Characterization of epitope and Fcγ receptor specificity of HCV and HBV neutralizing antibodies, and retrospective analysis of HCV infections in general population

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology

by

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Certificate of Approval

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Asma Ahsan

Publications

1. **Ahsan A**, Khan AZ, Javed H, Mirza S, Chaudhary SU, Shahzad-Ul-Hussan S. Estimation of hepatitis C prevalence in the Punjab province of Pakistan: A retrospective study on general population. *PLoS One*. 2019 Apr 3;14(4):e0214435. doi: 10.1371.

Contents

Summary

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections occur in approximately 257 million and 170 million people, respectively in the world accounting for an immense economic burden. These are blood borne infections characterized by liver inflammation that can develop into liver cirrhosis and fibrosis. In Pakistan, around 20 million people are affected by these two infections. Vaccine is available against hepatitis B and under current regulations every child is vaccinated. However, an effective vaccine against HCV remains to be developed. In recent years, several direct acting antiviral drugs have been approved to treat HCV infections, which can clear the virus. However, their effectiveness to lower the global disease burden is limited by their cost ineffectiveness, unavailability to low-income populations, associated side effects and emergence of drug resistant viral variants. The current project is focused on three different aspects of HCV and HBV infections including understanding the epitope specificity of antibodies present in the sera of chronic HCV patients and spontaneous resolvers of the infection, estimation of HCV prevalence in the Punjab province of Pakistan on the basis of retrospective analysis and characterization of different classes of FcγR activation by HBsAg specific antibodies present in Hepatitis B vaccinated individuals and self-resolvers of the infection.

HCV envelope glycoprotein, E2 is the primary target for immune recognition. The recombinant E2 protein based vaccines have not been successful possibly due to a highly glycosylated nature of E2 and the presence of highly variable regions that cover the more conserved parts of the protein. The E2 protein also incorporates few highly sequentially conserved regions that

constitute receptor-binding site of the virus as this protein mediates binding of the virus to its receptors. In chronically infected patients, antibodies targeting some of these conserved epitopes are produced that can neutralize a broad range of viral variants. Some of these antibodies recognize linear amino acid sequences, called linear epitopes despite they adopt specific structural and conformational features when recognized by the antibody. Although most of the identified HCV neutralizing antibodies are conformational epitope specific but some broadly neutralizing antibodies specific for linear epitopes have shown promising protection in animal models.

Around 25% of the HCV infected patients can clear the virus without any treatment. Cellular immunity has been described to be responsible for such viral clearance. However, the role of antibodies in spontaneous virus clearance has not been clear. Therefore, to delineate the role of antibodies in such HCV clearance has a high significance in particular in vaccine design perspective. In this project we aimed to elucidate the conserved linear epitope specificity of antibodies present in the sera of chronic patients and self-resolvers to understand the role of these antibodies in viral clearance.

We used combination of peptide ELISA, pseudo-typed HCV neutralization assay (HCVpp) and peptide competition HCV neutralization assay to identify the presence of conserved linear epitopes specific antibodies in the sera of chronic patients (CP) and self-resolver (SR) individuals. We observed that both groups of sera showed reactivity for peptides corresponding to different conserved linear epitopes. However, unlike the CP sera only 46% of the SR sera showed reactivity to any of the peptide corresponding to conserved linear epitopes. In most of these cases this reactivity was primarily for the epitope corresponding to the E2 region spanning amino acid 434-446 (the 434 epitope). Most of the CP and SR sera neutralized the virus in HCVpp assay, however the overall ED_{50} (effective dilution with 50% neutralization) values of the CP sera were much higher as compared to the SR sera suggesting much higher titer of total neutralizing antibodies in the CP sera. In peptide competition assay, the neutralization activity of the CP sera was hardly affected by the presence of any competing peptide corresponding to linear epitope. However, neutralizing activity of many of the SR sera was significantly decreased by the presence of a competing peptide corresponding to the 434 epitope suggesting the viral

neutralization was primarily due to the presence of 434 epitope specific antibodies in those sera. These results suggested the role of antibodies specific for the 434 epitope in spontaneous viral clearance.

Similarly, we also analyzed specificity of antibodies targeted to different epitopes present in the NS3 protein of HCV in the two groups of individuals. We observed antibody response against three epitopes present in the helicase domain of NS3 primarily in chronic patients. Similarly, antibody response against a specific epitope present in the protease domain of NS3 incorporating several highly conserved residues of the catalytic site was significantly higher in self-resolvers as compared to chronic patients. This interesting finding suggests the role of antibodies targeted against a specific epitope present in the protease domain of NS3 in spontaneous clearance of the virus.

Despite the availability of effective treatment regimens against HCV the disease burden in Pakistan has not significantly lowered. Due to an asymptomatic nature of hepatitis C and lack of routine medical examinations, numerous HCV infected individuals with low-grade viraemia remain unaware of their infection status for years and therefore, do not pursue treatment until the symptomatic stage of liver impairment. These individuals then also contribute to the spread of the virus to general population. Such situation hampers the efforts for controlling the HCV infections even with the availability of effective treatments. Population based studies to identify specific socio-demographic groups with high HCV prevalence and an analysis of contributing factors is therefore needed to control the disease in general population.

We performed epidemiological analysis by testing blood samples from over 66,000 participants from all major cities of the Punjab province for anti-HCV antibodies. Overall we observed positive response in 17.3% of the participants. The antibody-based seroprevalence was then associated with socio-demographic variables including geographical region, age, gender and sex, and occupation. Two geographical regions, Faisalabad and Okara districts, and an occupational group, farmers, were identified with significantly high HCV seroprevalence. These socio-demographic groups were identified as the potential focused groups for follow-up studies on

factors contributing to the high HCV prevalence in these groups towards orchestrating effective prevention, control and treatment.

The third project of this study was focused on characterization of different classes of FcγR activation by HBV surface antigen, HBsAg specific antibodies in Hepatitis B vaccinated and self-resolved individuals. A high number of individuals can clear hepatitis B virus (HBV) without any treatment as the case with HCV vaccinated individuals. In this study we investigated the role of different Fcγ receptors of HBV surface antigen specific antibodies in the viral clearance in the two groups of individuals. By using different types of BW5147 cells each expressing one of the three Fcγ receptors, CD16, CD32a and CD64, we studied the activation of these cells by the sera of the two groups of individuals. Results from 48 HBV vaccinated and 29 HBV self-resolved individuals indicated that vaccinated individuals could activate all three classes of Fcγ receptors including FcγRI, FcγRII and FcγRIII while SR individuals could activate primarily the FcγRI and FcγRIII receptors. These results describe the role of different Fcγ receptors in spontaneous viral clearance and indicate that the mechanism of spontaneous clearance of the virus could be slightly different than clearance through vaccine induced prophylaxis.

Project 1

Characterization of Epitope Specificity of Antibodies From Chronic Patients and Self-Resolvers of HCV Infection

1.1. INTRODUCTION AND BACKGROUND

1.1.1. Prevalence of HCV

HCV infections occur in approximately 3% of the world population [1]. These infections are the primary cause of liver damage associated mortality and the 7th leading cause of deaths in adults [2, 3]. Approximately 170 million individuals in the world are chronically infected, [4] with 71 million people are at the risk of developing liver cancer [5]. Approximately 3 million new cases are reported every year with a mortality rate of 0.4 million [6-8]. Egypt is the most affected country, with HCV prevalence in 22% of the population whereas in Pakistan 5% of the population is infected with HCV [6, 9]. In some cases the disease behaves like a silent killer, as it remains asymptomatic until the late stage of liver cirrhosis. However in many cases at the late acute phase, it become symptomatic with nausea, fever, joint pain, jaundice, abdominal pain and some flu like symptoms [10].

1.1.2. HCV transmission

After the discovery of HCV, it took three years until 1992 to uncover the fact that it is a blood borne virus, and contaminated blood, clotting factors and other blood products are the major source of its transmission [11, 12]. In developing countries, major route of HCV transmission is contaminated instruments, needles, infusion sets, syringes, catheters used for injection based therapies and other invasive procedures [13]. Factors that contribute to rapid progression of chronic phase are co-infection with HIV, age, fatty liver, high body mass index, metabolic syndrome and alcoholic drug abuse [14-16].

1.1.2. Diagnosis

Liver function test is generally performed as a part of routine physical examination to determine if the liver is healthy. In HCV infection, Serum Alanine Aminotransferase (ALT) is elevated within 8-10 weeks of initial infection. If serum ALT levels are found higher then specific diagnostic tests for HCV are recommended. [17]. The specific HCV diagnostic tests include serological test for the presence of anti-HCV immunoglobulin and PCR based nucleic acid tests (NAT) to detect viral RNA in the blood. It takes 7-8 weeks for the appearance of anti HCV antibodies in the serum of an infected patient therefore very early diagnosis is difficult [18]. Positive anti-HCV test needs to be followed by the NAT test to find if the infection is active or it was retrospective. Viral RNA starts to appear in blood after 1-2 weeks of initial exposure [14]. A

person with positive ELISA and positive nucleic acid tests (NAT) is considered serologically positive for hepatitis C. While a person with positive ELISA and negative NAT can be considered as a spontaneous resolver who has been infected sometime in the past and has clear the virus without any medical treatment [19]. To determine the degree of fibrosis, the LFT test with platelets count such as aminotransferase to platelet ratio index and fibrosis score is assessed [20].

1.1.3. About Hepatitis C Virus

HCV is a small, non-cytopathic hepatotropic virus that belongs to the *Hepacivirus* genus and the *Flaviviridae* family [21]. It is a positive sense single stranded RNA virus containing a 9.6 kb genome flanked by highly conserved 5′ and 3′ un-translated regions (UTRs). The genome encodes an uninterrupted ORF that translates into a polyprotein of around 3010 to 3033 amino acids. 5′ UTR is a highly structured part of the HCV genome and it is responsible for viral replication and proper initiation of the polyprotein translation at Internal Ribosomal Entry Site (IRES) [22, 23]. The polyprotein is cleaved by viral and host proteases at various junctions into three structural and seven non-structural proteins of the virus [24]. Three structural proteins located at the N terminus of polyprotein include the core protein and the E1 and E2 envelope glycoproteins. The core protein not only forms viral nucleocapsid but also interacts with viral RNA during assembly process. It is involved in the process of apoptosis, lipid metabolism and development of liver carcinoma [14]. E1 and E2 are trans-membrane glycoproteins that form the envelope of the virus particle and are heavily glycosylated at specific sites that are highly conserved across different genotypes.

Non-structural proteins include p7 (ion channel), NS2 (protease), NS3 (protease and helicase), NS4A (cofactor), NS4B (membrane anchor protein), NS5A (phosphor-protein) and NS5B (RNA dependent RNA polymerase) [25]. NS1 (p7) is an ion channel protein that prevents acidification and assists in viral assembly. NS2, a cysteine protease, plays a role in viral assembly and release, and promotes HCV induced retention of lipids in the liver [26, 27]. NS3, protease/helicase, is responsible for the catalysis of RNA binding and unwinding during replication. Its protease activity cleaves polyprotein precursor to produce nonstructural proteins. It also modulates host cellular defense process by cleaving the Mitochondrial Antiviral-Signaling (MAVS) protein

which is responsible to induce IRF3 mediated IFN1 production. NS4A is cofactor for NS3 protease. NS4B is involved in the formation of membranous web to assist viral replication in the cell. NS5A is multipurpose protein and plays role in viral pathogenesis, replication, modulation of cell signaling pathways and IFN response. NS5B is a RNA dependent RNA polymerase (RdRp) and generates a diverse swarm of viral quasi species due to error prone replication process [14, 28]. Moreover, there is another protein synthesized by ribosomal frame shifting in the core protein region. This protein is named as alternative reading frame protein (ARFP)[29]. It's a small protein, suppresses RIG 1 mediated interferon signaling and provides a defense mechanism to HCV [30].

1.1.4. HCV Genotypes

HCV is antigenically more diverse than HIV [19, 31]. Based on nucleotide sequence variability HCV is classified into 7 genotypes (GTs) (variability index 30-35%) and 67 subtypes (variability index 10-25%)[32-34]. These genotypes are geographically distributed and show differences in their pathogenicity and response to treatments. Genotype 1a is the most prevalent genotype in the world and is responsible for 46% of the HCV infections especially in East Asia, and America while GT3a is second most prevalent with 30% of the total cases worldwide [35]. GT4 is prevalent in Egypt, GT5 in South Africa, and GT6 in South East Asia. Due to an error prone replication process, viral quasi-species are accumulated after each replication cycle in an infected individual, which are the leading contributor for the progression of infection to chronic phase [31].

1.1.5. Models to study HCV

Efforts to understand different aspects of viral life cycle *in vitro* started with the cloning of full length HCV cDNA from GT1a. This cloned DNA was infectious in chimpanzee [36] but unfortunately inefficient to replicate *in vitro*. In 1999, sub-genomic bicistronic replicons were constructed from HCV GT1b RNA isolated from an infected human liver. These RNAs had a good replicative potential in the Huh7 hepatoma cell line [37]. This cell line was later modified to an interferon resistant cell line named as Huh7.5 [38]. This system helped to decipher different host factors involved in amplification of viral RNA. In 2003, discovery of pseudo particles based infectivity assay (HCVpp) with functional envelope proteins of HCV made it easy for in detail

characterization of early steps involved in viral entry within the host cells as well as to study different monoclonal antibodies and their neutralization potential [39].

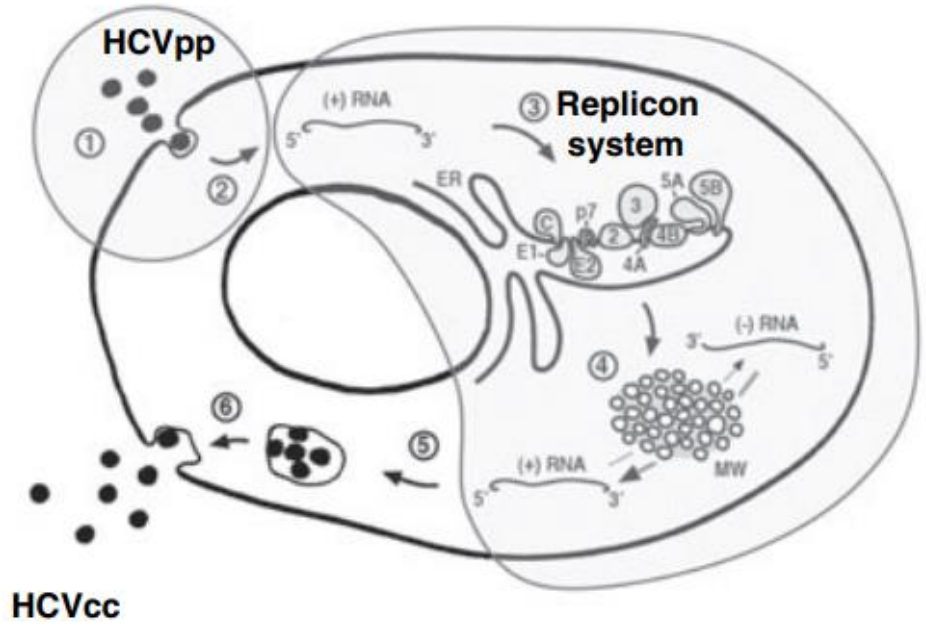


Figure 1.1. Depiction of different cell culture based systems to study HCV. HCV replicon based assay helps to understand viral RNA amplification, HCVpp helps to describe events involved in viral entry in the cells while HCVcc produces infective virions and help to study viral replication and infectivity [40].

To study different steps involved in viral infectivity, replication or secretory pathways, HCV cell culture (HCVcc) based assay has been designed that is based on replication of HCV genome from JFH1 strain (from GT2a) in human hepatoma cell line (Huh7 cells). JFH1 strain had high replication capacity and produce infectious virions without any adaptive mutations. This system is being used to study viral replication, infectivity, efficiency of drugs and drug resistance mechanisms (Figure 1.1) [41].

1.1.6. HCV life cycle

Hepatocytes are the main targets for HCV because of the presence of different receptors required for viral entry. Two most important cellular entry receptors are Scavenger receptor B1 (SR-B1)

[42] and receptor from tetraspanin receptor family (CD81) [43]. The viral E2 protein initially binds to LDLr and GAG followed by interaction with SR-B1 and CD81 receptors. The virus then interacts with tight junction protein named as occludin (OCLN) and claudin (Cldn). Other receptors involved in modulating the entry process are epidermal growth factor receptor, ephrin receptor type A2 and cholesterol absorption receptor Niemann–Pick C1-like 1 (NPC1). The step-wise binding of the virus to different cellular receptor ultimately results in viral entry through clathrine mediated endocytic pathway [44, 45]. Some structural conformational changes mediated by the acidic environment of endosomes lead to the fusion of viral and endosomal membranes and ultimately viral RNA released into the cytoplasm. The RNA is translated to viral polyprotein on endoplasmic reticulum, which is then cleaved into constituent proteins by viral and cellular proteases [46, 47]. NS5B use the positive sense RNA to synthesize a negative sense stranded RNA, which acts as a template for positive strand synthesis. This RNA is transported to the membranous web where NS5B starts to replicate viral genome. After a multistep process viral particle starts to assemble on ER as initiated by the assembly of core and NS5A protein [48] followed by encapsulation of newly synthesized viral RNA. Envelope proteins are encapsulated over nucleocapsid. This mature virion is released by liposomal mediated pathway (Figure 1.2) [46, 47, 49].

