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HDV DIAGNOSIS

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Abbreviations:

HDV	hepatitis delta virus
HD-Ag	hepatitis D antigen
Anti-HDV	antibody to hepatitis delta virus
Anti-HD IgG	IgG antibody to hepatitis delta virus
Anti-HD IgM	IgM antibody to hepatitis delta virus
HDV-RNA	hepatitis delta virus genome
HDV-1,2,3	hepatitis delta virus genotypes
CHD	chronic hepatitis delta
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
Anti-HBc	antibody to hepatitis B core
Anti-HBc IgM	IgM antibody to hepatitis B core
HBeAg	hepatitis B e antigen
Anti-HBe	antibody to hepatitis B e antigen
cccDNA	covalently closed circular DNA
pgRNA	pregenomic RNA
HBV-D,A,B,C,F	hepatitis B virus genotypes
HCV	hepatitis C virus
HIV	human immunodeficiency virus
AIDS	acquired immune deficiency syndrome
PEG-IFN	pegylated interferon
LAM	lamivudine
ADV	adefovir

Abstract

The first step in the diagnosis of Hepatitis Delta Virus Infection is testing HBsAg-positive individuals for the antibody to the HD antigen (anti-HD).

In anti-HD-positive subjects, the next step is testing for HDV-RNA in serum to determine whether the antibody reflects an ongoing active HDV infection (HDV-RNA positive) or represents a serological scar to past HDV infection (HDV-RNA negative). In the HDV-positive individual with liver disease, it is critical to distinguish acute HDV/HBV coinfection from chronic HDV superinfection in HBsAg carriers; the course, prognosis and management of the two conditions are different. The differential diagnosis can be achieved through the scrutiny of the battery of HDV and HBV markers, which combine in patterns characteristic for each condition.

Standardized competitive and μ -capture commercial assays are available to determine the IgG and IgM antibody to HDV. Several in-house assays were developed to determine HDV-RNA in serum; the sensitivity threshold of current PCR-based assays is 10 copies of HDV-RNA/ml.

Unfortunately, HDV-RNA assays are not yet standardized and the results from different laboratories often are not comparable due to different sensitivities. The development of an International reference HDV-RNA standard remains an unmet diagnostic need.

Introduction

Indicative of HDV infection are the replication of the viral RNA with the expression of the HD-Antigen (HD-Ag) and the specific immune reaction of the infected host (1). In the immunocompetent host, HDV infection elicits an antibody response to the HD-Ag (anti-HD), first of IgM then of IgG class (2). Active HDV infection is diagnosed on the finding of the HDV-RNA in serum and/or the HD-Ag in serum and liver.

As hepatitis D results from a double infection with the HBV, the virologic assessment requires the consideration not only of HDV but also of the concomitant HBV infection. In the clinical setting efforts to diagnose HDV infection are appropriate only in the presence of the HBsAg in serum; HBsAg-negative patients do not harbour productive HDV infections.

This review summarizes the virologic profiles diagnostic of HDV infections and provides an analysis of each marker, relevant to the diagnosis of hepatitis D.

Serologic patterns of HDV infection

The HDV is acquired either through simultaneous coinfection with the HBV by HBV naïve individuals or through superinfection on a pre-existing and persisting HBV state i.e. on individuals carrying the HBsAg (3). The clinical outcome is different, coinfection usually runs, a self-limited course that terminates with the clearance of HBV/HDV, superinfection usually induces a chronic hepatitis D that advances to cirrhosis and liver failure.

The distinction of coinfection from superinfection is critical to the management and prognosis; it is made possible by the scrutiny for HDV and HBV markers, which discloses serologic patterns typical for the two conditions (fig. 1).

In coinfection (fig. 1A) the expression of HDV is determined by the virulence of the concomitant HBV infection (4). With diminished HBV virulence, HDV expression is short-lived, viremia transient and the anti-HD response brief or abortive. Coinfections with large HDV expression exhibit a full serological pattern, consisting of early viremia and HD antigenemia, followed by seroconversion first to IgM and then to IgG anti-HD; HD-Ag is

expressed in liver and the HD-RNA peaks to high titers in serum. The antibody response may be delayed for several days; therefore prolonged monitoring for antibodies to HDV is required to exclude or confirm HDV infection in patients with an acute HBsAg-positive hepatitis. The IgM antibody to the core antigen of the HBV (IgM anti-HBc) as well as HBV-DNA are present in high titers; the HBeAg may also be detectable at onset of disease, but rapidly clears from serum followed by seroconversion to anti-HBe.

