Chronic hepatitis C: new diagnostic tools and therapeutic strategies

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Citation for published version (APA):

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CHARACTERISATION OF THE EUROHEP HCV-RNA PLASMA STANDARDS IN A COLLABORATIVE STUDY

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Eighty-six laboratories participated in a collaborative study and tested the second EUROHEP HCV-RNA reference panel. The coded panel was comprised of 4 HCV-RNA positive plasma samples, 6 HCV-RNA negative plasma samples and two dilution series of HCV-RNA genotype 1 and 3 plasma standards. The 86 laboratories submitted 136 coded data forms for evaluation. Of these data sets 99 were tested using a PCR assay developed in-house, 28 using the AMPLICOR HCV assay (Roche) and 9 using other amplification methods. Twenty-two data forms (16%) had faultless results, 39 (29%) missed the weak positive sample only and 75 data sets (55%) had false positive and/or false negative results. Similar results were found in the other EUROHEP/EGRVD quality control studies on HCV-RNA (1992), HBV-DNA (1993) and CMV-DNA (1995). In 56 proficient laboratories the 50% and 90% PCR detection endpoints in the dilution series of the HCV genotype 1 plasma standard were approximately 600 genome equivalents per ml (geq/ml) and 7750 geq/ml respectively according to standards applied in the bDNA Quantiplex 1.0 assay (Chiron). The values were close to those calculated from statistical analysis of the limiting dilution PCR results. The results from this study demonstrate that a commercial quantitative HCV-RNA assay underestimates the HCV-RNA concentration by a factor 10 or more. A number of laboratories used a 1:1,000 and 1:10,000 dilution of the EUROHEP genotype 1 plasma standard (3640 and 364 geq/ml respectively) as a run control and demonstrated a 10-fold increase in sensitivity of the AMPLICOR HCV assay when using a modified RNA extraction procedure.

This study demonstrates that an international quality assessment program is a powerful instrument for characterization of viral nucleic acid plasma standards and validation of amplification assays.
The technique used most commonly to detect HCV-RNA is reverse transcription of the viral RNA and subsequent amplification of the copy DNA (cDNA) by the polymerase chain reaction (PCR). However, other amplification techniques and a sensitive method involving hybridization with branched DNA probes (bDNA) can also be used. In a person with antibodies against HCV, a negative PCR result may reflect a resolved infection or non-specific anti-HCV reactivity while a positive PCR indicates ongoing infection. PCR is also used for monitoring the effect of antiviral treatment of HCV infection. Apart from sensitive, qualitative detection of HCV-RNA, quantification and genotype determination of HCV-RNA have also become important. PCR and other amplification tests have major consequences for the management of patients with viral infections and identification of infected blood donors. More recently, PCR is also used for the virological safety testing of plasma products. Since the monitoring of laboratory performance is a prerequisite for ensuring reliable amplification test facilities, the European Expert Group on Viral Hepatitis (EUROHEP) and the European Group for Rapid Viral Diagnosis (EGRVD) continue their international program for quality assessment and standardization of viral RNA/DNA detection. From quality control studies on PCR in leukaemia research and detection of human immunodeficiency virus, it has become apparent that PCR results are often false-positive or false-negative. In the first EUROHEP quality control study for HCV-RNA detection in 1992, only 16% of the laboratories produced faultless results (Table 1). Even these laboratories reported 100-fold differences in sensitivity by testing dilutions of the EUROHEP genotype 1 standard. Thereafter, similar EUROHEP/EGRVD studies have been organized for detection of HBV-DNA (1993), HCV-RNA (1994) and CMV-DNA (1995) (Quint et al., unpublished observations, March 1996).

