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REVIEW PAPER OPEN ACCESS

A review on advancement towards hepatitis c virus genotyping and novel assays approach for determination of HCV Genotypes

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Abstract

HCV is an epidemic pathogen worldwide and thousands of people die each year by infectious effects of HCV which is ultimately effect liver and damage liver functioning by liver cirrhosis. Currently, vaccine for HCV is not developed due to its 6 major genotypes and more than 67 subtypes. Therefore, for each viral genotype treatment is different. HCV is globally distributed in the world. Thus, each viral genotypes and subtypes are widespread in the particular areas of world. In Pakistan, the most prevalent subtype of HCV is 3a. There is a great challenge for identification of type of viral genotype in each patient for proper treatment of viral infection. Therefore, different types of assays are used for determination of type of viral infection. Nucleotide sequencing method for genotype and subtype determination is an appropriate method for diagnosis which require sequencing of whole genome of HCV, but this method is more expensive and time consuming. Versant assay or Line Probe assay (LiPA), Sentosa SQ HCV genotyping assay, HCV genotyping 9G test and Cobas HCV genotyping are appropriate and less time-consuming method of screening of HCV genotyping and can be suitable and can meet whole requirements of identification of type of viral infection.

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Introduction

Hepatitis C is an infectious disease that effects about 185 million people each year and can cause the death of more than 35,000 people each year (Lee *et al.*, 2014). Vaccine for hepatitis C is currently unavailable and liver cirrhosis may also be developed due to infection caused by hepatitis C virus in many people. Hepatitis is epidemic pathogen worldwide and hepatitis C virus is the main cause of liver diseases and liver damage. By the world-wide survey, it is stated, that about 71 million people are infected with hepatitis C virus and 400,000 people die each year due to hepatocellular carcinoma and liver damage. Hepatitis C is the main cause of liver diseases like hepatocellular carcinoma and liver cirrhosis and cause liver failure (Lavanchy, 2011). Hepatitis C virus has different genotypes and treatment for each genotype is different. Different combination therapies are used for different genotypes. These different genotypes are present in different ratios in different areas of world. Most commonly present genotype is HCV 1 (46.2%), HCV 3 (30.1%) HCV 4 (8.3%), HCV 2 (9,1%), 5.4 percent of hepatitis C virus and 0.8 percent of hepatitis C virus 5 (Messina *et al.*, 2015). Hepatitis C virus 6 is major reason of liver infection in South China, Cambodia Vietnam, Myanmar, and Laos, (Yamada *et al.*, 2015). Hepatitis C virus also the reason of virtually twenty percent of infections in Thailand (Wasitthankasem *et al.*, 2015).

HCV is a transportable agent causing major damage to the liver. It was extracted from the serum of patient without hepatitis-A and hepatitis -B by Choo *et al.* in 1989. Afterward brief examination it is stated that, the major cause of 90% of hepatitis C in US is a newly discovered virus. Chronic and fatal liver disease is caused by hepatitis C virus and it leads to liver cancer. Universally, it is the core basis of death and illnesswith main world health problems in 180 million people in all the world and 10 million people suffering in Pakistan. More than 3–4 million peoples are caused by hepatitis C virus every year. The 25% infected with hepatocellular carcinoma 27% people are infected with HCV and have liver cirrhosis (Cooke *et al.*, 2013).HCV infection is a common cause of chronic liver disease that can lead to end-stage liver damage or liver cancer, which includehepatocellular carcinoma. Some patients also suffer from various disease that can be cured (Naeem *et al.*, 2019).HCV in patients with severe infection (15– 25%) can be treated by using effective drug therapies.

Among those patients having therapeutic cure the total number of diseased people, 50–85% develops chronic infection after 6 months of determined infection (Hua *et al.*, 2018). If individuals with chronic infections or long-lasting infection are not cure at time, 10–20% of the patients will produce cirrhosis after 20 years of HCV infection, and 1–3% will develop cancer in liver 30 years after viral infection (Marcellin & Boyer, 2003).

Hepatitis C virus strains are subdivided into 7 subclasses genotypes (1-7) on the base of sequence analyses and phylogenetic analysis of complete viral genomes study. HCV infection which are belong to different genotypes vary at 30-35% of nucleotide sites in the genome of HCV. Each genotype of HCV is further sub-divided into 67 subtypes confirmed and 20 temporary subtypes. Strains which are belonging to the same genotype are different at less than 15% of nucleotide sites of HCV genome (Smith *et al.*, 2014). The main cause of variation in genotypes is due to the contoinuous mutation in the genome of HCV. 11 genotypes and about 70 subtypes have been discovered till that time. People of various genotypes varies by 30 percent at nucleotide sequence with others. Whereas, nucleotide sequences of different genotypes are different about 30%. Whereas, the genotypes from 7 to 11 are controversial because genotype 10 nucleotide sequence is more relevant to genotype 3 and we can say that the genotype 10 is the subtype of genotype 3. Same as with genotypes 7,8,9 and 11 which are phylogenetically relevant to genotype 6 and we can say that genotype 7,8,9 and 11 are the subclasses of genotype 6. Even every genotype suffers subtle genomic differences through infection. The total gathering of mutation in nucleotide sequence is not extreme which is sufficient to alteration in genotype.

Therefore, research revealed that hepatitis C virus diseased people remains suffered with the similar genotype over time (Weck, 2005).Various disease that can be cured by using herbal products(Usman *et al.*, 2019). Although, screening of genotype and subtype of HCV is a great challenge now a days for proper treatment of HCV infection, many screening methods are used. Like nucleotide sequencing method for genotype and subtype determination is perfect method for screening which need sequencing of infected HCV genome. As HCV infection spread throughout the world and by seeing the financial and economic conditions of our country it is not possible to use such expensive screening method for genotype determination of millions of patients infected by viral infection. The aim of this study is to put light on other assays which are quick and less expensive method and can be affordable method for HCV infected persons. In this literature, we study the comparison of HCV genotyping test performed by the Nucleotide sequencing method and Versant or line probe assay with Cobas genotyping method (Fernandez *et al.*, 2017).

