is distributed along the Western coast of sub-Saharan Africa, genotype F is dominant in South and Central Americas (and Alaska as well), and genotype G has been reported in France and the United States but does not seem to have genuine locus of high frequency. Genotype H is present in Mexico and Central America. Genotype I was initially identified in Vietnam, and is also present from North Eastern India (Harunachal Pradesh) to South China [59]–[61]. Genotype J was isolated from an 88-year-old hepatitis patient living in Okinawa, Japan who had resided in Borneo during the World War II[47], [62]. Phylogenetic analysis placed this genotype between human and ape (chimpanzee, gorilla, gibbon, and orangutan) HBV, and it is probably an example of a recombinant between a human and an ape viruses as also observed in Subsaharan Africa [63].

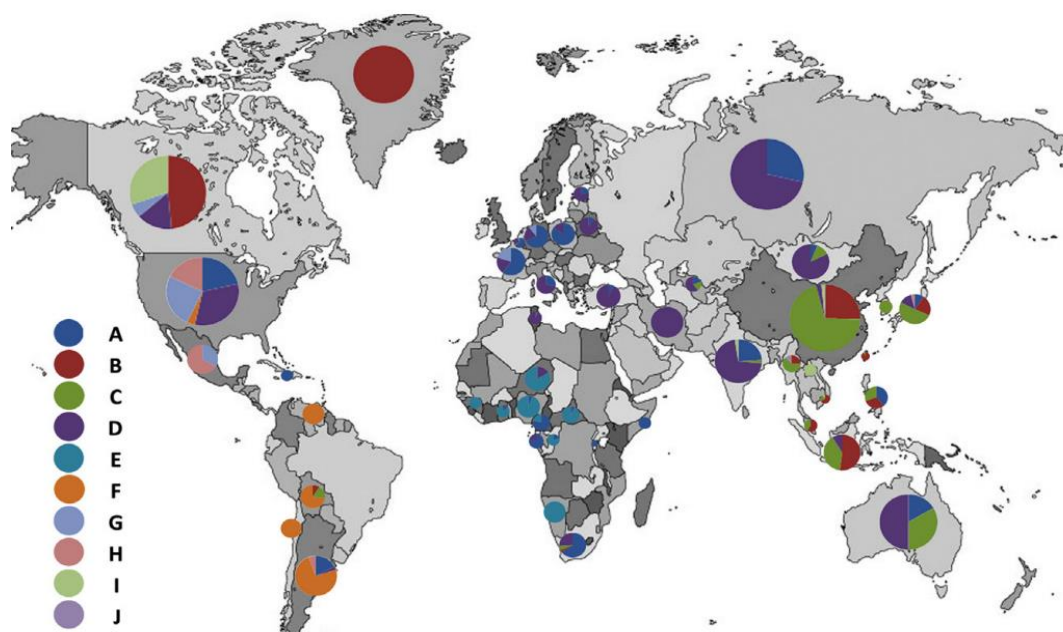


Figure 7: Geographic distribution of HBV genotypes

HBV subtypes

HBV genotypes A, B, C, D, F, and I have been further classified into subgenotypes. Subgenotypes of HBV strains E, G, H, and J have not yet been reported [64] – [66].

Subgenotype A1 is found in Asia and Africa [61], [66]. Subgenotypes of genotype B primarily cluster in Asia; B1 is prevalent in Japan, B2 can be seen in all countries, but especially in China [65] and groups with subgenotypes B3, B7, B8, and B9 which can be found almost exclusively in Southeast Asia, especially Indonesia and Malaysia [61]. The subgenotype B5 was reported in the Philippines, and B4 was found in Vietnam and Cambodia [66]. For Genotype C, the subgenotype C1 is present in South Asia and Southeast Asia, C2 is common in East Asia, North America and Oceania, and C6 prevails in Indonesia and Papua New Guinea [61]. Concerning genotype D, the subgenotype D1 has a worldwide distribution but is highly prevalent in Asia; D3 appears to be present worldwide, and D5 is found only in certain Asian countries [61], [65]. There are four subgenotypes of genotype F, F1–F4, all of which can primarily be found in Central and South America, as well as Alaska. Lastly, the two subgenotypes of genotype I, I1 and I2, are found in Laos, Vietnam, China, and India [65].

The geographic distribution of the various HBV genotypes and subtypes is, to some extent, associated with their principal routes of transmission. Genotypes B and C are common in highly endemic regions where perinatal exposure dominates. Other genotypes are, by contrast, more commonly found in regions with horizontal transmission [58]. The different genotypes are also associated with variation in disease progression, clinical outcomes, response to treatment, and disease prognosis. For example, subgenotype B1 is associated with fulminant HBV infections in Japan, while subgenotype B2 is associated with HCC or HCC recurrence in young patients in East Asia. Similarly, patients infected with subgenotype C2 are at higher risk of HCC [66].

1.6. Diagnostic

Table 2: Hepatitis B Virus Serological and Virological Markers		
Diagnostic tests	Clinical Interpretation	Typical Scenarios for its Use
HBsAg	Marker of acute and chronic HBV infection	<ul style="list-style-type: none"> - At the onset of infection; - Annually in patients with inactive infection. - Concern about sero-reversion (in immune-suppressed patients)
Quantification of HBsAg	<ul style="list-style-type: none"> - Defines the phase of infection, -Aids in identifying the response to peginterferon therapy - Determines HBV reactivation 	<ul style="list-style-type: none"> - To differentiating patients with HBeAg-negative chronic HBV infection from inactive carriers. - Prediction of mother to child transmission - Determine the frequency of ALT measurement and elastography tests for patients with inactive infections; - Identify patients for discontinuation of NA therapy. - Identify patients unlikely to respond to or continue peginterferon therapy.
Anti-HBs	Marker of immunity (natural or with vaccination)	Present upon presentation and persists when loss of HBsAg has been documented
Anti-HBc	Marker of HBV exposure	Present upon presentation
HBeAg	Related with high levels of HBV DNA, marker of infectivity	<ul style="list-style-type: none"> - At first present. - Every 6 months for patients on treatment with HBeAg-positive. - HBV flares (changes in levels of ALT and HBV DNA).
Anti-HBe	Associated with lower levels of HBV replication and infectivity;	<ul style="list-style-type: none"> - At initial presentation. - Every 6 months in HBeAg-positive patients receiving treatment.

	marker for treatment response	- HBV flares (changes in levels of ALT and HBV DNA)
HBV DNA	Detects HBV infection, used to define phase of infection and need for HBV therapy	<ul style="list-style-type: none"> - At initial presentation. - Every 6–12 months for untreated patients. - Every 3 months for treated patients until undetectable, then every 6 months. - For patients with ALT flares. - Anti-HBc positive individuals at risk for HBV reactivation.

The diagnosis of HBV infection is primarily based on laboratory tests, as clinical symptoms are not sufficient for distinguishing hepatitis B from hepatitis caused by other viral agents[67]. Different markers used in laboratory tests for hepatitis B infection appear in different stages of disease progression (see details in [Table 2](#))[68]–[70].

PART II – HEPATITIS B INFECTION

1. Routes of transmission

The hepatitis B virus is transmitted primarily by percutaneous or mucosal contact with infected body fluids such as blood, serum, wound exudate, semen, vaginal secretions, and saliva, where the concentrations of HBV are high to moderately high [71]. In highly endemic areas, vertical transmission from mother to child at birth is the most common route of transmission in addition to horizontal transmission, especially for children in the first five years of life through their close contact with parents or other family members or friends [67], [72].

Pseudo-Vertical or Perinatal or mother-to-child transmission (MTCT) is recognized as hepatitis B surface antigen (HBsAg) or HBV-DNA positivity in a 6–12-months-old infant [73]. The transmission generally occurs during delivery or in the early post-natal period. Infection during pregnancy is uncommon. Globally, transmission from HBsAg-positive mothers is responsible for about 50% of the global disease burden. The rate of MTCT is regulated by the hepatitis B e antigen (HBeAg) status of the mother, with a transmission rate of 70% to 90% for HBeAg-positive mothers, compared to 10% to 40% for HBeAg-negative infections. Intrauterine transmission can only occur in about 3-8% of cases and is the main factor in the failure of immunoprophylaxis. [73], [74].

Pseudo-vertical transmission of HBV during the natural delivery of the newborn accounts for around 35% of transmission events. It can occur via the microtransfusion mixing of maternal and fetal blood, direct contact with infected fluids in the maternal genital tract, and swallowing of infected fluids [74], [75]. There is no evidence of transmission during breastfeeding by HBV-carrying mothers. However, there are insufficient data to conclusively rule out transmission from an HBV-infected mother with nipples that are cracked and bleeding. Considering that the gastrointestinal tract is not the primary route of HBV transmission, and the many benefits associated with breastfeeding, the guidelines promote and encourage breastfeeding following the administration of passive and active immunoprophylaxis to the newborns of HBsAg-positive mothers [74]–[77].

Horizontal transmission in adults is primarily through sexual contact or through the reuse of contaminated needles and syringes or other sharp objects either in health care settings,

or among persons who inject drugs, tattooing, piercing, etc. [67], [78]. In addition, because HBV can survive outside of a host for at least 7 days [67] some horizontal transmission may also occur through household or close contact via bites, skin lesions, and unsanitary practices. However, this mechanism of transmission remains poorly studied, and further investigation is still warranted to document precisely the circumstances of contamination [78], [79]. Transmission risk appears to increase with increasing HBV-DNA concentrations in body fluids. For example, risk becomes significant when HBV copy number reaches $5.9 \log_{10}$ copies/mL in saliva, $4.3 \log_{10}$ copies/mL in urine, around $2 \log_{10}$ copies/mL to $6 \log_{10}$ copies/mL in tears, $5.2 \log_{10}$ copies/mL in sweat, $5.6 \log_{10}$ copies/mL in faeces, and $3.2 \log_{10}$ copies/mL in semen. Based on information of HBV-DNA level, transmission of HBV through saliva and tears may occur with household/close contact [78].

In addition, HBV genotype is also related to transmission rate. In southeast Asia, genotype C is associated with higher perinatal transmission rates than genotype B. However, both perinatal and horizontal transmissions depend on risk factors such as HBV-DNA level, HBeAg status, and hygiene practices [74].

Hepatitis B virus transmission in Southeast Asia

In Southeast Asia, approximately 35% (25.0-45.0%) of HBs(+) mothers are HBeAg positive and thus have high circulating HBV DNA loads ($>1.0 \text{ E}+05 \text{ IU/mL}$). Therefore, the risk of HBV transmission and the establishment of chronic infection is very high at delivery (85.0%-90.0%) when newborns are highly susceptible. The susceptibility of infants and preschool children to chronic infection with HBV tends to decrease thereafter to around 25%. Other family members are susceptible to transmission during this period, particularly from a positive mother or among siblings. Transmission risk is generally lower with fathers or other adults who are more physically distant from their children. Intrauterine transmission is rare and occurs only in HBeAg(+)mothers (2.5-5%). Other risks of natural transmission include sexual contact according to any modality (2.5-5.0% of chronicity), exposure to non-sterile needles, skin piercing and multiple blood transfusions (Figure 8) [80], [81].

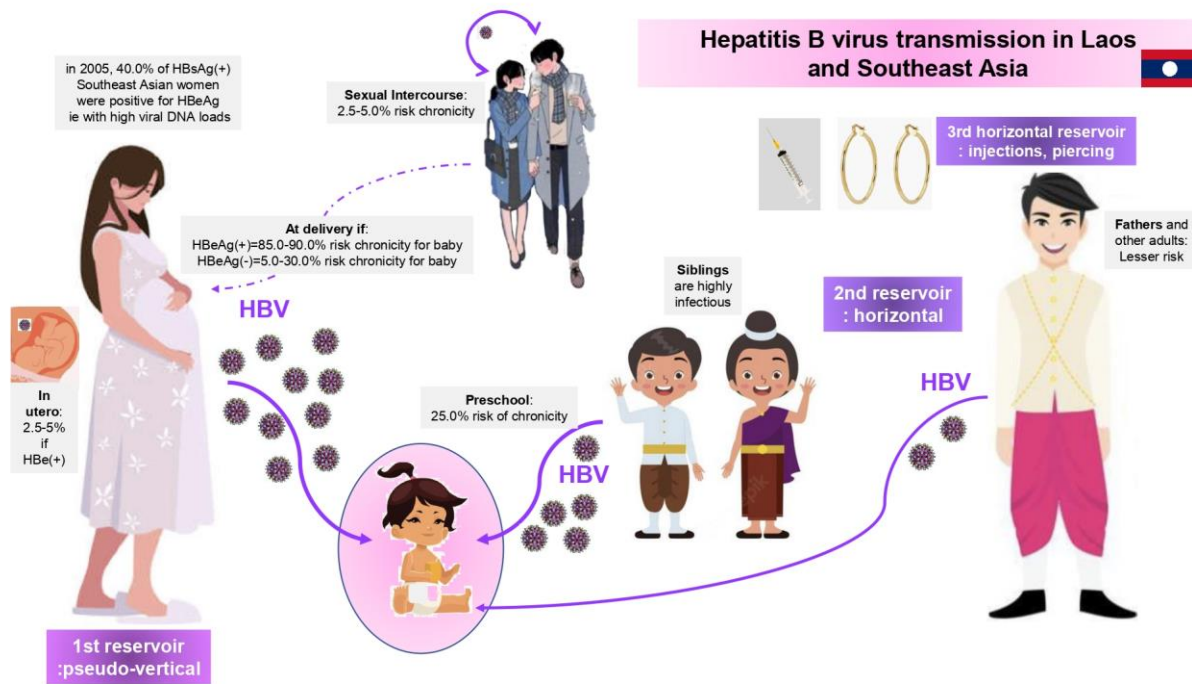


Figure 8: Transmission routs of Hepatitis B virus in Southeast Asia

2. Natural history of infection

2.1. Clinical outcomes

Hepatitis B virus infection can cause acute or chronic infection:

- Acute infection may remain asymptomatic, develop features of acute viral hepatitis or progress to acute liver failure. Liver failure is marked by the elevation of serum levels of liver enzymes. For acute infections,

the virus is cleared within six months [46].

- Chronic infection remains largely asymptomatic, and patients fail to clear the virus. However, after decades of exposure to virus-induced injury, liver fibrosis may occur and progress to liver cirrhosis if left unchecked. Patients may develop complications of cirrhosis, such as ascites, variceal bleeding, and hepatic encephalopathy (decompensation). Ultimately, an HCC may develop generally on cirrhotic tissue [70]. Without treatment, 20–30% of chronically-infected patients will develop liver cirrhosis and/or cancer [82].

Overall, the course of any particular HBV infection depends primarily on the patient's age at the time of infection. Infections during infancy remain asymptomatic and carry more than a 90% risk of progressing to chronic infection. About 20% of infections established in patients between 1 and 5 years old develop into chronic infections. After the age of 5, and particularly in adults, more than 90% of patients develop acute hepatitis and clear the virus in 6 months. A small proportion of adults will develop a chronic infection lasting more than 6 months [80].

In the case of acute HBV infection, the serological pattern is illustrated in (Figure 9). First, HBsAg appears 2–10 weeks after infection. One to two weeks later, total anti-HBc and IgM anti-HBc increase and total anti-HBc continues to be positive. HBsAg and anti-HBc IgM antibodies disappear within 6 months, and anti-HBs IgG antibodies appear. In most individuals, anti-HBs persists for life and provides long-term immunity.

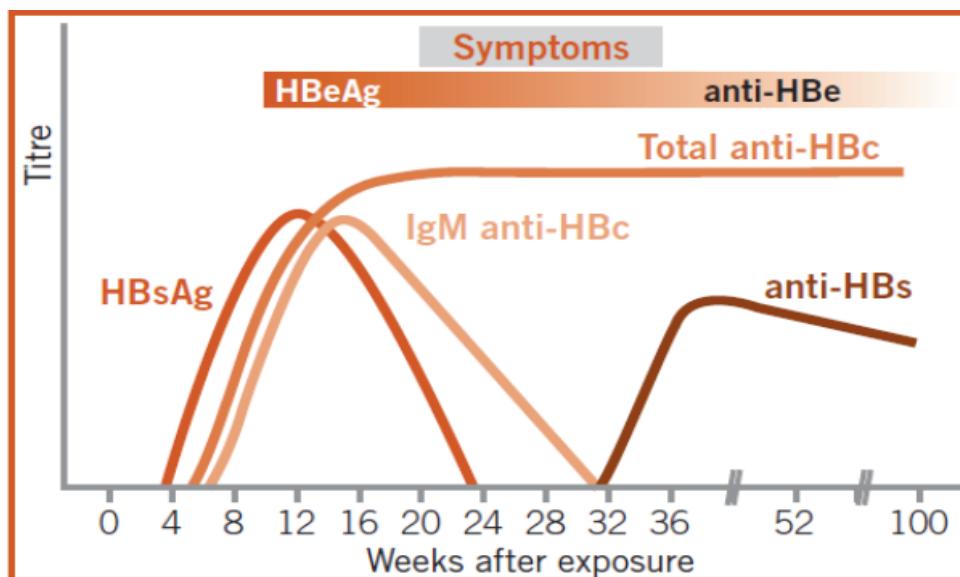


Figure 9: serological pattern of acute HBV infection[70].

2.2. The different phases of Chronic hepatitis B

Chronic hepatitis B (CHB) is defined as the presence of HBsAg detection in the blood or serum for more than six months, with or without related active viral replication and evidence of hepatocellular injury and inflammation. The 2015 WHO CHB guidelines depict five clinical or virological phases of infection and/or hepatitis (Table 3) [70] and Figure 10 [83]).

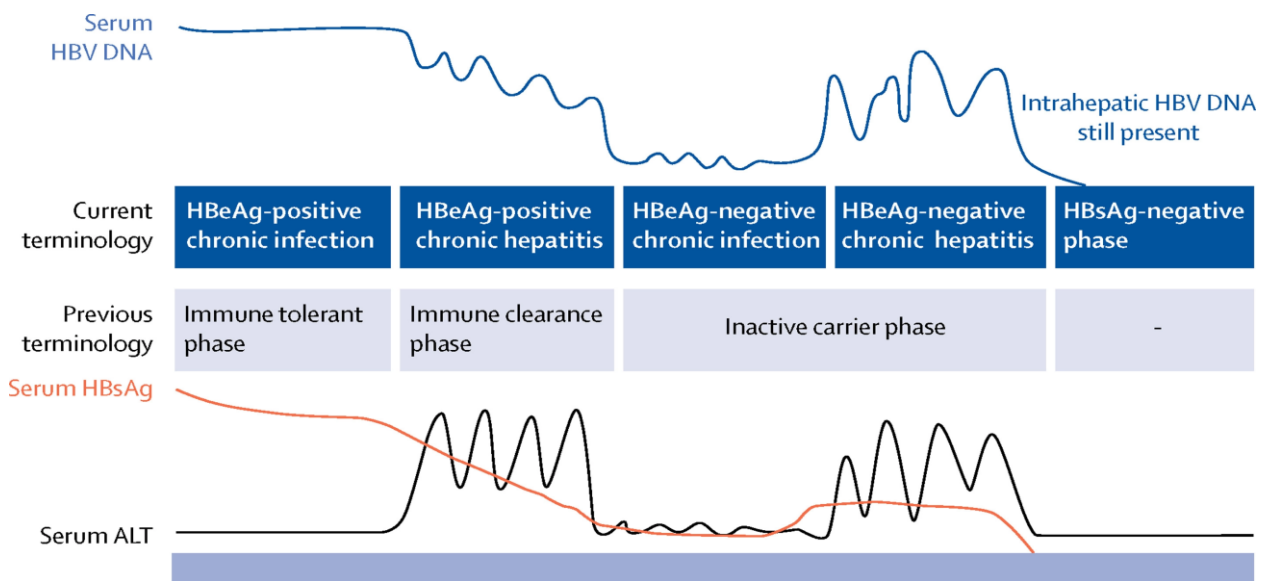


Figure 10: Phases of chronic HBV infection

Table 3: Phases of chronic HBV infection

Phase	HBeAg serological status	Pattern
Immune-tolerant Chronic infection	HBeAg positive	<ul style="list-style-type: none"> - Present in many HBeAg-positive children and young adults (infected at birth) - HBV DNA levels >200 000 IU/mL - Persistently normal ALT - Minimal histological disease
Immune-reactive Chronic hepatitis	HBeAg positive; may develop anti-HBe	<ul style="list-style-type: none"> - Abnormal/periodically abnormal ALT - HBV DNA levels >2000 IU/mL) - Histological necroinflammatory activity present - HBeAg to anti-HBe seroconversion possible, with normalization of ALT leading to “immune- control” phase

Inactive chronic hepatitis "Immune control" Chronic infection	HBeAg negative, anti-HBe positive	<ul style="list-style-type: none"> - Persistently normal ALT - HBV DNA levels <2000 IU/mL - Risk of cirrhosis and HCC reduced - Can be develop HBeAg-negative disease
Immune escape - Chronic hepatitis	HBeAg negative, with or without being anti-HBe positive	<ul style="list-style-type: none"> - HBeAg negative and anti-HBe positive - Abnormal ALT (persistent or periodically abnormal) - HBV DNA levels >20 000 IU/mL) - Older persons especially at risk for progressive disease (fibrosis/cirrhosis)
HBsAg seroconversion- occult HBV infection (OBI)	Anti-HBc(+), anti-HBs (+) or (-)	<ul style="list-style-type: none"> - Persistently normal ALT - HBV DNA levels<10 IU/mL
Reactivation" or "acute- on- chronic hepatitis	HBeAg positive or negative	<ul style="list-style-type: none"> - Can occur spontaneously or be precipitated by immunosuppression - Abnormal ALT - Moderate to high levels of HBV replication - Seroreversion to HBeAg positivity can occur if HBeAg negative - High risk of decompensation in presence of cirrhosis

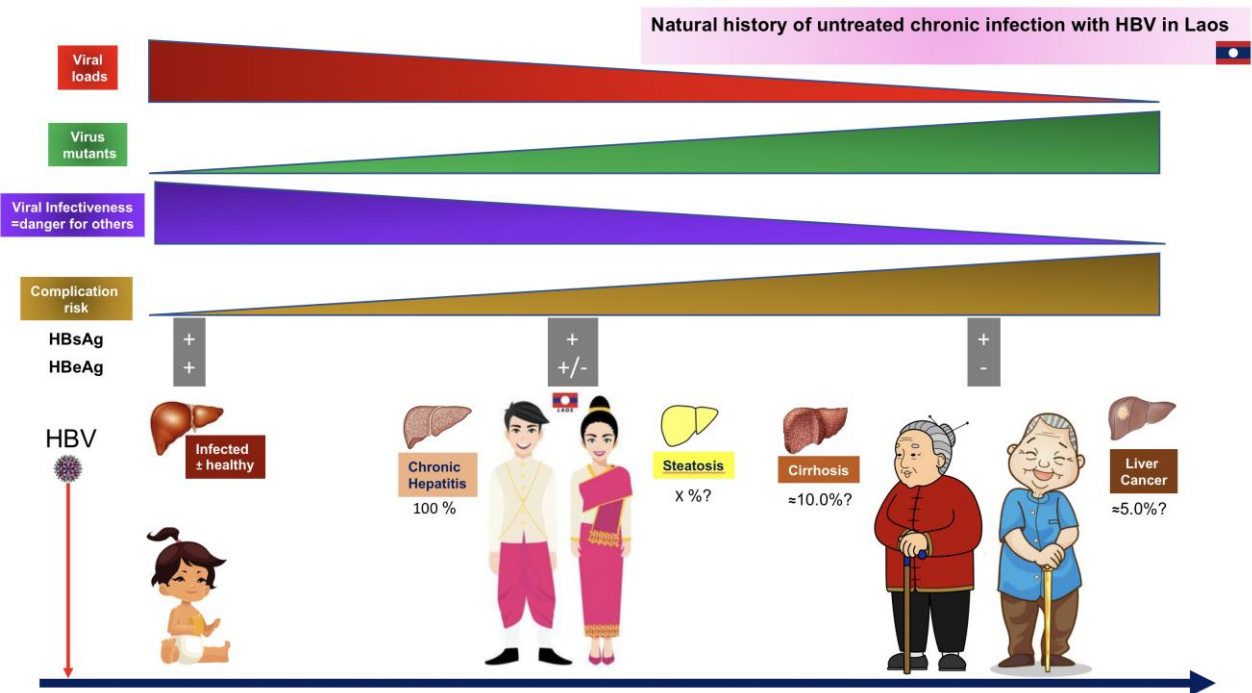


Figure 11: Natural history of untreated hepatitis B chronic infection in Laos.

2.3. Natural history of infection with HBV in Southeast Asia

In Southeast Asia, the natural history of hepatitis B presents some specificities that differentiates it from the disease course observed in sub-Saharan Africa, in Europe, or in Indigenous populations of Latin America. Most Southeast Asian countries are in areas of high (>8.0%) to high-intermediate (5.0-7.9%) endemicity for chronic infection with HBV. Southeast Asians are classically infected as infants (<1.0 year) or very young children (<5 years) when exposed pseudo-vertically at birth or horizontally through poorly defined modalities during the perinatal period and the years after. In the latter circumstances, older siblings are important vectors of infection for the last born in a family. This setting of transmission accounts for the observation that Southeast Asian children are classically immune tolerant, and at first present with very high viral loads (>1.0 E+06 IU/mL) and minimal liver inflammation. The immune tolerant phase is generally protracted with an HBeAg-positive phase that can last for decades. Anti-HBe seroconversion occurs commonly in the fourth decade of life and 15% of elderly patients (>60 years) are still HBeAg(+). In Asian patients, seroconversion is often associated with ALT flares, serious histological changes and increased AFP levels. Importantly, there are differences among HBV genotypes concerning their stability of HBeAg production. Infection with genotype B, due to specificities in its encapsidation signal sequence, allows more frequent and earlier seroconversion to anti-HBe than infection with genotype C. Unfortunately, this protracted phase of HBeAg(+) immune tolerance together with a late immune clearance reduces the duration of the next step i.e., the anti-HBe inactive carrier state. Furthermore, it has been shown that late anti-HBe seroconverters are more susceptible to early relapse of HBeAg(-) chronic hepatitis and of progression toward liver cirrhosis. In anti-HBe(+) patients from Asia-Pacific, the risk of subsequent death from HCC or cirrhosis is tightly linked both to HBV DNA loads and ALT levels. For the reason mentioned above, patients infected with genotype B develop a less progressive disease than those infected with genotype C[84].

2.4. Occult B infection in Southeast Asia

Occult infection with HBV or occult B infection (OBI) is defined as the presence of HBV DNA in the liver of a patient without the presence of HBsAg in the blood. HBV DNA can be found in the bloodstream at very low levels, generally under 20 IU/mL. Although anti-HBc is frequently present in cases of OBI (>75.0%), there lacks a well-defined serological signature

enabling the detection of this infection type with sufficient confidence. OBI is more prevalent in groups at high risk of infection or among patients with liver diseases, such as those infected with hepatitis C virus. Higher risk is also associated with HIV infection and in patients receiving haemodialysis. OBI is also found in a substantial proportion of patients with severe liver diseases such as cirrhosis or HCC developed in HBsAg(-) patients or initially tagged as cryptogenic cases. The causal role of OBI in such cases remains debated. Still, its frequent detection suggests, at the very least, that this form of infection might be important in a frequently multifactorial context [85].

The combination of invariably low to undetectable HBV DNA loads in the blood and the infrequent access to liver tissue, makes the diagnosis of OBI difficult. Furthermore, due to the high cost of routine nucleic acid testing (NAT), detection in patients in low-and-middle-income countries is often restricted to those involved in research projects. Its clinical importance is far from negligible in blood transfusions, considering that blood (8-30% of cases) or organ donations with OBI can transmit HBV infection. Understandably, in areas with high endemicity of HBsAg prevalence, OBI has attracted little attention from researchers, clinicians, and public health authorities in Southwest Asian countries. Furthermore, while some authors consider that OBI prevalence is very low in blood donors from countries highly endemic for HBsAg [86], other investigators observed that the levels of OBI mirror endemicity of HBV and can reach 1.0% in areas highly endemic for HBsAg [87]. It has been suggested that overlooking OBIs in LMICs might prevent these countries from reaching hepatitis B control goals in 2030.

While some Southeast Asian countries (Brunei, Cambodia, Timor, Philippines, Myanmar) have not published on OBI, this topic has been covered in others. For example, in Vietnam, blood donation screening estimated a post-transfusional risk of between 66 to 250 blood units per million. Interestingly, the authors observed that the fraction of potential OBI donors increased with age in both genders [88].

In Indonesia (Java and Sumatra), a survey conducted 12 years ago detected 8.0% of OBI in blood donors. On third of these occultly-infected donors presented HBV strains with a mutated “a” determinant changing its immunogenicity [89]. In Borneo, 4.6% of healthy young adults presented an OBI. Worryingly, most of them were anti-HBs(+) but could not be regarded as non-infectious. Mutations of the S gene were found in many cases (n=7/9, 77.7%) [90]. A similar result was reported from Java where a startling rate of 13.9% of anti-HBs(+)/anti-HBc(+) blood donors presented an OBI [91].

In continental Malaysia (Kuala-Lumpur), anti-HBs(+)/anti-HBc(+) OBI were found in 5.5% of

blood donors (n=1000). All of them were escape mutants. A total of 105 amino acid mutations were found in these isolates: 85.7% (90/105) were outside the major hydrophilic region (MHR) and 14.3% (15/105) were within the MHR (aa100–160), of which 40% (6/150) were within the a (aa 124–147) determinant region. As in Indonesia, a substantial proportion of OBI was found in vaccinated individuals (n=35/35, 63.6%) [92]. OBI was also reported from Singapore [93].

OBI were also found in blood donors from Thailand with a low proportion (4.2%) of anti-HBs(+) individuals [94]. In Laos, 10.9% of HBsAg-negative, anti-HBc and/or anti-HBs-positive donors were occultly infected with HBV. In this series, 65.7% (n=23/35) of OBI were anti-HBs(+). This latter observation implies that a sizable proportion of apparently safe blood donors in Laos are in reality potentially infectious. NAT should be seriously considered for blood donations in this country [95].

Overall, OBI exists in Southeast Asia albeit its true magnitude is poorly known in most countries. The consequences of this kind of infection for public health, including national blood donation systems, remain to be properly assessed.

3. Treatment

3.1. Therapy endpoints

The main objectives of hepatitis B treatment are to improve the quality of life and survival of patients by preventing disease progression (to fibrosis, decompensated cirrhosis, end-stage liver failure, HCC and death), and to prevent transmission of HBV to others. Nevertheless, chronic HBV infection cannot be completely eradicated due to the persistence of covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes. Therefore, the recommendations for criteria indicative of successful treatment are as follows:

- Induction of long-term suppression of HBV DNA levels to undetectable levels.
- Induction of HBeAg loss, with or without anti-HBe seroconversion, representing partial immune control of chronic HBV infection.
- A biochemical response, defined as ALT normalization, that is achieved in most patients with long-term suppression of HBV replication.

- Loss of HBsAg, with or without anti-HBs seroconversion, as it indicates profound suppression of HBV replication and viral protein expression [96]–[98].

