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A novel next generation sequencing assay as an alternative to currently available methods for hepatitis C virus genotyping

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ABSTRACT

Chronic HCV infection is one of the leading causes of liver-related death and in many countries it is a primary reason for having a liver transplant. HCV genotype identification has long been used in the clinical practice, since different genotypes have different response rates and required different doses and durations of IFN/RBV treatment; moreover both the frequency and the pattern of resistance to different Direct-Acting Antivirals (DAAs) classes are subtype specific. Hence the necessity to make an accurate HCV subtyping becomes a fundamental tool to optimize current and future clinical management of HCV infected subjects. In the present study the performance of a next generation sequencing (NGS: based on the Ion Torrent Platform-Vela Sentosa SQ 301 sequencer) HCV genotyping assay has been evaluated. The current method targets a region of the NS5 B gene and it is the unique NGS based market CE-IVD assay. As a comparative method a commercial method based on the detection via reverse hybridization of 5′UTR and core regions (Versant HCV Genotype 2.0 Assay, LiPA, Siemens) was selected. A total 207 plasma samples from HCV infected individuals were used. No selection was made for these samples that were submitted for routine HCV genotyping. The results show Vela NGS assay assigns major number of HCV subtypes with respect LiPA. Concerning genotype 1 and 3, the discrepancy of assigned subtypes for LiPA with respect to Vela NGS assay is not relevant (1.8% and 2%, respectively); in contrast, the difference of assigned subtypes for genotypes 2 and 4 is very high (96.6% and 100%, respectively). The resistance mutations data, except for 1a and 1b subtypes, remain scarce; the future relevant challenge will be to identify subtypes-specific drug resistance mutations, which are essential to create highly personalized therapeutic pathways.

1. Introduction

Hepatitis C virus (HCV) is a globally prevalent and genetically diverse virus that infects approximately 71 million people worldwide, about 1% of the world population (Mohd Hanafiah et al., 2013; Thomas, 2013). Chronic HCV infection is one of the leading causes of liver-related death and in many countries it is the primary reason for having transplant (Verbeeck et al., 2008). HCV is classified into 7 major genotypes based on phylogenetic analysis, with an average 35% nucleotide divergence between strains belonging to different genotypes (Sandres et al., 2000). Each genotype can be further subdivided into related subtypes (67 confirmed), with a nucleotide sequence divergence varying from 15% to 30% between diverse subtypes (Smith et al., 2014). The seven genotypes are differently distributed worldwide and are associated with different severity of disease (Chen and Morgan, 2006). HCV infection is a highly dynamic process: the virus has a half-life of few hours and it is estimated that in a patient with chronic infection up to 10^{12} new virions are generated per day. In the replication cycle, the synthesis of viral genome is performed by a viral RNA-dependent RNA polymerase, lacking proof-reading mechanism, that causes an error rate of about 10^{-3} base substitution per site (Hoofnagle, 2002). This inaccurate replication mechanism, coupled with the high rate of replication, results in HCV having extremely high sequence diversity (Susser et al., 2011). Consequently, in a chronic HCV infected individual, the virus exists as an heterogeneous population of virions, termed "quasispecies" (Scheel and Rice, 2013). Nowadays new direct-acting antiviral agents (DAAs) have been designed. Combination therapies based on different DAAs improve treatment response and reduce the duration of treatment, thus achieving the eradication of HCV infections in 3–6 months of treatment (Sarrazin et al., 2012). Genotype identification has long been used in the clinical practice, since different genotypes showed different response rates and required different doses

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and durations of IFN/RBV treatment. In contrast, until recently, subtype identification was mainly used in the frame of epidemiological studies. Nevertheless it has been proved that, at least for genotype 1 and 4, both frequency and the pattern of resistance to different DAAs classes are subtype specific (Wyles et al., 2014; Schnell et al., 2015). Moreover, co-infection with two or more HCV strains of different genotypes or subtypes is a common finding among some high-risk groups (Schnell et al., 2015; Grebely et al., 2012). Hence the necessity to make an accurate HCV subtyping becomes a fundamental tool to optimize current and future clinical management (Pham et al., 2010; Gregori et al., 2014; Avò et al., 2013; Bouchardeau et al., 2007; Cai et al., 2013; Chevaliez et al., 2009; Gonzàles et al., 2013; Hong et al., 2012; Noppornpanth et al., 2006). Recently, alternative regions have been proposed for HCV genotyping; the widely accepted reference method for HCV genotyping is based on the sequencing of NS5B, the viral gene coding for viral RNA-dependent RNA polymerase (Quer et al., 2015).