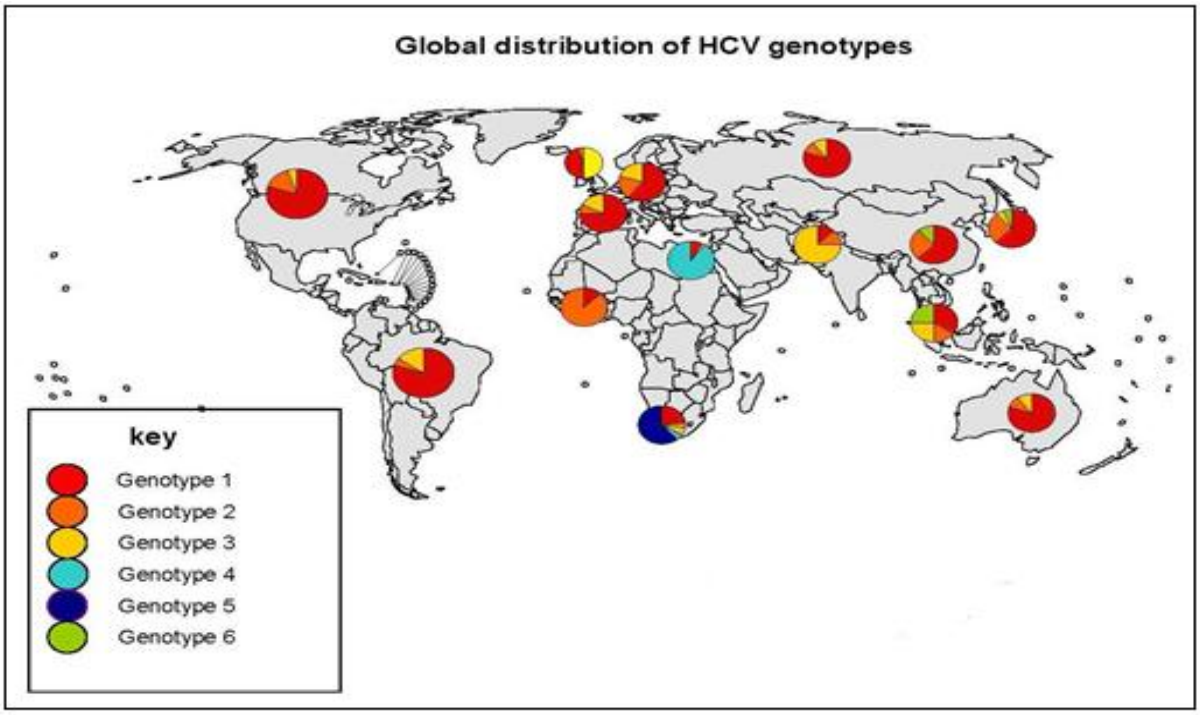

Fig. 1. Most prevalent genotypes in different areas of world.

Hepatitis C Virus

Global distribution of HCV genotypes

Most commonly present HCV genotypes throughout the world is genotype 1, genotype 2 and genotype 3 while genotype 4, genotype 5 and genotype 6 exist in firm parts of world. HCV is present very high frequency in European countries and there present 25 million positive cases of HCV infection. In Pakistan frequency of infection of HCV is more in Khyber Pakhtunkhwa province and the major cause of liver cancer (Khan *et al.*, 2014). HCV-4 is widespread among Africa and Middle East producing about 80% of HCV infections and has newly spread to many European countries. Egypt has the maximum occurrence of HCV globally (15%) and the highest occurrence of HCV-4 cause for about 90% of contagions and a main cause of long-lasting chronic hepatitis, hepatocellular carcinoma, liver cirrhosis, and liver transplantation in the country. Though HCV-4 is the basis of more than 20% of the 170 million cases of hepatitis C throughout the world. Hepatitis C virus infection is the major reason of liver transplantation in the United States and is a hazard issue for liver cancer in US, the majority of cases of HCV infection lower from top point of 0.2 million to 17,000 in 2007. More than 85% of patients of HCV

failed to cure from hepatitis by the use of different therapies and become chronic to infection which later on can cause the major cause of liver cancer or liver damage. Hepatitis C virus infection is the main reason of liver transplantation in United States due to liver infection caused by HCV (Nouroz *et al*., 2015).

Structure of HCV genome

Genome of virus which cause the hepatitis C consist of one strand, RNA with a diameter 50 nm. Virus related to the family of Flaviviridae and genus hepaciviral. Its genome contains 5' untranslated region (5' UTR) and 3' untranslated region (3' UTR) and these are the flanking regions of single open reading frame, which encodes three structural proteins including two glycoproteins of envelop and one core protein. 7 non-structural proteins which also encodes by genome of hepatitis C genome which includes P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. These are non-structural proteins that encodes by viral genome (Li *et al*., 2015). Various disease that can be cured by using neutraceuticals (Usman *et al*., 2019).

Hepatitis C virus genome is single stranded RNA which is the only viral genome that can cause the chronic infection in addition to retrovirus. Mutation is continuously accruing in the genome of HCV so on the basis of variation in the genome of HCV it can be divided into & subtypes and more than fifty subtypes (a, b, c…). Hepatitis C genotypes 1,2 and 3 are widely distributed throughout the world.

Genotypes 4 and 5 are found primarily in Middle East and Africa. Genotype 6 and its subtypes are found largely in Southeast Asia, whereas genotype 7 is only present in Congo, Africa (Murphy *et al*., 2007). Amongst structures of proteins, main protein (21 kDa) contains of 191 amino acids sequences, that are major parts of nucleocapsid of virus envelop. The main function of core protein is to modulate cell proliferation, gene transcription, cell death, metabolism may be lead to the oxidative stress and the major function is finally the hepatocellular carcinoma and liver steatosis. HCV genome has two envelop protein that are commonly glycosylated and provid a main part in cell entrance.

The protein of viral genome P7 played important role ion channeling and viral assembly after transcription of viral RNA. NS5B provided a vital role in vital viral genome replication. The majorette of anti-viral drugs is NS5B, NS3 and structural proteins (De *et al*., 2003).

The viral genome is about 9.5 Kb there is a specific region which encodes a single precursor polyprotein of about 3010 amino acid sequences which is separated into smaller fragments of 4 structural core, E1, E2, and P7, and 6 non-structural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Khan *et al*, 2014).Liver also suffer from toxic metabolites (Muhammad Naeem,, *et al* 2019). Core proteins forms the capsid of viral envelop which is surrounded by lipid bilayer contains glycoproteins E1 and E2.

Critical HCV infection is rarely analyzed as the majority of highly tainted people are asymptomatic. In the transfusion setting, severe HCV infection are known to be 70– 80% asymptomatic and 20– 30% of adults displayed clinical manifestations. Chronic hepatitis C is set separately by the constancy of HCV-RNA in the blood for no more than a half year after start of severe infection. Hepatitis C virus is a self-constrained in just 15– 25% of patients, whom HCV-RNA in the serum ends up imperceptible and ALT levels came back to ordinary.

Roughly 75– 85% of tainted patients do not clear the infection by a half year and chronic hepatitis created in them. The rate of chronic HCV contamination is influenced by numerous components, including the age at time of disease, sexual orientation, ethnicity and advancement of jaundice amid acute disease (Chen and Morgan, 2006).

Route of transmission of HCV

HCV can pass by intravenous drug utilize (IVDU), birth to an infected mother, during organ transplantation, use of contaminated and unsterilized

syringes, use of contaminated instruments for nose and ear penetrating or for tattoos (Table 1.4) and that's why Pakistan is suffering from HCV. In creating nations, sexual contact with more than one and blood contacts, inflammation beverage cause HCV transmission. (Maheshwari & Thuluvath, 2010).Hepatitis is the major cause of liver cirrhosis (Ghani *et al.*, 2019).

Indications can show up whenever from 2 weeks to a half year after a man is tainted with the infection. Side effects incorporate jaundice, weariness, dim shaded stool, joint agony, stomach torment, shortcoming, anorexia, bothersome skin and dim pee. Gentle psychological issues and weakness are the significant side effects of constant hepatitis C.

Table 1. Different route of transmission of hepatitis C virus identified different studies.

Route of transmission	Percentage (%)
IVDU	60
Tattoo	46.3
Ear piercing	30.1
Nail trimming	24.3
Sexual transmission	15
Shaving	14.5
Blood transfusion	10
Surgical treatment	7.6
Needle stick	2

An investigation led in Egypt demonstrated the manifestations of HCV as well as history of weariness, looseness of the bowels and stomach torments. Another examination achieved on 77 Spanish and Italian youngsters uncovered that Hepatitis C virus was for the most part asymptomatic among them (Jara *et al.*, 2003).

Prevalence of HCV genotypes

HCV prevalence varies in different locations of the world and between various communities. Genotype 1a is most common in the Northern Europe and United States, while 1b is the most widely known genotype around the world. In Japan and Europe, genotype 2b and 2a are mostly predominant, while 2c subtype is mutual in Northern Italy.