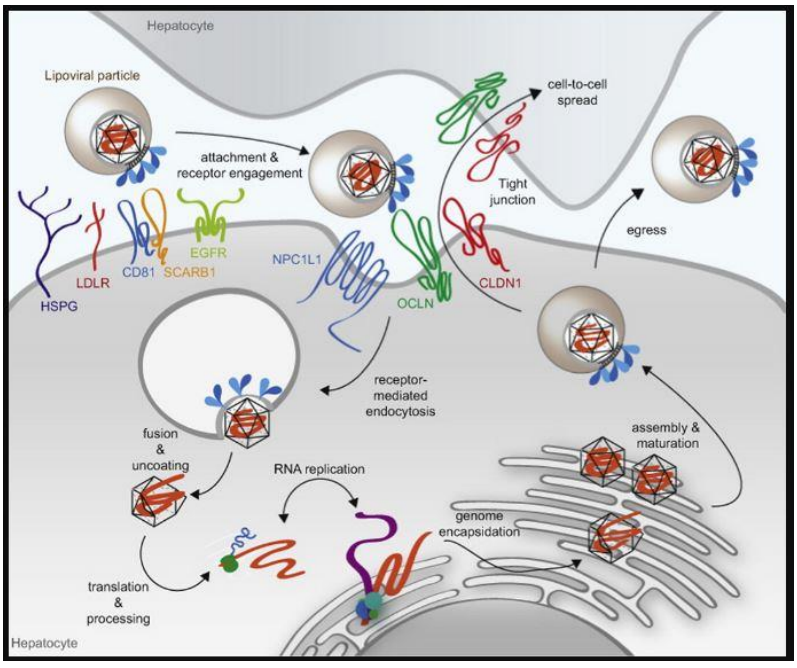


Figure 1.2. **Replication cycle of HCV**. A number of receptors including CD81 and SR-B1 mediate the viral enter into hepatocytes by endosome-mediated pathway. This is followed by uncoating and release of viral RNA in the cytoplasm. After replication of RNA and translation of viral proteins, viral RNA is packaged into nucleocapsid and secreted out from the cell [50].

1.1.7. Treatment history

In the early years of HCV emergence, efforts were focused to treat the infection through passive immunization. In this regard, hyper-immune serum against HVR1 region of envelope protein was raised in mice and its neutralization capacity was determined in chimpanzees, which showed cross neutralization of different variants [51]. Subsequently, Gamma gard, an IGIV product prepared from anti HCV screened plasma was used but it was withdrawn in 1994 when HCV was transmitted to the recipients [52]. First line of drug for HCV treatment was Interferon α (IFNα) that was approved by FDA in 1999 [53, 54]. But emergence of drug resistant variants and limited efficacy led the inclusion of viral protease inhibitor, Ribavirin along with IFNα [55, 56]. Later, another NS3-4 protease inhibitor, Ciliprevir was approved for clinical trial. It reduced viral loads up to four fold after 48 hours of administration [57, 58]. Combined treatment with PEG-IFNα, ribavirin and second-generation protease inhibitors provided 75-90% SVR with less side effects [59]. In the current decade many new direct acting antiviral (DAAs) targeting NS5A and NS5B have been approved by FDA, which include Daclatasvir, Sofosbuvir, Velpatasvir and Ledipasvir. These are used in different combinations and have been successful in treating HCV infections. These are also prescribed for HCV-HIV co-infected patients, for recurrent HCV infections, decompensated cirrhosis and end stage liver disease [60-63] and have over 95% SVR rates. However, some of these therapies have genotype specific response and are not cost effective. Moreover, due to a fast mutation rate in the virus, resistant variants have already been reported against most of these treatments. Another approach to treat these infections is targeting the host factors to develop host-targeting antivirals (HTAs). This approach however, has severe associated side effects [64, 65]. Some of the latest DAAs developed in last few years are highly efficacious and have pan genotypic antiviral response [66, 67].

1.1.8. Role of antibodies in HCV clearance

Antibodies against many viral infections including HCV exert a strong antiviral response to clear the virus. Passive immunization using anti-HCV plasma provided protection in chimpanzees with chronic HCV infection [68]. These antibodies have also shown their effect to delay the progress of infection [69, 70]. In some cases, anti-HCV neutralizing antibodies are produced earlier after the onset of the infection and have the potential to clear the virus. For example, spontaneous clearance of HCV during acute phase has been associated with early appearance of antibodies in high titer within 7-8 weeks of infection. These antibodies are usually strain specific, while production of cross neutralizing antibodies take more time, up to 33 weeks [3, 33, 71-74]. An important aspect associated with neutralizing antibodies is their epitope specificity. Usually antibodies specific for conserved epitopes of envelope glycoprotein are highly potent and broadly cross reactive to capture and clear the virus. A number of different monoclonal antibodies have been isolated from experimentally infected mice, chimpanzees and chronic and acute HCV patients. Among these, envelope specific monoclonal antibodies are highly potent and broadly neutralizing [75].

1.1.9. The E2 envelope protein

HCV envelope is a non-covalent heterodimer of E1E2. It is involved in receptor attachment and cellular entry process and is the primary antigenic site to elicit neutralizing antibody responses. The E2 protein incorporates two types of regions on the basis of sequence conservation and variability. Some sequential regions exhibit high variability index across genotypes and are referred to as hyper variable regions (HVR) including HVR1 (aa384-410), HVR2 (aa460-480) and IgVR (aa570-580)[76]. Two extra HVRs, HVR495 (aa495-501) and HVR575 (aa575-578) are present in only in GT3a [77]. Some regions of E2 are highly conserved in amino acid sequence across different genotypes, which include the regions spanning aa412-424, aa434-446, aa524-535 and aa611-617. The HVR1 region is a major antigenic determinant and antibodies against this region have good neutralization potential [74, 78-81] however due to high sequence variability of this region, such antibodies are strain specific. Selection pressure from neutralizing antibodies drives viral evolution during acute phase therefore these anti-HVR antibodies fail to neutralize the HCV HVR1 variants [82]. Broadly neutralizing antibodies are specific for conserved epitopes of E1E2. Two types of conserved epitopes on the HCV envelope have been

described, one consisting of continuous amino acid sequences called linear epitopes and the other formed by multiple sequential regions called conformational epitopes.

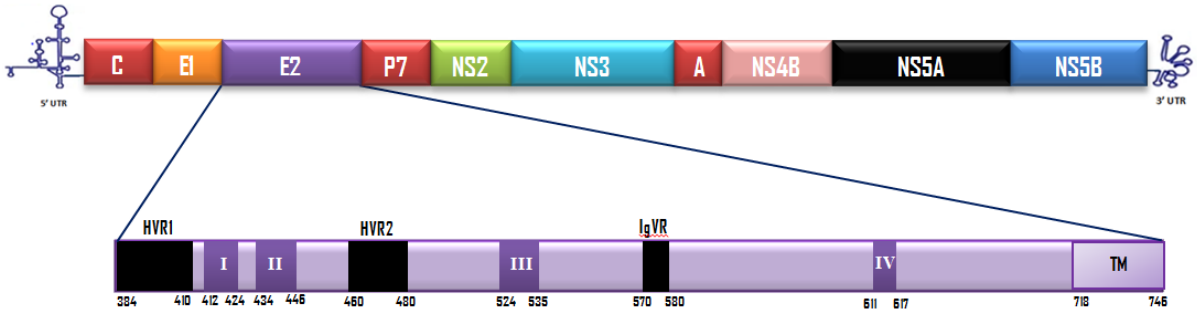


Figure 1.3. HCV genome structure and E2. HCV genome exhibits a single open reading frame that is translated into a polyprotein. This polyprotein is cleaved into 3 structural and 7 nonstructural proteins. The core protein (C), and the envelope proteins E1 and E2 form structural component of virus while non-structural protein-3 (NS3), NS4, NS5A and NS5B are responsible for viral replication. Viral envelope consists of the E1 and E2 proteins. The E2 protein exhibits highly variable regions including hyper variable region 1 (HVR1), HVR2 and inter-genotypic variable region (IgVR), and highly conserved regions spanning aa412-424, aa434-446, aa524-535 and aa611-617 labeled as I, II, III and IV, respectively.

Linear epitopes of antibodies on the viral envelope

Several anti-HCV antibodies specific for linear epitopes have been identified many of those are targeted against highly conserved linear epitopes and are able to neutralize a broad range of viral strains. One of the highly conserved linear epitopes is epitope I (aa412-424) that is highly flexible in terms of structural characteristics. AP33 is a mouse monoclonal antibody that binds to β-hairpin conformation of this epitope and interacts with L413, N415, G418, and W420 amino acids. This antibody can neutralize HCVpp panel from different genotypes with an IC_{50} value of 0.6-32 μg/ml [83]. HCV1 is human mAb that binds to hydrophobic face of β-hairpin conformation of this epitope and interacts with L413 and W420. This antibody has shown neutralization activity against different HCV genotypes including GTs 1a, 1b, 2b, 3a and 4a [84].

Antibody 3/11 is a rat mAb specific for this epitope. The epitope does not adopt β-hairpin conformation on binding to this mAb but adopts an extended peptide conformation when bound by the 3/11 mAb [83, 85-88].

The linear epitope II encompasses aa434-443 of E2 and exist in short alpha helical conformation with extended loops. Antibodies directed against this epitope can be neutralizing or non-neutralizing depending on the conformation of this epitope and the amino acids of the epitope involved in interactions with the antibody. For example neutralizing antibodies interact with the W437 and L438 residues in C-terminal α-helix, and E431 and N434 in N-terminal extended loop region, as in case of the mAb#8 antibody. These bifurcated interactions with the epitope help the antibody to orient itself in a way to interrupt viral entry process. Some non-neutralizing or interfering antibodies specific for this epitope have also been reported [89].

Epitope III encompasses the aa524-531 region, which also includes residues such as W529, G530 and D535 involved in interactions with the CD81 receptor. The 2/64a antibody targets this epitope and can inhibit HCV entry to the target cell [90]. The A8 and 1:7 antibodies specific for this epitope can neutralize all genotypes of HCVpp and HCVcc with IC_{50} value ranging from 60 to 560 ng/ml. These antibodies have been isolated from chronically infected patients [91].

Example of an antibody targeting E1 linear epitope is the IGH526 antibody that has been isolated from individual who had cleared the infection after IFN therapy. It binds to one face of alpha helical peptide of E1 encompassing aa314-324 with some additional contacts with residues on E2. It has cross neutralization activity against HCV GT 1a, 1b, 4a, 5a, and 6a [92, 93].

Conformational antibody epitopes on the HCV envelope

Most of the Abs that neutralize a broad range of HCV strains are directed against conformation dependent epitope present on the HCV envelope. The majority of these Abs discovered to date recognize their epitope encompassing CD81 receptor binding site and neutralize by inhibiting HCV interaction with the CD81 receptor [94]. Antibody competition and mutagenesis studies have suggested that the CD81 binding site is formed by several conserved regions present in the envelope glycoprotein E2. These include the regions spanning residues 412–423, 432–447, 528–

535 and 544–551. The specific residues within these regions that play critical roles in CD81 binding include W420, Y527, W529, G530 and D535 and the 436GWLAGLFY443 motif [95]. Examples of potent bNAbs directed against conformational epitope of the CD81 binding site include, AR3A-D, e20, e137, 1:7, CBH5 and A8, and NAbs with intermediate cross neutralization potential directed against this region include H35 and H48. Some of the bNAbs namely A8, 1:7, e20 and H48 recognize their key residues present in just one domain and other recognize their epitopes encompassing different domains of the CD81 binding site [96]. More recently a class of conformational epitope targeted bNAbs, HC84-related class has been reported. These Abs are directed against a conformational epitope formed by two discontinuous regions of E2, aa413-446 and aa613-616 and few contact residues of these Abs lie within the CD81 binding site [97].

1.1.10. Resistant variants

Many HCV neutralizing antibodies (NAbs) have been identified that are highly efficacious. But still viral variants emerge in the presence of these antibodies by using different evasion mechanisms. For example substitutions at the Ab binding site may affect the binding affinity of antibodies. Some of these substitutions are well tolerated by the virus while others may prove to be deleterious for the survival of the virus. For example under selection pressure of CBH-2 antibody, escape mutants develop D431G or A439E substitutions and these variants are completely tolerated by the virus. The HC-11 antibody escape mutants exhibit L438F and N434D or T435A mutations depending on antibody titer. Viral fitness is compromised after adopting these substitutions [98].

Glycan shifting is another immune evasion mechanism by the virus. For example, AP33 and HCV1 bind to β-hairpin structure of epitope I. The substitution N417S shifts the N-glycosylation site from N417 to N415 as the latter is not the glycosylation site in the absence of this substitution. This glycosylation site shifting disrupts the binding of the antibody to the epitope [99, 100].

1.1.11. Non-Structural 3 (NS3) protein

Nonstructural protein 3 (NS3) is a multifunctional protein of 70 kDa protein. It consists of the N-terminal protease domain and the C-terminal helicase domain. The protease domain is a serine protease belonging to trypsin/chymotrypsin superfamily of proteases and is responsible for cleavage of viral polyprotein into its component proteins. While the helicase domain occupies two third part of NS3 belonging to DExH superfamily of helicases. It is responsible for RNA folding and genome encapsidation. N-terminal 180 amino acids of NS3 also serve as a cofactor for the NS2 protein [101].

NS3 plays crucial roles in HCV replication and evasion of host defense by the virus. For example mitochondrial antiviral signaling protein (MAVS), and Toll-IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF or TICAM-1) inhibit HCV by stimulating IFN-β gene. Both of these proteins are cleaved by the protease domain of NS3 [102]. In addition, helicase domain generates reactive oxygen species (ROS) by activating the iNOS gene. ROS induces double stranded breaks in host DNA. Extensive double stranded breaks (DSBs) in most of the cellular genes of HCV infected cells followed by overwhelmed DNA repair mechanisms leads chromosomal aberrations. These aberrations are responsible for neoplastic transformations in hepatocytes [103]. It also plays role to establish persistent infection by blocking apoptosis and inhibiting p53 [104-107]. NS3-specific antibodies are produced in early stage of viral infection. Protective roles of NS3 targeted antibodies in other viruses of the *Flaviviridae* family have been described, however, such role of HCV NS3 specific antibodies still be delineated.

1.1.12. Research Question

Around 25% of the HCV infected patients clear the virus without any treatment, and cellular immunity has been described to be responsible in such viral clearance. However, the role of antibodies in spontaneous viral clearance has not been clear. Therefore, delineating the role of antibodies in such HCV clearance has a high significance in particular in vaccine design perspective. In HCV infected patients two types of antibodies exist on the basis of the properties of their target sites. Some antibodies recognize linear epitopes despite they adopt specific structural and conformational features when recognized be the antibody. The other type of antibodies are conformational epitope specific where the epitope is formed by different

sequential regions. Although most of the identified HCV neutralizing antibodies are conformational eptiope specific but some broadly neutralizing antibodies specific for linear epitopes present in the conserved region of the E2 protein have been reported. This is an important question to understand if linear epitope specific antibodies play any role in spontaneous viral clearance. In the context of immunogen design as potential vaccine, linear epitopes have high significance, as such epitope based immunogen design is less challenging as compared to conformational epitope based immunogens. Chronically infected people generally have high titer of antibodies specific for a diverse epitopes. In this project we aimed to understand the specificity of antibodies present in the sera of self resolvers and chronically infected patients for linear epitopes present in the viral envelope and the NS3 protein of the virus. Such information is highly significant towards vaccine desing against HCV.

1.1.13. Detailed Objectives

Following are the detailed objectives of this study

- Synthesize peptides with sequences corresponding to conserved epitopes present in the envelope E2 protein
- Synthesize peptides with ovelapping amino acid sequences corresponding to various regions of NS3
- Collect serum samples from chronic HCV patients and spontaneously resolved HCV individuals after informed consents
- Analyze antibody response against the E2 and NS3 peptides in two patient groups using ELISA technique
- Develop HCVpp assay
- Determine neutralization potential of sera from the two patient groups
- Determine viral nuetralization potential of particular epitope-specific antibodies in the sera by performing peptide competition assay to define the role of that epitope spcific antibodies in the viral neutralization.