In the present study, the performance of HCV genotyping CE-IVD marked assay based on Ion Torrent NGS Platform (Vela Sentosa SQ 301 sequencer) has been evaluated. The method assigns genotype on the basis of NS5 B region. The novel assay has been compared with a CE-IVD marked commercial method based on the detection, via reverse hybridization, of the 5′UTR and core regions (Versant HCV Genotype 2.0 Assay, LiPA, Siemens). A total of 207 plasma samples from HCV infected patients were included in the study.

2. Material and methods

2.1. Clinical samples

The study was performed on 207 clinical plasma samples from HCV infected individuals collected during the daily routine workflow at the Unit of Microbiology of the Hub Laboratory of the Greater Romagna Area (Pievesestina FC, Italy) from March to September 2014. All the samples used in this study have been anonymized following the anonymization procedure approved by the ethical committee of the AUSL Romagna. The samples were randomly selected, not considering the prevalence of some specific genotypes; the unique criterion for selection was a HCV viral load > 1000 UI/ml determined by the Abbott RealTime HCV assay, without choice based on age, sex or disease severity. The samples were genotyped with both Versant HCV Genotype assay 2.0 (LiPA, Siemens) and Sentosa SQ HCV Genotyping Assay 4 × 16 (Vela Diagnostics).

2.2. Versant HCV genotype 2.0 assay (LiPA)

HCV RNA was extracted (starting from 500 µL of plasma EDTA) and purified via magnetic beads technology with the Abbott *m*Sample Preparation System on the $m2000_{sp}$ automatic platform. The Versant HCV amplification 2.0 kit (LiPA), was used for the reverse transcription and amplification of 5′ untranslated (5′UTR) and core regions of HCV, using a Veriti Dx 96-Well Thermal Cycler BioRad, as suggested by manufacturer. Versant HCV Genotype assay 2.0 (LiPA) (Siemens Healthcare Diagnostics) utilizes the reverse hybridization technique with immobilized oligonucleotide probes specific for 5′UTR and core region of different HCV genotypes.

2.3. Sentosa SQ HCV genotyping assay

Sentosa SQ HCV Genotyping Assay is a ready to use kit that allows the automated library preparation using the Sentosa SX101 instrument and it is intended for the HCV genotyping and subgenotyping.

The workflow automates nucleic acid extraction from samples to the PCR set-up on the Sentosa SX101 instrument, and it is then followed by an off board PCR amplification. After the completion of the PCR amplification, the workflow continues with the library preparation and IonTorrent deep sequencing.

2.3.1. Extraction and PCR amplification

In detail, the Sentosa SQ HCV Genotyping Assay workflow starts with the extraction and purification, by magnetic beads technology, of nucleic acids from 530 µL of human plasma on the Sentosa SX101 instrument using the Sentosa Virus Total Nucleic Acid Plus II Kit in a complete automated way. After extraction, the genes NS3, NS5A and NS5 B are amplified via RT-PCR reaction (Veriti 96 well thermal cycler, Applied Biosystem). NS3 and NS5A are not useful for genotyping but just used for subsequent variant analysis for the detection of SNP's potentially relevant in the DAA resistance.

2.3.2. Emulsion PCR and enrichment

All individual PCR reactions are normalized via magnetic beads to obtain an even concentration of PCR fragments. Because of the defined concentration of beads, only a defined concentration of PCR fragments will bind, then surplus fragments are washed away by a magnetic separator. After normalization, the PCR fragments will be cut into smaller pieces (200 bp). For this step, a DNA shearing enzyme buffer is used. Each sample is barcoded via ligation, pooled together in one single tube and clonally amplified by emulsion PCR (Sentosa ST401i). Water-in-oil micro-reactor stochastically contains one strand of library DNA, one bead (ISP beads) and PCR components (Sentosa™ ST Template Kit). Ideally the PCR products are clonal beads population; polyclonal and empty beads will be filtered away in the successive steps. To avoid empty beads, enrichment step is performed using streptavidin-coupled Dynabeads (streptavidin coated magnetic enrichment bead). Positive template ISP will bind to enrichment beads, allowing to discard no template ISP. The enrichment step is performed automatically using Sentosa ST401e (as suggested by manufacturer).