3.2. Treatment indicators

Treatment indicators are mainly based on the combination of three criteria: serum HBV DNA levels, serum ALT levels, and liver disease severity (based on clinical, liver biopsy or noninvasive methods). Indicators should also consider age, co-morbidities, family history of HCC or cirrhosis and extrahepatic manifestations. Common recommendations of Asian-Pacific (APASL) and European Association for the Study of the Liver (EASL) and clinical guidelines for treating a patient are as follows:

Asian-Pacific (APASL)	European Association for the Study of the Liver (EASL)
<ul style="list-style-type: none"> - HBsAg positive, decompensated patients with detectable HBV DNA (IU/ml). - Patients with compensated cirrhosis and HBV DNA (IU/ml) >2000 - Severe chronic HBV reactivation, detectable HBV DNA (IU/ml) and high ALT. - Non-cirrhotic HBV-infected patients with persistent ALT >2 xULN and HbeAg positive with HBV DNA (IU/ml) >20,000 or HbeAg negative with HBV DNA (IU/ml) >2000. - Patients with elevated HBV DNA levels ([20,000 IU/ml if HbeAg positive and [2,000 IU/ml if HbeAg negative) but ALT <2 xULN. If age >35 years or if there is family h/o HCC or cirrhosis. Consider treatment if biopsy shows moderate to severe inflammation or significant fibrosis*. - HbeAg positive patients with HBV 	<ul style="list-style-type: none"> - HbeAg positive or negative chronic hepatitis B, defined by HBV DNA >2,000 IU/ml, ALT > ULN and/or at least moderate liver necroinflammation or fibrosis. - Patients with compensated or decompensated cirrhosis, with any detectable HBV DNA level and regardless of ALT levels. - HBV DNA >20,000 IU/ml and ALT >2xULN should start treatment regardless of the degree of fibrosis. - People with HBeAg-positive chronic HBV infection, defined as persistent normal ALT and elevated HBV DNA levels, can be treated if they are over 30 years of age, regardless of the severity of histological liver damage. - Chronic HBV infection with HBeAg-positive or HBeAg-negative, a family history of HCC or cirrhosis, and extrahepatic manifestations can be treated even if the typical indications

<p>DNA <20,000 IU/ml. If age >35 years or if there is a family h/o CHC or cirrhosis. They should be considered for treatment if biopsy shows moderate to severe inflammation or significant fibrosis*.</p> <ul style="list-style-type: none"> - HBeAg negative patients with HBV DNA <2000 IU/ml. If age >35 years or if there is a family h/o CHC or cirrhosis. They should be considered for treatment if biopsy shows moderate to severe inflammation or significant fibrosis*. <p>*Moderate to severe inflammation on liver biopsy means either hepatic activity index by Ishak activity score [3/18 or METAVIR activity score A2 or A3; significant fibrosis means F C2 by METAVIR fibrosis score or Ishak fibrosis stage C3. Significant fibrosis by noninvasive markers means liver stiffness C8 kPa (by Fibroscan) or APRI C1.5. Cirrhosis by noninvasive markers means liver stiffness C11 kPa (by Fibroscan) or APRI C2.0</p>	<p>for treatment are not met.</p>
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According to the Lao National Guidelines 2022 (unpublished), the recommendations for HBV treatment in **Laos** roughly follow the WHO guidelines, with the "persons to be treated" being as follows:

- Patients with clinical evidence of compensated or decompensated cirrhosis (or cirrhosis based on APRI score* >2 in adults) should be treated, regardless of ALT levels, HBeAg status or HBV DNA levels.
- Aged more than 30 years (in particular) and have persistently abnormal ALT levels (normal ALT have been defined as below 30 U/L for men and 19 U/L for women) and evidence of high-level HBV replication (HBV DNA >200,000 IU/mL) and regardless of HBeAg status.

Note: When HBV DNA testing is not available: Treatment can be considered on the basis of persistent abnormal ALT levels alone, regardless of HBeAg status. [70], [99].

***APRI CALCULATION.**

$$\text{APRI} = (\text{AST/ULN}) \times 100 / \text{platelet count (10}^9\text{/L)}$$

For APRI, ULN signifies the upper limit of normal for AST in the laboratory where these investigations were undertaken. For example, in a patient with an AST of 82 IU/L (where laboratory ULN for AST is 40 IU/L) and a platelet count of 90x10⁹/L, the APRI would be: (82/40) x100/90 = 2.28. This value is >2 and is consistent with the presence of cirrhosis.

Online calculators can be accessed for APRI at:

<http://www.hepatitisc.uw.edu/page/clinical-calculators/apri>

3.3. Available medicines

Currently, the available drugs and treatment options for chronically infected hepatitis B patients in Laos are adapted to the country situation. They are presented in the tables below:

Table 4: Drugs for the treatment of chronic hepatitis B and their doses in adults

Drug	Dose
Tenofovir disoproxil fumarate (TDF)	300 mg oral one daily
Tenofovir alafenamide fumarate (TAF)*	35 mg oral one daily
Entecavir (adult with compensated liver disease and lamivudine naive)	0,5 mg oral once daily
Entecavir (adult with decompensated liver disease and experience use of lamivudine)	1mg oral once daily

* Tenofovir alafenamide fumarate is an orally bioavailable prodrug of tenofovir with reduced renal and bone toxicities compared to tenofovir.

Table 5: Drugs for the treatment of chronic hepatitis B and their doses in children

Drug	Dose
Tenofovir (in children ≥12 years old, and weight > 35 kg)	300 mg once daily
Entecavir (in children 12 years of age or older and weighing at least 10 kg. The oral solution should be given to children with a body weight up to 30 kg)	Recommended once-daily dose of oral solution (ml)

Table 6: Antiviral options for management of Antiviral resistance

Antiviral Resistance by Genotypic Testing	Switch Strategy (Preferred)	Add Strategy: 2 Drugs Without Cross-Resistance
Lamivudine resistance	Tenofovir* (TDF or TAF)	Continue lamivudine; add tenofovir (TDF or TAF) (or alternative emtricitabine-tenofovir)
Telbivudine resistance	Tenofovir* (TDF or TAF)	Continue telbivudine; add tenofovir (TDF or TAF)
Adefovir resistance	Entecavir or Tenofovir* (TDF or TAF)	Continue adefovir; add entecavir
Entecavir resistance	Tenofovir* (TDF or TAF)	Continue entecavir; add tenofovir (TDF or TAF) or alternative emtricitabine-tenofovir
Tenofovir resistance	Entecavir*	Continue tenofovir (TDF or TAF) and add entecavir
Multidrug resistance	Tenofovir	Combined tenofovir (TDF or TAF) and entecavir*

* Similar efficacy between switching to an antiviral with a high genetic barrier to resistance and adding 2 drugs without cross-resistance with follow-up to 5 years. Thus, switching is the preferred strategy except if HBV is multidrug resistant [70], [99].

As we know, there are two main treatment options for chronically infected hepatitis B patients:

- Nucleos(t)ide analogues (NAs) that aim at stopping hepatitis and disease progression by inhibiting viral replication
- PegIFNa which rely on the induction of a long-term immune control by finite treatment

Nucleos(t)ide analogues (NAs): NAs directly inhibit HBV polymerase activity in cytoplasmic capsids. Third-generation NAs, including the deoxy guanosine analogue entecavir (ETV), deoxy adenosine analogues tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF), lead to elimination of HBV viremia in virtually all treated patients without significant selection of resistant variants in naive patients. NA has no direct effect on cccDNA levels, cccDNA chromatin organisation, cccDNA transcription

and viral protein production. A decrease in the total amount of intrahepatic cccDNA may be observed after several years of treatment due to: (i) inhibition of the intracellular recycling pathway, (ii) decrease in the rate of infection of new cells, and (iii) dilution of cccDNA by hepatocyte turnover, as cccDNA may be lost through cell division. NA treatment is generally accompanied by a rapid reduction in hepatocyte death and liver inflammation which could, at least theoretically, result in a decrease in hepatocyte turnover and a slower reduction in the cccDNA pool. However, the rate of HBs loss is low (10% at 5 years) and treatment is often required for life, particularly in patients with advanced liver disease. Clinically, effective treatment with NA is associated with regression of fibrosis, absence of disease progression and a reduced risk of HCC after 5-6 years of treatment.

Pegylated interferon (PEG IFN) (alpha 2a/2b) promotes innate antiviral immune activity and can lead to a durable off-treatment response in a finite treatment period. However, pegIFN is often poorly tolerated and response rates are limited with highly variable and genotype-dependent responses. IFN α reduces cccDNA transcripton by imposing repressive epigenetic modifications on viral chromatin in both in vitro and in vivo models. These observations may explain the impact of IFN-based regimens on HBs levels but not why only a minority of patient responds. Indeed, HBs loss is achieved in 11% of treated patients and 32–36% achieve e antigen (HBeAg) seroconversion after a year of theatment.

NAs in future combination therapies: Given the number of steps in viral replication and immune clearance that can be potentially targeted, the options for combination therapy are considerable. Adding NA-suppressed patients, pretreatment with NAs (sequential combination) or combination with other DAAs and/or immunomodulatory therapies are all possible in theory and there is only limited knowledge or experience to make informed choices.

Pre-treatment with NA or in combination with NA could achieve a complete suppression of DNA polymerase and, indirectly, contribute to reduce the recycling of mature capsids to the nucleus (reduction of the cccDNA pool) and limit viral spread to uninfected hepatocytes:

- Partially restore HBV specific CD4 and CD8 T cell function.
- Reduce the risk of hepatic flares and limit liver damage in case of efficient immune

restoration.

- Reduce the risk of resistance to the experimental drug.

The combination of NA with DAAs or multiple DAAs is likely to lead to more effective or even complete suppression of viral replication. However, unless complete elimination of cccDNA or sustained cccDNA silencing is achieved, functional or complete cure of HBV within a reasonable treatment period will require immune control.

4. Severe complications of chronic HBV infection: cirrhosis and/or HCC

The risk of progression to cirrhosis and/or HCC in cases of infection with HBV is not homogeneous throughout world and depends on different factors including the host's immune response and the co-occurrence of liver injury due to concomitant toxic exposure (alcohol, tobacco, aflatoxin). However, it appears that the most important factor conditioning complications in case of infection with HBV is the early age at infection that is associated with worse outcomes in addition to its effect of increasing the probability of chronic HBV infection [100].

Due to their frequent infection during infancy and early childhood, Southeast Asians are particularly exposed to the worst aspects of chronic infection by HBV.

4.1. Liver cirrhosis

Associated factors found to increase the risk of progression to cirrhosis in case of persistent infection with HBV are habitual alcohol intake, concurrent infection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV), high levels of HBV replication, and patients who had HBeAg reversion, HBV genotype C/B and a higher proportion of BCP mutation. The risk factors for HCC are similar but also include a family history of HCC, underlying liver cirrhosis, chronic hepatic necro-inflammation, older age, male sex, as well as obesity, diabetes, and smoking [96], [97]. Importantly, being "Asian" is considered at higher risk of HCC in case of infection with HBV.

Based on WHO data in 2016, age-standardized (>15 years) mortality rates for liver cirrhosis in 11 South-East Asian countries were high (Figure 12). In Laos, the liver cirrhosis death rates were 38.7 and 18.3 in males and females respectively.

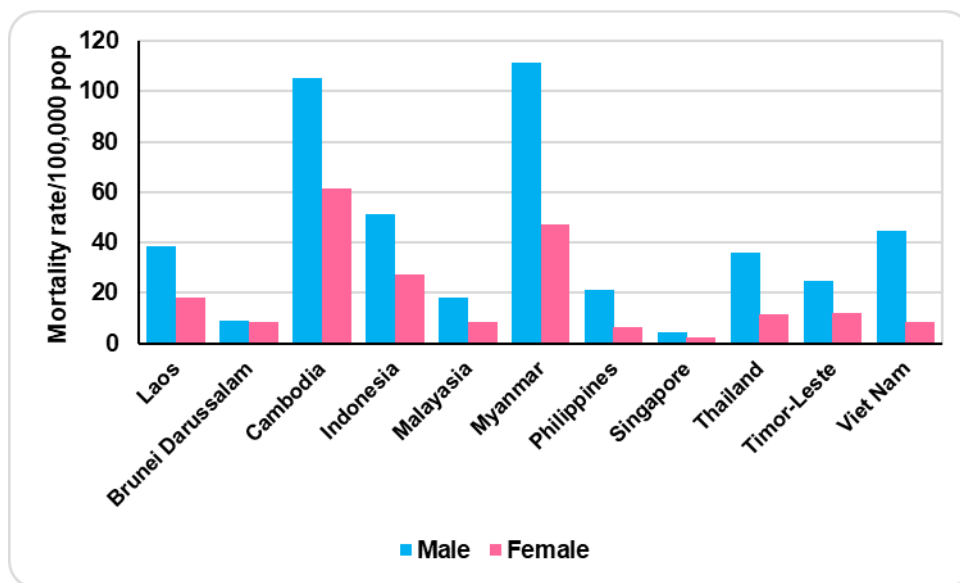


Figure 12: The mortality rate of Liver cirrhosis, age-standardized (15+), per 100,000 population. [[Liver cirrhosis, age-standardized death rates \(15+\), per 100,000 population \(who.int\)](http://www.who.int)]

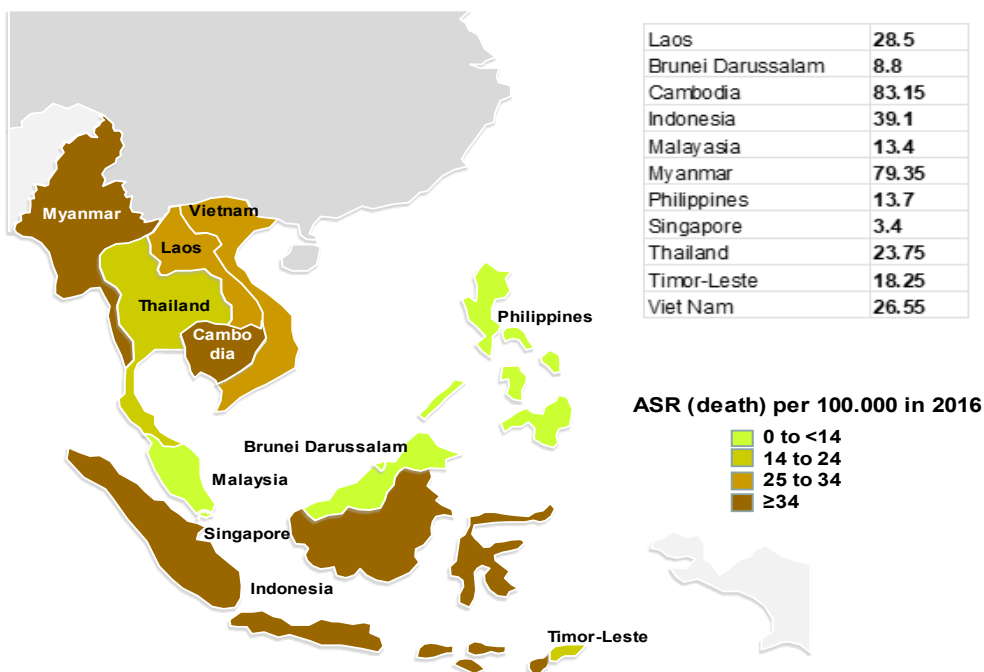


Figure 13: Age standardized rate of death from Liver cirrhosis for both sexes and any cause [[Liver cirrhosis, age-standardized death rates \(15+\), per 100,000 population \(who.int\)](http://www.who.int)]

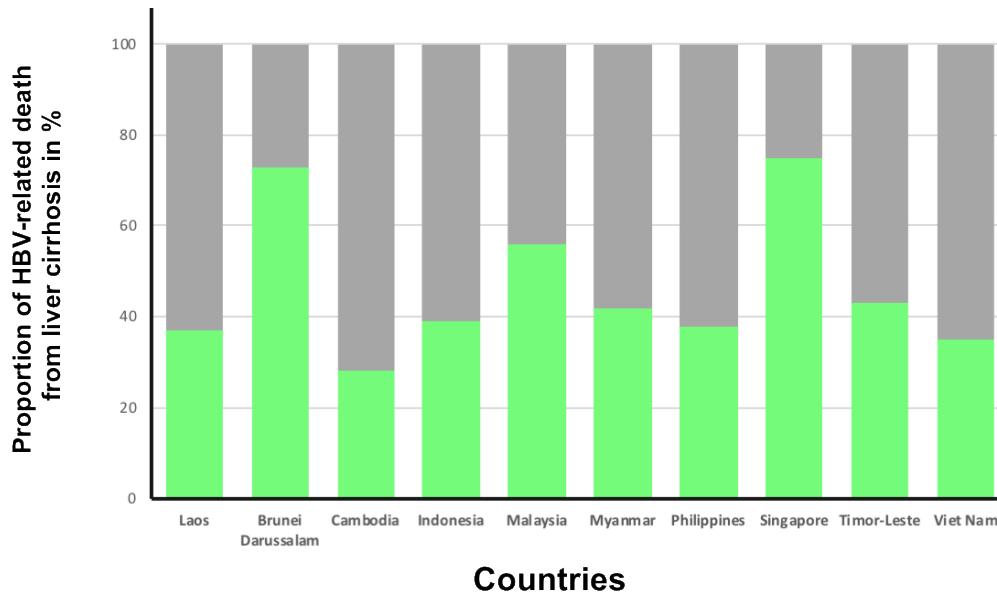


Figure 14: Proportion of HBV-related deaths from liver cirrhosis in Southeast Asian countries [101].

In Southeast Asia, liver cirrhosis is frequently caused by chronic infection with HBV (28.0-75.0% of cases, depending on the country). HBV is the most common cause of liver cirrhosis-related death in seven countries (Malaysia, Thailand, Indonesia, Myanmar, Timor-Leste, Brunei, and Singapore) and the second most common, after alcohol abuse, in Cambodia, Laos, Philippines and Vietnam [101]. This observation is somewhat at odds with the seroprevalence of HBsAg in the region, which is at its highest in Indochina and the Philippines. In the region, we can also observe a gender imbalance in mortality with a median M:F sex ratio around 2.0 (42.0/100,000 in males vs 20 for 100, 000 in females). In males, HBV occupies the first rank among causes of death (36.5%) while it is second for women (26.0%).

These mortality rates are high compared to the rest of the world, coming only after sub-Saharan Africa and Central Asia. By comparison, the prevalence of liver cirrhosis is remarkably low in Southeast Asia (750 cases/1.0E+05 in males, 350 cases/1.0E+05 for females) occupying the penultimate position among the WHO world regions. This observation suggests that despite its high prevalence in the region, HBV infection is usually not cirrhogenic but that the detection of the disease, and the management of its decompensation, should be improved in Southeast Asia [102]

4.2. Hepatocellular carcinoma in Southeast Asia

The 544 million inhabitants of the eleven Southeast Asian countries make up 6.8% of the world population, but nearly 11.0% (10.96%) of liver cancer diagnoses (Globocan, 2020, <https://gco.iarc.fr/>). Eight of the countries display an age-standardized rate of liver cancer higher than the world average. The three exceptions are insular countries located south of the region: Indonesia, Malaysia, and Timor-Leste. The most affected countries are Vietnam, Cambodia, Laos, and Thailand in the Indochinese peninsula.

The proportion of HBV-related liver cancer deaths among all liver cancer cases ranges from 21.0% in Indonesia to 57.0% in Malaysia (median=41.0%). HBV is the first cause of death from HCC in all countries in the region except Laos (1st: Alcohol) and Indonesia (1st: Hepatitis C) [101]. There could be, however, considerable localized regional variation of risk factors prevalence. For example, in Northern Vietnam HBV infection was involved in 81.3% of incident HCC cases for the period 2010-2017 [103].

HCC remains a multifactorial disease “*par excellence*” where the concomitance of several risk factors often participates to liver carcinogenesis. Our review, in annex of the current document, emphasizes the potential role of various risk factors on the liver of patients in Laos [104]. In this regard, it was observed recently that ethnic Vietnamese and Hmong HCC patients combine preferentially different risk factors (viral, lifestyle, or metabolic) [105]. Finally, a novel and more unexpected player of liver tumorigenesis has been recently identified in Southeast Asia: the molecular fingerprint of aristolochic acid, a potent mutagenic compound contained in various plants of the *Aristolochia* and *Asarum* genera used in herbal teas, has been found in liver tumors and other tissues from patients originating from Vietnam, Cambodia, and Myanmar [106]. It is well known that viruses are not responsible for the mutagenic signatures observed in liver cancer but that chemicals such as aflatoxin B1 and benzo-a-pyrene are responsible, instead, for the observed mutation spectra [107]. Aristolochic acid might be another important driver of liver carcinogenesis in Southeast Asia. The M:F sex ratio of incidence in all countries indicate a strong male predilection (median=2.9, range=2.3-4.9). Male patients tend to be diagnosed earlier than females, most probably due to the larger proportion of HBV-associated cases in men [108].

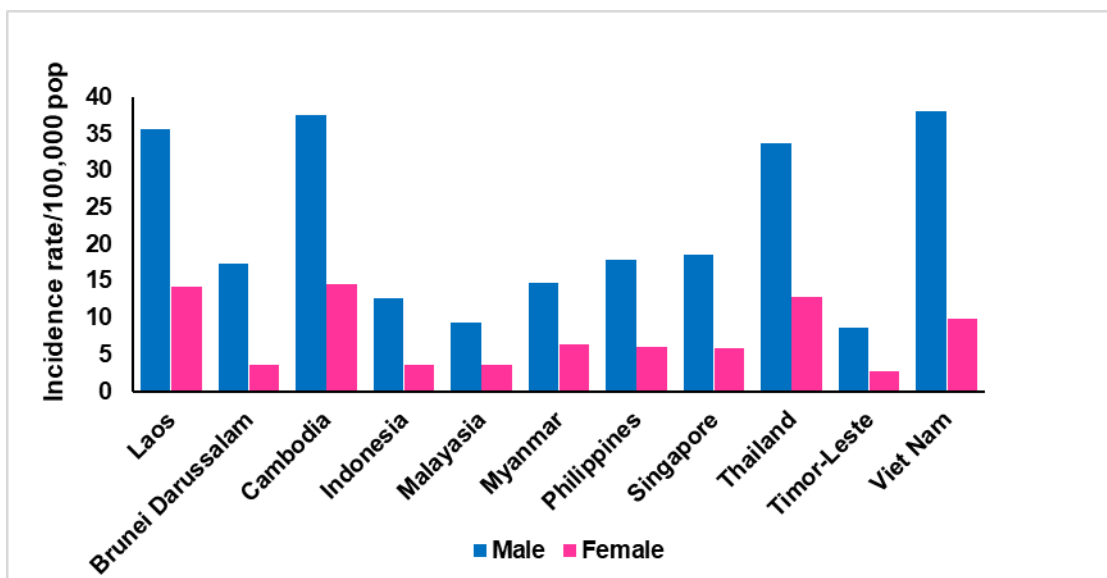


Figure 15: The incidence rate of Liver cancer in 2020, per 100,000 population

The age of diagnosis tends to increase in Asia, but Thailand displays the earliest onset of liver cancer with a peak at 45.0 years.

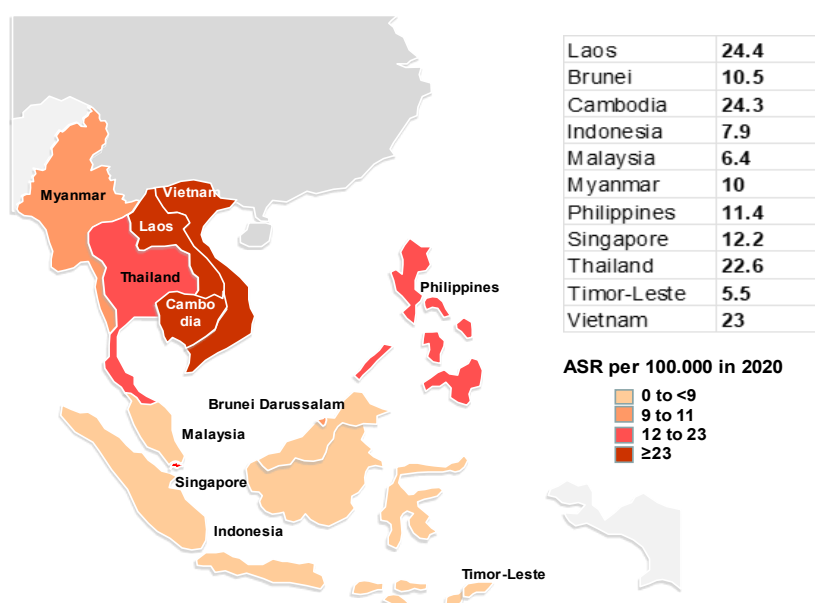
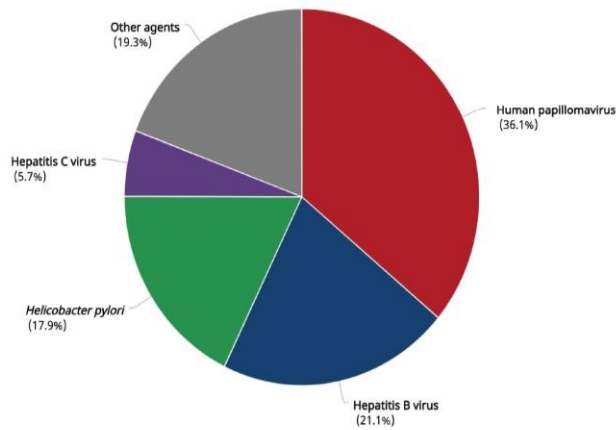


Figure 16: Liver cancer incidence in the 11 Southeast Asian countries (both sexes). Data: Globocan, 2020.

Concerning HBV-related HCC in South-East Asia in 2020, the International Agency for Research on Cancer [<https://gco.iarc.fr/causes/infections>] showed that HBV is responsible for 21% of the infection-associated cancer incidence, a figure four-fold higher than hepatitis C virus-associated cancers (Figure 17-1).



Total attributable cases: 180 000

Data source: de Martel C, Georges D, Bray F, Ferlay J, Clifford GM (2020)
 Graph production: Global Cancer Observatory (<http://gco.iarc.fr/>)
 © International Agency for Research on Cancer 2022

International Agency for Research on Cancer
 World Health Organization

Figure 17-1: Cancer cases (all infectious agents) in 2018 attributable to infections, in South-East-Asia.

According to the Coalition for Global Hepatitis Elimination [globalhep.org] modelling and the International Agency for Research on Cancer [<https://gco.iarc.fr/today/online-analysis-table>] data, in 2019 the percentage of liver cancer deaths attributable to Hepatitis B virus was comparable to other causes in South-East Asia (Figure 17-2). In Laos, hepatitis B-associated mortality from liver cancer was highest (9.6%) compared to other countries such as Thailand (8.7%), Viet Nam (8.5%), and Cambodia (7.5%).

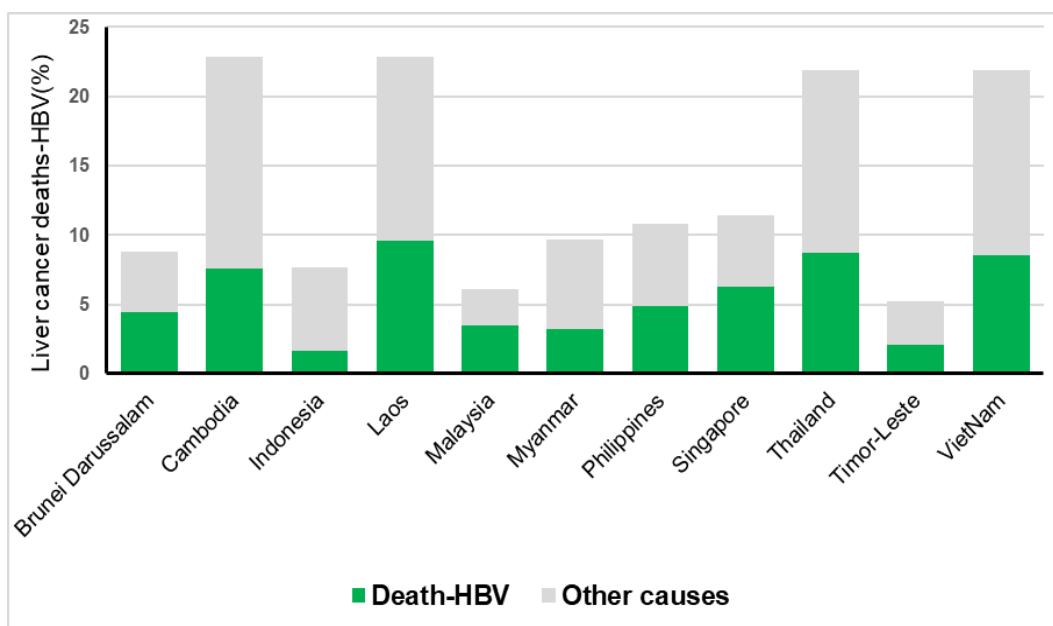


Figure 17-2: Percent of liver cancer deaths attributable to Hepatitis B virus compared to other causes in Southeast Asia countries. (Coalition for Global Hepatitis Elimination)

PART III – HEPATITIS B IN SOUTHEAST ASIA

In part III, I shall focus only on Southeast Asia, a region with characteristics justifying a targeted analysis of its epidemiological situation. Indeed, populations in the region, despite intrinsic heterogeneity, share large parts of their culture and lifestyle as well as genome architecture [109]. Likewise, hepatitis B genotypes prevailing in this region are very similar throughout the 11 countries [66]. Therefore, it is fitting to analyze the commonalities and differences encountered regarding the natural history of HBV infections in this region. Another important aspect lies in the fact that all countries in Southeast Asia share the same objective regarding the WHO strategies aiming to reduce HBV prevalence to less than 1.0% among children under 5 years (under a vaccination program) and eliminating chronic viral hepatitis as a public health threat by 2030 [110].

1. Epidemiology and health burden

The World Health Organization (WHO) estimates that in 2019, there were 296 million people living with a chronic hepatitis B infection despite the availability of a highly efficacious vaccine. In this same year, there were 820 000 HBV-related deaths, mostly from cirrhosis and hepatocellular carcinoma [67]. In the 11 Southeast Asian countries of Laos, Vietnam, Cambodia, Thailand, Myanmar, Philippines, Indonesia, Malaysia, Singapore, Brunei Darussalam and Timor-Leste, HBsAg endemicity in the general population has been classified (Figure 18) in four levels [111]:

- A weak prevalence is HBsAg positivity is lower than 2%
- A lower-intermediate prevalence is HBsAg positivity located between 2 and 4.99%
- A higher-intermediate prevalence is HBsAg positivity located between 5 and 7.99%
- A high endemicity prevalence is HBsAg positivity higher than 8%

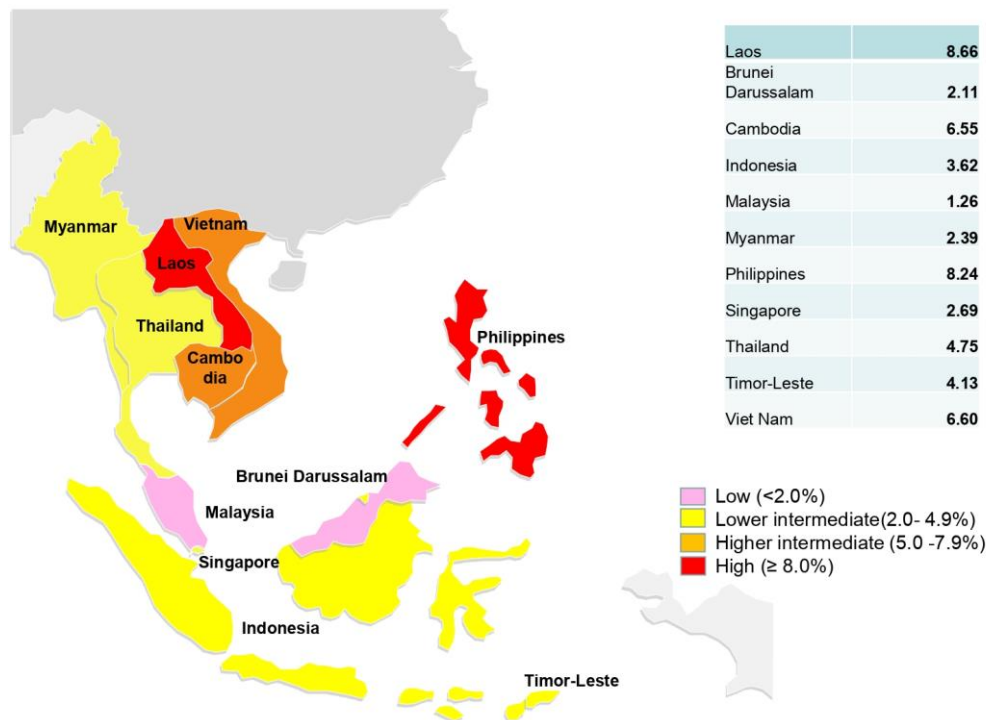


Figure 18: Prevalence of chronic HBV infection (HBsAg+) in Southeast Asia 2019 according to the Coalition for Global Hepatitis Elimination.

We shall not consider in this review of the literature the Western part of New Guinea, called Irian Jaya, albeit it is a part of Indonesia. Actually, this region is mostly inhabited by people of Melanesian descent with a genome architecture substantially different from that of other people living more in the West and the North of the region. Furthermore, Melanesians live beyond several important biogeographic lines (eg Wallace line) and are mostly infected with HBV genotypes substantially different from those found in the other countries of Southeast Asia [112], [113].

According to the modelled prevalence of chronic hepatitis B in 2019, continental countries located in the Indochinese peninsula (Laos, Cambodia, Thailand, Vietnam) are in general more affected than insular countries located in the southern part of the region (Malaysia, Indonesia, Singapore, Brunei-Darussalam, Timor-Leste). Myanmar, located on the western part of the region and anthropologically more similar to the Indian subcontinent and the Philippines, represent an exception to this pattern. The reasons for such wide heterogeneity are not completely understood although the timing of the implementation of Public Health policies in each nation could obviously play a role in current endemic levels of HBsAg (Figure 18).

Interestingly, there are major inconsistencies between national rates of HBsAg seroprevalence and HBV-associated morbidities and mortalities. In 2019, the HBV-related global rate death in Southeast Asia was highest in Thailand (18.88/100,000), followed by Cambodia (14.73/100,000) and Myanmar (12.15/100,000) even though HBsAg prevalence was relatively low (<8%) in this latter country. In contrast, in the countries where HBsAg prevalence is high, such as Laos and Philippines, estimated HBV-related death rates were much lower (7.14/100,000 and 5.40/100,000 respectively, see [Figure 19](#)).

