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Performance of Two Real-Time RT-PCR Assays for Quantitation of Hepatitis C virus RNA: Evaluation on HCV Genotypes 1–4

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Accuracy for monitoring of the concentration of hepatitis C virus (HCV) RNA represents a major challenge throughout the management of patients with chronic hepatitis C. To investigate the genotype-independent efficiency and the accuracy of two real-time detection reverse transcription-polymerase chain reaction (RT-PCR) assays; the Cobas Ampliprep/Cobas TaqMan (CAP/CTM); and the Abbott RealTime HCV (ART), a total of 184 samples with different HCV subtypes were examined; 1b (n = 58), 2a (n = 39), 2b (n = 26), 3a (n = 20), and 4 (n = 41). A robust linear correlation was observed between the two assays applied to genotypes 1b, 2a, 2b, and 3a [the correlation coefficient (R) ranged from 0.99 to 0.98], but not to genotype 4 specimens (R = 0.78). A significant difference in measurements of HCV RNA using CAP/CTM and ART in serum samples with genotypes 1b and 4 was observed (0.72, −0.53 log IU/ml, $P < 0.0001$, 0.01, respectively). A robust correlation was observed between the HCV core antigen and HCV RNA values by either of the HCV RNA quantitation assays applied to all genotypes with exception of genotype 4, for which R was higher with ART (R = 0.95) than with CAP/CTM (R = 0.80). The lower limit of detection of CAP/CTM and ART were 41.4 and 28.5 IU/ml using the WHO standards, respectively. In conclusion, two RT-PCR assays had a high efficiency and accuracy for quantitation of HCV RNA of genotypes 2a, 2b, and 3a, but the mean values of HCV RNA differed for genotype 1b and 4. ***J. Med. Virol. 82:1878–1888, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; real-time PCR; genotype 4; HCV core antigen; Cobas Ampliprep/Cobas TaqMan

INTRODUCTION

Hepatitis C virus (HCV) infection is a health problem worldwide. Chronic infection with HCV is a risk factor for the development of cirrhosis of the liver and hepatocellular carcinoma [Alter et al., 1989; Kiyosawa et al., 1990]. Numerous studies have suggested that successful treatment with interferon-based therapy reduces dramatically the risk for hepatocellular carcinoma [Hino et al., 2002; Yu et al., 2006].

Sustained virological response which is defined as undetectable HCV RNA 24 weeks after the completion of combined antiviral therapy [pegylated interferon + ribavirin] is the goal for the treatment of chronic hepatitis C [Davis et al., 1998; McHutchison et al., 1998b]. Several virological factors have been claimed to predict sustained virological response including the infecting HCV genotype, the base line viral load, and the early virological response to the HCV antiviral drugs [McHutchison et al., 1998a; Ferenci et al., 2005; Kanwal et al., 2007; Mangia et al., 2009].

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On the basis of the nucleotide sequence homolgy, HCV has been classified into six major genotypes (e.g., genotype 1, genotype 2, genotype 3) and within each genotype, closely related variants are grouped into numerous subtypes (e.g., subtype 1a, 1b, 2a, 2b) [Simmonds et al., 2005]. Molecular epidemiology studies have indicated a geographical restriction for some HCV genotypes (e.g., genotype 4 to the Middle East and Egypt, genotype 5a to South Africa and genotype 6 to Southeast Asia), while others are distriubuted globally, for example, genotypes 1a, 1b, 2a, 2b, 3a [Mellor et al., 1995; Simmonds et al., 1996; Smith et al., 1997].

HCV genotype and pretreatment HCV RNA level are important predictors for the response to interferon-alpha and ribavirin combination therapy [Lee and Abdo, 2003]. Lower rate of sustained virological response to the interferon-alpha and ribavirin combination therapy in patients infected with HCV genotype 1 or genotype 4 than those infected with HCV genotypes 2 or 3[Hnatyszyn, 2005; Shiratori et al., 1997].

HCV RNA monitoring early in treatment may help in identifying patients with little or no chance of achieving sustained virological response as well as identifying patients with a high chance of achieving sustained virological response who may benefit for a shorter treatment programme [Chevaliez and Pawlotsky, 2007].

The detection of viremia in patients infected with HCV can be achieved either directly by the nucleic acid amplification-based techniques (NAT) through qualitative and/or quantitative detection of serum HCV RNA [Beld et al., 2002] or indirectly by the measurement of the HCV core antigen (HCVcAg) [Orito et al., 1996]. Regarding the NAT, different methods based on branched DNA (bDNA) and polymerase chain reaction (PCR) technology assays have been developed [Pawlotsky et al., 1999]. Real-time detection reverse-transcription (RT) PCR (RT-PCR) based assays have replaced endpoint PCR in the routine monitoring of HCV RNA level throughout the management of chronic hepatitis C patients [Chevaliez and Pawlotsky, 2005].

In the present study, the performance of two real-time RT-PCR assays, CAP/CTM (Cobas Ampliprep/Cobas TaqMan, CAP/CTM; Roche Molecular Systems, Pleasanton, CA) and Abbott RealTime HCV (ART; Abbott Molecular Inc., Abbott Park, IL) was examined for the quantitative measurement of HCV RNA concentrations in samples harboring different HCV genotypes including the genotypes 1–4. In addition, we analyzed the genotype-wise correlation between the serum concentrations of HCV RNA obtained by the two assays and serum HCVcAg levels measured by a newly developed and highly sensitive chemiluminescence microparticle immunoassay (CMIA; Abbott-ARCHITECT HCV Ag assay, Tokyo, Japan).

PATIENTS AND METHODS

Clinical Specimens

The present study includes 184 patients with chronic HCV infection. Clinical serum samples were collected from 184 chronic hepatitis C patients infected with HCV genotypes 1b (n = 58), 2a (n = 39), 2b (n = 26), 3a (n = 20), and genotype 4 (n = 41) including 38 patients infected with genotype 4a, 2 with genotype 4o, and 1 with genotype 4m. The serum samples were separated from whole blood by centrifugation, divided into aliquots containing the appropriate serum volumes needed for the two assays measuring the HCV RNA (850 μl for CAP/CTM assay and 1,000 μl for the ART) and (150 μl) for measuring HCVcAg. Prior to processing by different assays, the aliquots were stored at −80°C.

