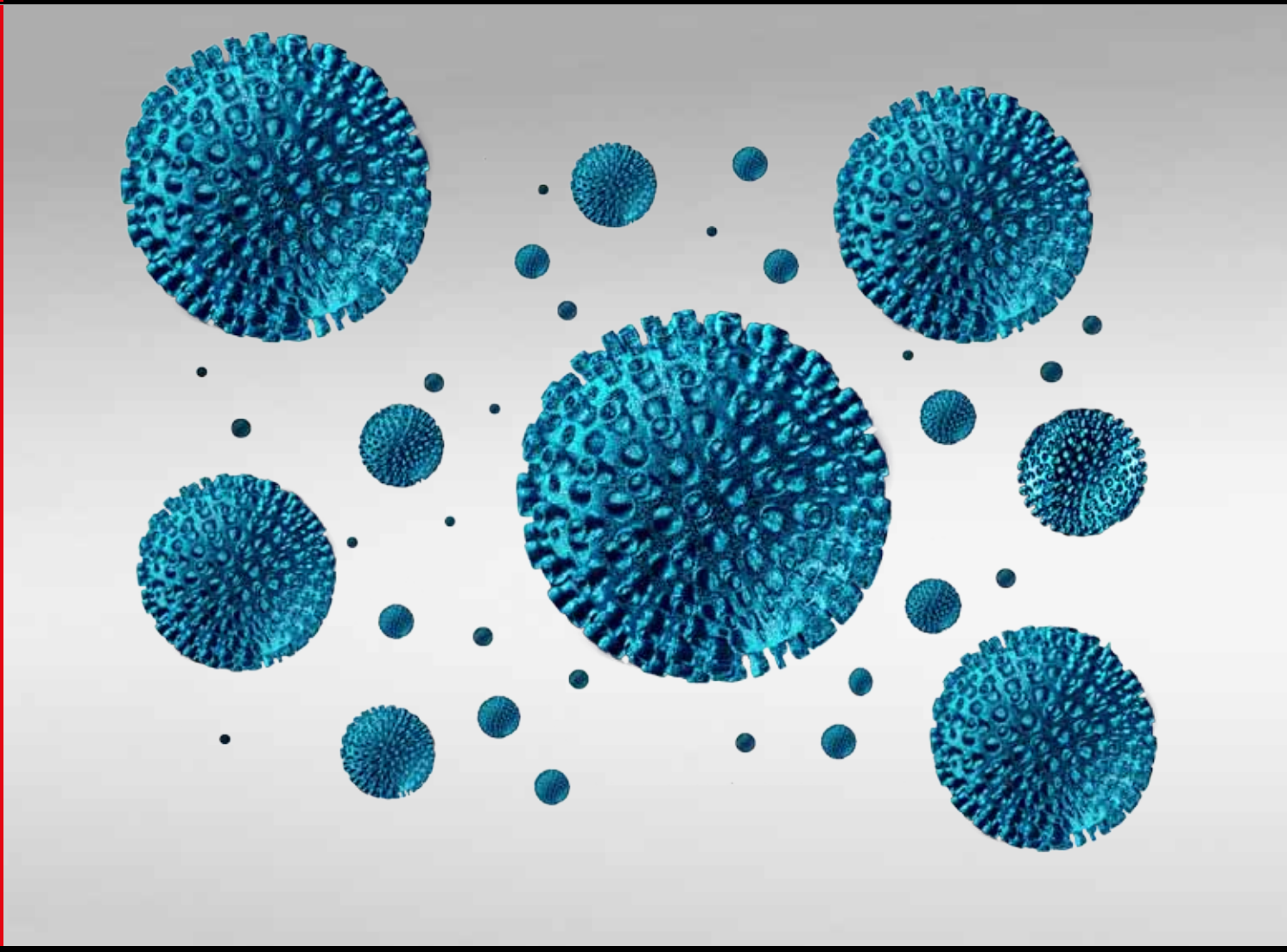
Improved Management of Hepatitis D: Standardized Quantification of HDV RNA

