Review Article

# A Comprehensive Overview of Hepatitis Virus Genotyping Methods

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## Abstract

The identification and differentiation of the various genotypes of hepatitis viruses are of great importance and can contribute to both clinical procedures and scientific research. There is a wide range of typing methods, which can be mainly divided into phenotypic and genotypic methods. Here, we focused on the available genotyping methods for hepatitis virus typing and tried to review and categorize them. This study aimed to study various hepatitis virus genotyping methods. In this study, to obtain a comprehensive overview of hepatitis virus genotyping methods, a perfect search was performed using related keywords in major journals and databases. Information was extracted from articles, analyzed, categorized, and compared. In analyzing the various articles, genotyping methods were divided into three different categories: Sequencing methods, hybridization methods, and methods based on DNA binding patterns. Sequencing-based methods were cited as the gold standard and the accuracy of other methods was compared to them. Hybridization-based methods, which were also used in commercial kits, include several methods. DNA binding pattern-based methods were mainly based on PCR with genotype-specific primers or RFLP. Although molecular methods allow accurate, sensitive, and reproducible genotyping, there is still much room to be exploited using emerging methods.

**Keywords:** Molecular Typing, Genotyping Techniques, Hepatitis Viruses, Polymerase Chain Reaction (PCR), Sequence Analysis, Polymorphism

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## Introduction

**Why typing is important?** Improving a general understanding of the diverse world of microbial taxonomy has shown that taxonomic rank (phyla, families, genera, and species) is not sufficient to describe microbial diversity[1].

It has been shown that microbes (bacteria, fungi, and viruses) can differ genetically within the same species and perform very different functions. Thus, microbial diversity below the spice level proved to be important and was called strain[1]. From a microbiological point of view, a strain is a result of culturing a single isolation in a pure culture, which ultimately consists of a series of cultures derived from the initial colony[2]. A strain can also be determined among other strains of the same species by exhibiting stable and heritable genotypic (molecular characteristics) and/or phenotypic (biological, serological characteristics) traits[3,4]. The strain is the

result of culturing a single isolation in a pure culture, which ultimately consists of a series of cultures derived from the initial colony[3,4]. The strain can also be determined among other strains of the same species by exhibiting stable and heritable genotypic (molecular characteristics) and/or phenotypic (biological, serological traits) traits. Genomic variations between microbial strains can range from single nucleotide variants (SNVs) to acquisition/loss of genomic elements including genes, operons, or plasmids [5,6].

SNVs occur particularly in viruses during viral replication because almost all viral polymerases (RNA/DNA polymerases) can be proofread. This leads to the emergence of different genotypes [7].

Identification and differentiation of specific strains by a defined pathogen species are of great importance and can contribute not only to effective measures for prevention, control, and treatment of infections, but also to the study of pathogen biology, pathogen-host interaction, modulation of immune homeostasis, and pathogenic potential[4-6].

The process of strain differentiation, termed "typing," is, therefore, a fundamental component of microbiological studies[8].

**Typing aims & strategy:** The goal of typing studies may include clinical, environmental, or industrial exploitation. Typing can be considered either comparative (comparing outbreak-related and unrelated isolates) or definitive (library) typing [8].

Previously, typing was based on serological characteristics or phenotypic differences, especially for bacteria and fungi. Based on differences in traits and growth requirements such as shape, size, staining characteristics, kinetics, metabolic function, and phage resistance, phenotypic typing is divided into serotyping, biotyping, bacteriophage or bacteriocin typing, protein typing, and antimicrobial susceptibility profiles, which can be assessed independently of genomic evaluation [9-11]. In other words, phenotypic methods examine the products of gene expression. Because gene expression is affected by many spontaneous or environmental stimuli, the results obtained are not reproducible. They also lack discriminatory power between closely related strains[8].

The development of new DNA-based analysis technologies in the late 1990s, called molecular analysis, led to the development of a more accurate typing method, molecular typing [10,11].