The reasons for such discrepancies between prevalence and death rates are unknown. Several hypotheses may shed light on this issue. The most trivial explanation concerns the poor quality of medical records, inappropriate sampling or even the absence of studies in some countries. Another explanation concerns medical aspects of HBV-associated chronic liver diseases. It is well known that HBV interacts with lifestyle (alcohol consumption) or dietary regime (aflatoxin contamination) [114], [115]. Hence, these two variables depend heavily on the national culture (e.g. Malaysia and Brunei-Darussalam are predominantly Muslim countries with a tradition of temperance concerning alcohol) and ecological and climatological variables responsible for variation of aflatoxin contamination of food between countries (WHO, Global status report on alcohol and health 2018) [116].

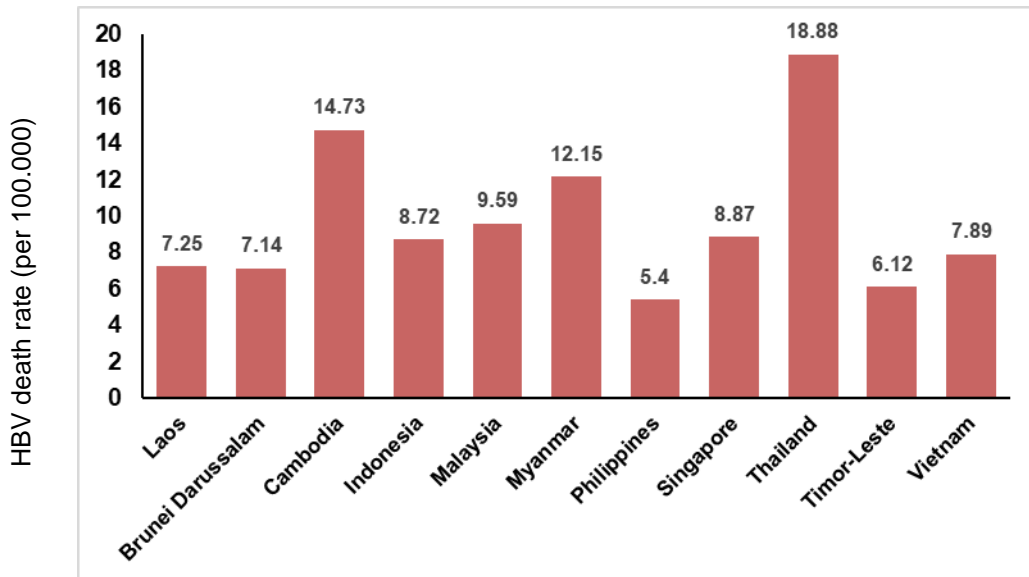


Figure 19: Hepatitis B related death rate (per 100.000) in 2019 according to the Coalition for Global hepatitis elimination in 2019.

2. Chronic Hepatitis B prevalence in different conditions in Southeast Asia.

Due to the cost and the complexity of the study design, unbiased determination of HBsAg seroprevalence in a given population is rarely estimated. As a consequence, the published seroprevalences are more or less realistic estimates of the national situations. These studies necessitate various procedures of adjustment to get closer to the epidemiological reality. Traditionally, selected groups, considered as good indicators of the prevalence or of the circulation of HBV in a given population are investigated: “general population”, blood donors, attending mothers, healthcare workers, HIV-infected patients, and patients affected with severe liver diseases (liver cirrhosis and hepatocellular carcinoma). Occasionally, additional groups such as prostitutes, male homosexuals, and intravenous drug users are investigated. However, these groups are rarely studied in Southeast Asia and will not be included in the present manuscript.

2.1. Chronic Hepatitis B prevalence in segments of the general population

In this section, we shall examine what is designated under the term “general population” by the authors albeit these studies usually target only a small segment of the true general population. Furthermore, these studies are mostly single-center surveys focusing on a given region/city providing inherently only a partial view of the seroprevalence of chronic hepatitis B in the country.

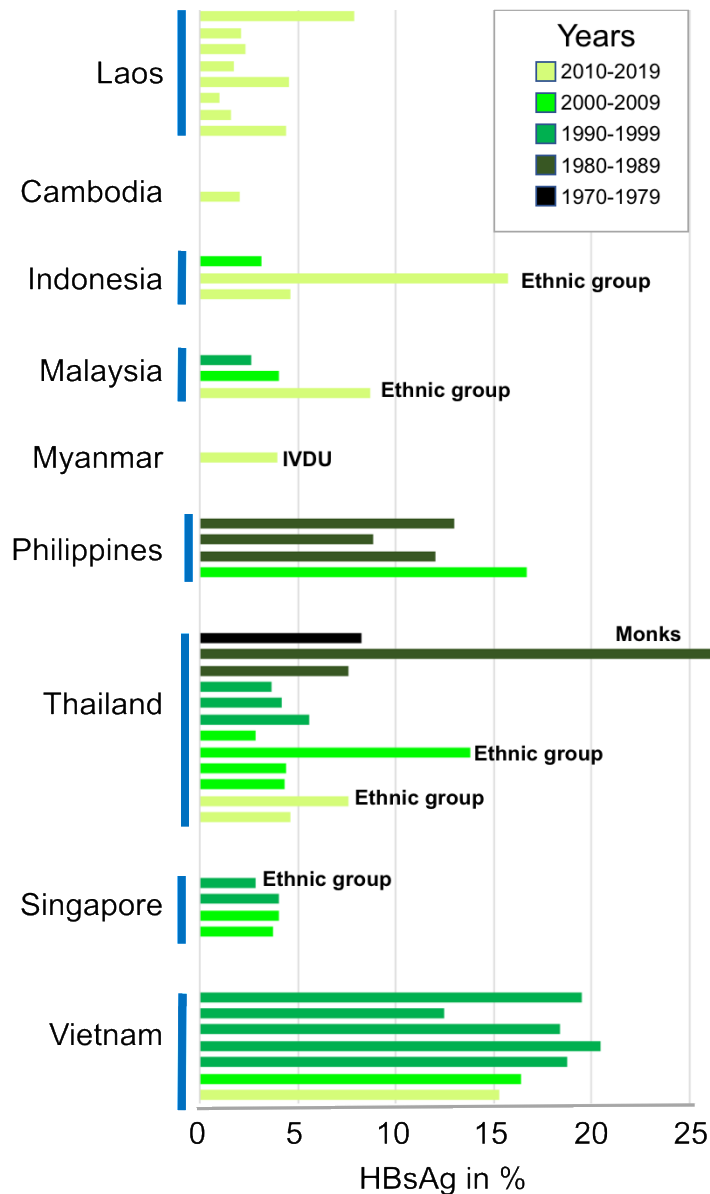


Figure 20: Seroprevalence of HBsAg in general populations of nine Southeast Asian countries [11], [117]–[149].

No studies were found for Brunei-Darussalam and Timor-Leste. Additional indication (Monks, Ethnic Group, IVDU for intra-venous drug users) indicate when the population is not “really” general.

Several remarkable features can be noticed from Figure 20. Within a given country, the seroprevalence of HBsAg tends to be rather stable or to slightly decrease with time. The seroprevalence tends to be higher for specific ethnic groups in each country (except for the Gurkhas from Singapore originating from Nepal ie a different epidemiological context). Surveys conducted in Laos and Cambodia are all recent (2010’s decade), a situation that

might explain why the observed seroprevalence is low. In addition, in both countries all surveys were performed on participants less than 19 years of age, thereby reinforcing the impression of low seroprevalence as universal immunization was implemented in both countries at the beginning of the XXIst century (2001 and 2005).

Vietnam and the Philippines show the worst situation in the region. It should be noted that the Vietnamese studies were all conducted in rural areas, a plausible reason for the observed very high HBsAg prevalence. It is known that the implementation of infection control activities in rural settings is less than in urban centers. Thus, most authors agree that to improve HBV control in Vietnam, universal HBV infant vaccination is required and its implementation in countryside areas will be key to its success [118]–[120]. The HBsAg prevalence among adults in the Philippines was high for reasons that still remain unclear. Universal infant HBV vaccination was first introduced into the national immunization program as early as 1992. However, because of insufficient resources, this program was not fully implemented until January 2007 but no large survey has been conducted after this date [133].

2.2. Hepatitis B prevalence in pregnancy

Pregnant women are usually considered a healthy component of populations. Unfortunately, given the higher susceptibility of males to chronic hepatitis B infection, studies tend to systematically underestimate HBsAg prevalence in young adult women who are likely to become mothers. Despite the importance of women's health in Southeast Asian nations the number of surveys dedicated to pregnant mothers is rather low, with seven of the eleven SE Asian countries having only published once on this topic (see Figure 21). Thus, it is rather difficult to develop an accurate picture of HBsAg prevalence in this segment of the population. The three countries of former French Indochina provide the bulk of data about pregnancy and HBV prevalence. In countries that published surveys conducted in different decades (Laos, Indonesia and Vietnam), a noticeable lack of decreasing seroprevalence is conspicuous probably because most participating mothers were born before the implementation of universal immunization programs (in 2001, 1997, and 1997 respectively). As for surveys of the general population described above, Vietnam (8.0-10.0%) and the Philippines (7.5%) exhibit the worst situation (albeit the survey from the Philippines is very old, 1980's). There is also concern for chronically HBV-infected mothers in Laos, where substantial variation in HBsAg prevalence in pregnancy (range 2.9-8.2%) has been

observed. Countries located in insular Southeast Asia, i.e. Indonesia, Malaysia, Brunei-Darussalam, and Timor-Leste, appeared as relatively spared by the problem.

The routine vaccination program for Lao infants still needs to be strengthened. A recent study among HBsAg-positive pregnant women showed 40% of them were HBeAg positive and the rate of mother to child transmission of HBV was high (53%) among those born from HBeAg positive mothers. This worrying situation is linked to the high rate of adolescent pregnancies in Laos. With a rate of 83/1000, the country is the most affected by this social phenomenon. These young women are more likely to be in the first phase of chronic infection with HBV, the immune tolerant phase, characterized by the presence of high loads of an unmutated and highly replicative virus [ref. UNPFA]. Furthermore, only 61% of infants born from HBsAg-positive mothers acquired sero-protection against infection by HBV (Anti-HBs positive) [150]. Factors associated with HBsAg(+) children were maternal infection and birth outside a healthcare facility [146] [148].

Recent studies conducted on mother-child pairs in Haiphong, Vietnam, showed a high HBsAg prevalence in mothers (10.6%) and consequently a very high mother-to-child transmission (8%). An important subset (23.0%) of the mothers were HBeAg(+) [151]. In Thai Nguyen province (Northern Vietnam), HBsAg prevalence in mothers was 7.8% and 40.3% of them were HBeAg positive. Consequently, 14% of their infants became HBsAg(+), most of them (85%) born from HBeAg(+) mothers. As in Laos, only 62.4% of infants enrolled in this survey subsequently developed anti-HBs >10 mIU/mL [152]. Confronted with such disappointing results, some authors propose that to decrease transmission an improved national plan including increased access to preventive measures should be implemented and that a tight monitoring of its outcome is necessary [151]. In contrast, the HBsAg prevalence among mothers in most other countries was lower (Figure 21). In Cambodia, HBsAg prevalence in mothers was (4.7%-5.7%)[153], [154] but mother-to-child transmission (MTCT) remained high (9.5%). Causes for this relative failure are unclear but according to the authors, it suggests that the strategy of prevention, control, and surveillance of mother-to-child transmission of HBV should be improved [154].

Overall, and despite an imperfect efficiency, we see that regardless of prevailing circumstances immunization programs help to reduce HBsAg carriage of more than one Log₁₀ in the region. Thus, there are more reasons to be optimistic than pessimistic concerning MTCT in Southeast Asia.

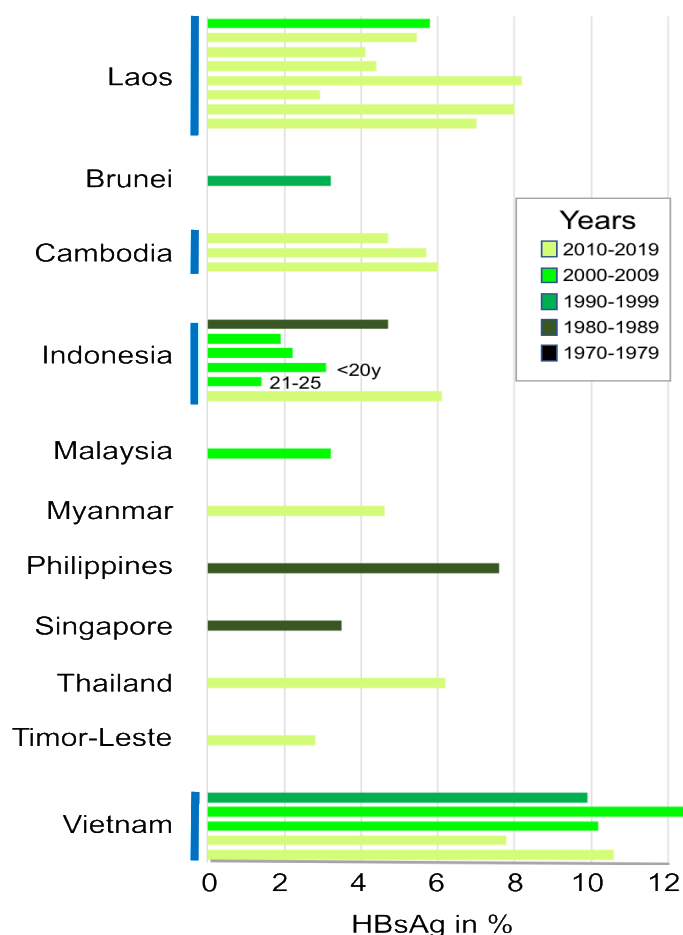


Figure 21: Seroprevalence of HBsAg in pregnant mothers of 11 Southeast Asian countries [145]–[167] .

2.3. Chronic Hepatitis B virus infection prevalence in blood donors

Blood donors traditionally represent one of the most important sources of information concerning HBsAg prevalence in a population. Peculiarities intrinsic to each national transfusion system are, however, susceptible to bias measured prevalence, as when donors are remunerated or if replacement donations for relative or friends are commonly practiced. These circumstances lead to a greater prevalence of HBsAg in donations than the general population. Consequently, all Southeast Asian countries have implemented policies to recruit only non-remunerated, voluntary blood donors (Table 7) and important progresses in this direction has been made in the last two decades. Indeed, remunerated blood donations no longer exist in Southeast Asia. However, in 2013 WHO reported that replacement donations in some of the Southeast Asian countries remained significant. In addition, throughout the region men, who have higher rates of chronic hepatitis B infection, donate blood at higher rates than women.

Countries	%
Laos	>90
Brunei	>99
Cambodia	>25
Indonesia	>50
Malaysia	>99
Myanmar	>50
Philippines	>50
Singapore	>50
Thailand	>99
Timor-Leste	>50
Viet Nam	>90

Table 7: Proportion of voluntary non-remunerated donations by country, 2013.

(Global Status Report on blood safety and availability, 2016, WHO)

Thus, measurement of HBsAg prevalence in blood donors in certain countries of Southeast Asian countries likely overestimate the real seroprevalence in age-equivalent segments of the populations.

All 11 Southeast Asian countries have surveyed HBsAg prevalence in blood donors (Figure 22), however most of the studies are from Laos, Thailand, and Vietnam. Furthermore, Brunei, Cambodia, Indonesia, Thailand, and Timor-leste have not conducted a blood donor survey in the last decade. It is thus difficult to have an accurate appraisal of the current situation and or its evolution with time.

Based of the most recent studies, it appears that the seroprevalence of HBsAg in blood donors ranges between 5.6 and 13.1% in Laos, and between 9.0% and 18.1% in Vietnam, the countries that are most affected by infections in blood donors. By contrast, prevalences in Malaysia, Myanmar, and Philippines range from 1.0 to 5.0%, despite the persistence of replacement donations in the latter two countries.

Vietnamese investigators propose to prioritize HBV screening of blood donors, along with more extensive vaccination, as a strategy to limit future cases of serious liver disease in the country [168]. In addition, if affordable, nucleic acid testing (NAT) for HBV should be seriously considered for blood donation screening in Vietnam to improve safe transfusion and reduce the threat represented by OBI [169]. In Malaysia, the prevalence OBI (HBV DNA+) among blood donors was 5.5% with 100% of these samples positive for Anti-HBc. The authors suggest the reliability of anti-HBc as a primary screening tool for occult hepatitis

B infection. They insist that this approach could be implemented in Low-and-Middle Incomes countries as an alternative to NAT, which may be prohibitively expensive, or in low endemic settings (due to the unsustainable rate of blood donation rejection in highly endemic countries where anti-HBc prevalence is high) [170]. The problem of anti-HBc prevalence and of the consequence of the rejection of anti-HBc(+) blood donations is well illustrated in Cambodia. In rural areas of Cambodia, the prevalence of HBsAg(-)/ anti-HBc(+) blood donations is around 50.0%. While the, actual estimates of OBI prevalence remain unknown, due to the lack of NAT, it is already clear that rejection of anti-HBc(+) donations would be unsustainable for the country [171]. In Laos, there are currently no serious discussions about a potentially high OBI rate among blood donors. The cost-benefit analysis of NAT in a resource-limited country such as Laos will require further careful evaluation [12], [172], [173].

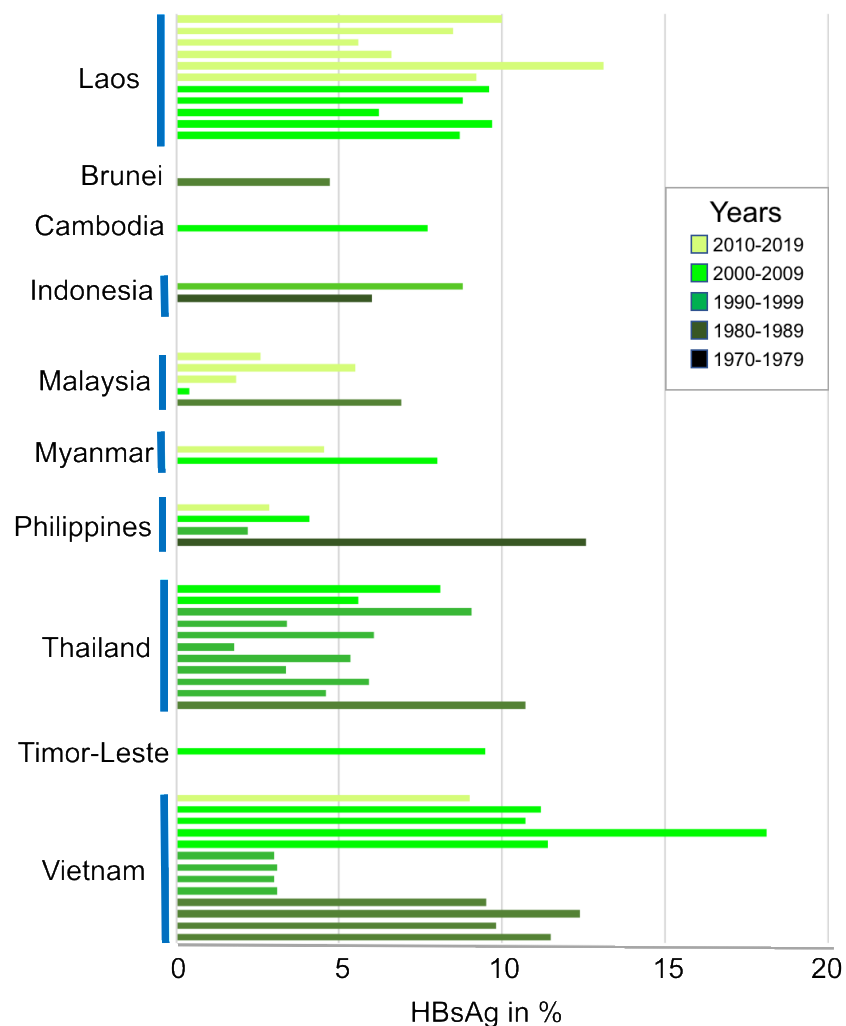


Figure 22: Seroprevalence of HBsAg in blood donors of 10 Southeast Asian countries [12], [168]–[195].

2.4. Prevalence of chronic Hepatitis B infection among health care workers

Healthcare workers (HCW) are inherently more exposed to HBV than the general population as evidenced by their higher rates for positivity for serological markers. A recent meta-analysis demonstrated that the global community of HCW is affected by an intermediate level (2.0-8.0%) of chronic infection [196]. Additionally, infected HCWs risk to transmit the virus to their patients.

HBV infections of HCW has not draw much attention in the region. Only seven Southeast Asian countries conducted a survey of HBsAg prevalence in HCW (Figure 23). Some of these studies are very old, especially in insular countries, making difficult the comparison with more recent estimates from Indonesia and Laos. Estimates obtained from 2010-2019 reported HBsAg seroprevalence in HCW was high, ranging between 3.8% and 7.1%. Although these estimates are lower than the 15.0% reported in the Philippines in the 1980's or 11.5% in Vietnam in the 1990's. Unfortunately, follow-up studies have been discontinued in most countries, preventing any educated conclusions about the evolution on chronic infection with HBV among HCWs.

In Laos and Indonesia the prevalence of HBsAg in HCW is high and the serological protection is low (43.0% in Indonesia but as low as 13.6% in Laos). Thus, there is an urgent need for a targeted HCW vaccination policy [197], [198], [199], [200]. A similar situation was observed historically in the Philippines, which had a very high HBsAg prevalence in HCWs. HBsAg screening and anti-HBV immunization were requested for HCW [201], [202]. Unfortunately, there has been a lack of recent studies to assess the evolution of this worrying situation. Likewise, there is no evidence currently available concerning the situation of HCWs in Singapore. A single survey from 1988 was found and it mentioned the requirements for screening and vaccination of all categories of dental personnel [203]. However, we expect that the situation has largely improved in the city-state simply because it has the second largest GDP per capita in the world and the second greatest healthcare expenditure per capita in Asia after Japan (<https://apps.who.int/nha/database/Select/Indicators/en>).

Concern for HCW remains in the region. In medical students from Thailand, those vaccinated during childhood had a 2.2 fold greater risk of remaining anti-HBs(-) following re-boosting by an anti-HepB vaccine than medical students without previous vaccination (OR = 2.2, 95%CI = 1.4-3.5). Thus, it seems that following the waning of the initial anti-HepB Ig

response, it can be more challenging to develop a protective immunity following a re-boosting immunization [204]. This observation asks many questions about the long-term efficiency of anti-HepB immunization in populations of HCW living in highly endemic countries and who are frequently exposed to the virus.

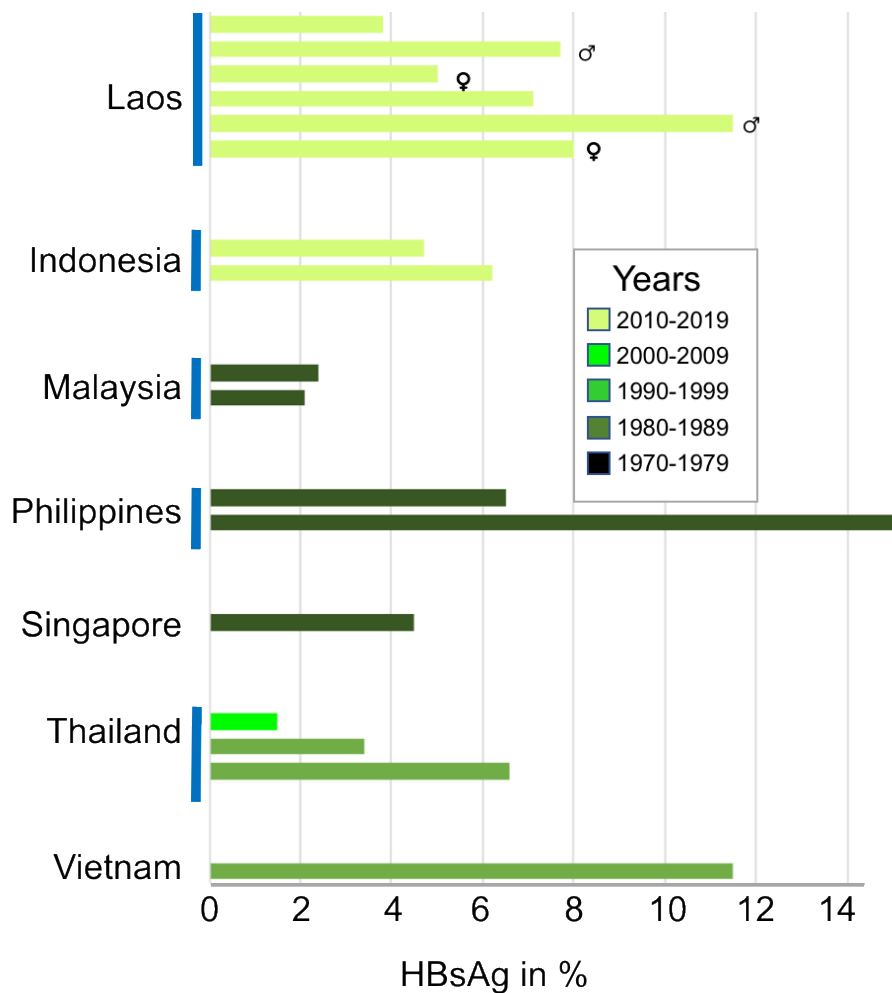


Figure 23: Seroprevalence of HBsAg in healthcare workers of seven Southeast Asian countries[197], [198], [201]–[210].

2.5. HBsAg prevalence among HIV(+) patients

HBV-HIV coinfection is of special importance. These patients will experience a quicker progression of their liver disease and higher liver-related morbidity and mortality despite an apparently milder disease, notably characterized by a reduced increase of circulating liver enzyme, hallmark of tissue injury [211]. Another consequence of co-infection with HIV

concerns the transition to the inactive carrier state i.e. the third phase of the disease. This transition is restrained in coinfecting patients with a lower seroconversion to anti-HBe and the frequent maintenance of high viral loads. The immune active phase (2nd step of the chronic infection) is thus prolonged with its inherently deleterious consequence for the liver. For patients who manage to enter in the inactive carrier stage, the deepening of immune suppression by HIV frequently leads to a rapid reactivation of HBV replication corresponding to the fourth and most dangerous phase of the disease. It is estimated that 25% of HIV-HBV coinfecting patients will develop a liver cirrhosis or an HCC, which explains why liver-related mortality is almost 20-fold higher in co-infected than in HBV mono-infected patients.

The prevalence of HBsAg in HIV-infected patients has not been explored in Laos or the two insular countries of Brunei and Timor. Remarkably, while HBV infections are constantly high throughout the region, it is not in the most affected countries (Vietnam, Philippines) where we see the greatest prevalence of HIV-HBV coinfection. Indeed, it is in Malaysia and Indonesia where co-infection rates are highest (13.0-15.0%, see [Figure 24](#)). The reason of this apparent discrepancy is unknown and not adequately discussed in the literature. Additional surveys are needed to verify these preliminary observations. In a country highly endemic for chronic infection with HBV like Vietnam, authors reported that rates of coinfection with HBV among HIV-infected Persons Who Inject Drugs (PWID) and Female Sex Workers (FSW) are only marginally above those measured in the general population (8.0-10.0%). Therefore, HIV prevention programs are not designed to prevent additional contamination with HBV. This observation is likely because HBV is primarily transmitted during infancy and early childhood in Vietnam while HIV is mostly transmitted at later ages [192]. In Thailand, after 25 years of universal anti-HepB vaccination, 5–10% of newly diagnosed HIV patients present with an HBV coinfection. National guidelines specify the routine screening of hepatitis B and C in HIV-infected patients and populations at increased risk of HIV, including Men who have Sex with Men (MSM) and Transgender Women (TGW), who represent sizeable segments of the local population [212].

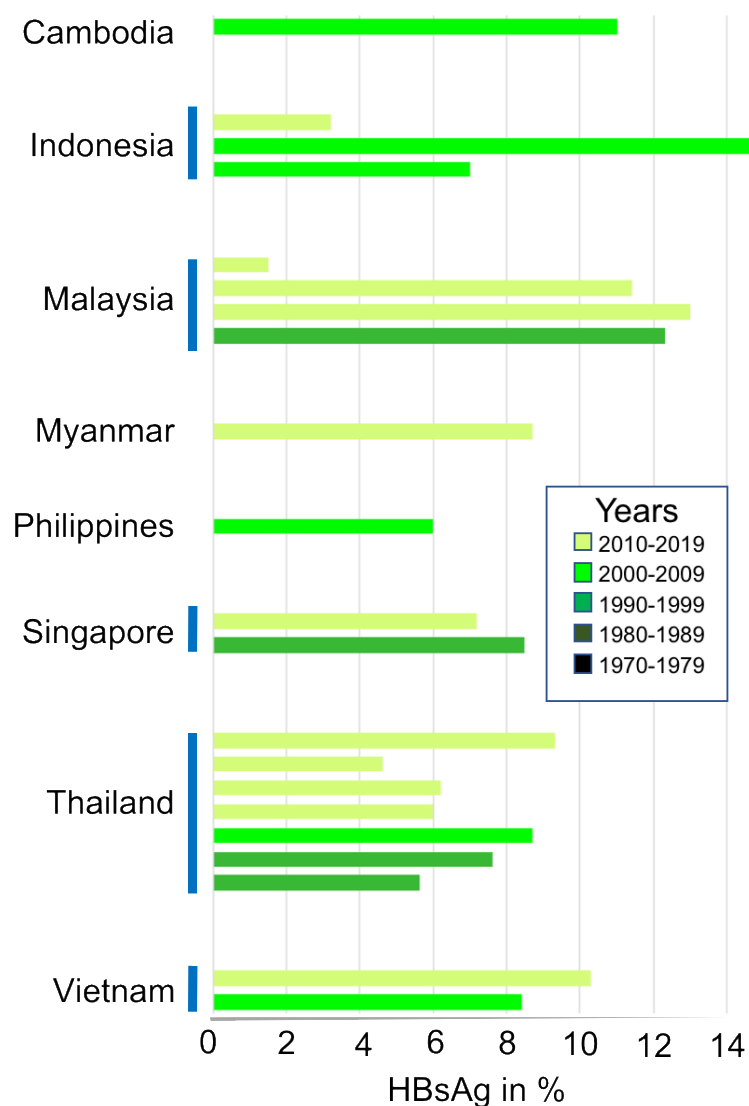


Figure 24: Seroprevalence of HBsAg in HIV-infected patients of 8 Southeast Asian countries [199], [200], [212]–[222].

3. National strategies for Hepatitis control in Southeast-Asia

3.1. History of vaccination

Immunization as a strategy to prevent disease dates back several hundred years. In 17th century China Buddhist monks would drink snake venom to confer immunity to snake bite and used variolation (inhaling small skin squams from a patient with smallpox) to confer immunity. In the west, the wider acceptance and use of the practice followed the 1796 discovery by Edward Jenner that immunity to smallpox was conferred to milkmaids who

were apparently naturally immunized against smallpox through their occupational contact with cowpox (and thus the name 'vaccination' from the Latin word 'vacca' for cow [223]).

The first generation of inactivated-virus vaccine against hepatitis B was developed in 1976 by Professor Maupas in Tours. Initially based on the purification of HBsAg from the blood of chronically HBV-infected patients, it was widely used before the creation of recombinant vaccines. Safe and effective hepatitis B vaccines have been commercially available since 1982 [224]. Hepatitis B vaccine was included in the Expanded Programme on Immunization (EPI) since 1992. The World Health Organization (WHO) recommended universal immunization of infants, with three doses of hepatitis B vaccine at 6 weeks, 10 weeks and 14 weeks, following a first dose given as soon as possible after birth (within 24 hours) [225]. Focusing on the South-East Asian Region, in 2018 the coverage of the third dose of hepatitis B vaccine was high (89%) and the coverage of the birth dose was intermediate (48%). In the Western Pacific Region, the coverage of the birth dose and third dose of hepatitis B vaccine was high overall (83% and 90%, respectively). The WHO objective for 2030 is to increase vaccination coverage to 90% to eliminate hepatitis B [67], [226]

3.2. Current immunization strategies adopted in Southeast Asian countries

All 11 countries in Southeast Asia adopted the policy of Hepatitis B immunization advocated by the WHO. The initial goal was to reduce chronic hepatitis B infection prevalence among children to less than 2% by 2012 and less than 1% by 2017. In 2030, the key impact is reducing new chronic infection by 90%, which is **equivalent** to reducing HBsAg prevalence among children < 5 years to 0.1% [227]–[229].

The vaccination schedule depends on the epidemiology of each region. In areas with high HBV transmission at birth, recommendations include HepB-BD within 24 hours of birth, followed by three additional doses at 6, 10 and 14 weeks. In settings with low transmission at birth, recommendations include HepB-BD within 24 hours, followed by two additional doses at 6 and 14 weeks [225]. Laos, Cambodia, the Philippines and Timor-Leste have all adopted the schedule corresponding to a high-prevalence of maternal infection (0, 6, 10 and 14 weeks). Details for the other countries are shown in [Table 8](#).

