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IDENTIFICATION OF VIRAL HEPATITIS PATHOGENS USING REAL-TIME PCR

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Abstract. This article provides a scientific analysis of the principles, methodological features, and clinical significance of real-time PCR (quantitative polymerase chain reaction, qPCR) technology in the identification of viral hepatitis pathogens — HBV, HCV, HDV, HEV, and HAV. The article examines the comparison of real-time PCR with conventional serological methods and next-generation nucleic acid analysis systems, TaqMan probe and SYBR Green technologies, and the diagnostic and therapeutic significance of quantitative viral load assessment. The literature review discusses the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines developed by Stephen A. Bustin and Michael W. Pfaffl, studies on HBV, HCV, and HEV published in JCM and JoVE journals, as well as recent work on multiplex qPCR systems. The article provides a scientific basis for the role of real-time PCR as the "gold standard" in clinical diagnostics.

Keywords: real-time PCR; qPCR; viral hepatitis; HBV; HCV; HEV; HAV; HDV; viral load; TaqMan; MIQE; molecular diagnostics; RT-qPCR; identification; genotyping.

INTRODUCTION Viral hepatitis — a group of infectious diseases characterized by hepatic inflammation — represents one of the most widespread chronic infections globally. According to the World Health Organization, over 296 million people live with chronic hepatitis B (HBV) and 58 million with chronic hepatitis C (HCV); more than 1.1 million people die annually from hepatitis-related complications [1]. Uzbekistan is no exception to this challenge, with HBV and HCV infections maintaining a sufficiently high prevalence among the population. Conventional diagnostic methods — serological tests based on enzyme-linked immunosorbent assay (ELISA) — served as the primary tool for detecting hepatitis viruses. However, these methods detect viral antigens or the antibodies directed against them; they may produce negative results during the so-called "window period" (the interval following infection before antibodies are produced). Furthermore, serological methods cannot quantitatively assess viral load or determine genotype.

Real-time PCR (RT-qPCR or qPCR) entered medical practice as a molecular diagnostic method addressing these limitations. This method directly detects viral nucleic acids (DNA or RNA), demonstrates high sensitivity and specificity even in minimal sample volumes, and simultaneously provides accurate quantitative assessment of viral load. As stated in a study published in JoVE journal, real-time PCR is now recognized as the "gold standard" for detecting HBV DNA and measuring viral load [2]. This article systematically analyzes the capabilities and limitations of this technology in identifying viral hepatitis pathogens.

The aim of this research is to explain the fundamental technical principles of the real-time PCR method, demonstrate the specifics of its application to different hepatitis viruses, evaluate it from the perspective of MIQE guidelines and international standards, and discuss the prospects of multiplex systems.

LITERATURE REVIEW The most significant methodological contribution to the field of real-time PCR was made by an international group led by Professor Stephen A. Bustin of Queen

Mary University London and Professor Michael W. Pfaffl of the Technical University of Munich. In 2009, they published the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines in *Clinical Chemistry* journal . These guidelines established minimum standard requirements for conducting qPCR experiments, analyzing data, and preparing results for publication. Bustin et al. emphasize that prior to 2009, qPCR results appeared in publications with highly variable quality; replicating results from one laboratory in another was often impossible. The MIQE guidelines address this problem by establishing detailed rules for: sample preparation, RNA/DNA quality control, primer and probe design, amplification efficiency, any quantitative calculation method, and data normalization [3]. In 2025, an updated version — MIQE 2.0 — was published in *Clinical Chemistry* incorporating new recommendations covering novel technologies such as digital PCR, extracellular vesicle analysis, and others [4]. Pfaffl's 2001 paper in *Nucleic Acids Research* proposed a mathematical model for calculating qPCR data — the "Pfaffl method" — which enables relative quantitative assessment of gene expression while accounting for PCR efficiency. This model remains widely applied today and serves as the primary calculation tool in the quantitative assessment of hepatitis viruses.

