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Comparison of Genomes of the *Hepadnaviridae* Family

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Abstract

Background: The *Hepadnaviridae* family is composed of small hepatotropic DNA viruses, divided into two main genera: *Avihepadnavirus*, which infects birds; and *Orthohepadnavirus*, which infects mammals. The human hepatitis B virus (HBV) is a member of the latter family and contains a relaxed circular double-stranded DNA genome of approximately 3 kb, and the objective of this study is to evaluate the genetic diversity of the genome of the *Hepadnaviridae* family. **Materials and Methods:** For this study, we evaluated the 26 NCBI reference sequences of the *Hepadnaviridae* family. **Result:** The two main genera, *Avihepadnavirus* and *Orthohepadnavirus*, show low similarity between them. The *Orthohepadnavirus*, to which HBV belongs, has two important hosts, monkeys and bats, while the genus, which infects humans, has greater diversity but is similar to the viruses that infect monkeys. The *Paraepadnavirus* isolated from the white sucker fish and the *Herpetohepadnavirus* from the Tibetan frog showed strong similarity with the *Avihepadnavirus* found in birds. The *Metahepadnavirus* isolated from the bluegill fish was the *Hepadnaviridae* which had the greatest difference, with less than 20% similarity using CLC Sequence Viewer. **Conclusion:** The *Hepadnaviridae* genomic replication cycle involves a late reverse transcriptase (RT) step. This polymerase, however, does not have proofreading activity, resulting in genetic variability in the *Hepadnaviridae* family.

Keywords

Hepadnaviridae, Hepatotrophic, Oncogenic, Hepatitis B

1. Introduction

All members of the *Hepadnaviridae* family have double-stranded DNA genomes, which are replicated through reverse transcription from a pre-genomic RNA template [1]. The taxonomic classification of these viruses includes the following levels: Kingdom *Viruses*, Phylum *Riboviria*, Class *Pararnavirae*, Order *Blubervirales* and Family *Hepadnaviridae*. Within the *Hepadnaviridae* family, two main genera stand out: *Orthohepadnavirus*, which infects some species of primates, rodents and bats; and *Avihepadnavirus*, which infects certain species of birds. Descriptions of members of the *Hepadnaviridae* isolated from fish, reptiles and amphibians have been published over several years, in different places around the world [2].

Each member of the *Hepadnaviridae* family exhibits a narrow host range, largely determined by the interaction between the virus and a specific cell surface receptor found on the host's hepatocytes. Despite significant genomic diversity among viral species, especially among *Hepadnaviridae* genera, all hepadnaviruses share several common features. Among them, the fact that all members have an extremely small (between 3.0 and 3.3 kb) and compact DNA genome that encodes overlapping open reading frames (ORFs) stands out [3]. Hepatitis B virus (HBV), which infects humans, is one of the prominent members of the *Hepadnaviridae* family. This virus is mainly hepatotrophic, with a relaxed circular double-stranded DNA genome having length of at least 3.0 kb, whose replicates use reverse transcription [1]. In turn, HBV, belonging to the *Orthohepadnavirus* genus of the *Hepadnaviridae* family, is approximately 3.2 kb long and has four overlapping open reading frames (ORFs), encoding polymerase (P) along with the core protein (C), surface antigen (S) and protein X. Based on intergenotypic divergences of at least 8% in the genome, HBV has been classified into at least 10 different genotypes [4]. Only limited cell culture systems are available to study the life cycle of *Hepadnaviridae*. In general, members of this virus family can only directly infect hepatocytes in the liver of their respective hosts, or highly differentiated primary hepatocytes *in vitro* derived from those hosts. This has hindered the ability of researchers to investigate natural HBV infections [3].