Atypical patterns of acute coinfection were reported in hepatitis D occurring among drug addicts (5). In some patients, strong and early repression of HBsAg resulted in an HBsAg-seronegative hepatitis with positive HDV serology, followed by the return of the HBsAg after the clearance of HDV (6); in a few patients, superinfection terminated the HBsAg state with seroconversion to anti-HBs (7). In a recent single case report, acute hepatitis D has terminated also chronic HCV infection (8). In other patients coinfection run a byphasis course, with two hepatitis bouts accompanied by sequential expression of either the HBV or HDV (9-10).

In superinfection, the virological pattern is more uniform. HDV-viremia appears early and the IgM and IgG anti-HD response is brisk (11). Both antibodies rapidly increase in superinfection progressing to cronicity and remain detectable in high titers along with the persistence of viremia and the progression to chronic hepatitis D (CHD); in the liver, the nuclei of hepatocytes are loaded with HD-Ag.

In most of the patients replication of HBV is repressed; serum HBV-DNA is null or present at low titers. The IgM anti-HBc is usually negative (Fig. 1B) (12). The patterns diagnostic of the various clinical forms of HDV infection are summarized in table 1.

The role of quantitative HBsAg testing in CHD remains under investigation. A correlation was observed between the decrease in serum of HBsAg and the decline of serum HDV-RNA in patients treated with Peg-IFN or with lamivudine; levels of HBsAg decreased also in patients treated with Peg-IFN in combination with Adefovir but not in patients treated with Adefovir monotherapy (13). The decrease of serum HBsAg might provide a valuable marker prognostic of the decline and ultimate clearance of HDV infection (14).

Antibodies to HDV

a) IgG anti-HD

Total antibodies to HDV are detected with a Enzyme Linked Immunosorbent Assays (ELISA). Commercial tests are available (ETI-AB-DetlaK-2 Sorin, Saluggia, Italy; Abbott IgM anti-HD). The method is a simultaneous competitive assay. The format includes a solid-phase (plastic wells) coated with recombinant HD_{Ag}, test sample, anti-HD negative control, an enzyme tracer (anti-HD-Fab human-conjugated with horseradish peroxidase) and a chromogen substrate to develop the colour of the enzyme tracer that binds to the solid phase. Anti-HD in the sample and labelled anti-HD compete for the fixed quantity of HD_{Ag} bound to the solid phase; the amount of enzyme activity bound to the solid phase is inversely proportional to the concentration of anti-HD in the sample.

Competitive assays measure all types of antibodies to HDV; however, antibodies other than IgG are a minority of the total reactivity and in practice the measure of total antibodies by competitive assays is synonymous with the measure of IgG antibodies (2, 12). The IgG antibody is not protective; it is present in all immunocompetent patients with HDV infection, coexisting with active HDV infection and hepatitis D, and may not be raised in immunocompromised patients who acquire HDV such as patients with AIDS (15). The IgG antibody is usually short-lived in coinfecting patients who clear HBV-HDV. Exception are the drug addicts; in a retrospective analysis of blood from 27 Swedish drug addicts drawn 5-13 years after the acute episode of HDV coinfection, 26 were clear-cut positive and one still gave a borderline positive result for anti-HD (16).

Anti-HD may persist for years as a serological scar in superinfected patients who clear HDV infection and remain HBsAg-positive; rarely it was also detected in HBsAg-negative subjects, who were superinfected in the past and cleared HBV with the loss of the HBsAg (17).

b) IgM anti-HD

The IgM antibody to HD_{Ag} (IgM anti-HD) is measured with μ -capture immunoassays; titration is made by tenfold dilutions (18). Assays are available

commercially(ETI-Delta-IgM-K2 assay, Sorin, Saluggia, Italy; anti-HD EIA Abbott, Anti-HD IgM Kit, Serono, Italy). In chronic infection the antibody is composed mainly of monomeric 7S IgM molecules while 19 S pentameric molecules are prevalent in primary HDV infection (19).

