



BRIEF REPORT

Challenges to Differentiate Hepatitis C Genotype 1 and 6: Results from A Field-Study in Cambodia

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ABSTRACT

Introduction: We aim to report on results and challenges of different methods used for hepatitis C (HCV) genotyping in a Cambodian HCV/HIV coinfection project.

Methods: Samples of 106 patients were available. HCV genotyping was initially (63 samples) done by the LightPower Taqman real-time PCR

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method (Viet A Corp.) and quality controlled using the Versant 2.0 line probe assay (Siemens Healthcare). Next, following interim quality control results, all 106 samples were (re)genotyped with Versant 2.0, complemented with 5'UTR/core sequencing for uninterpretable/incomplete Versant results.

Results: Using Versant, 103 (97.2%) of the 106 HCV-coinfected patients had an interpretable genotype result: 1b (50.5%), 6 non-a/non-b (30.1%), 1a (6.8%), 6a or b (4.9%), 2 (3.9%), 1 (2.9%) and 3 (1.0%). For 16 samples that were interpreted as genotype 1 or 1b per Versant's current instructions, it could not be excluded that it concerned a genotype 6 infection as the core region line patterns on the Versant test strip were unavailable, inconclusive or atypical. Upon sequencing, seven of these were genotyped as 1b and nine as genotype 6. Combining Versant and sequencing results, a definitive genotype was assigned in 104 patients: 1b (44.2%), 6 non-a/non-b (39.4%), 1a (6.7%), 6a or b (4.8%), 2 (3.8%) and 3 (1.0%). Genotyping by LightPower and Versant was discordant for 23 (of 63) samples. The LightPower assay misclassified all genotype 6 non-a/non-b samples as genotype 1, which indicates that this assay is only using 5'UTR information.

Conclusions: HCV genotype 1b and genotype 6 non-a/non-b were most common. With Versant 2.0 (using 5'UTR and core information), genotype classification (1 or 6) remained inconclusive in 15% of samples. The locally available

method (LightPower assay) failed to identify genotype 6 non-a/non-b, which highlights that methods using 5'UTR information only should not be used in Cambodia. Regional/national guidelines should be explicit about this.

Trial Registration: This study was performed as part of a larger cross-sectional study on the burden of hepatitis C coinfection in HIV patients in Cambodia (Clinical.trials.gov: HCV-Epi NCT02361541).

Keywords: Diagnostic methods; Genotyping; Hepatitis C; Misclassification; Southeast Asia

Key Summary Points

Why carry out this study?

Though HCV genotyping is not an upfront requirement before starting HCV treatment anymore, it may be important in case of treatment failures or population-level therapeutic decisions.

HCV genotyping was less straightforward than initially thought in our HCV care program for a Cambodian patient cohort. We were confronted with a locally available commercial genotyping test kit that did not perform well and misclassified most genotype 6 samples as genotype 1. The package insert and regional HCV guidelines did not provide a warning about this.

We therefore decided it was important to document our experience.

What was learned from the study?

Our study highlights the importance of external quality control for laboratory procedures.

We add to the limited information on HCV genotype distribution in HCV/HIV coinfecting patients in Cambodia.

Our experience underscores that genotyping methods solely relying on 5'UTR sequences are not suited for the Southeast Asian region. This problem had been described already in the literature, but was not noted in the international guidelines, package inserts or HCV diagnostic reviews, although it is of major importance for several countries in Southeast Asia.

INTRODUCTION

With the advent of pan-genotypic direct-acting antiviral (DAA) treatment regimens, genotyping is not an upfront requirement before starting a patient on hepatitis C (HCV) treatment anymore. However, for epidemiologic reasons and population-level therapeutic decisions, it remains important that each country or specific context disposes of solid baseline information on HCV genotype and subtype diversity. Also, for certain individual treatment choices (e.g., in case of treatment failure), it remains important to have a good genotype testing strategy in place.