Molecular typing focuses on the use of DNA sequence information and the genetic content of microbial elements, so it benefits from the accuracy, high resolution, and uniqueness of the genetic profile that can be used as a microbial fingerprint, but it requires specialized materials and equipment [12]. Therefore, genotyping has become an important basis for studying subspecies diversity and the dynamic nature of the prokaryotic genome.

Genotyping is especially important for viruses that are too small to be typed by phenotypic methods.

**Typing of Hepatitis Viruses:** Among microorganisms, viruses vary in their genomic material from single-stranded RNA/DNA to double-stranded RNA/DNA. Hepatitis viruses are an excellent example of the diversity of viral genomic material[13]. Viral hepatitis is a public health threat and about 2.3 billion people around the world are infected with at least one of the hepatitis viruses. There are five hepatitis viruses, hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). Approximately 90% of deaths from viral hepatitis are caused by HBV and HCV[14].

Studies around the world have shown that there are 7 genotypes of HAV (I- VII), 6 genotypes of HBV (A-F), seven genotypes of HCV[1,2,6,7], genotypes of HDV, and four genotypes of HEV (1-4), all divided into subtypes[15-17].

Genotyping of hepatitis viruses using reliable methods is of great importance for clinical trials because differences in genotype lead to differences in disease outcomes and response to different therapies. Therefore, hepatitis virus genotyping is now a part of pretreatment testing[18,19].

Hepatitis virus genotyping should generally consist of two phases. First, the most conserved parts of the viral genome are used for diagnosis and detection of the virus, and then variable genomic regions are used for genotyping. The 5' UTR is the most conserved genomic region in both HAV and HCV genomes. In HAV, the C-terminus of VP3, the N-terminus of VP1, and the VP1-2A junction region, and in HCV, the E1 and E2 regions are used to identify and genotype

different isolates[20]. The pre-S1 and S genes are the most type-specific regions used for HBV genotyping[21].

According to Wenjun Li et al, the results of molecular typing can be divided into three main categories, including DNA sequencing, DNA binding pattern, and DNA hybridization methods [12]. If we divide the different strategies into these 3 categories, we are dealing with different genotyping methods that differ in terms of accuracy, sensitivity, and reproducibility.

It is worth mentioning that as the first step of genotyping, PCR and RT-PCR are widely used to amplify and amplify the total amount of viral genomes on a DNA and RNA basis, respectively. Therefore, almost all genotyping methods depend on PCR-amplified fragments and inevitably have all the advantages and disadvantages that PCR itself has[22].

For the conclusion of this review article, the most important journals and databases such as Nature, ScienceDirect, Scopus, and PubMed were consulted. The most relevant articles from 1980 to the present were examined using keywords such as genotyping, molecular typing, hepatitis viruses, etc. Various articles were reviewed from a technical point of view. Different molecular typing techniques for hepatitis viruses were extracted from the articles, analyzed, categorized, and compared with each other, and finally summarized and divided into different sections.

**DNA sequencing-based methods:** Genotyping using DNA sequencing methods are based on the study of sequence homology between isolates and reference sequences available in GeneBank. Therefore, these methods are necessarily followed by phylogenetic analysis (such as neighbor-joining or MEGA4 methods). Although whole genome sequencing provides comprehensive data on genotypes and subtypes, it is time-consuming, cumbersome, and ultimately unsuitable for clinical purposes. Partial sequencing, i.e., sequencing of specific fragments of the viral genome, such as the 5' UTR, core gene, or NS5B for HCV, surface antigen gene/overlapping polymerase gene for HBV, VP1/2A cross for HAV, and C termini of HDAg for HDV, benefits from the advantages of sequencing-based methods and is not as cumbersome as whole genome sequencing [20]. As mentioned earlier, PCR/RT-PCR amplification of the fragment of interest is a prerequisite for the sequencing method. Sanger sequencing is the most widely used method, which is slowly being replaced by next-generation sequencing (NGS). NGS has advantages over traditional Sanger sequencing in predicting small differences between species. The Sentosa SQ HCV genotyping assay, which uses next-generation sequencing technology, is the standard approach for genotyping HCV and agrees well with Sanger sequencing, according to the study by Rodriguez et al.[20].