The IgM anti-HD is considered a marker of HDV-induced liver disease and may be a useful surrogate to determine HDV replication if molecular tests for HDV RNA are not available. The decrease and clearance of this antibody in chronic hepatitis D is a predictor of spontaneous or therapy induced disease remission (2,20,21). In 38 Italian HDV patients treated with Peg-Interferon and Ribavirin, all positive for IgM anti-HD at baseline with a mean titer of 10^4 , the antibody titer remained unchanged in virologic non-responders and in those in whom HDV-RNA decreased but did not clear from serum; in 5 patients who cleared HDV-RNA the mean antibody titer diminished by 1 log¹⁰ and it disappeared in 2 patients who achieved a sustained response 6 months off from therapy (22).

The HD-Antigen

The HD Antigen is expressed in the liver cell nuclei where it can be detected by immunochemistry (2,4). With the advent of molecular assays for HDV-RNA in serum, tissue immunochemistry has become obsolete (23); it requires an invasive liver biopsy and is less sensitive than molecular assays. The percentage of cells expressing HDAg diminishes with the progression of chronic hepatitis D to cirrhosis making immunohistologic testing inaccurate in patients with advanced disease. It can be used for the diagnosis of active HDV infection if molecular tests for HDV-RNA are not available.

After disruption of the HDV virion with detergent, the HD-Antigen is exposed and can be measured in serum with assays based on its capture by anti-HD fixed in a solid phase and subsequent binding to it of anti-HD conjugated with an enzyme tracer (2,12). Commercial assays are available (ETI-Delta-2, Sorin, Saluggia, Italy; HDAg Sanofi, Pasteur). However, immunocompetent patients exposed to HDV invariably develop in a short-while anti-HD, which binds and masks the serum antigen released from the virion, rendering it unavailable to the format. Serum HD antigen is detectable only transiently in blood specimens collected early at the onset of hepatitis D, before the rising of antibodies, and its finding is therefore diagnostic of primary HDV infection. Occasionally the HDAg can be detected for long in

serum of patients with an impaired immune system who do not raise antibodies, such as patients with AIDS or transplanted patients under pharmacological immunosuppression (15,24).

HDV RNA

Serum HDV-RNA was first determined with nucleic acid hybridization assays that used the cloned HDV genome or cDNA constructs of it (25). With the advent of Reverse Transcription (RT) - PCR techniques, HDV RNA has been measured with qualitative or semiquantitative RT-PCR assays. Sensitivity has markedly improved, with current detection limits of 1000 genome/ml for simple PCR and of 10 genome/ml for nested PCR (26) (fig. 2). A first method for the quantitation of HDV-RNA, based on the application of Real-Time PCR technique, has been described by Yamashiro in 2004 (27). In this assay, primers are designed on the template of the HD-Ag coding region; this is a highly conserved region with a homology of 98% between genotype 1 and genotype 2. In the assay of Le Gal (28) the design of primers and probe is targeted to a conserved region, located within the ribozyme. The assay allows the detection of all HDV genotype; a further forward primer specific for HDV 3 genotype was used to determine HDV-3 infection. With the procedure described by Hoffmann (29), genotype 1 can be distinguished from genotype 3 by the analysis of the results from a melting curve performed after the polymerase chain reaction. Mederacke has established an in-house real-time PCR assay, exploiting the utility channel of the Cobas TaqMan apparatus (Roche Diagnostics) (30); the use of a real-time PCR platform, in association with a system for the automatic extraction of RNA, provides more reproducible results. In the assay developed by Pollicino (31), HDV-RNA has also been quantified by real-time PCR with the utility channel of Cobas TaqMan instruments; with this assay, HDV - RNA can be quantified in serum and in liver. Table 2 and 3 report the principal technical features of in-house Real Time PCR methods and of commercial assays published in the last decade.

Unfortunately the results from different laboratories are often not comparable due to the diverse sensitivity of the assays; variance is caused by the use of different set of primers

and by the variability of the RNA region amplified.

To reduce false negative results and optimize specificity for all HDV genotypes, primers and probes should be targeted to the most highly conserved regions, located within the ribozyme. The standards utilized in different assays are heterogeneous; they include synthetic HDV-RNA, HDV cDNA plasmid, serum from HDV infected patients or Armored RNA (complex of MS2 bacteriophage coat protein and RNA). With a cDNA standard it is not possible to check the reverse transcription step while simple optical density measurements are not sufficient to quantify synthetic RNA standards, whose performance is variable for the presence of secondary structures different from natural HDV-RNA. To calibrate quantitative assays, it would be desirable that WHO develops an HDV-RNA reference preparation quantified in International Unit and makes it available as an international standard.