When we were preparing for an HCV prevalence and pilot HCV treatment project among HIV patients in Phnom Penh, data on HCV viral diversity (genotypes and subtypes) in Cambodia were still scarce and, to some extent, conflicting [1]. Three small-scale studies (number of samples with genotype results: 28, 9 and 25, respectively) among Cambodian residents reported genotype 1 (68%; 33.3%; 40%) and genotype 6 (25%; 66.7%; 60%) as the most prevalent [2–4]. All genotype 1 samples with assigned subtype were genotype 1b. Genotype 2 (7%) was only reported in the HIV/HCV co-infected cohort evaluated by Lerolle et al., genotype 3 in none of them. Among 25 Cambodian immigrants in Thailand, genotype 6 (56%) was the most frequent, followed by genotype 1 (24%) and 3 (20%) [5]. However, physicians involved in HCV care in Phnom Penh reported having consulted almost only patients with genotype 1 and very rarely with genotype 2, 3 or 6 HCV (unpublished data).

In addition to the above scarcity of epidemiologic data, the efficacy of new HCV treatment regimens is less well studied in patients with regionally confined genotypes, such as genotype 6. Careful monitoring of real-life cohorts is therefore warranted and requires correct genotype assignment. Subtyping of HCV has also proven important; for genotype 1 lower cure rates have been observed for subtype 1a compared to 1b because of a lower resistance barrier for multiple classes of direct-acting antivirals [6–8]. More studies on the impact of subtype diversity, especially in such a highly diverse genotype as genotype 6, may reveal similar concerns.

Therefore, as part of the HCV pilot project in Phnom Penh [1, 9], we determined the HCV genotype distribution among the co-infected HIV patients from Sihanouk Hospital Center of Hope. The project was intended to be a replicable model of care, and therefore we opted, in the first instance, for a locally available and affordable commercial genotyping assay with re-testing (as quality control) by a commonly used CE-marked commercial assay most suited for genotype 6 identification [10, 11]. Sequencing and phylogenetic analysis are too complex and time consuming to be operationally feasible in low-resource settings.

HCV genotyping in our project was however less straightforward than initially thought. We were confronted with important shortcomings of the initially intended genotype testing strategy and considered it important to share the results and lessons learned from our project. We report on the HCV genotype and subtype distribution among HIV/HCV co-infected persons in Phnom Penh (Cambodia) and on the limitations of the different methods as implemented in our project.

METHODS

We studied the HCV genotype distribution among a cohort of HCV/HIV co-infected patients in Cambodia as part of a larger cross-sectional study on the burden of HCV in HIV patients (Clinical.trials.gov: NCT02361541), the main results of which were published elsewhere. Key populations (men having sex with men,

injecting drug users, commercial sex workers) were rare in this cohort. Iatrogenic HCV transmission was considered more probable [9]. The study was approved by the Institutional Review Board of the Institute of Tropical Medicine of Antwerp, the Ethics Committee of the Antwerp University Hospital (Belgium) and the Cambodian National Ethics Committee for Health Research. All procedures performed in the study were in accordance with the 1964 Helsinki Declaration and its later amendments. No patient identifiers were included in the dataset used for this analysis.

A total of 3045 adult patients from the HIV cohort followed up at Sihanouk Hospital Center of Hope in Phnom Penh were screened for HCV between November 2014 and May 2016. Plasma samples of all patients with current HCV infection, i.e., HCV IgG antibody positive and HCV viral load detectable, were further analyzed to determine the genotype.

Applied Genotyping Strategies (Fig. 1)

As per the initial study protocol, genotyping was performed at a local private laboratory by means of a Taqman real-time PCR method and LightPower HCV genotype rPCR kit (Viet A Corp., Ho Chi Minh City, Vietnam). For the purpose of quality control, eight HCV RNA-positive plasma samples (stored at -80°C) were randomly selected in the beginning of the study and re-tested using the reverse-hybridization line probe assay (LiPA) and Versant HCV genotype 2.0 (Siemens Healthcare, Tarrytown, NY, USA) at the Antwerp University Hospital, Belgium. The LightPower HCV genotype assay provided genotype results, whereas the Versant HCV genotype 2.0 assay additionally assigned subtypes or subtype ranges, as per the manufacturer's instructions.