Commercial kits for HCV and HBV based on direct sequencing of the 5'UTR for HCV and both strands of the surface antigen gene/overlapping polymerase gene for HBV are available. Although these kits are no longer used for HCV because the sequencing of the most conserved sequence (5'UTR) results in misclassification compared with NS5B sequencing, the kits for HBV provide a powerful and time-saving tool[23]. Therefore, it is important to select regions within a viral genome that are not fully conserved for sequencing-based genotyping methods that truly affect the results obtained.

According to Serin et al. who used S-gene cycle sequencing for HBV genotyping in Turkey, sequence-based genotyping methods are best suited for genotyping viruses among other microbes because polymerases in viruses are error-prone and make mistakes during replication that lead to differences in individual nucleotides and interfere with other genotyping methods[24].

Sequencing has also made inroads into HDV genotyping. The resulting fragments of nested RT-PCR on partial delta antigen or another specific region can be subjected to cyclic sequencing and finally phylogenetic analysis as defined in several studies [25].

Whole genome sequencing and phylogenetic analysis used to be common methods for HEV genotyping. However, due to the time-consuming procedures, attempts are shifting to partial sequencing of the genome and searching for specific genomic regions that can serve as representatives of the whole genome. The introduction of a region in the RNA-dependent RNA polymerase domain and the ORF structure are the results of attempts in this area[26,27].

Some developments in conventional sequencing-based

genotyping methods will lead to new, more efficient methods. For example, because of some biased results in high-throughput sequencing and the inability to identify mixed infections, the amplification step has been cited as the main cause of these problems. Therefore, attempts have been made to use NGS assays independently of the PCR step as a solution, referred to as direct sequencing [28]. Another example in this area is the 2017 findings of Bai X et al. They presented a novel method for clustering HBV genotypes based on the frequency of word patterns from NGS reads instead of alignments in the pre-S region. They succeeded in presenting the performance of word patterns in clustering HBV and predicting the disease and also showed a correlation between these two methods[29]. Deep sequencing, i.e. multiple sequencing of a specific genomic region, is also a type of NGS that, if accompanied by phylogenetic analysis, could solve the problems of identifying indeterminate subtypes in mixed infections and could find its way into clinical diagnostics [30].

Special materials, instruments, and skills are always needed for sequencing. Recently, however, Virtanen et al. proposed a new stable and robust sequencing-based genotyping method using one-step reverse transcription and PCR amplification of the 5' UTR. This method uses simple Sanger sequencing followed by a web-based genotyping and analysis tool such as Chromas/4peaks or Standard Nucleotide BLAST. This method does not require any special analytical skills or tools and is very robust [31].

Apart from the time-consuming and complicated procedures that make them unsuitable for large numbers of samples, sequence-based methods are the gold standard in genotyping and are the most widely used due to their accuracy and improvement in experimental techniques[32].

Sequencing-based methods are more powerful than other genotyping methods in identifying and genotyping mixed infections. From a clinical perspective, the identification of mixed infections is very important for infection management, but most of the available methods are not able to do this[33].

**DNA banding pattern-based methods:** The naming of these methods arises from the fact that all methods based on DNA binding patterns involve an electrophoresis step (agarose gel/polyacrylamide gel). The components used in electrophoresis can be obtained either by type-specific PCR or by digestion with restriction enzymes, which are presented below.

**PCR-based methods:** Okamoto et al. proposed for the first time a PCR-based method for HCV typing. To obtain more accurate results, they performed the PCR procedure in two steps (nested PCR), the first step on HCV cDNA by universal primers and the second on the products of the first step by universal sense primers and four type-specific antisense primers. Examination of 44 HCV isolates for their nuclear gene yielded universal and type-specific sequences, any region of which served as a PCR primer. The final products were subjected to agarose gel electrophoresis so that different sizes of PCR products were expected to be representative of different HCV types[34]. This method was also used by Chayama et al. for the NS5b region of HCV[35].