Table 8: Current hepatitis B vaccine schedule in Southeast Asian countries [230], [231].

Weeks of injections	0	4	6	8	10	12	14	16	20-24	24	72
Laos	✓		✓		✓		✓				
Brunei Darussalam	✓			✓				✓		✓	
Cambodia	✓		✓		✓		✓				
Indonesia	✓			✓		✓		✓			✓
Malaysia	✓	✓								✓	
Myanmar				✓				✓		✓	
Philippines	✓		✓		✓		✓				
Singapore	✓	✓							✓		
Thailand	✓			✓				✓		✓	
Timor-Leste	✓		✓		✓		✓				
Vietnam	✓			✓		✓		✓			

Universal Hepatitis B vaccination in Southeast Asian countries was introduced from 1985 to 2008. Singapore was the first to introduce HepB3 vaccine in 1985, follow by Brunei Darussalam and Malaysia in 1988 and 1989 respectively. Philippines, Thailand, Vietnam and Indonesia introduced HepB3 during the 1990s, while the remaining countries of Laos, Myanmar, Cambodia and Timor-Leste introduced it during the 2000s. In seven countries in this region (Brunei Darussalam, Malaysia, Thailand, Indonesia, Myanmar, Cambodia and Timor-Leste) the HepB-BD was introduced at the same. The HepB-BD was introduced between two to 16 years later in Singapore, Laos, Vietnam and Philippines (detail in [Figure 25](#)) [230]–[232].

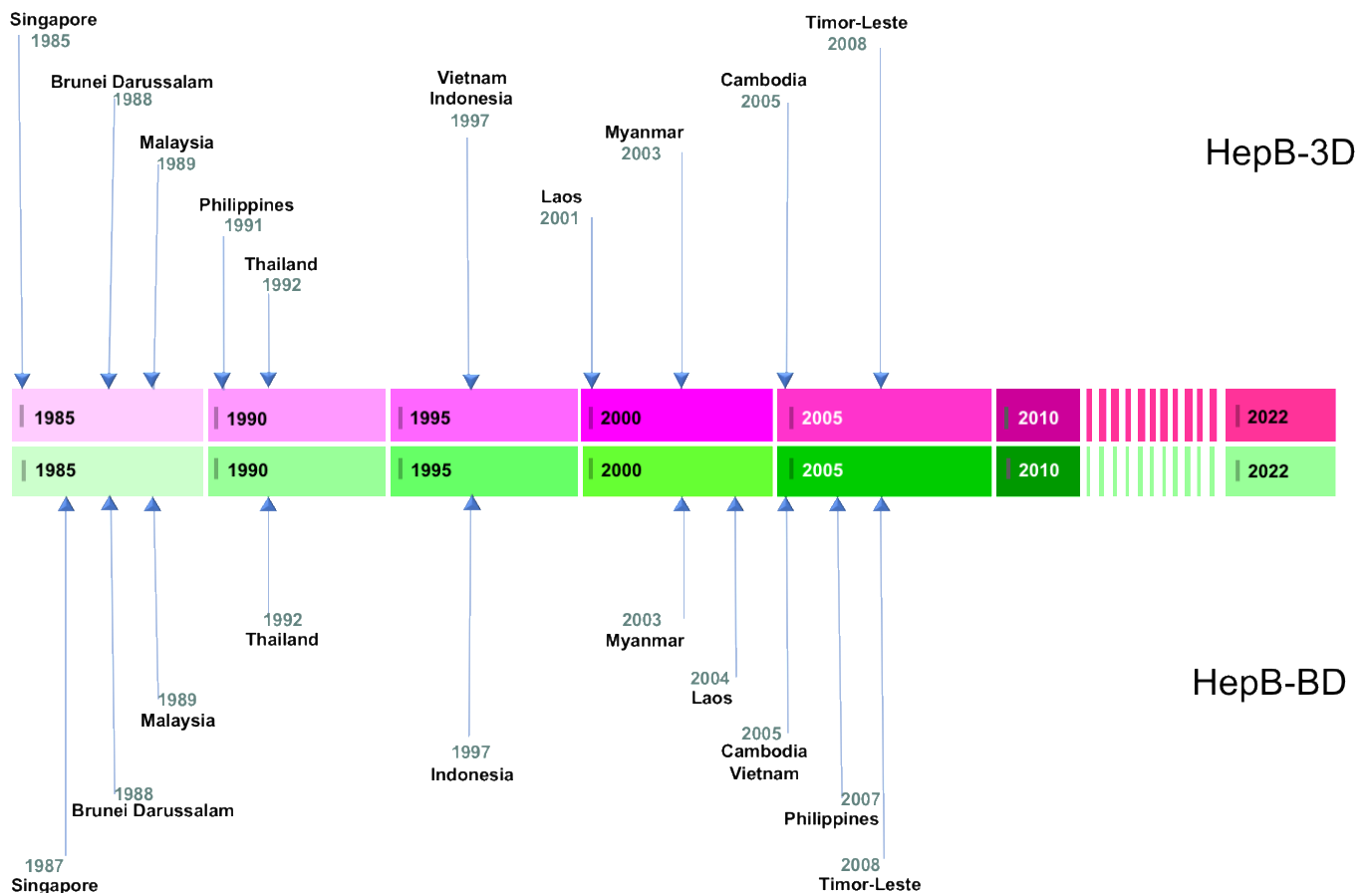


Figure 25: HepB-3 and HepB-BD introduced nationwide in Southeast Asia

We will now examine anti-HepB immunizations policies implemented in each Southeast Asian country, according to their order of introduction in medical practice.

Singapore. Overall, literature about the Singapore’s immunization strategy, including its outcome, is scarce, suggesting that Singaporean authorities currently consider the issue of Hepatitis B as residual. Nevertheless, Singapore is a lower intermediate (2.0-4.9%) hepatitis B endemic country (Global hepatitis elimination in 2019) of more than 5 million habitants (worldometers/world-population, 2020). A national childhood HBV immunization program was implemented on 1st October 1985 on a voluntary basis. It was extended to all newborns on September 1st, 1987 [233], [234]. Hepatitis B vaccine in Singapore is billed and paid through Medisave (MediSave is a national medical savings scheme that helps individuals set aside part of their income to pay for their personal healthcare. (Ministry of health Singapore, www.moh.gov.sg)), permanent residents are charged 50% and foreigners pay for full costs [235].

Beside anti-HepB vaccination for those born since 1985, Hepatitis B vaccine is

recommended for all Singapore residents if:

- Birth in intermediate and high endemic areas
- Young adults
- Healthcare workers
- Pregnant women
- Contacts of subjects with chronic hepatitis B virus infection (family, household and sexual contacts)
- Persons with multiple sexual partners/history of sexually transmitted diseases
- Individuals with chronically elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST)
- Individuals infected with hepatitis C virus or HIV
- Men who have sex with men
- Subjects with high-risk behaviours (IV drug users, sex workers) [236]

Brunei Darussalam is a lower intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 437,483 habitants (worldometers/world-population, 2020). We did not find any publication about the Brunei Hepatitis B national immunization strategy. However, based on a 2020 WHO report, an HBV vaccination program was implemented in 1988 for the BD and three dose vaccination schedule [231].

Malaysia is a low hepatitis B endemic country (Global hepatitis elimination in 2019), with 32,365,998 habitants (worldometers/world-population, 2020). The hepatitis B vaccination program for all neonates was implemented free of charge in Malaysia in 1989 [237]–[239]. The vaccine is also recommended for all adults in Malaysia, especially in high-risk groups, such as healthcare workers in public clinics and hospitals, but is not free of charge [238]. There used to be nationwide campaigns aiming to promote blood testing and access to hepatitis B vaccination under the auspice of a non-governmental organization (Malaysian Liver Foundation, MLF) with the aid of local physicians. Together, they also provided health information to the public. The ministry of health also supports hepatitis research and provides a subspecialty in hepatobiliary in tertiary care hospitals [240].

Philippines is a high hepatitis B endemic country (Global hepatitis elimination in 2019), with around 110 million habitants (worldometers/world-population, 2020). The hepatitis B vaccination program was initiated with a phased introduction (phasing modalities were not

explicit) into the national immunization program since 1992 [241]. HepB-BD was introduced 15 years later in 2007 [242]. The vaccine is free, together with other basic vaccines in the national program for infants and children under 5 years old [243].

Thailand is a lower intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 69,799,978 habitants (worldometers/world-population, 2020). Their Hepatitis B vaccination program was introduced in 1992 as part of the national expanded program on immunization (EPI) [244]. An earlier vaccination program had been adopted in 1988 and tested in a few provinces. The program was extended to newborns in 10 additional provinces in 1990 [245], [246].

Thailand has an Advisory Committee on Immunization Practice (ACIP) established by the Ministry of Public Health (MoPH) in 1970. Its role is to provide advice leading to the reduction in the incidence of vaccine-preventable diseases. The purview of ACIP includes:

- providing advice and guidance on vaccines and immunization to the MoPH;
- making recommendations on the inclusion of vaccines into the EPI, appropriate immunization schedules and target groups, and methods for administering the vaccines;
- suggesting areas of research related to vaccines and the epidemiology of vaccine preventable diseases, and suggesting possible sources of funding for this research;
- proposing immunization policies to the MoPH and National Vaccine Committee (NVC); and appointing working groups to study specific issues.

ACIP members do not have fixed terms. While there is no formal review process, all members are appointed, and nominees are proposed by the Secretariat to the full Committee for approval. Final approval is given by the Minister of Public Health [247]

Indonesia is a lower intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 273,523,615 habitants (worldometers/world-population, 2020). The anti-hepatitis B vaccination for all newborns was integrated into the National Immunization Program in 1997 [248], [249] .

Vietnam is a higher intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 97,338,583 habitants (worldometers/world-population, 2020). Anti-Hepatitis B vaccination in Viet Nam was tested in 1997 and included into the national EPI in 2002. The birth dose was implemented in 2005 [250], [251]. Since 2010, the hepatitis B vaccine is

given as a part of a pentavalent DTP-Hib-Hepatitis B vaccine schedule at ages 2, 3, and 4 months [250], [252]. Anti-Hepatitis B immunoglobulins (anti-HBV IG) at birth are not routinely recommended under the current strategy in Vietnam [253].

Myanmar is a lower intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 54,409,800 habitants (worldometers/world-population, 2020). Myanmar decided, in line with the WHO's global hepatitis strategy, to take actions to prevent, diagnose and treat viral hepatitis. To this aim, a set of comprehensive strategic directions defined in 2015 were followed [254]:

- 1: Prevention of Transmission of Viral Hepatitis
- 2: Diagnosis, Clinical Care and Treatment of Viral Hepatitis
- 3: Workforce Development to combat viral hepatitis
- 4: Surveillance, Research, and Strategic Information about Viral Hepatitis

Concerning Interventions strategy, several measures were implemented:

- 1) Advocacy, Education and Communication for public awareness;
- 2) Prevention of mother-to-child transmission of hepatitis B and hepatitis B birth dose;
- 3) Hepatitis B vaccination for high-risk populations and vulnerable adults;
- 4) Infection control precautions and safe injection practices in healthcare settings;
- 5) Harm reduction program for PWID and PWUD;
- 6) Implementation of blood safety strategies;
- 7) *Access to safe food and water (not applicable to HepB);*
- 8) Prevention and control measures for acute viral hepatitis outbreaks and
- 9) Promotion of safe sex through condom program.

The EPI is one of the National Health Projects under the Diseases Control Program in Myanmar. It was implemented in 1978 in 104 townships. The Hepatitis B vaccine program was introduced by EPI in phases from 2003 and covered the whole country by 2005 [255], [256]. The vaccine safety with quality control (QC) trial started in 2005 and completed in 2016. The recombinant vaccine found to be safe and the immunogenicity in newborns and adults was 100%. In the national immunization schedule, hepatitis B vaccine is currently provided as part of the pentavalent vaccine (DTP-Hib-HepB). Other commercial vaccines are imported and are available in the private sector [254].

The 2017-2020 action plan strategy in Myanmar to prevent HBV transmission especially

targets mother-to-child transmission. The birth dose was increased 5% per year (75% in 2017-90% in 2020) and the 3rd dose was increased 2% per year [257].

The HepB-BD vaccination program prioritized expansion in areas of greatest need in a manner that is explicitly pro-poor. With support for the HepB-BD from GAVI Vaccine Alliance, in 2003 Myanmar achieved 33% coverage across the country. Unfortunately, after the end of GAVI support in 2009, the HepB-BD was available only in large hospitals [256]. National coverage of HepB-BD between 2010 and 2014 ranged between 20-25% for hospital-based births [254]. In addition, according to the WHO, national coverage with the HepB-BD was 1% in 2017, 7% in 2018, and 17% in 2019, but population-based estimates of coverage, inequity, or maternal and health system factors associated with vaccination were unknown [258]. In 2016, HepB-BD was thus mainly administered for institutional deliveries in hospitals. Despite a HepB-3D vaccine coverage of 90% in 2019, only a minority (17%) of the population received HepB-BD. Additionally, only 36% of births in Myanmar occur in healthcare facilities, creating an obstacle to the delivery of HepB-BD vaccination [259].

According to the government of Myanmar, the cost of the vaccine is still a barrier in adults. It remains important to increase the availability and access to this vaccine particularly in vulnerable populations. The priority populations for vaccination outreach include:

- Health care providers
- Migrants (internally and internationally)
- Institutionalized population (e.g. prisoners)
- People living with HIV & AIDS
- Patients with chronic liver disease due to causes other than HBV
- Patients with chronic renal disease and patients on renal dialysis
- People who inject drugs & people who use drugs
- Men having sex with men
- Commercial sex workers [254].

The political situation in Myanmar makes the implementation of this strategy difficult.

Cambodia is a higher intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 16,718,971 habitants (worldometers/world-population, 2020). The national immunization program follows the WHO schedule implemented in 1992. According to the WHO, all member states shall introduce hepatitis B vaccine into the Universal Childhood

Hepatitis B vaccination by 1997 [260]. Hepatitis B vaccine in Cambodia was initiated in 2001 and included in the national immunization program in 2005 [261].

Timor-Leste is a lower intermediate hepatitis B endemic country (Global hepatitis elimination in 2019), with 1,318,445 habitants (worldometers/world-population, 2020). The country obtained its independence only 20 years ago (May, 2002) after centuries of Portuguese colonization (until 1975) and annexation by Indonesia (in 1976). Documents concerning anti-hepatitis B strategy adopted in Timor-Leste are non-existing on the internet.

Laos is highly endemic for chronic hepatitis B (Global hepatitis elimination in 2019), with 7,275,560 habitants (worldometers/world-population, 2020). Historically, understanding of the Hepatitis B situation in Laos started from a retrospective review of Indochinese refugees (Laos, Vietnam and Cambodia) from 1975-1981 in the United States of America (USA). At that time, the prevalence of HBV surface antigen (HBsAg) in these populations was uniformly high (>12.0%) [262]. In the following decade, it was recognized that Lao people established in the USA were often members of specific ethnic groups (Hmong, Mien) and had a very high prevalence of HBV (>14.0%) [2]. The worrying situation of hepatitis B in Lao refugees persisted until recently [3]. Taken together, these data indicate that chronic hepatitis B was and still is a serious health problem in many segments of Lao society and perhaps for the entire population.

Viral hepatitis in Laos is a major concern, along with other communicable diseases such as HIV, tuberculosis and malaria [263]. Laos joined the World Health Organization (WHO) Western Pacific Region in 2015, to endorse the Regional Action Plan for Viral Hepatitis in the Western Pacific 2016-2020, with the aim of eliminating hepatitis as a public health threat by 2030. This plan, as part of the Sustainable Development Goal, has benchmarks of stated by Laotian government of a 30% reduction in new cases of chronic viral hepatitis B and C infections by 2020 up to 90% by 2030; and a ten percent reduction in viral hepatitis B and C deaths by 2020, up to 65% by 2030 [264], [265]. The specific strategies against hepatitis B are mentioned in the country's five year health sector development plan. They include:

- HBV vaccination program.
- Improved capacity building and tools for prevention, treatment, and follow up patients with Hepatitis B.
- Strengthen the knowledge of laboratory and monitoring patients with Hepatitis B by conducting supervision visits and onsite training on sample collection and

transportation to Vientiane Capital from Northern, Central and southern parts [266]. The Ministry of Health (MOH) of Laos is committed to improving access to hepatitis treatment, care, and prevention of hepatitis for the people in Laos. For that reason, the Department of Health care and Rehabilitation (DHR), the Ministry of Health, together with National Committee on Hepatitis and HIV/AIDS care and treatment, and partners from many sectors, such as experts from central hospitals, central laboratories, and international experts, have joined to develop the national guidelines for diagnostic, treatment and prevention of Hepatitis B and C infection, in keeping with WHO treatment guidelines and recommendations and the current situation in Lao PDR. These guidelines will be distributed in 2022 to healthcare providers at central, provincial, district levels and to each clinician who manages persons with hepatitis B and/or C viral infections [99].

In Laos, Hepatitis B vaccine was introduced in 2001, and is included in the routine vaccination schedules such as Bacillus Calmette-Guerin (BCG), Diphtheria-Tetanus-Pertussis-Hepatitis B (DTP-HepB), DTP-HepB-Hib (Haemophilus influenzae type B), polio (OPV), and measles (MCV1). Vaccines administered free of charge to all children under one year old in Laos. Hepatitis B is a part of the tetravalent diphtheria-tetanus-pertussis-hepatitis B (DTP-HepB), followed by DTP-HepB-Hib (Haemophilus influenzae type B) vaccine. The national goal is to achieve at least 95% coverage with all vaccines included in the national immunization program by 2025 [8], [231].

Currently, HBV routine screening is performed only in pregnant women during antenatal care. Anti-Hepatitis B immunoglobulins are not available in Laos [267].

4. Hepatitis B vaccine coverage in Southeast Asia

4.1. Hepatitis B Birthdose (HepB-BD) coverage

While representing the most important intervention against the maintenance of chronic hepatitis B as a major health burden in highly endemic countries, universal administration of the HepB-BD remains the most difficult step in combating the disease [268] (see [Figure 26](#)). This situation is primarily due to the intrinsic obstacles faced by most Public Health systems in the region to reach the particularly numerous delivering mothers.

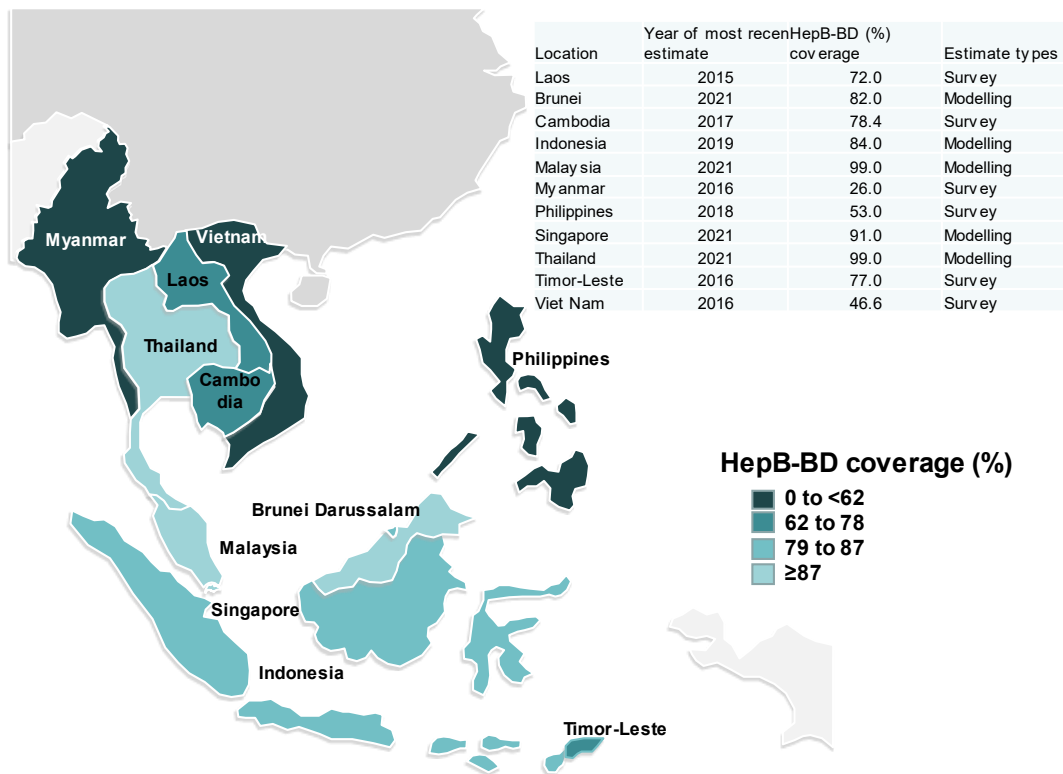


Figure 26: HepB-BD coverage in Southeast Asia. The percentages provided correspond to the last survey published [230], [231], [241], [258], [261], [269]–[271].

Some Southeast Asian countries are vast and fragmented (Indonesia and Philippines count respectively 17,000 and 7,000 islands, respectively). Furthermore, most of these countries are not equipped with a macadamized road network. In addition, in most of the countries, with the notable exception of the Singaporean city state, large subsets of the population are spread across rural communities that are sometimes difficult to reach. This situation partially explains why delivering a birth dose remains challenging in the region.

We can see in Figure 27 that SE Asian countries have gradually improved HepB-BD coverage. Those with a long practice (Brunei, Malaysia, Singapore, Thailand) perform better than countries with a more recent implementation (Laos, Cambodia, Timor-Leste). However, this is not the case in Myanmar, which has been affected by civil unrest for years, and in the Philippines and Vietnam as well, two countries with very different political systems but whose progress seems lower than one would expect (Figure 26).

Other significant correlations can be found. The strongest correlation concerns the improved BD coverage with the percentage of births attended by a qualified healthcare personal (see Figure 27). As expected, HepB-BD coverage declines with an increasingly rural population, and increases alongside increasing GDP per capita. For all of these correlations, Myanmar,

Vietnam, and Philippines behaved as outliers.

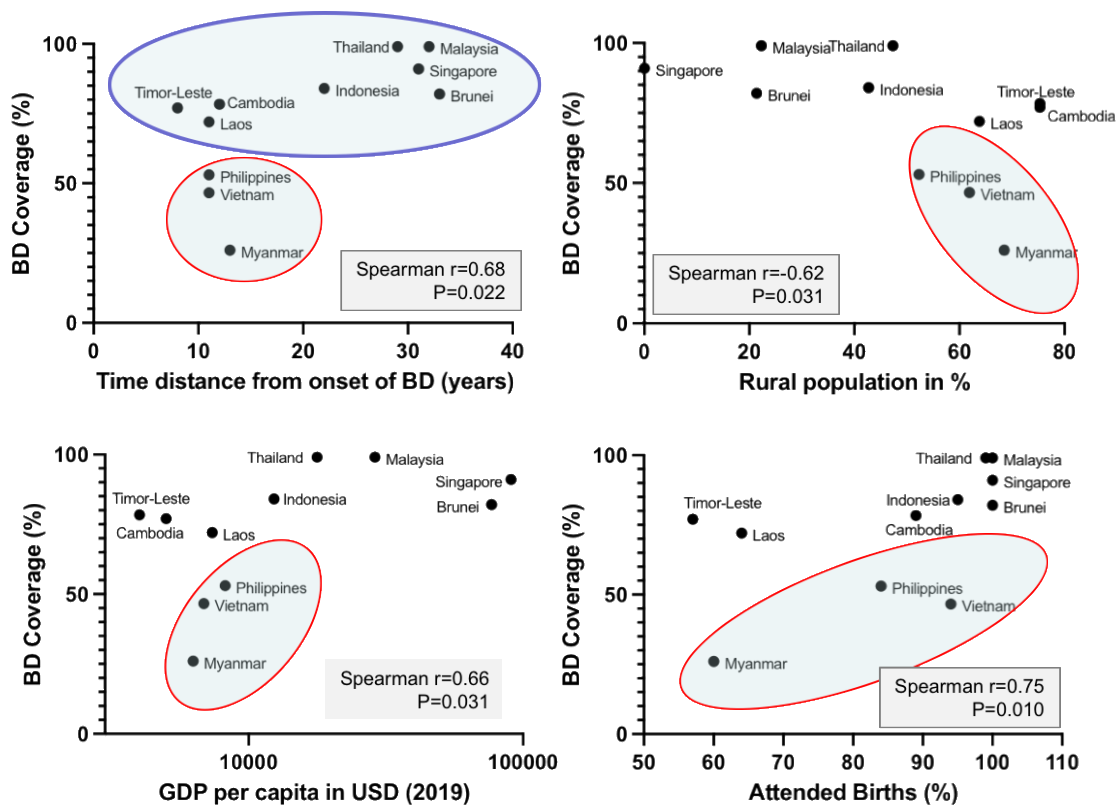


Figure 27: Correlation of anti-Hepatitis B Birth-Dose coverage and various demographic and economic parameters.

Data: https://www.theglobaleconomy.com/rankings/rural_population_percent/, <https://www.unfpa.org/>, <https://ourworldindata.org/>, <https://www.imf.org/>.

In contrast to some reports from the region, we did not find any correlation with the mean education levels in Southeast Asian country (mean number of schooling years, proportion of the population that received a secondary education) [270] and BD coverage. Interestingly, previous authors have not attempted to present general explanations capable of describing the heterogeneity in BD coverage mentioned above.

4.2. Anti-Hepatitis B 3-dose (HepB-3) Coverage

In Southeast Asia, anti-HepB-3 coverage tends to be slightly, albeit non-significantly, higher (median=87% vs 78%) than the BD. This situation is expected as anti-HepB-3 doses are administered in local health centers where mothers are commonly bringing their babies. However, B-3 has been notoriously associated with low compliance, a situation that in some circumstances leads to a reduction in immunization program efficiency [272]. In addition,

reports from sub-Saharan Africa have shown that theoretical coverage provided by personal immunization cards of children were only poorly correlated with true levels of sero-protection [273].

As for the BD, the Philippines appears as one of the less performant nations although Timor-Leste is apparently doing even worse concerning this anti-hepatitis B immunization step. Laos is also positioned in the lower quartile of B-3 administration (see Figure 28).

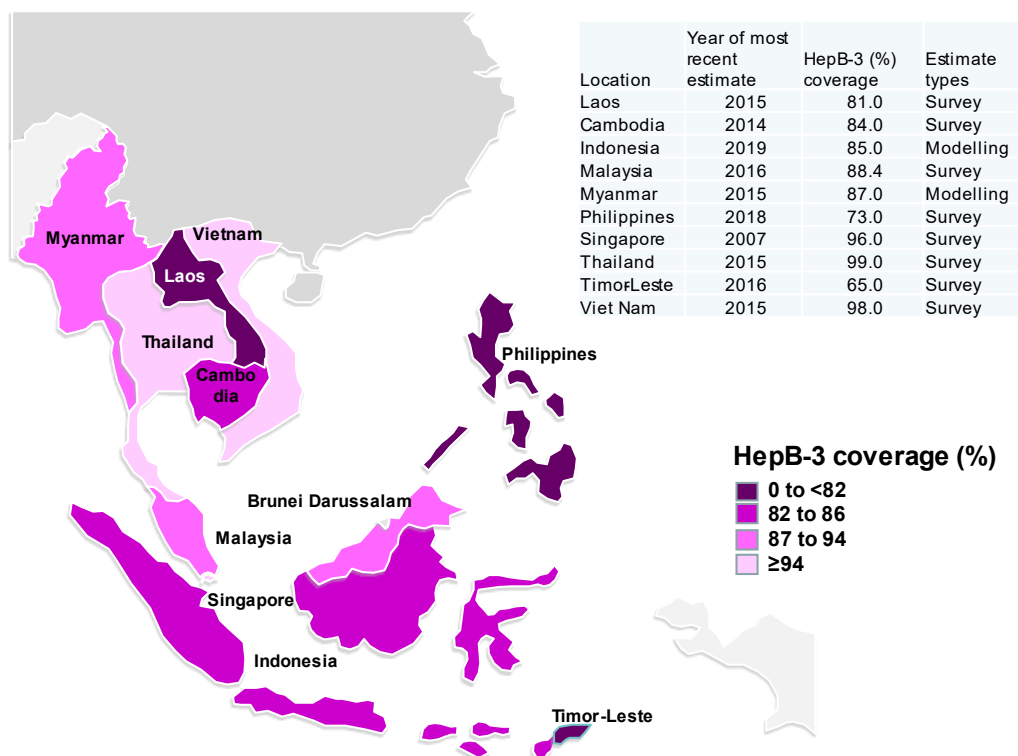


Figure 28: HepB-3 coverage in Southeast Asia [230]–[232], [235], [241], [274]

Overall, when we examined the potential reasons explaining the observed differences in B-3 coverage, the same variables as for the BD (time distance in years since the onset of the universal immunization campaign, rural population, GDP per capita, and of course birth percentage attended by a skilled health worker) were found significantly associated as well (see Figure 29).

However some additional variables, were sometimes more closely related to anti-HepB-3 vaccination rates in Southeastern Asian countries. For example, as the proportion of the population <15 increased (see Figure 28) B-3 coverage decreased, suggesting that in presence of a large annual birth cohort, the system present signs of overload that may explain why more infants are not properly immunized.

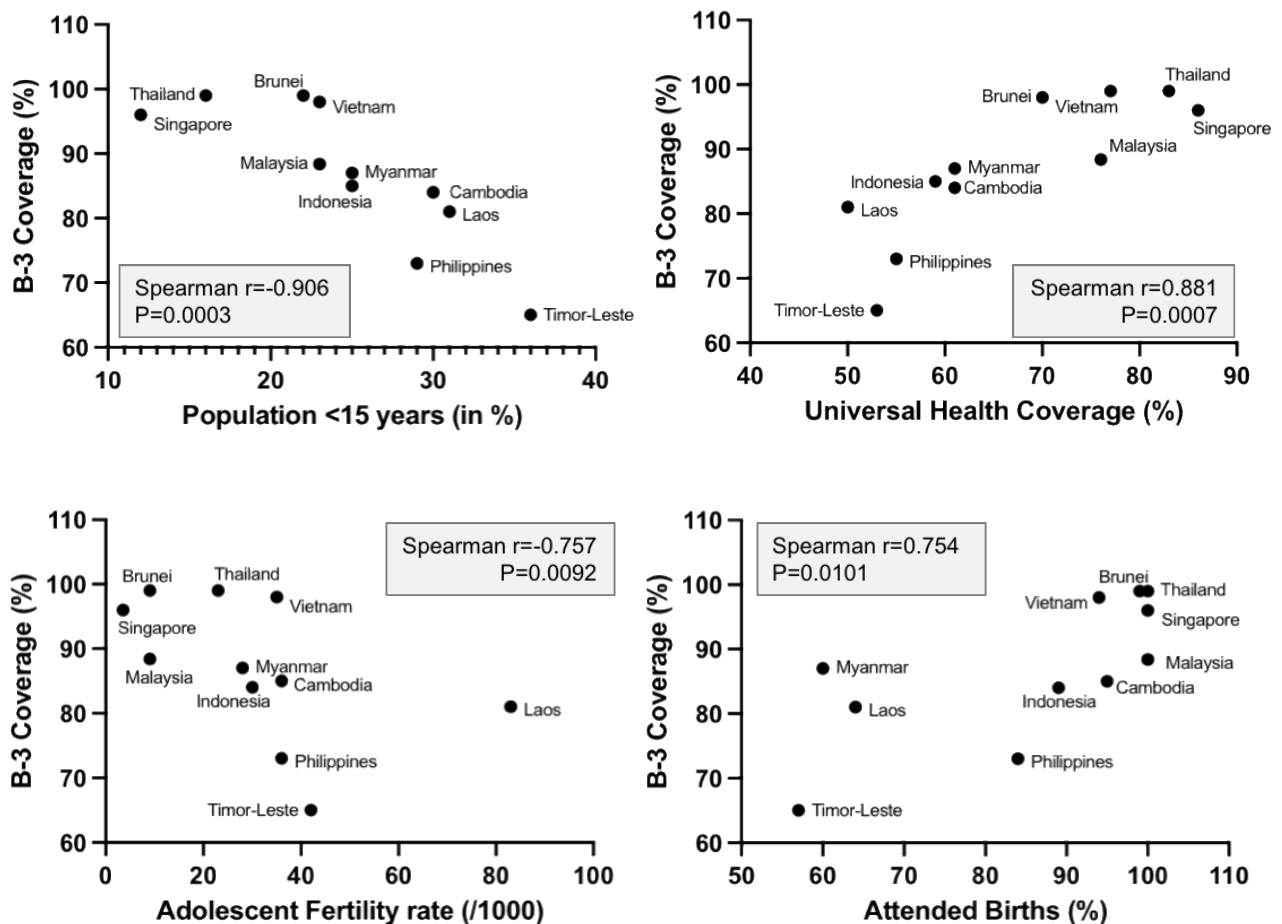


Figure 29: Correlation of anti-Hepatitis B-3 coverage and various demographic and/or economic parameters. Ref as in Figure 27.