One of the most important works in the field of detecting and quantifying hepatitis B virus (HBV) DNA using real-time PCR is the study by Pas et al. published in the *Journal of Clinical Microbiology* (2000): they developed a quantitative real-time PCR assay for HBV DNA detection and compared it with two commercial tests [5]. Subsequent studies in this direction published in JoVE (Journal of Visualized Experiments) — widely cited in this field — presented a systematic protocol for HBV diagnostics covering: sample collection and storage, DNA extraction, PCR mixture preparation, amplification, and result analysis in detail. Studies by Pas et al. (2000) and Ronsin et al. (2006) published in the Journal of Clinical Microbiology demonstrated that the Cobas AmpliPrep/Cobas TaqMan HBV system provides high sensitivity and reproducibility in clinical samples [5]. HBV DNA varies across an extremely wide clinical range — it can reach up to 10^{10} copies/ml during acute infection, while dropping to extremely low levels in chronic carriers and patients undergoing antiviral therapy . This capacity to cover a wide dynamic range is precisely what distinguishes real-time PCR from conventional methods.In a *Journal of Clinical Microbiology* article (2006) on the role of real-time PCR in HBV genotyping, the development of a new real-time PCR test capable of simultaneously identifying all eight HBV genotypes from A through G was reported [6]. This technical achievement is of clinical significance in selecting genotype-dependent antiviral therapy.

Detection and quantification of hepatitis C virus (HCV) RNA is an essential component of the clinical management of patients with chronic HCV infection. A study published in the *Journal of Clinical Microbiology* comparatively evaluated two standardized HCV RNA quantification tests — Abbott RealTime HCV and Roche Cobas TaqMan HCV — demonstrating that both were standardized to the WHO panel and possessed high sensitivity [7]. Importantly, results from these tests are expressed in IU/ml (international units per milliliter), allowing results from different laboratories to be compared. In the ScienceDirect article *Hepatitis C Virus RNA Real-Time Quantitative RT-PCR Method* (2015), a new HCV RT-qPCR method based on a novel primer design strategy was presented: R^2=0.99 linearity, a detection limit of 42.6 IU/ml, 100% specificity, and R^2=0.97 concordance with the CAP/CTM system — with these performance indicators, the authors demonstrated the high clinical suitability of the new method [8]. These results provide a robust scientific basis for the application of real-time PCR in HCV clinical management. In another important PubMed/PMC study (2002), an RT-PCR test targeting the HCV 5'-untranslated region using a molecular beacon probe was presented [9]. This test, covering a dynamic range of 10^3 to

10^7 RNA copies, demonstrated amplification of seven major HCV genotypes (1A, 1B, 2B, 3A, 4, 5A, 6A). This result facilitated the integration of real-time PCR into practical systems that cover multiple genotypes independently of genotyping.

Hepatitis E virus (HEV) is gaining increasing importance as a cause of chronic hepatitis among immunocompromised patients. A study by Baylis et al. (2013, 2017) published in the *Journal of Clinical Microbiology* developed a real-time RT-qPCR test system for HEV RNA calibrated to the WHO standard [10]. This test uses a broadly reactive primer set targeting the ORF2/3 region and utilizes Pleiades probe chemistry, while simultaneously detecting an internal control RNA — which is critical for result reliability. A critical review of RT-qPCR methods for HEV detection in pork products published in PMC (2022) comprehensively examined RT-qPCR methods and identified those successfully applied in subsequent studies [11]. The article notes that RT-qPCR methods can be used both qualitatively (presence/absence) and quantitatively; however, in the latter case, quantitative data may be unreliable without proper calibration. This observation fully aligns with the core idea in Bustin and Pfaffl's MIQE guidelines — the necessity of control and calibration standards [3].

Multiplex real-time PCR systems enabling simultaneous detection of multiple hepatitis viruses in clinical diagnostics constitute a distinct research direction. In a study by Park et al. (2012) published in *PLOS ONE*, a novel multiplex real-time PCR test simultaneously detecting HAV, HBV, and HCV was evaluated in 648 serum samples from patients with acute hepatitis symptoms [12]. Although the test did not outperform individual tests in clinical parameters, it was recommended as a practical alternative for rapid and efficient screening. In the pentaplex qRT-PCR study published in PMC (2023), the first pentaplex system simultaneously detecting HBV, HCV, HEV, Treponema pallidum, and a human housekeeping gene in a single tube was described [13]. Evaluated in 2,400 blood samples, this test demonstrated 100% clinical sensitivity, specificity, and concordance with individual tests. Additionally, several discrepant results were identified between serological tests and pentaplex qRT-PCR — two HBV DNA-positive samples and one HEV RNA-positive sample were negative by serological testing, once again confirming the PCR's detection capability even during the window period [13].