In the present study we assessed the laboratory performance on the second EUROHEP HCV-RNA reference panel and compared the results with those of other EUROHEP/EGRVD quality control studies. We also used the results of proficient laboratories to

<table>
<thead>
<tr>
<th>Performance</th>
<th>HCV-RNA 1992 (n=22)</th>
<th>HBV-DNA 1993 (n=43)</th>
<th>HCV-RNA 1994 (n=136)</th>
<th>CMV-DNA 1995 (n=63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faultless</td>
<td>16%</td>
<td>23%</td>
<td>16%</td>
<td>19%</td>
</tr>
<tr>
<td>Missed weak positive only</td>
<td>23%</td>
<td>21%</td>
<td>29%</td>
<td>24%</td>
</tr>
<tr>
<td>Total sufficient quality</td>
<td>39%</td>
<td>44%</td>
<td>45%</td>
<td>43%</td>
</tr>
<tr>
<td>False pos/neg results</td>
<td>61%</td>
<td>56%</td>
<td>55%</td>
<td>57%</td>
</tr>
</tbody>
</table>

*Zaaijer et al. 1993
*Quint et al. 1995
*Damen et al. 1996
*Quint et al. 1996, personal communication
characterise the EUROHEP HCV-RNA genotype 1 and genotype 3 plasma standards. Concurrently we examined the sensitivity and detection efficiency of qualitative and quantitative amplification methods for these two HCV genotypes. Finally, we demonstrated the suitability of the EUROHEP genotype 1 plasma standard as a run control in routine laboratory practise.

MATERIALS AND METHODS

HCV-RNA reference panel
The panel consisted of 26 coded plasma samples with and without HCV-RNA: 10 undiluted samples and 2 dilution series of 8 samples each. The 10 undiluted samples originated from 6 HCV-RNA negative donors and 4 HCV-RNA positive patients (one weakly positive). Two reference laboratories tested the samples before release of the panel. The participants were not able to deduce the HCV-RNA status from the anti-HCV status, since 5 out of the 6 HCV-RNA negative samples were anti-HCV (false) reactive. The dilution series were prepared from the EUROHEP genotype 1 and 3 plasma standards. The following dilutions were included: genotype 1 plasma; 100, 1000, 4000, 16000, 64000, 256000, 1024000, 4096000; genotype 3 plasma; 10, 100, 1000, 4000, 16000, 64000, 256000, 1024000. Genotype detection was performed at the Central Laboratory of the Blood Transfusion Service (CLB) using restriction fragment length polymorphism (RFLP) analysis.

Quality scoring of participants
The panels, together with result and method forms, were sent to 97 investigators of whom 86 submitted results (1-4 data sets per laboratory). In total 144 data sets were received, of which 136 were suitable for evaluation. To ensure confidentiality, all laboratories sent their results to Prof. S.W. Schalm (University Hospital Rotterdam), who assigned a code to each participant. The anonymous results were analyzed at the coordinating laboratory (CLB). Proficient laboratories were those whose results were faultless or missed only the weak HCV-RNA positive undiluted sample. Dilution series were considered to be tested correctly if one or more of the least diluted samples were found to be positive and all higher dilutions were found to be negative.

Characterization of the EUROHEP HCV-RNA plasma standards
To characterise the EUROHEP HCV-RNA genotype 1 and 3 plasma standard and to study the sensitivity of the qualitative assays applied, we used the results of proficient laboratories only.

The HCV-RNA concentrations in the undiluted EUROHEP standards were statistically determined by assessing the laboratories results on the dilution series. The 50% and 90% laboratories’ detection endpoints were calculated for different qualitative amplification methods (see statistics). Furthermore, the HCV-RNA concentration in the undiluted EUROHEP genotype 1 and 3 plasma standard was repeatedly measured at CLB with 3 commercial quantitative assays: the bDNA assay (Quantiplex HCV-RNA assay, Chiron Corporation, Emeryville, CA, USA), the AMPLICOR HCV MONITOR Kit (Roche Diagnostic Systems, Branchburg, NJ, USA) and an experimental, quantitative, self-sustaining isothermal RNA amplification method (QT NASBA).
Assay validation using the CLB HCV-RNA run control