Hepadnaviridae has a partially double-stranded genome, with a diameter of 42 nm, which contains a relaxed circular DNA (rcDNA), genome composed of complete negative strands and incomplete positive strands. It has a host-derived outer surface lipid layer (envelope), which contains a surface antigen composed of large (L-HBsAg), medium (M-HBsAg) and small (S-HBsAg) proteins, in addition to an internal core protein called hepatitis B core antigen (HBcAg). The pre-S1 domain of L-HBs has a key role in determining infectivity [5]. All mammalian hepadnaviruses have a gene encoding an X protein, whose essentiality for viral replication and oncogenic properties has been demonstrated. Furthermore, all hepadnaviruses employ a unique genome replication strategy, in which the virus copies its DNA, from an intermediate RNA through reverse transcription using the reverse transcriptase activity of the viral polymerase. These viruses

stand out from almost all others that utilize reverse transcription for viral replication, due to several unique features, including the use of a DNA instead of RNA genome, and the fact that viral replication does not rely on integration of the hepadnavirus DNA genome in the host cell genome, for viral replication [3].

Polymerase is an extensive protein, composed of approximately 800 amino acids encoded by ORF-P. Functionally, it is divided into three distinct domains: the terminal protein domain, responsible for encapsulation and initiating negative strand synthesis; the reverse transcriptase (RT) domain, which catalyzes genome synthesis; and the ribonuclease H domain, responsible for degrading pregenomic RNA and facilitating replication [6]. HBV encodes three envelope proteins, known as surface antigens (HBsAg), from the ORF-S, which constitute the viral envelope: medium surface antigen (M-HBsAg); small surface antigen (S-HBsAg); and large surface antigen (L-HBsAg), derived from the pre-S1, pre-S2 and S regions, respectively (**Figure 1**). L-HBsAg is produced from its own mRNA transcript, regulated by the pre-S1 promoter. The small protein or S, is 226 aa long and composes a shared C-terminal region of the two longer envelope proteins. The medium protein contains the S sequence with 55aa N-terminal extension, called preS2. The large envelope protein, contains S, preS2, and an additional 108aa N-terminal extension called preS1 [3]. The surface antigen S-HBsAg is the main product of the S gene, while the L and M proteins are considered secondary species. Each surface protein contains a glycosylation site in the S domain. Additional modification of the L and M proteins occurs in the pre-S2 domain, with an N-linked oligosaccharide and myristic acid at the amino-terminal glycine residue of the pre-S1 domain. The distribution of these three envelope glycoproteins varies between types of viral particles, with little or no L and M proteins in the 20 nm particles, but having a relatively greater amount of L protein in the Dane particles [6]. The core antigen protein is also known as the nucleocapsid, while the 22 kDa pre-core protein, containing 183 amino acids, is called HBeAg. The precore-core open reading frame (ORF-C) includes two start codons that code for the precore (p25) or HBc (p21). The precore protein, translated from the precore mRNA, has the same sequence as HBc, but with an N-terminal extension of 29 amino acids. p25 is further processed in the endoplasmic reticulum (ER) and secreted as HBeAg through the secretory pathway of the ER-Golgi apparatus, where two proteolytic cleavage events contribute to the production of HaeAg [7]. The HBV X antigen protein (HBxAg) is encoded by ORF-X and performs several functions that are essential for various stages of viral replication, including signal transduction, DNA repair, activation of transcription pathways and inhibition of protein degradation, in addition to contributing to the oncogenic potential of HBV [5]. Studies indicate that the hepatitis B virus X protein (HBx) is recognized as a viral oncoprotein and plays a crucial role in the initiation and progression of hepatocellular carcinoma (HCC) [8].