Upon receipt of the first quality control results (July 2015), the genotype testing strategy was adapted, and testing with the LightPower HCV genotype rPCR kit was discontinued. All samples were (re)genotyped manually with the Versant HCV genotype 2.0 assay and interpreted according to the manufacturer's interpretation chart at the Belgian National HCV Reference Centre (NRC—a consortium of the

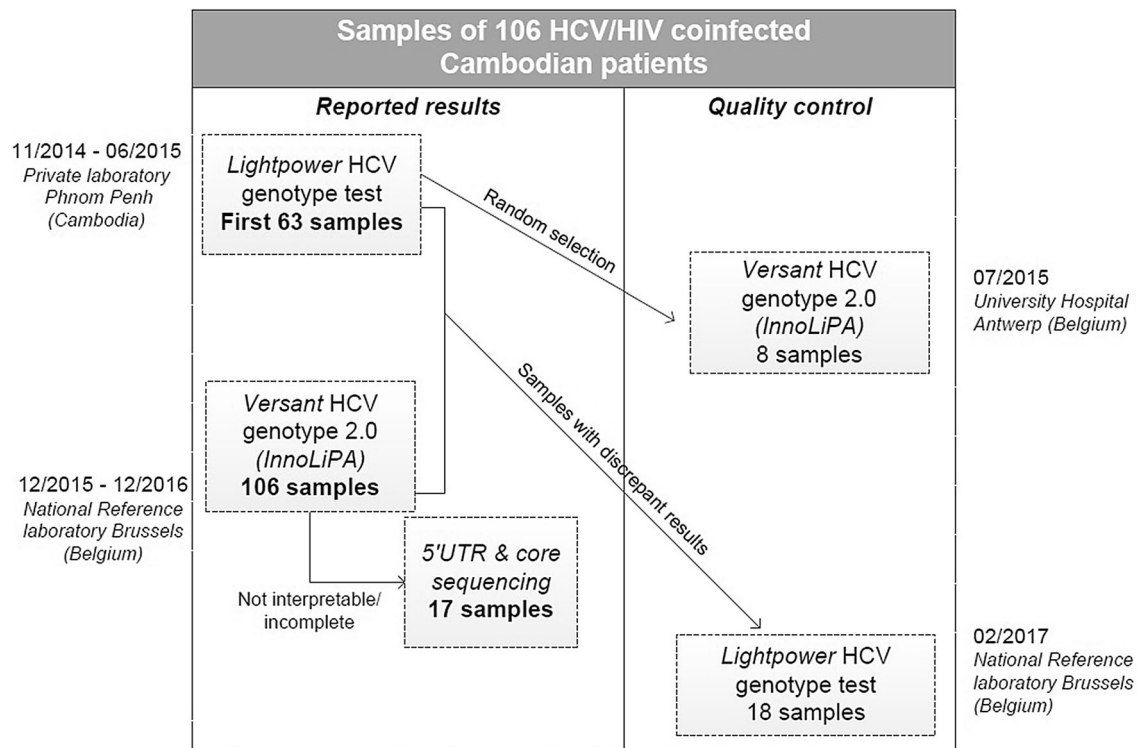


Fig. 1 Genotype testing and quality control strategies applied during the study

Institute of Public Health and Université Catholique de Louvain).