The 1:1,000 and 1:10,000 dilution of the EUROHEP genotype 1 plasma standard were used as run controls in every HCV-RNA test run at CLB. The performance of three different PCR test protocols used over the years was evaluated by the positive scores of these run controls. The following assays were evaluated: in-house developed HCV cDNA-PCR (period 1993-1994), Roche AMPLICOR HCV assay (period 1994-1995) and the Roche AMPLICOR HCV assay with a modified RNA extraction procedure (protein K digestion and phenol/chloroform extraction) (period 1995-1996). Furthermore, these run controls were used by many laboratories as a stop or go reagent named ‘PELISPY’. We evaluated the performance of the different test protocols used by these laboratories (in-house PCR, AMPLICOR and modified AMPLICOR) by the positive scores of the run controls. The results were obtained by a volunteer questionnaire.

Statistics
The 50% and 90% detection endpoints of the different PCR methods were calculated by probit analysis. Briefly, the method is as follows: in the dilution series the proportion of laboratories with a positive result is plotted against the log of the dilution. From this dose-response curve the 50% and 90% detection rates are calculated. The concentration difference between the genotype 1 and 3 standards as measured in the different PCR protocols, can be calculated from the shift between the curves if they are parallel. Curves were considered to be parallel if the results of the Pearson Goodness-of-fit test and Pearson Parallelism test were not significant ($P>0.05$).

The HCV-RNA concentrations in the EUROHEP plasma standards were estimated statistically according to the method of Strijbosch et al by assessing the limiting dilution PCR response rates which follow a Poisson distribution. For this estimation of the HCV-RNA content in the EUROHEP genotype 1 and 3 reference preparations it was assumed that the PCR is able to pick-up one copy per assay, even though the efficiency of the RT-PCR procedure is probably less. For this analysis we selected 16 proficient laboratories using the same PCR protocol (AMPLICOR). The HCV-RNA concentration in the EUROHEP standards was back-calculated from the equivalent of the plasma volume that was subjected to the amplification reaction (5μl).

The percentage positive scores on the run control (PELISPY) in the different laboratories and with the different amplification methods were compared in a chi-squared test for trend.

RESULTS

Amplification test protocols
In total 136 data forms suitable for evaluation were submitted by 86 laboratories. The following PCR protocols were used: in 99 data sets cDNA-PCR developed in-house, in 28 the AMPLICOR HCV test (Roche Diagnostic Systems, Branchburg, NJ, USA) and in 3 the HCV bDNA assay (Chiron Corporation, Emeryville, CA, USA). In 6 cases other protocols were used, such as experimental NASBA protocols or combinations of different protocols.

Performance on 2nd EUROHEP HCV-RNA reference panel
Overall, 22 of 136 result forms (16%) were fully correct and in 39 (29%) only the weakly positive sample was missed. In 75/136 data forms (55%) false-positive and/or false-negative
results were reported in the undiluted samples or the dilution series. As is shown in Table I these results are comparable with the results in other EUROHEP/EGRVD quality control studies on viral detection by nucleic acid based assays.

**Sensitivity in dilution series**

The response curves in fig. 1A and 1B show the detection rates of 56 sufficiently performing laboratories in the genotype 1 and 3 dilution series respectively. The results of nested-PCR developed in-house (n=33), single-PCR (n=7) and the commercial assay (AMPLICOR) (n=16) were analyzed separately. As shown in the figures nested amplification protocols tended to be more sensitive than single round PCR protocols. The individual endpoint titres achieved by participants varied between 1:100 to 1:1,024,000 and 1:10 to 1:64,000 in the genotypes 1 and 3 standard dilutions, respectively. This variation can be explained by differences in sensitivity of the applied PCR protocols, but also by the nature of PCR detection in limiting dilutions, which ought to follow a Poisson distribution. Table II shows the dilutions of the genotype 1 and 3 standards at the 90% and 50% laboratories.