1.2. MATERIALS and METHODS

1.2.1. Enzyme Linked Immunosorbent Assay (ELISA)

Materials

1. 100 mM carbonate bicarbonate buffer (pH 9.6).
2. PBS (1X).
3. Alkaline Phosphatase buffer (Cold spring harbor protocols) (pH 9.5).
4. Washing Buffer I: 0.05% Tween 20 in PBS.
5. Washing Buffer II: 0.02% sodium azide in PBS.
6. Blocking Buffer: 5% skimmed milk in washing buffer II.
7. ELISA Plates: Thermoscientific Immulon 4HBX Flat bottom microtitre plates Part No: 3855.

Methods

1. Peptides to be used as antigens, corresponding to highly conserved regions of the E2 envelope protein and the NS3 protein of HCV were synthesized by commercial facilities and obtained in lyophilized powdered form.
2. Peptide stock solutions of 1 mg/ml were prepared in distilled water and diluted in 50 mM sodium carbonate–bicarbonate buffer (pH 9.0) to get a final concentration of 5 µg/ml. ELISA plate were coated with 100 µl of peptide antigen and incubated overnight at 4^0 C.
3. Next day peptide solution was removed from the plate and plate surface was blocked using 200 µl of blocking buffer and incubated at room temperature for 1-2 hours.
4. These plates were washed with 200 µl of washing buffer II.
5. Primary antibodies (Ap33) as well as patient sera were diluted in PBS and 50 µl was added in each well. This plate was incubated at room temperature for 1 hour. BSA (5 µg/ml) coated well and blank well (carbonate buffer only) served as negative control for nonspecific antibody binding.
6. Plate was washed and incubated with secondary antibody (Goat anti-mouse IgG-Alkaline Phosphatase sc-2008), diluted 1000 folds in PBS/blocking buffer at room temperature for 1hr.
7. Plate was washed three times with 200 µl of washing buffer I.
8. PNPP (p-Nitrophenyl Phosphate) substrate (1 mg/ml) was prepared fresh and 50 µl of which was added in each well.

9. Reaction was stopped with 50 µl of 0.5 N NaOH and absorbance was recorded at 405 nm on Synergy HTX multimode reader.
10. Data was plotted using graph pad software.

1.2.2. Generation of HCV pseudo-typed particles

Materials

1. HEK293T cells (ATCC).
2. Huh7.5 cells (NIH).
3. HEK293T/Huh7.5 cells growth medium: Dulbecco's modified Eagle medium (DMEM, Gibco), supplemented with 2 mM L-glutamine (Gibco), 1× nonessential amino acids (Sigma-Aldrich) and 10% heat-inactivated fetal bovine serum (Gibco).
4. Tissue culture dishes (T25, T75) (Corning).
5. 96-well white flat bottom tissue culture plates (Corning).
6. 0.45 micron filters (Millipore).
7. 37 °C, 5% CO_2 incubator.
8. Luciferase Assay System reagent (Promega).
9. Luminometer.
10. Virus-G (VSV-G) pseudoparticles.

Methods

1. ***Day 1***: To synthesis HCVpp, 9.5×10^6 HEK293T cells were seeded into a T75 cell culture flask in HEK293T growth medium and incubated overnight at 37 °C and 5% CO_2.
2. ***Day 2*:** Next day confluency of the cells was checked that should be around 50% and evenly distributed over the plate.
3. For HCVpp to be produced in T75 flasks, 6µg of phCMV-1a (envelope plasmid), 18µg of pNL4.3Luc Luciferase reporter construct diluted in Opti-MEM medium was used to transfect the cells using lipofectamine 3000 as a transfection reagent.
4. Generation of control pseudoparticles:
    a. To generate mock pseudoparticles (control for nonspecific entry), step 3 was repeated but without addition of phCMV-1a construct.

b. To generate VSV-G-enveloped pseudoparticles (control for nonspecific neutralization), step 3 was repeated but phCMV-1a expression plasmid was replaced with VSV-G-envelope expression plasmid.

5. The reaction mix was incubated at room temperature for 30 min.
6. During this time, growth medium was removed from the cells with a serological pipette, and replaced with DMEM (containing 5% FBS).
7. Plasmid–lipofectamine mix was added to the cells drop-wise to ensure even distribution and incubated for 6 hr at 37 °C and 5% CO_2.
8. Opti-MEM was replaced with 10 mL of complete DMEM medium and incubated at 37°C in a humidified incubator for 52 hr.
9. ***Day 5*:** Medium was removed from the cells, centrifuged at 2000 rpm for 10 min and filtered it through a 0.45µ filter. The supernatant containing the pseudoparticles was stored at -80°C.

1.2.3. HCV neutralization assay

1. ***Day 1*:** Huh7.5 cells (1.5×10^4 cells per well) were seeded into a 96-well flat bottom tissue culture plate (Corning) in 100 µl of growth medium and incubated overnight at 37 °C and 5% CO_2.
1. ***Day 2*:** Plasma or serum samples to be tested for neutralizing activity were heated at 56 °C for 30 min followed by centrifugation at 1200 g for 5 min. Clarified serum was diluted in 293T cells medium.
2. HCVpp were diluted into HEK293T growth medium to a concentration that should result in infection in the linear range of the infectivity assay. VSV-G-enveloped pseudoparticles were diluted to similar level of infectivity like HCVpp to use as a control for nonspecific neutralization.
3. Serum dilutions were incubated with HCVpp for 1.5 hr at 37 °C and 5% CO_2 in a total volume of 100 µl.
4. After incubation serum-HCVpp mix was transferred to Huh7.5 cells and incubated for 5h at 37 °C and 5% CO_2.
5. The dilution mix was replaced with complete growth medium and incubated the cells at 37 °C and 5% CO_2 for 72 hr.

2. ***Day 5*:** Medium was removed from the plate and cells were lysed by adding 50 µL of 1× GloLysis buffer (Promega) to each well. Plate was frozen at -80^{0}C for 30 min followed by thawing at 37^{o}C for 30 min on shaking at 300 rpm.
3. Cell lysate 45 µl was transferred to white plate and centrifuged for 3 min at 2000 rpm to remove any bubbles.
4. Luminescence was detected by adding 50 µL of luciferase reagent (Promega) per well and quantitate relative light units (RLU) for 1–5 s after a 0.2 s delay using a luminometer.
5. Neutralization potential for each serum dilution was calculated using HCVpp infectivity in the presence of test serum, (RLUtest) relative to infectivity of HCVpp alone (RLUcontrol).
6. Neutralization values can be expressed as percent neutralization
   Percent neutralization = (1 – RLUtest/RLUcontrol) × 100).
7. Fifty-percent inhibitory concentration (IC_{50}) of an each serum dilution was calculated using the graphpad software.

1.2.4. Peptide competition assay

1. ***Day 1*:** Huh7.5 cells (1.5×10^4 cells per well) were seeded into a 96-well flat bottom tissue culture plate (Corning) in 200 µl of growth medium and incubated overnight at 37 °C and 5% CO_2.
2. Peptide stock 1 mg/ml was prepared in autoclaved distilled water.
3. Serum dilution 1:50 was prepared in PBS and heated at 56^{o}C for 30 min followed by centrifugation at 2500 rpm for 15 min. Clear serum was separated leaving the pellet at the bottom.
4. Peptide working stock (100 µg/ml) was prepared in PBS and incubated with 100 µl of serum at 37^{o}C for 2 hr on gentle rocking.
5. The mix was centrifuged at 15000 rpm, 37 ^{o}C for 10 min, supernatant was separated leaving the pellet intact.
6. Supernatant 100 µl was incubated with HCVpp (100 µl) and incubated for 1.5 hr at RT and 5% CO_2 (control: HCVpp 100 µl +PBS 100 µl).

7. After 1.5 hr, medium in Huh7.5 cells was replaced with 100 µl of the pseudoparticle/antibody mixture per well. This was incubated for 5 hr at 37 °C and 5% CO_2.
8. After incubation, medium was replaced with a complete growth medium and cells were incubated at 37 °C and 5% CO_2 for 72 hr.
9. ***Day 5*:** Medium was removed from the plate and cells were lysed by adding 50 µl of 1× GloLysis buffer (Promega) to each well. Plate was frozen at -80^{0}C for 30min followed by thawing at 37 ^{o}C for 30 min on shaking at 300 rpm.
10. Cell lysate 45 µl was transferred to white plate and centrifuged for 3 min at 2000 rpm to remove any bubbles.
11. Luminescence was detected by adding 45 µl of luciferase reagent (Promega) per well and relative light units (RLU) were quantitated using a luminometer.
12. Neutralization potential for each serum dilution was calculated using HCVpp infectivity in the presence of test serum, (RLUtest) relative to infectivity of HCVpp alone (RLUcontrol).
13. Neutralization values can be expressed as percent neutralization
    Percent neutralization = (1 – RLUtest/RLUcontrol) × 100).
14. IC_{50}) of every serum dilution was calculated using graphpad software.

1.3. RESULTS

1.3.1. Linear epitopes to delineate specificity of sera

Specificity of sera for conserved linear epitopes present in the E2 envelope of HCV and overlapping epitopes present in the NS3 protein of HCV was studied. In this regard three highly conserved regions of E2 including the regions spanning residues 412-424 (the 412 epitope), residues 524-535 (the 524 epitope) and residues 434-446 (the 434 epitope) were selected (Table 1.1). In some of the peptides with poor predicted solubility, lysine amino acid was added at the termini to increase their solubility index. For NS3 epitopes, 30 peptides each consisting of a 20 amino acids sequence corresponding to various overlapping regions of NS3 were designed using online available tools (Table 1.2). All these peptides were synthesized through commercial facilities.

Table 1.1. Peptides with amino acid sequences corresponding to conserved linear epitopes present in the E2 envelope protein

Epitope name	Length (aa)	Amino acid sequence	Sequential position
412	16	KKQLVNTNGSWHINKK*	412-423
434	14	SLNTGWLAGLFYKK*	434-446
524	14	RSGAPTYSWGANKK*	524-535

* Lysine residues at one or both ends are added just to increase the solubility of the peptide

Table 1.2. Peptide fragments of the NS3 protein with overlapping amino acid sequences

Epitope name	Length (aa)	Amino acid sequence	Sequential Position
1	20	GREVLLGPADDYREMGWRLL	1-20
2	20	DYREMGWRLLAPITAYAQQT	11-30
4	20	RGLLGTIVTSLTGRDKNVVT	31-50
5	20	LTGRDKNVVTGEVQVLSTAT	41-60
8	20	VIWTVYHGAGSRTLAGAKHP	71-90
9	20	SRTLAGAKHPALQMYTNVDQ	81-100

11	20	DLVGWPAPPGAKSLEPCACG	101-120
12	20	AKSLEPCACGSSDLYLVTRD	111-130
13	20	SSDLYLVTRDADVIPARRRG	121-140
14	20	ADVIPARRRGDSTASLLSPR	131-150
15	20	DSTASLLSPRPLACLKGSSG	141-160
16	20	PLACLKGSSGGPVMCPSGHV	151-170
17	20	GPVMCPSGHVAGIFRAAVCT	161-180
18	20	AGIFRAAVCTRGVAKSLQFI	171-190
19	20	RGVAKSLQFIPVETLSTQAR	181-200
20	20	PVETLSTQARSPSFSDNSTP	191-210
21	20	SPSFSDNSTPPAVPQSYQVG	201-220
22	20	PAVPQSYQVGYLHAPTGSGK	211-230
23	20	YLHAPTGSGKSTKVPAAYVA	221-240
27	20	FMSRAYGIDPNIRTGNRTVT	261-280
28	20	NIRTGNRTVTTGAKLTYSTY	271-290
35	20	ATATPPGSITVPHSNIEEVA	341-360
37	20	LGSEGEIPFYGKAIPIALLK	361-380
38	20	GKAIPIALLKGGRHLIFCHS	371-390
39	20	GGRHLIFCHSKKKCDEIASK	381-400
40	20	KKKCDEIASKLRGMGLNAVA	391-410
47	20	DPTFSIETRTAPQDAVSRSQ	461-480
48	20	APQDAVSRSQRRGRTGRGRL	471-490
49	20	RRGRTGRGRLGTYRYVASGE	481-500
50	20	GTYRYVASGERPSGMFDSVV	491-510

1.3.2. Blood collection and serum separation

The project was approved by institutional review boards (IRBs) of three collaborating institutes, Lahore University of Management Sciences (LUMS), Shalamar Hospital Lahore and Hepatitis

Prevention and Treatment Clinic (*HPTC*)-Pakistan kidney and liver institute (PKLI) Lahore. Collectively, we obtained serum samples from 84 individuals in collaboration with two hospitals. Among these, 30 sera were collected from chronic HCV patients, 49 from spontaneously resolved (SR) individuals and 5 were from healthy donors as negative control. Subjects were assigned spontaneous resolvers status if they had undetectable HCV RNA but showed a detectable anti-HCV antibody in serological test. Individuals with chronic HCV infections were considered viraemic if they remained positive for HCV RNA for a period of more than one year. Unique serial numbers were assigned to patients and their demographic data was collected and saved in excel sheets. From every subject, 5 ml blood sample was collected in plain vacutainers and transferred to our laboratory at LUMS at room temperature. After collection, the blood was kept undisturbed at room temperature for 30 minutes in biological safety cabinet, to let it clot. Subsequently, the samples were centrifuged at 1,000–2,000 g for 10 minutes in a refrigerated centrifuge. Following centrifugation, the supernatant (serum) was transferred into a clean eppendorf tube in 500 µl aliquots using a Pasteur pipette and stored at -20^{o}C.

1.3.3. Response against conserved linear epitopes present in the envelope region

To profile antibody response in the sera of spontaneously resolved individuals and HCV chronic patients against conserved linear epitopes of HCV envelope we performed ELISA. In this regard we coated the surface of 96-well plates separately with 5 µg/ml of the each peptide from E2 envelope protein. ELISA experiments were performed with the sera of five healthy donors, 30 chronically infected patients and 49 spontaneous resolvers using three dilutions of each serum as 10-fold, 100-fold and 1000-fold against all three highly conserved linear epitopes. Cutoff was defined as mean OD from healthy sera also considering standard deviations of three values.

In most of the chronic patients' sera, a robust response was observed against the 412 and the 434 epitopes, however, only few of the sera showed reactivity with the 524 epitope. Around 66% of the chronic patient's sera were positive against the 434 epitope while in 43% and 23% sera, positive response was observed against the 412 and 524 epitopes, respectively (Figure 1.4). Most of the sera showed reactivity with more than one epitopes, however, in 32% of the cases very specific response was observed against a single linear epitope, which was primarily against the 434 epitope – 71% of the single epitope specific sera were specific for the 434 epitope (Figure

1.4, Table 1.3). Very few sera from both groups showed reactivity to the 524 epitope. The response observed against this epitope was always weaker that could be attributed to the fact that this highly conserved epitope is masked within the hyper variable core of E2 during most part of the viral life cycle, due its direct involvement in binding to the CD81 receptor of the virus.

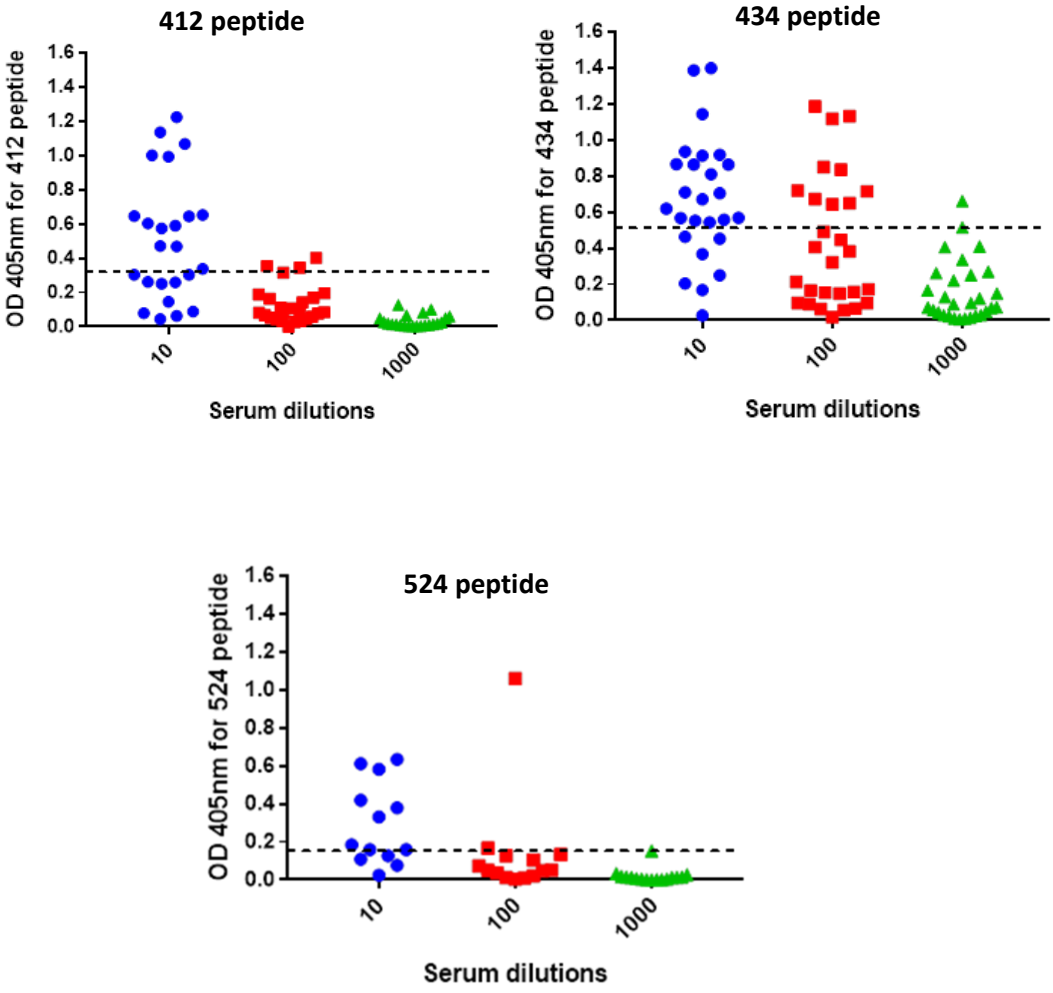


Figure 1.4. ELISA reactivity to determine eptiope specificity of chronic HCV patients' sera. Enzyme Linked Immunosorbent Assay was performed using three conserved linear epitopes of the E2 envelope protein, the 412 epitope, the 434 eitope, and the 524 epitope using three different serum dilutions (10 fold, 100 fold, 1000 fold) of sera from chronically infected HCV patients (CP). Dotted line represents the cutoff value (Mean from healthy controls).