HDV-RNA quantification does not seem to correlate with the clinical stage of liver disease (3). It is useful to monitor treatment response in patients undergoing antiviral therapy and may be useful in longitudinal studies to assess the dynamics of HDV replication over time in infections with other hepatitis viruses.

HDV Genotypes

Eight major clades of HDV-RNA have been identified, differing each other for about 40% of the nucleotide sequence (32). Genotypes may be determined by Restriction Fragment Length Polymorphism (RFLP) analysis or by direct sequencing (33). Genotyping is currently performed only in research laboratories. It has been valuable in tracing source and spreading of HDV infection (34); in areas where both genotypes 1 and 2 are present, it may help to identify patients infected with genotype 1, which has a worse outcome than genotype 2. In an Italian cohort (31), 21 HDV patients were infected with HDV-1 and HBV genotype D; among 22 HBV-monoinfected controls, genotype HBV-D was found in 18, genotype HBV-A in 4, genotype HBV-C in 1. The genotype of HBV seems to have no clinical relevance in the context of HDV infection, with the exception of the combination of HBV genotype F and HDV genotype 3 in South America, which was correlated with severe forms of hepatitis D (35). In the Western world the prevalent HDV-1 genotype combines with genotype D, A and C without clinical differences; new HBV genotypes incoming with immigration, such as

genotype B from China and genotype E from sub-Saharan Africa are unlikely to have an impact in the clinical expression of HDV infection.

IgM antibody to the Hepatitis B core antigen (IgM anti-HBc)

The IgM anti-HBc is a surrogate marker of liver disease induced by HBV replication and its determination is useful to distinguish whether an acute HDV-positive hepatitis is caused by HBV-HDV coinfection (IgM anti-HBc positive) or by HDV superinfection in an inactive HBsAg carrier (IgM anti-HBc negative) and whether the concomitant chronic HBV infection is also pathogenic in CHD (IgM anti-HBc positive) (2,4).

Interactions of HDV with HBV, HCV and HIV

Due to the dominance of HDV over HBV, the majority of patients with CHD have in serum anti-HBe and null or low HBV-DNA. Exceptions are drug-addicts and immigrants; a fair proportion have fresh CHD accompanied by active HBV infection with serum HBeAg and consistent levels of HB viremia (3).

Triple infections with HBV/HDV/HCV are common in drug addicts. In Germany, 29% of a total of 254 HDV patients were also positive for anti-HCV (36). Most of the German-born patients were drug-addicts and 54 % had antibody to HCV; only 8% of the HDV patients born in Turkey and 24% of those born in Eastern Europe had anti-HCV.

In multiple viral infections (HDV/HBV/HCV/HIV), HDV is usually dominant and inhibits the others viruses. In the study of the European cohort of HIV (EuroSIDA) (37), anti-HD was detected in 62 (14.5%) of 422 HBV/HIV; serum HDV-RNA was detectable in 87% the antibody positive patients with a median titer of 1.76×10^7 copies/ml. Infection with HDV was associated with a severe chronic hepatitis/cirrhosis and a consistent mortality risk.

In multiple infections viral dominance may change and alternate over time, with serum HBV-DNA and HCV-RNA reaching occasionally high levels. In a bimonthly surveillance of the levels of HDV/HBV/HCV during 12 months in 15 Italian patients with triple infection (38), 8 exhibited alternating peaks of HBV and/or/HCV replication. Similar fluctuations were observed in HDV cohorts from Germany and Spain and (3,39), suggesting that serum levels of HDV, HBV and HCV should be measured longitudinally in order to tailor antiviral

therapy.