Samples with uninterpretable or incomplete (i.e., genotype 1 or 1b, but genotype 6 not excluded as the core region information was unavailable or inconclusive/atypical) Versant HCV 2.0 results were analyzed further and sequenced at the Belgian NRC laboratory with an in-house method for the 5' untranslated region (UTR) and core region sequencing. Briefly, the assay consisted of separately sequencing the 5'UTR and the core amplicons obtained by Versant HCV genotype 2.0 using the BigDye[®] Terminator v3.1 cycle sequencing kit and ABI 3500 Genetic Analyzer (Thermo Fisher Scientific-Applied Biosystems, Foster City, CA, USA) with a set of four primers, the 5'UTR forward primer 5'-GCAACAGGGAAY YTDCCUGGTTGCTC-3', 5'UTR reverse primer 5'-CTATCAGGCAGTACCACAAGG-3', core forward primer 5'-GTGCCCGGGAGGTCTCG TAG-3' and core reverse primer 5'-CCAAGGGT ACCCGGGCTG-3'. The obtained sequences were aligned with the Geneious 4.0 bioinformatics software (Biomatters, Auckland, New

Zealand), and the HCV genotype and subtype were determined by using the online BLAST tool [12].

To assure reliability of the results obtained with the LightPower HCV genotype test kit in the laboratory in Cambodia, double re-testing (two runs) with the same test kit was organized in February 2017 in the Belgian NRC for 78.3% (18/23) of the samples with discrepant results for the Versant 2.0 and LightPower HCV genotype test. No leftover remained for the other five samples. All assays were thus performed (or quality controlled) and interpreted according to the manufacturer's instructions in accredited laboratories adhering to Good Clinical Laboratory Practices (GCLP).

Data Analysis and Reporting

Descriptive statistics included median with interquartile range (IQR), and frequencies with percentages when appropriate.

The agreement between the genotype results obtained with the LightPower test and Versant

2.0 was measured by calculating Cohen’s κ coefficient.

RESULTS

Among the 3045 screened adult patients of the HIV cohort of Sihanouk Hospital Center of Hope in Phnom Penh, 106 were identified with current HCV coinfection (i.e., HCV IgG positive and HCV viral load detected). The median age of the HCV/HIV coinfecting patients was 48 years (IQR: 42-53); 61 were female; none reported commercial sex work, being homosexual, or current or past injecting drug use [9]. All were HCV treatment naïve; median HCV viral load was 2,000,000 IU/ml (range 985–21,000,000).

Results of Genotyping with the Versant HCV Genotype 2.0 Assay

With the Versant 2.0 assay, 97.2% (103/106) of the samples gave interpretable results, 1.9% (2/106) failed to amplify, and 0.9% (1/106) amplified but gave uninterpretable results. The distribution of genotypes and subtypes, interpreted as per the current interpretation chart, is shown in Table 1. The predominant genotypes

Table 1 Genotype (and subtype) distribution in 103 Cambodian HCV/HIV co-infected subjects as classified by Versant 2.0 (Siemens)

Genotype (N, %)	Subtype (N)
Genotype 1 62 (60.2%)	1a 7
	1b 52 ^a
	Unassigned 3 ^b
Genotype 2 4 (3.9%)	2 1
	2a or 2c 3
Genotype 3 1 (1.0%)	3a 1
Genotype 6 36 (35%)	6a-b 5
	6 non-a/non-b 31

^a For 13 of the samples typed as genotype 1b, genotype 6 could not be excluded

^b For three genotype 1 samples without assigned subtype, genotype 6 could not be excluded

were genotype 1 (60.2%) and 6 (35%). For genotype 1, subtype 1a was assigned in seven samples (11.9%), subtype 1b in 52 samples (83.9%), and the subtype was indeterminate in 3 samples. Genotype 6 could not be excluded in 16 samples with unavailable ($n = 3$) and inconclusive/atypical ($n = 13$) results in the core region on the strip, but they were scored, as per the manufacturer’s instructions, as genotype 1/1b based on the information of the 5’UTR region. The line patterns are detailed in Fig. 2. For genotype 6, the majority ($n = 31$, 86.1%) were subtyped as ‘6 non-a/non-b.’ Genotypes 2 and 3 were rare, accounting together for < 5%.