Apart from its advantages, modified versions of this method were needed to improve its sensitivity and specificity. Okamoto et al. themselves again proposed a modified version in which both the sense and antisense primers in the second PCR were type-specific[35]. Another modified version was developed based on more type-specific primers for HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a, which improved the sensitivity and accuracy of the result compared to Okamoto in identifying 23 isolates [36].

Type-specific nested PCR for HBV has also been performed with type-specific primers designed for the region between the pre-S1 and S genes or for the S region itself as type-specific regions, based on the fact that the length of the amplicons is type-specific and the amplicons can be separated by electrophoresis. Type-specific PCR for HBV showed high accuracy, consistent with sequencing results in [37].

Multiplex PCR was considered to make type-specific PCR more effective. This method, first proposed for HBV, uses different genotype-specific primers simultaneously in one reaction and is robust, sensitive, suitable for large-scale analyses, and can also detect mixed infections, but is expensive[38,39].

To avoid subsequent PCR manipulation and ultimately save time, real-time PCR has found its way into diagnostics. Real-time PCR, also known as quantitative PCR, has found its way into genotyping.

This method uses both type-specific primers and fluorescent probes such as TagMan or cyber green simultaneously, which speeds up the analysis. This technique has recently been used for HCV genotyping based on analysis of the former 5' UTR and the NS5B region and has also been successful in subtyping, as confirmed by sequencing[40].

Significantly, real-time PCR is a sensitive, reliable, and cost-effective tool for both HBV diagnosis and genotyping in the form of multiplex real-time PCR performed for genotyping and quantification of different genotypes at the same time using type-specific TaqMan probes[41,42].

Genotyping by quantitative reverse transcriptase PCR (RT -qPCR) based on single nucleotide polymorphism (SNP) was proposed by Coudray-Meunier et al. for the 5'-UTR region of HAV. This method, which is suitable for subtyping, primers, and hydrolysis probes is designed to span the SNP site using software such as Beacon Designer. The hydrolysis probes were labeled at the 5' and 3' ends with fluorescein and quencher, respectively. The presence of each type leads to the separation of fluorescein and quencher from the probe and finally to the generation of a fluorescent signal. The results were in complete agreement with the sequencing. They believed that this method, developed to detect small amounts of HAV from contaminated samples, can be useful not only in identifying genotypes from human samples but also in identifying mixed infections[43].

As mentioned above, the detection of mixed infection is very important from a clinical point of view, but type-specific PCR is not a reliable detection method in this case, as non-specific primer annealing can lead to a false positive result[44].

To enable accurate, specific, and simultaneous genotyping of mixed infections, primer-specific extension analysis (PSEA) and primer-specific mismatch extension analysis (PSMEA), a modified version of type-specific PCR, have been introduced and have proven to be very powerful. Both of the aforementioned techniques select genotype-specific primers with a genotype-specific nucleotide at the 3' end. PSEA exploits the inefficiency of Taq DNA polymerase in extending a mismatch at the 3' end of a primer in such a way that the efficiency of primer extension is affected by the complementation between the 3' end of a primer and the template. Whereas PSMEA uses a DNA polymerase that has a 3'→5' exonuclease-correcting activity like pfu (Pyrococcus furiosus) DNA polymerase, which starts with the exonuclease and avoids primer extension when a mismatch is present. The extension product is then visualized by electrophoresis[45,46].

PSMEA is practical, rapid, inexpensive, the most sensitive method for detecting mixed genotypes and has also been practically recruited for large cohort studies testing for HCV[63,64].

No report was found on the use of the banding pattern-based method based on PCR for HDV and HEV.

**Restriction Enzymes-based method:** Nakao et al. first proposed a restriction enzyme-based genotyping method for HCV typing in 1991, leading to the identification of 2 types (K1 and K2). They used RT-PCR products of part of the NS5 gene for RFLP (Restriction Fragment Length Polymorphism) by 3 restriction enzymes[47]. In 1994, McOmish et al. modified the proposed RFLP method by using radiolabelled nucleotides in PCR before RFLP. in 1996, Pohjanpelto et al. omitted the step of incorporating radiolabelled nucleotides and used ethidium bromide again to simplify the procedure. They also used the diagnostic PCR products of 5' UTR for RFLP [48].