HCV Genotype Determination and Sequence Analysis of the 5′ Untranslated Region (5′-UTR) of the HCV Genome of Genotype 4

The infecting HCV genotype was determined by PCR amplification and direct sequencing of a partial genome; in structural (core/E1) and non-structural (NS5B) coding regions followed by phylogenetic analysis as described previously [Tanaka et al., 2002].

For samples harboring genotype 4, further amplification of almost the full length of the 5′-UTR was carried out by a single PCR using the forward primer KK30 (5′-CTGTCTTCACGCAGAAACGC-3′) and reverse primer Primer-1 (5′-AACAAGTAAACTCCGCCAACGATC). PCR was initiated by the hot start protocol. The PCR reaction was undertaken for 40 cycles (95°C for 1 min, 60°C for 1 min, 72°C for 1 min) followed by extension reaction at 72°C for 10 min. Sequencing was performed using the Prism Big Dye (Pekrin Elmer Applied BioSystems, Foster City, CA) in an ABI 3100 DNA automated sequencer according to the manufacture's protocol. The nucleotide sequence data described above will appear in the EMBL/DDBJ/GenBank sequence databases with accession numbers: AB548316–AB548326 and AB550013–AB550041.

Measurement of HCV RNA Using the Two Real-Time RT-PCR assays; Cobas Ampliprep/Cobas TaqMan assay (CAP/CTM); and Abbott RealTime HCV test (ART)

Abbott RealTime HCV test (ART). Abbott RealTime HCV test is based upon RT-PCR followed by real-time fluorescent detection of HCV RNA (RT-PCR assay). The assay starts with the fully automated RNA extraction by the m2000sp instrument from 1,000 μl of the sample (sample volume required, 500 μl). A 500 μl of the internal control (IC) is also added to the bottle of lysis reagent to monitor the efficiency of the process. After the completion of the HCV RNA extraction, the RT and amplification of both the IC and the target are performed simultaneously in the Abbott m2000rt instrument. The detection of the amplification product is done by the addition of two different dual-labeled fluorescent oligonucleotide probes that bind IC and HCV RNA. Hybridization of the probe to its complementary target sequence either HCVRNA or IC leads to separation of the quencher molecule and allows the fluorescence emission and detection. The amplification cycle at which

the fluorescence signal is detected by m2000rt is proportional to the log of the HCV RNA present in the original sample. Concentration of HCV RNA is calculated from the stored calibration curve and results are reported automatically on m2000rt workstation.

The assay has adopted the second international WHO standard for HCV RNA (code 96/798) for calibration. HCV RNA concentration is expressed in IU/ml. ART assay has a lower limit of detection (LOD) of 12 IU/ml with a linear quantitation range of $12–10^7$ IU/ml.

Cobas Ampliprep/Cobas TaqMan assay (CAP/CTM). Cobas Ampliprep/Cobas TaqMan assay is based upon RT-PCR followed by real-time fluorescent detection of HCV RNA from 850 μl serum. The assay starts by an automated glass particle-mediated RNA capture and purification system using the automated Cobas AmpliPrep (CAP). HCV RNA quantitation standard is added to the sample in order to achieve the full process control. After preparation of the samples, each sample is transferred and processed for RT and amplification in the Cobas TaqMan 48 analyzer (CTM 48). Detection of the amplification products is done by the addition of two different dual fluorescent probes that bind the HCV target amplicon and quantitation standard amplicon, respectively. Hybridization of the probe to its complementary target sequence leads to separation of the quencher molecule with fluorescence emission and detection. The larger the original HCV RNA concentration in a specimen, the earlier the fluorescence of the reporter dyes rises above the certain assigned fluorescence level (the critical threshold value). For a constant concentration of the RNA quantitation standard, the reporter dye fluorescence appears at the same cycle for all specimens. By comparing the critical threshold values obtained for the target HCV RNA and quantitation standard RNA, the original HCV RNA concentration in a specimen is calculated.

CAP/CTM is standardized against the first WHO International standard for HCV RNA (code 96/798). HCV RNA concentration is reported in IU/ml. CAP/CTM assay has a LOD of 15 IU/ml with a linear quantitation range of $43–6.9 \times 10^7$ IU/ml.

Sensitivity of the CAP/CTM and ART Assays

In order to assess the analytical sensitivity of the two HCV RNA quantitation assays, panels of the Third International WHO HCV RNA standard were diluted serially to the following concentrations: 100, 50, 25, 12.5, 6.25, and 3.125 IU/ml. Dilutions were stored at −80°C. Each concentration panel was tested in parallel by both assays for 20 replicates in a single run.

In addition, the LOD of each assay was calculated using serial dilutions of clinical serum samples harboring HCV genotypes, 1b and 2a. Panels of serum samples harboring HCV genotype 1b were prepared with the following concentrations: 100, 50, 25, 12.5, and 6.25 IU/ml, and of serum samples with genotype 2a with the following concentrations: 100, 50, 25, 12.5, 6.25, and 3.13 IU/ml. The original HCV RNA concentrations used for calculation of the dilutions were measured by CAP/CTM. Aliquots of each concentration were tested in parallel by both assays (CAP/CTM and ART) for 20 replicates in a single run.

Quantitative Detection of Hepatitis C Core Antigen (HCVcAg)

Quantitative determination of HCVcAg was done in serum samples by the chemiluminescence microparticle immunoassay (CMIA; Abbott-ARCHITECT HCV Ag assay) according to the manufacturer's instructions [Morota et al., 2009].

Data Analysis

Statistical differences were evaluated by use of the independent *t*-test for continuous variables using the SPSS software package (SPSS version 16, SPSS, Inc., Chicago, IL). *P*-values (two-tailed) <0.05 were considered significant statistically. Correlation coefficient (R) was assessed by the Spearman's correlation coefficient implemented in STATA software version 8.0 (StataCorp. LP, College Station, TX). Bland–Altman plot analysis was performed to assess the agreement level between CAP/CTM and ART assays for the quantitation of HCV RNA concentration [Bland and Altman, 1986]. *Probit* analysis (SPSS version 16) was used to determine the LOD of CAP/CTM and ART assays by assigning serial known dilutions of the WHO standards and clinical samples as independent variables and the numbers of detectable samples as dichotomous variables. The 95% probability of obtaining positive HCV RNA results is defined as the LOD.