Liver HDV-RNA

HDV-RNA can be detected in liver with qualitative Northern hybridization assays (25). The advent of qPCR technology has provided quantitative assays for the measure of nucleic acids in small biopsy samples, overcoming the limitation of sufficient material for the extraction; however, the need for an invasive liver biopsy restricts the clinical use of this procedure. The molecular interplays of HDV and HBV were investigated in serum and liver with sensitive qPCRs in 21 coinfecting HDV/HBV patients and in 22 HBV-monoinfected patients (31). In HDV patients the median level of serum HBV-DNA was $5 \log^{10}$, and of intrahepatic relaxed-circular DNA and cccDNA was $2 \log^{10}$, significantly lower than in HBV-monoinfected. Although pgRNA and pre-S/S RNA in liver were also diminished (by $1 \log^{10}$) in HDV-patients, the serum HBsAg concentration was comparable between the two groups; large deletions in the basal core promoter/precore region were detected in 5 HDV patients, all of whom had low levels of serum HBV.

These findings provide a molecular basis to the discrepancy between the biological help that HDV requires from HBV and the repression that it regularly exerts on the helper virus; the HDV is smart enough to selectively suppress some HBV functions while maintaining the capacity of HBV to synthesize the large amounts of envelope proteins necessary to the formation of HDV virions and to virus survival.

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Legends

Fig. 1A

Serological pattern of HDV/HBV coinfection: in severe HDV infection appear an early viremia and HD antigenemia followed by a seroconversion first to IgM and then to IgG antibodies to HDV. Simultaneously there is the expression of HBsAg and a primary response to HBV highlighted by the presence of IgM anti-HBcAg.

Fig. 1B

Serological pattern of HDV superinfection evolving to chronicity: HDV-RNA in serum appears early followed by a sudden IgM and IgG antibodies appearance. In most cases HBV replication is low or repressed and IgM antibodies to HBc antigen are negative.

Fig. 2

Increase of HDV-RNA sensitivity with the different tests developed over the last 30 years

Table 1. HDV and HBV markers in different clinical forms of HDV infection

Table 2. List of in-house real-time PCRs for HDV-RNA detection

Table 3. List of commercial assays for HDV-RNA detection

Table I: Serum patterns diagnostic of the different clinical forms of HDV infection

Serum	HDV Markers			HBV Markers				
	anti-HDV IgM	anti-HDV IgG	HDV-RNA	HBsAg	HBeAg	anti-HBe	anti-HBc IgM	HBV-DNA
Acute Hepatitis								
Coinfection	+	+	+	+	+	-	+	+ (> 20.000 IU/ml)
Superinfection	+	+	+	+	-	+	- / +-	- / +- (<2.000 IU/ml)
Chronic Hepatitis	+	+	+ (10 ⁵ ó 10 ⁷ copies/ml)	+	-	+	-	- / +- (<2.000 IU/ml)
Chronic active HDV/HBV Hepatitis	+	+	+ (10 ⁵ ó 10 ⁷ copies/ml)	+	-	+	-	+ (>2.000 IU/ml)
Cirrhosis	+ / -	+	+ / - (10 ³ ó 10 ⁷ copies/ml)	+	-	+	-	- / +- (<2.000 IU/ml)
HDV recovery	-	+	-	+	-	+	-	- / +- (<2.000 IU/ml)
Liver	HDAg		HDV-RNA	HBsAg		HBcAg		HBV-DNA
Chronic Hepatitis	+		+	+		- / +		- / +

Table II: principal technical features of published in-house Real Time PCR methods for HDV-RNA quantification