Table 1.3. Details of antibody response against three conserved epitopes of E2 in chronic patients. Sera with negative response for all three epitope are excluded from the table.

Serum No	Response against 412	Response against 434	Response against 524
68	-*	+	+
191	+*	+	+
193	-	+	-
201	+	+	-
336	++*	+	-
341	-	+	-
366	+	-	-
475	++	+	+
574	+	+	-
843	++	-	-
978	-	++	++
226	++	++	+
545	-	+	+
679	-	+	-
762	+	+	-
603	-	+	+
604	+	+	-
21	-	+	-
35	+	+	-
2469	-	++	-
2959	+	+	-
2967	++	++	-

*Non responders = "-", OD < 0.5 = "+", OD ≥ 0.5 = "++"

In case of spontaneously resolved sera, the observed responses were more specific. In 53% of the sera no response was observed against any of the epitope. While from the positive sera 82% of

the sera showed reactivity to the 434 epitope alone or in combination with other epitopes. In 21% and 13% of the positive sera the response was observed against the 412 and the 524 epitopes, respectively. Contrary to chronic patients, most of the positive sera from self-resolvers were specific for a single epitope primarily the 434 epitope as 82% the positive sera showed reactivity specifically to the 434 epitope alone (Table 1.4, Figures 1.5 & 1.6). Interestingly, there was not a single serum that showed reactivity to all three epitopes together. Overall response in both groups of sera is biased more towards the 434 epitope.

412 peptide **434 peptide**

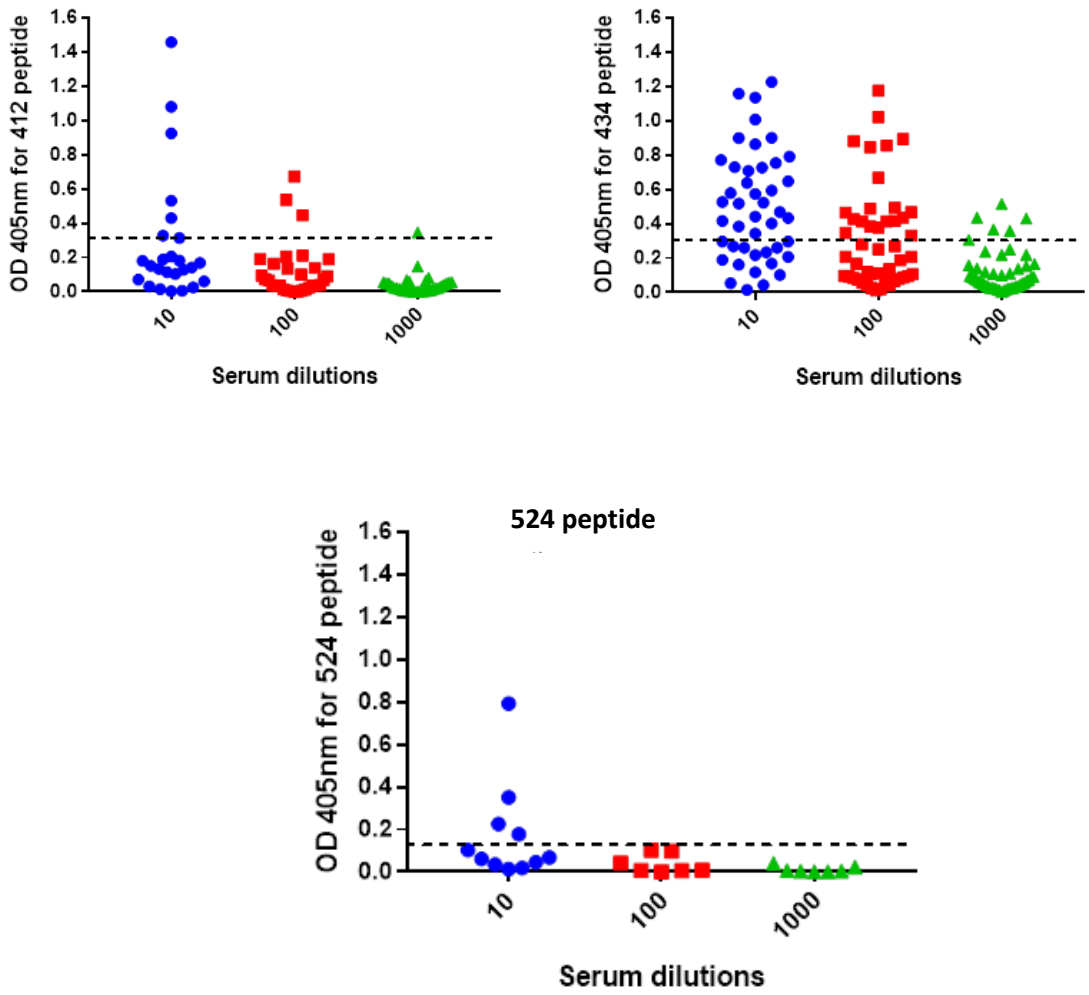


Figure 1.5. ELISA data representing eptiope specificity of self resolvers' sera: Enzyme Linked Immunosorbent Assay was performed using three conserved linear peptide epitopes of E2 envelope protein (the 412 epitope, the 434 epitope and the 524 epitope) against three different serum dilutions (10 fold, 100 fold, 1000 fold) of sera from self reslosolvers. Dotted line represents the cutoff value using the mean value from healthy serum controls.

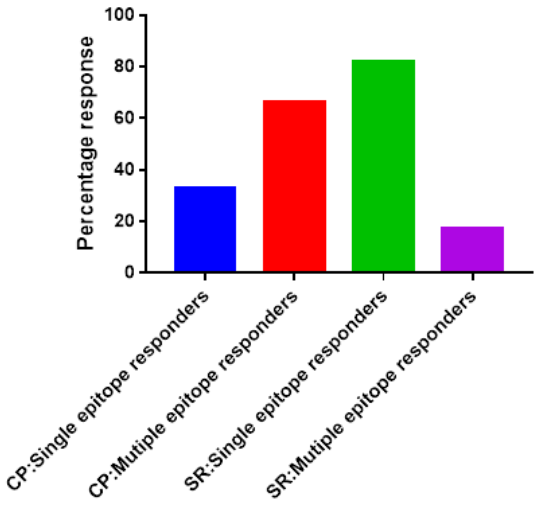


Figure 1.6. Comparison of the CP and the SR sera for the presence of single epitope and multiple epitopes specific antibodies.

Table 1.4. Details of antibody response against three conserved epitopes of E2 in spontaneously resolved individuals: Sera with negative response for all three epitope are excluded from the table.

Serum No	Response against 412	Response against 434	Response against 524
PK1	-*	++*	-
PK2 `	-	+*	-
PK3	-	+	-
PK4	-	+	-
PK6	-	+	-
PK7	-	+	-
PK8	-	-	+
PK9	+	-	-
PK13	-	++	-
PK14	++	-	-
PK16	-	+	-
PK18	-	+	+

PK20	-	++	-
PK25	-	+	-
SH8	-	+	-
SH15	-	+	-
SH16	-	+	++
SH18	++	+	-
SH19	++	-	-
SH504	-	++	-
SH508	-	+	-
SH510	+	+	-
SH511	-	+	-
SH513	-	+	-

*Non responders = "-", OD < 0.5 = "+", OD ≥ 0.5 = "++"

Within each group of sera, we compared the response against individual epitopes quantitatively using the mean value of optical density as an indicator of antibody titer. Within chronic patients' sera, the mean of the OD values for all three peptide epitopes were not significantly different from one another. Similarly same trend was observed in spontaneous resolvers' sera. This shows that although a lower number of sera had response against the 412 and the 524 epitopes but titers of antibodies were comparable to that observed for the 434 epitope, in both groups of sera. This specific data corresponds to 10-fold serum dilution (Figure 1.7).

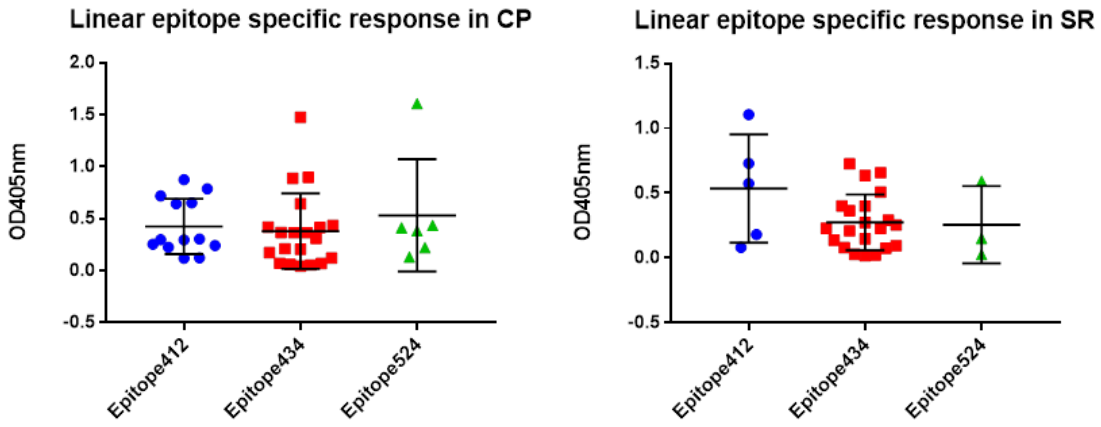


Figure 1.7. Comparison of different linear epitope specific antibody titers. Comparative response against all three epitopes in spontaneously resolved individuals and chronically infected patient using 10-folds serum dilution. Data points for all three epitopes are comparable to each other. Date from positive responders was included only. Each data point represents OD450 value after subtracting the value of the control.

While comparing antibody titer against individual epitopes in two groups of sera, CP and SR, the mean OD values for all three epitopes were not significantly different in the two groups (Figure 1.8). This suggests the antibody titer against any specific epitope was not different in two groups of individuals, despite self-resolvers generally have very short exposure time of active viraemic phase as compared to chronic patients. This further indicates that if antibodies are elicited in early phase of the infection their titer is high enough to clear the virus.

When we do comparison by setting a threshold of OD≥0.5 for high titer, a small proportion of individual epitope specific sera show OD≥0.5 in chronic patients. In this case 38% of the 412 specific sera have OD≥0.5, while that proportion for the 434 and 524 epitopes is 20% and 14%, respectively. This indicates that although higher percentage of chronic sera shows antibody response against these linear epitopes but a small proportion of these produces antibodies in high titer. In self-resolvers, 66% of the 412 specific sera, 33% of the 524 specific sera and 22% of the 434 specific sera show OD≥0.5. However, in SR group total number of 412 and 524 specific sera was very small (Table 1.5). Interestingly, both serum groups showed higher titer against the 412

epitope. This indicates that although just a smaller percentage of individuals produce antibodies against the 412 epitope but these specific antibodies are produced in high titer.

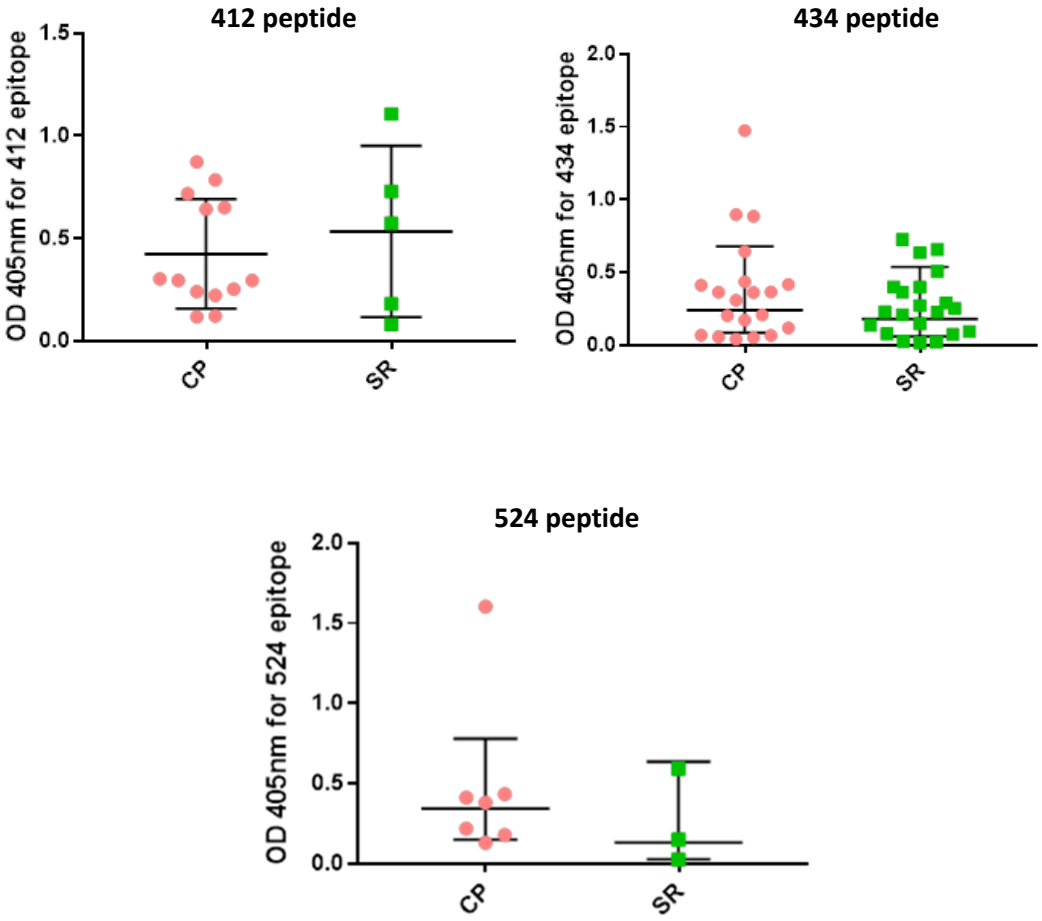


Figure 1.8. Comparison of antibody responses against individual epitopes in two groups of sera. Each data point represents OD450 value after subtracting respective values for healthy controls. Only 10-fold dilution of sera was used in this analysis and data for only positive responders was included in these plots.

Table 1.5. Summary of the antibody response against three conserved linear epitopes in the E2 protein from the sera of chronic patients and self-resolvers.

	Chronic HCV patients			**Spontaneously resolved individuals**		
	Non responding		30%	Non responding		53%
	Responding		70%	Responding		47%
Name of epitope	**Positive serum**	**Serum OD <0.5**	**Serum OD>0.5**	**Positive Serum**	**Serum OD <0.5**	**Serum OD>0.5**
Epitope 412	43%	62%	38%	21%	40%	60%
Epitope 434	66%	80%	20%	82%	78%	22%
Epitope 524	23%	86%	14%	13%	66.6%	33.3%

1.3.5. HCV neutralization

To test HCV neutralization activity of the sera containing these linear epitope specific antibodies we performed HCV pseudo-typed particles based neutralization assay. Initially we used 1:50 serum dilution for screening purpose. Sera with more than 30% neutralization activity in the initial screening were subjected to further experiments to measure effective dilution with 50% neutralization (ED_{50}) through dose response curve. ED_{50} values of chronic patient sera were observed in a range between 1:50 to 1:8000 (Figure 1.9). In case of self-resolver's sera, out of total 49 individuals, 30 sera showed >30% neutralization activity in the initial screening with 50 folds serum dilution. Dose response curve of these sera showed ED_{50} value ranging from 1:4 dilution to 1:850 dilution (Figure 1.10). Although, we observed comparable titers of antibodies specific to three linear epitope in two groups of individuals in ELISA, but the range of ED_{50} values in the two groups to neutralize the virus was markedly different. SR sera neutralized the virus only at low dilution. After the infection antibodies specific for various epitopes are elicited particularly most of the neutralizing antibodies in chronic patients are conformational epitope specific. Chronic patients generally have an active infection phase consisting of a long period of

time therefore high titers of different antibodies are present in their sera. Spontaneously resolved individuals, on the other hand, experience active infection for short period of time therefore overall antibody titer is low in these individuals as compared to chronic patients. However, in ELISA based study conserved linear epitope specific antibodies were observed in comparable titer in the two groups of sera suggesting such antibodies possibly exist in larger proportion of the total antibodies in self-resolvers as compared to in chronic patients. The lower ED_{50} values in chronic patients as compared to self-resolvers could be attributed to the presence of several types of antibodies in high titer in these patients.