In Middle East and Africa, the most commonly present genotype is 4. Genotype 5 and 6 are most regular in Asia and South Africa (Simmonds, 2004). HCV Genotype 3a is found in Pakistan in contrast with 1a and 3b.In the nucleotide arrangement of genome, the six primary genotypes are around 30–35% not quite the same as each other, while roughly 20– 25% subtypes are not the same as each other (Simmonds, 2004). Genotype 1 is more typical on the planet counting 83.4 million cases (42.6% of aggregate HCV cases), of which around 1/3 are available in East Asia. Afterward genotype 1, genotype 3 is more typical overall including 54.3 million cases (30.1% of HCV cases).

G-2, G-4 and G-6 are in charge of an aggregate 22.8%of entire cases, while G-5 incorporates the rest of the rate (>1%). In many nations G-1 and G-3 are predominant in contrast with different genotypes while G-4 and G-5 are dominant in less created nations (Messina *et al.*, 2015). Hospital based survey of different Pakistani urban areas showed that the pervasiveness rate of HCV is 2.45% in Rawalpindi, 5% in Faisalabad, 5.31% in Islamabad, 20.89% in Mardan, 4.0% in Multan, 25.7% in Northern zones and 4– 6% in Buner.

A comparative report led in Bangladesh showed that G-3 was the most dominant genotype in contrast with different genotypes (Islam *et al.*, 2015).

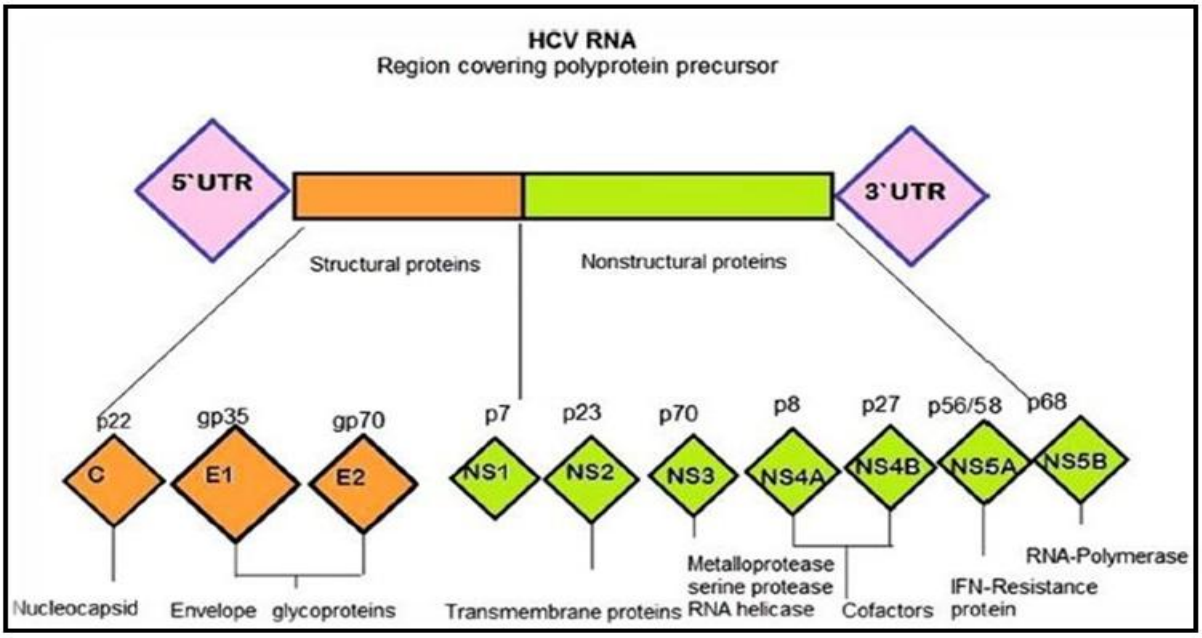

Fig. 2. Proteins encodes by HCV. All the structural & N-S proteins are given.

Identification of hepatitis C genotypes is most important for the proper treatment of disease. Each genotype is genotypically different from other genotype and efficacy of any antiviral drug depends upon the genome of virus. Different drugs are used for the treatment of HCV (Smith *et al.*, 2014). For each genotype there will be the different drugs are used for different interval of time. Mostly combination therapy is used for the treatment of HCV. Each combination therapy is specific for the treatment of specific genotype. So, identification of genotype of hepatitis viral infection most necessary for the effective treatment of viral infection and know about which type of viral infection is.

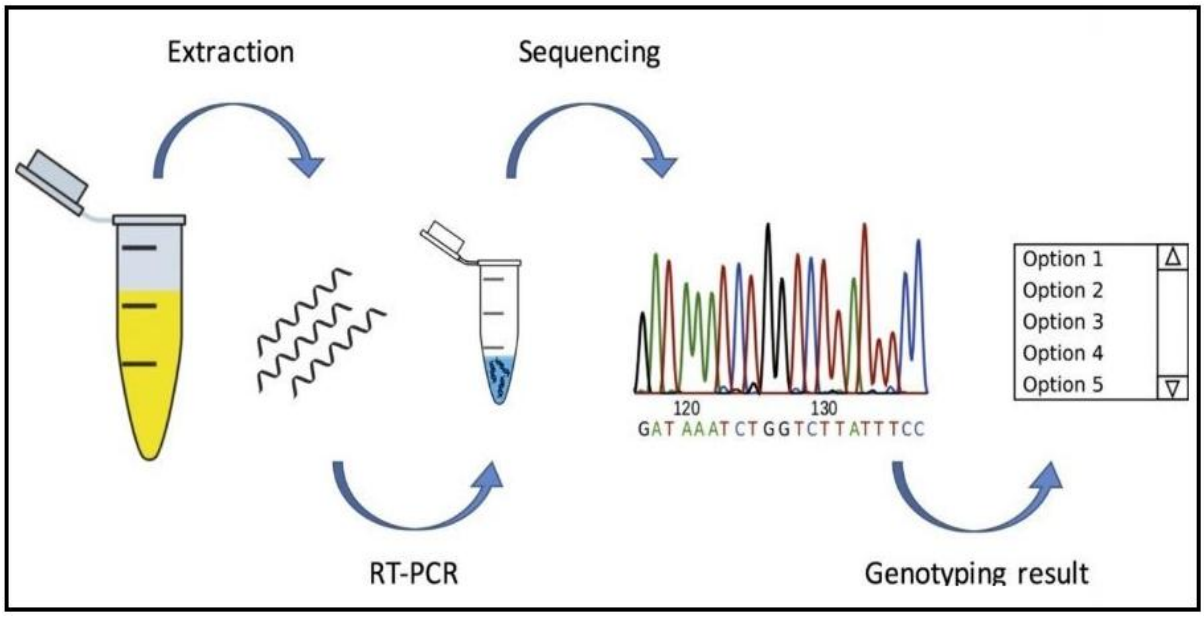

Fig. 3. This figure shows the methodology of nucleotide sequencing method.

It is also important for the development vaccines against the viral infection and necessary for the improvement of diagnostic tests (Nelson *et al.*, 2011).

Genotyping tests

Nucleotide sequencing is the most appropriate and most precise process of genotyping of hepatitis C

virus genome. It requires the sequencing of genome of HCV by sanger sequencing method. But it is more expensive, time consuming and need highly experienced professional for test to check the genotyping test. For the implementation of genotyping test in laboratories we need such test which are not time consuming, cheap and give accurate results of HCV genotyping. Because genotyping test are required for the effective treatment. Mostly combination therapy is used for treatment of HCV. We need simple, rapid and precise tests for genotyping (Firdaus *et al.*, 2015).

Fig. 4. Versant kit PCR Molecular system.

9G test and line probe assay are tests for nucleic acid tests and rapid and cost-effective tests for genotyping test (Cai *et al.*, 2013). The most common and important assay for HCV genotyping are 9G test, Versant HCV 2.0 assay (LiPA 2.0), and Cobas assay.

