

Analytical and biological variables influencing quantitative hepatitis C virus (HCV) measurement in HIV-HCV coinfection

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The present review considers issues pertaining to the precision and variability of quantitative hepatitis C virus (HCV) measurement in general, outlines the characteristics of HCV RNA in HIV-HCV coinfection and evaluates those factors which may affect this measure. The clinical relevance of accurate HCV measurement in HIV-HCV coinfection is discussed.

Key Words: HCV RNA; HIV; Variability

As a consequence of shared modes of transmission, HIV and hepatitis C virus (HCV) are often found concurrently (1,2). Each chronic viral infection influences the other in terms of clinical manifestations and disease progression (3,4). Although the clinical significance is uncertain, it is well documented that quantitative HCV RNA levels are higher in HIV-HCV coinfection than HCV alone (5-9). Antiretroviral therapy also influences HCV RNA levels in HIV-HCV infected patients but the size, direction and long-term consequences of this influence remains contentious (10). In fact, there are studies suggesting an increase, decrease or absence of change in HCV levels with prolonged HIV drug treatment. Although at first bewildering, the heterogeneity of these results can be better understood if the multiple factors increasing HCV RNA measurement variability and, thereby, reducing precision are considered (see Table 1 for definition of terms). The present review will consider analytical and biological variables effecting the accuracy and reproducibility of quantitative HCV RNA measurement, outline the characteristics of HCV RNA level in HIV-HCV coinfection and illustrate the clinical relevance of these issues.

ANALYTICAL VARIABLES INFLUENCING QUANTITATIVE HCV RNA MEASURES

There are multiple laboratory-related parameters which contribute to variability and loss of precision in quantitative measurement of a value. Sample processing, operator technique, type of quantitative assay used, assay platform and interfering substances (ie, endogenous and exogenous material found concomitantly within the specimen which may alter quantitative

Les variables analytiques et biologiques influant sur la mesure quantitative du virus de l'hépatite C (VHC) en cas de co-infection par le VIH et le VHC

La présente analyse évalue des problèmes reliés à la précision et à la variabilité de la mesure quantitative du virus de l'hépatite C (VHC) en général, traite des caractéristiques de l'ARN du VHC en cas de co-infection par le VIH et le VHC et évalue les facteurs qui peuvent influencer sur cette mesure. La pertinence clinique des mesures précises de VHC en cas de co-infection par le VIH et le VHC est exposée.

results) may each introduce variability and reduce precision in reported HCV RNA levels. Historically, interpretation and comparison of reported HCV RNA measurements in the published literature were hampered by the use of different assay technologies (reverse transcriptase polymerase chain reaction [RT-PCR], branch DNA [bDNA] and nucleic acid sequence-based amplification [NASBA]), different reported units of measurement, problems with the determination of HCV RNA for nongenotype-1 isolates and periodic changes in the dynamic range of some assays (eg, Roche monitor version 2.0 [Roche Diagnostics, USA]) (11-17). Some of these difficulties have been resolved by the development of newer generation assays and the adoption of a universal unit of HCV RNA measurement (ie, IU/mL). The problems with quantification of nongenotype-1 samples were corrected with a new generation RT-PCR assay (16). Despite these advances problems remain. For example, the reported values of the Cobas Amplicor HCV monitor version 2.0 (LaRoche, USA) (RT-PCR) may be lower than the Versant HCV RNA 3.0 assay (Bayer Diagnostics, USA) (bDNA) for samples with HCV RNA levels greater than 10^5 IU/mL despite the common use of IU/mL units (18). The reverse may be true for levels between 5×10^2 IU/mL and 5×10^5 IU/mL (19).

Currently, the Cobas Amplicor HCV monitor test and Bayer Versant HCV RNA assay are widely used in research and in clinical practice. Several recent reports evaluate and compare the characteristics of these two assays. The correlation between the HCV RNA results produced by these two assays is high ($r=0.924$) with similar slopes reported within the shared linear ranges (18). The reproducibility, linearity and analytical sensitivity across genotype of the Versant HCV RNA 3.0 assay