B-3 coverage also decreased with increasing adolescent fertility. This could possibly reflect the lower levels of education and possible additional difficulties in the daily lives of very young mothers [275]. Finally, the Universal Health Coverage (UHC) as defined by the WHO (i.e. access to needed health services, when and where needed, without financial hardship. UHC includes the full range of essential health services, from health promotion to prevention, treatment, rehabilitation, and palliative care) was also in tightly correlated with B-3 coverage (Figure 28). In this regard, the positive impact of public healthcare facilities on B-3 immunization in the poor countries of the region has already been highlighted [258].

5. Current seroprevalence after universal Anti-Hepatitis B vaccine introduction in Southeast Asian Countries

The variation in the prevalence of HBsAg in children <5 after universal anti-hepB immunization implementation appears more difficult to explain than that of the two previous variables (BD, B-3). The situations in Southeast Asian countries relative to the initial WHO aim to contain HBsAg prevalence under 1.0% are highly variable. In addition, the observed substantial variation (see Figure 30) of HBsAg prevalence from one study to another in such socially and geographically heterogeneous countries can (see Figure 31) make the reality rather difficult to grasp.

A prudent interpretation of the situation, would be that only four countries can reasonably be considered to have met the WHO objectives: Brunei [231], Malaysia [231], Philippines [241], and Singapore [276]. Four other countries (Indonesia [249], Vietnam [250], Timor-Leste [230], and Myanmar [230]) have failed according to all available evidence. Finally, for Laos[146], Cambodia[154], and Thailand [277], the situation is less clear as prevalence estimates fluctuate around the targeted 1.0% threshold (Figure 31).

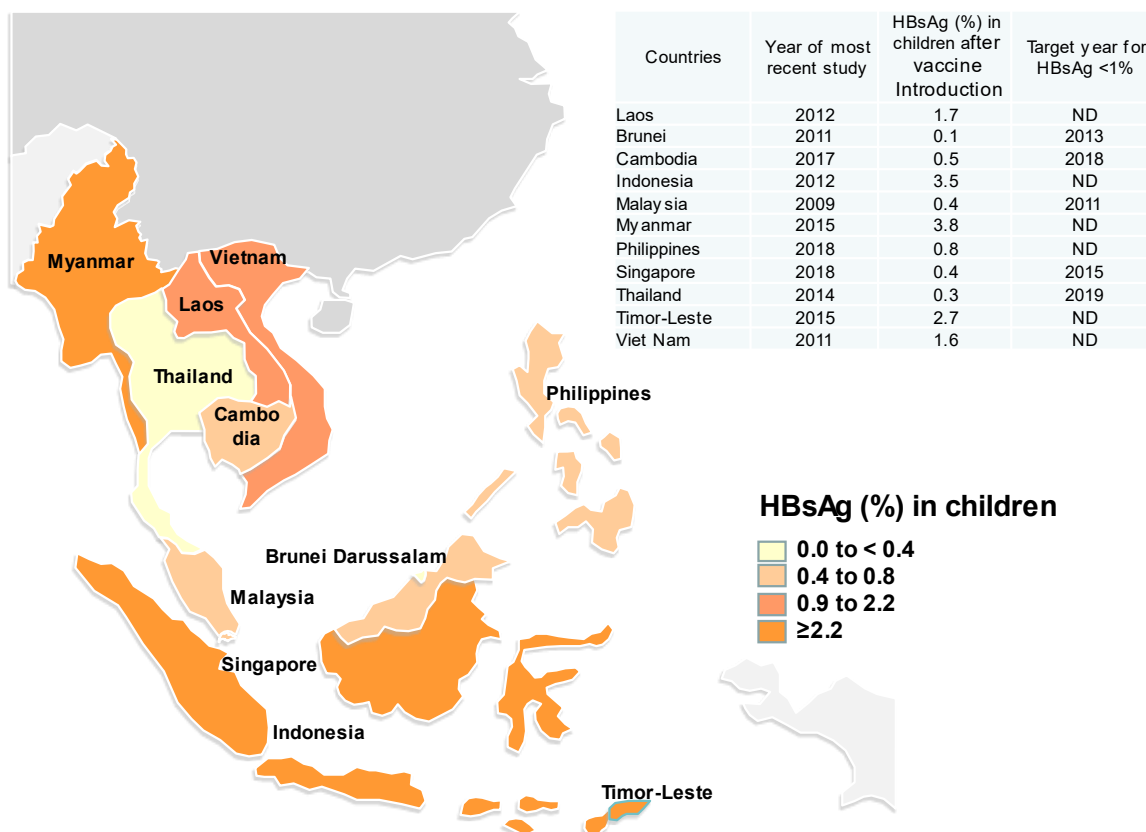


Figure 30: Seroprevalence of HBsAg in children’s cohorts after introduction of hepatitis B vaccine in Southeast Asian countries. The figures in the table and colors of the map correspond to the most recent publications.

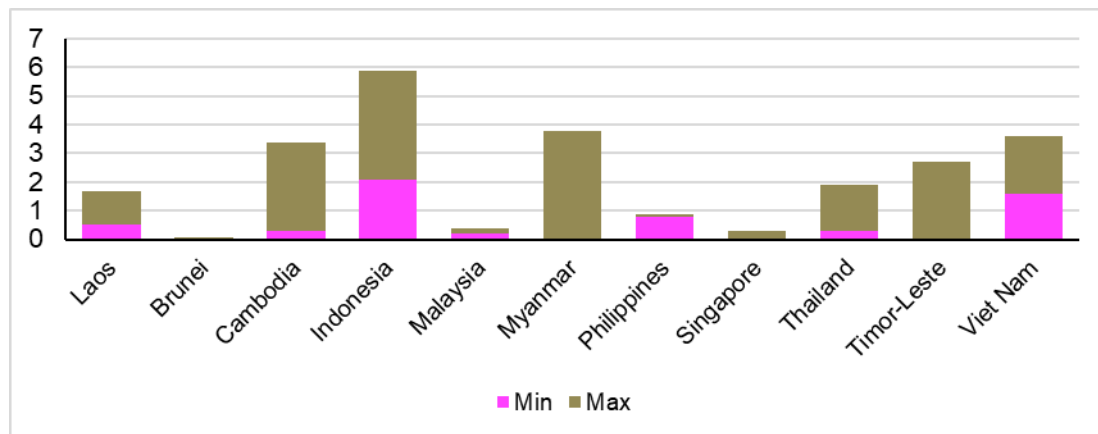


Figure 31: Lower (pink) and upper (brown) values of HBsAg seroprevalence (in %) following introduction of universal anti-hepatitis B immunization in the 11 Southeast Asian countries. [146], [149], [230], [231], [241], [249], [250], [276]–[281]

There is currently no clear explanation to this situation. The variables used to explain immunization coverage are less efficient in explaining the prevalence of HBsAg in children. However, it seems that the rate of births in presence of a qualified healthcare person (Spearman $r=-0.77$, $P=0.007$), the time distance separating the prevalence survey from the implementation of anti-hepB-3 (Spearman $r=-0.74$, $P=0.010$), and the population percentage benefiting from Universal Health Coverage (Spearman $r=-0.72$, $P=0.014$) account for significant parts of the variation observed (see Figure 32 below). By contrast with most similar analyzes done previously, unexpected outliers (e.g. Indonesia with attended births) are conspicuously uncovered by these correlation analyses.

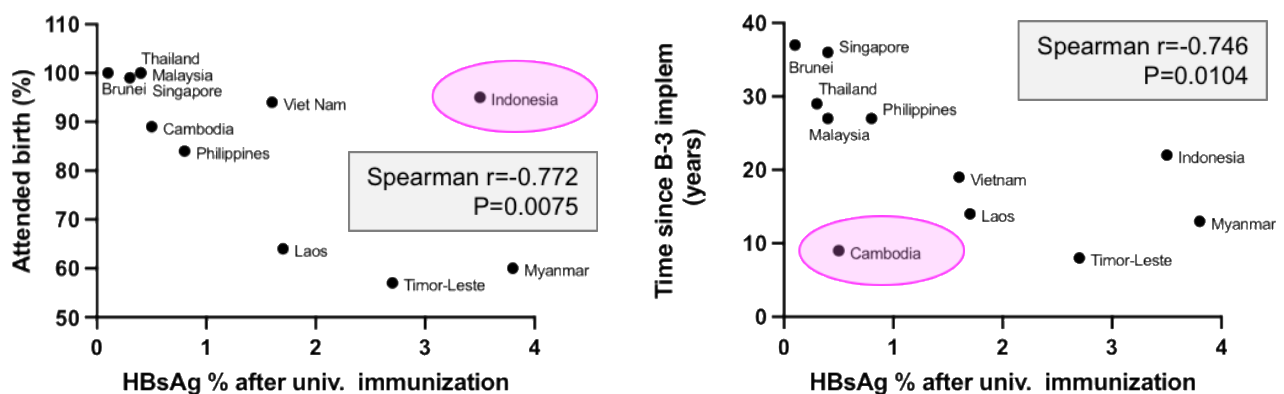


Figure 32: Correlation of HBsAg seroprevalence in children <5 in the 11 Southeast Asian countries with the rate of birth attended by a qualified healthcare worker and with the time separating the survey from that date of universal anti-HepB-3 implementation. Refs as in Figure 27.

CHAPTER II: RESEARCH PROJECT

Hypothesis and objectives

For the 1st study, as mentioned in the previous sections, Laos is considered a highly endemic country for persistent hepatitis B virus (HBV) infection with over 8.0% of the national population infected [4]. The country is also frequently mentioned for its high incidence of primary liver cancer resulting from either HBV or liver fluke infections [104], [282]. To eliminate the burden of disease linked to HBV, the Lao government decided to implement gradually the universal immunization of newborns against the virus hepatitis B starting from 2001 [8]. However, based on the results of past seroprevalence surveys in Laos, the situation of chronic infection with HBV is still only partially characterised. Therefore, we wished to conduct a large serological survey to get faithful representation of HBV seroprevalence in the country, with the avowed aim of providing information to the Lao Public Health authorities a documented tool to develop an appropriate strategy or policy against HBV in keeping with the frame established by WHO to reduce HBV infection of 90% by 2030 [67]. Our study has, of course, some limitations. The most noticeable is the fact that samples from blood donors were not clearly identified as first-time or recurrent donations, a lack of precision that might have some consequences concerning our estimation of HBsAg prevalence in the country. Further studies are needed to focus on the subset of Laotians born after the implementation of universal anti-hepatitis B immunization to identify the effectiveness of the HBV vaccination program in Laos. In particular, after vaccination, the seroprotection (anti-HBs antibodies ≥ 10 IU/L) needs to be confirmed.

The 2nd original study presented in this PhD. thesis manuscript corresponds to a clearly identified blind-spot of HBV research in Laos. There is a large number of studies describing HBV strains circulating in Southeast Asian countries. In Laos, the distribution of the HBV genotypes is grossly in line with our neighboring countries. The most prevalent is genotype C followed by genotype B while genotypes I comes well behind [13]. However, there is a striking lack of information connecting local HBV strains and the clinical presentation of liver pathology. We described, therefore, a series of 125 HBsAg(+) patients aged more than 40 years routinely followed-up at the Center Infectiology Lao-Christophe Mérieux (CILM) in Vientiane for their clinical, biochemical, or hematological characteristics and connected these different features with HBV isolates (DNA loads, genotypes, subtypes, clinically-relevant variations). In addition, we provide the near-complete (n=48) and partial sequences (n=31) of HBV genomes.

First Study: Estimating the burden of hepatitis B virus infection in Laos between 2020 and 2021: A cross-sectional seroprevalence survey

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Estimating the burden of hepatitis B virus infection in Laos between 2020 and 2021: A cross-sectional seroprevalence survey

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Summary

Background Laos is considered highly endemic for persistent infection with hepatitis B virus (HBV). To eliminate this burden, it has gradually implemented universal anti-hepatitis B immunisation of newborns over the past two decades.

Methods Using VIKIA[®] HBsAg, a rapid test for the qualitative detection of the HBV surface antigen, we conducted between Sep 1st, 2020 and Aug 31st, 2021 the largest prospective prevalence survey ever in Laos. This survey included blood donors (BD, $n = 42,277$), patients attending care in capital and provincial hospitals ($n = 37,347$) including attending mothers ($n = 20,548$), HIV-infected patients ($n = 7439$, recruited from 2009 to 2020), students from the Health Sciences University ($n = 609$), and outpatients ($n = 350$) coming for diagnosis at the Center Infectiology Lao-Christophe Mérieux in Vientiane. In total, 88,022 persons were tested, representing approximately 1.22% of the national population. To reach a reasonable estimate of HBsAg prevalence in Laos, we segmented the population according to three variables, age (≤ 20 years as a cut-off), sex, and geographical origin. BD values were used to estimate HBsAg prevalence in patients aged < 20 while hospital survey prevalence was used to estimate the prevalence in those aged older than 20 years.

Findings We observed an HBsAg seroprevalence ranging from 2.6% in blood donors to 8.0% in HIV-infected patients. In BD, men were significantly more at risk to be carriers than women ($RR = 1.2$, $P = 0.00063$). For BD, attending mothers, or HIV-infected patients, HBsAg was significantly more prevalent in northern Laos (5.1–8.4%) than in central (2.0–8.1%) or southern parts of the country (2.2–6.9%), thereby delineating a North-to-South gradient.

Interpretation We considered that HBsAg prevalence probably ranges between 5.0% and 6.0% of the total population. Thus, we consider that Laos may no longer be highly endemic for chronic HBV infection but rather a country with intermediate endemicity.

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Keywords: Hepatitis B virus; Southeast Asia; Laos; Endemic country; Universal Immunization

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Research in context*Evidence before this study*

According to a PubMed search (2007–2021) using “Hepatitis B surface antigen” [MeSH] AND “Laos” [MeSH], Laos is considered as a country highly endemic for persistent infection with hepatitis B virus (HBV) with more than 8% of the national population infected. The country is also frequently mentioned for its high incidence of primary liver cancer that results either from infections with HBV or with liver fluke. To put an end to the endemic circulation of HBV, the Lao government decided two decades ago, in 2001, to implement gradually the universal immunization of newborns against the virus.

Added value of this study

In the largest serological survey ever conducted in Laos, we screened, in 2020–21, the sera of around 80,000 participants (1.22% of the total population) for the presence of HBV surface antigen (HBsAg). We observed a North-to-South decreasing gradient of chronic infections in the country and a global rate of HBsAg carriage ranging between 5.0% and 6.0%.

Implications of all the available evidence

According to our survey, it appears that Laos might be no longer a country where HBV is highly endemic but rather a country of intermediate endemicity. This remarkable achievement, obtained within one generation by the poorest country of southeast Asia, represents an example to follow, and a source of hope for many similar countries affected by a heavy burden of persistent HBV infection around the world.

Introduction

Laos is a tropical landlocked country in Southeast Asia belonging to the lower-middle income countries (LMICs), ranking 99th in the Global Health Security Index.¹

With a little over 7.2 million inhabitants, it is underpopulated (32 inhabitants/km²) in relation to its neighbours and life expectancy at birth is one of the lowest in the region (67.9 years).² While the population living with HIV (0.3%) or the incidence of tuberculosis (149/100,000 inhabitants) are low by regional standards, infection death rates before five years (488/100,000 inhabitants) is the highest in Southeast Asia.³

Historical estimates of hepatitis B in Laos came from infectious disease screening surveys of immigrants to the United States of America around four decades ago. During these campaigns, that frequently mixed participants originating from different countries of the region (Laos, Cambodia, Vietnam), the rate of hepatitis B virus

(HBV) surface antigen (HBsAg) positivity was very high (e.g. 12.6–20.0% in the 1980s).⁴ In the following decade, however, it appears that Lao people established in the US were often members of specific ethnic groups (Hmong, Mien) that could be considered as under-served in comparison to the rest of their homeland populations.⁵ The worrying situation of hepatitis B in Lao refugees persisted until recently.⁶ Taken together, these data indicate that chronic hepatitis B was and still is a serious health problem in many segments of Lao society and perhaps for the entire population.

Laos is, thus, considered to be a country with a high (>8%) prevalence of chronic infection with hepatitis B virus (HBV) in the adult population.⁷ As a consequence, severe liver diseases including cirrhosis and cancer are estimated to be common in the country.^{3,8} This is especially true for liver cancer, for which the age-standardized incidence rate is one of the highest in the world, both for women and men (14.2 and 35.7 cases for 100,000 respectively in 2021).⁹ This major Public Health issue prompted the Lao government to progressively implement a nationwide anti-hepatitis B immunisation campaign of newborns, starting in 2001. Laos is currently applying the Expanded Programme of Immunisation of the WHO. In this frame, newborns should receive a first injection of anti-hepatitis B vaccine at birth. It is followed at weeks 6, 10, and 14 by three additional injections of vaccine.¹⁰ Importantly, there is no difference of procedure for newborns from carriers or non-carriers of HBsAg. Only 34% of newborns were covered by the birth-dose administration by 2011 while it was ranging between 58% and 66% in 2020.^{11,12} This same year the coverage of newborns by the three doses of anti-hepatitis vaccine was 83%.¹² There are therefore still substantial efforts to be made to protect Laotian babies from birth. To improve coverage, it was shown that it was possible to store anti-hepatitis B vaccine outside the cold chain to extend its availability.¹³ Significant dropouts were also noticed, particularly in provinces with higher rates of poverty.¹⁰ Despite the imperfect immunisation status of children, investigators found they had a much lower HBsAg prevalence than in mothers (1.0% vs 7.0%) and observed within a few years a two-fold reduction of HBsAg carriage in toddlers (0.75% vs 1.82%).¹⁴ The situation for children has improved recently due to the systematic anti-hepatitis B immunisation of newborns that started to be implemented two decades ago.^{7,15} In addition, serological evidence of improved protection against hepatitis B is already detectable in older schoolchildren or young students (11–18 years) in both the capital and provinces.¹⁶

The elimination of hepatitis B and C has been scheduled for 2030 by the World Health Organization,¹⁷ which necessitates a massive diagnostic effort to identify previously undetected virus carriers and correctly assess the disease burden in the country. Despite many seroprevalence surveys conducted in the last decade, the

situation of chronic infection with HBV is still only partially characterized in Laos, and unexpected results, such as geographical differences, are observed.¹⁸

To this aim, we conducted a large serological survey using the VIKIA point-of-care rapid test that explored the HBsAg status of 1.22% ($n = 88,022$) of the total Lao population.¹⁹ This survey included five categories of participants: blood donors, hospitalized patients, HIV(+) patients at the Center Infectiology Lao-Christophe Mérieux (CILM), students from the University of Health Sciences in Vientiane, and persons from the general population coming to the CILM for diagnostic purposes.

Methods

Study design and participants

This study was conducted between Sep 1st, 2020 and Aug 31st, 2021 except for HIV(+) cohort. Laboratory staff were trained in the proper use and interpretation of the diagnostic kit and good data collection at each study site. The study included all ages. For minors (below 18), an oral informed consent of the parents was obtained. Healthcare professionals explained the procedure to each participant and allowed them to freely decide if they wished to participate in the study. After obtaining oral informed consent, the doctor gave information about hepatitis B, its prevention, and treatment, and then conducted a face-to-face interview where data such as sex, age, occupation, address, and place of birth were recorded. Blood was then drawn and used to detect HBsAg as well as other parameters requested by the clinician. The HBsAg test result was given to the patient by a doctor at the next visit.

Five distinct cohorts were investigated with VIKIA[®] HBsAg (Biomérieux, Paris, France) (see Table 1):

- Hospital-based survey (Sep 1st, 2020- Aug 31st, 2021): Patients ($n = 37347$) primarily coming from

prenatal care clinics ($n = 20,548$), internal medicine departments, and emergency units, conducted in 8 provincial hospitals (Oudomsay, Sainyaboury, Saisomboun, Vientiane, Khammouane, Savannakhet, Salavan, and Champasak), and five central hospitals (Mahosot, Mittaphap, Mother and Child, Sethathirath, and 103-Hospital). Persons included were those willing to participate. No specific selection of patients with symptoms or with suspected HBV infection was introduced. For persons under 18, the consent of both the parents and the participant himself was sought. Persons whose physical or mental conditions prevented informed consent were excluded.

- Blood donors (Sep 1st, 2020- Aug 31st, 2021): All 42,277 blood donors who presented themselves at blood banks during the study period were tested for HBsAg. Eighteen blood banks participated in the survey, one in the capital and in each province of Laos. According to the routine procedure implemented in the Blood bank network of Laos, another rapid test, SD Bioline (Standard Diagnostics, Yongin, Korea), was used to confirm positivity of VIKIA[®]. Out of 2161 positive donors, 59 were serodiscordant. A commercial ELISA test (HBsAg ultra sens, Human GmbH, Wiesbaden, Germany) was performed for further validation or invalidation of these 59 samples (see Supplementary material for details about sensitivity and specificity of the tests). As an outcome, 56 samples were invalidated, and three were confirmed. In addition, another series of 1268 randomly-chosen negative samples were tested by ELISA (HBsAg ultra sens), 9 were found positive by ELISA (0.7%). In this Laotian series, the overall sensitivity of VIKIA[®] was 99.6%, while its specificity was 95.7%.
- Students from the University of Health Sciences, Vientiane: Volunteer students ($n = 609$) in the 2nd

	Blood donors survey ($n = 42,277$)	Hospital survey ($n = 37,347$)	HIV survey ($n = 7439$)	University of Health sciences survey ($n = 609$)	CILM survey ($n = 350$)
Sex ratio (M:F)	2.42 (29,929/12,348)	0.28 (10,672/26,675)	1.27 (4173/3266)	0.79 (269/340)	0.69 (136/214)
Age (years)					
mean±SD	26.6±8.9	33.6±14.1	17.8±15.9	21.5±1.3	33.8±14.2
median (IQR)	24 (19–32)	30 (24–40)	9 (5–31)	22 (20–23)	33 (23–43)
HBsAg(+) (%)	2.6 (1131/45,858)	6.4 (2380/37,347)	8.0 (595/7439)	3.3 (20/609)	10.0 (35/350)
Blood banks (n)	19	na	na	na	na
Hospitals	na	18	na	na	na
Diagnostic centre (CILM)	na	na	1	1	1
Provinces (n)	18	10	8	1	1
North (%)	20.7 (8775)	16.0	10.7	na	na
Center (%)	71.1 (30,023)	64.7	77.8	na	na
South (%)	8.2 (3479)	19.2	11.5	na	na

Table 1: Demographic, biological, and geographical features of the different series analyzed.

to 5th years of the curriculum were recruited between Jan 1st, 2021 and Apr 30th, 2021.

- At CILM: Inhabitants of Vientiane ($n = 350$) were referred to our laboratory (Jan 3rd, 2021–Jun 21st, 2021) by their family practitioners for free first-time HBsAg tests. As for hospital survey, no specific selection of patients with symptoms or with suspected HBV infection was introduced.
- HIV samples: All single patients positive for HIV followed-up in our centre ($n = 7439$) between Jun 1st, 2009 and Oct 7th, 2020 for HIV load were tested for HBsAg retrospectively. This cohort corresponds to half of the total estimated population of HIV carriers in the country, with patients coming from 8 different provinces. As HIV(+) samples were retrospectively analysed, informed consent was not obtained specifically for the current study. However, patients agreed that their sera could be used later for scientific purposes at the time of sampling.

The study was approved by the Lao National Ethics Committee for Health Research (reference NECHR 364/July 2020). The study adhered to the STROBE reporting guidelines.

Procedures

VIKIA[®] HBsAg is a rapid immuno-chromatographic (or lateral flow) test for the qualitative detection of Hepatitis B surface Antigen (HBsAg) in human serum, plasma or whole blood. It can be used for prenatal or transfusion screening and during acute infection or chronic carriage of the hepatitis B virus.¹⁹ According to the manufacturers' recommendations, capillary blood by fingerstick was used, dispensing 75 μ l of whole blood on the device, then one drop of buffer was added. Readings were available within 15 min, though for negative samples, it was recommended to wait up to 30 min to ascertain the result.

Data analysis and statistical tests

To estimate HBsAg prevalence, we combined three main parameters: age, sex, and geographical origin of participants. Relying on ≤ 20 years blood donor data, we estimated HBsAg seroprevalence in the general population ≤ 20 by region, and sex. Estimates of HBsAg seroprevalence in older blood donors do not reflect the true prevalence in these age categories as the carriers of HBsAg, once identified by blood banks, are discouraged to do some further donations. Older and, thus, potentially recurrent blood donors do not represent, therefore, an appropriate category to assess HBsAg carriage in the general population. The geographical distribution of sampling in the hospital-based survey was not comprehensive enough (10 regions out of 18) to rely on observed regional differences with sufficient

confidence. To get an estimate of HBsAg carriage in the general population > 20 of Laos, we calculated the mean hospital-based HBsAg prevalence for women (5.6%) and men (9.6%) between 20 and 69 years.

Statistical analyses were performed using the Prism 9.3.1 statistical package (GraphPad, San Diego, CA, USA). Continuous variables are presented by their mean and standard deviation or median and interquartile range depending upon the distribution type. Accordingly, they were compared either by a Student's t-test (Gaussian distribution) or a Mann-Whitney U test (non-Gaussian). Categorical variables were summarized as frequencies and compared either by Fisher's exact test or the Chi-Square test. All the tests were two-sided, and the level of significance was set at $P < 0.05$.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. PS, ED, PhPa, and PaPi had access to the dataset and had final responsibility for the decision of publication.

Results

With 80,082 tests, this was the largest seroprevalence survey of the hepatitis B virus surface antigen (HBsAg) ever conducted in Laos. The participants' demographic features and geographic origins are summarized in Table 1.

The structures of each series regarding age and sex were significantly different. Women were under-represented among blood donors while they were over-represented in the hospital-based survey (71.4%) due to our targeting of maternity departments and antenatal clinics.

HBsAg seroprevalence was 2.6% in the whole series of blood donors. We observed that it tends to decrease in blood donors older than 17–20 years of age (where it peaks above 3.0%) (Figure 1A). We observed overall prevalence of 6.4% and 8.0% in the hospital-based series and the HIV-infected cohort. In contrast with blood donors, we observed an increasing seroprevalence with age that reaches a plateau of around 8.0% in adults above 20 years in these two series.

The hospital-based series was, however, heterogeneous and hid significant differences in HBsAg prevalence. Mothers attending antenatal clinics or obstetrical departments are inherently healthier on average than other patients admitted to the hospital. Accordingly, HBsAg seroprevalence was 5.0% in attending mothers ($n = 1044/20,548$) while it reached 7.1% in children below 15 years ($n = 56/782$) and 8.0% in the remaining adult participants ($n = 1280/16,017$) (Figure 1B).

Concerning sex differences in prevalence, males were systematically more often infected than females

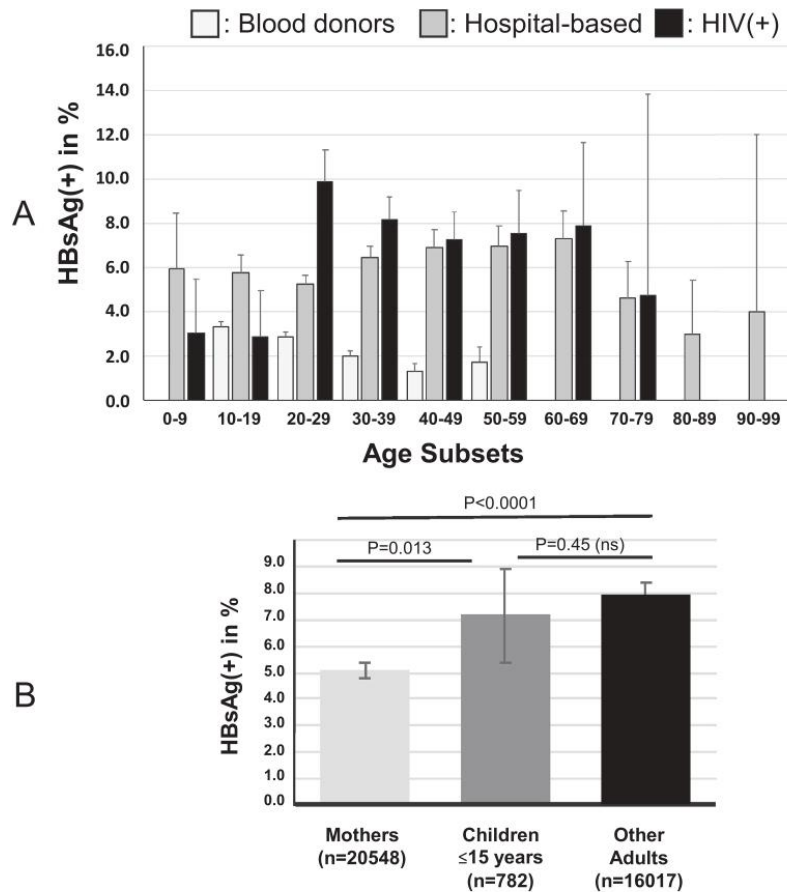


Figure 1. Prevalence of Hepatitis B virus surface Antigen (HBsAg) in the different subsets of participants and in the Hospital-based survey. A. Bar chart representing HBsAg prevalence by % in the 42,277 blood donors, the 37,347 patients attending care in the different participating hospitals, and the 7439 HIV-infected patients, according to the different age classes. B. Heterogeneity of HBsAg prevalence in the different components of the hospital-based series. It should be noticed that all patients except those infected with Human Immunodeficiency Virus, HIV(+), were recruited between September 2020 and August 2021. The Human Immunodeficiency virus-positive series [HIV(+)] was enrolled between 2009 and 2020.

(1.2–1.7 fold), and this difference reached the level of significance for blood donors, the hospital-based series, and the HIV(+) cohort (Figure 2A).

Geographic differences in HBsAg prevalence were remarkable. For blood donors and mothers attending maternity departments, HBsAg prevalence was significantly higher in northern than in southern and central regions of the country (Figure 2B and Figure 3A-C).¹⁸ Occupations of the 42,277 blood donors (BD) were known, and when we analysed the prevalence of HBsAg

in the nine major categories, the carriage rate was significantly higher in the North than in the Centre or the South (Supplementary Table 2) in six categories.

Realistic assessment of the current situation for HBsAg prevalence in Laos is crucial to assess any progress in the two decades following the implementation of an anti-hepatitis B immunisation campaign in newborns. An important demographic feature in Laos is the young age of the population, with 42.9% aged 20 or below (median age = 23.0 years).² In contrast to

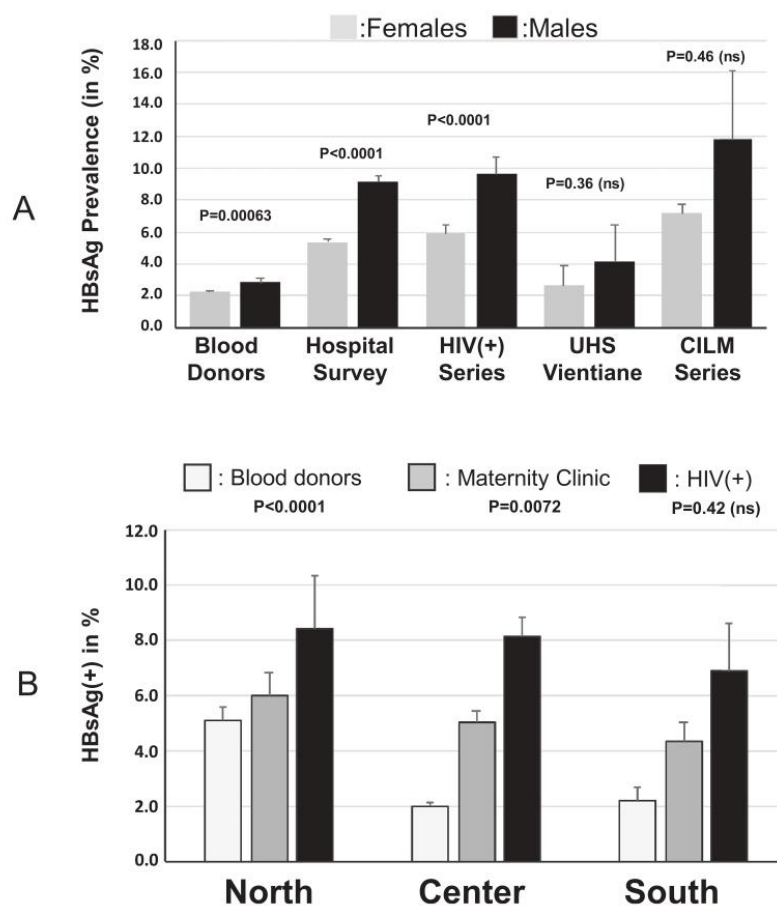


Figure 2. Differential Hepatitis B virus surface Antigen (HBsAg) prevalence according to sex or geographic origin. A. HBsAg seroprevalence according to sex in the five population subsets investigated. B. HBsAg seroprevalence according to the place of sampling in blood donors, mothers attending care in maternity departments, and Human Immunodeficiency Virus-infected patients [HIV(+)].

previous generations, blood donors between 17 and 20 would have benefited from the anti-hepatitis B immunisation program, which began in 2001. In addition, they could, for obvious reasons, be considered as first-time donors and thus, HBsAg seroprevalence in this age group should be relatively less biased than in older segments of the population. We observed that men from the northern region of Laos were particularly affected by HBV infection (1.8–2.1 fold the prevalence for women) (see Figure 4A). Sex differences were less pronounced in the Centre (1.0–1.2 fold) and the South (1.2–1.6 fold).

