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A detailed study on the probabilities of designing diagnostic molecular markers for Hepatitis C virus

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Abstract:

Hepatitis is the inflammation of liver caused by virus. It is occurring all over the world infecting a large population. Hepatitis B and hepatitis C are considered to be pandemic. Two billion people have been infected by Hepatitis B, 350 million have chronic infections and some 620,000 die each year from this disease. 170 million people are infected by Hepatitis C Virus, with 400,000 deaths each year, mainly from associated liver cancer and cirrhosis. There are five strains of hepatitis known namely A, B, C, D and E. out of the five strains, A, B and C are more common.

Introduction:

The word "hepatitis" means inflammation of the liver. This can be caused by a number of things, such as chemicals, alcohol, drugs and infection by viruses.

The symptoms of acute viral hepatitis include fever, headache, lethargy, nausea, dark urine, pale stools and jaundice. The most commonly encountered viral hepatitis are type A, type B and type C.

Hepatitis A

Hepatitis A is an infection of the liver caused by hepatitis A virus. The disease is generally mild, but severity tends to increase with age. Asymptomatic disease is common in children. Jaundice may occur in 70–80% of those infected as adults.

Fulminant hepatitis can occur but is rare. The overall case– fatality ratio is low but is greater in older patients and those with pre-existing liver disease. There is no chronic carrier state and chronic liver damage does not occur.

The virus is usually transmitted by the faecal–oral route through person-toperson spread or contaminated food or drink. Foodborne outbreaks have been reported following ingestion of certain shellfish (bivalve molluscs such as mussels, oysters and clams that feed by filtering large volumes of sewage-polluted waters) and salad vegetables. Transmission of hepatitis A has been associated with the use of factor VIII and factor IX concentrates where viral inactivation procedures did not destroy hepatitis A virus. The incubation period is usually around 28–30 days but may occasionally be as little as 15 or as much as 50 days.

There is no specific treatment for hepatitis A, however, rare complications such as extreme drowsiness, fluid retention, or blood abnormalities can be treated. The most important way to prevent hepatitis A is to use good personal hygiene, particularly careful hand washing and sanitary disposal of feces. An infected person may be restricted from work during the period of infectiousness (usually for one week after onset of jaundice). Persons who have been exposed to hepatitis A should be immunized with immune serum globulin (ISG). ISG works even when given as late as two weeks after a person has been exposed because the disease usually takes four weeks to appear.

Hepatitis A virus (HAV) is classified as Enterovirus type 72. It belongs to the genus Hepatovirus in the Picornavirus family (Yokosuka, 2000). The HAV genome comprises 7.5 kb single stranded RNA and is divided into three functional regions, P1, P2 and P3 (Rueckert et al., 1984). The P1 region encodes 4 capsid polypeptides (VP1, VP2, VP3 and

putative VP4). The P2 and P3 regions encode non-structural proteins necessary for various essential functions including viral replication. HAV is divided into at least 7 genotypes based on the VP1- P2A region (Robertson et al., 1991; Robertson et al., 1992; Taylor et al., 1997). However; genotype VII was recently reclassified as a sub-genotype of genotype II (Costa-Mattioli et al., 2002; Lu et al., 2004). HAV genotypes I, II, III and VII were isolated from humans, whereas genotypes IV, V and VI were isolated from several simian species. Most human HAV strains belong to genotypes I and III. HAV subtype IA is responsible for the majority of HAV infections worldwide including cases from Thailand (Theamboonlers et al., 2002; Poovorawan et al., 2005). HAV subtype IB is endemic in the Mediterranian region (Nainan et al., 2006; Pintó et al., 2007). Phylogenetic analysis of various human HAV genomes suggested an association between nucleotide sequence homology and geographic distribution of HAV. (Jansen et al.,1990; Robertson et al., 1991). Hence,characterization of HAV genotypes by partial genome sequencing can determine both source and evolution of the virus and may be used when comparing among future outbreaks of HAV. The incidence of HAV infection is higher in developing countries than in developed countries. Faecal-oral contamination transmits HAV, causing sporadic cases or epidemics of acute infectious hepatitis (Lemon et al., 1985; Cuthbert, 2001). In developing countries where HAV infection is endemic, most of the people get infected and become immune to HAV during childhood. Improvement of public sanitation and hygiene reduces the HAV infection rate in children and increases tThe number of adults susceptible to HAV infection

The Hepatitis A virus (HAV) is responsible for around half the cases of hepatitis diagnosed worldwide and is recognized currently as one of the most important human food-borne pathogens, as it is the cause of most outbreaks reported in the Western world. It is not possible to distinguish HAV strains by serotyping, but seven genotypes can be differentiated with molecular methods [1]. HAV infection is present in a worldwide distribution, although its endemicity varies significantly at both international and national levels [2]. Genotype I is the most prevalent genotype, comprising at least 80.0% of circulating human strains. The geographical origin of the genotypes correlates with the virus isolates. Subgenotype IA has been defined as the major HAV in the population in America. In Europe, a more heterogenous pattern is observed with co-circulation of genotypes IA and IB [3]. The detection of HAV is important for diagnosis and epidemiological studies of hepatitis A. Because of the slow and non-cytopathic replication of wild-type (wt) HAV strains, detection of HAV normally utilizes reverse transcription (RT) coupled to polymerase chain reaction (PCR) [4].

There are two products for immunisation against hepatitis A. An immunoglobulin provides rapid but temporary immunity. The vaccine confers active immunity but response is not immediate. Vaccines are available as either monovalent, or combined with either typhoid or hepatitis B.

HEPATITIS B

Hepatitis B (HBV) is a viral infection that causes swelling of the liver. The incubation period for hepatitis B ranges from 45-180 days, and onset is insidious. Clinical illness associated with acute infection is age-dependent with jaundice occurring in under 10% of children 5 years of age and under and in 30%-50% of older children and

adults. The case-fatality rate for reported acute cases in the United States is approximately 0.5%-1% although most result in complete recovery. Approximately 30%-90% of young children and 2%-10% of adults who are infected with HBV develop chronic infection and most of the serious sequele associated with HBV occur in these persons.

Symptoms of HBV include feelings of weakness and vague illness, loss of appetite, fever, and headaches. Less common symptoms include muscle pain, darkened urine, jaundice, nausea, abdominal discomfort, rash, depression, and irritability. Symptoms can begin as soon as six weeks or as long as six months after infection with HBV.

HBV is spread through infected blood (including dried blood), semen, saliva, and vaginal fluids. It can also be transmitted from mother to child during delivery. Sharing items such as toothbrushes and razors, (because of possible blood contact) with infected people Although HBV can be found in all body fluids, blood has the highest concentration and saliva the lowest. HBV in desiccated blood remains infective for at least one week and the antigen remains detectable for several years.

HBV consists of double-stranded DNA, enclosed within a nucleocapsid core (HBcAg) surrounded by an outer lipoprotein envelope into which the surface antigen (HBsAg) is embedded. A third antigen, HBeAg, is soluble and is released from liver cells with active HBV infection. The presence of HBeAg in the blood indicates a high degree of infectivity (ie, an actively Immunisation Handbook 2011 87 replicating virus). However, in some people a mutation in the HBV genome results in failure to produce HBeAg despite active replication of the virus. These people with a 'stop codon mutation' in the HBV genome may be highly infectious but HBeAg negative.

Measurement of HBV DNA levels is thus a more reliable measure of the concentration of HBV in the blood. The antigens are identified as indicated above, while their respective antibodies are designated anti-HBc, anti-HBs and anti-HBe.

The incubation period varies between six weeks and six months (average two to three months). The variation is related to the dose of virus in the inoculum, the mode of transmission and host factors. Blood from experimentally inoculated volunteers has been shown to be infectious many weeks before the onset of the first symptoms, and it remains infective through the acute clinical course of the disease and during the chronic carrier state in those who fail to eradicate infection.

The virus infects liver cells, multiplying there and releasing large amounts of HBsAg, which is always present in the blood of people with active infection. The virus itself is not cytopathic; it is the host's immune response that leads to death of the infected liver cell. Almost all people who acquire infection after early childhood will mount an effective immune response that leads to eradication of infection within a matter of months. Adults with acute infection may be asymptomatic (approximately 20 percent) or have symptomatic hepatitis with jaundice, anorexia, nausea and malaise (approximately 80 percent) with a small but significant risk of acute liver failure (1 percent), of whom almost half will die or undergo emergency liver transplantation.

In contrast, people who acquire infection at birth or in early childhood usually fail to mount an immune response that is adequate to eradicate infection.2 In these people, asymptomatic chronic infection stimulates persistent immune responses that may eventually lead to cirrhosis, which itself then increases

the risk of development of hepatocellular carcinoma. In the early years of chronic infection, high rates of viral replication are common, and both HBeAg and high levels of HBV DNA are present in the blood. In later years the rate of viral replication is lower, HBeAg may be absent from the blood, and HBV DNA levels are usually lower. It can be difficult to distinguish acute HBV from an acute exacerbation of

chronic HBV infection (so-called 'acute-on-chronic' hepatitis B). Such flares in chronic HBV infection reflect either spontaneous HBeAg seroconversion in HBeAg positive patients, or spontaneous flares in HBeAg negative patients (produced by mutations in the HBV, resulting in new targets for the host immune response). Therefore HBeAg may be positive or negative and HBV DNA may be high or low, or even undetectable at the time of onset of jaundice. The only way to distinguish acute HBV infection from acute-on88

Immunisation Handbook 2011 chronic hepatitis B is through previous documentation of HBV infection (ie, HBsAg positive more than six months earlier). Note that while the presence of anti-HBcore IgM is characteristic of acute infection, it may not be diagnostic because it can be positive in both situations. In New Zealand, most cases of acute hepatitis B in Caucasians reflect true adult-acquired infection, whilst most cases in Màori, Pacific Islander and Asians reflect acute-on-chronic hepatitis B.

HEPATITIS C

Hepatitis C is a liver disease caused by Hepatitis C virus (HCV). Transmission occurs from precutaneous exposure to contaminated blood or plasma derivatives. Possible methods of transmission include contaminated needles and syringes, occupational exposure and sexual exposure although the risk is considered low.

Transmission occurred through transfusions and transplants before screening of HCV. Its onset is insidious, with anorexia, vague abdominal discomfort, nausea and vomiting, progressing to jaundice less frequently than Hepatitis B. Diagnosis depends on demonstration of antibody to hepatitis C virus (anti-HCV weCommunicability is from 1 or more weeks before onset of first symptoms and may persist indefinitely. Sixty to seventy percent develop chronic hepatitis.

Hepatitis C is a complex liver disease. Its medical importance and the need to rapidly identify new therapeutic approaches has resulted in intensive study of its causative agent, hepatitis C virus (HCV). Humans are the only known natural hosts of HCV. Even after two decades since its discovery, HCV continues to bea major cause of concern and a huge burden on public health systems worldwide. The WHO estimates that a minimum of 3 per cent of the world's population is chronically infected with HCV1,2. HCV is a prototype member of the Hepacivirus genus and is further classified into at least seven major genotypes that differ by about 30 per cent in their nucleotide sequence. These genotypes (1, 2, 3, 4, 5, 6 & 7) show differences with regard to their worldwide distribution, transmission and disease progression3, 4. These genotypes have been further classified into sub-types (a, b, c, d, etc). In fact, HCV circulates in infected individuals as a population of diverse but closely related variants referred to as "quasispecies". HCV is most commonly spread by direct contact with infected blood and blood products. Availability of injectable therapies and drugs has had a remarkable influence on HCV epidemiology. The incubation period of HCV, though ranging up to several months, averages 6-8 wk. HCV infection is often asymptomatic, making it a very difficult to detect it at an early stage.

Review of litreture:

Hepatitis C infection has two phases. The first one, the acute phase, which last for 6 months from the time of exposure to the hepatitis C virus (HCV) till the onset of the symptoms. It is during the acute phase that the virus (HCV) finds its way to live and reproduce inside of liver cells. Quite often the acute phase is not recognize because most people have no early signs and symptoms. Some people, up to 20 percent, may experience flu-like symptoms; fever, chills, headache, fatigue, and muscle aches with nausea, vomiting, fatigue, poor appetite along with elevated liver enzymes (ALT) that may be associated with some degree of liver injury. Rarely, the acute hepatitis C results in severe or fatal liver condition called fulminant hepatitis. During the acute phase, first 6 months, only 15 percent of people are able to clear the hepatitis C virus (HCV).

Chronic hepatitis C

Approximately, 85 percent of people, whose immune system failed to clear the virus within 6 months, will have the virus for the rest of their lives, and will move to the second phase called chronic phase. During the chronic phase few people have symptoms. Some may complaint of bouts of fatigue, pain or discomfort on the right upper side of the abdomen, nausea, and join pain. The presence of symptoms does not always indicate the degree and severity of the infection to the liver. Progression Hepatitis C is a very slow progressive disease that affects people in many different ways. Its progression, manifestation, and outcome can greatly vary among people over a period of 20- 30 years. Here are possible outcome associated with the progression of Chronic hepatitis C:

25% of people have no symptoms or serious liver damage with normal levels of liver enzymes (ALT).

Usually liver biopsy shows some degree of chronic inflammation, but the degree of injury is usually mild, and the overall prognosis in these people is fairly good. 40-50% of people may have few or mild symptoms with mid to moderate elevated liver enzymes, but not enough to be severely detrimental to their health. In these people, progression of liver disease is difficult to predict. Up to 20 % of people will have severe hepatitis C symptoms, elevated liver enzymes, and who are at risk to develop cirrhosis (scar tissue between liver cells), which can lead to endstage liver disease. 1-5% of people with cirrhosis are at risk to develop liver cancer each year. Liver cancer (hepatocellular carcinoma) may develop on an average 20- 30 years later after being diagnosed with chronic hepatitis C. In the U.S., hepatitis C is the leading cause of liver transplant. Although it is difficult to predict how someone will progress with chronic hepatitis C, researchers have identified factors that may influence and accelerate the progression of the disease. These factors include; male gender, age at time of exposure to the HCV (greater than 40 years old), consumption of alcohol, and co- infection with hepatitis B or HIV.

replication and production of authentic infectious hepatitis C virus in cell culture remained a major obstacle for innovative and costeffective therapy. A breakthrough in the field came with the development of a complete in vitro cell culture system for HCV (JFH1) in 20057,8. JFH1 viral genome (genotype 2a), cloned from a Japanese patient with fulminant hepatitis could not only replicate efficiently in cell culture but could also generate viral particles. HCVcc (for HCV grown in cell culture) has allowed researchers for the first time to study the complete life cycle of HCV. Yet, owing to the limited host range of HCV, the

development of a small animal model (to study viral replication and pathogenesis) is still a big challenge in the field.HCV virions exhibit a wide range of densities,

although the most infectious fraction has a density of 1.15-1.17 g/ml8,9. Present inside the outer envelope, there is a (30-35 nm) inner core which encapsulates the single-strand viral RNA (positive-sense), which is approximately 9.6 kb (Fig. 2). The HCV genome does not enter the cell nucleus. HCV- RNA replication occurs in the cytoplasm of hepatocytes. The genomic organization of HCV is shown schematically in Fig. 3. The viral-RNA genome harbours a single ORF (open reading frame) which is flanked by 5' and 3' non translated RNA segments (NTRs). The cis-acting replication elements or CREs are located in both the 5' and 3' NTRs and in the NS5B coding sequence10. RNA in the multiplication of RNA viruses.