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TABLE 1
Definition of terms

Term	Definition
Accuracy	Ability of an assay to determine the true value of an analyte
Sensitivity	Ability of an assay to measure virus. This determines the lower limit of detection
Linearity	The degree to which assay standard curve approximates a straight line. A measure of how accurately changes in viral load over the dynamic range of an assay are measured
Dynamic range	Quantitative range over which an assay can reliably report results
Precision/ reproducibility	Ability to obtain the same answer when tested repeatedly. Reproducibility of result irrespective of laboratory, day, time, operator, instrument or assay used
Within assay variability	Difference in results between a split specimen performed in the same laboratory, on the same instrument, using the same assay and by the same operator
Between assay variability	Difference in results between a split specimen run at different times at the same laboratory. Assay batch and operator are additional sources of increased variability if they differ
Coefficient of variation (CV)	An expression of the SD as a proportion of the mean [CV=(SD/x)×100]. The CV allows for direct comparison of SD when distributions have means of different magnitudes

has been demonstrated (20). All non-1a genotype transcripts had relative recoveries within 1.5-fold of the genotype-1a test sample. With the exception of unconjugated bilirubin, greater than or equal to 20 µmol/L, and protein greater than or equal to 90 g/L, no endogenous or exogenous interfering substances were identified. Within-run and between-run reproducibility of the Versant HCV RNA 3.0 assay was assessed by testing specimens in replicates of six in two different runs (18). The estimated assay variability was within 0.10 log₁₀ SD. The coefficient of variation for quantification ranged from 4.9% to 9%, which is indicative of high precision. The within-run and between-run precision of the COBAS Amplicor assay was 0.10 log₁₀ SD and 0.14 log₁₀ SD, respectively, for log₁₀ HCV RNA copies/mL, which is likewise indicative of high precision (13). This is an excellent example of how different approaches to the reporting of assay performance (ie, coefficient of variation versus log₁₀ SD) make direct comparisons of results difficult.

Although assay performance has not been evaluated specifically in HIV-HCV coinfecting cohorts, it is unlikely to differ from HCV monoinfected individuals. Of note, HIV-1 was not found to act as an inhibitor of the Bayer Versant HCV RNA 3.0 assay (20).

The methods used to process and store specimens may influence HCV RNA quantification. In the past, a reduction in the measured HCV RNA was described if blood was not spun to remove the HCV-containing plasma within 6 h (15). More recent investigations evaluating the effects of temperature, duration of storage before measurement and type of blood collection tubes used suggest that there is minimal, if any, decline in HCV RNA levels in blood and plasma specimens, assuming the use of appropriate collection tubes. Little change in HCV RNA was noted in specimens drawn with EDTA-containing tubes and stored at 4°C or room temperature for up to 120 h (21). HCV RNA levels did not decline for at least 96 h at room temperature in whole blood specimens drawn in

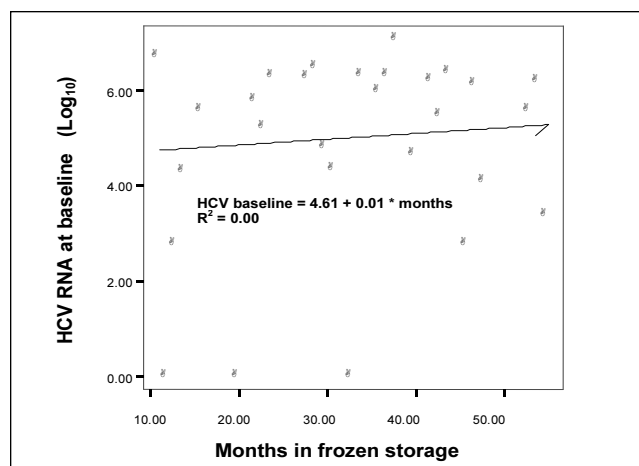


Figure 1) The duration of frozen storage (−70°C) has no influence on quantitative measurement of individual hepatitis C virus (HCV) RNA specimens in 50 HIV-HCV coinfecting subjects. A single measure was taken for each specimen. Each specimen was drawn immediately before the initiation of antiretroviral therapy

ETDA-containing tubes or in nucleic acid stabilization tubes (22). This knowledge is critical for Canadian-based clinicians and investigators given that specimens are often transferred long distances to central laboratories for measurement.

Many studies report results on specimens held in frozen storage for months or even years before measurement of HCV RNA. Halfon et al (23) suggested that frozen storage may influence HCV RNA measurement, because a 10% decline in levels was noted in specimens stored at −80°C for six months. Other studies (24,25) suggest that the HCV RNA levels remained stable while in varying periods of long-term frozen storage, assuming appropriate prestorage handling. Krajden et al (26) demonstrated that multiple freeze-thaw cycles had a small, clinically insignificant effect on HCV RNA levels. Furthermore, delays of up to 96 h in quantitative measurement of HCV RNA following removal of specimens from frozen storage appear to have little effect on precise quantification (27). In our own study, frozen plasma (−70°C) specimens were used to determine HCV RNA level in 50 combination antiretroviral treatment-naïve HIV-HCV coinfecting subjects. The duration of frozen storage did not influence accurate quantification ($\beta=0.01$, $P=\text{not significant}$) (Figure 1).