For the population between 20 and 69 years we decided to rely of the hospital survey. We consider that

the 21–69 years subset of patients attending care at the hospital represents approximately a random sample of the corresponding general population that did not benefit from the systematic anti-hepatitis B immunization campaign implemented in the country. The mean HBsAg prevalence for women was 5.6% and while it was 9.6% in men (see Figure 4B). Seroprevalence in the hospital series was not so different from that observed in the HIV-infected cohort (6.2% and 9.6%), which represents a population of patients often considered to be in the upper range of HBsAg prevalence in Southeast Asia (Figure 4C).²⁰

Overall, once normalized for the population structure and size in each region, according to our estimates,

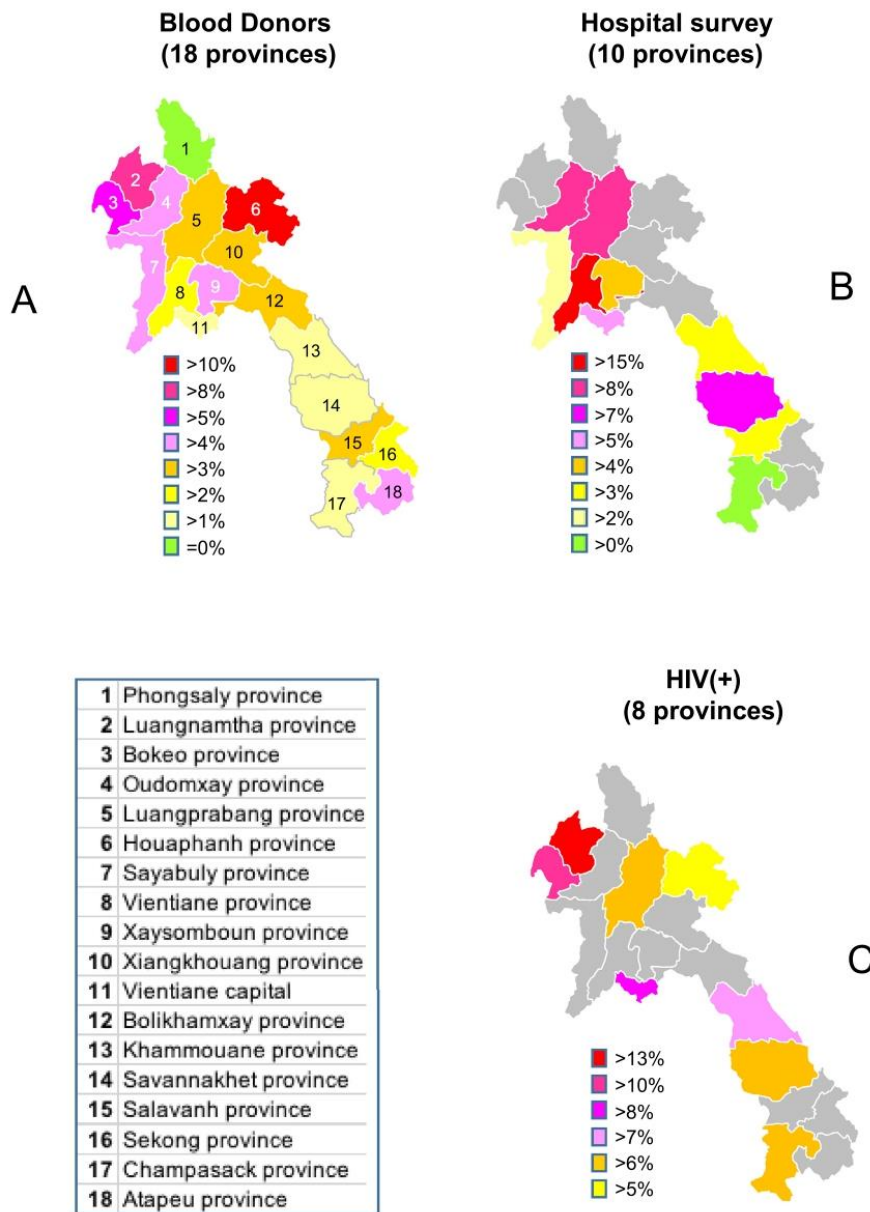


Figure 3. Map of Laos representing the prevalence of Hepatitis B virus surface Antigen (HBsAg) in the different provinces A. In blood donors, B. In patients attending care in hospitals, C. In Human Immunodeficiency Virus-infected patients [HIV(+)].

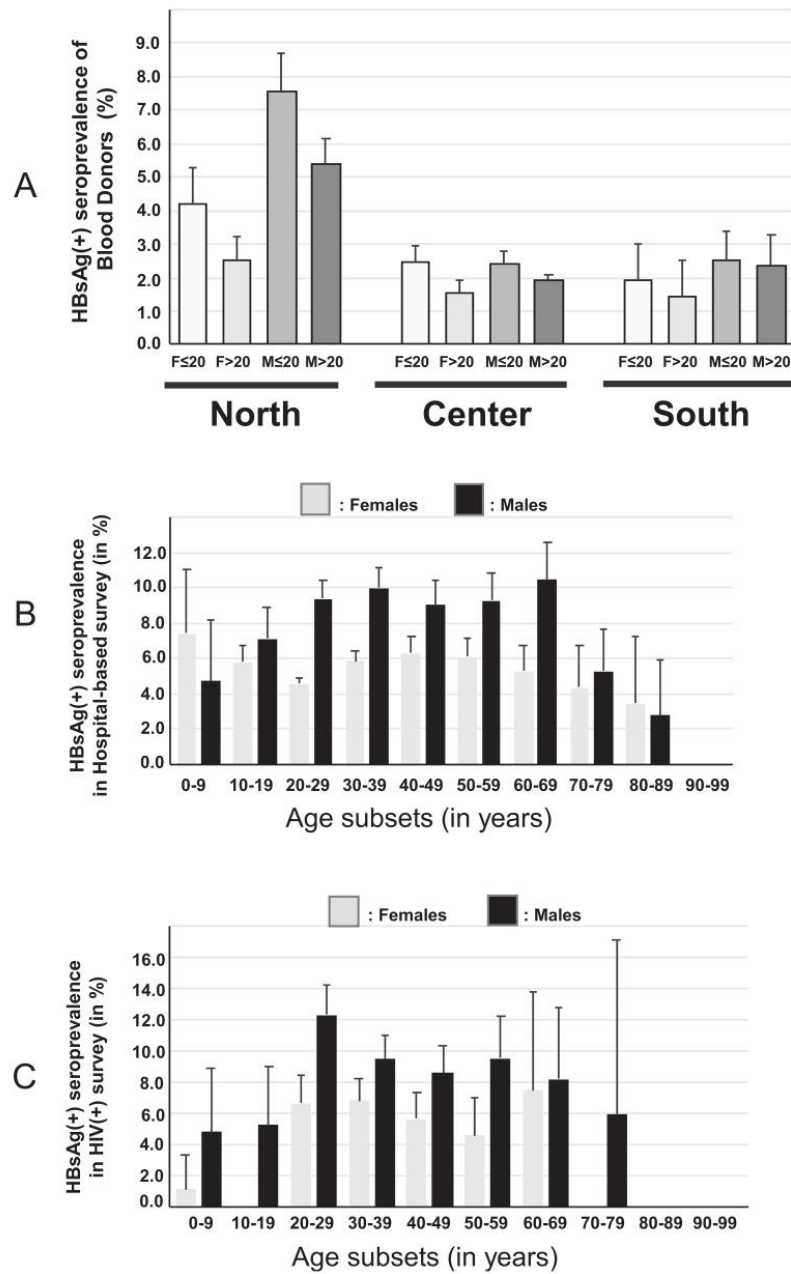


Figure 4. Bar-Charts differentiating Hepatitis B virus surface Antigen (HBsAg) prevalence according to age and sex. A. Bar chart representing HBsAg prevalence in blood donors according to sex, age, and region of origin. B. Bar chart representing HBsAg prevalence according to sex and age in the hospital-based cohort. C. Bar chart representing HBsAg prevalence according to sex and age in Human Immunodeficiency Virus-infected patients [HIV(+)].

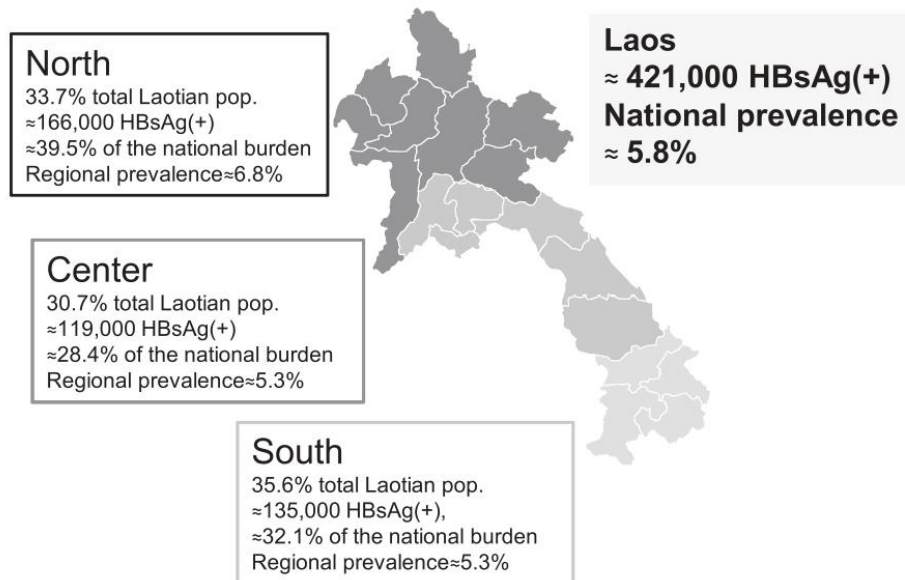


Figure 5. Estimated distribution and seroprevalence of Hepatitis B virus surface Antigen (HBsAg) in the three large regions of Laos.

HBsAg seroprevalence decreases along a north to south gradient for all ages, ranging from 6.8% in the North to 5.3% in Central and Southern Laos. The overall HBsAg prevalence in Laos is estimated to be 5.8% (see Figure 5).

Discussion

Our study conducted between 2020 and 2021 confirmed the existence of a decreasing North-South gradient of HBs antigen prevalence in Laos.¹⁵ Moreover, the participation of more than 42,000 blood donors allowed us to highlight a reduction in the prevalence of HBsAg in donors aged 20 and under. This last observation suggests that the immunisation policy for newborns implemented from 2001 has undoubtedly already borne fruit and reduced the burden of hepatitis B in the youngest segments of the population.

The different series investigated were characterized by substantial heterogeneities in sex-ratio. The observed under-representation of women among blood donors was expected as the high prevalence of anaemia affecting young women²¹ makes them less likely to donate blood. The earliest survey of HBsAg in blood donors in Laos was conducted 17 years ago on 14,000 first-time donors in Vientiane capital and Bolikhamsay province (Central region).²² The overall prevalence in these donors from the pre-immunisation era was 8.7%, much higher than observed in our series (2.8%). This

difference is probably due to recurrent blood donors in the current survey. It is known that blood banks discourage people already known to be infected with HBV to donate their blood resulting in a lower proportion of infected persons in recurrent donors.¹⁸ However, if we focus on first-time donors aged 17–20, we observe a 2.4-fold decrease from 8.5% to 3.3% ($P < 0.0001$, OR = 2.9, 95%CI = 2.5–3.3) in HBsAg prevalence between 2003–2005 and 2021. The sex difference observed at that time (6.2% HBsAg in females and 9.7% in males, 1.56-fold) is similar to what we observed (2.7% vs 3.6%, 1.33-fold). The difference of seroprevalence between males and females is a hallmark of chronic infection with HBV. It was described early on.^{23,24} The patho-physiological bases of this difference are not clearly known. Regarding viral factors, a role of the androgen-responsive elements (ARE) present on HBV genome and capable to stimulate HBV replication is sometime mentioned.²⁵ But, overall, it is considered that the higher susceptibility of males to infection with HBV is due to the lesser immune competence that characterize males in human species.²⁶

Additionally, HBsAg(+) donors tend to be older in the current study (24.6 ± 7.7 years) than previously (22.3 ± 6.6 years), suggesting the progressive ageing of HBsAg carriers. The large difference between first-time and other donors was already observed in 2006 when 17–20 years old donors in Vientiane and Bolikhamsay ($n = 342$) born before the immunisation era had an

HBsAg prevalence of 9.3%.²⁷ A recent first-time donor survey conducted between 2013 and 2015 observed, as in the current study, that HBsAg carriage was much higher in the North (13.1%) than in the rest of Laos (6.6% in the Centre, and 5.6% in the South).¹⁸ These rates were, however, much higher than those observed in first-time donors (17–20 years) in the present study (6.2%, 2.4%, and 2.3%, respectively). We assume that the discordance between that study and ours is because Nouanthong *et al.* includes individuals born in the pre-immunisation era.

In the past decade, a survey of mothers ($n = 965$) recruited throughout the country yielded an estimated HBsAg prevalence of 2.9% ($P = 0.00027$, OR = 0.5, 95%CI = 0.3–0.7),²⁸ much lower than the overall rate observed in our work (4.9%, $n = 20,156$). The reason for this difference may possibly be that participants in the present study were in hospital maternity departments, an at-risk medical context, while the previous ones were in villages in a potentially healthier context. Our results also differed from those of other studies conducted in antenatal departments in different provinces, such as a study in Vientiane, which measured HBsAg prevalence at 8.0–8.2% ($n = 388$, $P = 0.0067$, OR = 1.7, 95%CI = 1.1–2.5).¹⁵ We are, however, in closer agreement with the results of a serological survey of three maternity departments in central provinces and Vientiane Capital. In that study, HBsAg prevalence estimates were 4.1% ($n = 911$), 5.9% ($n = 3000$), and 5.4% ($n = 13,238$), none of which are significantly different than the 5.1% in our work ($P = 0.16$, $P = 0.22$, and $P = 0.30$ respectively, ns).²⁹ Overall, it seems that there could be considerable variation in HBsAg prevalence in Lao child-bearing women. These differences presumably depend upon the geographical locations of the recruitment centre as we and other have shown that there is a decreasing North to South gradient of HBsAg prevalence.¹⁸ The structures of recruitment might represent another modulator as attending mothers at risk of complications are primarily found in regional hospitals rather than in village health centres.

Few hospital surveys have been conducted in Laos. Recent work in the southern province of Saravan involving 2463 patients observed an overall HBsAg prevalence of 3.8%, characterized by a significant difference by sex (2.4% in females, 5.7% in males). The prevalence of HBsAg was low (2.8%) in the 11–20 years age group ($n = 643$) compared to the 4.8% observed for those aged above 21 years ($n = 1562$).³⁰ Our survey conducted in Saravan and Champasack ($n = 3657$) yielded higher values with overall HBsAg carriage at 6.5% (5.0% for females and 6.8% for males). As in the study of Xaydalasouk and co-workers, HBsAg prevalence was much lower in patients aged 20 and lower (3.3%) than in older ones (7.1%). These results clearly indicate that the immunisation program first implemented 20 years ago has already benefited populations seeking care at the

hospital. We observed that children attending care in hospitals were significantly more infected than young mothers (Figure 1B). This situation is most probably due to the fact that diseased children in Laos are logistically more at risk to be HBV-infected than the healthy segments of the population to whom belong the young mothers.

To the best of our knowledge, there has been no survey of HIV-HBV co-infections in Laos. However, the prevalence of HBsAg in the current retrospective Lao cohort (8.0%) was lower than those observed in studies in Cambodia (10.7%), Vietnam (11.9%), Thailand (11.9%), and more similar to observations from Myanmar (7.6–9.0%).^{20,31–34} The higher representation of men in participants with HIV infection plausibly reflects the fact that men who have sex with men are more infected with HIV than other groups in Laos.³⁵ The low rate of hepatitis B in HIV-infected patients is most probably due to the young age of the series (mean = 17.8±15.9 years), implying that most were born during the immunisation era. It represents further evidence of the efficacy of the immunisation policy conducted in Laos.

A remarkable feature of our work was the North-South decreasing gradient of HBsAg prevalence in the country. There are no well-established explanations to this observation recently reported by other investigators.¹⁸ It is noteworthy, however, that the ethnic composition of the northern part of the country differs substantially from that of the center and the south.³⁶ In the North are established communities speaking Hmong-mien languages described for a long time as affected by higher HBsAg prevalence.⁶ Other ethnic communities, speaking Tibeto-Burman languages are also established in the North of the country. Hence, these latter communities are considered by some authors to be the reservoir of the recently described genotype I known to be present in Laos.^{37,38}

Our study has some limitations that might have some consequences concerning our estimation of HBsAg prevalence in Laos. The principal problem came from the blood donors that were not identified as first-time or recurrent. It is reasonable to assume, however, that 17–20-year-old are presumably first-time donors, and this study is, therefore, able to provide a fair assessment of HBsAg seroprevalence in populations born after the implementation of universal anti-hepatitis B immunisation. Concerning older segments of the population, our estimation relies on the hospital survey that mechanically tends to overestimate the prevalence of HBsAg as its carriers are more often in position to seek for healthcare than healthy people. In addition, due to a less comprehensive sampling throughout the country, we were not capable to differentiate reliably Northern, Center, and Southern regions to produce estimates.

In conclusion, we observed a significant difference in HBsAg prevalence throughout the country, with

northern provinces being affected by a heavier burden of infection than central or southern regions of the country. New studies are needed both to definitively establish the existence of these differences and to better understand their causes. Given the Lao age pyramid (43% of the population is ≤ 20 years) and the implementation of universal anti-hepatitis B immunisation 20 years ago, we consider that Laos is probably no longer highly endemic ($>8.0\%$) for chronic hepatitis B virus infection. From now on, it seems reasonable to consider Laos as a country with high-intermediate endemicity (5.0–7.0%).⁷ However, it is fair to keep in mind that our work is only an approximation and that more data is needed to truly demonstrate a significant decrease in the AgHBs prevalence in young people and a modification in the status of Lao from high endemic country to an intermediate one. Finally, our work suggests that a developing country with a predominantly rural population can emerge from high endemicity for chronic hepatitis B virus infection within a single generation. This situation is primarily the result of the demographic dynamism of the populations whose many children are protected from infection by public health measures applied with determination. It is, therefore, a message of hope for many other countries facing similar circumstances.

Contributors

PhPa and ED designed the project. PS, VL, CS, VS, MM, SP collected the data. PaPi analysed and interpreted the data. PS and PaPi generated figures and tables. PaPi wrote the manuscript. ED, PhPa, AM, and PS edited the manuscript. NS, PV, and SB critically read the manuscript. All authors contributed substantially to the conception, writing and revision of the work and approved the final content of the manuscript. PS, ED, PhPa, and, PaPi had access and verified the data reported in the manuscript.

Data sharing statement

These data will be available with publication with investigator support, and after approval of a proposal.

Declaration of interests

All authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.eclinm.2022.101582.

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Supplementary information

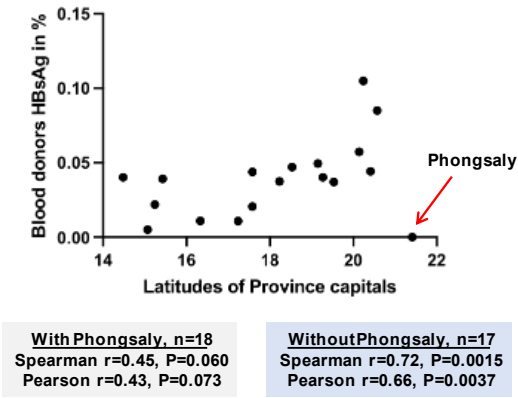
Supplementary table 1: sensitivity and specificity of the test used in the study in blood bank network of Laos.

Test	Specificity	Sensitivity
VIKIA [®] (in Asia)	99.8%	99.05%
SD Bioline	99.0%	100.0%
HBsAg ultra sens	99.6%	100.0%
Supplemental table1		

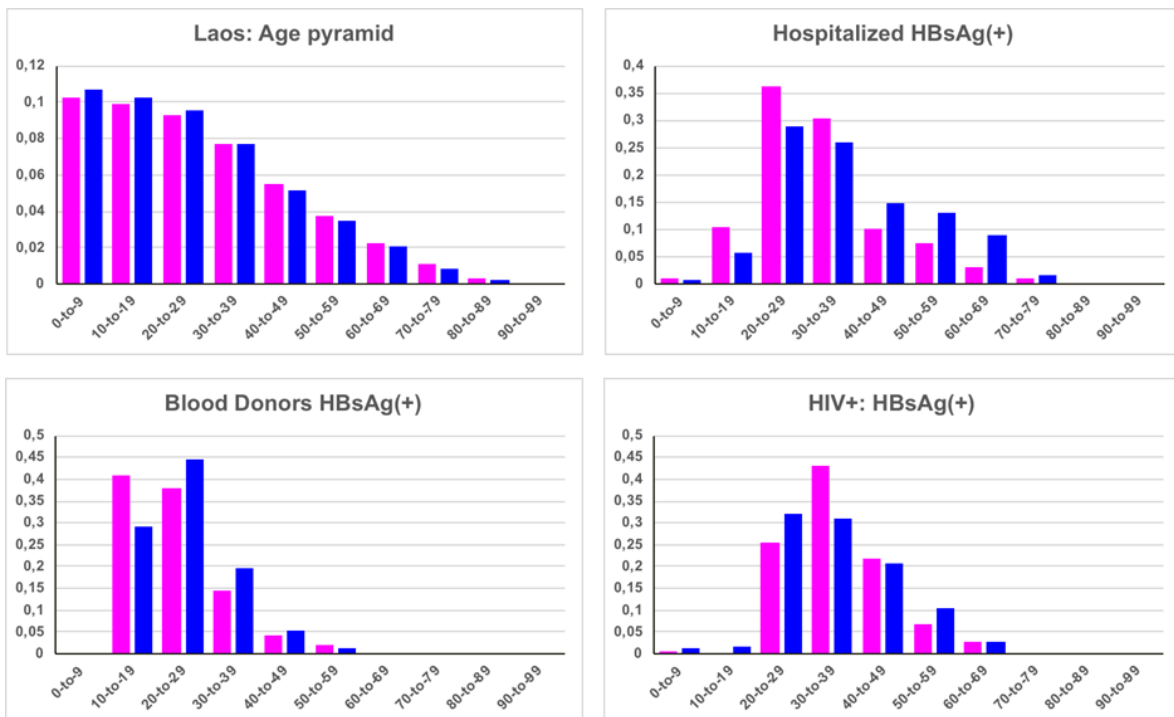
Supplementary Table 2: HBsAg prevalence according to occupations and geographical origin of the participant

Occupations	Global HBsAg prevalence (in %)	Region	HBsAg prevalence (%)	n HBsAg(+)/n total	OR	95% CI	P-Value
High School Students	3.1	Center	2.23	117/5242	Ref		
		North	7.15	132/1846	3.2	[2.6-4.3]	<0.0001
		South	2.24	23/1026	1.0	[0.6-1.5]	0.98(ns)
University Students	2.9	Center	2.56	201/7827	Ref		
		North	5.81	87/1496	2.3	[1.8-3.0]	<0.0001
		South	2.44	25/1024	1.0	[0.7-1.5]	0.80 (ns)
Soldiers	3.0	Center	2.83	98/3457	Ref		
		North	5.04	55/1090	1.8	[1.3-2.5]	0.0004
		South	1.99	9/452	1.4	[0.7-2.4]	0.35 (ns)
Police Officers	2.2	Center	1.49	10/667	Ref		
		North	4.59	9/196	3.1	[1.3-7.9]	0.0216
		South	0.00	0/7	na	na	1.00 (ns)
Civil Servants	1.4	Center	1.00	83/8330	Ref		
		North	2.85	57/1996	2.9	[2.0-4.1]	<0.0001
		South	1.50	10/664	1.1	[0.5-2.0]	0.74 (ns)
Farmers	4.0	Center	1.95	31/1582	Ref		
		North	5.81	76/1307	3.0	[2.0-4.6]	<0.0001
		South	4.14	8/193	2.1	[1.01-4.5]	0.064 (ns)
Workers	2.6	Center	2.42	49/2022	Ref		
		North	5.20	5/96	2.2	[0.9-5.5]	0.094 (ns)
		South	3.57	1/28	1.5	[0.1-8.1]	0.501 (ns)
Traders-Merchants	2.0	Center	1.36	10/735	Ref		
		North	3.26	15/460	2.4	[1.05-5.3]	0.036
		South	3.44	1/29	2.6	[0.23-16.2]	0.34 (ns)
Monks	4.0	Center	8.33	6/72	Ref		
		North	4.85	13/268	0.56	[0.2-1.5]	0.25 (ns)
		South	5.35	3/56	0.62	[0.16-2.3]	0.73 (ns)

Occupations of the 42,277 blood donors (BD) were known, and when we analysed the prevalence of HBsAg in the nine major categories, the carriage rate was significantly higher in the North than in the Centre or the South in six categories.



Supplementary Figure 1: dot plot correlating the latitude of provincial capitals of Laos and the prevalence of HBsAg in blood donors. Phongsaly is the small rural province in the north of Laos where we got zero HBsAg positive case because, there were not many voluntary blood donors, the majority were repeated blood donors coming for their family members. Of course, it created a major bias. However, it is clear that there is a positive South-North gradient of HBsAg prevalence in blood donors that follows the increase of latitude (if cut out Phongsaly).



Supplementary Figure 2: Age pyramids of the different populations tested for HBsAg. None of them corresponds to the age pyramid of Laos that is dominated by the youngest age categories. Such important differences are too rarely taken into account in the conclusions of surveys exploring HBsAg prevalence in general population.

Second study: in preparation

In Laos, infection with Hepatitis B Virus genotype C is associated with liver steatosis and obesity

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Abstract

Background: Laos is still considered a country with a high endemicity of chronic infection with hepatitis B virus (HBV). Accordingly, the incidence of death from primary liver cancer is one of the highest worldwide. While the molecular description of HBV strains circulating in Laos has been reported, no connections between local isolates and presentation of liver pathology is available.

Methods: We analyzed a series of 125 HBsAg(+) patients aged more than 40 years (median=51) monitored at the Center Infectiology Lao-Christophe Mérieux (CILM) in Vientiane. Plasma HBV DNA loads were measured by droplet digital PCR and circulating viral sequences were characterized by Sanger sequencing.

Results: HBV DNA was detectable in 91 samples (72.2%, HBV DNA load median=3.7 log₁₀ copies/mL). We obtained sequences from 79 isolates. The most frequent genotype was genotype C (49.4%), followed by genotypes B (31.6%), I, and D (each 6.3%), and undetermined (2.5%). Three strains (3.8%) originated from genotypes C and D recombination. Remarkably, patients infected with genotype C were more often obese (33.3% vs 11.4%, P=0.031). Patients displaying HBV isolates with pre-S deletions presented significantly higher concentrations of AFP (5.1 ng/mL vs 2.3ng/mL, P=0.013) suggesting a more aggressive infection and a more important stem cell niche solicitation for the liver of these patients.

Conclusion: In Laos, the dominant genotype C of HBV is frequently associated with metabolic alterations affecting livers of its carriers. In addition, mutations affecting HBV envelope-encoding genes might predispose concerned patients toward a likely evolution towards liver cancer.

Introduction

Chronic infection by hepatitis B virus (HBV) still represents a major disease burden in many countries, primarily those located in intertropical regions. According to the most recent estimates, 296 million people are chronically infected by the virus [1]. In the absence of proper antiviral treatment, it is estimated that HBV is directly responsible for the death of 40.0% of chronically infected patients. Mortality from all causes is increased in these patients but the most important fatality-associated complication remains hepatocellular carcinoma (HCC), while liver cirrhosis (LC) ranks second. In chronic HBV carriers, infection-associated death is significantly greater in males than the females [2].

Laos is highly endemic for chronic HBV infection with more than 8.0% of the general population affected. Consistent with this worrying situation, the incidence of liver cancer is very high: according to GLOBOCAN, Laos ranks fifth worldwide for liver cancer incidence [3]. Liver health is a major issue in Laos as in addition to HBV the population is exposed to a carcinogenic liver fluke, *Opisthorchis viverrine*, that infects a significant subset of the population [4].

HBV strains circulating in southeast Asian countries have been described by different authors. The most prevalent HBV lineage is genotype C followed by genotype B and the recently identified genotype I [5]. In Laos, the frequencies of HBV genotypes are similar to those observed in neighboring countries. However, there is still a lack of knowledge concerning HBV strains associated with liver disease progression. In the present manuscript, we describe the presentation of liver disease in 125 carriers of HBV surface antigen (HBsAg). Furthermore, we provide complete (n=40) and partial sequences (n=51) of HBV genomes and describe the molecular features of the virus associated with disease progression.

Material and Methods

Patients

A total of 125 patients followed-up for diagnostic purposes at the Center Infectiology Lao-Christophe Mérieux in Vientiane was included in the frame of the COCLICAN study. The criteria for inclusion were being HBsAg+ and 40 years old or older, as complications of infection with HBV have been shown to markedly increase after this age, especially in males [2]. Patients presenting anti-HCV reactivity were excluded from the study. No patients were positive for hepatitis delta or HIV. After obtaining their informed consent, patients were interviewed by a medical doctor and information concerning demography, and medical history was collected. All patients were examined by an ultrasound to assess the status of their liver tissue. Various blood parameters including aminotransferases (ALT, AST), complete blood count, and alpha-fetoprotein (AFP) were measured.

Ethics

The study was approved by the Lao National Ethics Committee for Health Research (reference NECHR 364/July 2020).

DNA extraction

DNA was extracted from 500 μ L of plasma-EDTA. After an initial overnight digestion at 37°C in presence of 2% sodium dodecyl sulfate (Euromedex, Souffelweyersheim, France), 10 mmol/L ethylenediaminetetraacetic acid (Merck), and 10 mg/mL proteinase K (Eurobio, Les Ulis, France), the solution was extracted twice by Tris-EDTA (TE)-buffered Phenol pH=8 (Interchim, Montluçon, France) and then once by chloroform-isoamyl alcohol 24:1 (Sigma and Prolabo respectively). DNA was precipitated overnight at -80°C in two volumes of absolute ethanol (Merck) in presence of sodium acetate pH=6 (0.1M final, Merck) and 4 μ L of glycogen 5mg/ μ L (Invitrogen, Villebon, France). Pellets were briefly rinsed in 500 μ L ethanol 70% at room temperature. After a short drying on the bench, DNA was resuspended in 50 μ L Tris-EDTA (TE).

Qualitative PCR and Sequencing

Six nested PCRs were carried out. External primers P1C and P2C were derived from those selected by Takahashi [6]. In brief, primers were made at the position of the HBV X region based on the sequences of HBV GT-C (AB014376). HBV DNA amplified by PCR resulted in a ~3,200 base-pair fragment, which contained the full-length HBV genome. The reaction mixture of the first round of amplification contained a 2µl DNA sample, 0.2 mM dNTPs, 1X Platinum II Taq Hot-Start Buffer (14966, Invitrogen), and 0.4µl of Platinum™ II Taq Hot-Start DNA Polymerase in a 50µl final volume. The amplification was carried out for 30 cycles 5 sec 98°C, 15 sec 60°C, 1 min 68°C after 2 min denaturation at 94°C for 2 min in a T1000 Thermal Cycler (Bio-Rad). Nested PCR with 6 primer pairs was performed using 2µl of the PCR in the same conditions with 30 sec of elongation time.

A volume of 4µl of PCR products were analyzed on 2% precast agarose gel (G800802, Invitrogen) then 20µl of PCR products were treated with 2µl exonuclease I (M0293, New England Biolabs, Ipswich, MA, USA), and 2µl of alkaline phosphatase (E70092Z, Cytiva, Vélizy, France) 15 min 37°C, 15' 85°C. Sequencing was carried out on Eurofins Genomics facility (Cologne, Germany) on 11µl of treated PCR product with 5 pmoles of the corresponding primer.