Monitoring of HDV RNA



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Improved Management of Hepatitis D: Standardized Quantification of HDV RNA

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Background

The hepatitis D virus (HDV) causes fulminant hepatitis and increases the severity of chronic hepatitis B virus (HBV) infection, leading to cirrhosis, liver failure, and hepatocellular carcinoma. HDV represents a major life threatening health risk in certain parts of the world, and always occurs in the presence of a co-existing HBV infection. Despite millions of HBV cases around the world, many of the major needs presented by HDV infection remain unmet. Hepatitis D infections urgently need to be confirmed and the viral load needs to be monitored to safeguard the treatment undergone by individual patients.

Hepatitis D: Key points of an exceptional target

HDV is a ~36 nm diameter enveloped RNA viral particle belonging to the Delta virus genus. Only one protein is known to be expressed by HDV, the hepatitis delta antigen (HDAg) in its small and large form that comprises a nucleocapsid-like structure within the genome. Together, these and the RNA genome are surrounded by a lipid envelope, which is embedded with HBV-derived surface antigen (HBsAg) proteins. Hence, natural HDV infections always occur in the presence of a co-existing HBV infection [1, 2].

HBV/HDV co- or superinfection leads to more severe forms of liver disease associated with a poorer prognosis than hepatitis B alone. Up to 80% of super-infection cases lead to chronic infection, of which approximately 60% to 70% of patients develop chronic cirrhosis and may do so some 15 years earlier than is the case in those with HBV monoinfection. Hepatitis D infection is associated with cirrhosis, liver failure or hepatocellular carcinoma [3, 4].

Over 350 million HBV carriers are at risk of contracting type D hepatitis. Therefore, HDV is a disease with a significant impact on global health affecting approximately 15 to 20 million people worldwide. HDV infection is spread in the same way as HBV, mainly through parenteral exposure. The virus shows high prevalence in the Mediterranean Basin, the Middle East, South and Central Asia, West Africa, the Amazon Basin of South America and certain South Pacific Islands [4]. Eight major genotypes (HDV-1 to HDV-8) are known and the genetic variability ranges from 20 to 35% between the genotypes. HDV-1 is the most widely distributed genotype throughout the world.

Improved management: HBV patients need HDV to be considered

Hepatitis D should be considered in any individual who is HBV positive or has evidence for a recent HBV infection. HDV diagnosis relies on detection of total anti-HDV antibodies by available radio- or enzyme immunoassays. Each of the markers of HDV infection, including IgM and IgG antibodies, disappear within months after recovery. In contrast, in chronic Hepatitis D virus RNA, HDAg, IgM and IgG anti-HD antibodies persist, suggesting nucleic acid amplification techniques (NATs) to be the only accurate tool for confirmation of HDV replication status due to possible sensitivities of detecting 5 to 25 IU/ml in serum of infected patients [5]. Furthermore, HDV RNA quantification is essential for monitoring viral load to verify patients' therapeutic success [6, 7].

The majority of NAT assays for HDV RNA are developed in house and based on Real-Time PCR technology. Usually, quantification of HDV RNA is based on internal standards of different origin, either *in vitro* transcribed HDV RNA or plasmid DNA containing HDV target sequences (rDNA). Due to their nature, these standards are often amplified and detected without the sample preparation step (nucleic acid extraction). Naked RNA can easily be degraded by ubiquitous RNases, and external DNA calibrators do not accurately reflect RNA isolation and reverse transcription steps. This issue was greatly improved with the establishment of the 1st WHO standard for HDV RNA, aiming the goal of standardization of HDV RNA quantification [8].