The LiPA 2.0 assay needs the use of explanation charts to get the outcomes. Trugene assay is accounted for to be work concentrated (Schutzbank *et al.*, 2006). For Hepatitis C virus genotyping Abbott Realtime HCV Genotype II assay use 4 characteristic groundwork sets 3 distinct reactions. Correlation of Trugene assay and Versant HCV 2.0 with arrangement investigation shows that these tests can neglect to separate HCV subtypes 1a and 1b, such outcomes would prompt basic critical errors in the right utilization of DAAs (Chueca *et al.*, 2016).

The Versant HCV 2.0 assay and the Abbott Realtime HCV Genotype II assay have constraints in distinguishing HCV genotype 6 (Yang *et al.*, 2014). The restricted accuracy of these three commercial assays is ascribed to the low single-nucleotide polymorphism (SNP) segregation proportion because of the high succession sequence similarity among the HCV genotypes.

Nucleotide sequencing of HCV genome

Genome of HCV contains untranslated region (UTR) the sequencing of this untranslated region gives more information which are sufficient for clinical purposes. Determination of subtype of genotype is not more necessary. NS5B region gives the information about the subtypes. We can determine the subtype by sequencing the NS5B region (Nakano *et al.*, 2012).

Sequencing of NS5B region is important for the genotype and subtype determination and it is an accurate method of genotype determination (Golemba *et al.*, 2013).

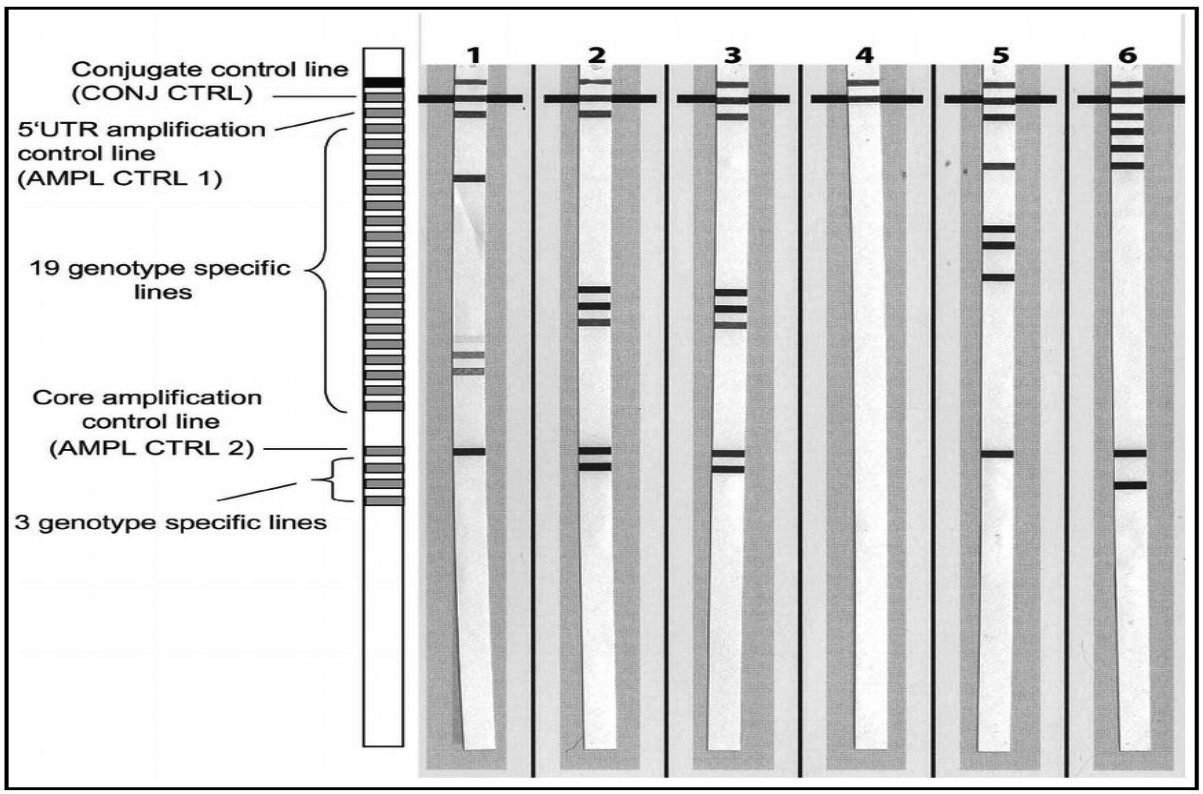

Fig. 5. Schematic representation of Versant HCV genotype assay (LiPA) 2.0 strip design and six strips that were processed according to the manufacturer's instruction.

Now, sequencing of the NS5B gene region of viral genome to examine HCV subtype and genotype is considered the more accurate method. However, mostly used small fragments of NS5B region to determine the genotype of HCV genome (Nakatani *et al.*, 2011). By using very short fragments does not give accurate results by using fragments of NS5B region they have certain drawbacks such as limited number of subtypes can be identify and low accuracy of results. By using nucleotide sequencing method, it is helpful to predict primer located in NS5B region by using the software PRIMER 5.0 and with the use of these potential primers we can amplify the other regions located on NS5B region which can be helpful in genotyping of HCV.

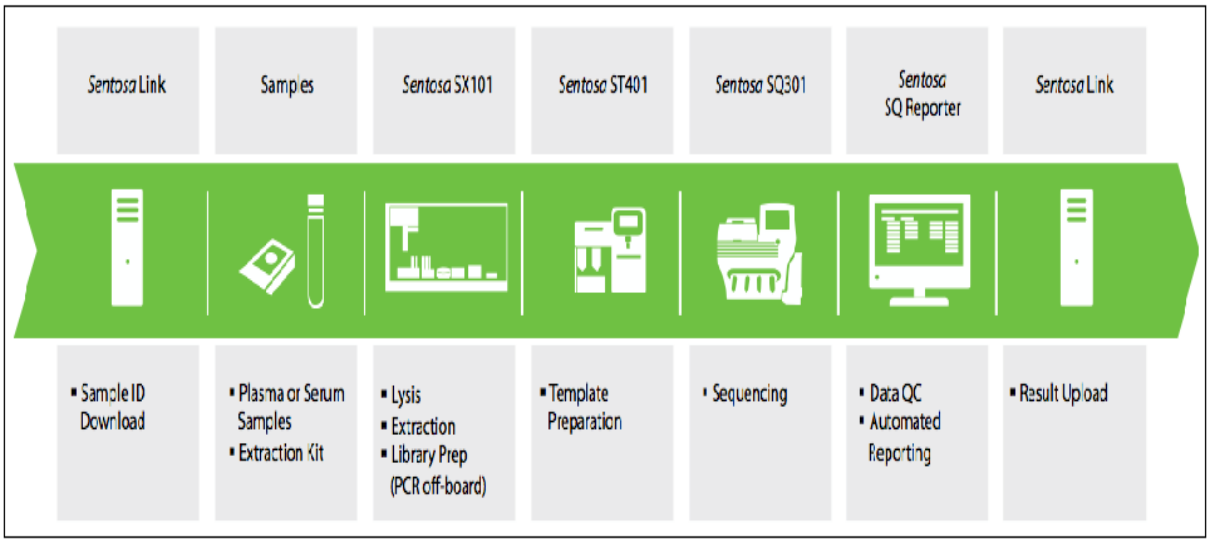

Fig. 6. Sequence wise methodology of Sentosa SQ assay.