RESULTS

HCV RNA Measurements by CAP/CTM and ART Assays

HCV RNA concentration was measured in 184 clinical serum samples in parallel by CAP/CTM and ART. In general, a robust linear correlation was observed between CAP/CTM and ART measurements ($R = 0.87$). The correlation coefficient (R) between the two assays measurements was 0.98 in serum samples harboring HCV genotypes 1b, 2a, 2b, 3a, and was 0.78 in serum samples harboring HCV genotype 4 (Fig. 1).

For serum samples harboring HCV genotype 1b, significantly higher HCV RNA values obtained by CAP/CTM assay (mean ± SD, 5.87 ± 1.12 log IU/ml) than that obtained by ART (mean ± SD, 5.15 ± 1.04 log IU/ml, $P < 0.0001$; Table I). In contrast, for serum samples harboring genotype 4, significantly lower HCV RNA values obtained by CAP/CTM (mean ± SD, 4.36 ± 0.95 log IU/ml) than that obtained by ART (mean ± SD, 4.89 ± 0.88 log IU/ml, $P = 0.01$; Table I). Interestingly, two serum samples harboring HCV genotype 4 showed measurements difference (CAP/CTM minus ART) of −3.75 and −1.68 log IU/ml (Fig. 2).

HCV RNA levels were significantly higher in patients infected with HCV genotype 1b than those infected

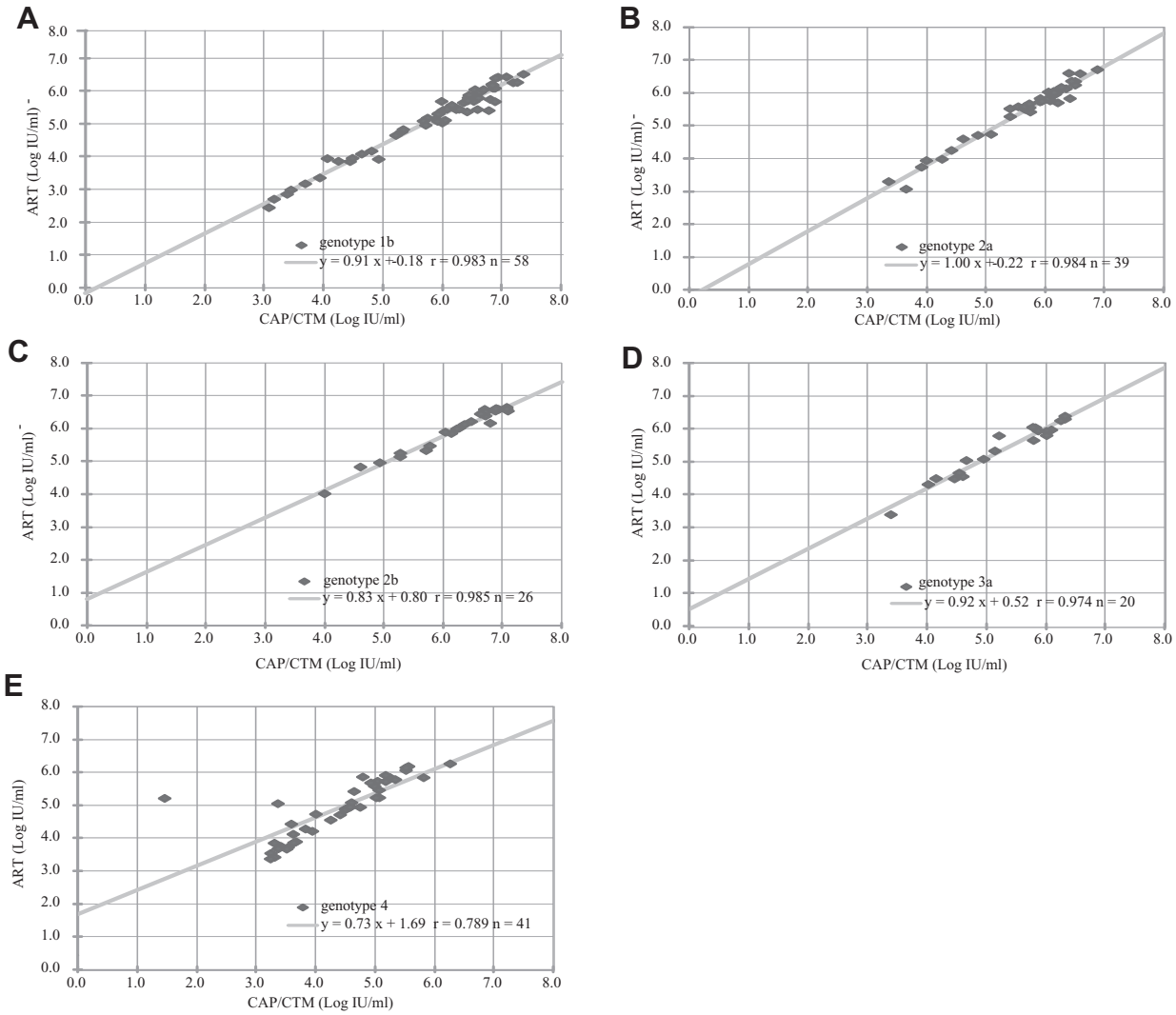

Fig. 1. Correlation between the HCV RNA measurements obtained by the two assays: CAP/CTM versus Abbott RealTime HCV in the same samples containing HCV genotypes (**A**) 1b, (**B**) 2a, (**C**) 2b, (**D**) 3a, and (**E**) genotype 4.

with HCV genotypes 3a and genotype 4 using HCV RNA values revealed by CAP/CTM assay ($P = 0.023$, <0.0001), respectively. However, such a significant difference was not observed using the HCV RNA values obtained by ART. A significantly lower HCV RNA level in patients infected with HCV genotype 1b than patients infected with genotype 2b was observed by use of ART ($P < 0.0001$).