The 5'- and the 3'-NTRs of the genome are highly conserved and contain control elements for translation of the viral polyprotein and replication. The 5′ UTR (+)

is ~341 nucleotides in length and contains an internal ribosomal entry site (IRES). The HCV IRES is folded into four stem-loop motifs which are called as I, II, III and IV. The IRES is required for cap-independent translation of viral RNA, which is carried out by host cell ribosome. The domain IIId of the IRES constitutes the key anchoring site for the 40S subunit11. The IRES domains III-IV have also been shown to be an activator of protein kinase R (PKR)12. However, this activation does not interfere with cap-independent translation of HCV viral proteins. HCV core protein was reported to interact with the 5'-NTR of plus-strand RNA13.

However, recent work with JHF1 viral RNA suggested that its 5'-NTR (+)

does not contain RNA packaging signals14 and authors further speculate that it may reside in the RNA region encoding the replicase. The 3'-UTR (+) is around ~200nt and is involved in RNA replication. Three different domains can be recognized in this U TR: (i) a poly (U/UC) tract with an average length of 80 nucleotides (nt), (ii) a variable region, and (iii) a virtually invariant 98-nt X-tail region made up of 3 stem-loops (3'SLI, 3'SLII and 3'SLIII). The 3'-UTR can robustly stimulate IRESdependent translation in human hepatoma cell lines15. Recent studies have recognized that various stemloop structures exist in the negative strand 3'-NTR. This region is recognized by the viral polymerase as the initiation site for plus-strand synthesis of the HCV genome16. A recent study identified a cellular factor

called Far-upstream element (FUSE) binding protein (FBP) which binds to 3'NTR by interacting with the poly(U) tract17. The importance of long-range RNARNA

interactions in the modulation of HCV lifecycle has been well documented. Within the 3'-end of the non-structural protein 5B (NS5B) coding sequence, a cis-acting replication element (CRE) was discovered18. This CRE is called as SL9266 (or 5BSL3.2) and its disruption blocks RNA replication19. Mutual longrange binding with both 5' and 3' sequences is suggested to stabilize the CRE at the core of a complex pseudoknot10. Non coding RNA molecules or microRNAs (miR) are important in the control of gene expression and regulation. MicroRNA, miR-122 is specifically expressed and is found to be abundant in the human liver20. A recent discovery showed binding of a miRNA (miR-122) to the 5'-UTR of HCV. Sequestration of miR-122 in liver cell lines strongly reduced HCV translation, whereas its addition stimulated translation via direct interaction of miR-122 with two sites in the 5'-UTR21. These studies

have generated a lot of interest in the role of miR-122 in HCV multiplication and its potential as a therapeutic target. A role for proteasome alpha-subunit PSMA7 in regulating HCV IRESmediated translation has also been demonstrated22. These host factors require further scrutiny to be considered as candidates for drug targets. HCV Structural Proteins

HCV encodes a single polyprotein (NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) which is approximately 3010 amino acids (Fig. 3). The structural proteins (core, E1 and E2) and the p7 protein are released from the polyprotein after cleavage by host endoplasmic reticulum (ER) signal peptidase(s).

The non structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are cleaved by viral proteases NS2-3 and NS3-4A. This proteolytic processing of the polyprotein during and after translation by host and viral proteases yield at least 10 mature viral proteins. Core: HCV core is a multifunctional protein which is highly basic in nature23. It forms the structural component of the virus particle. Core has been implicated in the development of hepatocellular steatosis and oncogenesis. The core protein is generated from a polyprotein encoded by the viral genome and

is processed by cellular proteases in the endoplasmic reticulum (ER)24. A recent study showed that core protein can self-assemble in HCV-like particles (HCVLPs)

in ER-membranes25. A region of core protein (spanning amino acids 112 to 152) is essential for association, not only with the ER but also with the outer mitochondrial membrane26. The HCV core protein is known to pass from the ER into mitochondria and is involved with Ca2+ regulation and apoptotic signals27. The HCV core protein was shown to affect the steady state levels of a subset of mitochondrial proteins, including

prohibitin (functions as a chaperon of mitochondrial proteins)28. This interaction of core with the chaperon protein resulted in an increased oxidative stress due to perturbation of normal interactions

between cytochrome c oxidase and prohibitin. Core has many intriguing regulatory functions with one of the most important being recruitment of non structural proteins to the lipid droplet-associated membranes29. Lipid droplets (LDs) are intracellular organelles involved in lipid storage and also take part in intracellular vesicular trafficking30. HCV makes use of lipid droplets for replication. In infected Huh-7 cells, the core protein is associated with the surface of lipid droplets31. Besides its structural and regulatory function, core plays an imperative role in the pathogeneses of liver steatosis. U pregulation of de novo fatty acid biosynthesis by HCV core protein in Huh7 cells has been reported. Core protein also interacts with apolipoprotein AII, a component of lipid droplets. HCV core protein is targeted to lipid droplets by its domain 2 (D2) and this association with lipid droplets is required for virus production. Disrupting the association of core protein with lipid droplets is deleterious for HCVcc production and this interaction

is thought to contribute to steatosis, via deposition of triglycerides in the liver30. These data are further supported by studies showing that the expression of core protein can lead to the development of steatosis in transgenic mice28. Clinical studies have reported that virus-induced steatosis is very severe with HCV genotype 3 than with other genotypes32. Interestingly, core protein derived from genotype 3a induced higher fatty acid synthase activity than core protein derived from genotype 1b. However, no genetic or functional differences were observed between genotype 3a core proteins from patients with and without HCV-induced steatosis, thus, suggesting a

possible role of other viral proteins in the development of hepatocellular steatosis33. HCVsubgenomic replicon systems (which allow HCV-RNA to replicate autonomously), have shown subcellular localization of core to be both cytoplasmic and nuclear26,34. A study with chronically HCV-infected liver also revealed a similar distribution, with core localized to both cytoplasm and the nucleus35. In the cytoplasm the core protein is mostly localized to the endoplasmic reticulum (ER). HCV core protein, circulating 'free' in non enveloped state has also been detected in HCVinfected patients36. Transgenic mice expressing the core protein develop HCC, indicating a direct part played by the core protein in this process37. A study found two mutations in the core gene (36G/C and 209A), which were linked with increased HCC risk38. The core gene sequence data may provide useful information about HCC risk and more studies should be performed to develop it further. However, it is important to consider the type of genotype involved, as it is clear that gene expression profile in hepatocytes is dependent on the HCV-core-genotype sequence39. Tumour suppressor protein promyelocytic leukaemia (PML) is known to be involved in antiviral response. Interestingly, a recent study suggests a potential mechanism for the development of liver cancer via the HCV-core mediated inactivation of the PML tumour suppressor pathway40. encoded by the +1 reading frame of the viral genome which overlaps with the core protein coding sequence. These additional proteins of unknown function are also called as core+1 or F1 and were recently identified41. Since then multiple mechanisms have been proposed for the expression of ARFPs, which include (i) frame shifting (ii) form transcriptional slippage, or (iii) from internal initiation in the +1 open reading frame (ORF) of the core

protein coding sequence. ARFPs have been shown to be associated with the ER and mitochondria.

Interestingly, these proteins are labile and very short lived42, 43. Bases on the ability of ARFPs to bind the proteasome subunit alpha3, it was suggested that it may regulate protein degradation in cells44. However, the functional role of ARFPs in the HCV lifecycle is not clear yet. Envelope glycoproteins: The HCV envelope glycoproteins, E1 and E2 are structural components of the virion. They constitute the outer coat of fine spikelike projections of the HCV particle (Fig. 2)9. They undergo post-translational modifications (N-linked addition of carbohydrate chains) while being translated in the endoplasmic reticulum (ER). Both, E1 and E2 envelope glycoproteins are required for host-cell entry via receptor binding. Insight into the mechanisms by which HCV gains entry into host cells is vital to understand primary HCV infection and re-infection post-transplantation. The E2 protein has the binding sites for human CD81, a tetraspanin expressed on various cell types including hepatocytes and B lymphocytes45. The binding of E2 was further mapped to the major extracellular loop of CD81. CD81 along with human scavenger receptor SR-BI, and tight junction molecules claudin-1 (CLDN) and occludin (OCLN) are the most important receptors that mediate HCV entry46. In addition, it is thought that HCV may utilize glycosaminoglycans and low density receptors on hepatocytes as initial attachment factors. Both CD81 and SR-BI were identified as candidate HCV receptors based on their physical interaction with a soluble version of E247,48. The HCVpp system was subsequently used to prove that they are required for viral entry49. HCVpp system (also called as HCV pseudoparticle) generates virus particles, which display E1-E2 glycoproteins of HCV on their surface. Cell entry of HCVpp is HCV

glycoprotein mediated. HCVpp are generated by co-transfection of 3 plasmids into 293T cells: (i) gag-pol genes of HIV or MLV, (ii) HCVgp, and (iii) a retrovirus genome with LTRs, packaging signals and a reporter gene. HCVpp system does not require productive HCV replication and hence is not restricted to Huh7 or Huh7.5 that support robust replication50. HCVpp closely resembles the cell entry properties of genuine HCV virions and was used to identify CLDN and OCLN. The discovery of OCLN provides an vital advance towards efforts to develop small animal models for HCV46. HCV-like particles (HCV-LPs) were isolated from insect cells, infected with recombinant baculovirus expressing HCV structural proteins(Core, E1 and E2)51. Isolated HCV-LPs were composed of all the structural proteins (E1, E2 and C). Further analysis of HCVLPs by cryoelectron microscopy (CryoEM) revealed that they are spherical particles with smooth surfaces. They were found to be consistent with the native HCV virions isolated from HCV infected patients. HCV-LPs for the first time allowed 3D structural analysis of HCV particles51, which further allows studying the complex mechanisms of HCV assembly and maturation. A hypervariable domain near the amino terminus of E2 is the most variable part of the viral polyprotein and called as HVR-1. It has been shown to be the target for neutralizing antibodies. It is interesting to note that HCV can associate with LDL and VLDL from infected patient serum. Amazingly, HDLs play a very active role in HCV entry. A complex interplay between HDL, SRBI and HCV envelope glycoproteins leads to enhanced HDL-mediated HCVpp entry in cells48. In addition, HDL can inhibit HCV-neutralizing antibodies in serum of acute and chronic HCV-infected patients52, 53. E2 has also been shown to bind with CD81 receptors

which are expressed on thyroid cell and induce a cascade of
signaling pathway leading to IL-8 release. It is thought that E2 protein may induce thyroidal inflammation, thereby triggering thyroiditis by a bystander activation
mechanism6. Finally, E2 protein has been shown to bind PKR and as a consequence perturb innate immune pathways.

HCV Non Structural Proteins

The non structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B are thought to be to be required for replication of the viral genome. The non structural protein, p7, can form ion channels, required for the production of infectious virus particles. It is now recognized that the cross-talk between structural and the non structural proteins of HCV is required for efficient virus particle production54. acid protein, positioned at the junction of the structural and non structural proteins. The p7 protein belongs to a family of viral proteins called as viroporins that form ion channels. It can oligomerize following its inclusion into a lipid membrane creating ion channels. The cleavages mediated by signal peptidases between p7 and NS2 occurs slowly and are partial at the E2/p7 and p7/NS2 sites55. This results in the formation of an E2p7NS2 precursor56. It is thought that this precursor may have a role in the regulation of HCV lifecycle. Many models have been proposed to explain the potential significance of these precursor forms, the simplest being, the necessity for a timely release of the individual proteins at the appropriate time in the viral lifecycle. However, the precise role and existence of these precursors in a natural HCV infection is not known. The p7 protein is highly hydrophobic in nature. It is localized in the ER-membranes when encoded by a replication-competent genome57. It has two amphipathic, transmembrane regions, TM1 and

TM2 (spanning amino acids 19-32 and 36-58) which are embedded in the ER-membrane. The N- and C-termini are exposed to the extracellular environment58. The 3- dimensional structure of p7-complex was determined by using single-particle electron microscopy. This hexameric (42 kDa) protein complex was found to depict flower-shaped architecture with protruding petals oriented toward the ER lumen59. The p7 ion channel protein serves an essential role in the production of

infectious virus particles during HCV lifecycle60. It appears to be dispensable for viral RNA replication, as replicons lacking the p7 gene replicate or make viral