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**Fig. 1.**

detection endpoints with the different PCR protocols, as calculated by probit analysis. The 90% genotype 1 detection rate was found at a higher dilution by the commercial assay than by the in-house assays. By contrast, the 90% genotype 3 detection rate was found at a lower dilution by the commercial assay.

Quantification of HCV-RNA in EUROHEP standards
We estimated a concentration of $25 \times 10^5$ cps/ml in the EUROHEP genotype 1 standard by statistical analysis of the results of 16 laboratories using the same method (AMPLICOR) in the standard dilution series (see methods).

Statistical analysis of the genotype 3 results was not possible, due to a non-Poisson distribution of PCR response rates. The probit analysis on the collaborative results of 56 qualitative PCRs of proficient laboratories revealed on average a factor 3.3 difference in HCV-RNA concentration between the genotype 1 and 3 plasma standards (see tab. II). According to this factor the HCV-RNA concentration in the type 3 standard is $25 \times 10^5$ cps/ml/3.3 = $7.6 \times 10^5$ cps/ml. In Table III the results of the quantitative methods on the undiluted genotype 1 and 3 HCV-RNA plasma standards are compared with the values obtained by the statistical quantification of the collaborative study results.

Table II.
Dilution at the 50% and 90% laboratories detection endpoint of nested-PCR, single-round PCR and the AMPLICOR assay in dilution series of the EUROHEP genotype 1 and 3 plasma standards, as determined by probit analysis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection endpoint</th>
<th>Genotype 1 series</th>
<th>Genotype 3 series</th>
<th>Factor difference$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested-PCR (n=33)</td>
<td>50%</td>
<td>1:7521</td>
<td>1:2978</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>1:346</td>
<td>1:282</td>
<td></td>
</tr>
<tr>
<td>Single-PCR (n=7)</td>
<td>50%</td>
<td>1:3350</td>
<td>1:1614</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>1:729</td>
<td>1:566</td>
<td></td>
</tr>
<tr>
<td>AMPLICOR (n=16)</td>
<td>50%</td>
<td>1:6060</td>
<td>1:1197</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>1:1137</td>
<td>1:117</td>
<td></td>
</tr>
<tr>
<td>All (n=56)</td>
<td>50%</td>
<td>1:6582$^b$</td>
<td>1:2299</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>1:624$^b$</td>
<td>1:270</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Factor difference between genotype 1 and genotype 3 series as determined by probit analysis.

$^b$ The concentration at the 50% and 90% detection endpoint was 600 geq/ml and 7750 geq/ml, as deduced from the bDNA measurement in the undiluted sample.

The factor difference between genotype 1 and genotype 3 concentration is also shown for the different methods. Instead of a 3.3 factor difference the commercial quantitative HCV-RNA assays showed a 7- to 43- fold concentration difference between the two standards.

Laboratories performance on the run control
The laboratories positive detection rates on the HCV-RNA run controls (1:1,000 and 1:10,000 dilution of the EUROHEP genotype 1 standard plasma) for different methods are
### Table III.

Quantitative HCV-RNA test results on the EUROHEP HCV-RNA plasma standards.

<table>
<thead>
<tr>
<th>Method</th>
<th>Genotype 1</th>
<th>Genotype 3</th>
<th>Factor diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCV-RNA concentration x10⁶:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genotype 1</td>
<td>Genotype 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=6)⁵</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>bDNA 1.0</td>
<td>36.4 geq/ml</td>
<td>≤3.5 geq/ml</td>
<td>&gt;10</td>
</tr>
<tr>
<td>bDNA 2.0</td>
<td>37.9 geq/ml</td>
<td>not reliable</td>
<td>not reliable</td>
</tr>
<tr>
<td>AMPLICOR MONITOR</td>
<td>2.8 cps/ml</td>
<td>0.0065 cps/ml</td>
<td>43</td>
</tr>
<tr>
<td>QT NASBA exp.</td>
<td>165 cps/ml</td>
<td>24 cps/ml</td>
<td>6.9</td>
</tr>
<tr>
<td>Statistical analysis on dilution series⁶</td>
<td>25 cps/ml (n=16)</td>
<td>7.6 cps/ml (n=16)</td>
<td>3.3⁷</td>
</tr>
</tbody>
</table>

* n=number of measurements

b not reliable results: 9.3, 5.3, ≤0.2, ≤0.2 x10⁵ geq/ml

c calculated by probit analysis on 56 sufficient performing laboratories

d Strijbosch et al 1988; the genotype 3 titre could not be determined by statistical analysis and is therefore deduced from the genotype 1 titre by probit analysis.

