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Clinical evaluation of IntelliPlex™ HCV genotyping kit for hepatitis C virus genotyping

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ABSTRACT

Genotyping of the hepatitis C virus (HCV) is crucial for determining the most efficient anti-viral therapy. The clinical sensitivity and specificity of the IntelliPlex™ HCV Genotyping Kit was determined by comparing the assay results of 307 specimens with the results obtained by Sanger sequencing. Out of 202 HCV-positive specimens tested, 8 samples yielded discrepant results between the IntelliPlex HCV Genotyping Kit and Sanger sequencing. For 5 of these discrepant samples, the IntelliPlex HCV Genotyping Kit classified the correct genotype but failed to show the same single or dual infected status as determined by Sanger sequencing. A total of 105 samples which tested negative for HCV by In-Vitro-Diagnostics (IVD)-approved viral load assay tested negative for HCV by the IntelliPlex HCV Genotyping Kit. The IntelliPlex HCV Genotyping Kit has a clinical specificity of 100% and a clinical sensitivity of 96.9% and is suited to be used in clinical laboratories to genotype HCV.

1. Introduction

With up to approximately 180 million infected patients, Hepatitis C virus (HCV) remains a major global public health problem (Petruzziello et al., 2016). HCV is the causative agent of hepatitis C infection and most cases of acute infections will progress to a chronic disease state (Morozov and Lagaye, 2018). Chronic HCV infection often results in liver cirrhosis, hepatocellular cancer, liver failure, and potentially death. HCV is also the leading cause for liver transplantation in Europe and the United States (Martini, 2018).

HCV is classified into seven major genotypes (1-7) and more than 60 confirmed subtypes (Smith et al., 2014). Worldwide, HCV genotype 1 is the most prevalent (42–49%), followed by genotype 3 (18–26%) (Petruzziello et al., 2016; Gower et al., 2014). However, significant regional differences in HCV genotype distribution exist. For example, the most dominant genotype in China and Taiwan is genotype 1b (56.8% and 45.5% respectively), followed by genotype 2 (24.1% and 39.5%, respectively).

Until 2011, pegylated interferon alpha in combination with ribavirin was the standard treatment regimen. With this regimen, the treatment dose, duration and the success rates varied significantly between genotypes, and patients were at risk of severe side effects (Manns et al., 2006). In 2011, the first direct-acting antiviral (DAA) was approved and multiple second generation DAAs have been approved since that time. These newer therapeutic agents represent a major advancement in the treatment of HCV and demonstrate sustained virologic responses (SVR) exceeding 95% for most genotypes. Recently, several pan-genotypic DAAs have been approved for HCV treatment in the USA and Europe (Carrion and Martin, 2018; Heo and Deeks, 2018; Cory et al., 2018). However, due to high drug costs and insurance restrictions, the use of HCV genotyping will remain important for treatment selection.

All current guidelines for testing management and treatment of HCV infection (e.g. the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases/ Infectious Diseases Society of America (AASLD/ IDSA), and the World Health Organization (WHO) recommend genotyping of HCV (including subtyping 1a and 1b) prior to treatment (Association E., 2016; AASLD/ IDSA HCV Guidance Panel, 2015; World Health Organization, 2016).

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The IntelliPlex HCV Genotyping Kit (Plexbio Co., Ltd; Taipei, Taiwan) is a new commercial assay intended to detect and differentiate genotypes 1 to 6 and subtypes 1a and 1b of HCV. The assay utilizes reverse transcription-polymerase chain reaction (RT-PCR) to amplify the viral RNA and multiplex probe hybridization in combination with propriety πCode (pi-Code; acronym for "Precision Image Code") MicroDisc technology for the detection of the genotype and subtype. In the IntelliPlex HCV Genotyping Kit, HCV sequences in the viral 5′ UTR, the Core- and NS5B-coding region are analyzed in an efficient multiplex fashion for discrimination between genotypes and subtypes.

The goal of this study was to evaluate the clinical performance of the IntelliPlex HCV Genotyping Kit using clinical specimens infected with various genotypes and subtypes or samples without detectable HCV levels. The IntelliPlex HCV Genotyping Kit results were compared to the results obtained by Sanger sequencing as the reference method to determine the clinical sensitivity and clinical specificity (Parikh et al., n.d.).