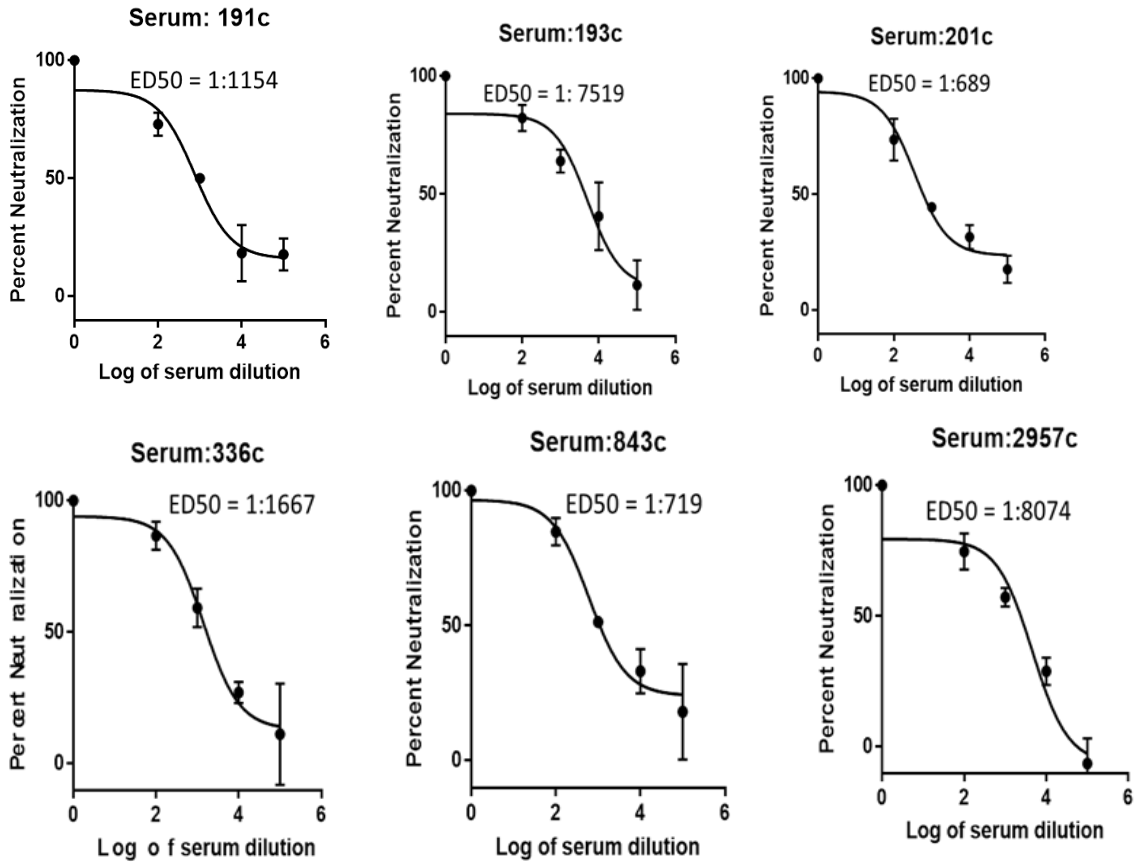


Figure 1.9. Dose response curve of sera from chronic HCV patients. Percent neutralization activity was measured for different dilutions of selected chronic sera and dose response curves were plotted using Graph pad software.

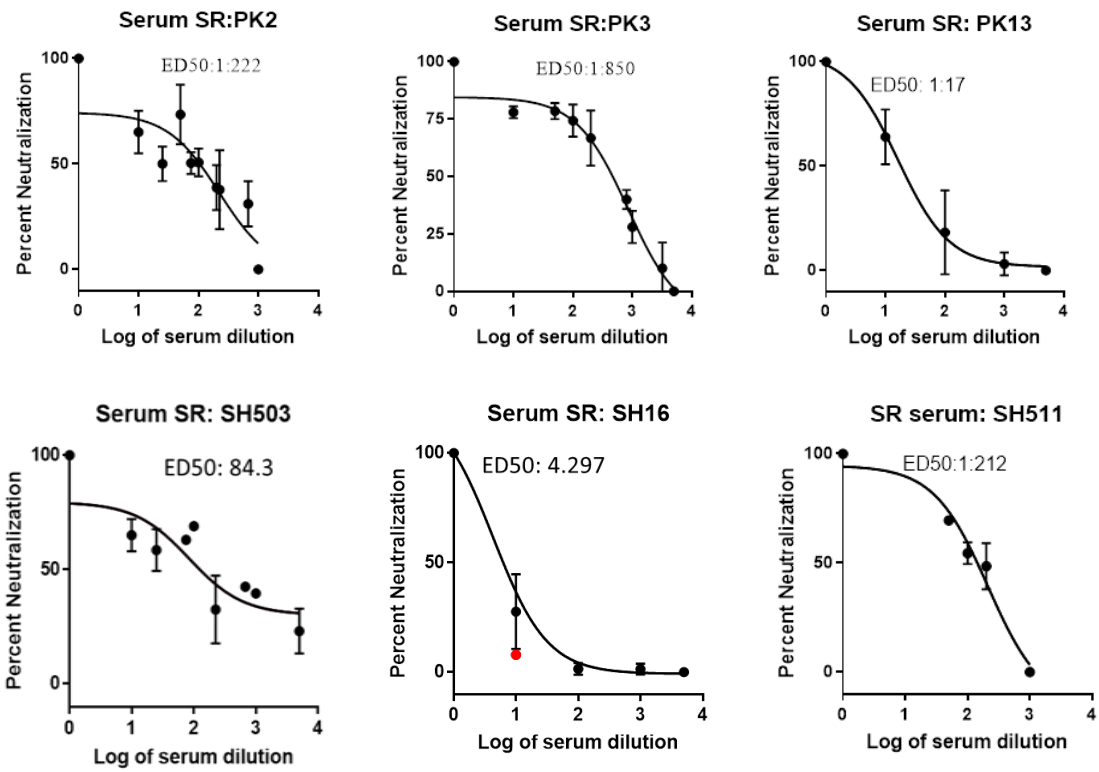


Figure 1.10. Dose response curve for sera from self-resolvers. Percent neutralization activity was measured for different dilutions of selected self-resolvers' sera and dose response curves were plotted using Graph pad software.

1.3.6. Peptide competition assay

To further clarify the role of conserved linear epitope specific antibodies in viral neutralization we performed competition assay where the selected sera containing high titer of linear epitope specific antibodies were first incubated with individual peptides corresponding to these epitopes before testing the viral neutralization activity. Sera with positive neutralization activity were selected for this competition assay. Among eight chronic patient sera tested in competition assay, only one showed significant decrease in viral neutralizing activity of the serum by the presence of competing peptides corresponding to the 412 and the 434 epitopes (Figure 1.11, serum number 201). This suggested the neutralization activity of this serum was primarily due the presence of these linear epitope specific antibodies. Viral neutralizing activity of no other chronic serum was affected by any other peptide despite binding of antibodies from these sera to different linear epitopes was detected in ELISA. These observations suggest that either the linear

epitope specific antibodies detected in ELISA are non-neutralizing or these antibodies are inhibited by the presence of interfering antibodies and the observed neutralization is due to the presence of conformational epitope specific antibodies or any other linear epitope specific antibodies. Neutralization interference by other antibodies is a very common phenomenon [7].

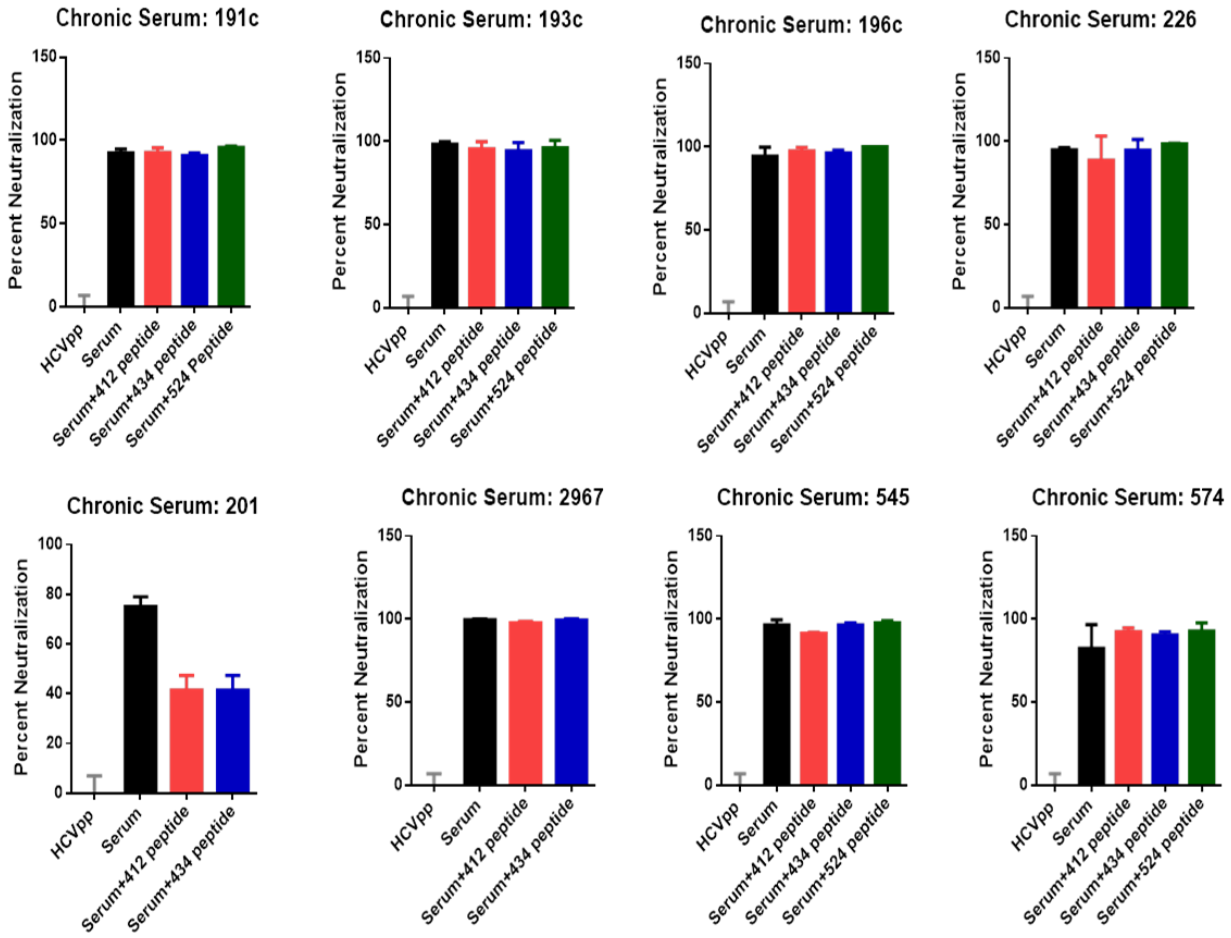


Figure 1.11. Peptide competition assay to elucidate the role of linear epitope specific antibodies in viral neutralization. Different chronic patient sera with good HIV neutralization activity at 1:50 serum dilution were incubated with different peptides (1mg/ml) at 37^{o}C for 2 hr before performing HCVpp neutralization assay. Column bar labeled with "HCVpp" represent percent 100% infection, column bar labeled with "Serum" represent HCV neutralization by the respective serum without adding peptide, while column bar labeled with "Serum+412",

“Serum+434” or “Serum+524” represent neutralization of sera after those ware incubated with respective peptides.

For self-resolvers’, out of 30 sera that had shown viral neutralization activity, 7 sera (PK2, PK3, PK4, PK7, PK13, SH503, SH16) were selected for competition assay. A significant decrease in viral neutralizing activity of two of the selected sera was observed by the presence of competing peptides. In both cases the competing peptide was corresponding to the 434 epitope. Moreover, the activity of another serum was slightly affected by the presence of peptide corresponding to the 524 epitope. Activity of no other serum was competed by any peptide. This suggest that either the linear epitope specific antibodies detected in ELISA are non-neutralizing or these antibodies are inhibited by the presence of interfering antibodies and the observed neutralization is due to the presence of conformational epitope specific antibodies or any other linear epitope specific antibodies. However, the exact mechanism remains to be elucidated. Nevertheless, the most striking finding of these experiments was the possible role of 434 epitope specific antibodies in viral neutralization and possible virus clearance.

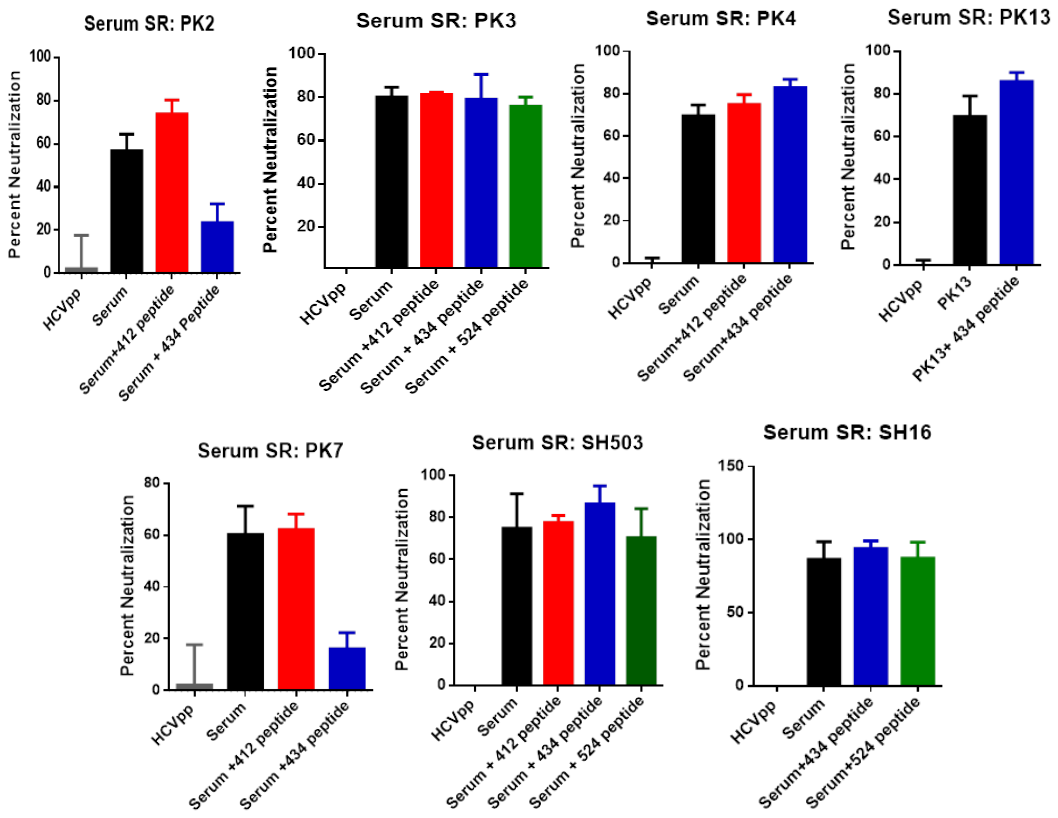


Figure 1.12. Peptide competition assay to elucidate the role of linear epitope specific antibodies in viral neutralization. Different self-resolvers' sera with good HIV neutralization activity at 1:50 serum dilution were incubated with different peptides (1mg/ml) at 37^{o}C for 2 hr before performing HCVpp neutralization assay. Column bar labeled with "HCVpp" represent percent 100% infection, column bar labeled with "Serum" represent HCV neutralization by the respective serum without adding peptide, while column bar labeled with "Serum+412", "Serum+434" or "Serum+524" represent neutralization of sera after those ware incubated with respective peptides.