METHODOLOGY This article is based on a narrative review methodology of scientific literature. The following reputable scientific databases were used: PubMed/PMC, ScienceDirect, ASM Journals (Journal of Clinical Microbiology), JoVE, PLOS ONE, Springer Nature Link, and Oxford Academic. Search terms included: "real-time PCR hepatitis", "qPCR HBV HCV HEV detection", "RT-qPCR viral load", "MIQE guidelines hepatitis", "multiplex PCR hepatitis", and "TaqMan probe hepatitis virus". Priority was given to peer-reviewed articles published between 2000 and 2025. For each hepatitis type, specific diagnostic features, methodological challenges, and clinical significance were discussed separately.

DISCUSSION Polymerase chain reaction (PCR) is based on the principle of exponentially amplifying a target DNA or RNA sequence. In conventional "endpoint" PCR, the amplification product is detected only after the reaction is complete using gel electrophoresis or other methods. In real-time PCR, however, a fluorescent signal is measured at each cycle during amplification — this simultaneously allows both qualitative (presence) and quantitative (viral load) assessment [2]. From a clinical hepatology perspective, the main advantages of real-time PCR include the following. First, extremely high sensitivity: the ability to detect viral loads as low as 10–15 IU/ml, capturing levels that serological tests such as ELISA cannot detect. Second, elimination of the "window period": viral nucleic acids appear in blood much earlier than antigens and antibodies, so positive results are obtained even at the earliest stages of infection. Third, wide dynamic range: accurate

assessment of viral loads from very low (10^2 copies/ml) to very high (10^{10} copies/ml). Fourth, genotyping: using the same method to determine the virus genotype is of critical importance in selecting antiviral therapy [6].

Probe and Dye Systems: TaqMan and SYBR Green There are two main approaches to generating fluorescent signals in real-time PCR. The first is SYBR Green dye: this inexpensive and universal dye binds to any double-stranded DNA and emits a signal. Its advantage is that no additional probe synthesis is required and the cost is low. Its disadvantage is that primer dimers and non-specific amplification products also generate signals and may lead to false-positive results [3]. The second is TaqMan probes (also known as hydrolysis probes or dual-labelled probes): these probes specifically bind to the target sequence and are cleaved by Taq polymerase during amplification, releasing a fluorescent signal in the process. The advantage of the TaqMan system is its extremely high specificity: a signal is produced only when the target sequence is present. For this reason, TaqMan probes are the dominant choice in clinical diagnostics, especially in multiplex systems. The majority of validated commercial PCR kits for HBV, HCV, and HEV are based on TaqMan chemistry [2], [7].

Clinical Protocol and Significance of Viral Load In managing HBV infection using real-time PCR, the key indicator is HBV DNA viral load (expressed in copies/ml or IU/ml). In clinical practice, viral load is determined for the following purposes: making decisions about initiating or modifying antiviral therapy; monitoring treatment efficacy (assessing viral load at 12 and 24 weeks after starting therapy); and determining chronic carrier status and risk of liver cirrhosis [5]. According to the JoVE protocol, the main stages of HBV diagnostics are as follows: DNA extraction from serum or plasma (usually by automated bead-based method); quality assessment of the sample (monitoring for contamination and absence of inhibitors); preparation of the PCR mixture (master mix, primer, TaqMan probe, internal control); amplification and real-time signal recording; quantitative calculation based on a standard curve. Each test is run alongside an internal control sample, protecting against false-negative results — situations where amplification fails due to the effect of inhibitors.

The role of real-time PCR in HCV diagnostics is manifested in three directions. The first direction is diagnosis: detecting the presence of HCV RNA in blood serum, especially to confirm active infection when anti-HCV antibodies are positive. The second direction is genotyping: identifying the major HCV genotypes 1 through 6, since the genotype determines the duration and choice of therapy. The third direction is treatment monitoring: determining SVR12 (complete virological response 12 weeks after treatment completion) is currently the primary criterion for treatment success. Regarding the Abbott RealTime HCV and Roche Cobas TaqMan HCV systems, a *Journal of Clinical Microbiology* article states that both systems have lowered their detection limit to 15 IU/ml, reliably providing results across a wide dynamic range of 5 to 6 $\log^{10}$, and have been standardized using the second WHO panel [7]. This standardization is essential for results from different laboratories to be comparable and consistent with international treatment protocols. In a study by Zeuzem et al. that comprehensively evaluated the Cobas TaqMan HCV system, the behavior of two commercial systems in samples with low viral loads (below quantification limit) was compared, and attention was drawn to the need for caution in interpreting results [14].