Figure 2 showed Bland–Altman analysis of HCV RNA levels measured by CAP/CTM and ART in the 184 serum samples with different HCV genotypes. The differences between the two measured values (CAP/CTM minus

TABLE I. HCV RNA Concentrations of Genotypes 1–4 as Measured by the Two Assays: CAP/CTM and Abott RealTime HCV

		Mean ± SD (log IU/ml)				
Genotype	N	CAP/CTM	ART	Average HCV RNA level	Quantitation difference (CAP/CTM-ART)	*P*-Value[a]
1b	58	5.87 ± 1.12	5.15 ± 1.04	5.51 ± 1.08	0.72 ± 0.21	<0.0001
2a	39	5.66 ± 0.88	5.46 ± 0.90	5.56 ± 0.89	0.20 ± 0.16	0.33
2b	26	6.25 ± 0.82	5.98 ± 0.69	6.12 ± 0.75	0.27 ± 0.18	0.20
3a	20	5.28 ± 0.87	5.38 ± 0.82	5.33 ± 0.84	−0.10 ± 0.19	0.73
4	41	4.36 ± 0.95	4.89 ± 0.88	4.63 ± 0.86	−0.53 ± 0.60	0.01

[a]HCV RNA measurements by CAP/CTM versus HCV RNA measurements by Abbott RealTime HCV.

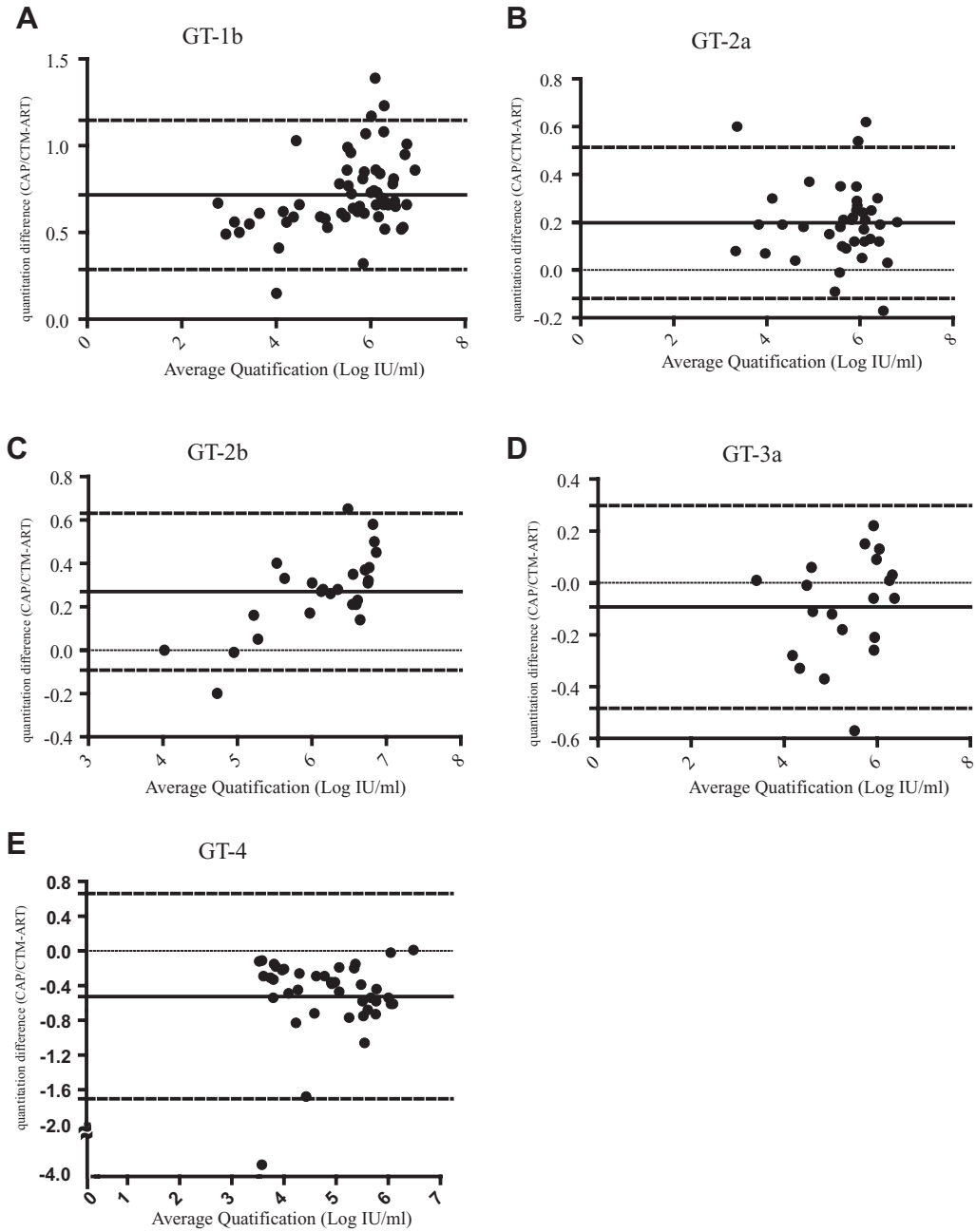

Fig. 2. Bland–Altman analysis of the genotype-specific HCV RNA levels difference in the HCV RNA measurements by the CAP/CTM versus that by the ART test in samples with HCV subtypes; 1b (**A**), 2a (**B**), 2b (**C**), 3a (**D**), and 4 (**E**). The average quantification of HCV RNA concentration is the function of the mean of the two HCV RNA measurements obtained by CAP/CTM and ART assays for each sample individually. The bold lines correspond to mean difference for the samples, the dashed lines to 95% limits of agreement and the dotted lines are the reference lines.

ART) were plotted against the average HCV RNA concentrations which is the function of the mean of the two measurements. Concordance in HCV RNA values between the CAP/CTM and ART assays was observed in serum samples harboring HCV genotypes 3a, 2a, and 2b specimens. For serum samples with HCV genotype 3a, the mean difference between the values of the assays was 0.10 log IU/ml and limits of agreement (defined as mean difference ± 2 SD) were −0.48 and 0.29 log IU/ml and 95% of differences fell within these limits (Fig. 2D, Table I). For serum samples with HCV genotype 2a, the mean difference was 0.20 log IU/ml and limits of agreement were −0.11 and 0.51 log IU/ml (Fig. 2B, Table I). For genotype 2b, the mean difference was 0.27 log IU/ml and limits of agreement were −0.09 and 0.63 log IU/ml (Fig. 2C, Table I).