Comparison of the Results of the Versant HCV 2.0 and the LightPower HCV Genotype Assay

The first 63 samples were also genotyped with the LightPower HCV genotype test. This test classified 58 (92%) samples as genotype 1, 3 as genotype 6 and 2 as genotype 2 (Table 2). Upon retesting with the Versant 2.0, genotype results were discordant for 23 samples. Twenty-two samples classified as genotype 1 with the LightPower test typed genotype 6 (‘6 non-a/non-b’) with the Versant 2.0. Only 3 of the 25 genotype 6 samples, subtype ‘6a or 6b’ by the Versant 2.0, were recognized as genotype 6 by the LightPower test. One sample determined as genotype 1 with the LightPower test was classified as genotype 3a with the Versant 2.0. Overall, poor agreement (63.5% concordance, $\kappa = 0.22$) between the two tests was obtained.

Results of the Versant HCV Genotype 2.0 Assay Complemented with 5’UTR and Core Sequencing

The Versant 2.0 amplicons of 16 samples with incomplete results (genotype 1 or 1b, but genotype 6 not excluded) were sequenced (Fig. 2). Seven samples were classified as genotype 1b and 9 as genotype 6 upon sequencing. All six samples with line pattern 23, 24 and 25 sequenced as genotype 6, while the three samples with line patterns 23, 24 and 26, as well as

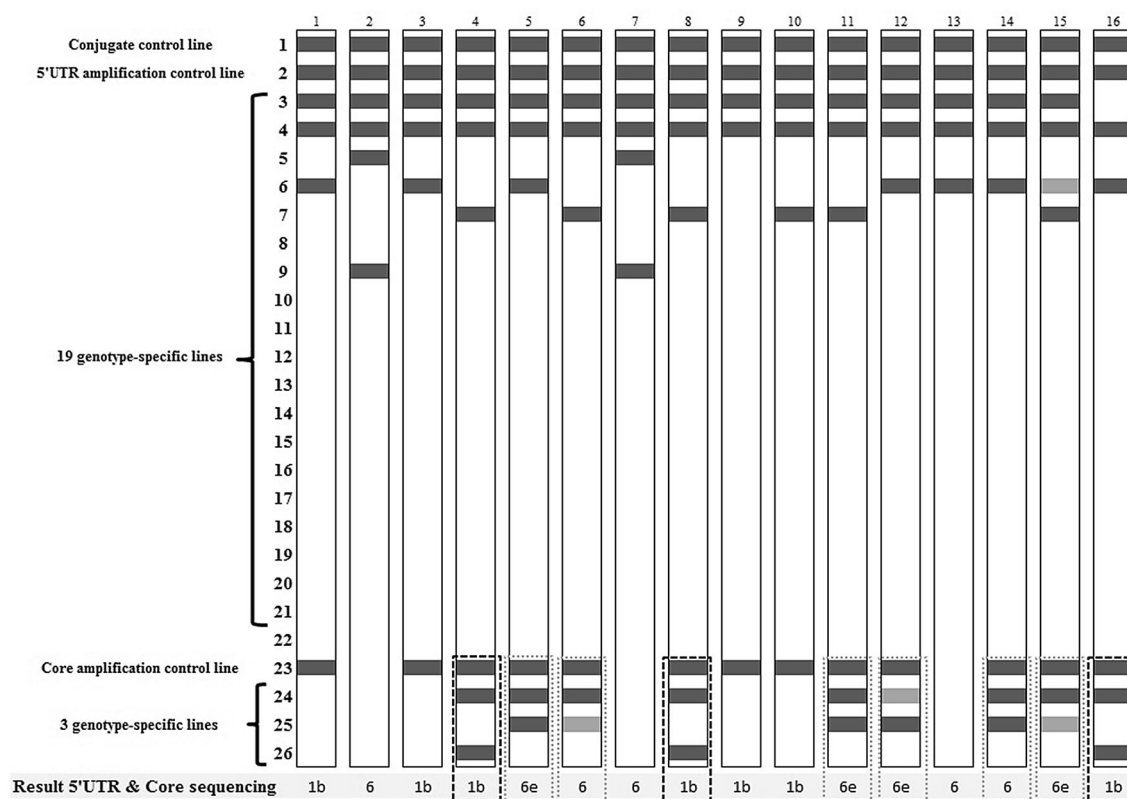


Fig. 2 Line pattern Versant 2.0 and results of 5'UTR and core sequencing for 16 samples scored genotype 1/1b by Versant 2.0 but for which genotype 6 could not be excluded. Faint colored bands are colored lighter gray