2. Methods

2.1. Clinical and genotyping samples

For the purpose of this study, a total of 307 clinical specimens were included. This sample population consisted of 259 fresh specimens (collected at National Taiwan University Hospital (NTUH), Chang Gung Memorial Hospital-Linkou (CGMF-LK) and Kaohsiung Medical University Hospital (KMUH)), with a total of 105 specimens obtained from HCV negative subjects. To ensure sample coverage of all genotypes, 48 purchased specimens with known genotype (as certified by the commercial supplier in the accompanying Certificate of Analysis (CoA)), including genotypes 3, 4 and 5 were spiked into the sample pool (samples were obtained from SeraCare Life Sciences Inc., [Cambridge, MA United States]; Discovery Life Sciences, Inc. [Los Osos, CA United States] and Biomex GmbH [Heidelberg, Germany]).

All negative samples were determined negative by anti-HCV serological tests and no HCV RNA was detected by CE-IVD and Taiwan FDA-IVD-approved viral load kit. All HCV positive samples showed detectable HCV RNA higher than 3000 IU/mL (95% of samples with viral loads $>10^4$ IU/ml). The sample pool consisted of specimens from 259 Asian subjects (fresh samples), 1 purchased standard specimen from 1 Asian subject, and 3 purchased standard specimens from 3 Caucasian subjects. Information on ethnic origin for the remaining 44 purchased specimens was not available from the supplier. The sample pool consisted of 264 serum and 43 plasma samples. The plasma samples were all from the sample group of purchased samples.

The study was performed in accordance with International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines: Guideline for Good Clinical Practice (GCP), applicable local regulations, and World Medical Association (WMA) Declaration of Helsinki (International Conference on, n.d.; General Assembly of the World Medical Association, 2014). All patient information was anonymized and de-identified prior to the analysis by the IntelliPlex HCV Genotyping Kit and Sanger sequencing.

2.2. HCV viral load

The viral load was determined by a third-party clinical laboratory (YiYang Clinical Lab, Taipei, Taiwan) using a CE-IVD and TFDA-IVD (Taiwan FDA) approved viral load kit (COBAS® AmpliPrep/COBAS® TaqMan® HCV Test, v2.0, Analytical sensitivity: 15 IU/mL, Linear range: 15–1.7×10^6 IU/mL).

2.3. HCV RNA extraction

All HCV RNA was extracted using QIAamp MinElute Virus Spin Kit Manual (Qiagen, Cat No. 57704). Samples that were tested for genotype by Sanger sequencing were extracted following Qiagen's instruction. Samples that were tested for genotype using IntelliPlex HCV Genotyping Kit were extracted following PlexBio's instruction.

2.4. Sanger sequencing

Sequencing-based genotyping of all samples was performed at a CAP -certified and TFDA-approved independent third-party clinical laboratory (YiYang Clinical Lab, Taipei, Taiwan). Sequences in the Core coding region were analyzed to classify the genotype. For some isolates, sequences in the 5′ UTR were utilized.

2.5. IntelliPlex HCV genotyping kit

HCV genotyping with the IntelliPlex HCV Genotyping Kit was performed according to manufacturer's instruction. The technology combines established molecular methods (RT-PCR) with state-of-the-art multiplexing πCode (piCode) MicroDisc technology. The πCode MicroDiscs are circular discs (diameter of 50μm) with an imprinted image pattern on the surface. The coding capacity of the discs accommodates over 16,000 distinct image patterns which can be used individually to tag nucleic acid capture probes. This allows for high-throughput, high complexity multiplexing applications for molecular diagnostic tests such as required for HCV genotyping. For HCV genotyping, a total of 96 samples (including controls) can be processed in less than 6 hours (including RNA extraction). The IntelliPlex HCV Genotyping Kit has been designed to differentiate genotype 1 to 6 and subtype genotype 1a and 1b from non-1a/-1b genotype 1 samples. The number of unique piCode MicroDics identities used for assignment of each genotype differs across genotypes; with a minimum of two piCode identities targeting genotype 5 and up to 12 unique piCode identities targeting classification of genotype 1b. The assay requires a minimum of 200 μL specimen (serum or plasma) and has a reported limit of detection of 250 IU/mL.

Following RNA extraction, regions of the HCV genome (5′ UTR, Core- and NS5B-coding region) were amplified by RT-PCR using biotinylated primers. Detection of the HCV genotype and subtype was achieved by hybridization of HCV-derived amplicons to genotype or subtype specific probes coupled to unique πCode MicroDiscs. Hybridized amplicons were labelled fluorescently with streptavidin-phycoerythrin conjugate. The resulting labeled πCode MicroDiscs were interrogated by optical imaging using the PlexBio™ 100 fluorescence analyzer. The analyzer uses a CCD camera to read the distinct image patterns under bright field microscopy and then the instrument automatically switches to a fluorescent reading mode to quantify the median fluorescence intensity (MFI) associated with each πCode MicroDisc. A schematic overview of the assay process is depicted in Figure 1. For more details, please visit www.plexbio.com.