The agreement of HCV RNA values was poor between CAP/CTM and ART in serum samples harboring geno-

type 1b and genotype 4 with a mean difference of 0.72 log IU/ml and −0.53 log IU/ml in HCV genotypes 1b and 4, respectively (Fig. 2A,D, Table I). The limits of agreement for serum samples with HCV genotype 1b were 0.28 and 1.14 log IU/ml (Fig. 2A), while the limits of agreement for serum samples with HCV genotype 4 were −1.70 and 0.65 log IU/ml (Fig. 2E).

In sera harboring HCV genotype 1b, The over quantitation by CAP/CTM was higher significantly in samples with high viral load and the mean difference (CAP/CTM minus ART) for samples with mean viral load of ≤5 log IU/ml and >5 log IU/ml was 0.57 and 0.76 log IU/ml, respectively ($P = 0.003$). Conversely, for serum samples containing HCV genotype 4, the mean difference was not different statistically in samples with mean viral load of ≤5 log IU/ml (mean difference; −0.55 log IU/ml) from samples with mean viral load of >5 log IU/ml (mean difference; −0.49 log U/ml).

Measurement of Hepatitis C Virus Core Antigen (HCVcAg) Using Abbott-ARCHITECT HCV Ag Assay

Hepatitis C virus core antigen was measured in all serum samples with HCV genotypes 1–4. The correlation between HCVcAg concentrations and HCV RNA titers obtained by CAP/CTM and ART was investigated individually. Consistency between HCVcAg and HCV RNA concentrations was observed across all the studied genotypes regardless the assay used for measuring the viral load. However, this was different for serum samples with HCV genotype 4 where a better correlation coefficient (R) was observed between the HCVcAg levels and the HCV RNA values revealed by ART ($r = 0.95$) than between the HCVcAg levels (Fig. 3D) and HCV RNA values revealed by CAP/CTM ($r = 0.80$; Fig. 3B). For serum samples with HCV genotype 1b, the relationship between concentration of HCVcAg in log fmol/l and HCV RNA in log IU/ml, was calculated to be that 1 log fmol/l of the total HCVcAg is equivalent to 3.53 log IU/ml of HCV RNA tested by CAP/CTM, and equivalent to 2.98 log IU/ml HCV RNA tested by ART.

For sera with HCV genotype 4, 1 log fmol/l of the total HCVcAg is equivalent to 2.0 log IU/ml of HCV RNA tested by CAP/CTM, and equivalent to 3.04 log IU/ml HCV RNA tested by ART.

CAP/CTM and ART Assays Sensitivity Using the WHO Standards, Genotypes 1b and 2a Clinical Samples

Sensitivity of CAP/CTM and ART was evaluated independently using the WHO standards and clinical samples with HCV genotypes 1b and 2a.

For the WHO standards, both the CAP/CTM and ART were capable of detecting HCV RNA concentration values of 100–50 IU/ml in all tested replicates (Table II). However, for a concentration level of 100 IU/ml, 7 out of

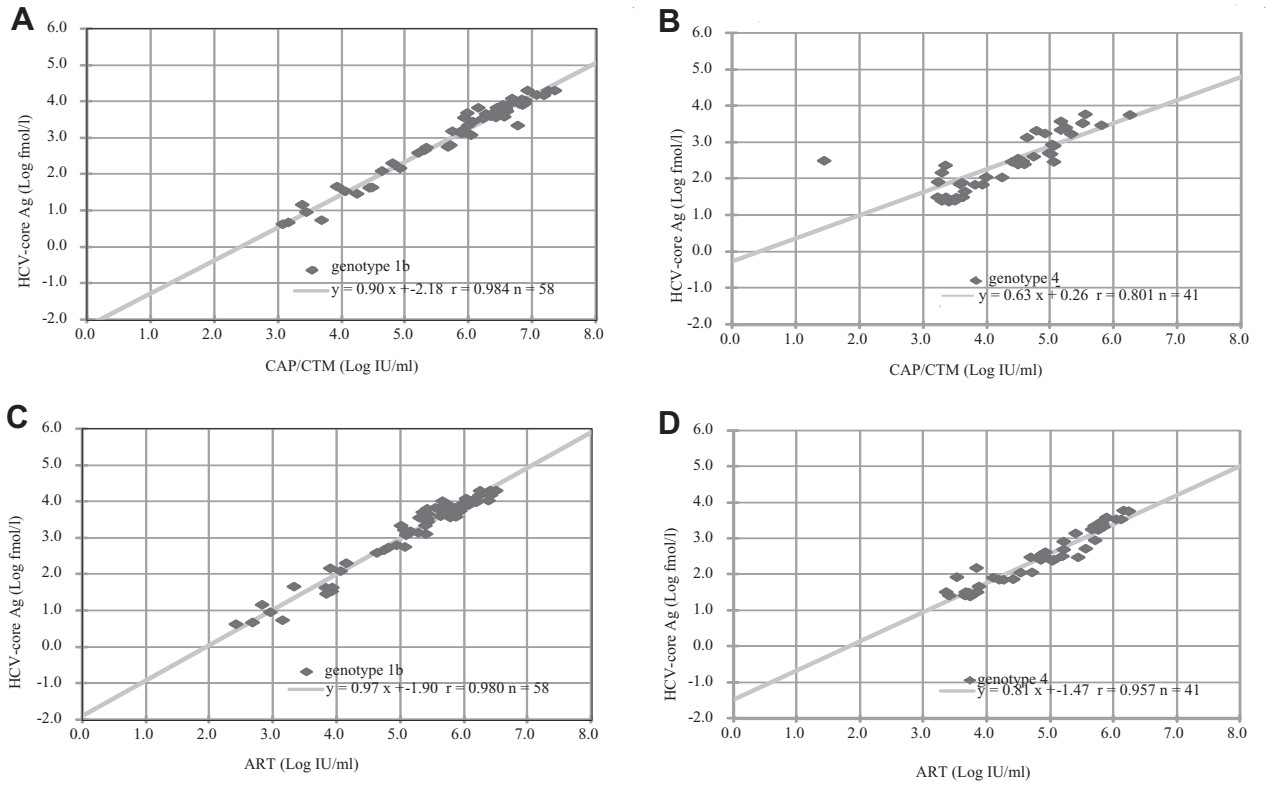

Fig. 3. Correlation between the HCVcAg level and the HCV RNA level measurements by CAP/CTM in samples containing HCV genotype 1b, and genotype 4 (**A**,**B**); and the HCVcAg level versus the HCVRNA level measured by Abbott RealTime HCV in samples containing HCV genotype 1b and genotype 4 (**C**,**D**).