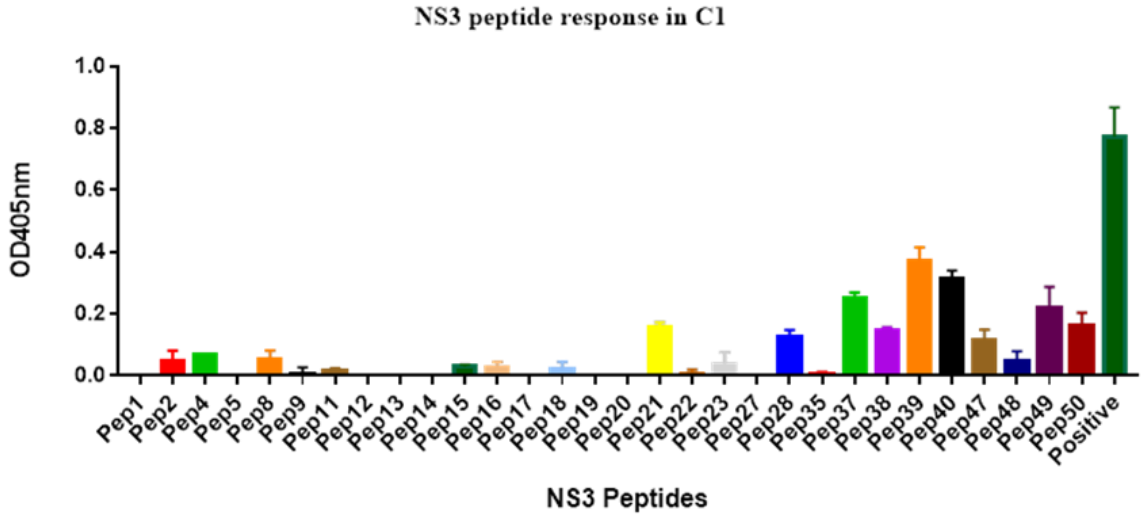
1.3.4. Antibody response against NS3-peptides

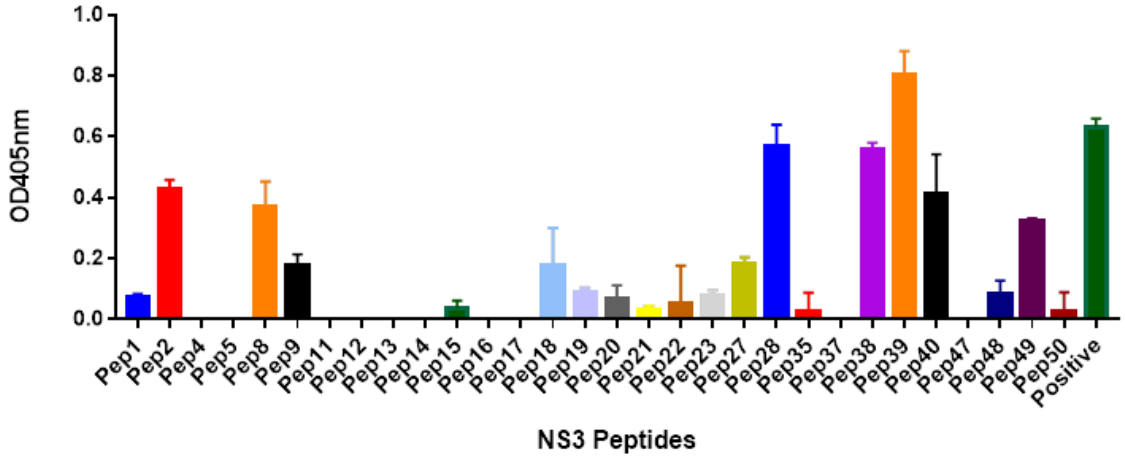
To understand the specificity of antibodies for epitopes present in the NS3 protein, ELISA was performed against thirty peptides with overlapping sequences spanning the full length NS3 protein. These include fourteen peptides from the protease domain and sixteen peptides from the helicase domain of NS3 in addition to using full length NS3 protein. Sera from 55 chronically

infected patients and 35 spontaneous resolvers at single optimized serum dilution of 1:50 were used.

In different sera a very diverse response was observed with respect to reactivity for different peptides. Data from some representative individual sera have been shown in Figure 1.13. Overall reactivity against the full length NS3 protein in chronic sera was significantly higher than self-resolvers (Fig 1.14), which could be attributed to longer exposure time in chronic infections. Interestingly, in chronic patients' sera the overall response against peptides from the helicase domain of NS3 was intense as compared to response against the protease domain (Figure 1.15). However, in self-resolver's sera the response against peptides from the two domains of NS3 is not significantly different (Fig 1.16). Helicase domain is reported to have high immunogenicity than protease domain that is probably the reason for higher response in chronic patient's sera [108].

By looking at the reactivity against individual peptides, certain peptides showed response in significantly higher number of chronic patients and with high titer as compared to in self-resolvers (peptide 35, 40 and 47 in Table 1.6). Response against some peptides was much higher in self-resolvers as compared to chronic patients (Table 1.6). For example peptide18 has significantly different response in two groups with higher mean value in SR group. Many of SR sera are not only responsive towards peptide18 but they also have significantly higher titer in comparison to CP (Figure 1.17). However, in some peptides no response was observed against certain peptides in both types of sera. Interestingly, the peptides, which have intense response in significantly higher number of self-resolvers, belong to the protease domain of NS3 while the peptide with higher response in chronic patients belongs to the helicase domain of the NS3 protein.


NS3 peptide response in C1
OD405nm
1.0
0.8
0.6
0.4
0.2
0.0
Pep1 Pep2 Pep4 Pep5 Pep8 Pep9 Pep11 Pep12 Pep13 Pep14 Pep15 Pep16 Pep17 Pep18 Pep19 Pep20 Pep21 Pep22 Pep23 Pep27 Pep28 Pep35 Pep37 Pep38 Pep39 Pep40 Pep47 Pep48 Pep49 Pep50 Positive
NS3 Peptides


NS3 peptide response in C7
OD405nm
1.0
0.8
0.6
0.4
0.2
0.0
Pep1 Pep2 Pep4 Pep5 Pep8 Pep9 Pep11 Pep12 Pep13 Pep14 Pep15 Pep16 Pep17 Pep18 Pep19 Pep20 Pep21 Pep22 Pep23 Pep27 Pep28 Pep35 Pep37 Pep38 Pep39 Pep40 Pep47 Pep48 Pep49 Pep50 Positive
NS3 Peptides

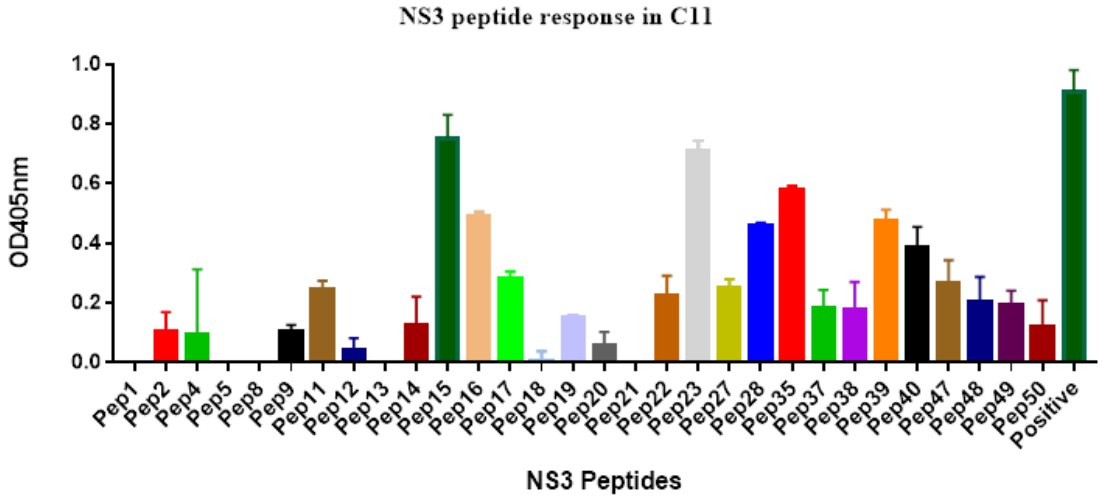

NS3 peptide response in C11
OD405nm
1.0
0.8
0.6
0.4
0.2
0.0
Pep1 Pep2 Pep4 Pep5 Pep8 Pep9 Pep11 Pep12 Pep13 Pep14 Pep15 Pep16 Pep17 Pep18 Pep19 Pep20 Pep21 Pep22 Pep23 Pep27 Pep28 Pep35 Pep37 Pep38 Pep39 Pep40 Pep47 Pep48 Pep49 Pep50 Positive
NS3 Peptides

Figure 1.13. Reactivity of chronic sera with different represntative peptides of NS3. ELISA was performed using 30 peptide epitopes of NS3 protein against one optimized serum dilutions of 50 fold from chronically infected HCV patients (CP). Full length NS3 protein served as positive control. The plot is drwan after substracting the OD values for control from the sample values.

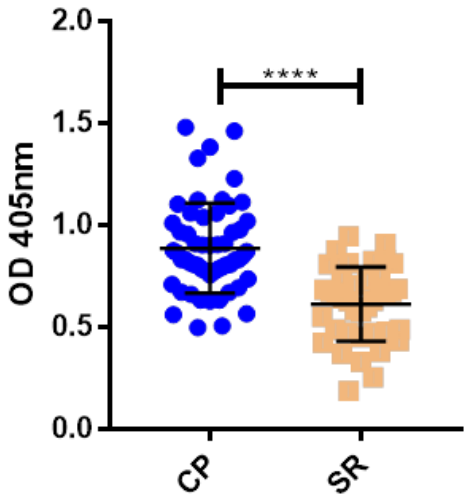


Figure 1.14. Comparative response against NS3 in spontaneously resolved individuals and chronically infected patient. Each point on the graph represents mean OD_{405} value from one serum. This data represent the mean OD values for NS3 protein from each patient in CP and SR group. Overall mean value from the whole data set of chronic patient is higher than the overall mean value from SR patient group.

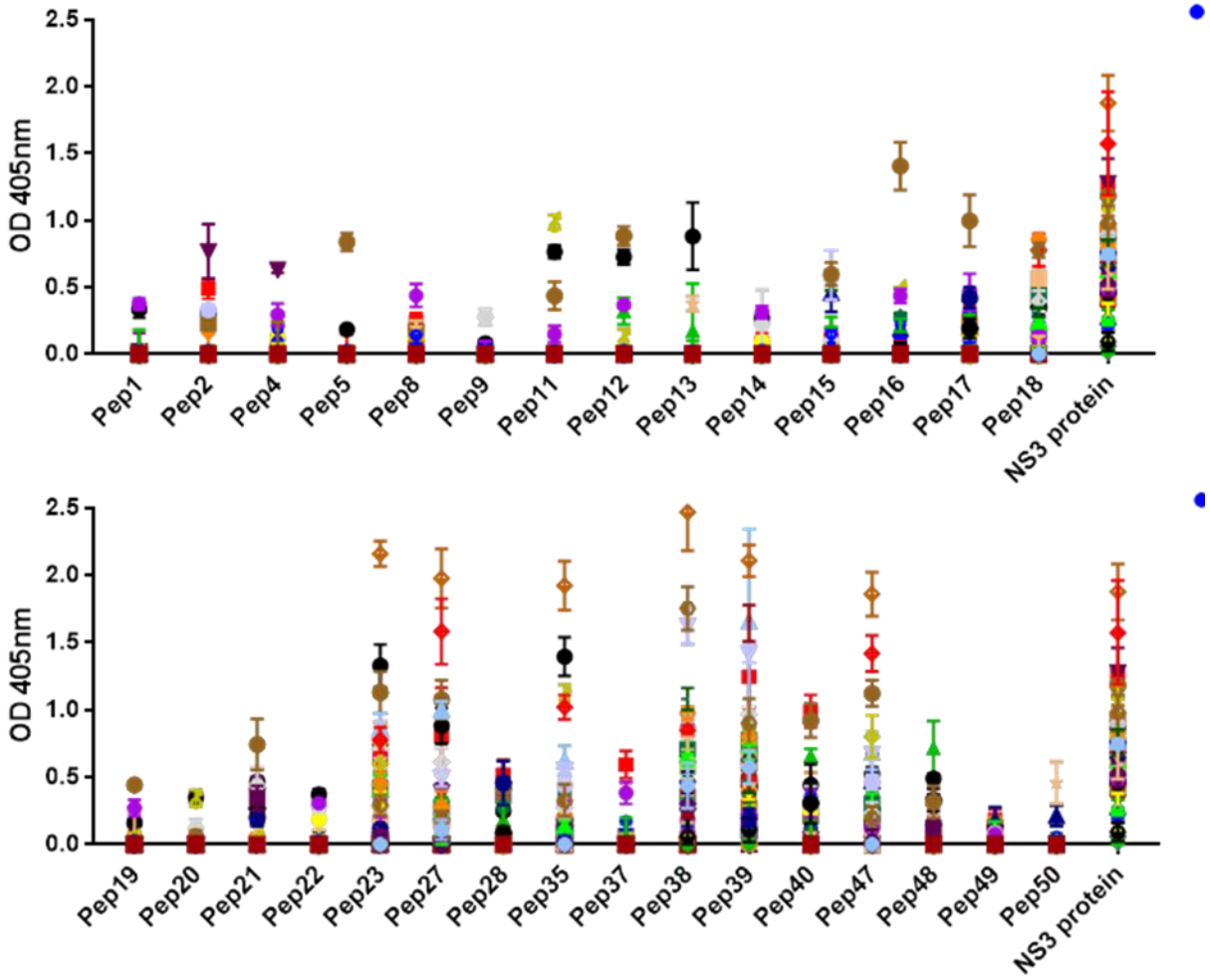


Figure 1.15. Antibody response against different sequential regions of NS3 in chronic HCV patients: Patients: Enzyme Linked Immunosorbent Assay was performed with fourteen peptides from NS3 protease domain (A) and sixteen peptides constituting NS3 helicase domain (B) using 1:50 serum dilutions of chronic HCV patient (CP). Symbols on the graph represent OD values from each chronic HCV patient against the peptides mentioned on X-axis. Full length NS3 protein was used as positive control.

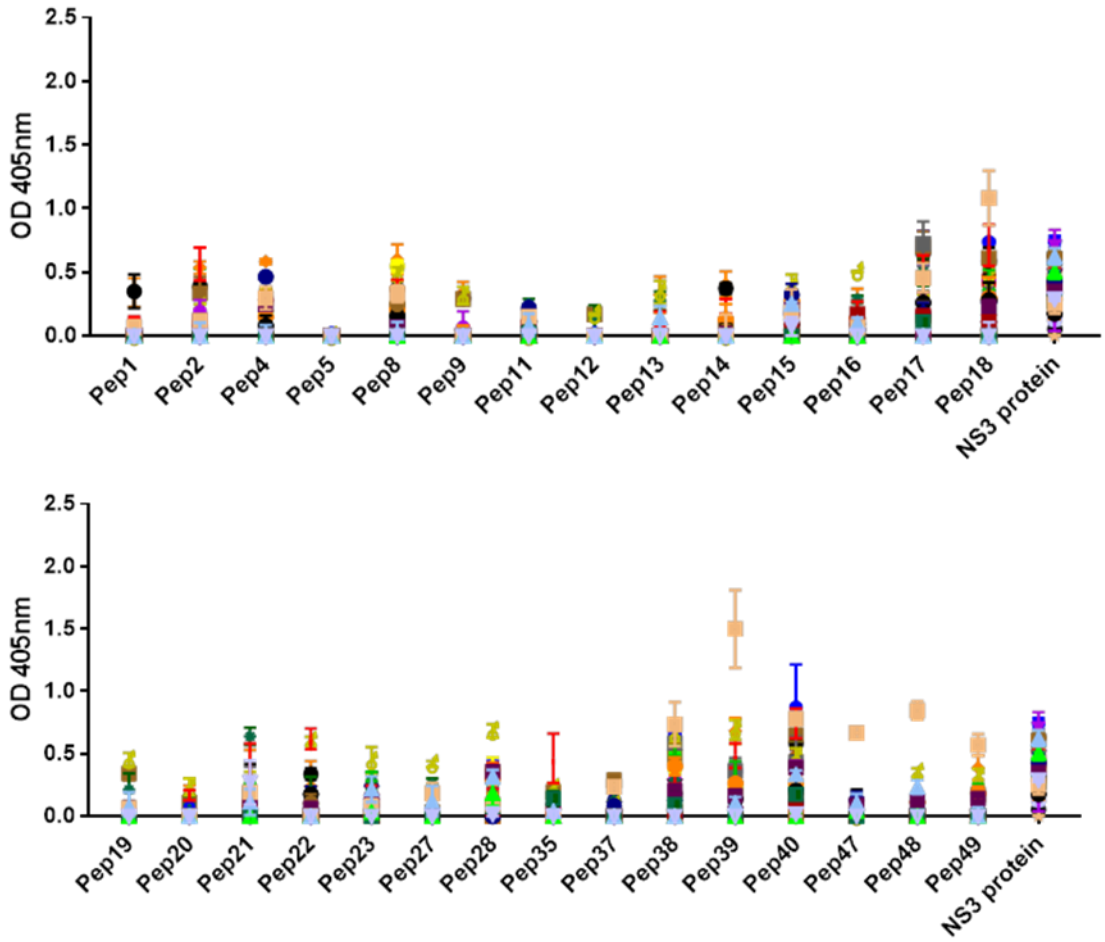


Figure1.16. Antibody response against different sequential regions of NS3 in HCV Spontaneous resolvers: Enzyme Linked Immunosorbent Assay was performed with fourteen peptides from NS3 protease domain (A) and sixteen peptides constituting NS3 helicase domain (B) using 1:50 serum dilutions of spontaneously resolved patients. Full length NS3 protein was used as positive control.