Table 2 Genotype concordance among 63 patients tested with LightPower HCV (Viet A Corp.) and Versant 2.0 (Siemens)

LightPower	Versant 2.0							Total (for LightPower)
	Genotype 1		Genotype 2	Genotype 3	Genotype 6			
	1a	1b	1	2a or 2c	3a	6a–b	6non-a/non-b	
Genotype 1	4	28 ^a	3 ^b	0	1	0	22 ^c	58
Genotype 2	0	0	0	2	0	0	0	2
Genotype 3	0	0	0	0	0	0	0	0
Genotype 6	0	0	0	0	0	3	0	3
Total (for Versant 2.0)	4	28	3	2	1	3	22	63

^a For six samples, genotype 6 could not be excluded by Versant 2.0

^b For three samples, genotype 6 could not be excluded by Versant 2.0

^c For two samples, the result 'genotype 1 or 6' was obtained in one of the test runs by LightPower testing

the four samples with line pattern '23 only' (core amplification control line), sequenced as genotype 1b. The sample with an

uninterpretable result by Versant 2.0 genotyping was sequenced as genotype 6. Subtype assignment was possible for five out of ten

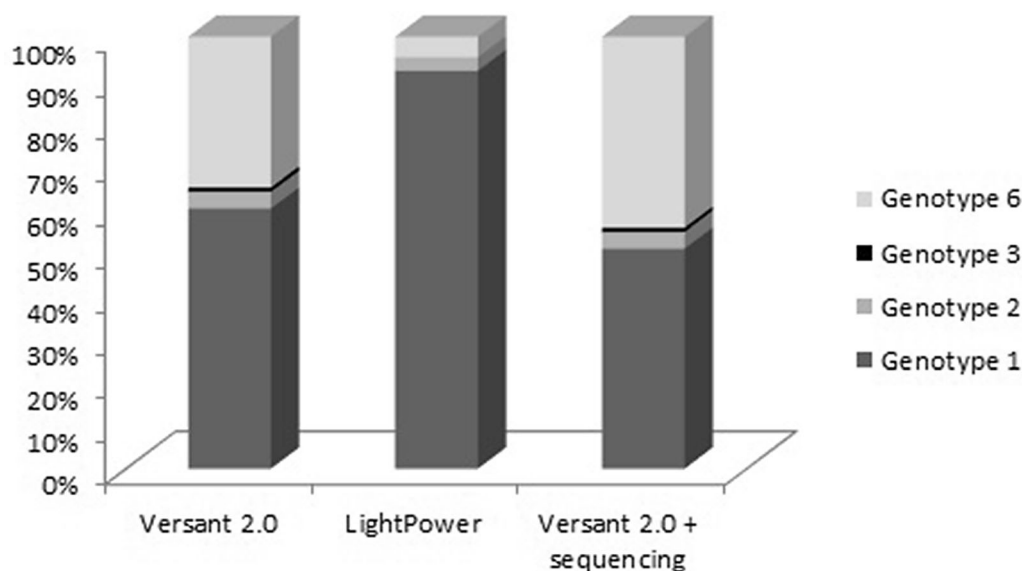


Fig. 3 HCV genotype distribution in an HCV/HIV coinfecting Cambodian cohort. Results of different testing strategies. The strategies are: (1) genotyping with the Versant 2.0 line probe assay ($N = 103$), (2) genotyping

with the LightPower HCV test ($N = 63$) and (3) genotyping results of the Versant 2.0 complemented with sequencing for samples with uninterpretable/incomplete results by Versant 2.0 testing ($N = 104$)

samples sequenced as genotype 6; all were genotype 6e.