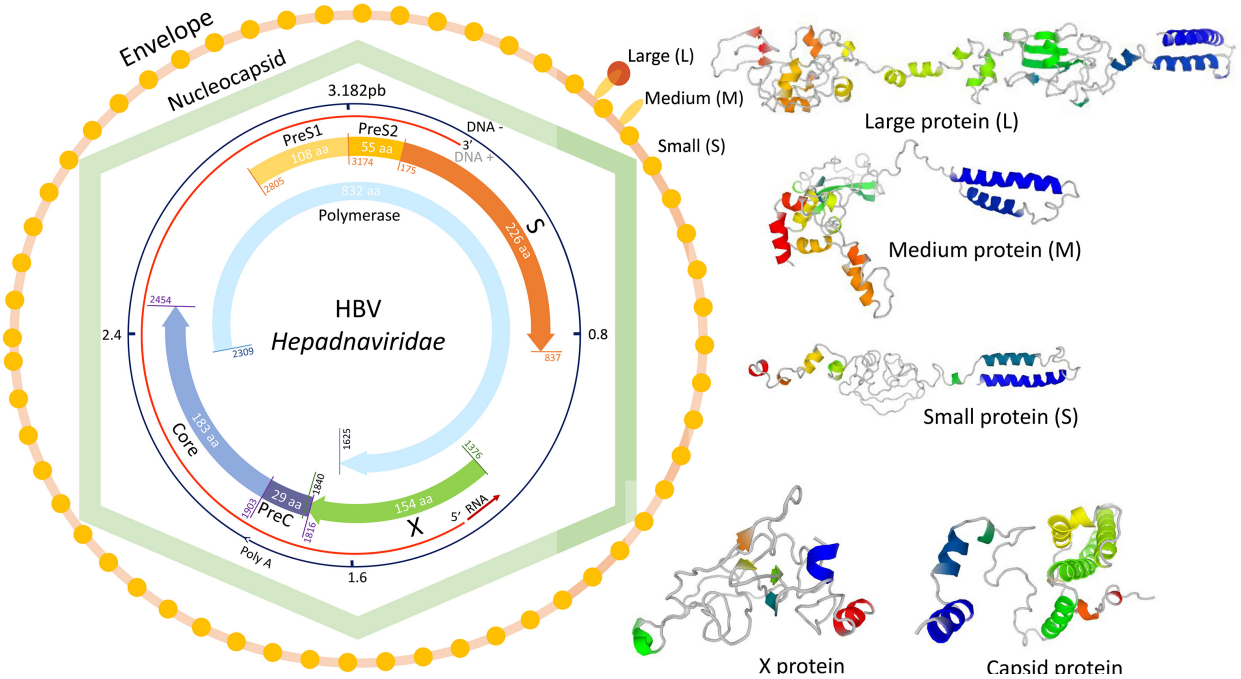

Figure 1. Structure and organization of the HBV genome strain *ayw* (NC_003977), belonging to the Orthohepadnavirus genus and the *Hepadnaviridae* family: ORF-P HBV reverse polymerase region; ORF-S pre-S1 region (large HBsAg protein), pre-S2 region (medium protein) and S region (small protein); ORF-X region X (Hbx); ORF-C precore region (HbeAg) and core region (HbcAg). GalaxyWEB was used to predict protein structures.

2. Materials and Methods

The National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database, is a compilation of taxonomically diverse, non-redundant, and highly annotated sequences. It offers a comprehensive, integrated and well-annotated set of sequences. Various levels of validation, additional annotation, and manual curation are applied to recording sequences in the RefSeq. Reference sequence is a synthesis of integrated information from multiple sources at a given time. These sequences provide a basis for integrating sequence data with genetic and functional information. They are created to establish reference standards for a variety of purposes, from annotating genomes to recording sequence variations in medical records [9].

Therefore, we downloaded the 26 reference sequences of the *Hepadnaviridae* family available until March 2024 from the NCBI, along with their main information, as shown in Table 1. The first step towards a phylogeny is to create a multiple alignment of several homologous DNA sequences. This process involves developing a hypothesis about how these sequences have changed over time. Phylogenetic trees aim to establish similarity between organisms based on their DNA sequences, helping to identify groups that are closely related and share common characteristics. For this purpose, we used the CLC Sequence Viewer v 8.0 (QIAGEN) to align and construct the phylogenetic tree of the *Hepadnaviridae* family, based on the NCBI reference sequences.