All primers present on the NS5b are located by sequencing the nucleic acid by PRIMER 5.0. These identified primers can accurately increase the sequences of Hepatitis C virus strain that can effectively identify the genotype if standard HCV strain. These results show that this method of sequencing the genome region of HCV can correctly identify the genotypes and subtypes of HCV patients' sample (Fakhr *et al*., 2013).

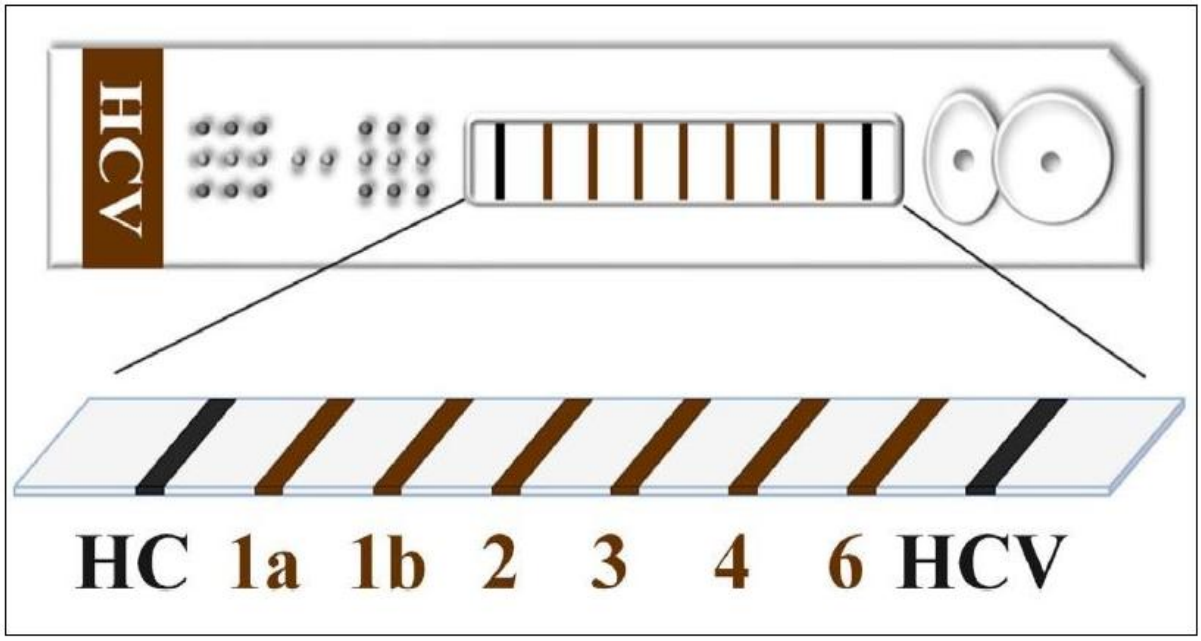

Fig. 7. Respective probes position corresponding to the HCV genotypes 1a, ab, 2, 3, 4 and 6.

Plasma preparations

Blood samples of 3-5 mL is from each HCV infected patients are taken before getting antiviral treatment taken before genotyping tests. Blood samples are taken in a tube with EDTA which act as anticoagulant. The patients comprised of patients with chronic hepatitis C, acute hepatitis C, hepatitis C cirrhosis and HCV acute infection. All the samples were centrifuged in centrifuge machine for five minutes at 3000 rpm. Supernatants from the sample is collected and stored at 70 degrees centigrade (Tong *et al*., 2015).

HCV RNA extraction

Mini spin kit is now being in use for the separation of Viral RNA from plasma by following the instruction made by Qiagen, Hilden, Germany. Plasma volume is adjusted on the basis of viral load for example, for HCV RNA _5000 IU/mL 140 µL volume is used and for HCV RNA <5000 IU/mL 280 µL volume is used, and then mixed with volume 40–120 µL of protease K and 560–1120 µL volume of AVL buffer added 5.6–11.2 µg of carrier transfer RNA. The mixture then incubates for ten min at normal room temperature, and then 560–1120 µL volume of ethanol was supplemented to each sample tube and stirrer by pulse vertexing for fifteen seconds. The sample mixture the put into a mini column and then centrifuge in centrifuge machine at 6000 rpm. Later, the mini column then washes away with 500 µL volume of AW1 buffer and 500 µL volume of AW2 buffer, separately and the centrifuge at 6000 rpm for one min. secondly wash with 500 µL volume of AW2 buffer was taking place in centrifuge machine at 20 000 rpm for short time of tree min, and then column dried with full speed of centrifuge machine for 1 min.

The RNA was taken by putting of Buffer AVE of volume 50 µL onto every column, then incubate for a short time of 1 min at normal room temperature and then centrifuge for 6000 rpm for one minute. The elution is repeat another time to increase the yield of final product. In conclusion, a total of 50 µL volume of HCV viral RNA was separated from 140–280 µL volume of plasma (Tong *et al*., 2015).

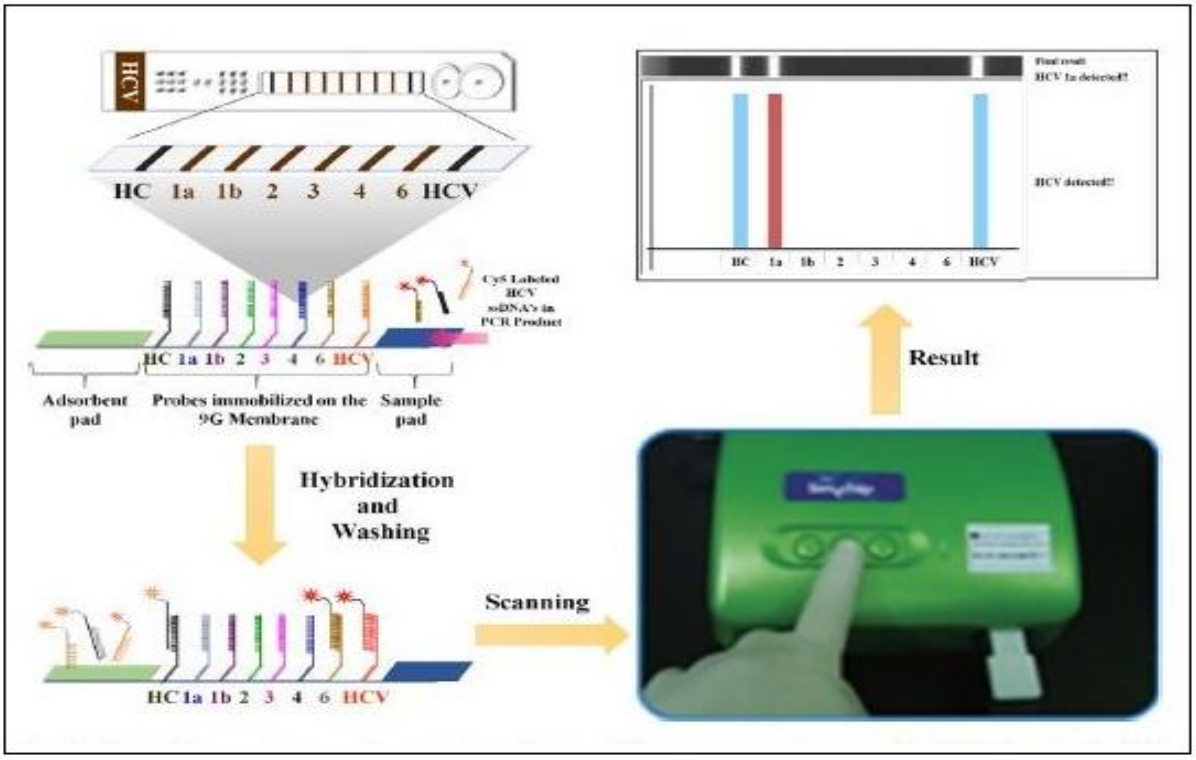

Fig. 8. Position of respective probes on 9G membrane and experimental protocol of 6 genotyping 9G test.

cDNA synthesis

Thermo scientific Revert Aid First strand complementary DNA produce complementary DNA from HCV RNA by following the instruction of manufacturer. HCV RNA is extracted from viral strain and dilute on the basis of viral load for example diluted 1:2 or 1;4 on the base of viral load. For HCV RNA <5000 IU/mL ratio will be 1:2. For HCV RNA _5000 IU/mL the diluted ratio will be 1:4 and mix with 20 µL of total RT reaction which is composed of random hexamer primer of 1 µL. The reverse transcription reaction is taking place in thermo cycler at specific condition stated below: give the specific temperature to sample mixture 5 minutes 25°C, for 60 minutes 42°C, for 5 minutes 70°C. By giving this specific temperature for specific time step wise reverse transcription is taking place (Tong *et al.*,2015).