TABLE II. Positive Hit Rates of CAP/CTM and ART for the WHO Standard Genotype 1a, Genotype 1b, and 2a Clinical Samples

		CAP/CTM			ART		
		Samples with detectable measurement (IU/ml)			Samples with detectable measurement at (IU/ml)		
HCV RNA concentration (IU/ml)	Sample no.	≥15	<15 (signal positive) or ≥15	Hit rate (%)	≥12	<12 (signal positive) or ≥12	Hit rate (%)
WHO standard							
100	10	3	10	100	10	10	100
50	20	0	20	100	3	20	100
25	20	0	13	65	0	18	90
12.5	20	0	10	50	0	15	75
6.25	20	0	10	50	0	8	40
3.125	20	0	2	10	0	9	45
HCV genotype 1b							
100	20	20	20	100	20	20	100
50	20	11	20	100	18	20	100
25	20	1	20	100	12	20	100
12.5	20	0	19	95	1	17	85
6.25	20	0	16	80	0	11	55
HCV genotype 2a							
100	10	8	9[a]	100	10	10	100
50	20	7	20	100	19	20	100
25	20	0	20	100	14	20	100
12.5	20	0	19	100	6	16	85
6.25	20	0	18[b]	95	1	14	45
3.13	20	0	12[c]	64	1	9	40

[a]Invalid sample 9/9 (100%).
[b]Invalid sample 18/19 (95%).
[c]Invalid sample 12/19 (64%).
Dark grey shaded portions are positive hit rates more than or equal to 95%. Light grey portions are positive hit rates less than 95%.

10 replicates could not be measured at ≥15 IU/ml by CAP/CTM. At a concentration of 25 IU/ml, 18 out of 20 (90%) and 13 out of 20 tested replicates (65%) were detectable by ART and CAP/CTM assays, respectively (Table II). At a concentration of 12.5 IU/ml, 15 out of 20 (75%) and 10 out of 20 (50%) tested replicates were detectable by ART and CAP/CTM assays, respectively (Table II). At a concentration of 6.25 IU/ml, 8 out of 20 (40%) and 10 out of 20 tested replicates (50%) were detectable by ART and CAP/CTM assays, respectively. At a concentration of 3.125 IU/ml, 9 out of 20 (45%) and 2 out of 20 (10%) were detectable by ART and CAP/CTM assays, respectively (Table II). Interestingly, the LOD for CAP/CTM assay was 41.4 IU/ml (95% CI: 27.1–166.2) while the lower LOD for ART was 28.5 IU/ml (95% CI: 21.2–50.3).

For clinical serum samples with HCV genotype 1b and at a concentration level of 12.5 IU/ml, positive hit rates of >95% were achieved only by CAP/CTM. At a concentration level of 6.25 IU/ml, CAP/CTM assay achieved positive hit rates higher (16 out of 20 tested replicates; 80%) than that achieved by ART (11 out of 20 tested replicates; 55%).

For clinical serum samples with HCV genotype 2a, and at a concentration of 6.25 IU/ml, positive hit rates of >95% were achieved only by the CAP/CTM assay (18 out of 19; 95%).

For clinical serum samples harboring HCV genotype 1b, the lower LOD was 12.3 IU/ml for CAP/CTM assay and 16.2 IU/ml for ART. For the clinical serum samples harboring HCV genotype 2a, the lower LOD was 10.3 IU/ml for CAP/CTM assay and 17.9 IU/ml for ART.

Sequence Analysis of the Target Samples Harboring HCV Genotype 4

A significant underestimation of HCV RNA concentrations by CAP/CTM assay comparing to ART assay was observed in serum samples harboring HCV genotype 4. In order to investigate the possible presence of nucleotide polymorphism that may contribute to mismatch of primers or TaqMan probe to its target viral sequence, nearly the full length of 5′-UTR of the HCV genome was sequenced in 40 samples containing HCV genotype 4 and aligned with respect to the consensus HCV genotype 1b sequence. The sequences were ranked in order of the increasing measurement difference values (CAP/CTM – ART).

Three samples showed measurement difference levels of more than −1 log IU/ml. Substitution of G145A was detected in the sample which exhibit the highest measurement difference level; −3.34 log IU/ml (Sample ID; T58-UTR; Fig. 4). A165T substitution was detected in 7.5% (3/40, Samples ID; T48-UTR, T72-UTR, and T69-UTR) with measurement difference levels of −1.68, −0.7, −0.7 log IU/ml, respectively (Fig. 4). Comparing the studied sequences in the present study to HCV genotype 4 consensus sequence, two nucleotide polymorphism varieties were found in considerable number of studied sequences; A/C insertion after nucleotide