BIOLOGICAL SOURCES OF VARIATION IN HCV RNA MEASUREMENT

During the acute period of HCV infection, the HCV RNA level spikes and then falls to undetectable levels in those who spontaneously clear infection or to a stable level in those who remain chronically infected. The initial HCV RNA levels may fluctuate dramatically before seroconversion (28). For those who eventually become chronically infected, the HCV RNA level remains relatively stable over time in the absence of HCV antiviral therapy or significant alteration in host immune status (12,23,29-36). Over a period of two to three months, HCV RNA levels fluctuate by no more than 0.75 log₁₀ (23,35). Arase et al (34), in a study of 212 subjects, reported HCV RNA level fluctuations of less than 1.0 log₁₀ in 93% of subjects over a two-year period. It is important to note that at least some of the important biological factors which could influence HCV RNA measurement (ie, HCV antiviral

drug exposure and the use of immune suppressants) were accounted for in that study. Subjects with antiviral or immune suppressant use during the study period were excluded from evaluation. In a small cohort ($n=14$) followed from 3.7 years to 6.6 years, HCV RNA level fluctuated by less than $1.0 \log_{10}$ in all but one individual (31). By contrast, in a study of 60 patients, followed a median of 40.4 months, the group mean HCV RNA level fluctuated by greater than $1.0 \log_{10}$ in 39% and greater than $1.5 \log_{10}$ in 17% (33). The reason for greater fluctuation of HCV RNA level in this cohort is unclear and again illustrates the importance of identifying sources of analytical and biological variability. Of note, each of the above studies used the Chiron Quantiplex bDNA assay (Bayer Diagnostics [formerly Chiron Corp], USA).

The stability of HCV RNA level is not as well described in HIV-HCV coinfecting subjects (7,30,32). HCV RNA stability, defined as group mean HCV RNA level fluctuations of less than $1.0 \log_{10}$, was reported over the initial two to five years of infection in a cohort of 13 coinfecting individuals (7). This was also observed in four HCV-HIV coinfecting individuals followed for over two years (30). These subjects were not on antiretroviral therapy. Over a mean follow-up period of seven years, mean HCV RNA levels in 175 HIV-HCV coinfecting subjects with minimal antiretroviral exposure was relatively stable between baseline ($6.67 \log_{10}$ copies/mL) and seven years ($6.54 \log_{10}$ copies/mL) (32). Individual variability and selective follow-up were not addressed, thereby limiting application of these results. Keeping these issues in mind, the best observations available suggest HCV RNA fluctuations due to analytical variability and normal biological fluctuations within the infected host generally do not exceed $1.0 \log_{10}$ in HIV-HCV coinfecting individuals.

The extent of immune deficiency, estimated by measurement of the absolute CD4 T lymphocyte count, may influence HCV RNA level in HIV-HCV coinfection. An inverse correlation between absolute CD4 T lymphocyte count and HCV RNA level has been described (7,32). Eyster et al (7) reported relative stability in HCV RNA level until profound immune suppression developed (ie, CD4 T lymphocyte counts less than 200 cells/ μ L). Daar et al (32), in a cross-sectional study, reported an inverse relationship between CD4 T cell count and HCV RNA level. The $0.028 \log_{10}$ copies/mL increase per 100 cells/ μ L change in CD4+ T cell count described in this paper is likely of no clinical significance (32). Beld et al (30) assessed nine HIV-seropositive subjects without profound immune suppression and reported a very weak inverse relation. Several other studies (5,6,37,38) evaluating sizable cohorts do not identify a correlation between CD4 T lymphocyte count and HCV RNA level. Overall, these data suggest that HCV RNA level is relatively stable in HIV-seropositive individuals, but that with advanced immune deficiency, some increase in HCV RNA level may occur.

Although the magnitude may not be fully resolved, it is well documented that the absolute HCV RNA level is higher in HIV-HCV coinfecting individuals when compared with HIV-seronegative cohorts (5-9,37,39,40). Cribier et al (39) compared 59 HIV-HCV coinfecting with 51 HCV infected subjects and demonstrated a greater than $0.5 \log_{10}$ higher HCV RNA level in the former. Similar results were reported (9) in a group of 343 HIV-HCV coinfecting hemophiliac patients in comparison with 42 HCV-seropositive but HIV-seronegative hemophiliac patients. In a study controlling for age, HIV RNA level and

CD4+ T lymphocyte count, Dragoni et al (5) also demonstrated higher HCV RNA level in HIV-HCV coinfection. These studies are limited by the fact that antiretroviral therapy, a biological factor that likely influences HCV RNA levels, was not fully described or controlled for by multivariate analysis.