Table 1. Hepadnaviridae reference genomes available at NCBI until March 2024.

NCBI	GENOME	GENUS	YEAR	PLEASE	HOST	REFERENCES	PubMed
NC_074953	3368 bp	*Orthohepadnavirus*	2009	Gabon	Roundleaf bat	[20]	24043818
NC_001486	3027 bp	*Avihepadnavirus*	-	Germany	Herons	[21]	3418788
NC_024444	3377 bp	*Orthohepadnavirus*	2009	Gabon	Horseshoe bat	[20]	24043818
NC_074954	3149 bp	*Orthohepadnavirus*	2011	Panama	Tent-making bat	[20]	24043818
NC_076022	3165 bp	*Orthohepadnavirus*	2017	China	Asian grey shrew	[22]	30884426
NC_076026	3128 bp	-	2015	Cote d'Ivoire	Duiker	[23]	30893858
NC_043528	3182 bp	*Orthohepadnavirus*	2012	Brazil	Capuchin monkey	[24]	29428874
NC_040719	3187 bp	*Orthohepadnavirus*	2016	Australia	Domestic cat	[25]	29772771
NC_038503	3278 bp	*Orthohepadnavirus*	2011	China	Pomona bat	[26]	25193071
NC_035210	3024 bp	*Avihepadnavirus*	2015	Germany	Tinamou	[27]	29148393
NC_030446	3138 bp	*Herpetohepadnavirus*	2010	China	Tibetan frog	[10]	27334580
NC_030445	3260 bp	*Metahepadnavirus*	2009	USA	Bluegill	[10]	27334580
NC_028129	3179 bp	*Orthohepadnavirus*	2003	USA	Woolly monckey	[28]	12829821
NC_027922	3542 bp	*Parahepadnavirus*	2014	USA	White sucker	[11]	26378165
NC_024443	3368 bp	*Orthohepadnavirus*	2009	Gabon	Roundleaf bat	[20]	24043818
NC_024445	3149 bp	*Orthohepadnavirus*	2010	Panama	Tent-making bat	[20]	24043818
NC_020881	3230 bp	*Orthohepadnavirus*	2008	Myanmar	Long-fingered bat	[26]	23631923
NC_016561	3048 bp	*Avihepadnavirus*	2012	Poland	Parot	[29]	22183110
NC_005950	3024 bp	*Avihepadnavirus*	1999	Germany	Snow goose	[30]	10489339
NC_005888	3018 bp	*Avihepadnavirus*	2004	-	Ross's goose	Shi *et. al.*, 2004	Unpublished
NC_005890	3051 bp	*Avihepadnavirus*	2005	USA	Sheldgoose	[31]	15708992
NC_001896	3179 bp	*Orthohepadnavirus*	-	USA	Woolly monckey	[28]	9576957
NC_004107	3323 bp	*Orthohepadnavirus*	1988	USA	Woodchuck	[32]	3336938
NC_001484	3311 bp	*Orthohepadnavirus*	1984	USA	Squirel	[33]	6086950
NC_001344	3027 bp	*Avihepadnavirus*	2000	USA	Duck	[34]	2235507
NC_003977	3182 bp	*Orthohepadnavirus*	1979	France	Human	[35]	399327

3. Results

From the phylogenetic tree, we observed high genetic diversity and noted that the most studied and known *Hepadnaviridae* are those of the genus *Orthohepadnavirus*, which infects some species of primates, rodents and bats; and the *Avihepadnavirus* genus, which infects birds, as can be seen in **Figure 2**. These two genera have low similarity. The *Metahepadnavirus* isolated from fish [10] was the *Hepadnaviridae* with the greatest difference (less than 20% similarity-0.810) The *Paraepadnavirus* (white sucker) and *Herpetohepadnavirus* (Tibetan frog) showed the greatest similarity with the *Avihepadnavirus* found in birds. The prevalence of liver tumors in these fish (white suckers) was 4.9%, and 2.4% of the fish were