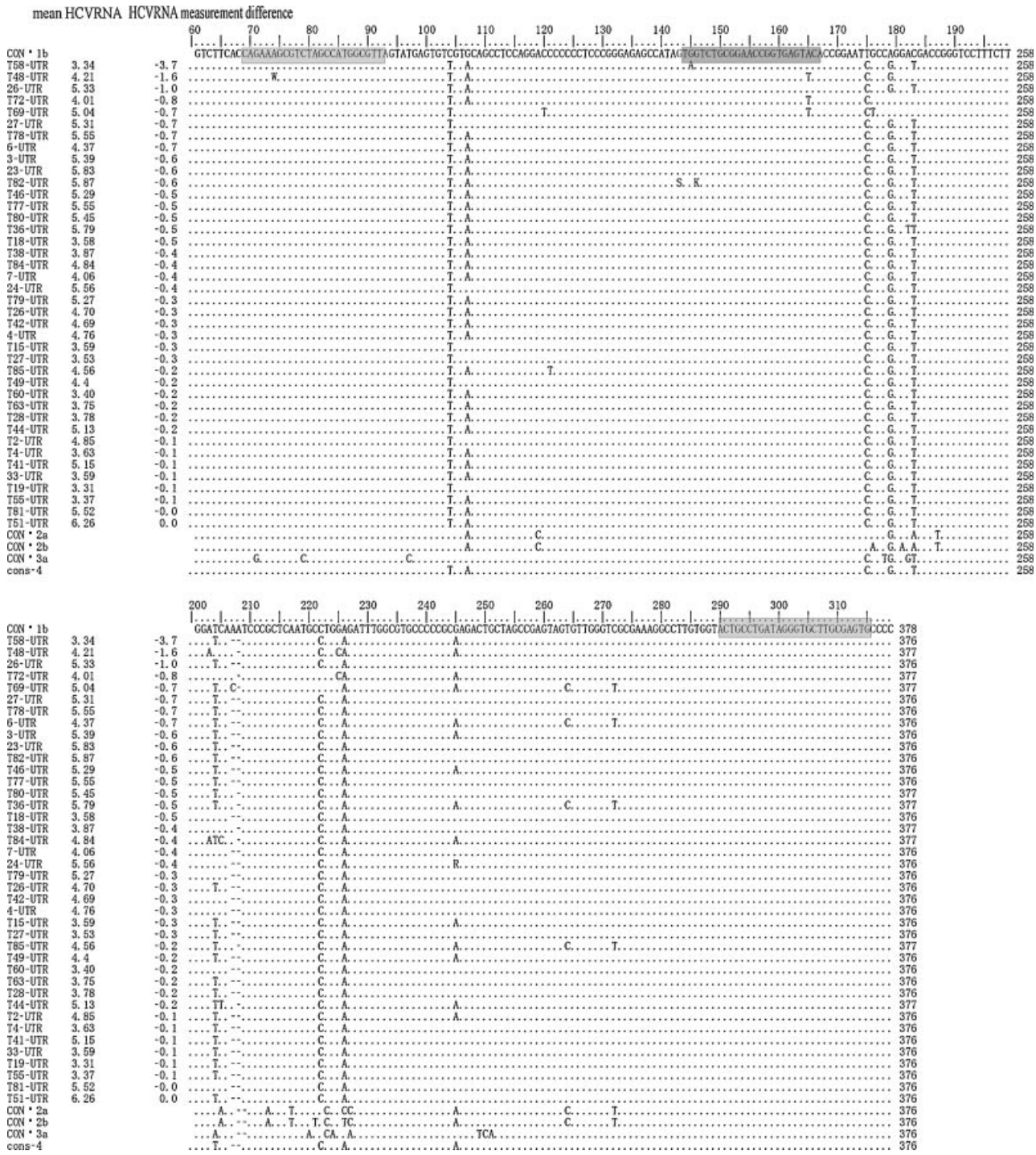

Fig. 4. The alignment of 5′-UTR sequences in samples containing HCV genotype 4 with respect to the consensus sequence of genotype 1b. The studied sequences of genotype 4 are ranked according to the increased measurement difference between the real-time RT-PCR assays (CAP/CTM – Abbott RealTime HCV) as represented in the third column. The second column represents the average RNA level in each sample individually calculated from results obtained by both assays. The estimated primer site sequences and the probe site sequence for CAP/CTM was indicated by light gray and dark gray boxes respectively.

position 206 which was observed in 22.5% (9/40) of samples and T204C substitution in 27.5% (11/40) of samples. In addition, one sample (sample ID; T48-UTR) with a measurement difference value of −1.68 log IU/ml, exhibited the two nucleotide polymorphism varieties; the insertion of nucleotide A after position 206 and T204C substitution. However, none of these two polymorphisms was detected in the sample with the highest measurement difference value (Sample ID; T58-UTR).

DISCUSSION

Despite the standardization of HCV RNA quantitation assays to the international unit [Saldanha et al., 2005b], still a significant variability in the HCV RNA concentration results within different assays [Caliendo et al., 2006]. Furthermore, the performance of an individual assay may differ within different HCV genotypes [Chevaliez et al., 2007]. In the present study, HCV RNA concentration values obtained by CAP/CTM assay were consistent with values obtained by ART in samples containing HCV genotypes 2a, 2b, and 3a. However, a discrepancy between HCV RNA concentration values obtained by the two assays were observed in serum samples harboring HCV genotypes 1b, and genotype 4 samples. The results in the present study are in line with those reported previously not only for CAP/CTM versus ART but also for CAP/CTM versus bDNA assay [Pittaluga et al., 2008]. The present data are emerging a question; which assay produces true and reliable results particularly that both assays are having a high degree of reproducibility and intra and inter-assay precision profile. For CAP/CTM, intra-assay variability experiments demonstrated that CVs ranged from 1% to 3% and CVs for inter-assay imprecision ranged from 1% to 5% [Sarrazin et al., 2008]. For ART, the intra-assay variability experiments demonstrated that CVs ranged from 0.76% to 7.68% while CVs for the inter-assay imprecision using high and low positive controls were 1.98% and 3.91%, respectively [Chevaliez et al., 2009b].

In an attempt to answer this question, two further steps were taken in this study. First, in all tested serum samples with different HCV genotypes, HCVcAg levels were compared to HCV RNA concentration levels measured by each assay individually depending on the higher stability and reproducibility of HCVcAg quantitation than PCR [Tanaka et al., 2003; Alvarez et al., 2004]. Of note, the HCVcAg assay was highly reproducible and assay precision was ranged from 4.4% to 9.5% of the total %CV using samples from low to high concentration of HCVcAg in the NCCLS precision performance study [Morota et al., 2009]. Interestingly, concordance between HCVcAg and HCV RNA levels was clear regardless of the assay used for measuring HCV RNA concentration in all genotypes but to less degree in samples containing HCV genotype 4. Better correlation coefficient was observed between HCVcAg and HCV RNA concentration values obtained by ART than those obtained by CAP/CTM, suggesting that ART may yield more reliable results than CAP/CTM in determination of HCV RNA concentration in the case of serum samples harboring HCV genotype 4. Experiments regarding the sensitivity of each assay using the WHO standards and clinical serum specimens harboring HCV genotypes 1b and 2a were also done in the present study. In sensitivity experiments and using the HCV genotype 1a Third International WHO standard, it was clear that ART was more sensitive than CAP/CTM in detecting and measuring small levels of the HCV RNA. In addition, the LOD of ART for the WHO standard was 28.5 IU/ml, which was comparable to that of CAP/CTM (41.4 IU/ml).