Alcoholism is a significant medical issue for many HIV-HCV coinfecting individuals (41). Excessive consumption of alcohol, defined as greater than 50 g/day (ie, three to four alcoholic beverages per day), increases HCV RNA level in a linear fashion (29,42-44). Although the mechanism is poorly understood, it is speculated that increased release of HCV RNA level from hepatocyte lysis, increased viral replication and impaired HCV clearance resulting from alcohol-induced immune suppression contribute to this phenomenon (45,46). Excess alcohol may blunt immune restoration following the initiation of highly active antiretroviral therapy (47) and/or accentuate antiretroviral-related hepatotoxicity with subsequent increase in HCV RNA level (48). These factors may influence the amount and direction of change in HCV RNA level following the initiation of antiretroviral therapy.

Other factors with potential to influence HCV RNA level include severity of hepatocellular dysfunction, HIV RNA level and HCV genotype (5,16,49,50). In immunocompetent hosts, HCV RNA levels do not differ between individuals with compensated cirrhosis and those with precirrhotic chronic liver disease (49). HCV RNA level is lower in those with decompensated liver disease. An increase in HCV RNA levels in HIV-HCV coinfecting patients with decompensated liver disease has been described (7). These subjects also had low CD4 T lymphocyte counts, thereby confounding this result. The major cell receptors and target cells of these two viruses differ, therefore, HCV replication is likely virologically independent of the level of HIV RNA. A lack of correlation between HCV and HIV RNA level suggests that this assumption is accurate (6,30). Both viruses may use CD81 cellular receptors but the relevance to HIV and HCV RNA quantification is uncertain (51). As in HIV-seronegative individuals, HCV genotype does not appear to influence HCV RNA level in HIV-HCV coinfecting individuals (8,16,39,49,50,52).

DISCUSSION

Because there are many analytical and biological variables which influence measurement of HCV RNA, researchers and clinicians should exercise caution when small mean changes in conjunction with antiviral therapy are reported. This is analogous to HIV RNA levels measurements, where the sum of biological and analytical variation is $0.5 \log_{10}$ according to the Centers for Disease Control and Prevention treatment guidelines for HIV infection (53). Given the limited and problematic data currently available, it is difficult to determine a similar value for HCV RNA level with great confidence. Nonetheless, taking analytical issues and natural biological variability into consideration, the sum of biological and analytical variation is likely no more than $1.0 \log_{10}$.

In terms of HCV drug therapy with pegylated interferon and ribavirin, HCV RNA declines of less than $2 \log_{10}$ following 12 weeks of therapy is highly predictive of failure to achieve a sustained virological response (54). This has been validated in HIV-HCV coinfecting patients (55). Currently, the entire approach to therapy is built on the '2 \log_{10} to 12 week rule'. Clearly, accurate and reproducible quantification is critical to use early virological response for clinical monitoring.

Clinicians and patients may be tempted to persist with therapy when smaller reductions in HCV RNA from baseline are identified. We recommend that clinicians remember to consider sources of analytical and biological variability before attributing small reductions in HCV RNA entirely to the treatment effect of pegylated interferon and ribavirin. An illustrative example of how biological variables can influence serial HCV RNA quantification and which is often seen in the clinic setting pertains to alcohol consumption. A pretreatment, baseline HCV RNA level is often measured at the initial clinic visit, well before HCV treatment is initiated. If alcohol is being used excessively at the time, the HCV RNA may be greater than when therapy is actually started (ie, once sobriety has been achieved). Keeping this scenario in mind, a reduction in HCV RNA level of as much as $1 \log_{10}$ may be observed that may not be a direct effect of HCV antiviral treatment.

Another issue in which analytical and biological variability is relevant is in evaluation of the long-term influence of antiretroviral therapy on HCV RNA levels. This issue is of relevance as it may influence the decision as to whether HIV or HCV drug therapy should be initiated first in those with HIV-HCV coinfection. If HCV RNA levels fall with antiretroviral therapy

then it would make sense to defer HCV treatment because lower HCV RNA levels predict an improved chance for achieving a sustained virological response. Currently, there are conflicting results (10) concerning change in HCV RNA following extended courses of HIV therapy. Most of these changes, irrespective of direction, are small and likely explained by unavoidable analytical variability and sources of background biological variability not accounted for in these reports. We have demonstrated that improved clarity is possible once major sources of biological variability, such as alcohol consumption, are considered (56). In this work we found that HCV RNA increased by over $0.5 \log_{10}$ in those consuming greater than 50 g of alcohol per day and declined slightly below baseline in those remaining free of alcohol use while on highly active antiretroviral therapy for 12 months.

These examples illustrate why knowledge of analytical and biological variability of HCV RNA measurement in HIV-HCV coinfection is relevant. These factors should be considered for interpreting both the biological significance of published research and for guiding laboratory-based decisions in the clinical setting.

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