Combining Versant 2.0 and available sequencing results, the definitive (sub)types assigned were as follows: 1b ($N = 46$, 44.2%), 6 non-a/non-b ($N = 41$, 39.4%), 1a ($N = 7$, 6.7%), 6a or b ($N = 5$, 4.8%), 2 ($N = 4$, 3.8%) and 3 (1.0%). An overview of the genotype distribution in terms of proportions with the three methods (Versant 2.0, LightPower and Versant 2.0 complemented with sequencing) can be found in Fig. 3.

When comparing the LightPower results for the first 63 samples to the combined standard (Versant 2.0 testing complemented with sequencing for samples with incomplete results), concordance further declined (57.1% concordance, $\kappa = 0.18$).

DISCUSSION

In this article, we described the results and challenges of implementing HCV genotyping in a small-scale HCV/HIV coinfection project in Cambodia.

In terms of genotype distribution, four genotypes (1, 2, 3 and 6) and six subtypes or subtype ranges of HCV were detected in this cohort of 106 HCV/HIV co-infected patients. Samples of 103 patients were amplifiable and interpretable with the Versant 2.0 genotype test. Applying the current interpretation rules of this method, 60.2% of the samples were scored as genotype 1 and 35% as genotype 6. An important caveat was, however, that among those scored as genotype 1, there were 16 samples (15% of amplified samples) for which genotype 6 could not be excluded by the Siemens method, as only information from the 5'UTR region was interpretable for these samples. Upon further analysis by sequencing (5'UTR and core), nine of these samples were typed as genotype 6 rather than genotype 1, resulting in equal frequency of genotype 1b (44.2%) and 6 (44.2%) in our cohort. The remaining were genotype 1a (6.7%), genotype 2 (3.8%) and genotype 3 (1.0%).

Our results confirm the predominance of HCV genotype 1 (subtype 1b) and 6 (mainly non-a/non-b subtypes), which was also found in the previously published small-scale studies in

Cambodia and among Cambodian migrant workers in Thailand [2–5]. The proportions of genotype 1 and 6 infections are similar to those documented in a recent study of the Pasteur Institute in Cambodia and Médecins Sans Frontières, with predominantly HCV mono-infected patients [13]. Genotype 3 was rare, in line with what was previously described in the studies among patients residing in Cambodia. The higher frequency of genotype 3 reported in the study among Cambodian migrants in Thailand might be explained by the fact that some of these migrant workers might have acquired their infection in Thailand where genotype 3 is more frequent [5].

The LightPower assay, which we chose as the first-line genotype test because it was locally available, affordable and already in clinical use, misclassified all genotype 6 non-a/non-b isolates as genotype 1. Though vigilant from the start (and therefore including a per-protocol quality control), we did not expect this kind of systematic misclassification or gross genotyping difficulties. Major HCV guidelines and published reviews on HCV diagnostics, even when focusing on the Asian region, are silent on this matter [14–16]. Through a focused literature search—once confronted with the problem—we found that many commercial genotyping methods are falling short in correctly classifying genotype 6 strains because they rely solely on the 5'UTR region for genotyping. The 5'UTR region has sufficient nucleotide sequence divergence to discriminate most genotypes, but not to differentiate HCV genotype 6 non-a/non-b from genotype 1/1b [17–19]. The misclassification pattern in our study, with the LightPower assay, is the same and had, as it was a frequently used test in Phnom Penh, fed the misperception among HCV medical care givers in Cambodia that genotype 6 is very rare. Later studies confirmed that genotype 6 non-a/non-b HCV infections are frequent in Cambodia [13].

The Versant HCV genotype assay 2.0 uses sequence information from both the 5'UTR and the core region, allowing an improved distinction between HCV genotype 1 and non-a/non-b subtypes of genotype 6. This test seems therefore currently one of the better suited commercial genotyping methods for use in

Southeast Asia [10, 11, 20]. However, relying solely on this method and its current interpretation chart would in our setting have led to unclear genotype assignment (1/1b, but 6 not excluded) in 15% of the samples and underestimation of genotype 6 prevalence. The sequencing results for the samples with atypical core line patterns (pattern 23, 24 and 25: genotype 6; pattern 23, 24 and 26: genotype 1b) suggest that these patterns could be added to the interpretation chart to further improve the performance of the Siemens method to differentiate between genotype 1 and 6. Furthermore, our data also suggest that genotype results based on the 5'UTR pattern of the Versant 2.0, in case of core pattern '23 only,' can be considered conclusive. Applying these additional interpretation rules would have decreased the proportion with inconclusive genotype assignment to 3.9%.