⁵ 16 proficient laboratories using the same RT-PCR protocol (AMPLICOR)

### Table IVa.

Detection rate of the PELISPY HCV-RNA run controls at the CLB during 3 test periods (1:1,000 and 1:10,000 dilution of the EUROHEP genotype 1 standard).

<table>
<thead>
<tr>
<th>Method</th>
<th>Period</th>
<th>Number (%) positive:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1,000 dil.</td>
</tr>
<tr>
<td>In-house</td>
<td>1993-1994</td>
<td>25/25 (100%)</td>
</tr>
<tr>
<td>AMPLICOR</td>
<td>1994-1995</td>
<td>209/235 (89%)</td>
</tr>
<tr>
<td>Modified AMPLICOR</td>
<td>1995-1996</td>
<td>71/71 (100%)</td>
</tr>
</tbody>
</table>

*Chi-squared test for Trend: p=0.0007

bChi-squared test for Trend: p<0.0001

### Table IVb.

Detection rate of the PELISPY HCV-RNA run controls in 11 laboratories (1:1,000 and 1:10,000 dilution of the EUROHEP genotype 1 standard).

<table>
<thead>
<tr>
<th>Method</th>
<th>Laboratories</th>
<th>Number (%) positive:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1000 dil.</td>
</tr>
<tr>
<td>In-house</td>
<td>n=3³</td>
<td>19/56 (34%)</td>
</tr>
<tr>
<td>AMPLICOR</td>
<td>n=6</td>
<td>45/53 (85%)</td>
</tr>
<tr>
<td>Modified AMPLICOR</td>
<td>n=2</td>
<td>6/6 (100%)</td>
</tr>
</tbody>
</table>

*Chi-squared test for Trend: p<0.0001

³Chi-squared test for Trend: p<0.0001

³Two labs had a low sensitivity.
shown in Table IV. Both at CLB and in other laboratories, the sensitivity of the AMPLICOR assay with modified sample preparation was approximately 10-fold higher than the regular AMPLICOR assay (p=0.0007; Chi-squared Test for Trend).

**DISCUSSION**

In the present and 3 other EUROHEP quality control studies on viral nucleic acid based amplification assays, 39-45% of the laboratories produced sufficient results and 55-61% generated false positive and/or false negative results. Only 16-23% of the participants had faultless results. In the present report we used the results of the proficient laboratories that participated in the second EUROHEP HCV-RNA quality control study to examine the sensitivity of the HCV-RNA amplification protocols applied.

The 50% laboratories detection endpoint of the different protocols taken together was 600 geq/ml according to standards applied in the bDNA 1.0 assay (Chiron). At the 90% detection endpoint in the genotype 1 standard dilution series the commercial assay (AMPLICOR) tended to be more sensitive than in-house PCR protocols. By contrast, the in-house methods were more sensitive at the 90% detection endpoint in the genotype 3 standard dilution series. However, the differences in sensitivity were not statistically significant. The results indicate that the EUROHEP HCV-RNA genotype 3 standard is detected with somewhat lower efficiency by AMPLICOR than by in-house PCR protocols. Also, in another study on dilutions of the NIBSC HCV-RNA genotype 3 plasma standard it was found that the AMPLICOR assay was less sensitive than in-house PCR tests.