The HCV NS5B gene fragments Amplification byPolymerase Chain Reactionthe viral complementary DNA template is need for the PCR amplification. The PCR performed following step-wise reactions: for 3 minutes at 94°C, for 30 seconds at 94°C, for 40 seconds at 56°C, for 60 seconds at 72°C, repeat these reactions for 35 cycles; for 10 min at 72°C. The primers are annealed with cDNA at the same temperature of annealing step as far as possible. The 56–60°C is the average or normal temperature Tm for all types of selected primers. Annealing is performed between the between the annealing temperature of 54°C to 62°C but the best annealing temperature for annealing step is 56°C that temperature can effectively amplify all the viral genome with only slightly decrease of productivity rate for R4, F3 and F1.

Then PCR product are analyzed in 1.2% agarose gel and electrophoresed for purification of PCR products and recognition by imaging. The primers for amplifying the NS5B region of gene fragment is is the conserved region of 3' non-coding region which is used as control.

The conserved region is annealed with forward primer and further amplified. Forward primer is given as 5'-GCGGAACCGGTGAGTACA-3' and reverse primer is given as 5'-CCTATCAGGCAGTACCACAAGG-3'. For DNA sequencing and purification product from agarose gel

is taken for sequencing. Sequence examination was achieved with the ABITM3130 Genetic tool (Tong *et al.*, 2015).

Phylogenetic analysis

After the sequencing of NS5B region by sequencing method, sequences are the analyzed by using international database provided by different database organizations. These analyses sequences are compared with database sequences of Hepatitis C viral genome sequences already present on database by comparing the NS5B region sequence we can determine the genotypes and subtypes of HCV genome. Different analyzing an aligning tool are present, like CLUSTRAL W 2.0 software MEGA software and other tools are available. Nucleotide sequences are aligned by using CLUSTRL W 2.0 software with already reported present HCV sequences in database which is provided by National Laboratory of Los Alamos. For analyzing pairwise evolutionary history of NS5B region sequences is analyzed by using the software of MEGA version 5.2 devised and organized by a university of USA Pennsylvania State University, University Park, PA, in 2012. Sequence distance matrices were analyzed with conservative statistical software SYSTAT, v13.0; Systat Software, Inc. USA using files resulting from MEGA software (Simmonds *et al.*, 2004).

Versant Assay or Line Probe Assay (LiPA) 2.0

Versant assay is widely use assay for determination of genotypes by using the information of core region of HCV genome and to determine the genotypes and subtypes of HCV genome. It is accurate and rapid method of HCV genotyping. it is not time consuming and it is useful for using at commercial level in contrast with nucleotide sequencing method because of it is time consuming and expensive method of genotyping. But versant assay is not more expensive and easily used for commercial level. Versant assay use core region for its information and detect the genotypes of HCV from 1 to 6 and can easily distinct between a and b subtypes of six genotypes of HCV. The principle of versant assay depending on reverse hybridization with specific probes and primers directing both core region and 5' UTR region of HCV genome (Verbeeck *et al.*, 2008).

Extraction and amplification

In vitro diagnosis use QIAamp DSP virus kit is used for RNA extraction from HCV stains taken from sample of HCV positive patients. Infection of HCV may be Acute or Chronic.QIAamp DSP virus kit in combination with QIAvac 24 Plus vacuum system used to extract HCV RNA from strain according to manufacturer's organization instructions Qiagen GmbH, Hilden, Germany. Versant HCV control 2.0 kit is used for negative and positive control kit which is placed between the extracted samples. The kit which is used for amplification of proper fragments of HCV genome of Core region and 5'UTR is the Versant HCV amplification 2.0 kit**.**

Methodology Versant HCV genotype assay (LiPA) 2.0

In versant genotype assay or line probe assay biotinylated product that is obtained from PCR amplification is reverse hybridize with the restrained nitrocellulose strains that are specific the core region and 5' UTR of genotypes of HCV. The probes that are bound to the nitrocellulose strips by poly T tail. After washing the strip, visible pattern on the strip are shown because of the reaction of 5-bromo-4-chloro-3-iodolyl phosphate (BCIP) with hybridized conjugates on lignocellulose that show a purple/brown precipitate on the stip. These patterns are specific for each genotype.

Nitrocellulose strip has 22 parallel DNA probe line and three control line. Probe on which target RNA sequence of HCV genome are hybridized are immobilized on nitrocellulose strips which are specific for each type of genotype.. Limits of determination of genotype is described in user manual are 2,106 IU/ml to _7.7 _ 106 IU/ml (Verbeeck *et al.*, 2008).

The main principle of versant HCV genotype 2.0 assay or line probe assay is reverse hybridization of biotinylated HCV genome PCR product with the DNA

probes that are immobilize on nitrocellulose strips by Poly T tail. These probes are specific for each type of genotypes from 1 to 6.

Amplified PCR product is then biotinylated and these biotinylated PCR products then hybridized with probes on nitrocellulose strips. Streptavidin an alkaline phosphatase labeled is bound with biotinylated hybrids. These patterns then compared by using interpretation charts to determine genotypes and subtypes (Cai *et al.*, 2013; Manee *et al.*, 2017).

Sentosa SQ HCV genotyping assay

Hepatis is caused by the infection of HCV and HCV has more than one genotypes. It is the possibility that more than one types of HCV infections can be present like different genotypes may be present in the same person. More than one genotypes and subtypes are commonly found in high risk group. These mixed infections mostly present in the person homosexuals and such persons that inject drugs (Qiu *et al.*, 2015).

All the assays for the determination of HCV infection are specific for specific type of genotype and subtypes. The methods having low sensitivity does not determine the minority strains and easily identify the majority strains because minority strains are more different from majority strains.

Determination of all genotypes and subtypes in mixed viral infection is essential for the effective treatment of infection (Cunningham *et al.*, 2015).

Many of the assays are used for the determination of HCV genotypes and subtype commercially diagnostics for rapid detection (Verbeeck *et al.*, 2008). These two regions of viral genome do not cover all the genotypes and subtypes. Only those subtypes that are related to these two regions can be identified by Versant assay but this method is still mostly used for genotyping because of its simplicity and its rapidity (Cabezas-Fernandez & Cabeza-Barrera, 2012).

Determination of HCV genotype is of major importance because of effective treatment of viral infection. This also provide guide line to physician to for effective treatment of HCV infection. Sanger sequencing and then determination of genotype is still the most effective method of identification.

A newly developed assay is for the second-generation sequencing method for the determination of HCV genome which is very sensitive perfect for daily routine diagnostic of genotyping that can perform 15 samples for per run. This is totally automated which is known as the name of Sentosa SQ HCV genotyping. This assay can able to detect RAVs as well as HCV genotyping. This assay targets many NS5B, NS3 and NS5A of viral genome and identify the subtypes and genotypes of HCV and additionally it determines the RAVs (Gryadunov *et al.*, 2010).