Interestingly for clinical serum samples containing HCV genotype 1b, the opposite was observed as CAP/CTM was more sensitive than ART in detecting small levels of HCV RNA concentration and this was also reflected by the lower LOD values.

CAP/CTM adopted the First International HCV RNA WHO Standards for the assay calibration, while ART adopted the Second International HCV RNA WHO Standard for calibration. Direct comparison of the two assays for measuring the WHO standards that carried out by Vermehren et al. [2008] revealed a consistently higher quantitation of the WHO standards by CAP/CTM and a lower quantitation of the WHO standards by ART HCV. The difference in calibration method or the WHO standards version used by each assay for calibration may be responsible for the discrepancy in the results [Vermehren et al., 2008]. In addition, the fact that both standards are prepared only from HCV genotype 1a in a pooled human plasma may play a role in difference in the efficiency of an individual assay across different HCV genotypes [Saldanha et al., 2005a].

When analysing the agreement between the two assays measurements by the Bland and Altman bias analysis, it was observed that variations in the measurement difference in HCV RNA levels (CAP/CTM minus ART) were independent from the viral load in samples containing HCV genotype 4. This may suggest the presence of an intrinsic factor regarding CAP/CTM assay itself may be responsible for underestimation of the HCV RNA concentration in specimens harboring HCV genotype 4. Improper binding of CAP/CTM primers and/or the probe to its target sequences in the 5′-UTR of the HCV genome either due to the presence of a specific genotype 4 nucleotide polymorphism or due to the secondary structure of internal ribosome entry site has been suggested as possible mechanisms that contributes to primers and/or probe mismatch [Chevaliez et al., 2007; Vermehren et al., 2008]. Although our sequence data revealed the presence of frequent nucleotide polymorphism varieties in the 5′-UTR of the samples containing HCV genotype 4, none of these nucleotide polymorphisms had explained the sample which exhibits the extreme difference of measurements between CAP/CTM and ART by more than 3 log IU/ml. Yet, another single substitution was observed at nt145 position and nt165 in samples which exhibit a measurement difference of more than 1 log IU/ml (samples ID; T58-UTR and T48-UTR). Recently, Chevaliez et al. [2009a] described the inability of CAP/CTM assay to detect HCV RNA in highly viremic HCV genotype 4 samples. Interestingly, the report described the presence of two nucleotide polymorphism at positions 145 and 165 in the 5′-UTR sequences of HCV genome may be related to this failure. This position was compatible with mismatch at TaqMan probe level, which may influence the sensitivity of CAP/CTM [Chevaliez et al., 2009a]. The present data provides another strong evidence regarding the presence of nucleotide polymorphisms

which are responsible for mismatches with the primer/and probe site and thus HCV RNA underestimation by CAP/CTM in the case of serum samples harboring HCV genotype 4. This evidence lacks the confirmation due to the refusal of the Roche Molecular Systems to provide the sequence or position of the primers or probes.

In the present study as well as previous comparative studies comparing the CAP/CTM with ART, and CAP/CTM with bDNA assay, the over estimation of the HCV RNA levels reached a significant values in samples containing HCV genotype 1b. The global over estimation of the HCV RNA level for all genotypes by CAP/CTM exclusively in the undiluted clinical samples have been described by Chevaliez et al. [2007]. The molecular basis for the HCV RNA overestimation by CAP/CTM in undiluted samples is unknown. However, possible explanation has been claimed for this phenomenon is a biochemical interaction with a blood component during one of the reaction steps, an interaction that vanishes when the concentration of this putative component is reduced in diluted samples [Chevaliez et al., 2007]. The results of the present study suggest that this putative interaction does not occur during the ART reaction.

The underestimation of HCV RNA concentration in samples harboring HCV genotype 4 has a major clinical impact in Egypt where infection with HCV genotype 4 is prevalent [Ray et al., 2000; Genovese et al., 2005]. In addition, recent reports indicated the high prevalence of HCV genotype 4 particularly among intravenous drug users and patients infected with human immunodeficiency virus in Western and European countries [Cantaloube et al., 2005; Nicot et al., 2005]. In patients with chronic HCV genotypes 1, 4, and 6 infection, a viral load decline by less than 2 log steps between baseline and week 12 of pegylated interferon–ribavirin combination therapy is a predicative factor for virological nonresponse [Zeuzem et al., 2006; Lukasiewicz et al., 2007]. In addition, undetectable HCV RNA at treatment week 4 is an indicator for the 12-week treatment course for patients with chronic hepatitis C [Mangia et al., 2009]. HCV RNA underestimation by CAP/CTM, particularly when it reaches extremely high values represents a critical issue throughout the monitoring of the HCV RNA concentrations in patients with chronic HCV genotype 4 infection particularly those receiving antiviral therapy.

In conclusion, the difference in measurement efficiency of the HCV RNA by CAP/CTM within different genotypes was indicated in this study, particularly in serum samples harboring HCV genotypes 4 and 1b. In addition, the ART assay may yield more reliable and accurate results in quantitation of HCV RNA in samples containing HCV genotype 4. A difference in the sensitivity properties was observed depending on the samples used for the test, for example, ART was more sensitive for measuring the low viral load specimens in the WHO standards and less sensitive than the CAP/CTM for testing the clinical samples containing HCV genotypes 1b and 2a. Whatever the underlying cause for the underestimation of the HCV RNA in samples harboring HCV genotype 4 by CAP/CTM, it represents an important critical issue that must be considered if this assay is used for monitoring HCV RNA level in patients with chronic HCV genotype 4 infection.

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