RNA efficiently61, 62. The ion channel blocker, amantadine, was thought to interfere with the ion channel activity of p7 based on studies with artificial lipid bilayer system. The p7 protein could form amantadine-sensitive ion channels

in this artificial system63. However, it became clear after clinical trials, that p7 ion channel function is not affected by amantadine64. Another study also reported

similar findings, where HCV strains were found resistant to the ion channel blocker, amantadine65. Nonetheless, dose-dependent reductions of virus titres

were achieved with iminosugars64,66. The absolute dependence of HCV on-ion channel function of p7 protein for infectivity makes it an attractive candidate target for antiviral intervention. HCV NS2: The non structural protein 2 (NS2) is a 23-kDa transmembrane hydrophobic protein. The membrane association of NS2 is p7-independent and occurs co-translationally67,68. NS2 is a membraneassociated

cysteine protease69,70, required for HCVcc infectivity71. The cleavage between NS2 and NS3 is absolutely required for persistent viral infection in a chimpanzee72. NS2 followed by

only 2 amino acids of NS3 produces a basal proteolytic activity in vitro. However, the N-terminal 180 aa of NS3 are required besides NS2 for a robust protease activity. Interestingly, all the active site residues (H952, E972 and C993), needed for the catalytic activity of the NS2/3 cysteine protease, are located entirely in NS273. This requirement of NS3 remained intriguing until the recent discovery showing that the zinc binding domain of NS3 could in fact stimulate the protease activity of NS2. The functional sub-domains in NS3 essentially function as its regulatory cofactor73 again highlighting tight regulation of the proteolytic processing. This process is undoubtedly vital for virus multiplication. NS2 interacts with itself forming homo-dimers. Moreover NS2 has also been shown to interact with all the other HCV non structural proteins74. U ntil recently the only known function for NS2 was its autocleaving activity at the NS2/3 junction. Expression of NS2 in Huh7 cells resulted in upregulation of transcriptional factor sterol regulatory element-binding protein 1c (SREBP-1c) and fatty acid synthase. These studies implicate a role of NS2 in promoting steatosis. NS2 protein can be phosphorylated by casein Kinase II on Ser residue at position 168. This phosphorylation event appears to regulate the stability of NS2 protein (at least from genotype 1a). NS2 also appears to be involved in a particle assembly step which happens post core, NS5A, and NS3 assembly75. The search for molecules or inhibitors targeting NS2, should without a doubt, advance the development of new therapeutics against HCV. HCV NS3: The non structural protein 3 (NS3) is a member of the superfamily 2 DExH/D-box helicases and its crystal structure has been determined. It is a 67 kDa tri-functional protein with a serine protease, an RNA helicase and NTPase activities (Fig. 4). The NS3 enzyme has a chymotrypsin-like

serine protease activity76. Along with its cofactor NS4A is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions. NS3/4A are localized in ER cisternae surrounding mitochondria77. However when co-expressed with p53 (tumour suppressor), it is both RNA and DNA substrates, although there is no DNA intermediate involved in HCV lifecycle. It couples unwinding of RNA strands (with extensive secondary structures) to ATP hydrolysis. A direct interaction between NS3 and NS5B occurs through the protease domain of NS3. RNA unwinding activity of NS3 helicase is modulated by this interaction with NS5B polymerase78. In addition to its role in viral polyprotein processing and HCV multiplication, another important function of NS3 involves antagonizing host innate-immune pathways. The induction of type I interferon (IFN) genes (Type I IFNs include several IFN-α subtypes and a single IFN-β subtype) is regulated at the step of transcription and is best understood for the IFN-β promoter. Innate immune defense mechanisms activated by alpha/beta

INFs represent an essential first line of protection against viral infections. Retinoic acid inducible gene or RIG-I is a cytoplasmic RNA helicase79. It is an essential pathogen recognition receptor (PRR) for HCV. RIG-I upon binding viral RNA undergoes changes in conformation and can then interact with IFN- β promoter stimulator-1 or IPS-1(IPS-1 is also known VISA, Cardif or MAVS)80. This interaction of RIG-I with IPS-1 can signal downstream activation of IRFs and NFkB to trigger alpha/beta-INFs. The localization of MAVS to the mitochondria is

critical for its ability to induce IFNs. This function of MAVS is abolished if the mitochondrial-targeting domain of MAVS is deleted81. HCV NS3/4A protease has remarkably evolved to target and cleave ISP-1 (or MAVS at

Cys-508), thereby halting alpha/beta interferon expression81. This activity may be necessary but does not appear to be sufficient for long-term viral persistence since there is (cytotoxic) T cellmediated clearance of NS3/4A-expressing hepatocytes in vivo82. Therefore, other HCV proteins are most likely responsible for interfering with the adaptive

immunity.HCV NS4B: The HCV non structural protein 4B (NS4B) is a 27-KDa polypeptide. It is a highly hydrophobic integral ER-membrane protein86. It contains four transmembrane domains and is palmitoylated at two C-terminal cysteine residues. Palmitoylation of 4B protein facilitates oligomerization which appears to be essential for HCV replication87,88. It has an amphipatic helix at the N-terminal and a C-terminal domain which are both associated with membranes89. Allelic variation in the NS4B sequence between closely related HCV isolates was found to drastically impact HCV replication in cell culture90. A characteristic feature of Plus-strand RNA viruses is their ability to induce alterations in cellular membranes and then utilize it to replicate their own genomes.replicon revealed a loss of the organization and other morphological alterations of the ER showing

convoluted cisternae and paracrystalline structures77. The replication of HCV is a very intricate process, occurring through protein–RNA and protein–protein interactions. HCV-replication-complex was first identified in Huh7 cells supporting sub-genomic replicon in 200393. These membranous webs are made up of small vesicles (80-180 nm in diameter) embedded in a membranous matrix and are found closely associated with the rough endoplasmic reticulum. NS4B colocalized within this web together with other structural and NS proteins92. Interestingly, NS4B alone could induce the

formation of membranous web. In vitro

expression studies of NS4B from all major genotypes have demonstrated the importance of N terminus of NS4B94. Disrupting the amphipathic-helix in the N

terminus of NS4B abolishes the ability of NS4B to rearrange membranes. Even before an important role in formation of membranous web like structures was

discovered for this interesting protein, a role for NS4B in malignant transformation of NIH3T3 cell in association with the Ha-ras oncogene had been proposed95. It is an important protein for HCV lifecycle. HCV NS5A: The non structural protein 5A (NS5A) has RNA-binding activity96. NS5A protein can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms in cells. P58 form of NS5A is hyperphosphorylated at additional sites that remain unmodified in the p56 form by casein kinase I-alpha97, 98. The first of its kind, the sub-genomic replicon utilized a HCV genotype-1b clone, called as Con1. This clone was engineered such that HCV-structural genes were replaced by a neomycin resistance gene.Following transfection of in vitro transcribed RNA into Huh-7 cells, antibiotic G418-resistant cells could be obtained in which the sub-genomic RNA replicated autonomously. Mutations that enhance the capacity of sub-genomic HCV RNA to replicate in cell culture (Huh-7 cells) were mapped to the NS5A-coding sequence and were called as adaptive mutations. The adaptive mutations alter the phosphorylation state of the NS5A protein. Loss of hyperphosphorylation (or NS5A-p58 form) stimulates RNA replication of the HCV genotype 1b replcion in Huh-7 cells. Human vesicle-associated membrane protein (hVAP) subtype A is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. Phosphorylation status of

NS5A is also known to affect the interaction of NS5A with VAP-A99. NS5A inhibitor (BMS-824) and the NS3 protease inhibitor block hyperphosphorylation of NS5A; however, the mechanism of inhibition remains unknown. Further, the involvement of a protease inhibitor in inhibiting p58 is quite intriguing100. NS5A has an amphipathic alpha-helix at its amino terminus with which it is anchored to the ER membrane. NS5A is further divided into three-domains which are separated by linker regions. The crystal structure of the conserved domain I (DI) was solved recently and revealed a unique fold. DI has a zinc binding motif and also forms a nucleic acid-binding domain. NS5A specifically binds to the G/U rich sequences in the 3' ends of HCV genomic RNA96. Domains II (DII) and III (DIII) are more variable among HCV genotypes.

Crystal structures for both DII and DIII are not available yet. NMR studies have shown DII of NS5A to be flexible and disordered. NS5A-DII contains the PKR and the HCV-NS5B binding domains. A role for NS5A-DIII in virus assembly was shown via phosphorylation of a serine residue at position 2433 by CKII101. NS5A domain III is not required for RNA replication as sub-genomic replicon lacking DIII, replicate in Huh7 cells. However, a recent study with JFH1 virus showed that DIII of NS5A could influence RNA replication. Genotype 2 HCV isolates have

a 19 residue insertion near the C terminus of DIII. Upon its deletion, authors observed a delay in both RNA replication and particle assembly102 thereby suggesting a role in viral multiplication. Full length HCV NS5A has a cytoplasmic

localization. Cleavage by caspase 3 and 6 leads to genearation of N-terminally truncated NS5A fragments which get localized within the nucleus. The C-terminal

domain of NS5A can associate with c-Raf kinase103. This interaction

appears to be essential for HCV replication in Huh7 cells, as sequestration of c-Raf by truncated NS5A into the nucleus, negatively impacts HCV replication104. Oxysterol binding protein (OSBP) can interact with the N-terminal region of DI. OSBP is co-localized to the golgi with NS5A and this functional interaction is suggested to play a role in HCV particle release105. A significant association between variations in sequences between 2209-2248 nucleotides of NS5Agene,

and response to interferon treatment has been proposed. This region of NS5A (amino acids 237−276) is called as ISDR or interferon sensitivity determining

region. The ISDR was also found to associate with the antiviral molecule, PKR. Patients with a mutant-type response (SVR) than those with the wild-type NS5AISDR. However, recent studies have questioned the existence of an ISDR in NS5A. Existence of an ISDR is an ongoing debate and controversial. Nevertheless,

NS5A can repress the PKR pathway. NS5A also has the ability to inhibit IFN-gamma production106. A remarkable study with NS5A transgenic mice suggested

that NS5A protein could impair both the innate and adaptive hepatic immune response107. Indeed, NS5A has attracted tremendous attention in the HCV field

and represents a very promising target for anti-HCV therapy. HCV NS5B: The HCV non structural protein 5B (NS5B) is a RNA dependent RNA polymerase (RDRP) containing the GDD motif in its active site108. It belongs to a class of integral membrane proteins termed tail-anchored proteins. NS5B initiates synthesis of complementary negative-strand RNA using the HCV genome (positive polarity) as a template. Subsequently it generates positive-strand RNA from this negativestrand RNA template. The NS5B crystal

structure shows the typical fingers, palm and thumb sub-domains. NS5B is anchored to the ER-derived "membranous webs" via its C-terminal 21 amino-acid residues109. Studies with sub-genomic replicon revealed that the membrane association is indispensable for viral RNA replication110. In vitro NS5B cannot distinguish between natural and synthetic templates. NS5A can directly bind to NS5B and modulate its polymerase (poly A template/poly U primer) activity111. NS5A (at substoichometric levels) stimulates replication by NS5B on templates derived from the 3' end of the positive strand112. NS5A stimulates NS5B during elongation; however the mechanism of action is not clear. It could either be due to the stimulation of its catalytic activity or due to a conformational change induced by NS5A binding112. There appears to be a difference in polymerase specific activity in vitro, depending on the type of HCV genotype. The JFH1 NS5B enzyme shows a 10- fold-higher specific activity when compared to J6 NS5B113. The termination of RNA synthesis in vitro is not understood well. NS5B lacks a "proofreading" function. Due to a high rate of error-prone replication, complex mutant swarms are generated. However, HCV must also maintain highly conserved genomic segments and a balance between conserved and variable viral elements is above all important to avoid "error catastrophe". Over the past few years numerous nucleoside and

non nucleoside inhibitors of the polymerase have been discovered and demonstrated clinical efficiency and advanced to clinical trials. NS5B nucleoside

inhibitors (NIs) or analogs (NM-107/NM-283, PSI- 6130/R7128, IDX184, MK-0608) compete with cellular ribonucleoside triphospahtes act as functional chain terminators114. Non-nucleoside inhibitors (NNIs) or analogs (ABT-333, GSK625433,

VCH-759, PF- 868554, GS-9190) act by allosteric mechanism. They bind to allosteric sites on NS5B and thereby inhibit RNA synthesis114,115.

An important lead was provided by a study which established that NS5B associates with cyclophilin A (CypA) via its enzymatic pocket and exploits the isomerase/chaperone activity of CypA to replicate in cells116. NS5B is phosphorylated by the protein kinase Crelated kinase 2 (PRK2) in cell culture117. It was further discovered that inhibitors of NS5B phosphorylation, HA1077 (fasudil) and Y27632 suppressed the activation of PRK2. The treatment of liver cell lines (stably replicating HCV sub-genomic replicon) with these inhibitors cleared viral RNA suggesting that PRK2 inhibitors act by suppressing HCV replication via inhibition of NS5B activity118. However, it would be interesting to see if resistant mutants emerge over a period of time. More critical would be the normal functions affected which are regulated by PRK2. The continuous generation and selection of resistant variants allows HCV to escape control by these inhibitors/antiviral drugs. A study where patients infected with genotype 1a were treated with ribavirin, led to the emergence of a Phe-to-Tyr (F415Y) mutation in the viral polymerase119. There is also evidence for recombination events leading to genetic variation in HCV120. Thus, very high rates of genetic variation ensure the survival of hepatitis C viruses under every changing hostile cellular environment.

Tools for identifying hepatitis c virus

Viruses are major factors of human infectious diseases. For example, influenza virus is responsible for more than 30,000 deaths each year in the United States [Fornek *et al.*, 2007]. During the influenza pandemic of 1918, tens of millions of people died

from the infection. Understanding of the structure-function correlation in viruses is important for finding potential anti-viral inhibitors and vaccine targets. Although virology was slower to embrace bioinformatics [Kellam and Albà, 2002], this situation has changed dramatically in recent years. Databases and bioinformatic tools that contain genomic, proteomic, and functional information have become indispensable for virology studies. A timely review of these virus-related bioinformatics applications would be not only helpful for laboratory end-users, but also important for bioinformatics researchers to make further progress in the field.

Bioinformatic analysis on viruses involves the general tasks related to the analysis of any novel sequences, such as gene identification, gene functional annotation, and analysis of phylogenetic relationships. However, specific features in virus biology determine specific challenges to those general bioinformatics tasks. For example, many viruses have overlapping open reading frames or translational frameshifts. In addition, the extent to which related viruses undergo recombination often makes it impossible to achieve the success of classical phylogenetic analysis for evolutionary histories. Because of the volume and diversity of sequences available in virus databases such as strains, isolates, mutants and quasispecies, it is necessary to have the required data organized and integrated in virus-specific repositories. In addition, homology searches among viral genomes often require tools that have been optimized for virus families. In this review, some tools and sources that were built specially for viral studies are discussed, focusing on viruses from eukaryotic hosts. Most of the tools and sources mentioned here are freely available on the Web.

In this section, commonly used bioinformatics tools are introduced

with descriptions of why and how they are used in virology research. These general bioinformatic analysis steps in virology are summarized in a workflow diagram in Fig. 1. These are important steps usually used for structure-function analyses or systems analyses, which are the crucial studies for understanding viral diseases and finding potential anti-viral agents.

Open reading frame (ORF) identification and gene prediction

ORF finding is the basis for further homologous search, functional analysis, and identification of viral proteins for possible utilities such as antiviral agents or vaccine targets. From genomic DNA or RNA sequences, ORFs can be identified for candidate genes. If an ORF is a surface protein (which can be checked through the transmembrane domain programs listed below) and unique to the organism, it may cause immune responses and become a candidate for a vaccine.