3. Results

3.1. Impact of sample matrix

The IntelliPlex HCV Genotyping Kit is intended to be used with serum or plasma specimens. Prior to the genotyping phase of the study, 63 specimens were collected as both serum and plasma samples. Of the 63 samples 30 were obtained from HCV negative subjects. This was done to confirm that sample matrix did not have an impact on results.

All 63 plasma and all 63 serum samples were analyzed with the IntelliPlex HCV Genotyping Kit and the results showed an agreement of 100%. All 30 samples from patients that tested negative for HCV using a CE-IVD and TFDA-IVD approved viral load kit also tested negative for the virus using the IntelliPlex HCV Genotyping Kit. All of the remaining 33 samples tested positive for HCV and the same genotype/subtype was determined for each serum/plasma pair derived from the same patient using the IntelliPlex HCV Genotyping Kit (Table 1). The

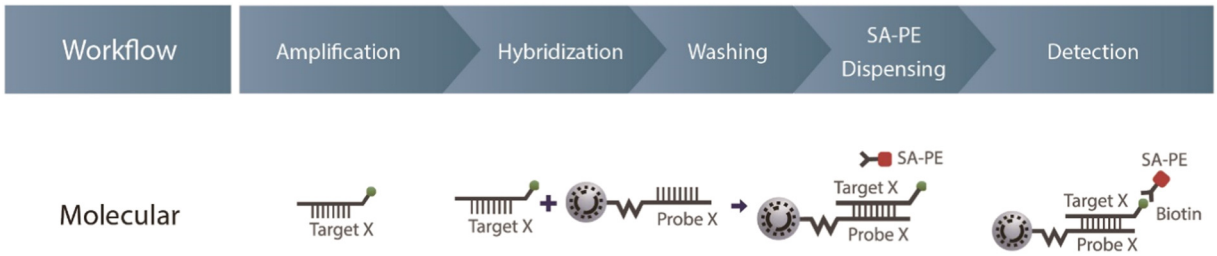

Figure 1. Workflow of IntelliPlex HCV Genotyping Kit. A schematic representation of the steps on the molecular level is shown.

results confirmed that the sample matrix of serum or plasma did not affect genotyping determinations made by the IntelliPlex HCV Genotyping Kit in this study.

3.2. Clinical Specificity

Clinical Specificity was evaluated by testing 105 specimens that showed negative anti-HCV serological results and tested negative for HCV RNA utilizing a CE-IVD and TFDA-IVD approved viral load kit (Parikh et al., n.d.). Sanger sequencing failed to determine the HCV genotypes for all 105 samples. The IntelliPlex HCV Genotyping Kit results were negative for all 105 samples. The clinical specificity obtained in this study was 100% (Table 2)

3.3. Clinical Sensitivity

Clinical Sensitivity was determined testing the remaining 202 samples (Parikh et al., n.d.). Both the Sanger sequencing method and the IntelliPlex HCV Genotyping Kit detected HCV in all 202 samples, resulting in a clinical sensitivity for HCV detection independent of the genotype of 100%. The overall clinical sensitivity of IntelliPlex HCV Genotyping Kit for correct detection of HCV genotype was 96.9% (Table 2; IntelliPlex HCV Genotyping Kit correctly identified the genotype for 192 out of 198 samples; four samples detected with dual-infections are excluded from clinical sensitivity as discussed below). The clinical sensitivity for each genotype and subtype is also given in Table 2.

The genotypes determined by Sanger sequencing are shown in column "Total Number" in Table 3. The IntelliPlex HCV Genotyping Kit classified 69 of the 73 genotype 1 specimens correctly (94.5%) with 7 of 7 genotype 1a (100%) samples and 62 of 66 genotype 1b (93.9%) samples being measured. For one genotype 1b sample, the IntelliPlex HCV Genotyping Kit detected HCV but was unable to classify any genotype. For two genotype 1b samples, the IntelliPlex HCV Genotyping Kit detected dual infections which included genotype 1b. The IntelliPlex HCV Genotyping Kit misclassified only one genotype 1b sample as genotype 1a.

All 54 genotype 2 samples were correctly identified by the IntelliPlex HCV Genotyping Kit (100%). Twenty three of 24 genotype 3 samples were detected (95.8%) while one sample was classified as genotype 3 sample with a genotype 1b co-infection. All 7 genotype 5 were correctly identified (100%). The agreement for detection of genotype 6 was 94.1% (16 of 17). One genotype 6 sample was falsely classified as genotype 1 by the IntelliPlex HCV Genotyping Kit. Sanger sequencing detected 4 dual infection (all genotype 1a plus genotype 1b) and the IntelliPlex HCV Genotyping Kit confirmed 2 of the 4 results while classified the other 2 dual-infection as genotype 1 only (Table 3).