Table 1.6. Comparison of NS3 protein specific peptide epitope response against selective peptides between CP and SR: Positive responders are bifurcated to higher (OD>=0.5) and lower responders (OD<0.5. Peptides highlighted in red are the one with biased response towards one patient group.

	CP			SR		
Peptide name	**Positive responders**	**OD<0.5**	**OD >0.5**	**Positive responders**	**OD<0.5**	**OD>0.5**
	%	%	%	%	%	%
Peptide17	43	92	7	62	72	27
Peptide18	47	73	26	60	80	19
Peptide21	50	93	6.	60	95	4
Peptide23	78	76	23.	40	100	0
Peptide35	43	75	25	31	100	0
Peptide38	56	64	35	65	82	17
Peptide39	90	58	42	60	95	4
Peptide40	56	82	17	80	82	17

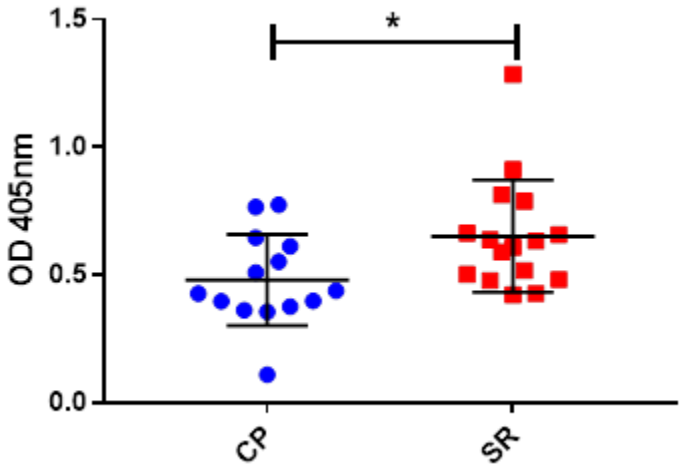


Figure 1.17. Response against NS3 peptide18 between CP and SR group: Data point in both patients groups were compared using unpaired T test.

1.4. DISCUSSION

Peptide or small protein subunit based-immunogens as vaccine candidates are more advantageous as compared to whole surface protein or attenuated virus owing to very specific response of antibodies against immunogens. Viral surface or surface proteins incorporate epitopes of neutralizing, non-neutralizing and interfering antibodies [7]. Elicitation of all kinds of antibodies by a vaccine in particular interfering antibodies could mask neutralizing epitopes limiting the efficacy of a vaccine. Moreover, surface exposed regions of a viral surface protein exhibit higher sequence variability and antibodies targeting these epitopes are not broadly neutralizing. Sequentially conserved epitopes, which are generally less exposed, elicit broadly neutralizing antibodies. In recent years, small peptides corresponding to conserved epitopes of antibodies have been considered as potential vaccine candidates. Different approaches have been used to efficiently present peptide epitopes to immune system, increase their half-life, decrease their renal clearance and restrict these in a specific conformation.

Around 25% of the HCV infected patients clear the virus without any treatment and the role of antibodies in spontaneous viral clearance is not clear. A major part of HCV envelop protein E2 is highly variable but the regions involved in interaction with the CD81 receptor are highly conserved across genotypes. Antibodies directed against these conserved epitopes from chronic patients or immunized mice have been reported to have broad neutralization[83, 88]. These conserved epitopes could be linear or conformational epitope. Although most of the identified HCV neutralizing antibodies are conformational eptiope specific but some broadly neutralizing antibodies specific for conserved linear epitopes have been reported. In this project we aimed to decipher the role of linear epitope specific antibodies in spontaneous viral clearance to obtain essential information for immunogen based vaccine design.

In this regard, we profiled antibody response in the sera of spontaneously resolved individuals and HCV chronic patients against three highly conserved linear epitopes of HCV envelope. As the first step we performed ELISA with the sera of five healthy donors, 30 chronically infected patients and 49 spontaneous resolvers against all three highly conserved linear epitopes of HCV envelope.

We observed that both groups of sera showed reactivity for peptides corresponding to different conserved linear epitopes. However, unlike the chronic sera 70% of which were reactive, only 46% of the self-resolvers' sera showed reactivity to any of the conserved linear epitopes. In most of these cases, this reactivity was primarily for the 434 epitope. In viral neutralization assay most of the chronic patients' and self-resolvers' sera neutralized the virus, however overall ED_{50} (effective dilution with 50% neutralization) values of chronic patients' sera were much higher as compared to self-resolvers' sera suggesting much higher titer of neutralizing antibodies in chronic patients. After the infection, antibodies specific for various epitopes are elicited particularly most of the neutralizing antibodies in chronic patients are conformational epitope specific. Chronic patients generally have an active infection phase consisting of a long period of time therefore high titers of different antibodies are present in their sera. Spontaneously resolved individuals, on the other hand, experience active infection for a short period of time therefore overall antibody titers are low in these individuals as compared to in chronic patients. However, in our ELISA based study conserved linear epitope specific antibodies were observed in comparable titers in the two groups suggesting such specific antibodies possibly exist in larger proportion of the total antibodies in self-resolvers as compared to in chronic patients. The lower ED_{50} values in chronic patients as compared to self-resolvers could be attributed to the presence of several types of antibodies at high titers in these patients.

In peptide competition viral neutralization assay neutralizing activity of many self-resolvers' sera was significantly decreased by the presence of competing peptide corresponding to the 434 epitope suggesting the viral neutralization by the sera was primarily due to the presence of 434 epitope specific antibodies in those sera. These results suggested the role of the 434 epitope specific antibodies in viral clearance in self-resolver individuals. However, the neutralization activity of chronic patients' sera was hardly affected by the presence of any competing peptide corresponding to linear epitope, despite the reactivity of these sera for all three epitopes were detected in ELISA.

The 524 and the 412 epitopes are the most conserved regions of the HCV envelope protein in different genotypes. Several broadly cross-reactive antibodies targeting the 412 epitope have been identified; some of these antibodies have shown protection against HCV in Chimpanzees

[84]. However, only a very few antibodies targeting the 524 region as a linear epitope have been reported earlier but several antibodies specific for a conformational epitope involving residues from the 524 region have been reported[109]. The 434 epitope has been described to be the target site for neutralizing, non-neutralizing and interfering antibodies depending on which particular residues of this region are involved in recognition. This region has also been reported to be the part of conformational epitopes of several HCV neutralizing antibodies [110]. A possible role of the 434 epitope specific antibodies in spontaneous viral clearance defined in this study is one of the most significant findings. Viral neutralization by the sera reactive for the 524 and the 412 epitopes from both groups of individual were not competed by the presence of respective peptides. This suggests the viral neutralization observed by these sera was due to the presence of conformational epitope specific or any other linear epitope specific antibodies in these sera and the antibodies specific for these two linear epitopes possibly have been inhibited by the interfering antibodies.

Several previous studies have reported the interference of 434 epitope targeted antibodies in viral neutralizing activities of antibodies specific for other epitopes particularly the epitope present in the 412 region of the E2 protein [7, 8, 111]. Kachko et al recently elucidated the role of interference antibodies in vaccinated individuals. They analyzed the sera from 112 individuals participating in phase-1 clinical trials of a recombinant E1E2 protein based HCV vaccine for the presence of different epitope specific antibodies. In their ELISA based study, 22 sera were found to contain double-positive antibodies specific for the 412 and 434 epitopes. By removing the 434 specific antibodies from the sera, the neutralization potential of over 70% of the sera significantly increased [7].

The 412 and 524 regions are sequentially highly conserved across different HCV genotypes and constitute the CD81 receptor-binding site. Structural studies have shown that a small portion of the 434 epitope is also the part of the CD81 receptor-binding site[112]. It suggests that the 434 region is structurally adjacent to the 412 and 524 epitopes and binding of certain antibodies to the 434 epitope could sterically interfere with and prevent the binding of antibodies to neighboring epitopes. Nevertheless, the extent of interference will depend on relative affinity of the two antibodies. Also, the 434 epitope is sequentially adjacent to the 412 region and some

antibodies specific to this region can potentially mask the latter. Moreover, conformational changes in the envelope protein resulting from the binding of 434 epitope specific antibodies could also interfere with the binding of other antibodies to their respective epitopes.

Seven chronic patients and three self-resolvers also showed reactivity to the 524 epitope in our ELISA screening. This epitope is the most conserved region, and previously very few antibodies targeting this linear epitope have been reported. Presence of antibodies specific for this highly conserved epitope in different sera in particular in self-resolvers' sera is of high significance as these antibodies from these individuals could be isolated and characterized in vaccine design perspective.

Antibody response against different peptide fragments of the HCV NS3 protein was also evaluated using ELISA. In different sera a very diverse response was observed with respect to reactivity for different peptides. Interestingly, in chronic patients' sera the overall response against peptides from the helicase domain of NS3 was intense as compared to response against the protease domain. However, in self-resolvers' sera the response against peptides from the two domains of NS3 was not significantly different. The helicase domain has been reported to exhibit high immunogenicity as compared to the protease domain that is probably the reason for its higher response observed in chronic patients' sera [108]. Three peptides including peptide 35, 40 and 47 (Table 1.6) all belonging to the helicase domain of NS3, showed reactivity in significantly higher number of chronic patients and with high titers as compared to self-resolvers. Similarly, a peptide from the protease domain of NS3, peptide 18, showed reactivity in significantly higher number of self-resolvers and with higher titers as compared to chronic patients. This differential NS3 epitope specificity in chronic patients and spontaneous resolvers indicates a possible role of NS3 epitopes in the spontaneous viral clearance.

Peptide 18 is an NS3 fragment from the active site of the protease domain and incorporates several highly conserved residues – Arg-155 is highly conserved residue of the active site and involves in the formation of catalytic triad with Asp-81, Phe-154 and Ala-157 constitute the specificity pocket while R-155, K-156, A-157, V-158, A-166, V-167, D-168 interact with the

substrate. The presence of antibodies specific for peptide18 specifically in spontaneous resolvers' sera suggests a possible role of peptide18 specific antibody in viral clearance.

NS3 has been reported to be more immunogenic than the E2 protein of HCV [113] and elicits an early humoral immune response during early steps of viral replication [114-117]. Although NS3 incorporates primarily the T-cell epitopes, but very early appearance of anti NS3 antibodies indicates that peptide fragments of NS3 may be incorporated on the infected cell surface or secreted out from the cells during early stage of the viral infection. NS3 specific antibodies are produced even before envelope specific antibodies therefore their presence is used as a diagnostic indicator [114]. After gene immunization of mice with conserved linear peptides of protease and helicase domain, a strong humoral response is developed in most of the vaccinated mice [118]. The NS3 specific antibodies have a potential to delay the progress of infection in case of dengue virus [119, 120] and bovine viral diarrhea virus (BVDV)[121]. Moreover, production of these anti NS3 antibodies during convalescent phase of DENV infection also indicates a protective role of these antibodies [122]. Studies from NS3 protein of other flaviviruses represent their protective role but for HCV, much information needs to be unveiled. Multifunctional capacity of NS3, early appearance of NS3 antibodies, protective role of anti-NS3 antibodies of other flaviviruses indicates the significance of humoral immune response against the NS3 protein. However, these antibodies are not expected to be neutralizing but their mechanism of protection or viral clearance could be based on antibody-dependent cell cytoxicity (ADCC) that has been observed in case of murine retrovirus clearance by specific antibodies [123].

Project 2

Estimation of hepatitis C prevalence in the Punjab province of Pakistan:

A retrospective study on general population

2.1. INTRODUCTION

Hepatitis C virus (HCV) infections progressively lead to liver impairment, cirrhosis and hepatocellular carcinoma [124]. Since the discovery of HCV in 1989, these infections continued to propagate across the globe despite extensive research to understand various aspects of the virus and the disease [125]. The prevalence of hepatitis C in Pakistan is possibly the second highest in the world with an estimated 10 million people (~5% of the population) affected [126-133]. Factors contributing to the high HCV infection rates in Pakistan include, unsafe practices of medical equipment by healthcare providers and dentists, unnecessary clinical use of injections, unhygienic state of instrumentation at barber salons, sharing of needles by drug users and unsafe blood transfusion [134]. Additionally, lack of awareness amongst general population regarding factors associated with viral transmission is another underlying factor for the spread of the disease [133]. Several efficacious direct acting anti-HCV treatments have become available to general population in Pakistan as part of Government's hepatitis control programs [135]. However, due to the asymptomatic nature of hepatitis C and lack of routine medical examinations, numerous HCV infected individuals with low-grade viraemia remain unaware of their infection status for years and therefore, do not pursue treatment until the symptomatic stage of liver impairment [136]. These individuals then also contribute to the spread of the virus to general population. Such situation hampers the efforts for controlling the HCV infections even with the availability of effective treatments. Population based studies to identify specific socio-demographic groups with high HCV prevalence and an analysis of contributing factors is therefore, needed to control the disease in general population. In recent years several HCV related epidemiological studies have been conducted in Pakistan, which provide an overview of HCV prevalence. However, these investigations were limited to small population size [137, 138] or only to high risk groups (IDUs, blood donors, health care workers) [139, 140] covering very small geographical regions [141].

In the current study, we have measured the seroprevalence of anti-HCV antibodies to perform retrospective analysis of HCV infections in general population of major cities of the Punjab province that accommodates 53% of the total population of Pakistan. Aim of this analysis was to identify socio-demographic groups with higher HCV prevalence so that these groups could be further investigated for factors contributing to higher HCV infections. Findings from the study will help in better management of hepatitis C prevention and treatment strategies in the country.

2.2. MATERIALS and METHODS

A total of 66,086 individuals participated in this study by visiting screening camps established for a week by Punjab AIDS Control Program (PACP) in year 2017, in 80 different towns across the Punjab province after a campaign was run through print media and by displaying posters at public places to encourage general public to test for HCV. Institutional review board of PACP and Lahore University of Management Sciences approved this study. No pre-selection criterion was applied for participation. For measuring serological response to HCV surface antigens, RAPID ICT test kit (One Step Hepatitis C Virus Test, Alere, Cat. No 02FK10) was used according to manufactures instruction. Informed consent was obtained from every participant before collecting samples. Each collected sample referred to a distinct individual as every individual was given a specific identification number (ID) that was related to their national ID card number ruling out the possibility of retesting. To ensure reproducibility and quality of the data approximately 5% of the samples were re-tested through ELISA to validate the results after collecting samples in EDTA vacutainer tubes. Participants' socio-demographics were recorded, which included, age, sex and gender, occupation and city of residence. Indeed, complete information about socioeconomic data and characteristics of the regions (rural or urban) would have been much better. However, information about only four demographic variables was collected at this stage due to budget, time and staff limitations. Some socioeconomic information could be indirectly derived from this data. For example, the occupation category "farmers" means the participants were from rural area, and a "student" means the participant is in a school or college.

