Serological and Molecular Diagnosis of Hepatitis Delta Infection: Results of
a French National Quality Control

Running title: French National Quality Control for HDV Diagnosis

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Disclosures

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Abstract (Word count 53)

A French national quality control for serological and molecular diagnosis of hepatitis Delta virus (HDV) was organized. All participants detected total HDV antibodies; 8/14 failed to detect low titers of IgM; 6/11 laboratories failed to quantify and/or underestimated RNA viral load in several samples. These discrepancies are likely related to HDV molecular diversity.

Key words: HDV; diagnosis; viral load; quality control
Hepatitis Delta virus (HDV) is a satellite of hepatitis B virus (HBV). The HDV genome is composed of a small negative single-stranded RNA (1679 to 1697 nt) with a rod-like structure related to an extensive intramolecular complementarity. This genome is closely associated to the two isoforms of the Delta protein, the small (s) and the large (L) HDV antigens (Ag), and forms a ribonucleoprotein enveloped by the HBV surface antigen (HBsAg).

Eight genotypes (HDV-1 to -8) and several subgenotypes have been described, defined respectively by nucleotide divergences of >20% and >10% considering the complete genome sequence (1-3). Due to population migrations, most genotypes circulate worldwide and especially in France (4-8).

HBV/HDV co- or super-infection leads to more severe liver disease. HDV diagnosis relies on the detection of total anti-HDV antibodies (Ab). Anti-HDV IgM Abs usually persist in patients chronically infected with HDV. Some authors consider that anti-HDV IgM are a surrogate marker of HDV replication (9), however they may be lacking in some African patients ((10) and E Gordien personal observations). Therefore, HDV RNA detection/quantification is currently the only accurate diagnostic tool to confirm HDV replication status and to allow optimal management of infected patients (11, 12).

Commercial tests for HDV serology have been available for many years and are used routinely worldwide. Currently, a few commercial tests are available for HDV RNA quantification, but they perform poorly, at least for HDV-non-genotype-1 samples (13).

Numerous “in-house” assays have been developed with very different protocols (11, 14-20). To our knowledge, these assays have not been evaluated on a large panel of clinical samples of various genotypes and viral loads (VL).

In 2012, the French National Reference Center for HDV (F-NRC) in Avicenne Hospital (Bobigny, France) organized an unprecedented French national quality control (FNQC) for
the diagnosis of HDV infection by serology and molecular biology. A total of 28 laboratories participated in this study, including 22 university hospital laboratories, 2 private laboratories and 4 foreign laboratories (from Greece, Switzerland, the United Kingdom and the USA). They performed either total HDV Ab (n=24) and/or IgM Ab (n=14) detection and/or HDV RNA viral load (VL) quantification (n=11), which are performed routinely for their patients, using either in-house or commercial assays. The serology panel consisted of one negative sample and 3 undiluted sera from blood donors selected for their strong or weak reactivity performed in 3 different experiments with our commercial assays (Diasorin, Antony, France).

The molecular biology panel consisted of 11 plasma samples of various genotypes and VL, comprising: 3 HDV-1Eu/As (Europe/Asia), 3 HDV-1Afr (Africa), 2 HDV-5, 1 HDV-6, 1 HDV-7, 1 HDV-8 and a negative control (table 1). Quantification was performed blindly 3 times by different technicians in our lab using the consensus real–time RT-PCR assay described elsewhere (11). The mean of the 3 values was considered as the expected value for each sample (see Table 1). Strains were genotyped by direct sequencing of the amplicon of the R0 region (nt 889-1289) exactly as previously described (1).

The samples were sent in dry ice to the participating laboratories by an accredited transporter according to regulatory standards for the distribution of infected samples. Commercial assays were used by all labs for total and/or IgM Ab detection: Diasorin (Antony, France) (n=20), Aldatis (Rome, Italy) (n=2) or Diagnostics BioProbes (Milano, Italy) (n=2). Diasorin (n=14) was used for IgM detection. No discrepancies were found for total Ab detection, whatever the test (Table 1). The negative control (D) and the sample with high IgM Ab titer (B) were correctly identified by all labs. However, the samples with low IgM Ab titers (A and C) were identified as negative by 8 and 5 labs respectively. These results strengthen arguments for the use of HDV RNA as the sole reliable replication marker.
According to data provided, 10 laboratories used an in-house assay based on real-time RT-PCR protocols for HDV RNA quantification and the remaining one the Lightmix Roche commercial kit (Meylan, France). Primers and probes were located in the HDAg, the ribozymes or the inter-ribozyme coding regions. The labs used either an RNA or a DNA standard to quantify HDV viremia. The amount of extracted sample as well as the declared lower limits of quantification (LLOQ) ranging from 40 to 1000 copies/mL, were also different across assays. It is noteworthy that 2 labs used the same assay as the F-NRC. Overall results are shown in Table 1. Detailed results for each sample are shown in Figure 1. Two laboratories found a positive signal for the negative sample (sample 12) raising the problem of specificity for these assays, as well as the interpretation of very weak signals obtained in the real-time RT-PCR assays. Moreover 5 laboratories failed to detect one or several positive samples. Six laboratories underestimated several samples by more than 1 log copies/mL, and among them 3 underquantified all the samples. These results were obtained whatever the genotype of the infecting strain. As equally described elsewhere (13), poor results were obtained with the commercial test (Lightmix Roche). Overall, 5 laboratories found similar results (<±1 log copies/mL) to those of the F-NRC. Interestingly, all but one use primers and probes designed in the same conserved ribozyme region of the HDV genome and the remaining one in the antigen-coding region. This FNQC provided an overview and a comparison of the performances of the different HDV tests, including those of labs outside France. Commercial total anti-HDV-Ab ELISA kits seem to be the tools of choice for HDV screening in HBsAg-positive patients. IgM anti-HDV-Ab assays may fail to detect low titers of Abs and thus should not be used to evaluate HDV replication status. Therefore, HDV RNA, providing accurate detection/quantification, should be considered as the main tool for patient management.
Previous work in our lab shed light on the importance of HDV genetic variability (13). In France, 40% of the spreading strains belong to HDV-1Eu/As, 35% to HDV-1Afr and 25% to HDV-5 to -8; HDV-2, -3 and -4 remain anecdotal.