4. Discussion

Phylogenetic analysis of the complete HCV genome or certain validated regions is considered the gold standard for HCV genotyping (Smith et al., 2014; Firdaus et al., 2015; Murphy et al., 2007). However, this method is expensive and time-consuming, requires highly trained personnel for execution and does not allow for high-throughput sample screening. To address this laboratory need, several approved assays have been developed and commercialized. The IntelliPlex HCV Genotyping Kit (Plexbio Co., Ltd.), a newly-available commercial assay, is intended to detect and differentiate genotypes 1 to 6 and subtypes 1a and 1b of HCV. The goal of this study was to evaluate the clinical performance and to determine the clinical sensitivity and specificity of the IntelliPlex HCV Genotyping Kit in comparison to the established method of Sanger sequencing.

The 105 samples that tested negative for both HCV serological markers and by approved viral load kits also tested negative for HCV by the IntelliPlex HCV Genotyping Kit, resulting in a clinical specificity of 100%.

The IntelliPlex HCV Genotyping Kit showed a clinical sensitivity for HCV genotyping of 96.9% in this study. The overall performance is therefore comparable to the published results of other commercial HCV genotyping assays. Published results for the Cobas® HCV GT (Roche), RealTime HCV Genotype II assay (Abbott) and VERSANT® HCV Genotype 2.0 Assay (Siemens) all reported agreement of genotype detected by the respective assay in comparison to sequencing as the reference method between approximately 90% and 99%, depending on the study

Table 1
Comparison chart of IntelliPlex HCV Genotyping Kit results utilizing either plasma or serum derived from 63 specimens. (NT = Not Tested)

Genotype	Total Number of samples	IntelliPlexTM Results (Serum)	IntelliPlexTM Results (Plasma)	Percent correlation Serum/Plasma
G1	21	21	21	100%
G1a	1	1	1	100%
G1b	20	20	20	100%
G2	6	6	6	100%
G3	2	2	2	100%
G4	NT	NT	NT	-
G5	NT	NT	NT	-
G6	3	3	3	100%
G1b+G6	1	1	1	100%
Negative	30	30	30	100%
Total	63	63	63	100%

Table 2
Clinical Specificity and Sensitivity for the IntelliPlex HCV Genotyping Kit. Clinical sensitivities to detect HCV (independent of genotype) and to detect each genotype/subtype are shown.

Clinical Specificity (%)	
100	
Clinical Sensitivity (%)	
ALL	96.9
HCV G1	94.5
HCV G1a	100.0
HCV G1b	93.9
HCV G2	100.0
HCV G3	95.8
HCV G4	100.0
HCV G5	100.0
HCV G6	94.1

Table 3
Overview of IntelliPlex HCV Genotyping Kit results.

Genotype	Total number (based on reference/sequencings[a]	Total number of eligible results[b]	Number of intelliplex results in agreement with sequencing	Number of intelliplex results with wrong results	Number of intelliplex results with no results[c]	Percent correctly identified (accuracy)
1 (all)	73	73	69	3*	1	94.5
1a	7	7	7	0	0	100
1b	66	66	62	3*	1	93.93
2	54	54	54	0	0	100
3	24	24	23	1**	0	95.8
4	23	23	23	0	0	100
5	7	7	7	0	0	100
6	17	17	16	1	0	94.1
All genotypes	198	198	192	5	1	96.9
Dual	4	4	2	2***	0	50
Negative	105	105	105	0	0	100

[a] Total Number" reflects all samples genotyped by Sanger sequencing.
[b] Total Number of Eligible Results" excludes non-eligible IntelliPlex results (any result with failed controls).
[c] Results with "Unrecognized Pattern" or "No HCV detected" are considered "No Result".
* IntelliPlex detected samples as dual infection including genotype 1b.
** IntelliPlex detected sample as dual infection including genotype 3.
*** For two samples, IntelliPlex only detected genotype 1 instead of genotype 1a + 1b dual infection.

and sample pool tested (Chueca et al., 2016; Liu et al., 2015; Benedet et al., 2014; Mokhtari et al., 2016; Manee et al., 2017; Fernández-Caballero et al., 2017; Némoz et al., 2018; Nieto-Aponte et al., 2017; Ceccherini Silberstein et al., 2016).