Droplet Digital PCR

For HBV DNA detection by ddPCR, 2µL of extracted DNA were amplified using ddPCR Supermix for Probes (No dUTP) kit (Bio-Rad). Probes were 3'-modified by Iowa Black Quencher (IBQ, Integrated DNA technologies, Coralville, Iowa, USA) that produced less background noise. Droplets were generated and read on the QX200 AutoDG system (Bio-Rad). RT and PCR amplification were carried out in an ICycler PCR instrument (Bio-Rad) with the following steps: 1 cycle at 95°C/10min, and 40 cycles [94°/30sec, 59°C/1mn] and a termination step of 98°C/10min. All cycles were performed with a ramping rate of 2°C/sec. All ddPCR reactions were performed in presence of 2.5 units of Csp6I restriction enzyme (Thermo Fischer) as recommended by ddPCR kit manufacturer. Determination of the limit of detection (LoD) was based on the establishment of the limit of blank (LoB). LoB is defined as the maximal number of positive droplets obtained on genomic DNA samples extracted from uninfected tumour cell lines, and calculated according to a modified version of the procedure described by Armbruster and Pry [7-9]. The LoB were derived from the analysis of 100 ddPCR replicates containing a mean droplet number of $XXX \pm XXX$ and generated from XX different DNA samples. Accordingly, LoD was set at 5 droplets.

Cellular free circulating DNA concentrations were assessed by amplifying a human gene, *RPP30* (ribonuclease P/MRP subunit p30, locus 10q23.31) using a commercial assay (dHsaCP2500350, Bio-Rad).

Sequence and phylogenetic analyses

Sequences were analyzed with SeqScape software v3.0 (4474978, ThermoFisher Scientific) and MEGA 11 software (*) for phylogenetic analysis [10]. The output fasta format from the SeqScape alignment was imported into the MEGA software. The phylogenetic tree was constructed by using the Maximum Likelihood method and Tamura-Nei model concomitantly with genotyped HBV sequences from GenBank database [11].

Data analysis and statistical tests

Analyses were performed using the Prism 9.3.1 statistical package (GraphPad, San Diego, CA, USA). Continuous variables are presented by their mean and standard deviation or median and interquartile range depending upon the distribution type. Accordingly, they were compared either by a Student's t-test (Gaussian distribution) or a Mann-Whitney U test (non-Gaussian). Categorical variables were summarized as frequencies and compared either by Fisher's exact test or the Chi-Square test. All the tests were two-sided, and the level of significance was set at $P < 0.05$.

Results

Demography and outline

Laos is considered a hot-spot for liver cancer incidence worldwide [12]. While HBV strains circulating in Laos have been described, very little is known about liver disease progression associated with the different genotypes and the relevant molecular determinants present on HBV isolates [5]. To this end, we conducted a survey of 125 chronic carriers of HBsAg followed-up at the CILM at Vientiane. The demographical, clinical, biological characteristics of the participants are described in **(Table 1)**.

In brief, because of inclusion criteria, all patients were more than 40 years (median 51). The age at which the disease is contracted - at birth, perinatally, or in infancy - generally predicts its clinical consequences. Severe forms of the disease were infrequent in our study population that was not selected for the severity of symptoms. A small subset of patients was diagnosed for liver cirrhosis (8.8%, n=11) while another more significant proportion

(24%, n=30) was suspected to be affected by significant liver fibrosis, according to non-invasive scoring systems (API, APRI, FIB-4). Ultrasound detection of liver steatosis was particularly frequent (52.3%). Around three-fourth of the patients were overweight or obese according to Asian standards (BMI>23) [13]. A remarkable proportion of patients reported a familial history of liver cancer (21.4%). Toxicant consumption (either alcohol or tobacco) was frequent (53.3% and 33.3% respectively). A majority of patients reported having eaten raw fish, a food habit known to be responsible for opisthorchiasis in the region.

HBV DNA was analyzed from the inaugural samples that had been used to determine the presence of a chronic infection; at that moment all patients were naïve from any antiviral therapy. HBV DNA was detectable by dd-PCR in 91 samples (72.2%).

Clinical and pathological correlations

Despite the absence of significant differences associated with age, HBV DNA prevalence, or viral load, disease expression and progression differed between the sexes. Men were showed greater exposure to fibrogenic toxicants (50.0% and 68.5% to tobacco and alcohol) than women (2.3% and 34% respectively, $P=0.0008$ and $P=0.0026$). Accordingly, significant fibrosis was present in 39.0% of men and in only 18.2% of women ($P=0.0173$, OR-2.88, 95%CI:1.1-6.7). Liver enzyme values and various blood parameters, including AFP, platelets, and Age-to-platelets index (API), were significantly worse in men. On the other hand, estimates of the de Ritis ratio (AST/ALT), Hepatic Steatosis index, BARD score and Systemic inflammation index were significantly worse in women.

As expected, patient age was an important predictor of disease progression. Liver fibrosis was more common among patients older than the median age (51.0 years) than younger participants (42.8% vs 20.6%, $P=0.0123$, OR=2.88; 95%CI:1.3-6.3). All indices and scores, that often include patient age for their calculation, were consequently degraded in elders.

Investigators have previously reported that long-term evolution of AFP is predictive of HCC occurrence [14]. Here, patients with AFP levels above the median (2.4ng/mL) were more likely to present with fibrosis (49.1% vs 17.3%, $P=0.0002$, OR=4.6, 95%CI:2.0-9.9). Furthermore, higher aminotransferase concentrations, and fibrosis scores (API, APRI, FIB-4), were associated with more severe liver tissue damage.

Patients with liver cirrhosis were slightly older (median=53.0 vs 50.0, $P=0.0483$), more often smokers (63.6% vs 30.7%, OR=3.9, 95%CI:1.1-12.5, $P=0.0422$) and more likely to report a familial history of liver cancer (54.5% vs 18.4%, OR=5.3, 95%CI:1.4-17.2, $P=0.0128$).

By comparison with other features, liver steatosis was mostly a benign condition in HBsAg

carriers. As expected, these patients were more often obese (34.8% vs 8.3%, $P=0.0005$, $OR=5.9$, $95\%CI:2.0-14.9$) and with higher BMI (median=26.3 vs 23.3, $P<0.0001$). However, ALT was the only laboratory feature significantly affected (median=44.0 vs 31.0 IU/mL, $P=0.0031$). By contrast, platelets (median=223 vs 209, $P=0.0224$) were well preserved as were most other biological variables suggesting a weakly fibrogenic liver disease. Regarding the already mentioned liver toxicants, tobacco consumption was associated with greater negative consequences than alcohol intake, possibly due to its intrinsic fibrogenic activity and its almost exclusive association with male sex.

Molecular characterization of HBV isolates

HBV DNA was detectable by dd-PCR in 91 of the 125 patient samples (72.2%). The median HBV DNA load was $3.7\log_{10}$ copies/mL. From these 91 samples, full-length or partial HBV genomes sequences were obtained in 79 cases (63.2%). The 12 samples for which we could not obtain amplimers for a subsequent sequencing step were characterized by significantly lower viral loads ($2.69\log_{10}$ copies/mL vs $3.91\log_{10}$ copies/mL, $P=0.0032$). The genotypes present were genotypes C (48.1%, $n=38$), B (31.6%, $n=25$), I (6.3%, $n=5$), D (6.3%, $n=5$), and C/D recombinants (5.0%, $n=4$). In two cases (2.5%), we could not accurately determine HBV genotype (Figure 1).

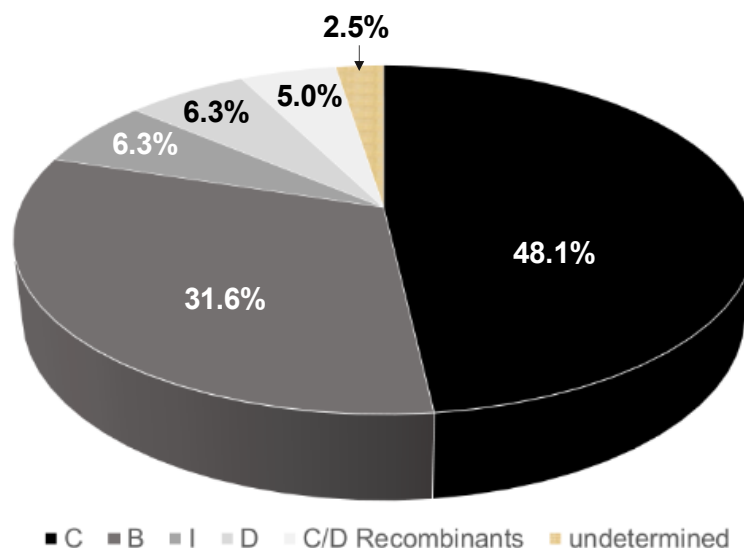


Figure 1: HBV genotypes in 79 chronically-infected patients from the CILM.

We focused on 31 HBV DNA mutation hotspots frequently described in the literature. The average alteration rate (mean \pm SEM) at these hotspots was $13.6\pm 2.6\%$ (see Figure 2). Concerning point mutations, the most frequently altered positions were nucleotides 2875

(preS1, c>a, Q10K, 52.9%), 1762 (enhancer 2, basal core promoter, a>t, 50.0%), 1764 (enhancer 2, basal core promoter, g>a, 48.3%) and 1896 (preC, g>a, 28Stop, 39.2%). The region most frequently affected with deletions was the preS1 domain (29.0%).

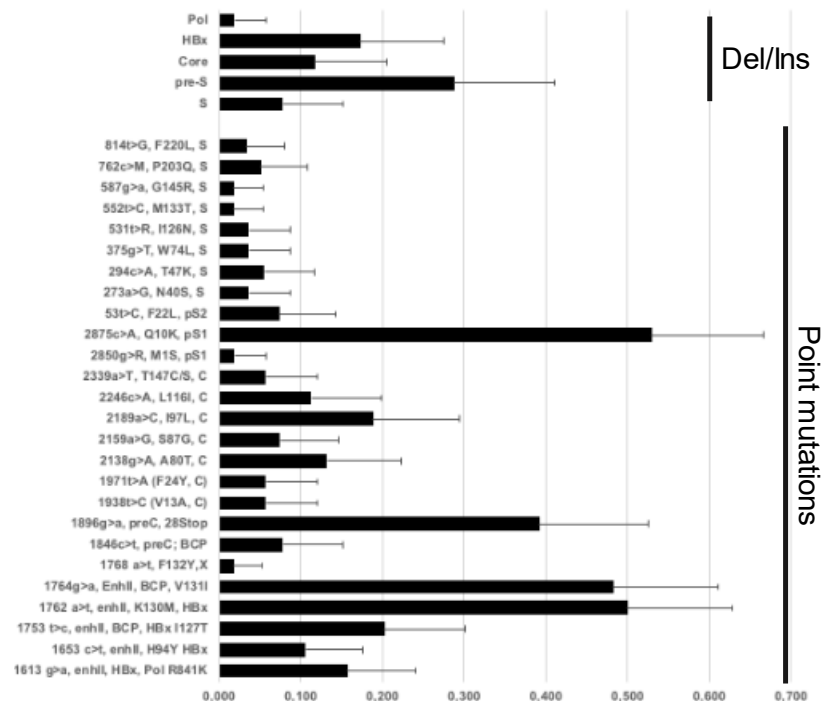


Figure 2: Prevalence of mutants at hotspots in 79 patients from the CILM

Clinical and Biological associations related to HBV infection

The comparison of HBV DNA(+) and HBV DNA(-) patients revealed, as expected, significant differences. Liver tissue was less likely to present as grossly normal in HBV DNA(+) patients (24.1% vs 42.8%, $P=0.0275$). Furthermore, values for aminotransferases (and more particularly AST) both at initial sampling and in 2020 were significantly worse among HBV DNA(+) patients, as was the case for APRI scores (1.01 ± 1.53 vs 0.56 ± 0.52 , $P=0.0089$). Patients with viral loads above the median value (>3.7 log₁₀ copies/mL) presented with important declines in some biological variables. The most important difference concerned primarily aminotransferase values measured at the time of diagnosis (e.g., median AST, 62 IU/mL vs 40 UI/mL, $P=0.0019$). But some differences were observable during the follow-up in 2020. There was an increase lymphocyte proportion in patients with high viral loads (median=38.0% vs 34.5%, $P=0.0159$) an observation that appeared largely dependent on an increased proportion of neutrophils (49.0 vs 53.0%, $P=0.0116$). Consequently, a prognostic scoring indexes such as the Neutrophils-to-Lymphocytes ratio (NLR, median=1.3

vs 1.5, $P=0.0039$) or Systemic Immune-Inflammation index (SII, median=230 vs 329, $P=0.0168$) were significantly lower in patients with high viral loads, a somewhat paradoxical situation.

We then examined HBV genotype-specific characteristics. Genotype C is generally considered responsible for more severe presentations of liver disease than other common regional genotypes of HBV [16]. In our study population, genotype C and its recombinant isolates were associated with higher rates of obesity (33.3% vs 11.4%, $RR=2.9$, $95\%CI=1.1-7.9$, $P=0.0311$) and higher values of the Hepatic Steatosis Index (median=34.9 vs 32.0, $P=0.0184$) a metric based on ALT, BMI, gender, and presence of diabetes. In addition, genotype C-infected patients tended to have a greater BMI, although this difference was not statistically significant (25.4 vs 24.6, $P=0.0788$). Genotypes B, D, and I were not associated with any salient clinical or biological characteristics. However, most patients infected with genotype D or I (80.0% vs 28.1%, $RR=2.8$, $95\%CI=1.5-4.3$, $P=0.0025$) were from Northern Laos (Huaphan, Phongsaly, Xienkouang).

We next examined associations between disease progression and mutations in mutational hotspots. Mutations at any hotspot located in the S gene were associated with a lower rate of liver steatosis (30.5% vs 75.8%, $RR=0.48$, $95\%CI=0.27-0.80$, $P=0.0018$) and a slightly higher Age-to-Platelet Index (API) score (median=5.0 vs 4.0, $P=0.0368$). Mutations in the Major Hydrophilic Region (MHR, located between 462 and 634 on HBV genome, amino acids residues 100-162 on HBs) were also associated with lower rate of liver steatosis (33.3% vs 66.6%, $RR=0.50$, $95\%CI=0.23-0.91$, $P=0.0405$) but a significantly higher rate of fibrosis (61.1% vs 30.5%, $RR=2.0$, $95\%CI=1.06-3.69$, $P=0.0423$) and more frequent presence of hypertension (33.3% vs 8.3%, $RR=4.00$, $95\%CI=1.21-13.30$). The API score was also worse in the case of mutations of the MHR (median=6.0 vs 4.0, $P=0.0302$). Deletions in the Pre-S region (S1 and S2 altogether) were associated with significantly higher levels of AFP (median=5.1 ng/mL vs 2.3 ng/mL, $P=0.0131$) suggesting a possibly important role of these molecular defects in liver tumorigenesis. A mutations in the pre-Core-Core mutational hotspots were more often found in patients reporting a familial history of cancer (36.6% vs 8.6%, $RR=4.21$, $95\%CI=1.21-16.12$). Finally, mutations in the HBx hotspot affected scores in the different liver fibrosis scoring systems (API, FIB-4, APRI, and PAAR index) indicative of the fibrogenic process in liver tissue (e.g. FIB-4 median=2.0 vs 1.2, $P=0.0048$). Systemic inflammation scoring systems (ALRI, ASPLI, ANRI) were also worsened above this mutation threshold (e.g. AST-to-Lymphocytes Ratio Index, ALRI, median=20.9 vs 13.4, $P=0.0097$). In reality, all these changes in metrics were connected

with a significant decrease in platelets count (186 G/L vs 212 G/L, $P=0.0174$) in combination with a sub-significant increase in AST (median=41.0 IU/mL vs 33.5 IU/mL, $P=0.0610$, ns). Neither the 1896g>a nor the 2875c>a mutants were associated with any notable clinical or biological change. By contrast, 1613g>a, located in the negative regulatory element (NRE), was the only mutant associated with increased inaugural viral loads (HBV DNA copies/mL median=157380 vs 5188, $P=0.0201$) [17]. In addition, BMI in 2020 was slightly higher in the case of 1613g>a mutation (26.4 ± 3.9 vs 24.6 ± 3.4 , $P=0.0499$). Meanwhile, 1762-4 ag>ta was only associated with higher initial AST values (median values, 70.0 IU/mL vs 44 IU/mL, $P=0.0392$).

No single mutation already present at diagnosis was associated with a specific clinical evolution. However, we observed that patients displaying more than two mutations at HBx hotspots and/or an MHR mutation at initial diagnosis were significantly more at risk to display worsened liver fibrosis scores (API, FIB4, APRI, PAAR) during follow-up. Altogether, significant fibrosis was suspected in 61.3% of these patients versus only 18.2% in the absence of HBV genomic alterations ($RR=3.3$, $95\%CI=1.5-8.7$, $P=0.0022$). This latter value was comparable to the proportion observed in patients without detectable DNA in plasma (17.1%). No significant difference in viral loads and time of follow-up was observed between both categories.

Table 1: The demographic, clinical, biological characteristics of the participants

Features of Participants to COCLICAN (n=125)	
<i>Demography</i>	
Age in years, median, (IQR)	51.0 (47.0-56.0)
Sexes M:F (n, %)	81 : 44 (64.8-35.2)
<i>Régional origin (birth)</i>	
North	48 (38.0)
Centre	67 (53.1)
South	11 (8.7)
<i>Liver diseases</i>	
HCC (%)	1 (0.8)
Cirrhosis (%)	11 (8.8)
Fibrosis (%)	30 (24.0)

Steatosis (%)	66 (52.3)
Grossly conserved liver (%)	36 (28.5)
<i>Viral features</i>	
HBsAg(+) in %	125 (100.0)
Detectable HBV DNA (%)	91 (72.2)
Baseline HBV DNA log 10 copies/mL, Median, (IQR)	3.7 (2.7-5.1)
anti-HCV(+) in %	0 (0.0)
HDV RNA (+) in %	0 (0.0)
Time since initial diagnosis (in years)	7.9±7.0
Received Hepatitis treatment (%)	72 (57.1)
Herbal medicine use (%)	64 (50.7)
<i>Spouses status (n=92)</i>	
Immunized	39 (42.4)
Tested	66 (71.7)
Not investigated	25 (27.2)
<i>Clinical features</i>	
BMI, 2020	25.1±3.5
Obesity (% , BMI>27.5)	27 (21.4)
Overweight (% , 23<BMI<27.5)	67 (53.1)
Type 2 Diabetes (%)	8 (6.3)
Hypertension (%)	18 (14.3)
Family history of cancer (%)	33 (26.2)
Family history of liver cancer (%)	27 (21.4)
Gallbladder abnormalities (%)	7 (5.5)
Kystic kidney (%)	14 (11.1)
<i>Other Risk factors</i>	
Tobacco consumption (%)	42 (33.3)
Alcohol consumption (%)	68 (53.9)
<i>Liver fluke (Opisthorchiasis)</i>	
Raw Fish consumption (in%)	
Often	5 (3.9)
Sometimes	58 (46.0)

<i>Rarely</i>	17 (13.4)
<i>Never</i>	45 (35.7)
Stool tested (n=115, %)	70 (60.8)
Positive <i>O. viverrini</i> diagnosis (n=72, %)	28 (38.8)
Deworming medication (%)	41 (32.5)
<i>Biochemistry (median, IQR)</i>	
Initial ALT (IU/mL)	49.0 (30.0-94.0)
Initial AST (IU/mL)	46.0 (29.0-73.0)
ALT (IU/mL)	38.0 (27.0-58.0)
AST (IU/mL)	33.0 (26.0-45.0)
AFP (ng/mL)	2.4 (1.5-4.2)
fcDNA-RPP30 log 10 copies/mL	4.0 (3.6-4.5)
<i>Hematology (median, IQR)</i>	
Hemoglobin (g/dL)	13.5 (12.4-14.5)
Mean Corpuscular volume (fL)	85.0 (73.0-90.0)
Leukocytes (/mL)	6400 (5400-7400)
Platelets (G/mL)	213 (177-241)
Neutrophils (%)	52.0 (45.0-58.0)
Lymphocytes (%)	36.0 (31.0-41.0)
Monocytes-Macrophages (%)	7.0 (6.0-8.0)
Eosinophils (%)	4.0 (3.0-7.0)
<i>Staging scores and indices (median, IQR)</i>	
APRI	0.50 (0.40-0.84)
FIB-4	1.33 (0.99-1.91)
API	4.0 (3.0-6.0)
de Ritis index, initial	0.93 (0.72-1.18)
de Ritis index	0.87 (0.68-1.11)
BARD score, 2020	2.0 (0.0-2.0)
PAAR Index	1.0 (1.0-1.0)
Gholam Model	23.7 (21.9-26.1)
Hepatic steatosis Index	33.0 (30.9-36.0)
Palekar index (partial)	2.0 (1.0-3.0)
Platelets-to-White Blood Cells Ratio (PWR)	33.0 (27.0-38.9)

Neutrophils-to-Lymphocytes Ratio (NLR)	1.4 (1.0-1.8)
Platelets-to-Lymphocytes Ratio (PLR)	93.4 (70.4-116.1)
Lymphocytes-to-Monocytes Ratio (LMR)	5.2 (4.0-6.5)
AST-to-Platelets Index (ASPLI)	0.15 (0.11-0.24)
AST-to-Lymphocytes Index (ALRI)	14.3 (10.7-22.2)
AST-to-Neutrophils Index (ANRI)	10.0 (7.7-15.4)
Systemic Inflammation Index (SII)	299 (200-418)
Systemic Inflammation Response Index (SIRI)	0.65 (0.43-0.89)
LIDISCO, initial (Mean±SD, median)	6.0 (5.0-8.0)

Discussion

The primary aim of this cross-sectional study of patients from Laos chronically infected with HBV was to describe at the molecular level viral isolates circulating in the country and to connect this putative molecular diversity with the clinical presentation of the disease. Such an analysis has not been previously reported despite the exceedingly high incidence of primary liver cancer in the country [18].

HBV genotypic diversity in the study grossly conformed to what was expected in a region characterized by a large diversity of endemic isolates. The dominant subtype C1 (formerly Cs for South), considered highly tumorigenic, is a typical subtype of Southeast Asia [19, 20]. It is apparently less dominant in Laos (47%) than in Cambodia (92%) or even in Guangdong (Southeast China, 100%). In Vietnam, C subtypes are generally much less frequent than genotype B [16, 21, 22]. Our study population was not selected based on presentation with severe disease, which may explain the relatively lower observed frequency of C subtypes; many studies focus on patients with severe liver disease which may enrich for subtype C1-infected patients. The presence of subtype C5, present in continental and insular (Indonesia, Philippines) Southeast Asia in a small subset of Laotian patients is the witness to the diversity of HBV lineages endemic in indigenous populations of the region [23-25]. The main B subtype in our study was B4 (around 18% of all strains) a value similar to that from a previous study performed on blood donors from Laos [5]. This subtype is also predominant

in Vietnam (63%) but only marginally present in Cambodia (4%) [16, 26]. Subtype B3, often considered a quasi-subgenotype, is another strain characteristic of insular countries in Southeast Asia (Indonesia, Philippines, and Malaysia) Also found in Laos and Yunnan, its north-to-south distribution most probably corresponds to migration routes of ancient populations [27]. Genotype I, the result of complex recombination events involving genotypes A, C and G, is also endemic to the region and believed to be the hallmark of population-speaking Tibeto-Burmese languages. Genotype I had spread in the mountain regions extending from Northeast India (Arunachal Pradesh) to Northern Vietnam, but most isolates are coming from South China. It is commonly isolated in Laos as well and was reported recently in Thailand, a country that counts a large minority of ethnic Lao people [28]. In our population, subtype I2 was more frequent than I1. The infrequent genotypes such as D and I were mostly isolated from patients originating from northern Laos, suggesting that greater strain diversity may be related to the higher HBsAg prevalence observed there [29].

As already mentioned, this cross-sectional study inherently lacks the power of case-control or true longitudinal surveys. However, with an average period of 7.7 ± 6.2 years between initial sampling and samples gathered in 2020, we have some insight of the impact of the different viral strains on disease evolution. Of course, our study describes, by definition, only those patients who were still followed up at the CILM in 2020, implying that those who died before being recruited, or who were potentially infected by more aggressive HBV strain, represent blind spots of our study. Another major shortcoming of our study regards the lack of data concerning some important serological markers, such as HBeAg/anti-HBe that are not routinely assayed in Laos.

Surprisingly, we observed that genotype C-infected patients were significantly more obese (34% vs 11%) or displayed a worsened Hepatic Steatosis Index (34.9 vs 32.0), while liver steatosis (66% vs 33%) or BMI (25.4 vs 24.6) were slightly increased, albeit without reaching the significance level [30]. Taken together, associations between genotype C and these four elements suggest this strain may cause major metabolic changes affecting lipid metabolism in the liver. However, the Laotian population is particularly diverse regarding ethnicity and genetics, and we cannot, therefore, exclude that obesity and HBV genotype C distribution overlap in particular subsets of the population [31]. For example, it has been shown by Chinese investigators that both the distribution of HBV genotypes and the prevalence of obesity vary significantly according to the ethnic minority considered [32-34]. The fact that

HBV in Southeast Asia is transmitted pseudo-vertically from mother to newborn in the vast majority of cases reinforces the links between this infection and familial or genetic lineages [35]. Consequently, it seems likely that the apparent connection between HBV genotype C and obesity in Laos is likely due to the confounding factor represented by ethnic diversity.

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DISCUSSION AND PERSPECTIVES

Anti-Hep B immunization and seroprotection in Southeast Asia (anti-HBs antibodies ≥ 10 IU/L)

Our study explored the seroprevalence of HBsAg in different Lao population groups, exposing populations where anti-hepB immunization did not take place or did not work. HBsAg is, thus, a biomarker for immunization failure (and its absence is an indirect marker for immunization success). To further assess the success of universal immunization, assays of seroprotective anti-HBs represent another more direct measure of the failure or success of public health measures.

The analysis of immunization efficacy can assess the immediate short-term protection in young children and the long-term protection of adolescents and young adults.

Concerning short-term protection, a study conducted in Central Laos in 2013-2014 focused on the levels of anti-HBs in Lao children aged from 9 to 50 months who received three doses of the anti-hepB vaccine. Worryingly, only 38% of these children presented with anti-HBs >10 mIU/mL, a concentration considered protective. The exact causes of this low protection are unknown but could include malnutrition or poor vaccine storage [145]. To further complicate the picture, infants enrolled in this study reacted disparately to the different components of the trivalent vaccine; unlike for the anti-hepB vaccine, a majority of children were found to have produced protective antibodies against tetanus (85%) and diphtheria (56%). Several hypotheses may explain this discrepancy. First, the stability of HBsAg might be lower than for diphtheria and tetanic antigens in tropical conditions. Given the well-known stability of HBV, this hypothesis seems unlikely. Second, the dose administered was maybe too low. Chinese authors recently considered that administering 20 μ g HBsAg instead of 10 μ g generates a stronger response [283]. Alternatively, the frequently anergic immunity to the administered HBsAg might be caused by the presence of HBV in the babies' environment. Indeed, the authors observed that the seroprevalence of anti-HBc in newborns was higher than the rate of chronic infection among mothers meaning that some infants were horizontally exposed to HBV by persons who were not their mothers and were overtly or occultly infected with HBV [284]. As mentioned above, the problem of horizontal transmission of HBV through casual (benign, everyday life) contacts is almost systematically

neglected in Southeast Asia and in Laos. As a consequence, most Laotians, including HCW, are not aware of the existence of this route of transmission. Overall, the study of Evdokimov suggests that in high endemic areas, immunized children remain frequently exposed to HBV and might develop an immune-tolerance, on the model of allergen desensitization, that prevents them from developing a protective humoral immune response. A search for the occult presence of HBV DNA in these children will have brought to light useful information in this regard.

However, a more recent 2017 study of children aged 8-28 months from Vientiane found a remarkable 86.9% developed anti-HBs ≥ 10 IU/L post-immunization, i.e. a 2.3 fold increase by comparison with the study of Evdokimov et al. It seems likely that the seroprotection rates in remote areas covered by local health centers is significantly lower than those obtained in central, provincial, and district hospitals. This difference may be related to lack of proper training of the HCW, or to difficulties in vaccines storage and/or transportation. Laos is primarily a rural country (64% of the population) with nearly 11,000 villages considered hard-to-reach communities not easily accessible by car. This situation might jeopardize the success of the universal immunization policy. Therefore, appropriate training of HCW and investment in securing an adequate cold-chain for delivery of pharmaceuticals are needed in remote health centers [10].

The low efficacy of anti-hepB immunization in Laos remains enigmatic. A study conducted in central Thailand in children who received full doses of anti-HBV vaccines reported that 98.5% had developed protective antibody titres [285]. Another study conducted in Malaysia on infants (age range 6.3–48 months) who completed the three doses of anti-HBV vaccination reported a similar proportion of 96.7% [286]. Not all vaccination campaigns are this successful. A 2009/2010 survey of infants in central Vietnam reported a rate of seroprotection of around 47.0%. The factors associated with the absence of protective anti-HBs were male sex, HBV infection, infants born to older mothers, and partial immunization course [287]. A slightly more positive 2016/2017 study in southern Vietnam, found that 68.3% of infants aged 12-24 months who had received all of the anti-hepatitis B vaccine doses within the schedule recommended by the EPI showed seroprotection. This rate is still rather low compared to rates in Thailand and Malaysia. To remedy the situation the authors suggest that anti-hepatitis B immunization should include an additional dose administered to infants between 12 and 24 months of age [288].

It should be emphasized that the design of vaccine efficacy studies has an obviously large impact on the results. For example, the systematic inclusion of mothers and newborns belonging to families (households) where there is no HBsAg carrier upwardly bias estimates of vaccines efficacy and the relevance of such studies for public health in a Southeast Asian context is questionable. In the region, we are unaware of any study examining the immune status of children born to HBsAg(+) mothers. In Laos and other Southeast Asian countries new studies are needed measuring anti-hepatitis B vaccine efficacy in newborns of HBsAg(+) mothers and/or newborns with HBsAg(+) siblings, both situations offering a very challenging context for immunization.

The evaluation of longer-term protection is also an important metric for the analysis anti-HepB immunization efficacy. A study conducted in 2018 compared the seroprevalence of anti-HBs between students born before and after 2001 and observed an increase from 13.2% to 21.9% ($p < 0.05$) in Vientiane and 3.0% to 19.7% ($p < 0.001$) in Bolikhamxay [11]. Thus, despite some improvement, the prevalence of anti-HBs remains very low, even for young adults who benefited from anti-hepB vaccination in infancy. Another survey conducted in Malaysia on medical students born 2-4 years after universal anti-HBV vaccine implementation in 1989 reported a far less favorable situation and was in this regard very similar to Laos. In that study only 28.0% of participants were protected 20 years post-vaccine administration although a single booster dose managed to reinstall seroprotection in 59.0% of previously seronegative participants. The authors recommended a systematic booster dose without necessarily testing for anti-HBs presence beforehand [289].

Anti-HepB immunization and Occult Hepatitis B infection (OBI)

Our study has shown that HBsAg prevalence in Laos in 2020-21 has decreased substantially compared to the (mostly) hypothetical values of 2000-01. This change is most likely the positive outcome of the universal immunization policy applied to newborns over the last 20 years. While results from studies of HBsAg anti-HBs prevalence are as yet far from ideal, it is commonly considered that Laos will slowly rid itself of chronic infection with HBV. This process will also inexorably take place due to the replacement of older “infected” generations by immunized younger generations. Presently, up to 41.0% of the Lao population was born after the implementation of universal anti-hepatitis B immunization and most members of these age categories received anti-hepatitis B vaccine. In addition, the age pyramid of Laos, dominated by the younger age categories (0-9 years=21.21% of the population,

>60=6.97%), combined with the unfortunately short life expectancy of Laotians (66.2 years for men, 70.9 years for women) will speed up the process of HBsAg disappearance.

However, an important point that should not be ignored is the problem of OBI that has the potential to change our vision of hepatitis B epidemiology. Prevalence and problems generated by OBI were mentioned in Part II, "Occult hepatitis B in Southeast Asia". OBI is a serious public health issue, especially in blood, solid tissue, or organ donation. In these therapeutics, OBI is responsible for cases of HBV transmission that can materialize in most cases in an overt acute or chronic hepatitis B infection [290]. However, we shall focus only on post-vaccination OBI. As the literature about OBI related to immunized subjects in Southeast Asian countries was rare, we collected information from throughout the Asia-Pacific region. An early study conducted in Taiwan in 2007, where the universal vaccination program is considered as successful [291], found that the prevalence of OBI was high (10.9%) in a population of children aged between 1-12 years. It was unclear if these children failed to respond to vaccine and were then later infected, or if they were infected prior to vaccination by escape mutants. An interesting but worrying study from India (2004-2009), reported that among 259 infants born to HBsAg(+) mothers who had received the full vaccine course including birth dose, the prevalence of OBI was a staggering 42%. OBI was even more common in infants who received anti-HBV immune globulins (anti-HBV IG). This study showed that the full administration of vaccine with anti-HBV IG at birth is not effective in preventing OBI in babies born to HBsAg(+) mothers. In this case, as in the previous study from Taiwan, vaccine escape mutants producing a modified HBsAg were likely transmitted from mother to newborns. These mutants are not recognized by some commercially available HBsAg detection assays. After active or passive immunization, HBV surface gene variants emerge or are selected by the immune pressure generated by vaccination of the host or by the anti-HBV IG injection [292]. A similar situation was reported in China between 1984 and 2009 in participants <18 years divided into two groups: unvaccinated cohorts (born before 1984) and vaccinated cohorts (born after 1984). The OBI prevalence tended to be higher in the vaccinated group of anti-HBc-positive participants (4.8% in vaccinated vs 1.7% in unvaccinated; $P=0.072$). as well as in participants seropositive for both anti-HBs and anti-HBc (5.6% in vaccinated vs 1.8% in unvaccinated; $P=0.025$). Thus, the OBI seemed to be more frequent in the post vaccination group than in the natural infections in unvaccinated group [293].