METHODS	HDV-RNA EXTRACTION	REVERSE TRANSCRIPTION	TARGET REGION	GENOTYPES DETECTED	REAL-TIME PCR INSTRUMENT	STANDARD	LINEARITY	LIMIT OF DETECTION
Yamashiro et al., J Infect Dis. 2004; 189: 1151-7	Acid guanidium phenol-chloroform method	Random hexamers (Takara Bio) RNase inhibitor (Promega) MMLV Reverse Transcriptase (Gibco)	HDAG-coding region Primer 1164 nt 1164-1192 Primer 1297 nt 1297-1268	1, 2a, 2b	Light Cycler System (Boehringer Mannheim)	Synthetic HDV-RNA standard	$10^3 \text{ } \delta \text{ } 10^9$ copies RNA/ml	10^3 copies RNA/ml
Le Gal et al., J Clin Microbiol 2005; 43: 2363-9	QIAamp MinElute virus vacuum (Qiagen)	Random Primers RNase inhibitor (Promega) SuperScript Reverse Transcriptase II (Life Technologies)	Ribozyme region Delta-F/ T3-Delta-F nt 693-709 Delta-R nt 891-907 Delta-P nt 858-872	1-7	ABI PRISM 7000 (Applied Biosystems)	Plasmid pCRII-dFr45-R01	$10^3 \text{ } \delta \text{ } 10^9$ copies /ml	10^2 copies/ml
Erhardt et al., Liver Int 2006; 26: 805-10	MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics)	One Step	HDAG-coding region	1-3	Light Cycler System	Patient serum calibrated on HDV plasmid pSVL LD3	<i>nr</i>	$1,5 \times 10^2$ copies/ml
Sheldon et al., Antiviral Therapy 2008; 13: 97-102	QiaAMP viral RNA extraction kit (Qiagen)	AMV Reverse Transcriptase (Promega)	<i>nr</i>	<i>nr</i>	Light Cycler PCR thermocycler (Roche)	DeltaF-R2 PCR DNA fragment cloned into a pGEMT vector	$10^{-1} \text{ } \delta \text{ } 10^8$ copies/ml	10^2 copies/ml
Zachou et al., Liver Int 2009; 430-7	<i>nr</i>	<i>nr</i>	<i>nr</i>	1	<i>nr</i>	HDV-cDNA plasmid	$1,1 \times 10^2 \text{ } -1,1 \times 10^5$ copies	$1,2 \times 10^2$ copies/ml
Mederacke et al., J Clin Microbiol 2010; 48: 2022-29	TINAI kit (Roche)	One step	HDAG-coding region F1: nt 837-853 F2: nt 837-854 R: nt 891-907 P: nt 858-872	<i>nr</i>	Cobas TaqMan 48 (Roche Diagnostics)	cDNA clone in a pSVL expression vector	$3 \times 10^2 \text{ } \delta \text{ } 1,5 \times 10^8$ copies/ml	3×10^2 copies/ml
Schaper et al., J Hepatol 2010; 52: 658-64	<i>nr</i>	One step	HDAG-coding region Primer DP1: nt 887 Primer DP2: nt 993 Probe HDV-FL: nt 921 Probe HDV-LC: nt 947	1	LightCycler system (Roche)	Quantified serum from a chronic hepatitis Delta patient	$10^3 \text{ } \delta \text{ } 10^7$ HDV-RNA equivalent/ml	10^3 equivalent/ml
Hofmann et al., Diagn Microbiol and Infec Dis 2010; 67: 172-9	QiaAMP Viral RNA mini Kit (Qiagen)	Primer Random (Roche) SuperScript III RT (Invitrogen)	HDAG-coding region Primer HD10: nt 469-485 Primer HD11: nt 839-819 Probe HD LNA: nt 703-720 Probe HD 13R: nt 722-740	Opportunity to identify HDV-3 by subsequent melting analysis	LightCycler 2.0 instrument (Roche)	Plasmid pSC-A HDV-1	$2 \times 10^3 \text{ } \delta \text{ } 10^8$ copies/ml	2×10^3 copies/ml
Pollicino et al., J.Virol 2011; 85: 432-9	TRIZol reagent (Invitrogen)	Oligo(dt)primers SuperScript Reverse Transcriptase kit (Invitrogen)	Primer DELTAF: nt 1063-1081 Primer DELTAR: Nt 1236-1216 Probe DELTA/FL: nt 1086-1108 Probe DELTA/LC: nt 1113-1137	1	Cobas TaqMan 48 (Roche Diagnostics)	Plasmid pCRII-delta-R0	$1 \times 10^1 \text{ } \delta \text{ } 1 \times 10^7$ copies/ml	1×10^1 copies/ml

nr : not reported

Table III: principal technical features of commercial assay for HDV-RNA quantification