ORF detection is listed at the top of the flow diagram of bacterial vaccine discovery research [Zagursky *et al.*, 2001]. In viral vaccine or drug intervention studies, ORF identification is also one of the first steps in analyzing an unknown viral genome for potential targets. National Center for Biotechnology Information (NCBI)'s ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) is a general ORF prediction tool. This program has been used in analyzing complete viral genomes, such as the Hz-1 virus (a new family of viruses distantly related to the Baculoviridae) with a length of 228,089 bp [Cheng *et al.*, 2002]. Through the use of ORF Finder together with other programs including the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) [Altschul *et al.*, 1990], 154 ORFs were found in the Hz-1 virus genome. The program GeneMark (http://opal.biology.gatech.edu/GeneM

ark/genemarks.cgi) also provides gene prediction tools for viruses [Besemer *et al.*, 2001]. In addition, the Gene Ontology (GO) (http://www.geneontology.org/) provides a controlled vocabulary for genome annotation.

Homology searching and sequence alignment

Homology searching against known or already annotated viral genomes, such as using the BLAST program, can also be used for predicting genes in unknown viral genomes. Homology searching is usually the next step for genome annotation and functional analysis after ORF finding in viral genome research. A high degree of homology between an ORF from an unknown genome and a known protein may suggest the new protein's similar function to the known one. A commonly used program for homology searching is BLAST. The program can be used for both nucleotide and amino acid sequence searching. The updated formats of NCBI's BLAST programs have some enhanced functions that make the searching more convenient. For example, certain formats can be selected to show detailed sequence alignments, as well as conserved and varied regions. NCBI provides interfaces that are specific for searching against viral protein databases. A useful interface is "Mega BLAST", which can search a batch of sequences using BLAST. The batch of sequences can be uploaded from files on the user's disk. This application is especially helpful for searches in viral genomes, as most of the time one needs to run BLAST for multiple viral sequences.

The sequence alignment program ClustalW (http://www.ebi.ac.uk/clustalw/) [Thompson *et al.*, 1994] has been used extensively in studying viral genomes. Nucleotide and amino acid sequence alignments are important in comparing

viral sequences in different species and strains. Such analysis is useful for identifying similarities, comparing conserved and non-conserved regions, establishing evolutionary relationships, and building phylogenetic trees.

Mining data derived from ClustalW alignments enables one to complete a series of comparisons between different viral genomes. For example, for genomes of varied strains of the same virus, one can compare sequence regions with less than 90% identity, and analyze functional differences within these regions through motif analysis (motifs will be discussed below). Such analysis might provide clues on how the structural differences affect functions, which may point to such utilities as potential antiviral agents or vaccine candidates. For instance, using ClustalW, researchers analyzed sequencing data of coxsackievirus A24 variants and found homologies that ranged from 97.7%-100% [Park *et al.*, 2006]. Phylogenetic trees were also built based on the analysis. A comprehensive list of programs for building phylogenetic trees is available at Phylogeny Programs (http://evolution.genetics.washington.edu/phylip/software.html). Phylogeny packages are grouped nicely at this site, according to the available methods such as maximum likelihood and Bayesian methods, or computer systems on which they work. For example, SimPlot (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) and SplitsTree (http://www.splitstree.org/) are computational tools for inferring recombination between ancestors of a given virus.

Pattern/motif/epitope recognition

A pattern (or a motif) represents common characteristics of a protein family, it constitutes a usually short but characteristic region within a protein sequence. Protein domains in a

particular family that are from a common ancestor usually share functional features. Thus pattern or motif searching is an important method to correlate genomic sequence structure to proteomics function. Functional motifs can also be potential targets for anti-viral inhibitors.

Motif searching has been used broadly in viral genome sequence analysis. Motif analysis tools usually search given protein sequences against the PROSITE database (http://www.expasy.ch/prosite/) [Hulo *et al.*, 2006] or other motif databases and predict functional regions. For example, the PROSITE database was used in analyzing White spot syndrome virus for studying the functions of the viral gene products [Huang *et al.*, 2002]. Twenty possible phosphorylation motifs were found in the newly identified protein VP466, a component of the viral envelope. In another study of cucumber mosaic virus (CMV), researchers used the PROSITE software to search for functional sites and compared plant symptom determinants with the PROSITE hits [Gellért *et al.*, 2006]. They found that all analyzed mutations were related to the modifications of the predicted phosphorylation sites. Based on their analyses they predicted the infectivity of the examined viruses. Besides the facilities described, this site provides PRATT, a tool for discovering patterns in a set of unaligned sequences. It allows for finding novel motifs (not listed in the database). Pfam (http://www.sanger.ac.uk/Software/Pfam/) [Finn *et al.*, 2006], another database for protein families and domains, is also frequently used.

Because viruses have characteristics different from other genomes and may have their own special motifs, in many cases one needs to use specifically defined patterns. In some programs such as ScanProsite (http://www.expasy.ch/tools/scanprosite/), user-defined patterns in the

PROSITE format can be used. Another program is Simple Modular Architecture Research Tool (SMART), which can be used for identification of signaling domains [Schultz *et al.*, 1998; Letunic *et al.*, 2006].

Epitopes are the parts of antigens interacting with receptors of the immune system and are important for understanding viral diseases and finding anti-viral targets. A useful program for analyzing epitopes is the Immune Epitope Database and Analysis Resource (IEDB) (http://www.immuneepitope.org/) [Peters *et al.*, 2005]. The database has data related to antibody and T cell epitopes for humans, non-human primates, rodents, and other animal species. It also has MHC binding data from a variety of different antigenic sources. Currently the database contains curated data about influenza, hepatitis B, and herpes viruses. Both query and browsing functions are provided by the resource.

Short tandem repeats

Short DNA repeat regions are potential sites of mutation and immunogenic variable regions. The detection and comparison of changes in short tandem repeat regions in viral genome sequences are useful in identifying viral lineages from distinct geographic regions. These findings can be helpful in epidemiological studies of viruses that are important pathogens. A popular and user-friendly tandem repeats finder is Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html) [Benson, 1999]. The program finds tandem repeats in user submitted DNA sequences. The output of the program has two files, a repeat table file that has information about each repeat including location, and an alignment file.

Transmembrane domains

Transmembrane (TM) domains are frequently found in surface proteins,

which can be recognized by the immune system. They are good candidates for inclusion in viral vaccines. TM identification programs can usually predict membrane-spanning regions and their orientation for given sequences. A program that can be used for this purpose is the TMpred (Prediction of Transmembrane Regions and Orientation) (http://www.ch.embnet.org/software/TMPRED_form.html) [Hofmann and Stoffel, 1993]. For example, TMpred and two other transmembrane domain prediction programs (SOSUI and DAS) were used in the analysis of tobacco mosaic virus (TMV) [Li *et al.*, 2006]. These researchers found that TMV recombinants that infected susceptible tobacco contained a transmembrane domain in the coat protein subunits and caused local necrotic responses.

Secondary and tertiary structural studies

Viral structural modeling can help clarify the structure-antigenicity relationship. For example, secondary structural analysis was done on a flavivirus protein [Leyssen *et al.*, 2002]. Researchers analyzed the putative folding patterns and found structural elements distinguishing the Modoc virus (MODV) untranslated regions (UTRs) from mosquito-borne and tick-borne flaviviruses.

Three-dimensional prediction models in different strains and segments can be compared to explore how alignment differences affect the actual protein structure and antigenic sites, folding surfaces, and functional motifs. These comparisons can be used for identifying candidate antiviral inhibitors and vaccine targets. For instance, the predicted 3-dimension (3-D) protein structure was analyzed in human respiratory syncytial virus (HRSV) [Sugawara *et al.*, 2002]. In this study, researchers observed significant structural differences related to the length of peptides

containing the cysteine noose, which showed good correlation with the immunogenic activity of the peptides.

The tool PredictProtein (http://www.predictprotein.org/) can be used for protein secondary structure modeling [Rost *et al.*, 2004]. This program generates predicted secondary structure and solvent accessibility, and possible transmembrane helices. The program also provides information about the expected accuracy of prediction methods.

For the prediction of 3-D structure, tools such as SWISS-MODEL (http://swissmodel.expasy.org/) can be used. Programs to predict the 3-D structure are usually based on homology searching using similar and known protein structures as templates. These templates mostly come from protein domains available in Protein Data Bank (PDB) [Reddy *et al.*, 2001]. The program output includes the template selected to be used, the sequence alignment between the query sequence and the template, and the predicted 3-D model. Currently when using SWISS-MODEL, after users have entered their query sequences, modeling results would be sent to users through email. The program can be more convenient to use if the predicted model can be shown and downloaded directly through the Web.

Pathway analysis

Through analyzing biochemical pathways and protein-protein interactions, one gains understanding of how these molecules interact with each other, as well as their functional roles at the systems level. Abnormal variations and interactions in these pathways may contribute to disease states. Pathway information can be useful for finding potential antiviral interventions and vaccine targets. A commonly used pathway database is Kyoto Encyclopedia of Genes and Genomes (KEGG)

(http://www.genome.ad.jp/kegg/kegg2.html), which includes information of metabolic pathways and regulatory pathways [Kanehisa *et al.*, 2006]. Although not specifically directed to virology analyses, the database contains graphical pathway maps, ortholog group tables, and molecular catalogs that might be helpful to the discerning virologists.

Pathway analysis plays an important role in understanding virus infection processes, and signaling pathways that enable viral growth are excellent targets for antiviral therapy. Virus infection can trigger a variety of signaling pathways evoking antiviral responses of the host cell. In the meantime, viruses are able to suppress these cellular responses to assure their own replication. For example, activation of the Ras/Raf/MEK pathway has been found to down-regulate IFN-induced antiviral response [Battcock *et al.*, 2006]. In addition, viruses can turn on and take advantage of cellular signaling pathways for their own proliferation.

Pathway databases can help elucidate the mechanism and processes of viral infections. For example, apoptosis signal-regulating kinase 1 (ASK1) and its downstream pathway are important in the regulation of apoptosis in the infection of viruses including influenza, AIDS, hepatitis C and others [Sumbayev *et al.*, 2006]. ASK1 is a component of the mitogen-activated protein (MAP) kinases,

Microarray data analysis

Microarray technology enables us to simultaneously examine the expression of thousands of genes. This technology is useful for studying functional genomics and for understanding host-virus interactions. The detection of genes that are differentially expressed under specific conditions may help us discover mechanisms for specific biological phenomena. Genes with similar expression patterns may be grouped or

clustered together, and can provide us insights of signal transduction pathways. For example, using microarray technology, researchers found that the pathogenicity and lethality of the 1918 influenza virus were associated with an aberrant and unchecked immune response [Fornek *et al.*, 2007].

Concomitantly, researchers can fall victim to the so-called "curse of dimensionality" on too much information. Several informatics sites may offer a balm from this curse of data overload. Microarray Informatics at European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/microarray) is a Web portal that contains information about managing, storing and analyzing microarray data. Its ArrayExpress is a database that contains data from more than 1300 experiments and 900 arrays, including more than 20 experiments about viral infection. Information such as experimental protocols and references is also included.

Gene Expression Omnibus (http://www.ncbi.nih.gov/geo/) is a gene expression and array database. It contains microarray data from more than 2,000 platforms and 70,000 samples. It can be browsed through platforms, samples, or series. It can be queried from datasets, gene profiles, and accession numbers. For example, Fig. 3 shows a screenshot of gene expression analysis results about human cytomegalovirus (HCMV)-infected foreskin fibroblasts submitted by Marc Kenzelmann at the University of Bern. The datasets were published in 2003 and can be downloaded from the database site [Kenzelmann and Mühlemann, 2000]. Various data analysis results based on the datasets are provided, such as the heatmap result of Euclidean Single Linkage analysis

Many general databases and query systems can be used in virology research. For example, GenBank and the Entrez query system can be used for searching nucleotide and protein

sequence information. PDB is a good source for 3-D biological macromolecular structure data.

National Center for Biotechnology Information (NCBI)

NCBI (http://www.ncbi.nlm.nih.gov/) currently contains several major virology resources, including Influenza Virus Resource, Retroviruses, and Viral Genomes. Influenza Virus Resource (http://www.ncbi.nih.gov/genomes/FLU/FLU.html) includes tools for searching the Influenza Virus Sequence Database. This site provides sequence analysis tools such as BLAST for sequence queries against the influenza database, and a tool for building clustering or phylogenetic trees for analyzing influenza sequence relationships. The Retroviruses page (http://www.ncbi.nih.gov/retroviruses/) contains information about complete reference genomes, including genomic and protein sequences, a Genotyping Tool to identify the genotype of a viral sequence, and alignment and BLAST tools. The Viral Genomes page (http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html) contains sequences for more than 1400 viral genomes. BLAST and alignment tools are provided at this site, which make it convenient for virus-related sequence analyses. A newly developed part is the collection of clusters of related viral proteins by certain categories. For example, dsDNA virus proteins are clustered by different functional categories, including movement proteins, proteins for DNA replication, proteins for repair and nucleotide metabolism, structural proteins, and proteins for regulation of cellular metabolism. Fig. 4 shows a homepage screenshot from the keyword query of "respiratory syncytial virus." Detailed information about this virus can be further retrieved from this page, including genome information, gene features, genome map, and links to other resources and publications.

Universal Virus Database of the International Committee on Taxonomy of Viruses (ICTVdB)

ICTVDB (http://www.ncbi.nlm.nih.gov/ICTVdb/index.htm) is developed for virus classification and nomenclature taxonomy that have been approved by the International Committee on Taxonomy of Viruses [Büchen-Osmond, 2003]. The taxonomic index can be browsed by alphabetical order, host categories, and family categories. The database provides detailed description of viruses, including images of electron micrographs, description of virus properties, genome organization and replication, antigenic properties, and biological properties such as transmission and geographic distribution. Although abundant information is available, this database would be more useful if they can provide user-friendly web-based analysis tools rather than a downloadable program called Intkey.

Virus Database at University College London (VIDA)

VIDA (http://www.biochem.ucl.ac.uk/bsm/virus_database/VIDA.html) collects some homologous protein families derived from ORFs from complete and partial virus genomes, including Arteriviridae, Coronaviridae, Herpesviridae, Papillomaviridae, and Poxviridae families [Albà *et al.*, 2001]. The database contains functional and taxonomy information, and links to DNA sequences and structures. An alignment of the conserved regions is provided for each virus family. The database can be searched through homologous protein families, virus names, GenBank protein numbers, and protein functions such as regulatory proteins or structural proteins such as capsid proteins. It can also be searched with just free text keywords.