2.3.3. IonTorrent sequencing

Positive template beads are then sequenced using the Ion Torrent technology. The sequencing chemistry is remarkably simple. Naturally, a proton is released when a nucleotide is incorporated by the polymerase in the DNA molecule, resulting in a detectable local change of pH. Each micro-well of the Ion Torrent semiconductor sequencing chip contains approximately one million copies of a DNA molecule. Sentosa SQ301 sequencer (Sentosa SQ Sequencing Kit was used) sequentially floods the chip with one nucleotide after another. If nucleotide complements the sequence of the DNA molecule in a defined micro-well, it will be incorporated and hydrogen ions are released. As a consequence the pH of the solution changes in that defined well and it is detected by the ion sensor. Primary data analysis is performed automatically using Sentosa SQ Suite software; secondary data analysis is performed using Sentosa SQ Reporter software. Genotyping is assigned by sequencing a region of NS5 B (685 bp), that shows a median coverage per sample of at least 200x. The analytical limit of detection is declared by the User Manual to be 1000 IU/mL for genotypes 1a, 1b, 2, 3 and 4, and 2000 IU/mL for genotypes 5 and 6 (Application Note by Ion Torrent Life Technologies).

2.4. Statistical analysis

The two methods were compared evaluating the concordance of the respective results by computing Cohen's Kappa coefficient.

3. Results

As shown in Table 1, 62 samples were subtyped as 1a by Vela NGS; the same result was achieved by LiPA. Vela NGS identified 47 samples as 1b, whereas LiPA was not able to identify one (2.1%) subtype 1b. One subtype 1 g was detected by Vela NGS but not identified by LiPA. Concerning genotype 2, the Vela NGS classified 25 samples as 2c and one sample as 2a, 2b, 2i and 2k, respectively. LiPA did not assign subtypes for seven (28%) genotype classified as 2c and one (100%) as 2k by Vela NGS. LiPA classified 18 (72%) samples as genotype 2a/2c,

Table 1
Comparison of HCV genotyping results from Versant HCV Genotype 2.0 Assay (LiPA) and Sentosa SQ HCV Genotyping Assay.

LiPA	Vela NGS Genotype												Total
Genotype													
	1a	1b	1g	2a	2b	2c	2i	2i + 2c + 2d	2k	3a	4d	1a + 4d	
1		1	1										2
1a	62												62
1b		46											46
2						7		1	1				9
2b					1								1
2a/2c				1		18	1			1			21
3										1			1
3a										50			50
4											5		5
4a/4c/4d											9		9
1a + - 4a/4c/4d												1	1
Total	62	47	1	1	1	25	1	1	1	52	14	1	207

which were further subtyped as genotypes 2c by Vela NGS. Two samples yielded LiPA patterns that were categorized as genotype 2a/2c are identified as subtype 2a and 2i by Vela NGS. One subtype 2b was identified by both methods. One sample that was classified by LiPA as genotype 3 (1,9%) was grouped as genotype 3a by Vela NGS. Among the 14 samples subtyped as 4d by Vela NGS, five (35,7%) could not be subtyped and 9 (64,3%) were inconclusive (4a/4c/4d) by LiPA. Interestingly, one sample that showed by NGS analysis a potential coinfection by genotype 2i + 2c + 2d was identified by LiPA as genotype 2, without sub genotyping result. It is higly unlikely to detect a 3 genotypes infection, in particular because genotype 2i and 2d are itself very rare. Similarly, LiPA detected one partially inconclusive co-infection (1a + 4a/4c/4d), which was found to be a mixed infection of HCV subtype 1a (21,490 reads, 70.43%) and 4d (9021 reads, 29.57%) by Vela NGS. Only one discrepancy was found at genotype level: LiPA assigned a genotype 2a/2c which was yielded 3a by Vela NGS. The discrepancy (expressed as percentage) of assigned subtypes for LiPA with respect to Vela NGS assay is as follows: concerning genotype 1 and 3, the discrepancy is not elevated (1.8% with Cohen's Kappa = 0,981C.I. 0,954;1.000 and 2%, Cohen's Kappa = 0,974C.I. 0,938;1.000 respectively) if compared to the difference of assigned subtypes for genotypes 2 and 4 (96.6% with Cohen's Kappa = 0,056C.I. −0,049; 0.161 and 100% Cohen's Kappa = 0 respectively). Statistical evaluation of the results shows that subtyping global concordance across all genotypes between the two methods is moderate, with a concordance coefficient of Cohen's Kappa equal to 0.715 (CI = (-0.668; 0.786)). Hence, it is possible to affirm that there is not a significant statistical difference between the two methods, or rather the number of assigned subtypes by Vela NGS is significantly higher than subtypes assigned by LiPA. Therefore Vela NGS assay showed to have a better performance with respect to LiPA in assigning a major number of HCV subtypes. It was observed that the viral load of the 46 samples showing discrepancy between the two methods are all > 10000 UI/ml. This value of viral load is abundantly higher then the limit of detection of both assays declared by the manufacturer (stated for both tests at 1000 UI/ml). Therefore it is unlikely that these discrepancies could be related to the viral load of individual samples.