This same epidemiological situation may be true for other European countries experiencing migration of populations from endemic areas, although HDV-1 remains largely predominant and ubiquitous (4-7, 17, 20-22). However, we demonstrated in this study that even HDV-1Eu/As strain VL could not be properly quantified by all labs.

A thorough verification of primer/probe design is necessary as extensive sequence alignments have shown mismatches that can predict quantification failure for some samples (Le Gal F. personal data).

Considering available data provided by the participants, other technical points could possibly explain some discrepant results, but were not assessed at in the present study: some protocols lack an internal control to validate all steps from extraction to amplification. Therefore, for some labs, we cannot exclude specific technical problems on particular samples. Additionally, some steps are performed manually, raising the question of the reproducibility of results, although we did not look at that issue in the present study.

Either cDNA or RNA samples were used as quantification standards, thus precise comparisons between tests were difficult in the absence at the time of the study of an international standard. This issue will be greatly improved with the now-available WHO HDV RNA standard (www.who.int/biologicals/expert_committee/BS_2227_HDV_RNA.pdf).

Nevertheless, this study did show that comparable results (coefficient of variation <20%) can be obtained with different assays (data not shown).

The management of an HDV-infected patient relies on an accurate estimation of HDV replication. Indeed, a false-negative HDV-VL result could lead to (i) misdiagnosis and failure
to identify a HDV-replicating patient; (ii) poor management with under treatment and (iii) failure in detecting early relapse (12, 13).

To conclude, we advise that HDV-VL should be monitored only in reference virology centers using well-evaluated techniques. Also, there is a need for international comparative studies comprising samples of all genotypes and subgenotypes spreading worldwide. Indeed, all efforts to improve and standardize quantification assays should be continued, including the use of the newly available WHO HDV RNA standard, with the goal of forwarding collaborative clinical studies and ultimately optimizing patient management.
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References


Figure 1 legend:

HDV RNA quantification results for the 12 samples, for all participants, expressed as log_{10} copies/mL. Horizontal bars represent the expected VL values, obtained by the F-NRC laboratory (mean of 3 different experiments). VL values < \pm 1 \log_{10} \text{ copies/mL} or > \pm 1 \log_{10} \text{ copies/mL} compared with the expected VL values are represented with dots or crosses, respectively. The grey area represents the lower limit of detection/quantification of the different assays. Genotypes of the different samples are indicated.

Table 1 legend:

<table>
<thead>
<tr>
<th>n: Number of concordant results</th>
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<td>N: Number of participants</td>
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* Mean of values of 3 different experiments performed by the F-NRC laboratory

** Concordance: less than \pm 1 \log_{10} \text{ copies/mL} compared to the expected values from F-NRC lab
Table 1: Serological and molecular biological results

<table>
<thead>
<tr>
<th>Samples</th>
<th>HDV-Ab</th>
<th>Expected results</th>
<th>Concordance n / N (%)</th>
<th>Molecular biological results</th>
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<td>Samples</td>
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<tr>
<td>A</td>
<td>Total Ab</td>
<td>POS</td>
<td>24 / 24 (100%)</td>
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<tr>
<td></td>
<td>IgM Ab</td>
<td>POS (weak)</td>
<td>6 / 14 (43%)</td>
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<tr>
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<td>24 / 24 (100%)</td>
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<td></td>
<td>IgM Ab</td>
<td>POS</td>
<td>14 / 14 (100%)</td>
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<tr>
<td>C</td>
<td>Total Ab</td>
<td>POS</td>
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<tr>
<td></td>
<td>IgM Ab</td>
<td>POS (weak)</td>
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<tr>
<td>D</td>
<td>Total Ab</td>
<td>NEG</td>
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<td>IgM Ab</td>
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