The site provides some useful viral sequence analysis tools, including WebORF and MultiSearch. WebORF can identify putative translated products in virus genomic sequences. MultiSearch can be used to search for similarities between sequences and the conserved domains in the homologous protein families (HPFs). The results of MultiSearch include a graphic representation of the distribution of the HPFs in all available complete genomes. Their website also has a table of HSV-1 genome annotation, including a list of HPFs, ORFs, protein products, biological processes, molecular functions, and interactive cellular components.

Viral Bioinformatics Resource Center (VBRC)

VBRC (http://athena.bioc.uvic.ca/index.php) provides databases of viral genomes and tools for comparative genomic analyses. Its central database Virus Orthologous Clusters (VOCs) is a downloadable database with tools for searching and analyzing the genes, gene families, and genomes of different virus families including Adenoviridae, Arenaviridae, Asfarviridae, Baculoviridae, Bunyaviridae, Coronaviridae, Filoviridae, Flaviviridae, Herpesviridae, Paramyxoviridae, Poxviridae, and Togaviridae. Information covered in their databases includes genome maps, viral structures, life cycle summaries, structural and nonstructural proteins, and links to other databases or sources.

A downloadable tool called Viral Genome Organizer (VGO) can be used for viewing and searching genomic information including ORFs, start/stop codons, and motifs [Upton *et al.*, 2000]. Other downloadable tools include BaseByBase, a whole genome pairwise and multiple alignment editor [Brodie *et al.*, 2004a]. Jdotter is a program for generating dotplots of

large DNA or protein sequences [Brodie *et al.*, 2004b]. ReHAB is a tool for finding new protein hits in repeated PSI-BLAST searches [Whitney *et al.*, 2005]. GFS is a program to map peptide mass fingerprint data directly to raw genomic sequence, to facilitate the identification of proteins in genomes for which annotation is lacking [Giddings *et al.*, 2003]. NAP computes an optimal global alignment of a DNA sequence and a protein sequence without penalizing terminal gaps [Huang and Zhang, 1996]. GraphDNA is a tool for generating graphical representations of raw DNA sequences. Hydrophobicity Grapher graphs the hydrophobicity/hydrophilicity of an amino acid sequence using a sliding window with sequence lengths that can be specified by the user [Parker *et al.*, 1986].

These programs would be more useful and convenient to use if they would provide user-friendly web-based interfaces. An interesting database on their site is The Database of Virologists (DoV), from which users can register their own information and search for other virologists.

GeneMark™ VIOLIN

GeneMark™ VIOLIN (Annotations of viral and phage genomes obtained by the GeneMark gene prediction programs, http://exon.gatech.edu/GeneMark/VIOLIN/) is a program for predicting genes in viral genomes [Besemer and Borodovsky, 1999]. There are two ways to use their program. One is to search their database with virus names or accession numbers. The output includes the beginning and the end of predicted genes, protein prediction, and links with GenBank records and BLAST results. Users can also use their heuristic model to search for predicted genes with the user's own sequences as input. The program would be more useful if they can be

integrated with other sequence analysis tools, such as combining gene prediction and phylogenetic programs.

The databases introduced above are mostly comprehensive databases or portals useful for obtaining a more global perspective of viral information. Other sources that contain information for specific viruses or specific aspects of viruses are introduced below.

Other databases and resources

1. VIrus Particle ExploreR Database (VIPERdb) (http://viperdb.scripps.edu/main.php) collects information about various icosahedral virus capsid structures in PDB. The database provides links to the PDB coordinates of virus structures, rendered structural images of viral subunits, capsids, and crystal contacts. Users can browse the database through virus names that are grouped by family, cryo-electron microscopy-based models, or crystal information such as space groups and inter-particle contacts. The site also has some tools such as Icosahedral Matrices data for generating a complete icosahedral particle given the transformed coordinates for each entry in the index of virus structures.
2. Recombinant Virus Database (RVD) (http://www.brc.riken.jp/lab/dna/rvd/) contains information focuses on viral DNA clones, recombinant retroviruses, and recombinant adenoviruses.
3. Subviral RNA Database (http://subviral.med.uottawa.ca/cgi-bin/home.cgi) is a database of "the smallest known auto-replicable RNA species," including viroids, viroid-like RNAs, and human hepatitis delta virus (vHDV) [Rocheleau and Pelchat, 2006]. The

database currently has more than 1,700 sequences.

4. The Influenza Sequence Database (ISD) at Los Alamos National Laboratory (LANL) (http://www.flu.lanl.gov/) is a database exclusively for influenza viruses [Macken *et al.*, 2001]. It contains influenza nucleotide and amino acid sequence information and provides query tools. Two other sites, Influenza Primer Design Resource and Biohealthbase also provide information and tools for influenza virus and other organisms (see links in Tab. 2). While some of these databases may have redundant information, NCBI's Influenza Virus Resource as mentioned previously is an integrated portal that provides comprehensive support for research on influenza viruses.
5. European Hepatitis C Virus database (euHCVdb) (http://euhcvdb.ibcp.fr/euHCVdb/) contains HCV information including sequences, genome maps, and links to some sequence analysis tools [Combet *et al.*, 2004]. Compared with LANL's HCV Databases discussed below, this database focuses more on genomic sequence information, while the later also has immunology and other information.
6. LANL's Hepatitis C Virus (HCV) Databases (http://hcv.lanl.gov/content/hcv-db/index) contain a HCV sequence database and a HCV immunology database [Kuiken *et al.*, 2005; Yusim *et al.*, 2005]. HCV Sequence Database provides nucleotide and amino acid sequence search and analysis tools. For example, N-GlycoSite is a program for analyzing N-linked glycosylation site patterns.

HCV Immunology Database contains search tools and epitope maps. For example, Epilign is a program that can be used to align epitopes and functional domains. These programs would be very useful for the understanding of HCV viral genomes and host-virus responses.

7. LANL's HIV databases (http://www.hiv.lanl.gov/content/index) contain data on human immunodeficiency virus (HIV) genetic sequences, immunological epitopes, drug resistance-associated mutations, and vaccine trials. HIV Drug Resistance Database (http://hivdb.stanford.edu) is a curated HIV database that includes data on genotype-treatment correlation, genotype-phenotype correlation, and genotype-clinical outcome correlations. These data are helpful for both experimental and clinical studies of anti-HIV agents.
8. Poxvirus Bioinformatics Resource Center (http://www.poxvirus.org/) provides information on poxvirus genomic sequences, and annotation and analysis of poxvirus genes. The site has BLAST tools for poxyvirus gene sequence comparisons, a repository of poxvirus species and strains, and a discussion forum.
9. SARS (Severe Acute Respiratory Syndrome) Bioinformatics Suite (http://athena.bioc.uvic.ca/database.php?db=coronaviridae) is an information portal about SARS. It contains a coronavirus database, and links to tools to analyze the genomes, genes and proteins of SARS and other related viruses.
10. A site concerned especially with plant viruses

(http://www.dpvweb.net/) has information about viruses, viroids and satellites of plants, fungi and protozoa [Adams and Antoniw, 2006]. It has a database that contains sequences and other major features of the sequences, such as annotations for the ORFs.

A special website worth mentioning is All the Virology on the WWW (http://www.virology.net/). It provides links to various virology information on the internet, including information about specific viruses, viral diseases, vaccines and treatments, groups of interest to virologists, and educational resources. In addition, some new user-generated formats are in development, such as BioDirectory.com's BioWiki (http://www.biodirectory.com/biowiki /Virology), which uses the advanced technology in Web 2.0 to facilitate virology research

Hepatitis c identification

There are a number of different methods for HCV genotyping and subtyping. The most frequently used typing methods are line probe assay (LiPA) and sequencing of the 5'UTR. The Versant HCV genotype assay (LiPA) manufactured by Innogenetics has been developed based on hybridization of 5'UTR amplification products with genotype specific probes. On the other hand, the TruGene HCV 5'NCR genotyping kit (Bayer Healthcare, CA) is based on semi-automated sequencing (Verbeeck et al. 2008; Chevaliez et al. 2009). However, it has been shown that genotyping methods using the 5'UTR, including LiPA, may not discriminate subtypes 1a from 1b in 5% to 10% of cases. Thus, other investigators have used different regions of the HCV genome using RFLP analysis or sequencing of the 5'UTR and NS5B for genotyping and subtyping (Zein 2000; Chen and Weck 2002; Zheng et

al. 2003; Martro et al. 2008; Qiu et al. 2009; Mora et al. 2010).

PCR-RFLP analysis of the nested RT-PCR amplified 5'UTR is generally used for the identification of HCV genotypes in the Philippines (Maramag et al. 2006). It has been suggested that as the virus continues to evolve and more HCV-infected individuals are tested, new subtypes such as HCV-1c will emerge (Ross et al. 2008; Verma and Chakravarti 2008; Utama et al. 2010). Until now, only three confirmed HCV-1 subtypes specifically 1a, 1b, 1c, and 10 provisional assigned subtypes specifically 1d to 1m have been described (Bracho et al. 2006; Bracho et al. 2008; Martro et al. 2011). Thus, it is likely that typing methods including PCR-RFLP analysis will have to be modified to accommodate the rapidly increasing database of information collected on HCV sequence heterogeneity (Davidson et al. 1995; Buoroa et al. 1999; Lee et al. 2010). In addition, there is little doubt that HCV typing methods require careful redesign.

The importance of identifying HCV genotypes and subtypes using bioinformatics tools transcends mere academic interest because, it will provide clinicians and scientists with invaluable information about HCV genomics, which can be used for epidemiologic studies. Furthermore, molecular characterization of HCV subtypes is likely to facilitate the development of an effective vaccine. From a clinical point of view, current therapeutic decisions for chronically infected HCV patients are made on the basis of genotyping and subtyping. Thus, accurate identification of subtypes will enable the clinicians to make the proper choice of new antiviral compounds which are likely to show distinct activities against isolates belonging to different subtypes of HCV (Chandra et al. 2007; Chevaliez et al. 2009; Koletzki et al. 2010; Panduro et al. 2010; Pickett et al. 2011).

Bioinformatics tools have been developed to generate, store, analyze, and visualize biological data. The challenge is to choose user-friendly tools that would give clear and meaningful biological information, without being overwhelmed by the complexity of the data. In this paper, we demonstrate how bioinformatics tools can be used to identify HCV-1 subtypes and we highlight selected freeware bioinformatics software and web-based software. We also report the accuracy of HCV-1 subtyping by 5'UTR PCR-RFLP analysis compared to direct DNA sequencing. Additionally, we compare the HCV-1 subtypes by phylogenetic analysis of the 5'UTR and non-structural 5B (NS5B) region.

Since the identification and molecular characterization of the non-A, non-B hepatitis virus in 1989 by Choo, [5] a variety of diagnostic tests based on the detection of anti-HCV antibodies in serum samples have been developed and refined. The first-generation anti-HCV test that was commercially available and widely used was an ELISA that incorporated the c100-3 epitope from the nonstructural NS4 region. [See Figure 1] Three generations of serodiagnostic anti-HCV antigen tests have been developed, with each new generation providing incremental improvements in the sensitivity to antiHCV antibodies. [See Table 1] Many of the screening tests employ enzyme-linked immunosorbent assays (ELISA), such as the ORTHO HCV 3.0 ELISA Test System (ELISA 3.0 HCV; Ortho Clinical Systems, Raritan, NJ), MONOLISA HCV (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France), and the Abbott HCV 3.0 (HCV EIA 3.0; Abbott Laboratories, North Chicago, Il). The third-generation ELISAs, recently approved by the Food and Drug Administration for blood donor screening, are designed to detect antibodies to four recombinant HCV

proteins [See Table 2]. The third-generation assay differs from the second-generation ELISA by substitution of the NS5 protein for the 5-1-1 antigen. However, this substitution does not appear to account for the increased sensitivity of the newer assay. [4] The average period for HCV seroconversion after blood transfusion has been shortened with each generation as well: 7 to 8 weeks with ELISA-3 as compared with 10 weeks with ELISA-2 and 16 weeks with ELISA-1. [4]

ELISA assays have many advantages in the diagnostic setting including ease of automation, ease of use, relative cost-effectiveness low variability and high sensitivity in screening before liver transplantation. Some of the major disadvantages include suboptimal sensitivity and specificity, an abundance of false-positives in low-prevalence populations and poor sensitivity in post-liver transplant patients because of immunosuppression [6]

Although false-positive ELISA results are a problem in low-prevalence settings, the accuracy of the third-generation test is very good in high-prevalence populations, and therefore, supplemental anti- HCV tests may not be necessary in high-risk patients with a positive anti-HCV screen. A study by Pawlotsky et al [1] determined whether a double ELISA determination and confirmation of positive ELISA results with immunoblot assays were still useful in clinical laboratories performing routine HCV diagnosis. The study showed that one single ELISA 3.0 determination was sufficient for diagnosis of HCV infection in clinical laboratories eliminating confirmation of positive or weakly positive ELISAs with immunoblot assays.

Automated systems can improve workflow in the clinical laboratory such as the Abbott AxSYM, an automated system that provides random- and continuous-access testing for immunoassays, 20 onboard

reagents, primary tube sampling, and a throughput of 80 to 120 tests per hour. The AxSYM incorporates three separate analytical technologies for processing immunoassays: microparticle immunoassay, fluorescence polarization immunoassay, and ion-capture immunoassay. The system incorporates both common and technology-specific subsystems controlled by a real-time software scheduling processor. Tests can be processed in one- or two-step sandwich or competitive formats, with variable pipetting steps, incubation periods, optical read formats, and wash sequences. Abbott expects to launch an updated version called AxSYM 2. This new system will use existing AxSYM tests, but will incorporate additional labor-saving features to simplify and improve the testing process for mid-volume laboratories. The AxSYM line is not available in the United States pending FDA approval. [7]

The PRISM (Abbott) is another automated, high-volume, donor screening immunoassay analyzer in which a sample is split between disposable reaction trays in a group of linear tracks. The system's pipettor uses noninvasive sensing of the sample volume and disposable pipet tips. Each assay track has (a) a conveyor belt for moving reaction trays to predetermined functional stations, (b) temperaturecontrolled tunnels, (c) noncontact transfer of the reaction mixture between incubation and detection wells, and (d) single-photon counting to detect a chemiluminescence (CL) signal from the captured immunochemical product. Multiple channels on the PRISM accommodate hepatitis and retrovirus assays and use either serum or plasma. [8] The assay methodology, a combination of microparticle capture and direct detection of a CL signal on a porous matrix, offers excellent sensitivity, specificity, and ease of automation. PRISM HCV is not

available in the United States pending FDA approval.