Using probit analysis on the EUROHEP study results of 56 proficient laboratories, we calculated an HCV-RNA concentration difference between the type 1 and type 3 standards of 2.8, 2.2 and 4.7 by assessing limiting dilution results of in-house single-PCR, in-house nested-PCR and AMPLICOR respectively (Tab. II). However, the concentration difference between the EUROHEP genotype 1 and 3 standards as measured by three quantitative assays (bDNA, Roche MONITOR assay and experimental NASBA) was more than 10-, 44- and 7-fold respectively (Tab. III). This demonstrates that HCV-RNA of genotype 1 and 3 are detected with different efficiency in the various quantitative assays. If we assume that limiting dilution nested-PCR is the most accurate and least genotype dependent quantitative method, the commercial quantitative assays underestimate the genotype 3 standard by a factor 2 to 16. Probably because of the application of genotype 1 based calibrator molecules, the Roche AMPLICOR MONITOR assay underestimates the genotype 3 HCV-RNA plasma standard by a factor 10 if compared to limiting dilution PCR with the same primers (qualitative AMPLICOR HCV assay)(see tables II and III).

Detmer et al reported that the new and more sensitive version of the bDNA assay (bDNA 2.0) detected HCV-RNA of 6 genotypes with equal efficiency. This was demonstrated by testing in vitro HCV-RNA standards quantified by physical and chemical non-hybridization dependent methods. However, since the newer version of the bDNA assay also is approximately 1,000-fold less sensitive than the PCR methods it was not possible to reliably quantify the EUROHEP genotype 3 plasma standard in the bDNA assay (Tab. III). The HCV-RNA concentration in the EUROHEP genotype 1 plasma standard, according to statistical analysis of the collaborative AMPLICOR detection rates in the dilution series, was very close to the HCV-RNA concentration measured by the bDNA 1.0 assay (25x10^5 cps/ml and 36.4x10^5 geq/ml respectively). Gerlich et al (personal communication, May
characterized the EUROHEP HBV-DNA standards (genotypes A and D) with limiting dilution analysis using a similar statistical method, whereas Zaaijer et al tested the same standards with the HBV-bDNA assay. It was found that the estimated HBV-DNA concentration according to the limiting dilution analysis was very close to the HBV-DNA concentration in the bDNA assay: $4.0 \times 10^9$ cps/ml and $4.3 \times 10^9$ geq/ml respectively in the genotype A standard and $3.6 \times 10^9$ cps/ml and $3.5 \times 10^9$ geq/ml respectively in the genotype D standard (historical bDNA values were corrected with a factor 0.65 according to Urdea et al (personal communication, May 1996)). Since the bDNA assay is widely accepted as an accurate and precise method for quantitative measurement of viral load, we decided to express the HCV-RNA concentration in the EUROHEP standards according to the standards applied in the bDNA assay.

We used the HCV-RNA genotype 1 standard in a 1:1,000 and 1:10,000 dilution as a run control (PELISPY HCV-RNA) in our routine laboratory practice and also distributed the 1:1,000 dilution with diluent to other laboratories. As established in the present collaborative EUROHEP study the 1:1,000 diluted standard lies approximately at the 90% detection endpoint of proficient laboratories whilst the 1:10,000 diluted standard lies below the 50% detection endpoint. Our studies show that the 1:1,000 and 1:10,000 run control (3640 and 364 geq/ml) are suitable for validation of routine HCV-RNA amplification assays in donor or patient specimens as well as in plasma pools. Laboratories using the PELISPY run control found a 10-fold increase in sensitivity of the AMPLICOR assay if a modified RNA-extraction method was used.

An external quality assessment program is essential for the characterization of standards and validation of nucleic acid amplification techniques. This study demonstrates that the characterized standards can then be used as reference materials for validation of new viral nucleic acid amplification tests.

ACKNOWLEDGEMENTS

This work was undertaken as a part of a joint quality assessment project of the European Concerted Action on Viral Hepatitis and the European Group for Rapid Viral Diagnosis. We thank S.W. Schalm (EUROHEP project leader, Rotterdam), and all participants for their efforts and co-operation.

REFERENCES