This method is also used for other hepatitis viruses. In this field, RFLP-based genotyping of HBV for the pre-S and S genes is performed by selected enzymes based on genotype sequences according to GeneBank. Several studies have shown that by modifying the factors involved in RFLP, such as the type of enzymes, the accurate typing of more genotypes is possible. A limitation of this method for HBV is related to the fact that the HBV genome is highly variable, which can lead to restriction sites being destroyed or new ones being created, leading to various misunderstandings. RFLP is also unable to detect mixed infections[49-51].

For HAV, B. Goswami et al. proposed an RFLP-based method in 1996 and felt that resolution could be significantly increased by combining RFLP with single-strand conformation polymorphism (SSCP) analysis. SSCP analyses the behavior of radiolabelled and denatured restriction fragments in acrylamide gel

electrophoresis[52].

Studies in the literature have shown that there is a link between the RFLP-based method and genotyping of HDV and HEV [53]. Here, Gouvea et al. proposed RFLP analysis for HEV on reverse transcribed fragments from four different regions of the viral genome (segments of the helicase, polymerase gene, and segments of the capsid gene), which was completely suitable not only for genotyping but also for subgenomic differentiation [54]. This method depends on both the PCR step before restriction enzyme digestion and the type of enzymes selected. The choice of enzymes is therefore of great importance and can influence the result of genotyping. Extensive sequence data is now available that can help in the selection of restriction enzymes concerning sequence differences [54].

**DNA hybridization-based methods:** When it comes to nucleic acid sequences, hybridization is one of the most powerful tools to aid in diagnosis and differentiation. Line probe hybridization (LiPA) is a probe hybridization method that uses type-specific probes to determine major genotypes at high levels of sensitivity. In this method, the probes detect variations in the genome. PCR amplicons, which can come from different parts of the genome of hepatitis viruses (HBV and HCV), are fixed on a nitrocellulose membrane and applied to labeled type-specific probes. A so-called reverse hybridization can also be carried out, in which type-specific probes are fixed on a nitrocellulose membrane and labeled amplicons, e.g. biotin-labeled amplicons, are applied as complementary sequences. This method is expensive, complicated, and time-consuming, but it is now the most commonly used method. It is also a sensitive method for the analysis of mixed genotypes[44].

Considering the advantages of DNA hybridization-based methods, several commercially available LiPA assays have been developed for different parts of the genome to allow accurate genotype detection. Kits based on both hybridization and reverse hybridization methods are available but are not fully suitable for subtyping[55-57].

It seems that DNA hybridization-based methods have attracted the attention of researchers as they have tried this method for HBV and HAV as well. LiPA for HBV, which was developed based on S-gene analysis, showed the same results as whole genome sequencing. However, for HBV, the sequencing-based kit is more sensitive than LiPA[58]. Although hybridization-based methods are used for the detection of HAV, there is no report on the use of this method for HAV typing[59].

Hybridization-based methods include a variety of technically different but essentially the same methods. A thorough search among studies shows that hybridization-based methods can change their appearance, although they retain their principles. For example, melting curve analysis and DNA immunoassay are two genotyping methods that use hybridization probes, even though they are widely used.

Melting curve analysis of probes and/or amplicons can be a cost-effective, rapid, and reliable method for genotyping. In this analysis, a pair of genotype-specific probes called the detection probe and the anchor probe, labeled with different fluorophores, are used for genotype differentiation in a single step in the light cycler. After the last PCR cycle, the products were heated in a special area for melting point Tm curve analysis. Hybridization of the genotype-specific probe with the PCR amplicons results in the convergence of the probes leading to fluorescence signals under specific excitation [60-62].