Supplemental or confirmatory tests for HCV

An accurate diagnosis of hepatitis C virus (HCV) is necessary before treatment and counseling of patients begins. As stated above, false-positive results following ELISA testing continue to be noted among low-risk blood donors. While elevated aminotransferases and high-risk factors for infection are indicative of active infection and hepatitis, additional testing for antibody specificity can further document HCV infection. A number of confirmatory and/or supplemental serodiagnostic tests are available to cross-check seropositive results obtained with ELISA screening tests. An HCV diagnosis can be confirmed by the recombinant immunoblot assay (RIBA). RIBA identifies antibodies to individual HCV antigens and has a higher specificity than ELISA. [3] Confirmation can involve the use of either a four-antigen RIBA (RIBA HCV 2.0; Chiron Corporation, Emeryville, CA) or strip

immunoblot assay (SIA). The second-generation RIBA, or RIBA-2, uses the same recombinant antigens as the ELISA-2. [See Table 2] A more sensitive third generation RIBA is in the process of obtaining FDA licensing and has recently been introduced in Europe. How do RIBAs compare with other diagnostic tests? RIBAs are technically more demanding than ELISA. Also, RIBA positivity is not always a true indicator of active infection by HCV because recovered patients may stay anti-HCV positive for years. Conversely, RIBAs are simpler, more standardized, and more reproducible than tests for HCV RNA, such as the branched chain DNA assay.

The third-generation RIBA has resolved many of the RIBA-2 interdeterminate samples. However, only 50% of the RIBA-3 positive blood donors are HCV RNA positive

by polymerase chain reaction (PCR) assay. [3, 9]

Supplemental tests can also indicate whether or not a patient will respond to a particular therapy, in this case, interferon (INF). INNO-LIA HCV Ab III is a confirmatory assay, like RIBA-3, and exposes several specific HCV peptides from two non-overlapping core regions (C1 and C2), E2, NS4 and NS5A regions, and recombinant NS3 of the HCV polyprotein. The HCV nonstructural 5A (NS5A) protein may contribute to the interferon-resistant phenotype of HCV. In the mechanism of HCV resistance to interferon therapy, the NS5A protein represses the action of PKR, a protein activated by interferon that shuts down viral protein synthesis thereby inhibiting replication of the HCV. A study by Frangeul et al [10] used both RIBA-3 and INNO-LIA HCV Ab III in determining which patients with chronic hepatitis C would respond better to interferon (INF). An association was found between a primary response to INF therapy and reactivity towards the NS5A antigen in the INNO-LIA HCV Ab III as well as the NS5 in the RIBA-3 with no differences between the two tests. As further studies become available, both RIBA-3 and INNO-LIA HCV Ab III could become simple predictive markers of INF response.

Molecular assays: detection and quantitation

While the diagnosis of HCV is currently based on the detection of antibodies via ELISA, the technique is less sensitive in the early phases of HCV infection and cannot differentiate between active infection and disease resolution. Also, immunocompromised patients such as those who are infected with HIV, or hemodialysis patients, produce fewer antibodies. [11] The direct molecular qualitative detection of HCV RNA by reverse transcription (RT) and PCR are considered the gold standard for the diagnosis of HCV infection and for assessing the antiviral response to

INF therapy. Quantitative assessment of HCV RNA levels, via signal amplification and quantitative PCR (Q-PCR), are valuable tools in the clinical management of patients before, during and after therapy. PCR-based assays are able to ascertain minute amounts of HCV RNA in serum or plasma. HCV RNA detection by PCR helps to resolve weakly positive or negative ELISA results when clinical signs and/or risk factors are compatible with HCV infection.

A variety of home-brew or in-house PCR assays to test for the seropresence of HCV RNA are available. However, numerous factors add to the variability of such PCR testing including the handling and storage of samples, DNA product contamination, correct design of amplification parameters, and efficacy of postamplification detection. To that end, only 5 of 31 (16%) laboratories scored perfectly on a standardized test. [12] Of these 5, a 100-fold difference in sensitivity was reported for the dilution series. Almost one-third of the laboratories produced false negative and/or false positive results. The investigators concluded that contamination was a major problem. Contamination can come from two areas: carry-over results in the amplification of PCR products that were synthesized during previous PCR reactions, and cross-contamination whereby one sample is contaminated by a positive sample at any given step in the procedure.

A reliable, standardized assay for HCV RNA can convey 1) whether a patient will likely respond to INF therapy, 2) if a virologic response has occurred and 3) promote a better comprehension of the relationship between viral load and the natural history of chronic HCV infection. [13] In studies by Davis et al [14] and McHutchison et al [15], HCV RNA levels correlated with rates of response to INF and/or ribavirin therapy. Late clearance of HCV RNA from serum during combination therapy was

associated with a sustained response, a phenomenon not typically seen in patients treated with INF alone. [McHutchison] The genotype and the pretreatment serum HCV RNA level were related; response rate was as high as 100% in patients with low HCV RNA at baseline and a genotype other than type. 1 [14]

The only standardized Q-PCR is the Roche Monitor assay but experience with this assay has been limited. The main strength of Q-PCR assays, however, is their high analytic sensitivity with reports as low as 1,000 RNA copies per milliliter. [4] The biggest disadvantages include high assay variability and limited linear range above 1 million RNA copies per milliliter. [4] In contrast, the branched chain DNA assay (bDNA), a signal amplification technique, is highly standardized. The second generation bDNA provides a modest increase in sensitivity compared to the previous generation and minimal bias in measuring HCV RNA levels for the major HCV genotypes.

Direct detection of as few as 1,000 hepatitis viral genomes is possible. [16] In a study [17] using the Quantiplex HCV RNA 2.0 assay (bDNA-2), the Quantiplex RNA assay (bDNA-1) and the Roche monitor assay, highly reproducible results were observed upon repeat testing of samples by both the bDNA-1 and the bDNA-2. A greater variability was observed in the Roche Monitor assay (correlation coefficient of 0.537, compared with 0.942 and 0.964 for the bDNA-1 and bDNA-2 assays, respectively). Significant differences in the efficiency of detection of genotypes 1, 2, and 3 were observed for the bDNA-1 and Roche Monitor assays, whereas the bDNA-2 assay and nested PCR at limiting dilution were able to quantify genotypes with equal sensitivity. These results suggest that many of the previous studies evaluating the effect of genotype and virus load on the response to INF

using methods such as the Roche Monitor assay and other competitive PCR methods require reinterpretation. Differences in efficiency of quantitation should be considered in future trials that investigate the relationship between genotype and virus load.

The clinical value of bDNA assay has been the object of several performance studies. Jacob et al [13] compared the relative sensitivities of first-and-second generation branched nucleotide assays (Quantiplex HCV RNA 1.0 and 2.0, respectively, Chiron, Emeryville, CA) for the detection of HCV RNA to that of a RT-PCR method (Monitor, Roche Molecular Systems, Nutley, NJ) in 53 patients with chronic hepatitis C. They concluded that both methods can be used to detect HCV RNA in patients who are infected with the genotypes that are most commonly encountered in the United States. The HCV RNA 2.0 bDNA assay may offer advantages when attempting to quantify high-level viremia. Lu et al [11] compared the Roche Amplicor Monitor to the Chiron bDNA assay in quantitative measurement of serum HCV in patients with chronic hepatitis C. The serum of the patients was qualitatively positive by RT-PCR. They concluded that the Roche Amplicor HCV Monitor test kits and the Chiron bDNA are equally sensitive in the quantitative measurement of serum HCV RNA in patients with chronic hepatitis C and can be reliably used in measuring HCV viremia clinically. Roth et al [18] observed that RT-PCR and bDNA in clinically significant agreement in measurement of HCV RNA concentrations, despite subtype-specific differences. However, they stressed that when monitoring an individual patient, kits and methods should not be interchanged. Comparing the bDNA with a semi-quantitative cDNA-polymerase chain reaction (cDNA-PCR) in monitoring HCV RNA levels, the bDNA assay was not as sensitive as cDNA-PCR, given its user

friendliness and quantitative results, but it is considered a useful test for monitoring HCV RNA levels in patients treated with INF.

However, patients who are non-reactive in the bDNA assay have to be re-tested by cDNA-PCR because low viral titers are not detected by the bDNA assay. [19] Actual sensitivities of the tests cannot be deduced from the manufacturers' stated HCV RNA cut-offs. Analytic sensitivity is estimated according to the smallest amount of HCV RNA detected and reliably quantified. Manufacturers of the Roche Monitor assign a cut-off of 1,000 copies per milliliter. Manufacturers of the Chiron bDNA 2.0 assay state the cut-off is 200,000 equivalent genomes per milliliter. This suggests that the Roche product is the more sensitive of the two assays. In reality, the Roche copy and the Chiron genome do not represent the same amount of HVC RNA in a clinical sample. [20] A global standardized system is needed that utilizes quantified standards, such as nucleic acid transcripts of the same nature, length and sequence.

Quasispecies

The RNA-dependent polymerase of RNA viruses is highly error prone and lacks proofreading capabilities. Consequently, within any given individual, HCV exists as a heterogeneous mixture of closely related viruses called quasispecies. In contrast to HCV genotypes, which vary by 31% to 35% of bases over the entire length of the genome, quasispecies vary from each other by 1% to 9% of bases. [21] The quasispecies nature of HCV has several potentially significant biological consequences. They are likely an important factor in the inability of acutely infected individuals to clear infection. Additionally, mutations in the viral populations likely contribute to drug "resistance" during INF treatment and to the ineffectiveness of isolate-specific vaccines. Both direct and

indirect methods of detecting and quantifying quasispecies within an individual exist. Early studies utilized cloned PCR products, a procedure that is relatively easy to perform and reliable, but labor-intensive. Indirect methods of measuring the number of different viral populations within an individual include single-strand conformation polymorphism (SSCP). In SSCP analysis, products of PCR are subjected to electrophoretic analysis under denaturing conditions, such that single-stranded RNA is obtained. Single nucleotide polymorphisms result in different mobilities of the single-stranded fragments. Depending on the quasispecies diversity, as well as the sensitivity of the RNA staining technique, a range of the most prevalent variants can be observed. Using SSCP, investigations of large patient cohorts with chronic HCV infection can be undertaken. [22]

In direct sequencing, PCR products obtained are not cloned from PCR fragments, but all RNA strands with varying sequences are directly submitted en masse to sequence analysis. The presumed sequence represents the master sequence and can show degeneration at certain positions, that is, certain positions may, for example, show both an adenine and guanine residue. Degeneration can only be observed when the minor sequence is observed in 20 percent or more of the RNA strands. [23]

Quasispecies measurement can be used to predict INF responsiveness in patients with mutations in HCV genotype. Polyak et al [24] used nucleotide sequencing to show that INF therapy frequently exerts pressure on the second envelope glycoprotein gene hypervariable region 1 (HVR1) in HCVinfected individuals. This pressure results in quasispecies distribution in such persons with genetic complexity significantly higher in transfusion recipients than in intravenous drug users. Laskus et al [25] used SSCP and sequencing to

determine that, in the presence of HIV-1 infection, viral sequence differences existed in the same tissue samples, arguing in favor of extrahepatic HCV replication that can interfere with HCV therapy.

Sequence determination and phylogenetic analysis

Sequencing of the E2 HVR1, followed by phylogenetic analysis is recommended for studying patientto-patient transmission (i. e., hematology ward), analysis of interspousal transmission, nosocomial infections in a hemodialysis unit, and in geographical regions with a high endemicity of only one subtype (e. g. subtype 1b in Belgium or Sicily). In a multivariate analysis of pretreatment parameters with a sustained virological response to treatment, three parameters appear to be independent predictors of a treatment response: a low viral load (P<.04), a low anti-HCV core IgM titer (P=.03) and a low genetic complexity of HVR1 major variants (P<.04). [24] However, the E2 HVR is too heterogeneous to be of value for classification of HCV genotypes. Instead, the 5' NCR, core, E1 and NS5B regions are frequently amplified for the purpose of genotypic classification. Phylogenetically analyzing a subtype needs to factor in, beyond simple sequence differences, the possibility of mutation and reversions. Nucleotide sequence analysis of hepatitis C virus (HCV) strains showed substantial variability leading to a classification into several genotypes and subtypes. The data correlating HCV genotypes and subtypes with hepatitis C viremia levels, demographic characteristics of patients (age, mode of transmission, duration of infection), and severity of liver disease conflict. The interpretation of clinical studies is further complicated because the molecular methods used lacked specificity for genotyping/subtyping and underestimated viremia levels,

especially in patients infected with HCV genotypes 2 and 3.

Zeuzem et al [26] sought a connection between phylogenetic analysis of HCV isolates and viremia, liver function tests, and histology. HCV subtyping was performed by sequence and phylogenetic analysis of the nonstructural (NS)-5 region and assessed serum HCV-RNA concentration by a validated genotype-independent quantitative reverse-transcription-polymerase chain reaction assay using an internal RNA standard. There were no significant differences between median serum HCVRNA concentrations in patients infected with different genotypes/subtypes. Although patients infected with HCV-1b were older, no biochemical or histological evidence was obtained that this subtype is associated with more severe liver disease. Furthermore, this study showed a lack of correlation between the serum HCV-RNA concentration, biochemical parameters, and liver histology. The median serum HCV-RNA levels in patients with chronic persistent hepatitis, chronic active hepatitis, and liver were not significantly different and no correlation was shown between HCV genotypes/subtypes, viremia, liver function test results, and histology.

Genotyping/Subtyping: Divergence within the human population

Not only do HCV quasispecies sequences express variability in different regions of the genomes, but isolates also differ among themselves. All isolates separate into phylogenetically related clusters called subtypes. One or several subtypes can be classified into several major types that show similarities over 65 percent to 75 percent over the total genome. The term genotype is used generically to refer to subtypes, types or both. Use of the term genotype to describe quasispecies variants is not appropriate. Eleven HCV genomes are known to exist [23] as well as more than 90 subtypes [27], with more

subtypes being discovered at a continuous rate. [23]

Genetic variation can determine the success of therapy in a patient with HCV. For example, INF-alpha therapy is more effective in genotypes 1a, 2, 3 and 5 than in 1b and 4 infections. Post-transplantation, recurrent subtype 1b and type 4 infections proceed much faster to chronic hepatitis in the new graft, while subtypes 1a and 2a show very similar, more benign recurrences. [23] Most importantly, HCV genotypes are distributed differently, depending on geography and etiology. For these reasons, genotyping is important.