Our study has some limitations as we assumed that the samples with conclusive Versant 2.0 results were correctly genotyped without further confirmation by sequencing. Underestimation of subtype 1a and genotype 6 prevalence in our current results can therefore not be excluded as other performance evaluations reported cases of deficient subtyping between 1a and 1b [21] and misclassification of genotype 6a samples as genotype 1b [22, 23]. Additional sequencing (including NS5B or whole genome) would have provided the firmest genotype and subtype information.

When implementing our HCV treatment project in Cambodia, the HCV genotype was still a key parameter for deciding on the treatment regimen and its duration. Currently, this is no longer the case. HCV pan-genotypic treatment is now more widely available. Nevertheless, our experience remains relevant to share, as the in-country HCV genotype testing capacity will remain important for some specific individual cases (e.g., treatment failure) and for overall treatment program monitoring as there might be slight, so far uncovered, differences in treatment efficacy along the very diverse subtypes of genotype 6.

Our experience also demonstrates the importance of having a well-defined and solid laboratory quality control program in place in

each research or operational health care project that involves laboratory testing.

CONCLUSION

The scale of our study and experience is too limited to enable translation into comprehensive guidance on HCV genotyping testing strategies for Cambodia or the Southeast Asian context.

However, based on our results and experience, we call for a careful selection of genotyping methods in the Southeast Asian context. Methods solely relying on 5'UTR sequences (as the LightPower assay) are not suited for this region as both genotype 1 and genotype 6 non-a/non-b are frequent. The 5'UTR sequences are highly similar between these two genotypes, and without additional information from the core or NS5B region these genotypes cannot be correctly differentiated. This problem has been described occasionally in the literature, but to our knowledge has not been drawn any attention to in international guidelines, package inserts or HCV diagnostic reviews although it is of major importance for several countries in Southeast Asia.

Also, the Versant 2.0 showed a high proportion of inconclusive results (genotype 1 or 6 non-a/non-b). Our data suggest however that additional differentiating line patterns could be inserted in the interpretation chart to decrease this problem.

Considering the shortcomings of commercial methods and the limited use of genotyping in HCV care nowadays, centralized sequencing facilities might respond best to the needs and be most apt, for example, to uncover specific genotype 6 subtype-related differences in treatment response.

Generally, research focused on regionally confined, but frequent genotypes such as genotype 6 should be accelerated in the field of both therapeutics and diagnostics so that, if need be, evidence-based context-tailored guidance can be provided to the end users, i.e., laboratories and clinicians.

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Authorship. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole and have given their approval for this version to be published.

Author Contributions. ADW conceived the study, but all authors, each with their specific expertise, were involved in the design and/or implementation of the study. ADW wrote the first draft of the paper, which was further improved by the input of all co-authors. All authors read and approved the final manuscript. ADW is the guarantor of the paper.

Disclosures. Anja De Weggheleire, Irith De Baetselier, Sokkab An, Sylvie Goletti, Vanessa Suin, Sopheak Thai, Sven Francque, Tania Crucitti, Lutgarde Lynen, Steven Van Gucht and Benoît Mukadi Kabamba declare that they have no competing interests.

Compliance with Ethics Guidelines. The study was approved by the Institutional Review Board of the Institute of Tropical Medicine of Antwerp, the Ethics Committee of the Antwerp University Hospital (Belgium) and the Cambodian National Ethics Committee for Health Research. All procedures performed in the study were in accordance with the 1964 Helsinki Declaration and its later amendments.

Data Availability. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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