Of the 202 samples testing positive for HCV, the IntelliPlex HCV Genotyping Kit showed 8 results to be discordant with the Sanger sequencing reference method. The most common difference observed was with the detection of a co-infection by the IntelliPlex HCV Genotyping Kit in samples that were classified as single infection by Sanger sequencing. Two genotype 1b specimens were incorrectly classified as dual-infections by the IntelliPlex HCV Genotyping Kit, one as genotype 1a and 1b and one as genotype 1b and 6 co-infection. One genotype 3 sample was reported as genotype 3 and genotype 1b co-infection. All three results detected the correct genotype determined by Sanger sequencing, but reported a second subtype/genotype in the sample which was not confirmed by Sanger sequencing. Sanger sequence methodology is known to detect subpopulations only if they are present in the sample at or greater than 20% (Mallory et al., 2017; Davidson et al., 2012). It cannot be ruled out that the subpopulations observed using the IntelliPlex HCV Genotyping Kit are below the detection threshold of Sanger sequencing and therefore missed by Sanger sequencing. Similar discrepancies between detection of dual- versus single-infection by Sanger sequencing have been reported for Abbott RealTime HCV Genotype II assay and VERSANT® HCV Genotype 2.0 Assay (Liu et al., 2015; Mallory et al., 2017; Minosse et al., 2016; Yang et al., 2014).

For one genotype 1b specimen, the IntelliPlex HCV Genotyping Kit confirmed the sample as positive for HCV but was unable to determine a genotype. In this case, the result reported was “Unrecognized Pattern”. This can be caused when the viral sequences differ from the published reference sequences (Smith et al., 2014). Similar issues have been reported for each of the other non-sequencing based commercial HCV genotyping assays (Benedet et al., 2014; Yang et al., 2014; González et al., 2013; Ciotti et al., 2010; Shinol et al., 2012; Verbeeck et al., 2008; Noppornpanth et al., 2006). The IntelliPlex HCV Genotyping Kit also classified one sample that was classified as genotype 1b based on Sequences in the Core coding region as genotype 1a.

Of the 17 genotype 6 specimens included in this study, only 1 sample was incorrectly reported as genotype 1 by the IntelliPlex HCV Genotyping Kit. Sanger sequencing utilized the Core coding region for genotyping and the results showed that the isolate misclassified as genotype 1 by the IntelliPlex HCV Genotyping Kit did not represent a genotype 6a or 6b sequence. Given the diversity of subtypes in the genotype 6 group and the similarity between genotype 1 and 6 in the 5′UTR and Core-coding region, it is not unexpected that probe-based HCV genotyping assays may classify genotype 6 specimens, and in particular, non-6a or non-6b subtypes, as genotype 1 and this problem is well documented (Liu et al., 2015; Manee et al., 2017; Mallory et al., 2017; Yang et al., 2014; Cai et al., 2013). One published report concluded that the Cobas® HCV GT assay is only able to detect genotype 6a and 6b and will miss all other subtypes (Yusrina et al., 2018). Several other reports showed that the VERSANT® HCV Genotype 2.0 Assay and RealTime HCV Genotype II assay also show discrepancies in classifying genotype 6 samples and have an overall agreement rate as low as 50% (Yang et al., 2014; Cai et al., 2013; Yusrina et al., 2018). The inability to detect genotype 6 correctly potentially impacts the usability of those assays in Southeast Asia, where genotype 6 infections are highly prevalent among the population. It should be noted that all 17 specimens with genotype 6 in this study represent HCV isolates naturally circulating in Taiwan. IntelliPlex HCV Genotyping Kit’s clinical sensitivity to detect genotype 6 is 94.1%.

Additional studies are needed to further address the performance of the IntelliPlex HCV Genotyping Kit with genotype 1a and genotype 5 samples as well as dual infected specimens. Side-by-side comparisons of the IntelliPlex HCV Genotyping Kit with IVD-approved devices such as the VERSANT® HCV Genotype 2.0 Assay or the RealTime HCV Genotype II assay are needed for further assay evaluation.

5. Conclusion

The IntelliPlex HCV Genotyping Kit is highly accurate and demonstrates overall good performance with genotype 1 to 6 and subtyping of genotype 1a and 1b. The assay allows for a rapid and sensitive HCV genotyping of up to 96 samples (controls included) in less than 6 hours with limited hands-on time. The operation of the IntelliPlex system is amenable for clinical laboratory workflow. The IntelliPlex HCV Genotyping Kit is suitable for use as a routine tool to genotype HCV.

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Competing interests

Yao-Yun Cheng, Jui-Yu Hu, Wen-Kai Liang, Peter Friebe, Stuart Palmer, and Chin-Shiou Huang are employed by PlexBio Co. Ltd.

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