Several screening tests have been developed to identify HCV genotype, and include reverse hybridization line probe assay (LiPA, Innogenetics, Zwijnaarde, Belgium), restriction fragment length polymorphism (RFLP) of the PCR amplicons, and nested PCR with genotype-specific parameters to the core region. The optimal genotyping region is reported to be the 5' untranslated region (UR) because of high conservation within, but considerable variation between, genotypes. LiPA technology is based on the reverse hybridization principle in that biotinylated PCR fragments are hybridized to a selection of highly specific immobilzed probes. In a second step, the biotin group in the hybridization complex is exposed by incubation with a streptavidin-alkaline phosphatase complex and the appropriate chromogen compounds. Previously, an LiPA was developed that allowed discrimination of HCV types and subtypes and was capable of detecting single nucleotide differences in the 5' UR. In a more recent study, 21 probes dispersed over seven variable 5' UR areas were applied to an LiPA and used to analyze 506 HCV-infected sera from different geographical regions resulting in an abundance of subtypes. The investigators concluded that the selected probes detected the corresponding signature motifs in the

seven variable regions with 100% reliability. [27] In addition, these motifs allowed correct type interpretation of samples collected worldwide, with the exclusion of Vietnam, Thailand, or Vietnamese patients residing in European hospitals. Finally, subtyping specificities vary according to geographical region, with 11 prototype subtyping patterns identifying the majority of samples from Europe and the Americas. These results indicate that the LiPA is a reliable assay applicable to routine typing and subtyping of HCV specimens. [28]

A study that used LiPA to determine HCV genotypes in tertiary referral centers through the United States found that the proportion of patients with HCV had types 1 (71.5%), 2 (13.5%) and 3 (5.5%). Patients with HCV type 1 had a longer estimated duration of infection compared to patients with HCV type 3 (P=.004) and type 4 (P<.05). Disease activity and viremia levels did not differ among patients with HCV types 1, 2 or 3 but patients with type 4 had a lower level of viremia than patients with type 1 (P<.05). [29]

In RFLP analysis, a single PCR fragment is amplified from a certain region of the HCV genome with universal primers. Restriction enzyme recognition sites present in the DNA fragment usually show subtype- or type-specific distribution. Thus, restriction fragments with varying lengths are created after cutting the PCR fragment with one or several restriction endonucleases. The electrophoretic separation of these fragments lets the observer infer the approximate lengths of the restricted fragments and, in turn, identify the genotype.

A Spanish study used RFLP analysis to identify the HCV genotypes in their country and to show a relationship between the genotype and disease severity. The results showed that genotype 1b was associated with advanced liver disease, including

hepatocellular carcinoma and cirrhosis in Spain. However, the investigators conceded that the results may be related to a cohort-effect caused by overrepresentation of genotype 1b in older patients with more advanced disease. [30]

Genotypes of HCV differ in their biologic effects. Variations in HCV genotype have major implications in the design of HCV vaccines and biotherapeutic agents. Antibody elicited by one genotype may not protect from reinfection with other variants, as has been observed with other enveloped viruses. Some HCV-infected individuals infected experience multiple episodes of acute hepatitis. It is unclear whether these episodes are due to reinfection with HCV or to reactivation of the original virus infection. Genotype-related differences may also include viral replication rates, mutation rates, histologic inflammatory activity, disease severity and INF response. The development and clinical application of HCV genotyping assays are the object of much research.

Serotyping

Determination of hepatitis C virus (HCV) genotype could be routinely run in the future to tailor treatment schedules for patients with chronic hepatitis C. The suitabilities of two versions of a serological, so-called serotyping assay (Murex HCV Serotyping Assay version 1-3 [SA1-3] and Murex HCV Serotyping Assay version 1-6 [SA1-6]; Murex Diagnostics Ltd.), based on the detection of genotype-specific antibodies directed to epitopes encoded by the NS4 region of the genome, for the routine determination of HCV genotypes were studied by Pawlotsky. [31] The NS4, E1 and a small variable region in the core yield type-specific antigenic determinants. Type-specific B-cell epitopes have also been reported in the NS4A and NS4B regions, thus, single or branched peptides obtained from the NS4A and NS4B regions can be used

for serotyping. For serological determination of HCV genotype in the study, SA1-3 and SA1-6 were compared to reverse hybridization line probe assay (LiPA). The results showed that SA1-6 had increased sensitivity relative to SA1-3 but remained less sensitive than the genotyping assay on the basis of PCR amplification of HCV RNA. Cross-reactivities between different HCV genotypes could be responsible for the mistyping of 8 percent (SA1-3) and 6 percent (SA1-6) of the samples. Subtyping of 1a and 1b is still not possible with the existing peptides, but discriminating between subtypes may not be necessary for routine use.

Liver biopsy

Grading and staging liver biopsy lesions is important in HCV, particularly for patients with necroinflammation, septal fibrosis and regions of modularity on initial biopsy who are at high risk for developing advanced necrosis in the ensuing decade. [32] Liver biopsy, when combined with periodic serum alanine aminotransferase (ALT) measurements, can be useful in determining the severity or activity of liver disease and the stage or degree of fibrosis. Rate of progression to cirrhosis has been seen to accelerate in patients whose initial biopsies showed high-grade and high-stage lesions. Liver biopsy is recommended prior to treatment to obtain a baseline disease stage and to exclude other forms of liver disease, such as concurrent alcoholic liver disease and iron overload. However, liver biopsy is costly and carries its own risk. Thus, monitoring patients who are being treated should involve serial ALT measurements and qualitative HCV RNA testing.

RT-PCR has been used to obtain molecular evidence for intrahepatic HCV replication occurring shortly after liver transplantation. The level of replication does not correlate with the development of recurrent hepatitis, which suggests that HCV can replicate

without inducing morphological evidence of liver damage as seen by biopsy. [33] RT-PCR can also be used to clarify the relationship between anti- HCV to HCV infection of the liver by detecting HCV sequences in liver tissue. These findings were found feasible in the study reported by Shieh, et al. [34] However, repeatedly negative RT-PCR tests for HCV RNA in serum does not indicate the absence of HCV from the liver. [35] The majority of long-term responders who have been on INF therapy do test negative for HCV RNA in the liver, suggesting definite eradication of HCV RNA infection. [36] RT-PCR with unlabeled primers followed by in situ hybridization (RT-PCR-ISH) and in situ RT-PCR with FITC-labeled probes (RT-PCRd) showed HCV signal in all liver biopsies of patient who had been treated with INF one year after treatment stopped. [37]

The TORDJI-22 MoAb (BioGenex, San Ramon, Calif) is specific for the C-100 protein of the hepatitis C virus, and was compared with RT-PCR of tissue for viral RNA. Immunohistochemistry with the TORDJI-22 monoclonal antibody was found to be a very specific, fairly sensitive diagnostic test for hepatitis C virus in fixed liver tissues. [38] The identification of hepatitis C virus antigen (HCVAg) in liver tissue indicates that the presence of viral antigens in hepatocytes of patients with transplants and recurrent HCV infection is a consistent finding one month or longer after transplantation. However, the relationship between the antigen and the development of pathologic changes remains to be investigated. [39]

Non-invasive markers of fibrosis or cirrhosis The synthesis and deposition of hyaluronic acid (HA) increases during fibrogenesis. Serum HA is a useful marker in chronic CV patients in that it can be used to monitor patients at risk of progressive fibrosis, in controlled clinical trials, as a measure of antifibrogenic response

and in those in whom liver biopsy is difficult or contraindicated. [40] Cirrhosis can be correctly diagnosed in over 90% of patients with chronic liver disease by observing serum HA concentration. [41] Serum procollagen-III peptide has been shown to be valuable in predicting the development of chronic active fibrogenic liver disease.

In chronic viral C hepatitis, the levels of HCV-RNA correlate directly with the severity of hepatic histology and inversely with response to INF therapy. Serum procollagen-III peptide provides a relatively noninvasive means of following disease progression. [42] Laminin is an extracellular matrix component that, when measured in the serum, correlates with severe complications of liver cirrhosis. Patients who present with elevated serum laminin have a high risk of developing severe complications.

Both HA and laminin can be used as prognostic markers in addition to the Child criteria in liver cirrhosis. [43]

Cryoglobulin

Among the many conditions that are spawned by HCV infection, mixed cryglobulinemia is by far the most closely linked to HCV infection. A high frequency of mixed cryoglobulinemia (types II and III) is seen in patients with chronic HCV infection. In HCV-positive patients, stage of liver disease correlate with the prevalence of cryoglobulinaemia. Patients with type II cryoglobulins showed a significantly higher risk of cirrhosis and of extrahepatic manifestations while patients with type III cryoglobulins had a significantly higher prevalence of hepatocellular carcinoma. In one study by Donada et al [44], type II cryoglobulin patients had an odds ratio of 11.9 of death from extrahepatic complications during follow-up while type III patients had an odds ratio of 3.4 of dying from hepatic disease.

Protocols:

DNA extraction from fecal samples

- 1 g stool, frozen at -70°C,is diluted in 10 mL of lysis buffer (Tris-HCL, 0.5 M; EDTA, 20 mM; NaCl, 10 mM; SDS, %0.1; pH 9.0) (TEN-9) in 50 mL tube.
- After vortexing for 5 minutes, samples were homogenized by shaking for 10 minutes.
- Samples were then diluted again (1/2) with 10 mL lysis buffer and homogenized for 5 minutes. Particulate materials were removed by centrifugation at 4500 ×g for 10 min.
- After transferring the supernatant to a new tube, approximately 10 mL of supernatant.
- DNA was precipitated by adding 5 ml ammonium acetate 7.5 M (half of the sample volume) and 25 ml of ice-cold ethanol 95-100% (twice the sample volume).
- Incubation at -20°C for 20-30 minutes will render a better precipitation.
- DNA was collected following centrifugation at 4500 ×g for 15 minutes at room temperature.
- In this step, precipitated DNA is not colorless and contains the bile salts. The DNA pellet was re-suspended in 600 µl of TE (pH 8) and incubated at 65°C for 15 minutes.
- Then, DNA was extracted organically and also purified using conventional single step phenol/chloroform/ isoamylalcohol protocol. 10.After isopropanol precipitation, the colorless DNA pellet was collected and dissolved in 300 µl of Tris-EDTA buffer following an overnight incubation at 37°C.

PCR

Setting up the PCR reaction

A fresh master mix tube which contains the dNTP and Taq DNA polymerase was taken and the following components are added

- Master Mix vial 10 µl
- Forward Primer 1.5 µl
- Reverse primer 1.5 µl
- Template DNA 2 µl
- Nuclease free water 5 µl

  Total 20 µl

(Master Mix contains 1U Taq DNA polymerase, 10X Taq buffer, 10 mM dNTPs and PCR grade water. Primer dye mix contains 10 uM forward primer, 10 uM reverse primer and PCR compatible dye with glycerol).

After addition of all the components the PCR tube is gently spun down in centrifuge briefly and is placed in the Thermal cycler. The thermal cycler was programmed as follows.

Program 1 (one cycle) (Initial denaturation)94°C for 2 minutes

Program 2 (30 cycles) (Amplification)

Step one (denaturation) 94°C for 45 seconds

Step two (annealing) 56°C for 1 minute

Step three (extension) 72°C for 1 minute 30 seconds

Program 3 (one cycle - final extension)

72°C for 5 minutes, then hold at 4°C

The programmed temperatures of the PCR reaction is represented in the diagram shown belowAgarose gel electrophoresis:

REQUIREMENTS -

- 1% Agarose gel.
- TBE buffer.
- Electrophoresis assembly.
- Ethidium bromide.
- Bromophenol blue etc.

COMPOSITON OF TBE BUFFER -

10X FOR 50 ml

- Tris base - 5.45 gm

- Boric acid- 2.75 gm
- EDTA- 0.23 gm

COMPOSITION OF LOADING DYE

-FOR 10 ml

- 25 mg brophenol blue
- 4 mg sucrose

PROCEDURE

- Prepare 1.2% agarose gel in TBE buffer by dissolving 0.24 gm agarose in 20 ml TBE buffer.
- Add 20 µl Etbr at 55˚c before setting gel
- Add gel into electrophoreses base plate and wait for solidification
- Remove comb carefully and pour TBE buffer into apparatus.
- Prepare samples to be electrophoresis and add 2µl of loading dye to sample
- Load different sample in different well and switch on the power supply
- Electrophoresis carried out at 100V
- Of f current when tracking dye moves ¾ the of gel
- Remove gel for visualization

ELISA:

Antigen Coating:

- Antigen diluted in the ELISA coat buffer is added into the Polystyrene wells. The dilution of the antigen should be in such a pattern that the amount of the antigen adsorbed to the polystyrene plate must be maximum at such dilution. Here the concentration taken is 10µg/ml.
- Incubation for 2 hours to over night at the temperature of 370C
- Store the antigen solution until the immunoassay is performed.
- Before performing the Immunoassay the wells should be washed in the following way.

- PBS-TWEEN wash: Add 300µl of the PBS-TWEEN buffer, mix it well and leave it for 2 minutes. After that aspirate the buffer from the wells.
- BSA-ELISA wash : Add 300µl of the BSA-ELISA coat buffer to the wells and incubate at room temperature for about 20 min.
- Repeat the PBS-TWEEN wash.
- Repeat the BSA-ELISA wash.Test sample addition:

- Add 300µl of undiluted antibody suspension.
- Incubate it for 4 hour at room temperature or for overnight at 4 ^{o}C.
- PBS-TWEEN washes for 3 times

Conjugate addition:

- Add 300µl of 1:5000 v/v diluted [in PBS_TWEEN buffer] conjugate.
- Incubate at room temperature for 2 hrs.
- PBS-TWEEN washes for 3 times.
- Add 300µl of 0.15M NaCl
- Mix the solution and leave for 1 to 2 minutes
- Aspirate the solution and follow the successive washes.

Substrate addition:

- Add 300µl of substrate to each well.
- Watch for the development of the color
- Here the substrate is present in the Sodium Carbonate buffer [pH 9.8] in the concentration of 1 mg/ ml where the buffer is also containing Magnesium Chloride at the molarity value of 1/1000M

ELISA Readings:

The color development is measure by the help of ELISA reader at the wavelength of 400nmn.