positive for both viruses and liver tumors, although this was not sufficient to correlate the presence of the virus with liver cancer in this fish species [11]. *Orthohepadnavirus*, the most studied genus and that to which HBV belongs, has two important hosts, monkeys and bats, with the HBV that infects humans being more similar to the species that infect monkeys (**Figure 2**, Group I). The diversity among those that infect bats is greater, as can be seen in Group II. The *Hepadnaviridae* NC_076026 isolated from duiker at the base of the tree does not have a defined genus, but has greatest similarity to *Orthohepadnavirus*. Three *Orthohepadnavirus* sequences are duplicated in NCBI reference sequences (NC_028129 e NC_001896; NC_024445 e NC_074954; NC_074953 e NC_024443). NC_028129 was deposited in 2018 as clone WMBV-2, and NC_001896 was deposited in 2023, both belong to the same bioproject (PRJNA485481). In general, only one complete RefSeq genome is created for each viral species. Occasionally multiple RefSeq records are created within a given viral species to reflect well defined genotypes or important laboratory and/or wild strains. Also, a separate RefSeq record is created for each segment in segmented viral genomes (Projeto NCBI Viral RefSeq).

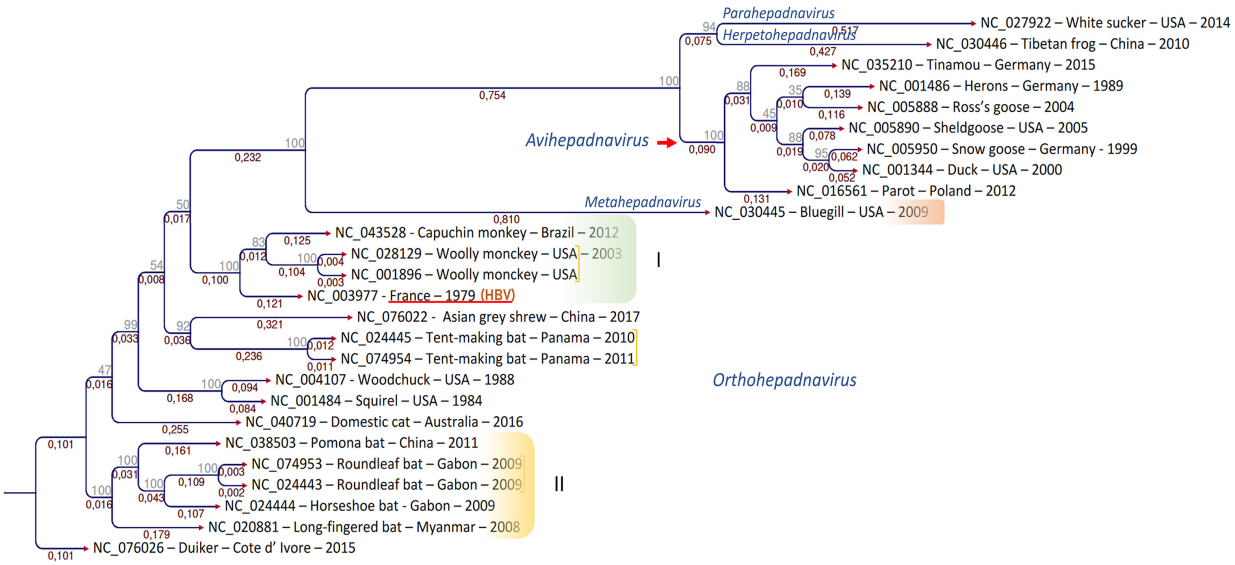

Figure 2. An unrooted neighbor-joining (NJ) phylogenetic tree illustrates the genetic relationships among all members of the *Hepadnaviridae* family (genetic distances are shown in red and bootstrap values are shown in gray on the tree).