It has been shown that melting curve analysis for genotyping of amplification products can also be performed with SYBR Green. In this type of analysis, presented by Fujigaki et al, PCR products were mixed with SYBRGreen (a double-stranded DNA binding dye), and melting point analysis was performed by heating the mixture in a special ring. When the temperature rises above the melting point of the PCR product, the fluorescence decreases because SYBRGreen can no longer bind to single-stranded DNA[63,64].

DNA enzyme immunoassay (DEIA) is another form of hybridization-based method. This method is based on monoclonal antibodies that are specific only for double-stranded DNA. The binding of the antibodies thus occurs when the single-stranded cDNA of the sample attaches to the oligonucleotide probes. Colorimetric detection is used to study the binding between antibodies and double-stranded DNA. This

method was first presented for HBV diagnosis and then for HCV genotyping [64-66].

Genotype identification is an important component of clinical testing, as different genotypes of the same species may result in different signs and symptoms and may respond quite differently to treatment strategies. In this article, we focus on different molecular-based typing methods for hepatitis viruses. Unlike HCV and HBV, which cause both acute and chronic hepatitis and can lead to the development of cirrhosis or liver cancer, HAV causes mild to severe disease and, like HEV, is self-limiting. HDV is HBV-dependent, so measures against HBV will limit HDV infection. Therefore, it is not surprising that most genotyping studies focus on HCV and HBV[67-9].

PCR plays an important role in viral genotyping. Genotyping of hepatitis viruses also relies mainly on this method. The fragments resulting from PCR can be sequenced directly, serve as the substrate for restriction endonucleases in RFLP, hybridize with genotype-specific probes, or serve as a PCR substance for re-amplification by genotype-specific primers.

The most acceptable genotyping methods are based on sequencing, so Sanger or newer NGS methods have been performed for all types of hepatitis viruses. Since whole genome sequencing is time-consuming, attention is drawn to partial genome sequencing. Considering its accuracy, partial genome sequencing itself is not suitable for large-scale analysis.

Methods based on DNA banding patterns can be used for genotyping if the length of the PCR fragments is taken into account. These PCR fragments, obtained using type-specific primers, can be evaluated directly or after applying the effect of restriction enzymes. These types of methods are more popular for HAV, HBV, and HCV than for HDV and HEV [69-71].

It is worth noting that the development of commercial kits for HAV, HBV, and HCV based on the hybridization method has greatly facilitated the genotyping procedure. Hybridization-based methods include a few different methods, all based on type-specific probes hybridized to a specific region of the viral genome obtained by PCR or RT-PCR. Other genotyping methods such as serotyping and ELISA are also available for some types of hepatitis viruses such as HCV. Apart from their advantages that make them suitable for a large number of analyses, they suffer from disadvantages such as lack of specificity and sensitivity that limit their use [44].

Therefore, attention has been drawn to molecular methods. Molecular typing methods have advantages such as accuracy, sensitivity, and high discriminatory power. Since these methods rely on the most stable molecule in the cell, DNA, the results obtained are also robust and independent of the conditions of cultivation and sample preparation, so the results are also reproducible. The accuracy of all these methods mentioned above can reach the same level as sequencing, depending on the part of the genome studied. Despite the wide-ranging advantages of these molecular typing methods, the need for specialized instruments and reagents may limit their use in well-equipped laboratories. On the other hand, the analysis of the numerous results can lead to time-consuming post-processing procedures. It is also clear that the selection of the region of the genome to be studied is very important, as it can influence the results. Overall, special attention should be paid to deciding on a genome region that is both conserved enough for routine PCR testing and variable enough for genotype discrimination. In this area, phylogenetic analysis helps researchers find the best regions [61,62].

## Conclusion

The advances in microbial typing using molecular methods, and the emerging techniques of micro/nanotechnology can also provide a better perspective. As advances in micro/nanotechnology lead to the development of a new generation of diagnostic methods, it is also hoped that they will lead to the development of a new generation of typing methods. Ultimately, it seems that further studies are needed, not only to introduce micro/nanotechnological methods but also to determine the best recommendation for each virus typing method.

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## Conflict of interest

The authors further declare that they have no conflict of interest.

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