4. Discussion

Genotyping of HCV has become an essential tool for the prognosis and the prediction of treatment duration and efficacy. Nowadays, the introduction of the DAAs drugs has improved the treatment response and its tolerability, reducing the duration of therapy and establishing interferon-free treatment options. However the introduction of these new treatments makes the HCV subtyping fundamental step in order to achieve the best for the clinical outcome (Sarrazin et al., 2012), since both the frequency and the pattern of resistance to different Direct-Acting Antivirals (DAAs) classes are subtype specific. The present is the first study that applies a CE-IVD marked NGS technique for the HCV genotyping in clinical samples (Sentosa SQ HCV Genotyping Assay), thus de facto introducing a next generation sequencing based assay for routine HCV genotyping. Sentosa SQ HCV Genotyping Assay identified the HCV subtypes in all the 207 samples, and was able to identify the presence of different HCV subtypes in two samples with mixed infection. In contrast, the LiPA did not identify HCV-1 subtype in 2/110 (1,8%) samples, HCV-2 subtype in 29/30 (96.6%) samples and HCV-3 subtype in 1/51 (2%) samples. Concerning the genotype 4, LiPA was not able to assign subtype in all 14/14 (100%) samples (see Table 2). These results underline that LiPA and NGS have similar performance in subtyping genotype 1 and 3. Genotype 1a and 1b are the most known and widespread in the European area; Siemens recently optimized the LiPA (Version 2.0) thus improving the capability to detect 1a and 1b genotypes, while in a previous release these were not well defined. Concerning genotype 3, the only subtype revealed in samples is 3a, therefore it is possible to affirm that LiPA has a similar performance to NGS in defining only subtype 3a. The capability of subtyping genotypes 2 and 4 of LiPA with respect to NGS is significantly low, this reveals the lack of specific probes defining 2 and 4 genotypes in the LiPA. In addition, the treatment duration for genotype 2 is reduced with respect to genotype 3a, hence this potential misclassification may have a detrimental effect on the therapeutic success rate (EASL, 2016). Naturally, the subtypes distribution of sample collection is influenced by the spread of the diverse HCV genotypes in Italy; this means that the most infrequent genotypes in European area (genotypes 5 and 6) were not included for the evaluation of the genotyping performance assays (Murphy et al., 2007). Furthermore, in the mixed infection (1a + 4a/4c/4d) LiPA did not identify subtype of the genotype 4. The NGS analysis assigned this sample to genotype 4d. Coinfections by more than 2 genotypes are extremely rare: the NGS method showed the possible contemporary presence of genotypes 2i,2c and 2d in one patient. Since genotype 2i and 2d are extremely rare therefore the confection may be considered as unlikely.

Consequently it is only possible to speculate that the computational assignment of some of the sequencing fragment to these rather rare sub type was not accurate in this sample. To clarify the hypothesis of this 3 genotype infection additional testing by PCR amplification and cloning of individual amplicons followed by sanger sequence should be performed.

Hence, at present NGS represents an effective tool to discriminate the minority variants in the mixed infection, and to study the diversity of the viral population within one single plasma sample.

At present, accurate HCV genotyping/subtyping is mandatory to select the most appropriate DAA and to reduce the risk of therapy failure and to this respect sequencing-based subtyping methods (direct sequencing and NGS) may be of relevant help.

4.1. Conclusions

The results obtained in the present study have confirmed that the NGS based method is more efficient for HCV subtyping than some of the most used commercial available methods (Abbott RealTime and LiPA), allowing identification of mixed infections and resolving genotype 1 and equivocal genotype samples.

Therefore NGS allows accurate identification of HCV multiple infections, and is able to rule out inaccurate assignment of mixed infections by commercial methods (Minosse et al., 2016). underline the importance and advantages that would been achieved by applying this technique in clinical laboratories, especially when looking for the identification of rare viral types, or while searching for resistance associated variants in patients experiencing a relapse and not achieving a sustained viral response (Trémeaux et al., 2016).

As of today, the NGS applications have been limited to research projects, but this study may encourage the use of the NGS based technique routinely in the procedure of HCV genotyping. The most relevant strength of this NGS based technique genotyping assay is enhanced by the semi-automatic system provided by the manufacturer and turnkey products. This represents a unique useful feature out of which clinical laboratories would gain a great benefit, nowadays unavoidable.

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Authors contributions: DG, PE, ME, DB, BB and GAR performed the experiments, PF and SV conceived the study, DG and PE wrote the manuscript, CMR and SV revised the manuscript.

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