Taken together, these studies warrant confirmation in Southeast Asian countries, and suggest that in the next decades, the epidemiology of infections with HBV will likely change.

They also suggest that HBV is unlikely to be eradicated from populations as was the case for the smallpox virus in the 1970's (e.g. 1974 in Indonesia). We forecast that the virus will circulate mostly through occult infections maybe for decades in Southeast Asia. While such a scenario would lead to lesser disease burden than the classical acute and chronic forms of hepatitis B, it is reasonable to predict that OBI will play a role in liver diseases such as non-alcoholic fatty liver disease just as it has played a role in liver diseases from HCV-infected patients. In addition, it seems reasonable to consider the introduction in HBV vaccines targeting frequently isolated escape variants observed in Southeast Asian OBI patients (eg G145R in the "a" determinant of HBsAg).

Improving HepB-BD coverage in Laos

Administration of the anti-Hepatitis B vaccine birth dose (HepB-BD) within 24 h after birth is paramount to reducing mother-to-child transmission of the virus, a problem particularly important in highly endemic areas such as southeast Asia where this route of transmission common. In addition, the HepB-BD is absolutely necessary to meet the WHO goal of reaching 0.1% HBsAg prevalence in five-year-old by 2030 and eventual HBV elimination [294]. Unfortunately, according to our survey of the literature in the 11 southeast Asian countries less than 80% of newborns are covered and in countries such as Myanmar, Vietnam and Philippines HepB-BD coverage is even worse (Figure 26). Many causes can prevent the timely administration of HepB-BD at community level, such as shortage of vaccine doses, limited cold chain capacity and reliable transportation system, lack of effective sensitization and training of HCW, limitation of financial resources from a year to another, and finally lack of will in policy or strategy implementation and practices [295]. In Laos, anti-hepatitis B vaccine is theoretically available at all healthcare services from central, provincial, district and local levels. In addition, vaccination services are systematically available in villages once a month, meaning that all infants are able to receive the HepB-BD within one month (ref. unpublished). This observation implies that for a certain proportion of births, HepB-BD is simply not available at the time of birth. As a consequence, a recent survey found that HepB-BD coverage was only 72% (Figure 26) either because of vaccine stockout or because a lack of available trained HCW in the place of births [9]. Healthcare workers report being unaware of home births and thus unable to conduct timely postnatal care home visits. A pilot study conducted in rural Laos in 2014, provided mobile phones and phone credits to village health volunteers (VHV) and healthcare workers (HCWs) to increase

HepB-BD accessibility, as well as birth notification and increased home visits. This strategy has improved HepB-BD coverage, however, supplying mobile phone and credits to all delivering mothers is prohibitively expensive for a country such as Laos [296].

Currently, the policy and vaccination strategy in Laos seems as good as possible. However, a major challenge is communicating to mothers or their spouses the importance of coming rapidly after delivery to healthcare facilities to receive vaccinations and other needed medical care and empowering local HCWs to provide post-natal visits in the villages under their responsibility. In Indonesia, a very practical and cost-effective strategy for delivering the HepB-BD relies on midwives. Trained midwives are present in every village where they store anti-hepB vaccine in their homes. With this strategy, HepB-BD coverage increased significantly in three provinces of Indonesia from 2000-2002 [297]. At the same time, in Timor-Leste, 41% of eligible children with documented vaccination histories missed an opportunity for vaccination (MOV). This situation was partly explained by the fact that 33% of HCW admitted an insufficient knowledge of immunization practices. Another explanation for the MOV was the lack of vaccines in stock and problems with cold chain equipment in some localities. It was suggested that HCW training, together with improvement of vaccines availability at more healthcare facilities and cold chain equipment replacement are keys to reduce MOV and to improve national vaccination coverage [271].

In Vietnam, proposed solutions were not drastically different. HepB-BD administration started more than 10 years ago but coverage has barely reached 46.6%. Better training and providing HCWs better information need to be implemented [270], [288].

Concerning the various experiences in HepB-BD coverage, anti-HBV seroprotection and OBI, the countries in the region are facing similar challenges and resource constraints. As a consequence, countries should adapt their attitudes and practices to fit local realities. In Laos, which until recently had a high endemicity for chronic HBV infection (Figure 18), mothers are not always screened for HBV infection and do not benefit from anti-HBV IG. Routine vaccination of all newborns with anti-hepatitis B vaccine is thus paramount. As already mentioned, the main barrier to the success of this policy in Laos is the difficulty for populations to access to healthcare services, especially in rural areas [296]. Therefore, in my opinion the key element needed to continue to increase vaccine coverage in our country is to provide up to date training of HCWs at all levels, particularly those who live close to village communities. HCWs can provide information to communities and encourage populations to come and get healthcare at local facilities. A second aspect is the

communication between villages and healthcare centers. At the village level, there are many village health volunteers (VHVs), members of community health committees, and traditional birth attendants [298]. We can imagine that VHVs communicate the list of pregnant women in their village and their provisional dates of delivery (via phone) to the local health centers to ensure enough vaccine is available at the time of birth and that VHVs or traditional birth attendants (like in Indonesia) will communicate new births through the same channel. The involvement of local community members might facilitate greater compliance to the program. It necessitates, however, the sensitization of tens of thousands persons, an arguably difficult task. Furthermore, as this system is based on volunteering there is no guarantee it can work efficiently at the national level. Other factors could also contribute to improving the situation. In 2010, there were only 860 health centers in the country (1/8500 habitants) implying a huge workload for each center and pointing to a need for a greater number of health care facilities. Lastly, better equipment for vaccine storage and improved infrastructure, including cold chain transport, for vaccine delivery is needed but necessitates maintenance and thus increases costs.

After completion of the vaccination schedule, seroprotection is an important biological marker of vaccination success. A national survey of vaccinated children is called for to evaluate the efficacy of the vaccination program. Furthermore, infants born to HBsAg positive mothers should be tested regularly to ensure that they have developed a *bona fide* immune protection capable to maintain itself until adult age and whether they should be boosted by extra-doses of vaccine.

The last issue that I would like to highlight concerns OBI in Laos. Knowledge about OBI is very scarce in Laos. In the context of Laos, assays for anti-HBc should be systematically performed alongside screening for HBsAg at healthcare facilities. In case of positivity, HBV DNA detection should be performed. Unfortunately, such a diagnostic approach is not possible due to resource limitations. As a surrogate, surveys should be conducted to at the very least provide some clues about the magnitude of the problem. We propose conducting serological (anti-HBc detection) and molecular surveys (HBV DNA) on representative subsets of blood donors, immunized children, and on in patients with liver disease apparently unrelated to HBV i.e. HBsAg(-).

Conclusion and perspectives

An initial survey of HBsAg prevalence in Laos showed that focusing on the national situation of the country would not allow for a realistic appraisal of the situation, or of the problems HBV infections and universal immunization. Thus, we decided to extend the scope of our work to try and capture generalities of the experiences recorded in the 11 countries that compose the vast population ensemble of the 681 million habitants of SE Asia. Overall, despite substantial heterogeneity, HBV remains a serious health burden in the region, as most SE Asian countries are either intermediate or highly endemic for HBV (Figure 18). Consequently, all nations have committed to the WHO program of universal immunization of newborns designed to reduce national HBsAg prevalence by one Log₁₀ (from around 10.0% to <1.0%) in 2030 [217]. However, it appears that Southeast Asia is, for different reasons, one of the most challenging places to conduct such an ambitious immunization strategy. Firstly, in this region, young chronic carriers of HBV, who represent the natural reservoir and maintain the circulation of the virus, are known to shelter very high viral loads during the initial (immune tolerant) phase of infection [70]. HBV DNA loads measured in Southeast Asia are statistically higher than those observed in sub-Saharan Africa, Eastern Europe, or Latin America [299]. Both the pseudo-vertical mother-to-child and the poorly defined horizontal transmission routes of early childhood are thus responsible for the massive historical infection rates in unvaccinated populations. Secondly, the implementation of newborn vaccination depends largely on the timely delivery of vaccines either at birth or during the first week of life to prevent predictably-easy transmissions from highly infectious relatives. The problem is that Southeast Asian nations are often composed of rural and hard-to-reach populations making the implementation of universal immunization difficult. In addition, we discovered that two additional parameters, the large number of newborns that can overwhelm the capacity of thinly stretched healthcare system, and a high rate of births among adolescent mothers who are often less able to take care of the health of their children, may combine to sabotage the program's success.

Overall, to improve our understanding of the factors or problems facing HBV control in Laos, which so far appears to have been successful, further studies should be conducted, including:

- 1) Continuing exploration of the Hepatitis B situation in the north of Laos. Based on our survey and that of others (Nouanthang et al.), HBV prevalence is higher in the north than the south of Laos. The reasons for this situation are unknown, but likely include

more limited access to health care facilities and populations living in relative isolation who often possess a certain distrust of representatives of the central state, both of which may limit or prevent people in these communities from receiving about HBV. Furthermore, specific cultural practices may heighten infectious risks ultimately leading to higher HBsAg prevalence in Northern Laos.

- 2) Implementation of a well-designed and properly balanced prevalence study, based on the country's age pyramid and geographic distribution of the population, is needed to generate realistic and less biased estimates of HBsAg. Most HBsAg seroprevalence studies conducted so far in the 11 Southeast Asian countries are partial and, in my opinion, not necessarily representative of the populations living in this region.
- 3) As we know, in most cases, HBV is transmitted between Lao family members following pseudo-vertical and horizontal routes. However, most studies in our region focus only on mother-to-child transmission without assessing the share of infections attributable to horizontal transmission. Therefore, to quantify the magnitude of the different transmission risks in Laos, we intend to conduct a study in families with at least one HBsAg(+) member, to explore the presence of HBV DNA in the household environment.
- 4) HBV seroprevalence in children under 5 years of age in Laos (Figure 30) is on track to meet the WHO targets for 2030. A study assessing seroprotection and/or OBI in children who completed the vaccination schedule might represent an important step in assessing the true efficacy of the HBV immunization strategy in Laos. Furthermore, such a survey would represent a crucial source of information to identify factors associated with immunization failure, such as issues with storage or transportation conditions, knowledge and corresponding performance of Health care workers, the role of the parents, and other contingencies such as isolation from the village community.

5) Concerning liver cancer, a double infectious burden with hepatitis B and *Opisthorchis viverrini* (OV) seems to be main cause of its high incidence in Laos [91]. However, the true national incidence is still unknown. Thus, we shall carry on our study of 125 HBsAg(+) patients followed-up for diagnostic purposes at the Center Infectiology Lao-Christophe Mérieux in Vientiane. These patients were included in the frame of the Collaborative project for the early detection of liver cancer (COCLICAN), a project bringing together researchers from three continents (Europe, South America, Asia) in 5 countries (Belgium, France, Laos, Lithuania and Peru) with the objective of improving the detection of liver cancer emergence in Laotian patients with viral hepatitis B. In our second publication (in progress) mentioned above, we identify clinical, pathological, and molecular features of HBV infection in these patients. Then, to substantiate our hypothesis concerning the role of HBV-OV co-infection in liver tumorigenesis of Lao patients, we will try to detect circulating DNA fragments from the liver fluke *Opisthorchis viverrini* (OV) by droplet digital PCR (ddPCR) and at the same time proceed with the detection of circulating molecular biomarkers of preneoplasia, such as *TERT* promoter and *TP53* Arg249Ser (R249S) mutations.

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APPENDICES

Published articles

The Threat of Multiple Liver Carcinogens in the Population of Laos: A Review

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Review

The Threat of Multiple Liver Carcinogens in the Population of Laos: A Review

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Abstract: Laos is a landlocked country in South East Asia, ranking fifth for primary liver cancer incidence worldwide. Risk factors that might explain this worrying situation are poorly known. We conducted a review of the literature concerning the etiologies of terminal liver diseases in Laos. A double infectious burden with hepatitis B and C viruses and the liver fluke *Opisthorchis viverrini* seems to be the main cause of the high liver cancer incidence. Moreover, it was also suggested that mutagenic substances frequently found in tobacco, alcoholic beverages, fermented fish, and mold-contaminated cereals or nuts, which are all substances heavily consumed by Lao people, lead to the accumulation of DNA mutations in the liver cell genome causing tumor processes. However, the respective proportions of liver cancer cases attributable to each category of infections and substances consumed, as well as the histological nature of the neoplasia are still not precisely documented in Laos. The international medical and scientific communities as well as public health stakeholders should urgently consider the alarming situation of liver health in Laos to stimulate both research and subsequent implementation of prevention policies.

Keywords: primary liver cancer; hepatocellular carcinoma; cholangiocarcinoma; hepatitis virus; *Opisthorchis viverrini*; mutagens

1. Introduction

Laos is the least densely populated country with 30 people/km² and with the lowest life expectancy of 66 years in mainland Southeast Asia/Indochinese peninsula. According to Coker et al., the endemic presence of severe infectious diseases is a factor both preventing population expansion and increase of life expectancy in the region [1]. Furthermore, Laos is affected by a worrying pattern of non-communicable diseases that includes a high age-adjusted death rate in the 15–59-years population, high levels of tobacco use in men of 64%, and a significant proportion of the population is overweight (>35%) [2].

It was estimated that Laos has an incidence of primary liver cancer (PLC) of 22.4 cases/100,000 people, which is currently the second highest rate in Southeast Asia, behind Vietnam (see Figure 1). It was estimated that around 1100 new cases occur yearly in the country, equally affecting men and women, an unusual situation as worldwide women are usually relatively less affected by this

disease. However, these figures are mere estimates from the World Health Organization (WHO), taking neighboring nations as references. There have been significant variations in estimates of the incidence of PLC in Laos, illustrated by the last two GLOBOCAN reports. These variations are not supported by any obvious changes in the epidemiology of risk factors. There is currently no comprehensive cancer registry in Laos and very few pathologists. For reasons that will be discussed below, it is thus highly plausible that PLC incidence is largely underestimated in Laos. The underestimation of PLC cases is common worldwide, even in high-income countries [3,4].

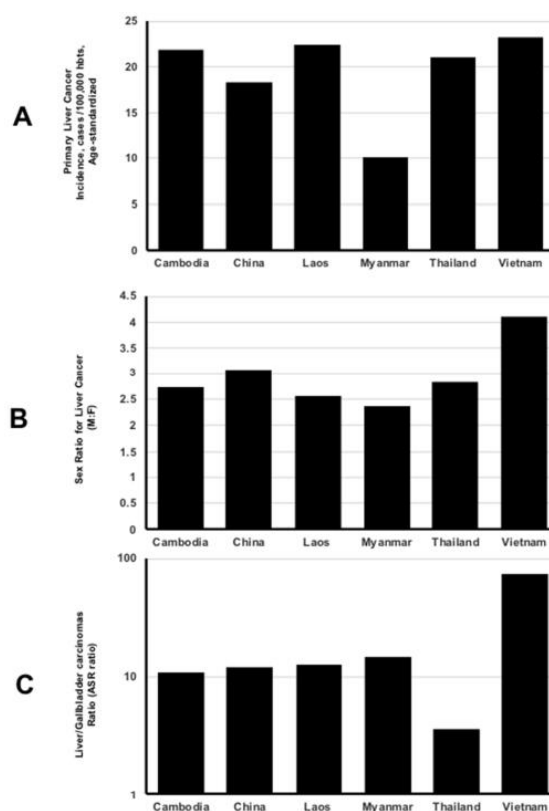


Figure 1. (A) Incidence of primary liver cancer (PLC) in Laos and neighboring countries. Myanmar is clearly less affected than all other countries of the region. (B) Male to female M:F sex ratios of PLC in the same countries. An increased sex ratio is observed in Vietnam suggesting a strong dominance of hepatitis B virus in PLC etiology. (C) Ratios of liver cancer/gallbladder cancer in the region. The low ratio observed in Thailand indicates the epidemiological importance of gallbladder and bile ducts carcinomas cases supposedly triggered by *O. viverrini*. In contrast, PLC is hyper-dominant over gallbladder in Vietnam, suggesting that the role played by flukes is less important in this country. All values have been calculated from Globocan 2018 <https://gco.iarc.fr/today/home>, accessed on 15 October 2020.

To explain the high incidence of PLC in Laos, we should first state that various infectious burdens, including chronic hepatitis B and C and frequent liver infestation with the fluke *Opisthorchis viverrini* (OV) afflict Lao people. The respective contributions of the different viruses and parasites to the local burden of PLC are, however, still unknown and therefore national incidences of cholangiocarcinoma (CCA), due to OV or hepatocellular carcinoma (HCC), due to hepatitis viruses are hypothetical. Furthermore, the impact of their interactions on liver carcinogenesis in case of co-presence has not yet

been properly characterized [5]. More remarkably, no description of PLC in the Laotian population is currently available in the medical literature.

2. Methods

Relevant literature included in this short review was collected in keeping with the guidelines proposed by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) [6]. Records were retrieved from relevant online databases' (Medline, PubMed, Google Scholar) collection of publications ending in October 2020. Our search was targeted on terms corresponding to liver diseases and carcinogenic factors in the region (hepatitis viruses B and C, *O. viverrini*, aflatoxin B1, nitrosamines, alcoholic beverage, fatty liver, liver cirrhosis, liver cancer, cholangiocarcinoma, hepatocellular carcinoma) and country names. We primarily focused our search on the literature treating Laos (or Lao People's Democratic Republic, Lao PDR, also frequently used in the medical literature), but when no record was available, we explored publications from immediately neighboring countries (Cambodia, Myanmar, Thailand, Vietnam). Relevant medical literature from China was only marginally approached and mostly focused on the Yunnan province, the only Chinese province to share a border with Laos. Articles were first selected after reviewing the title and abstract, and subsequently by reading the full text of papers initially retained.

3. Results

3.1. Viral Risk Factors

3.1.1. Hepatitis B Virus

In Laos, the situation of persistent infection with hepatitis B virus (HBV) corresponds to that of a highly endemic country. More than 8% of the population are chronically infected. A decade ago, 8.7% of blood donors were seropositive for HBV surface antigen (HBsAg) and this rate remained stable, but steadily increasing (at 9.6%), in a more recent study [7,8]. Additional evidence in adolescents from Vientiane and Luang Prabang confirms these preliminary estimates, detailing rates of 10.2% [9]. Furthermore, it was shown that more than 10% of HBsAg-blood donors present circulating HBV DNA, i.e., an occult hepatitis B infection (OBI). This observation implies that in reality, around 20% of the Laotian population might be chronically infected with HBV. Moreover, in a study conducted in Vientiane on 3857 HBV-infected patients, it was shown that 50% of patients were under 37 and almost 60% had a viral load higher than 10,000 IU/mL, the threshold value for high risk of developing liver cancer. Among the patients, 10.6% had both a high level of HBV DNA over 10,000 IU/mL and elevated aminotransferases. However, only 2.4% of them received treatment for chronic hepatitis [10,11].

Finally, several surveys conducted on mothers and immunized newborns indicated that hepatitis B vaccination is remarkably inefficient in Laos, and results in protection only in a minority of newborns [12–14]. In addition, despite mass vaccination campaigns, only a minority of newborns actually receive the birth dose of anti-hepatitis B vaccine [15]. Taken together, the data show that HBV will still be a significant public health problem in Laos in the next decades. Finally, it was demonstrated that the HBV strains circulating in Lao blood donors predominantly belong to subtypes C1 and B4. However, mixed infections are frequent and more importantly, recombinant viruses between B and C genotypes, including the rare genotype I, which is dominant in around 5% of cases [16]. This is surprising, as HBV is believed to be transmitted pseudo-vertically at birth from mother to newborn in Laos. The circumstances that explain multiple strain infections are poorly known, as are the consequences of this situation for disease progression.

3.1.2. Hepatitis C Virus

In comparison, hepatitis C virus (HCV) is a lesser problem, as seroprevalence for anti-HCV virus reaches "only" 1.1% in Lao people. In Laos, seropositivity for HCV affects older subsets of

the population, suggesting the virus circulated more widely in the past than now. In this regard, a case-control survey on acute hepatitis conducted two decades ago measured anti-HCV prevalence around 60% in patients over 50 years [17]. More recently, a retrospective study conducted on 1765 HCV-infected patients showed that, in Vientiane, 28% had high viral loads ($>6\log_{10}$). About 23% of patients had levels of aminotransferases indicative of liver damage >40 IU/mL, but less than 20% of them received antiviral drugs [10,11]. In Laos, circulating strains overwhelmingly belong to genotype 6 $>95\%$, the Southeast Asian version of HCV, with frequent viral isolates specific to the local population [18–20]. Overall, Laos exhibits the highest HCV genotype 6 diversity in Asia, suggesting that the country might be the place where this virus originated. Another specificity of Laos is that no rapid expansion of HCV subtype was observed in Laos during the 20th century, a situation that contrasts with neighboring countries. As for other HCV genotypes, the origin of genotype 6 entry in human populations is remote, dating back 1100–1350 years ago [18]. Its original host is unknown. During recent decades, a large number of novel hepacivirus species have been identified in various mammals including bats [21]. In this context, the well-known capacity of hepaciviruses to cross species barriers, illustrated by the recent transmission of equine hepacivirus to dogs, appears to be especially relevant [22]. In this regard, although animal hepaciviruses circulating in Laos are still unknown, there are a large diversity of chiropters [23]. These animals, well-known for their capacity to transmit viruses to humans, are occasionally consumed as bushmeat by the Laotian population, and also found in highly frequented Buddhist cave temples such the Pak Ou caves. These circumstances may possibly represent an appropriate configuration for the efficient crossing of species barriers by hepaciviruses [24,25].

Subsequent to its entry in the human population of Laos, HCV circulation could have been maintained for centuries through the practice of *Sak Yant* tattooing, Buddhist monk tattoos, or by variolation practiced by traditional healers for smallpox protection since its possible import in the early 19th century by the French [26,27].

Altogether, for both HBV and HCV, the medical literature describing disease presentation in Laos is scarce, while references describing the roles of the viruses in terminal diseases such as liver cirrhosis or PLC are non-existent [10,11].

3.2. Parasitic Risk Factors

3.2.1. Opisthorchiasis

The epidemiology of opisthorchiasis, responsible for bile duct inflammation cholangitis, cholecystitis, periportal fibrosis, and intra-hepatic cholangiocarcinoma, is well described in Laos. The prevalence of OV infection is high in Laos; an estimated 2 million people are infected. Southern provinces of the country, with a high proportion of populations living on the banks of the Mekong river are more heavily affected, with a 50% prevalence, while central areas like Vientiane are around 20%. A survey conducted in Saravane province showed that the prevalence increases with age, reaching 85% in older population subsets of 46–55 years, but tending to already reach a plateau of 70% as soon as 20 years of age [28]. This situation is primarily due to the consumption of raw, fermented, or brined cyprinoid fish *padek* infected with OV metacercaria in a context where absence of latrines facilitates the perpetuation of the parasite cycle by the daily shedding of parasites eggs in stools of infected patients.

3.2.2. Other Helminths

Furthermore, multiple parasitism with other helminths and protozoa is common in populations of southern Laos and might play some role in liver disease progression [29]. In this context, *Schistosoma mekongi* (Sm), responsible for hepatomegaly, liver fibrosis, and portal hypertension is present in the same region where OV prevalence is high and OV/Sm co-infestation was shown to be synergistic in liver pathology [30].

Overall, at a given time point, it is considered that more than 1% of OV-infected patients present liver lesions compatible with cholangiocarcinoma CCA [30–32].

3.3. Chemical/Environmental Risk Factors

It is well known that cancer is primarily a genetic disease characterized by the accumulation of mutations in a cell clone. Recent genomic data indicate that liver infections, rather than being mutagenic *per se*, instead create a tissue microenvironment that is favorable for tumor development [33]. Infected liver tissue becomes the target for decades of inflammatory infiltrates that kill both infected and uninfected cells. The resulting regeneration and DNA replication that occur on a daily basis as an emergency response to cell death are associated with intrinsically poor genome maintenance due to lack of time. During this accelerated tissue repair process, concomitant presence of mutagenic substances such as polycyclic aromatic hydrocarbons (PAH) produced by tobacco combustion, aldehydes, or carbamates present in alcoholic beverages, nitrosamines generated during fish fermentation (an important component of Laotian cuisine), aristolochic acid found in herbal teas or aflatoxin B1 (AFB1) present in cereals or nuts will invariably lead to accumulation of mutations that promote tumor processes [34–37]. As mentioned above, many of these mutagens are prevalent in Laos, such as PAH produced by tobacco combustion in a country where the majority of men and a substantial proportion of women are smokers [2]. Other mutagenic contaminants may be found in unrecorded spirits. Lao-Lao rice whiskey and Lao Hai rice wine consumption were studied only from an anthropological point of view and chemical contaminants in these home-made alcoholic beverages (Figure 2) are unknown [38,39]. It is known, however, that spirits resulting from rice fermentation may contain traces of carcinogenic substances such as ethyl carbamate, acetaldehyde, and/or arsenic [40–42].



Figure 2. Traditional alcohol production device still in use in the Sayaboury province of Laos.

Other important compounds, nitrosamines, are mutagens produced during fermentation of fish. Nitrosamines directly ingested with food or endogenously produced by OV-infected patients have been long suspected to play an important role in cholangiocytes transformation [43–46].

Another of the mutagens potentially present in Laos is AFB1, a carcinogenic mycotoxin produced by *Aspergillus* species. Unfortunately, there are no publications about AFB1 in Laos. However, the toxin has been found in food from all neighboring countries, Thailand, Cambodia, Vietnam, Myanmar, and China [47–50]. It seems, therefore, reasonable to assume that AFB1 is present in Laos as well. AFB1 is known for its molecular fingerprints in liver cancer, as it was shown to generate mutation due to transversion of G to T at codon 249 of *TP53* gene leading to the replacement of an ARG by a SER. Such mutations, clonally present in developing HCC, have already been found in neighboring Thailand [51].

Finally, it is worth briefly mentioning the issue of Agent Orange. Agent Orange (AO) was the principal herbicide derived from chlorinated phenoxyacetic acid used by the US Army during the Vietnam war (1955–1975). It was sprayed to remove the forest cover that was hiding North Vietnamese military units particularly along the Ho Chi Minh trail linking North to South Vietnam and occasionally passing through Laotian territory. Laos was thus affected to some extent essentially in the Savannakhet province. It was estimated that 1.8 million liters of AO were sprayed on Laos [52]. AO turned out

to be contaminated by dioxin (in fact tetrachlorodibenzodioxin, TCDD), an environmentally-stable non-mutagenic tumor promoter [53]. Recent surveys conducted in Laos on small series of breast milk, blood, and food samples observed only very low levels of contamination with dioxin [54,55]. US government acknowledges the fact that AO exposure is associated with some tumors in US veterans (lymphoma, soft tissue sarcomas, and possibly prostate cancer). Some Asian and European researchers found an epidemiological association of AO exposure with PLC development, while surveys conducted on US Army veterans did not reach similar conclusions [56–59]. It seems that the liver tumor-promoting activity of dioxin or phenoxyacetic acid derivatives develops primarily when combined with chronic HBV infection, a condition obviously more frequent in Indochinese populations than among US Army veterans [57]. We consider that molecular studies, rather than epidemiological ones, are warranted to uncover hypothetical fingerprints of dioxin exposure in Laotian patients with PLC.

3.4. Non-Communicable Liver Diseases

Chronic liver disease with an infectious origin or exposure to mutagens are not the only threats for liver health in Lao PDR. Non-communicable diseases (NCD) such as alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD) both characterized by the presence of significant steatosis in liver tissue emerged in the last decades as a major threat at a global level [60].

There is, actually, a dearth of data concerning both ALD and NAFLD in Laos. However, NAFLD is considered the most frequent chronic liver disease in Southeast Asia, with a 40% prevalence according to a recent meta-analysis [61]. Abdominal ultrasound assessment of the liver is the most convenient screening method to evaluate the consequences of *O. viverrini* infection. A recent survey observed a 12% prevalence of fatty liver changes in a series of 431 patients from southern Laos [31]. Consistent rates are also observed in similar patients from Thailand (12–19%) [62,63].

NAFLD is primarily associated with metabolic diseases (obesity, type 2 diabetes, metabolic syndrome) linked to excessive nutritional intake, lack of physical activity, and aging. The Laotian population is, however, far to have reached the affluence and food security observed in some of its neighboring countries and there is in the country a double nutritional burden [64]. A significant subset of the adult population is affected with malnutrition (underweight, body mass index, BMI < 18.5, 10%) while another segment is on the contrary affected by obesity with substantial variations in prevalence (6–25.2%) depending on which criteria (WHO or Asian-specific) are retained [2,65]. Overall, the prevalence of obesity in Laos is one of the lowest in the Western Pacific region [66]. Likewise, the prevalence of diabetes is mild in Laos (5.7%) [2,67]. As a consequence, the proportions of NAFLD only-associated deaths from liver cirrhosis (12–19%) or PLC (10%) can be considered as still limited in Laos [68,69].

The medical literature concerning ALD is similarly scarce in Laos. However, as most countries in Southeast Asia, alcohol per capita consumption increased significantly in Laos in the last decade. The annual consumption of alcohol by Laotians is 10.6 L/year, the highest in Southeast Asia. As a consequence, with a rate of 81/100,000, Laotian population is affected by the heaviest alcohol-attributable burden of death in the region [70]. According to a recent review, the alcohol-attributable fractions of death is 41% for liver cirrhosis and 39% for PLC, i.e., the highest proportions in Southeast Asia [68,69].

Overall, ALD represents the largest NCD affecting the liver and is currently a more worrisome health problem than NAFLD in Laos.

4. Conclusions

In conclusion, populations in Laos suffer from a heavy but imprecisely quantified burden of PLC. This situation is due to the pressure applied on Laotian populations by highly prevalent and deleterious risk factors such as chronic infections, either with hepatitis viruses or with carcinogenic trematodes from *Opisthorchis* genus, responsible for HCC and CCA, respectively (Figure 3). The disturbingly high level of alcohol intake *per capita* in Laos represents an additional contributor to the situation. The proportions of cancer cases, which can be attributed to each agent are unknown, just as are their

respective implications in liver cirrhosis or severe periductal fibrosis prevalence among Lao citizens. Likewise, virus or parasite subtypes/strains potentially endowed with an increased carcinogenic potency are unknown in Laos.

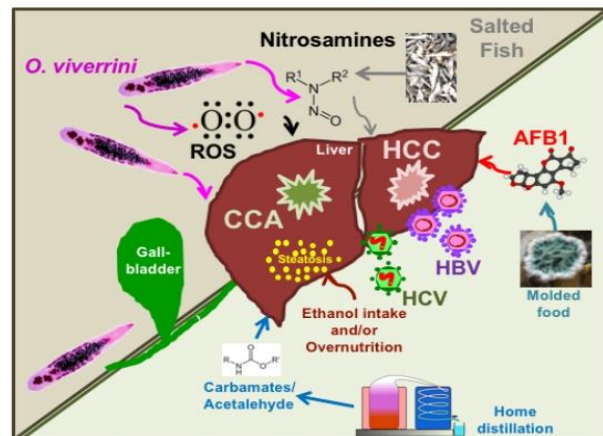


Figure 3. Schematic representation of the liver carcinogenic risk factors strongly prevalent in Laos. *O. viverrini*: *Opisthorchis viverrini*, ROS: radical oxygen species, HCC: hepatocellular carcinoma, CCA: cholangiocarcinoma, HBV: hepatitis B virus, HCV: hepatitis C virus, AFB1: aflatoxin B1.

Finally, carcinomas are invariably multifactorial, and the high incidence of PLC in Laos is also due to the exposure of infected patients to mutagens, e.g., aflatoxins, nitrosamines present in foodstuffs and acting synergistically with infectious agents to trigger liver tumorigenesis. Molecular fingerprints left by these chemicals in DNA have never been characterized in Laos.

Overall, the issue of severe liver diseases among Lao people could be tackled through a holistic approach gathering expertise from different fields. The production of data through sound clinical and translational studies is a crucial and mandatory milestone to design and implement prevention policies that will benefit the youngest and future generations of Laotian citizens.

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