Proven reliability: RoboGene® HDV Quantification Kit 2.0 (CE-IVD)

Originating from this international standardization progress, the RoboGene® HDV RNA Quantification Kit 2.0 is the first worldwide unique CE-IVD-certified Real-Time PCR kit for quantification of HDV RNA, applying the 1st WHO standard for HDV RNA. Analytik Jena's kit is intended for quantification of Hepatitis D Virus RNA in human plasma or serum samples using One-Step Real-Time PCR. Following extraction of viral RNA, applying the INSTANT Virus RNA/DNA Kit (Analytik Jena) and due to the internal RoboGene® extraction and TaqMan®- based amplification concept as well as high-quality quantitative standards, the RoboGene® HDV RNA Quantification Kit 2.0 features a perfect analytical and diagnostic specificity. Data represented below include the assays analytical sensitivity and specificity, linear range and diagnostic evaluation. The RoboGene® HDV RNA Quantification Kit 2.0 was evaluated according to the common technical specifications (CTS) for in vitro diagnostic medical de-vices (2009/886/EC).

Included high-quality quantitation standards consist of 8 tubes coated with given amounts of synthetic HDAg RNA that must be amplified in parallel to the specimens. A synthetic internal control (IC RNA) is included to control RNA extraction and to indicate inhibitory influences on detection. Thus, the risk concerning false-negative results is drastically reduced, yielding increase in diagnostic correctness. Amplification of HDV RNA in samples and standards and of IC RNA is measured independently at different wavelengths due to probes labeled with different fluorescence reporter dyes (HDV RNA: FAM, IC RNA: Yakima Yellow).

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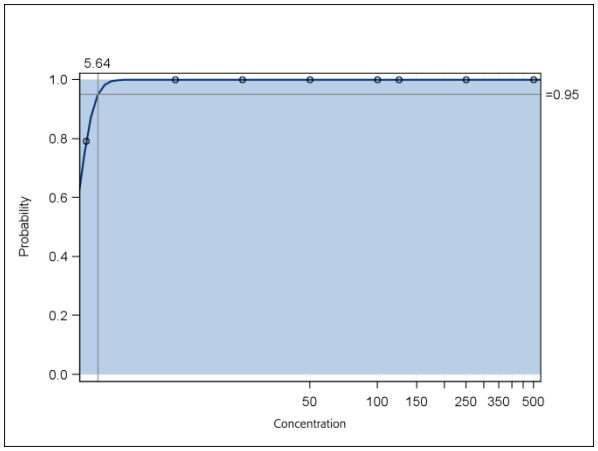

Fig. 1: Analytical sensitivity of the detection kit is subjected to the Real-Time PCR instrument and purification kit in use. Exemplarily a probit analysis on 7500 Fast is shown. Concentrations are given in IU/ml

Determination of the analytical sensitivity was performed by analyzing dilutions of the 1st WHO International Standard for HDV RNA following extraction of 400 µl of plasma using the INSTANT Virus RNA/DNA Kit and amplification on different Real-Time PCR platforms (Figure 1 and Table 1).

Tab 1: Detection limit using different Real-Time PCR platforms

Instrument	Detection limit
Rotor-Gene™ 3000	8 IU/ml
7500 Fast	6 IU/ml
LightCycler® 480	14 IU/ml

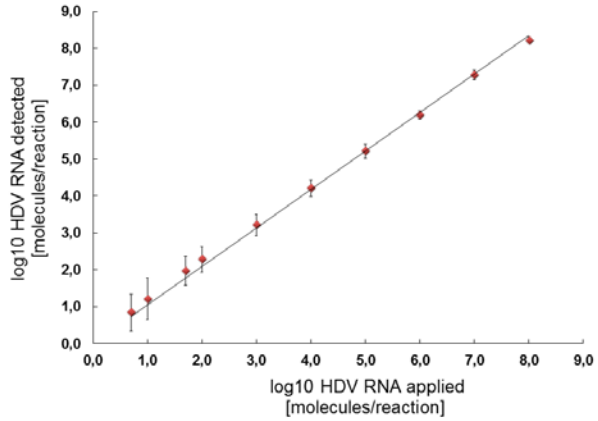

Fig. 2 : Linearity of the RoboGene® HDV RNA Quantification Kit 2.0 on 7500 Fast using synthetic HDV quantitation standard.