Immunoelectrophorosis:

- Prepare 5 ml of 1.0% agarose in 1X Assay buffer by heating slowly till agarose dissolves completely. Take care not to froth the solution.
- Clean the glass slide, electrophoretic apparatus with alcohol thoroughly to make it grease free and for even spreading of agarose. Mark the end of a glass slide that will be towards negative electrode during electrophoresis
- Place the glass slide on a horizontal surface. Pipette and spread 5 ml of agarose solution onto the slide. Take care that the slide is not disturbed and allow the gel to solidify.
- Punch ~4 mm diameter well with gel puncher or with the back of a 200 µl micropipette tip according to the template indicated in Figure and cut the trough with the help of a gel cutter or a scalpel. The distance between the trough and the edge of each well should not be more than 0.5 cm.
- Remove agarose gel plugs with a toothpick or forceps. Carefully remove the gel piece from the trough so as to not to damage or lift the gel from the glass slide.
- Transfer the glass slide containing the gel to the electrophoresis apparatus; ensure that the wells are towards the cathode.
- Gently lay the filter paper wicks over the ends of the gel (they should overlap about 3 to 4 mm) and allow them to become saturated with 1X Assay buffer.

- The wicks should be submerged in the buffer. Press lightly on the wicks to ensure good contact between the gel and the running buffer. If necessary, add more buffers, but do not cover the gel with buffer.
- Load 30 µl of antigen solution into the well as indicated by the diagram. Loading of wells should be carried out quickly to minimize diffusion from the well.
- Carefully, connect the cord of the electrophoresis apparatus; insert the cords into the power supply with the black cord in the black (negative) input and the red cord into the red (positive) input.11.Turn on and set the power supply for the required voltage (60 Volts). To ensure proper current flow check for bubbles formed on the electrodes.
- Electrophorese the antigen until the blue dye has migrated to the ends of the troughs.
- After electrophoresis is completed, turn off the power, unplug the power source, disconnect the leads and remove the cover.
- Discard the filter paper wicks and carefully remove the glass slide from the electrophoresis apparatus. Set the tray on a level surface and proceed with the diffusion steps.
- Add 75 µl of antibody to the appropriate trough. Ensure even spreading of the antibody solution along the entire length of the trough.
- Place the glass slide in a moist chamber box and allow diffusion to take place for over 24 to 48 hours, or until visible precipitates form in the gel. The chamber can be placed in a 37°C incubation oven or remain at room temperature.

- Observe for precipitin lines between antiserum troughs and the antigen well.

Rocket immumoelectrophorosis:

Material Required

- Standard antigen: BSA
- Antiserum: Goat anti BSA

Procedure:

- Prepare 10 ml of 1.0% agarose (0.1 g/10 ml) in 1X electrophoresis buffer by heating slowly till agarose dissolves completely.
- Allow the molten agarose to cool to 55°C.
- Add 1 ml of antiserum to 10 ml of agarose solution. Mix gently, ensure uniform distribution of antiserum.
- Pour the mix onto a glass plate placed on a horizontal surface and allow it to gel/solidify.
- Place the glass plate on the template holder provided (in ETS-2) and fixes the RIEP template.
- Punch 3 mm wells with gel puncher towards one edge of the plate.
- Place the glass plate in the electrophoresis tank; ensure that the wells are towards the cathode.
- Fill the tank with 1X electrophoresis buffer till it covers the gel.9. Connect the power cord to the electrophoretic power supply according to the convention: Red: anode and Black: cathode.
- Add each of the given standard antigen and test antigen to the wells. Loading of wells should be carried out quickly to minimize diffusion from the well.
- Electrophoreses the samples at 100 volts, till the rockets are visible or the dye front reaches the edge. This generally takes 1 to 11/^ hours. Electrophoresis

can be continued for an additional 15 minutes after the dye has run out of the gel. This ensures better visibility of the precipitation peaks.

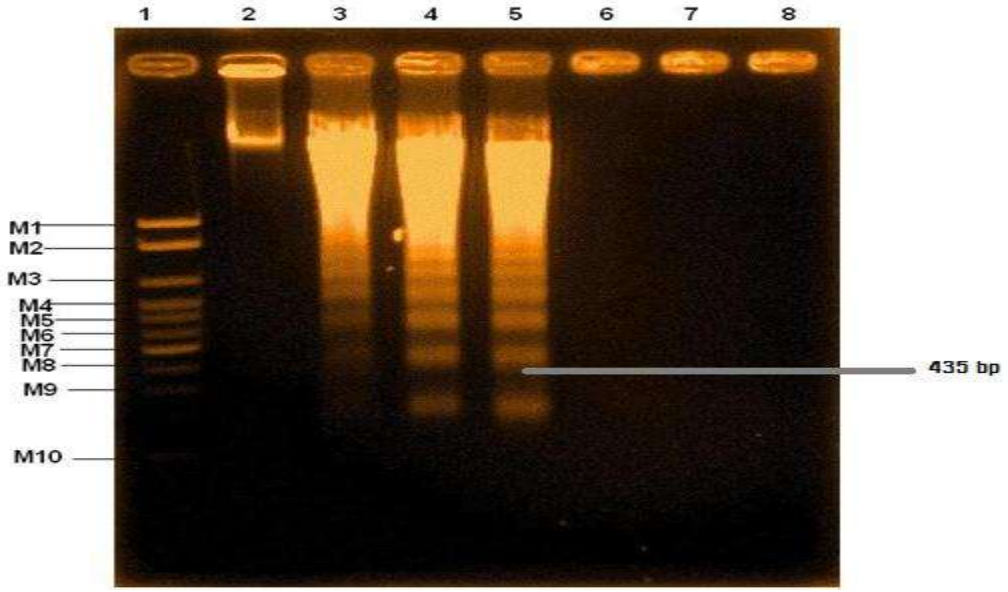
- Stop electrophoresis; remove the glass plate from the electrophoresis tank.
- Observe the precipitation peak or rocket formed against a dark background. If the rockets are still not clear; incubate the plate in a moist chamber at room temperature for 1 hour to overnight.
- Measure the rocket height from the upper edge of the well to the tip of the rocket.
- Construct a standard graph by plotting the height of the rocket on Y-axis (linear scale) against the concentration of antigen on X-axis (log scale) on a semi-log graph sheet.
- Determine the concentration of antigen in the test sample by reading the concentration against the rocket height from the standard graph.

Results:

PCR cycler

Agarose Gel Electrophoresis:

In this Agarose gel electrophoresis result can be observed between M8 and M9 with the help of the DNA marker.



M1 – 2000bp M2 – 1800bp M3 – 1600bp M4 – 1400bp M5 – 1200bp M6 – 1000bp

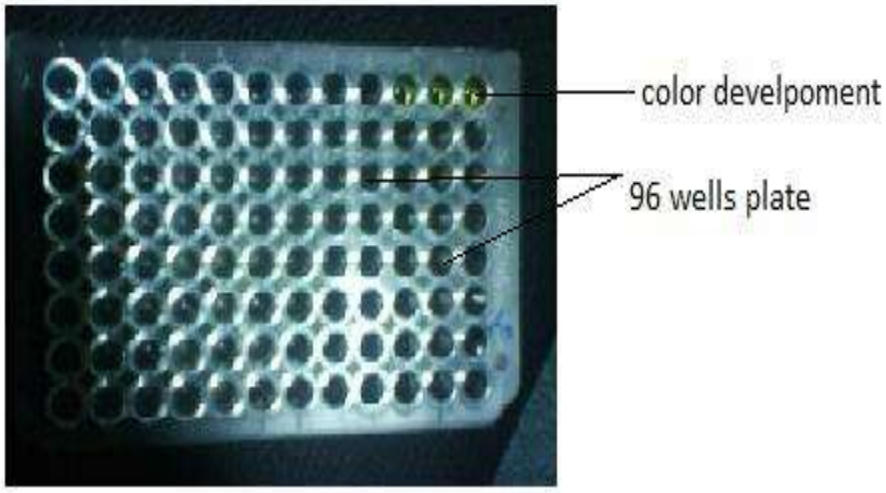
M7 – 500bp M8 – 400bp M9 – 200bp M10 – 100 bp

1- DNA Ladder

The gene of interest selected from the adeno virus is E1B-144R stereotype 34 which has a base pair length of 435 is seen to be observed.

ELISA

The extent of color development is inversely proportional to the amount of analyte in the Sample or standard. Development of color reaction through enzyme catalysis. ABTS (2, 2' Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) is used to detect HRP and Yields a water soluble green end reaction product. The green product has two major absorbance Peaks, 410 nm and 650 nm.



GenWay Adenovirus IgG antibody test kit (GWB-AEBE26) is used for ELISA test. The adenovirus antigen is coated to the titter wells and antibody ie the serum from the blood is added then ready-to-use anti-human-IgG peroxidase conjugate is added and the substrate (TMB) solution is added and the specificity of the IgG antibodies is directly proportional to the intensity of the color.

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Qualitative Immunoelectrophoresis

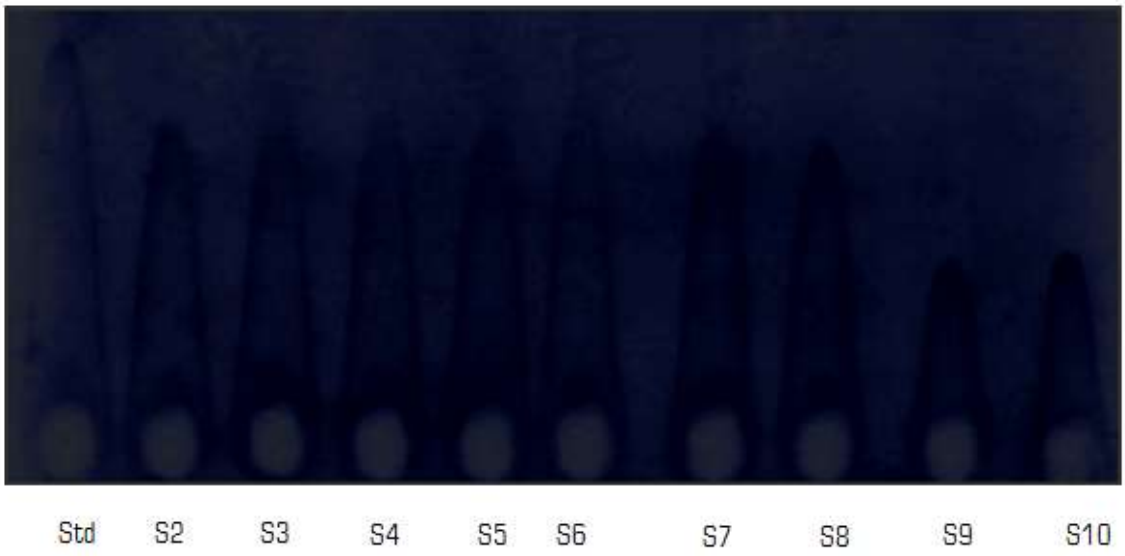
The formation of precipitin line indicates the presence of antibody specific to the antigen.

1. Homogeneity of the antiserum to the antigen is denoted by presence of a single continuous precipitin line.
2. Heterogeneity of the antiserum to the antigen is denoted by presence of more than one precipitin line which not only gives an indication of the number of immunodominant epitopes, but also the non-identical nature of such epitopes.

Display of precipitin lines

Rocket immunoelectrophoresis:

The height of the precipitin peak depends on the concentration of antigens loaded in the corresponding wells.



Precipitin peaks

By plotting the graph of concentration of antigens versus length of the precipitin peaks one can calculate the concentration of test antigen.

S2=Sample 2=49 mm

S3=Sample 3=43 mm

S4=Sample 4=40 mm

S5=Sample 5=38mm

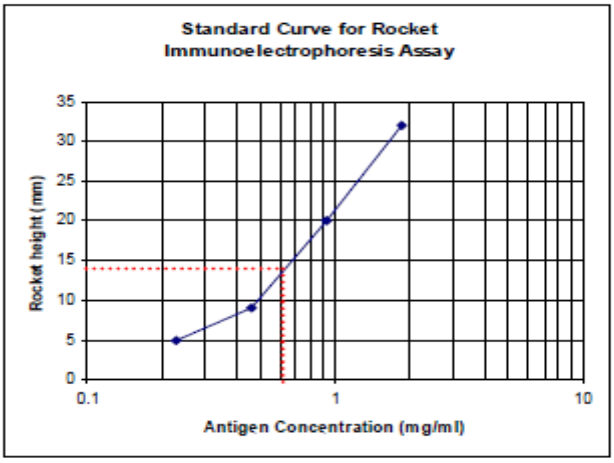
S6=Sample 6=34 mm

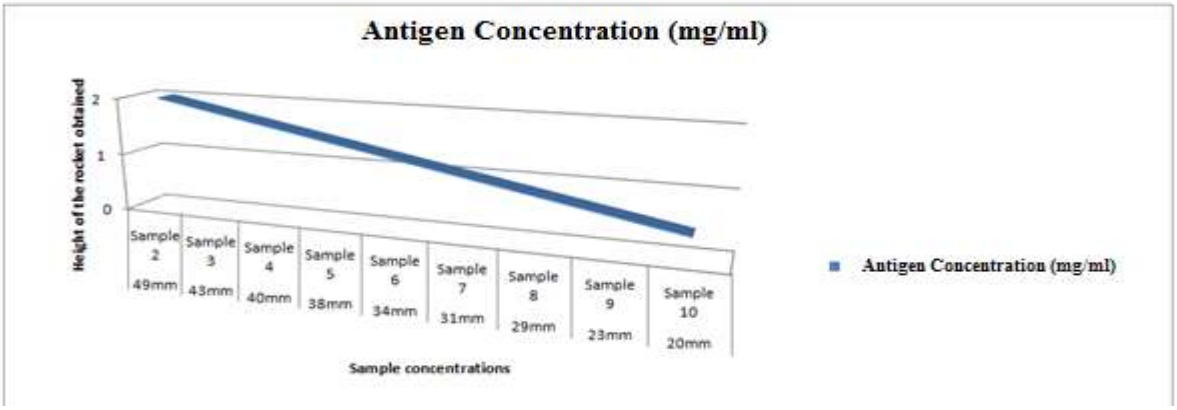
S7=Sample 7=31 mm

S8=Sample 8=29 mm

S9=Sample 9=23 mm

S10=Sample 10=20 mm





Qualitative Radial immuno diffusion:

Precipitin ring formation

The diameter of the precipitin ring depends upon the concentration of antigens loaded in the wells. By plotting the graph of concentration of antigens versus diameter of the corresponding precipitin ring one can calculate the concentration of any test antigen.

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