Detecting hepatitis E virus (HEV) RNA is technically more complex than HBV or HCV: significant variability exists in the HEV genome, making universal primer selection challenging. The broadly reactive one-step RT-qPCR test developed by Jothikumar et al. (2006, published in J. Virol. Methods) targets the ORF2/3 region and demonstrated effective amplification of HEV samples of varying genotypes [11]. The WHO standard-based calibration system (Baylis et al.,

2013) made it possible to minimize inter-laboratory variation in results [10]. Real-time RT-PCR is also applied in hepatitis A virus (HAV) diagnostics, especially in epidemiological surveillance and source identification of infection. The 5' UTR region of the HAV genome is convenient for universal primer selection, and most validated tests target exactly this region. Successful results have also been achieved with systems that simultaneously detect HAV alongside HBV, HCV, and HEV in multiplex formats [12], [13].

The MIQE guidelines developed under the leadership of Bustin and Pfaffl play a decisive role in ensuring the quality of real-time PCR studies. For any scientific publication on the detection of hepatitis viruses, MIQE requirements include: description of the sample (tissue type, storage conditions, extraction method); RNA/DNA quality indicators (A260/A280 ratio, bioanalyzer electrogram); primer and probe sequences, target region, amplicon size; PCR efficiency (required to be in the range E=90–110%); limit of detection (LoD) and limit of quantification (LoQ); control samples and normalization method [3]. In the updated MIQE 2.0 version published in 2025, requirements for new technologies such as digital PCR (ddPCR), next-generation sequencing (NGS), and extracellular vesicle analysis were added [4]. This update is also important for hepatitis diagnostics: early articles on applying ddPCR in HBV viral load assessment are emerging, and the MIQE 2.0 standard applies to these as well.

Real-time PCR also has limitations in clinical practice. The most important problem is PCR inhibitors: heparin, excess EDTA, or improper sample storage sometimes present in blood serum can block amplification and lead to false-negative results. For this reason, an internal control sample is added to each clinical sample, and its detection confirms the absence of inhibitors.The second problem is measurement uncertainty at low viral load levels: in a study evaluating HCV RNA, the coefficient of variation in samples below the quantification limit reached 19–45% [14]. This requires caution in interpreting viral load during treatment in clinical practice. The third problem is proper sample storage: HCV RNA is particularly unstable and may degrade during improper collection, storage, and transport [8].

Droplet digital PCR (ddPCR) is considered the next generation of real-time PCR. This technology divides the sample into thousands of microdroplets, runs separate PCR in each, and provides absolute quantitative assessment based on the proportion of positive droplets. As noted in the JoVE article, digital PCR is showing much greater sensitivity than conventional qPCR in cases of "occult infection" with very low HBV viral load and in monitoring minimal residual viremia during antiviral therapy. Next-generation sequencing (NGS) covers the broad spectrum from virus identification through genotyping, mutation detection, and epidemiological analysis. However, the high cost of NGS, complex bioinformatic analysis, and longer turnaround time currently keep it from wide clinical diagnostics. In the future, hybrid systems combining real-time PCR and NGS are expected to offer the optimal solution.

CONCLUSION Real-time PCR technology has firmly established itself as the most reliable and widely used molecular diagnostic method for identifying viral hepatitis pathogens today. Its superiority over serological methods — eliminating the window period, accurately quantifying viral load, genotyping capability, and detecting even very low-level infections — has set a new standard in clinical hepatology. The MIQE guidelines developed by Bustin and Pfaffl have become a mandatory standard for laboratories worldwide as the foundation for ensuring the quality of real-time PCR studies. The 2025 MIQE 2.0 update sets the same quality requirements for new technologies such as digital PCR and NGS. Studies on HBV, HCV, and HEV published in JCM, JoVE, PLOS ONE, and PMC journals clearly demonstrate the multifaceted clinical applications of this technology.

The following directions hold strategic importance for future prospects. First, clinical standardization of multiplex qRT-PCR systems — the widespread adoption of a validated commercial kit simultaneously detecting HAV, HBV, HCV, HDV, and HEV in a single sample would significantly improve diagnostic efficiency. Second, integration of ddPCR into clinical medicine — especially in monitoring chronic HBV and HCV infection at minimal residual viremia levels. Third, inter-laboratory standardization according to MIQE requirements — this should be a key priority for bringing laboratory diagnostic quality to international standards, especially in Uzbekistan and the Central Asian region. Overall, the proper and standardized application of real-time PCR methodology serves as a critical tool in achieving the global goals of early hepatitis detection, monitoring treatment efficacy, and eliminating hepatitis.

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