Genotyping samples

Samples are collected randomly from the HCV patients without patient background history. These samples are collected in the separate tubes and put EDTA solution to avoid blood clotting and preserved at the temperature of -80°C.. The reason is that we have to check the genotypes and subtypes that previously cannot be identified by another assay that also cover the mixed viral genotypes infection. As Sentosa SQ genotyping have ability to determine the minority strains of HCV viral infection and also the mixed genotyping assay (Manee *et al.*, 2017).

HCV RNA extraction

Different assays use different method of HCV RNA extraction. NucliSENS easy MAG is used by LiPA from 200 µL of blood samples according to manufacturer's command. Automatic method of RNA extraction is done by MPS in which 530 µL from 15 routine clinical blood samples. Biosystems® 3500 Series Genetic Analyzer is used by Sanger method that extract viral RNA from 400 µL of blood sample (Manee *et al.*, 2017).

Target region for massively parallel sequencing

This assay target on three region of HCV viral genome which are NS5B, NS5A and NS3 that targets at 684-bp, 604-bp and 944-bp respectively. These regions

then amplified and produce DNA PCR product. For NS5B region more than 30 primers are used for Sentosa assay to cover >98 percent of hepatitis C Viral strains (Manee *et al*., 2017).

Sentosa SQ HCV methodology

Sentosa is the sequencing method of HCV genotypes determination. It uses three major regions of HCV genome core region NS5B, NS5A and NS3 regions for sequencing to determine which type of viral infection is. Sentosa is the next generation automated method for genotypes determination the plate on which RT PCR reaction is taking place is taken from the Sentosa apparatus and this plate is then sealed with pierceable foil by using plate sealer which is named as PX1 PCR plate sealer by giving the temperature of 170 °C for just three minutes.

Veriti Dx 96 Well Thermal Cycler is used for off-board reverse transcription PCR amplification for 4 hours and 20 minutes. Then for library preparation reaction plate is then transferred back to the Sentosa SX101. The process is then started in Sentosa SX101 that produce 200 base pair base-nucleotide fragments of a library.

At a time Sentosa assay have ability to comprise 15 sample of HCV genome library. After viral RNA extraction and off board RT PCR amplification Sentosa ST401 automated instrument is used continuously used for the preparation of template. Then Sentosa SQ301 is used for the sequencing of HCV genome. SQ 301 is used for the sequencing of Viral genome by using the sequencing kit in Sentosa SQ 301 automated machine that is measured by hydrogen ion these hydrogen ions released when nucleotide sequences are bind with the sequencing kit and these hydrogen ions then converted into electrical signals that can be detected on Sentosa SQ reporter. These sequences aligned in the BLAST tool with available data on database. By aligning sequence data genotypes and subtypes are determined. Mixed infection is analyzed by making phylogenetic tree which accurately determine the genotypes and subtype (Manee *et al*., 2017).

HCV genotyping 9G test

This test for the determination of HCV genotypes and subtypes is known as the name of HCV genotyping 9G test that determine genotypes and subtypes that comprise genotype including 1a and 1b genotype 2,3,4 and genotype 6 which include subtypes (6a, 6b, 6i and 6n) form clinical blood samples in just 30 minutes. This is the easier and most comparable test for the determination of most abundantly present all over the world. It can be used for commercial determination because it is not more time consuming it gives results in short interval of time.

In 9G test viral RNA molecules are first extracted from infection blood samples then converted RNA molecule into cDNA molecule then PCR amplification is done in PCR then ssDNA or cDNA is attached with 9 consecutive Guanine and then immobilized with membrane of glass fibers at specific sites. Then immobilized probes cDNA molecules are then hybridized with Cy5 labeled complementary single stranded DNA residues. Hybridization of complementary DNA a probe is occur and then then analyzed by different techniques. The probes and cDNA show high SNP ration that results in the determination of HCV genotypes and subtypes. 9G test gives the physician with important information about the six genotypes of HCV viral infection which is complementary for the proper treatment of viral infection. And it gives the results in just 30 minutes.

Sampling for 9G test

More blood samples are taken from the patients of HCV infection which having previously history of HCV infection. Blood samples are taken from the patients with average age. Samples are taken randomly from different area of world because some specific genotypes of HCV infection are present more depends on locality but other genotypes are subtypes are not abundantly present on such areas.

Some blood sample are also taken from different areas with having not precious history of HCV infection. For example, we take 152 blood samples with 110 are HCV suspected and 42 are those samples

with healthy person with previous history of HCV infection all the samples are taken in the EDTA tube to prevent blood clotting and then the preserved at -80 degree centigrade.

HCV RNA extraction

HCV viral genome is comprised of positive sense RNA molecule which is extracted from NucliSENS easyMAG kit which is the automated method of HCV RNA extraction. 200 µL of plasma from each sample is taken then added with lysis buffer for the lysis of whole genome and to separate HCV RNA from the samples.

Silica partials are removed from the solution with washing buffers. Then HCV RNA residues are separated from the samples and put in the 50 µL of tris elution buffer. This whole method of HCV RNA extraction is used for all of the samples (152 samples) (Chantratita *et al.*, 2017).

Methodology of 9G test

For the detection of HCV viral infection of which genotype HCV 9G test is most commercially used test. It is rapid easy method of HCV genotype detection HCV 9G test include isolation of Viral RNA. Synthesis of cDNA, amplification of cDNA with PCR and PCR amplicons detection for the determination of 6 HCV genotypes and its subtypes (Nimse *et al.*, 2011).

9G test for the determination of six genotypes is performed by addition of 110 µL of hybridization solution which contains 0.1% of triton X-100, 25 percent of formamide and 6x SSC into the 20 µL on PCR product. Then we load 110 µL of this solution in the sample port of 9G kit for 20 minutes at the temperature of 25 °C.

After 20 minute we put washing solution of 200 µL in the washing port for just 8 minutes. Then we put 9g test strips and then scanned on the BMT reader for scanning. This scanner analyses the 9G strip and give the results automatically. Scanner interpret the strips and show results. Results depends on the intensity of the signal for respective probes.

The HC test fills in as an internal standard for hybridization control. The HCV test is used to perceive the closeness or nonappearance of HCV in the case. Right when 6 HCV Genotyping 9G test shows three signs contrasting with tests including the HC, HCV, and 1a or 2 or 3 or 4, an illustration is allotted as an HCV 1a or 2 or 3 or 4, independently. The case is recognized as HCV 1b if the test shows four signs for tests including HC, HCV, 1a, and 1b, separately. Exactly when the test exhibits three signs identifying with the tests HC, HCV, and 6, the illustration is genotyped as HCV 6a or 6f(Chantratita *et al.*, 2017).

The rule of 6 HCV Genotyping 9G test joins viral RNA isolation, complementary DNA (cDNA) mix, PCR amplification, and area of PCR amplicons. The foundations in the 6 HCV Genotyping 9G test increment the 5_UTR for genotyping of six HCV genotypes. The probes allow the division of HCV genotypes1a, 1b, 2, 3, 4, 6a or 6f, and 6i or 6n at 25◦C in less than 30 min after PCR. As showed up in Fig. 1, the HC (hybridization control) probe, HCV (probe specific for the distinguishing proof of HCV) probe, and six other probes specific to the HCV genotypes 1a, 1b, 2, 3, 4, and 6, were immobilized on the glass fiber movies to convey the 9G membranes according to the starting late uncovered system (Song *et al.*, 2013).