ASSAYS	HDV-RNA EXTRACTION	REVERSE TRANSCRIPTION	TARGET REGION	GENOTYPES DETECTED	STANDARD	LINEARITY	LIMIT OF DETECTION
RoboGene HDV RNA Quantification kit	INSTANT Virus RNA Kit, version HDV (AJ Innuscreen) or QIAmp Viral RNA Mini Kit (Qiagen)	One Step	HDAG-coding region	1 - 8	Synthetic HDV HDag RNA	1×10^1 ó 1×10^7 copies/run	1×10^1 copies/run
DIA.PRO HDV RNA Quantitation	QIAmp Viral RNA Mini Kit (Qiagen)	RNA retrotranscription Kit	<i>nr</i>	<i>nr</i>	External standard curve	10^3 ó 10^{12} copies/ml	1 copies/ μ l
PrimerDesign Quantification Kit for HDV genomes	<i>nr</i>	PrimerDesign <i>Precision</i> Reverse Transcription kit	HDAG-coding region	1 -7	Positive control template to generate a standard curve	1×10^2 ó 1×10^8 copies/ml	1×10^2 copies/ml

nr : not reported

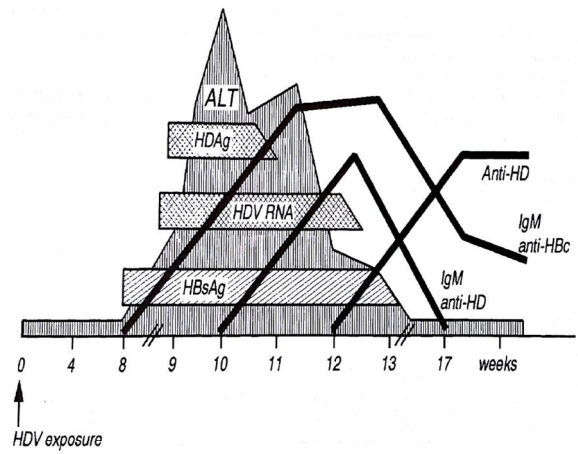


Fig.1A

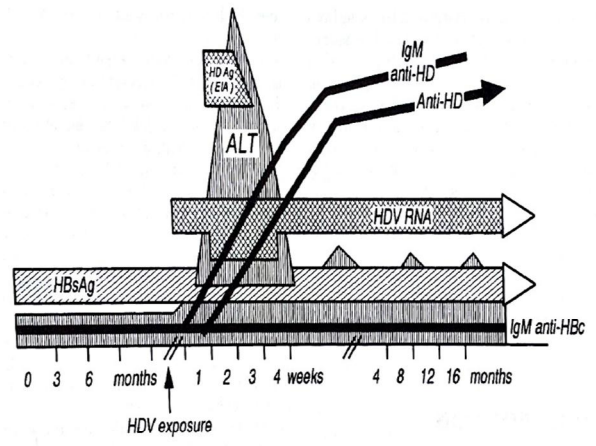


Fig.1B

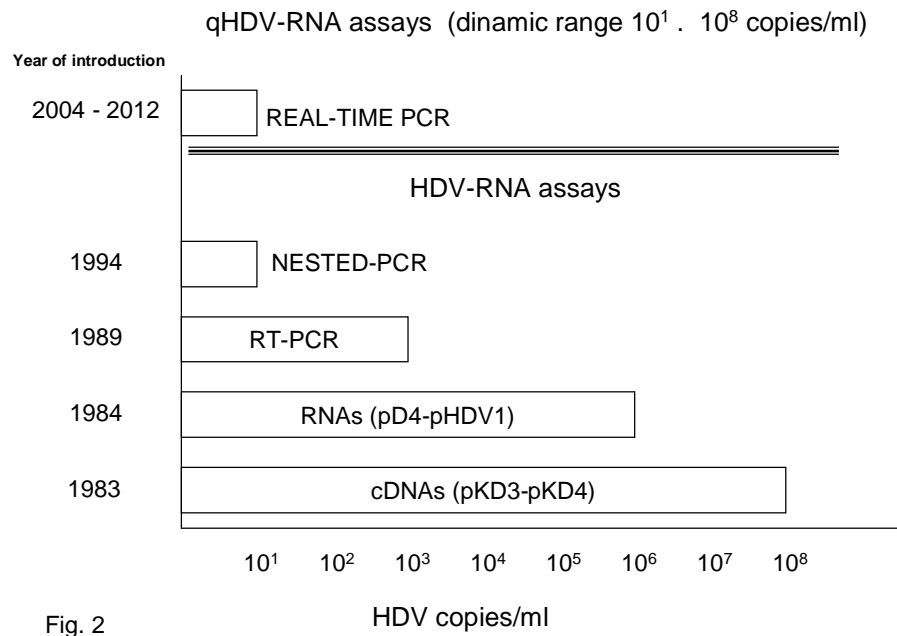


Fig. 2