4. Discussion

Sequence heterogeneity among *Hepadnaviridae* isolates is expected because of the absence of proofreading activities in reverse transcriptases (RTs), resulting in error rates that are much greater than those of cellular DNA polymerases, during chromosomal DNA replication. The complexity of the small genome limits the viability of mutants. All viral genes are overlaid by a second reading frame or receive regulatory signals such as enhancers, promoters and signals for RNA packaging and DNA replication. However, it is hard to extrapolate the actual natural mutation rate of hepadnaviruses from these results, because the degree of virus

replication varies among patients, due to differences in the immune response activity, against infected hepatocytes and other unknown parameters [12]. A crucial factor for calculating the evolutionary divergence of hepadnaviruses is the mutation rate inferred from the viral genome, which is defined as the number of base substitutions within the genome of each site per year, of continuous virus replication in the host. Virus mutation rates are often inferred by comparing the viral sequences of mothers and children, in cases acquired vertically or from chronic carriers during a specific time interval. However, these mutation rates are considered to have short terms and should be used with caution, since the HBV genome has a very complex structure, in which most genes are located in overlapping reading frames and viral regulatory elements incorporated into coding regions [13]. HBV genotypes/subgenotypes and HBV genetic variability are valuable for epidemiological studies, monitoring human migrations and predicting the risk of developing severe liver disease, along with the response to antiviral therapy. Furthermore, knowledge of the genotypes/subgenotypes is essential to implement preventive strategies. Hence, it is crucial to correctly assign new strains to their respective genotypes/subgenotypes, using consistent, unambiguous and generally accepted nomenclature [14]. Viral RNA transcription is fundamental for the persistence of these viruses in infected hepatocytes [12]. Eliminating viral covalently closed circular DNA (cccDNA) is still a challenge for researchers and pharmaceutical companies seeking to eradicate or control HBV infection. This requires detailed knowledge of the molecular mechanisms involved in cccDNA formation, as well as its intracellular stability and regulation during replication and transcription [15]. cccDNA is important for virus replication, it is synthesized in the hepatocyte nucleus in the form of stable minichromosomes, hindering the action of the immune system, and being difficult to degrade, it can be integrated into the host chromosome [16].

Three mechanisms have been proposed to explain the oncogenic properties of hepatitis B virus (HBV) infection: 1) inducement of chronic inflammation and cirrhosis; 2) expression of HBV oncogenic proteins; and 3) insertional mutagenesis in the genome of infected hepatocytes [17]. Like many viruses, HBV needs to optimize the cell environment for its replication. In the case of HBV, this involves inducing hepatocytes to emerge from quiescence and enter an active cell cycle. The status of cell proliferation pathways can have a significant effect on HBV replication [3]. It is noteworthy that patients suffering from chronic hepatitis B (CHB) infection have a 30-fold higher risk of developing hepatocellular carcinoma (HCC), which is the world's most common type of primary liver cancer and the third-most lethal cancer [18]. HBV infection can result in a range of liver diseases, varying from acute cases, including fulminant liver failure, to chronic forms such as hepatitis, cirrhosis and hepatocellular carcinoma. Many chronically infected people will experience a mild form of liver disease, with few or no symptoms of long-term morbidity or mortality, while other people with chronic HBV infection develop active disease, which can progress to cirrhosis and liver cancer [6]. HBV has

been identified in roughly 85% of HBV-associated hepatocellular carcinomas (HCC), and can integrate its complete or partial genome into the host's genomic DNA. The molecular mechanisms involving the integration of HBV DNA are still unclear. Indeed, several models have been described with regard to the relaxed circular DNA (rcDNA) or the double-stranded linear DNA (dslDNA) of HBV, and several genes have been identified that are affected by HBV DNA integration, such as genes related to cell proliferation, oncogenes, and long non-coding RNA genes [19].

5. Conclusion

The *Hepadnaviridae* genomic replication cycle involves a late reverse transcriptase (RT) step. This polymerase, however, does not have proofreading activity, resulting in genetic variability in the *Hepadnaviridae* family.

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Conflicts of Interest

The authors declare no conflict of interest.

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