All probes were picked by the summed-up probe selection method (Nimse *et al.*, 2011). Fig. 1 moreover depicts the fundamental experimental tradition of 6 HCV Genotyping 9G test to obtain last results. When the test exhibits three signs identifying with the probes, HCV, and 6, the case is genotyped as HCV 6a or 6f. Whereas, if the test demonstrates four signs contrasting with the probes HC, HCV,1a, and 6, the case is genotyped as HCV 6i or 6n. Exactly when, 6 HCV genotyping 9G test shows two signs contrasting with the HC and HCV probes, the case is recognized to contain the HCV genotype other than the HCV 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n.

In any case, for an HCV-negative case, the test exhibits only a solitary banner corresponding to the

HC probe. The method of 6 HCV Genotyping 9G test after satisfaction of cDNA association taken after by PCR upgrade is rapidly discussed here. 110 _L of hybridization course of action was added to each PCR tube containing 20 _L PCR thing. By then, 110 _L of this mix was loaded into the illustration port of the 6 HCV Genotyping 9G test and allowed to stay for 20 min at 25◦C. After 20 min, 200 _L of washing solution was stacked into the washing port of the 6 HCV Genotyping 9G test and allowed to stay for 8 min at 25◦C.

Comparison of 9G test with sequencing method

The evaluation of 6 HCV Genotyping 9G test for HCV genotyping and subtyping was composed on 152 patients' serum tests and the outcomes were separated and course of action examination. All representations were endeavored under blinded codes by the two frameworks until the satisfaction of tests. The similar inevitable results of 6 HCV Genotyping 9G test with the reference demonstrated 99.1% affectability and 99.7% specificity. The deferred results of the 6 HCV Genotyping 9G test with clinical cases undeniably exhibit that this test can exactly see and confine the HCV genotypes as HCV 1a, 1b, 2, 3, 4, HCV (6a or 6f), and HCV (6i or 6n). The most correct framework for genotyping is sequencing of HCV genome.

In this way, a speedy, correct, easy to utilize and even minded genotyping technique is more helpful. In any case, among Asian nations neighboring China, the dissipating and subtype relationship of HCV6 is essentially not the same as by and large proportion. For instance, HCV6a (24%) and HCV6n (39%) are inescapable in Vietnam and Myanmar, freely (Pham *et al.*, 2011). In Thai patients HCV6f is the most extensively seen HCV6 subtype (56%), trailed by subtypes 6n 22%), and 6i (11%) (Akkarathamrongsin *et al.*, 2010).

Cobas HCV genotyping

For the determination of HCV genotypes and subclasses hepatitis C virus genome sequencing is measured as main objective for identification. But being the more time consuming and not profitable process for HCV determination and it requires highly experienced professionals for sequencing and analyze the genotypes.

In short, this method cannot be applied for commercial use so we use other methods of HCV determination that are not expensive and time consuming. Versant HCV 2.0 assay or line probe assay is reverse hybridization method that is approved by United States Food and Drug Administration for detection of HCV genotypes from 1-6 and its subtypes. However, many studies on LIPA shows that certainly it gives incorrect results that may cause the decrease in efficacy of drugs in treatment and total treatment cost-effectiveness (Polilli *et al.*, 2016).

Clinical samples

All 97 blood samples taken from 97 HCV infected between March and July 2016 were included in this study. Samples collected at the National University Hospital NUH in Singapore from daily clinical tests by the Versant assay, according to the manufacturer's directions. Most of the samples received for this testing are from different counties (Yusrina *et al.*, 2018).

Viral extraction

Qiagen EZ1 Virus Mini Kit v2.0 (Qiagen, CA) is used for extraction of viral genome RNA from 400 µL of serum on the instruction of manufacturer's commands. The extracted RNA gnome is then eluted via 60 µL of elution buffer and test is done by using the LiPA and sequencing assays.

Methodology of Cobas HCV genotyping

Cobas 4800 system is completely automated procedure in which all the procedure is processed automatically from blood sample preparations, viral RNA extraction, PCR mixture preparation to real-time PCR amplification (RT PCR amplification) in this process core region, 5' UTR and nonstructural region of NS (NS5B) regions are used for identification of six genotypes from 1-6 and its subtypes specially 1a and 1b subtypes of viral genome.

The protocol is made according to the producer's procedure. The essential blood sample input volume of sample is 1 mL, though, the only used volume of sample used for test is 0.4 Ml.

This test for genotyping identification is use for the Cobas 4800 system. After automated sample processing, every sample is the amplified by RT PCR by using three reactions by using genotypes and subtypes primers which are specific to each genotype and subtype, and then oligonucleotide probes are labelled with fluorescent dye.

These probes are labelled with four different types of dyes, which allows the frequently detection of HCV genotypes and subtypes. Up to three subtype or genotypes are detected in each reaction. Cobas assay is CE labeled but not approved by FDA. It is an invitro diagnostic
test (Stelzl *et al.*, 2017).

Cobas versus LiPA 2.0

The Cobas assay effectively genotyped 73 % whch are 71 samples out of the 97 blood samples but unsuccessful to determine the genotype 25 (26%; which is known as indeterminate herein) samples with limited PCR amplification results. The remaining one sample (1%) not amplify. In contrast, the LiPA assayefficiently determine the genotypes 79% which are 77 samples and determine 7 (7%) HCV blood samples with genotype 1 because of unavailable core region of HCV genome result and unsuccessful to determine 13 genotypes which are 13% of overall samples know as indeterminate herein genotype. Overall, mutually assays had concordant results for 62 samples which are 64% samples.

The sample with a unsuccessful result by the Cobas assay is not repeat due to unavailable volume of sample. Particularly, in one of the blood samples, the Cobas assay determine it as a varied mixed infection of subtype 1a and genotype 3, but the LiPA assay determined it as an infection of subtype 1a not the infection of genotype 3. All the 33% samples with conflicting results are then checked with Sanger sequencing method (Yusrina *et al.*, 2018).

Sequencing method versus Cobas

Of the 32 conflicting and unspecified samples examined with the Cobas test, 7 (22%) had genotyping outcomes, and the 25 remains (78%) are unspecified. The outcome of 7 blood samples are cnflicting with the genotyping tests by core and nonstructural NS (NS5B) regions of viral genome. Upon performance the NS5B and core sequencing, 72% of the 25 unclassified samples are classified as subtype 6m 6xa, 6v or 6n. 16% blood samples comprised of 3 genotypes 6 but with unidentified subtype (i.e. N160622a, N160701c and N160329a) and one probable recombinant virus from subtype 1b and genotype 6 (N160322a).

The Cobas test for genotyping exhibited a 95% of confidence interval CI from 94% to 100% n=71, exclusive of blood samples with unclassified outcomes and accuracy of 100 percent. The LiPA assay presented a 95% Confidence interval CI from 92%-100% n=77, exclusive of blood samples with unclassified or genotype 1 with absent results correctness of 99%. There has no arithmetical variation in precision with different assays (Yusrina *et al.*, 2018).

Conclusion

It is necessarily important to determine HCV genotypes and subtypes before the prescription of physician with the direct acting antivirals (DAA), because treatment for each genotype and subtype is different and require different time interval. If genotype or subtype is determined by diagnostic test for genotyping then efficacy of drug taking increased by using the appropriate antiviral for infection. Different assay is used for the treatment of HCV infection. Most accurate diagnostic test for HCV genotyping is nucleotide sequencing by Sanger sequencing method. But it is more expensive and time-consuming method and it is not possible to use this for commercially for genotyping diagnostic test for hepatitis viral infection. Other tests or assay or devised for this purpose. These assays are less

accurate to nucleotide sequencing method but are more rapid and cost-effective method for genotyping.

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