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The linear range of the RoboGene® HDV RNA Quantification Kit 2.0 was determined by analyzing a dilution series of the synthetic HDV quantitation standard ranging from 5 to 1 · 109 copies per run. Each dilution was tested in replicates on Rotor-Gene™ 3000, 7500 Fast and LightCycler® 480 (n=10 for each concentration). The linearity of the assay was >8 logs as determined by linear regression of the log10 calculated with the log10 nominal concentrations for all used Real-Time PCR instruments (see example on 7500 Fast in Figure 2). Furthermore, linear range was determined using native HDV RNA of clinical material. Comparable results were achieved (data not shown).

Due to the lack of commercially available HDV genotype panels, the RoboGene® HDV RNA Quantification Kit 2.0 was only analyzed with a small amount of clinical samples qualitatively, representing six of the known eight genotypes. The assay is able to detect all eight major clades of HDV [9] by applying probes and primers specific for a subsequence to HDAg. The theoretical possibility for the detection of all eight genotypes was proved by analyzing numerous sequences, published at the NCBI database (data not shown).

By scientific definition, HDV replication is strictly dependent on the presence of HBV (i.e., HBV-negative samples are HDV-negative, as well). Thus, plasma samples tested negative for HBV using the Cobas® TaqMan® HBV Kit, were analyzed to determine the diagnostic specificity of the RoboGene® HDV RNA Quantification Kit 2.0, which is expressed as negative result in absence of the target. None of the HDV-negative samples (n=100) gave a positive result (IC RNA positive: n=100). Furthermore, analytical specificity was evaluated by analyzing non-HDV-positive specimens.

Tab 2. : Results obtained for analysis of analytical specificity

Control group	HDV-RNA (FAM)	IC-RNA (JOE/VIC)
Hepatitis B virus (HBV), n=10	0/10	10/10
Hepatitis C virus (HCV), n=10	0/10	10/10

The RoboGene® HDV RNA Quantification Kit 2.0 provided ideal analytical and diagnostic specificity. None of the analyzed samples gave positive test results for HDV RNA.

Finally, diagnostic sensitivity and linearity was analyzed with HDV RNA positive patient samples (n=109). Quantitative data were compared with results obtained at an accredited laboratory applying a certified in-house assay. Figure 3 shows 50 patient samples detected using the LightCycler® 480 Real-Time PCR instrument in both laboratories.

By consideration of the method of inherent imprecision as well as the variability of individual data points (Deming regression analysis), no significant statistical differences between both methods was detected (data not shown).

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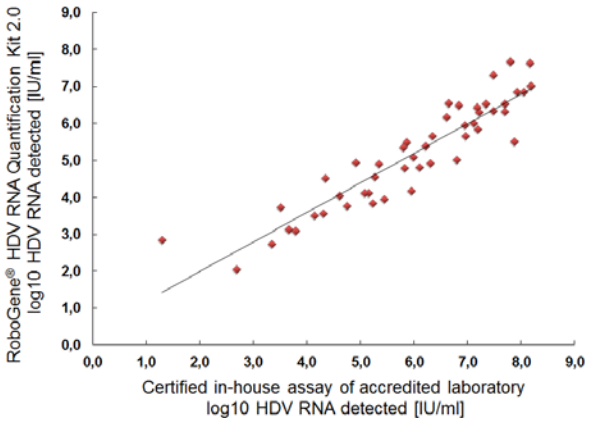

Fig. 3 : Linear regression of quantitative results from both tests (n=50) showed a very high degree of correlation

Future option: Introduction of standardized HDV RNA quantification procedure

Hepatitis D represents a major and life-threatening health burden in certain areas of the world. Confirmation of hepatitis infection, as well as standardized assessment of antiviral treatment effectiveness may improve patient management in the future. Demonstrating the first worldwide unique CE-IVD certified One-Step Real-Time PCR quantification kit, the RoboGene® HDV RNA Quantification Kit 2.0 is an optimal tool for improved management of HDV infection.

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Reference: Lit_0332_0001_en_150914.docx

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Certified Quality System mdc ISO 13485

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