Statistical analysis

Data was analyzed using IBM SPSS statistics version 23.0. Pearson's chi square test was applied to determine correlation between categorical variables using significance threshold of $P < 0.05$. Age as a continuous variable was categorized into groups of decades including <20, 21– 30, 31–40, 41–50, 51–60, 61–70 and >70 years for each of the three genders, male, female and transgender. Univariate analysis was performed to determine the anti-HCV antibody prevalence in relation to any of the four independent variables including age, geographical region, sex and gender, and occupation. Subsequently, bivariate and multivariate analyses were performed while controlling the effect of potential confounders. Prevalence ratios (PR) with 95% confidence interval (95% CI) were also calculated as a measure of association [142]. For multivariate

analysis, Fisher's linear discriminant analysis was performed and the classification function coefficients were used to construct the membership function of age, occupation, and sex and gender. Each independent variable was converted into a categorical variables and a linear classifier for HCV status was constructed. Box's model was employed for evaluating goodness of fit of the resulting model. Receiver operator characteristic curve was plotted to compare the sensitivity and specificity of the linear classification model. The predicted membership LDA functions for anti-HCV antibody response are given below:

Discriminant function LDAI*NonReactive*

= -13:362 + 1:553 (*Occupation*) + 7:070 (*Gender*) + 3:520 (*Age_Groups)*

Discriminant function LDAI*Reactive*

= -14:248 + 1:453 (*Occupation)* + 7:189 (*Gender*) + 3:904 (*Age_Groups)*

2.3. RESULTS

Study was conducted on a total of 66,086 subjects. Out of the total participants, sex and gender information of 22043 individuals (33%), age information of 18671 individuals (28.1%), occupation information of 19437 individuals (29.3%) and resident city information of 55207 individuals (83.5%) was available (Figure 2.1). Overall 17.3% of the participants demonstrated a positive response for anti-HCV antibodies. In terms of geographical regions a sporadic distribution of seroprevalence was observed ranging from around 5% to 45% in different areas of the Punjab province. Five districts of Punjab including Khanewal (45%, $P<0.01$), Nankana Sahib (37.1%, $P<0.01$), Sheikhupura (36.8% ($P<0.01$), Okara (31.2%, $P<0.01$) and Faisalabad (25.1%, $P<0.01$) were identified with significantly high seroprevalence (Table 2.1, Figure2.2) as compared to other districts. While analyzing the seroprevalence in different age groups, a significantly high prevalence was observed within 41–60 years of age as over 33% ($P<0.01$) of the tested individuals in this age group were positive for anti-HCV antibodies (Table 2.2.). In gender-based analysis, higher seroprevalence was observed in females as 18.7% ($P<0.01$) of the tested females demonstrated positive anti-HCV antibody response as compared to 17.5% and 14.5% in males and transgenders, respectively (Table 2.3). In bivariate analysis by combining age, and sex and gender, the age group with the highest seroprevalence in females was observed to be 51–60 years (38.8%) [PR 1.73, 95% *CI* 1.486–2.024]. However, in males and transgenders the highest prevalence was observe in age groups 61–70 years (31%) [PR 1.64, 95% *CI* 1.340–2.015] and 51–60 years (32.9%) [PR 2.87, 95% *CI* 1.867–4.414], respectively (Figure 2.3, Table 2.4).

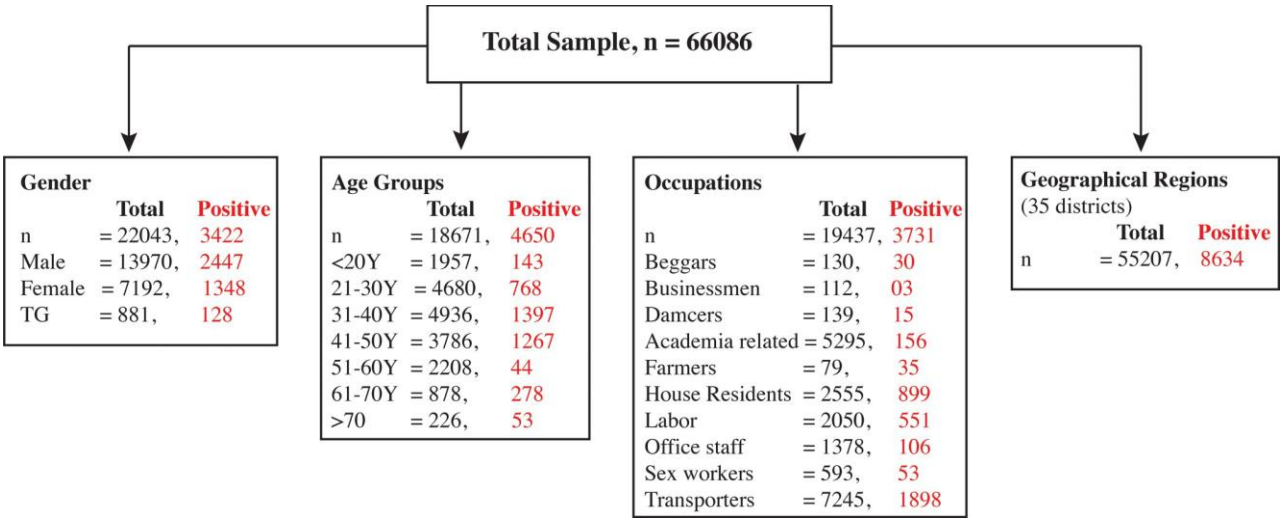


Figure 2.1. A summary of HCV seroprevalence in the Punjab province of Pakistan measured in year 2017. In the total population sample of more than 66000 individuals, information about the gender, age and occupation is available to analyze seroprevalence of HCV from 32 districts of Punjab.

Table2.1. Seroprevalence of anti-HCV antibodies in 32 districts of the Punjab province

	City	Total	Seropositive	% positive	p-value
1	Attock	2084	121	5.8	<0.001
2	Bahawal Nagar	68	4	5.9	0.027
3	Bahawalpur	3392	320	9.4	<0.001
4	Bhakkar	245	28	11.4	0.069
5	Chakwal	181	14	7.7	0.003
6	DG Khan	828	55	6.6	<0.001
7	Faisalabad	5734	1440	25.1	<0.001
8	Gujranwala	3247	703	21.6	<0.001
9	Gujrat	1209	211	17.4	0.079
10	Jehlum	776	79	10.2	<0.001
11	Jhang	1574	335	21.2	<0.0010
12	Kasur	1144	186	16.2	0.56

13	Khanewal	20	9	45	<0.001
14	Lahore	8040	1148	14.3	<0.001
15	Layyah	245	28	11.4	0.069
16	Mandi Bahauddin	537	75	13.9	0.284
17	Mianwali	3314	220	6.6	<0.001
18	Multan	3622	577	15.9	0.6
19	Muzaffar Garh	460	40	8.7	<0.001
20	Nankana Sahib	35	13	37.1	<0.001
21	Narowal	31	6	19.3	0.56
22	Okara	2705	845	31.2	<0.001
23	Pakpattan	526	99	18.8	0.44
24	Rahim Yar Khan	3023	421	13.9	0.008
25	Rajan Pur	231	25	10.8	0.043
26	Rawalpindi	5644	450	7.97	<0.001
27	Sahiwal	1795	375	20.89	<0.001
28	Sargodha	1958	297	15.2	0.56
29	Sheikhupura	114	42	36.8	<0.001
30	Sialkot	1733	323	18.6	0.1
31	T.T.Singh	654	140	21.4	<0.001
32	Vehari	38	5	13.2	0.67
		55207	8634		

Table 2.2. Anti-HCV antibody seroprevalence in different age groups of the total tested samples

Age groups	**Total**	**Seropositive**	**% +ve**	**PR**	***P*-value**	**95% CI**
<20 Y	1957	143	7.3%	0.238	<0.001	0.201-0.281
21-30 Y	4680	768	16.4%	0.592	0.001	0.552-0.635
31-40 Y	4936	1397	28.3%	**1.190**	<0.001	1.130-1.254
41-50 Y	3786	1267	33.5%	**1.517**	<0.001	1.430-1.608
51-60 Y	2208	744	33.7%	**1.532**	<0.001	1.412-1.663

61-70 Y	878	278	31.7%	**1.397**	<0.001	1.217-1.604
71-100 Y	226	53	23.5%	0.9	0.6	0.6-1.2
	18671	**4650**				

Table 2.3. Anti-HCV antibody seroprevalence in different genders

Gender	**Total**	**Seropositive**	**% +ve**	**PR**	***P*-value**	**95% CI**
M	13970	2447	17.5%	0.981	0.151	0.955-1.007
F	7192	1348	18.7%	1.065	0.011	1.015-1.118
TG	881	128	14.5%	0.785	0.01	0.65-0.94

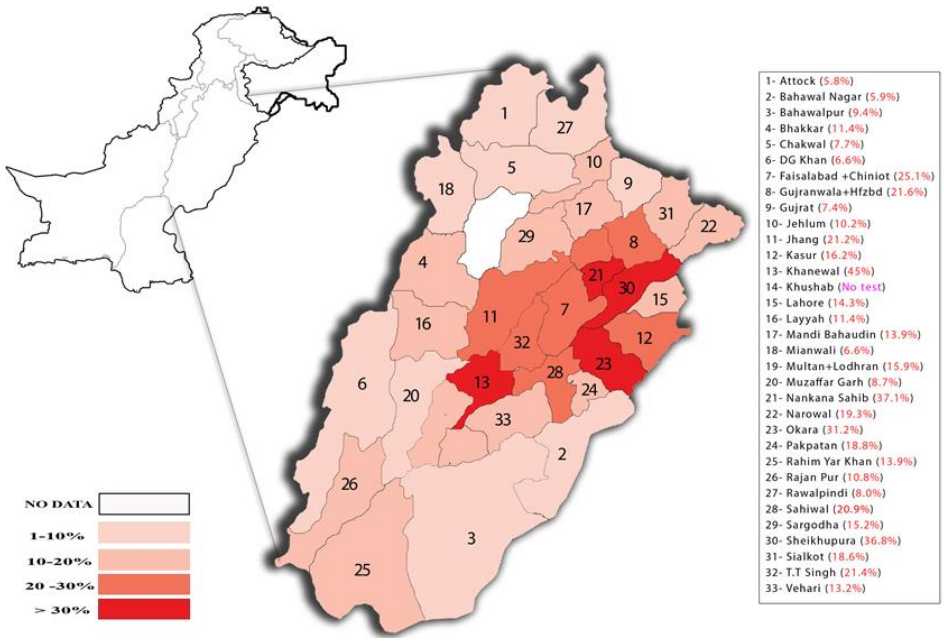


Figure 2.2. HCV seroprevalence in different geographical regions of the Punjab province. Seroprevalence in percentage of the total tested samples in the respective regions is color-coded. Data from three districts, Chiniot, Hafizabad and Lodhran were combined with neighboring larger districts as mentioned in the legend.

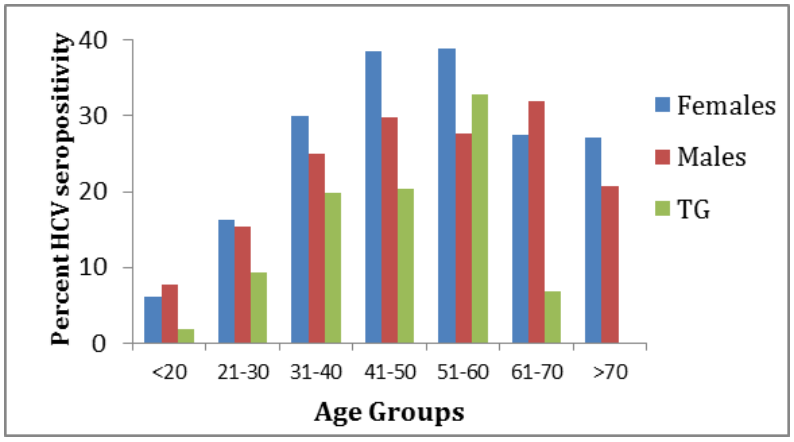


Figure 2.3. Gender specific HCV seroprevalence in different age groups in the province of Punjab analyzed in year 2017. Females in the age group 41-60year had highest seroprevalence. Men above 60Y had high seropositivity while TG have highest rate in the age group 51-60Y.

Table 2.4. HCV seroprevalence in different age groups of every sex and gender in the province of Punjab in year 2017. Prevalence ratios and P-values were calculated while comparing a specific age group with all other age groups of a specific sex and gender.

Gender	Age	Seropositive	%+ve	PR	p-value	95% CI
Females (4674)	<20	556	6.1	0.178	0.001	0.127-0.250
	21-30	1036	16.2	0.53	<0.0001	0.45-0.62
	31-40	1199	30	1.17	0.003	1.056-1.303
	41-50	973	38.5	1.714	<0.0001	1.533-1.917
	51-60	577	38.8	1.734	<0.0001	1.486-2.024
	61-70	263	27.4	1.03	0.824	0.792-1.341
	>70	70	27.1	0.937	0.946	0.604-

						1.718
Males (10706)	<20	1065	7.7	0.293	<0.0001	0.235-0.366
	21-30	2889	15.4	0.638	<0.0001	0.583-0.699
	31-40	2908	25	1.174	<0.0001	1.094-1.260
	41-50	2085	29.8	1.495	< 0.0001	1.377-1.622
	51-60	1243	27.6	1.34	<0.0001	1.193-1.504
	61-70	405	31.9	1.643	<0.0001	1.340-2.015
	>70	111	20.7	0.919	0.716	0.582-4.451
TG (877)	<20	110	1.8	0.108	<0.0001	0.543-1.523
	21-30	280	9.3	0.599	0.002	0.419-0.856
	31-40	196	19.9	1.454	0.017	1.081-1.955
	41-50	158	20.3	1.486	0.026	1.058-2.087
	51-60	79	32.9	2.871	<0.0001	1.867-4.414
	61-70	44	6.8	0.428	0.134	0.135-1.362
	>70	11	0	-	-	-

To measure HCV prevalence with respect to occupation, on the basis of available data occupation was categorized into 10 categories including beggars, businessmen, dancers,

academia associated (students and staff members of schools, colleges and universities), farmers, house residents (housewives, and retired and jobless individuals), laborers (security guards, electricians, motor mechanics, shopkeepers, technicians, plumbers etc), office staff, sex workers and transporters (drivers, truckers, conductors, helpers and loaders). Four occupations were identified with significantly high anti-HCV antibody prevalence, which included farmers (44.3%, P <0.01) house residents (35.2%, P <0.01), laborer (26.9%, P <0.01) and transporter (26.2%, P <0.01) (Table 2.5). However, the participants related to academia such as students and faculty or those working in close association with academia, or individuals involved in trade or businessmen demonstrated significantly lower HCV prevalence (2.9% and 2.7%, respectively).

Table 2.5. HCV seroprevalence in population according to their occupation measured in year 2017

Occupations	**Total**	**Seropositive**	**%+ve**	**PR**	**p-value**	**95% CI**
Beggars	130	30	23.07	1.263	0.252	0.841-1.896
Businessmen	112	3	2.7	0.116	0.0001	0.037-0.365
Dancers	139	15	10.8	0.511	0.012	0.300-0.872
Education related	5295	156	2.94	0.128	0.0001	0.109-0.149
Farmers	79	35	44.3	3.349	0.0001	2.151-5.212
House residents	2555	899	35.2	2.285	0.0001	2.125-2.458
Laborers	2050	551	26.9	1.547	0.0001	1.413-1.695
Office staff	1378	106	7.7	0.351	0.0001	0.289-

						0.426
Sex workers	593	53	8.9	0.413	0.0001	0.312-0.547
Transporters	7245	1898	26.2	1.494	0.0001	1.44-1.55

In bivariate analysis, anti-HCV antibody prevalence in different occupation was analyzed while controlling the effect of age as potential confounder. Farmers and house resident groups demonstrated significantly high prevalence (Figure 2.4).

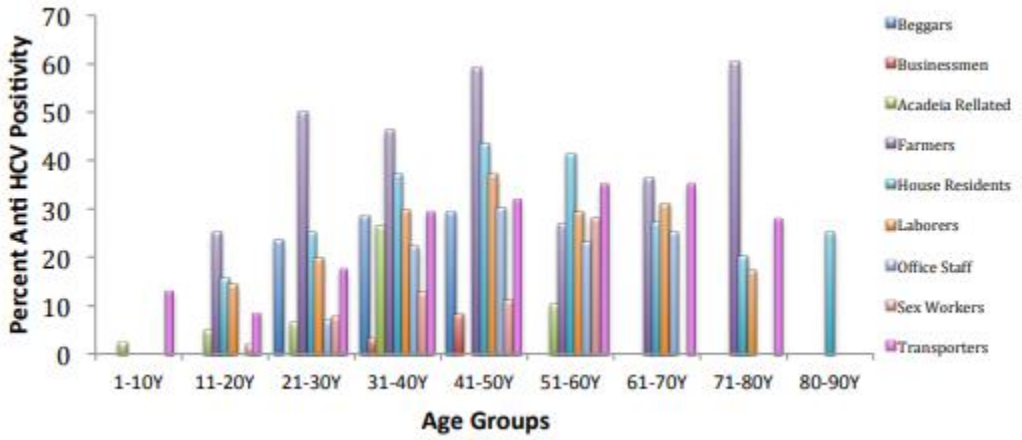


Figure 2.4. Bivariate analysis of age specific seroprevalence in different occupational groups: Farmers and house residents have highest seroprevalence in all age groups while academia related population sample had lowest seropositivity.

Fisher's linear discriminant analysis revealed that age, occupation, and sex and gender all are strong determinants of HCV prevalence (Table 2.6). ROC plot showed that the classification rate between reactive and non-reactive HCV has 63.4% accuracy, which can provide high-confidence classification for further HCV prevalence studies in the province (Figure 2.5). In addition to the socio-demographic variables discussed above, 52.0 of the participants declared themselves as Injecting Drug Users (IDUs), 46.0 (88%) of those showed positive response for the presence of anti-HCV antibodies.

Table 2.6. Fisher's linear discriminant functions for classification of 'Reactive' and 'Non-Reactive' cases. The coefficients of the classification function include Age Group, Sex and Gender, and Occupation. The values for each of these coefficients have been tabulated separately for both 'Non-Reactive' (no HCV) and 'Reactive' (with HCV) cases.

Classification of function coefficient		
	Response	
	Non-Reactive	Reactive
Age_Group	3.572	3.904
Sex_Gender	7.070	7.189
Occupation	1.553	1.453
Constant	-13.262	-14.248

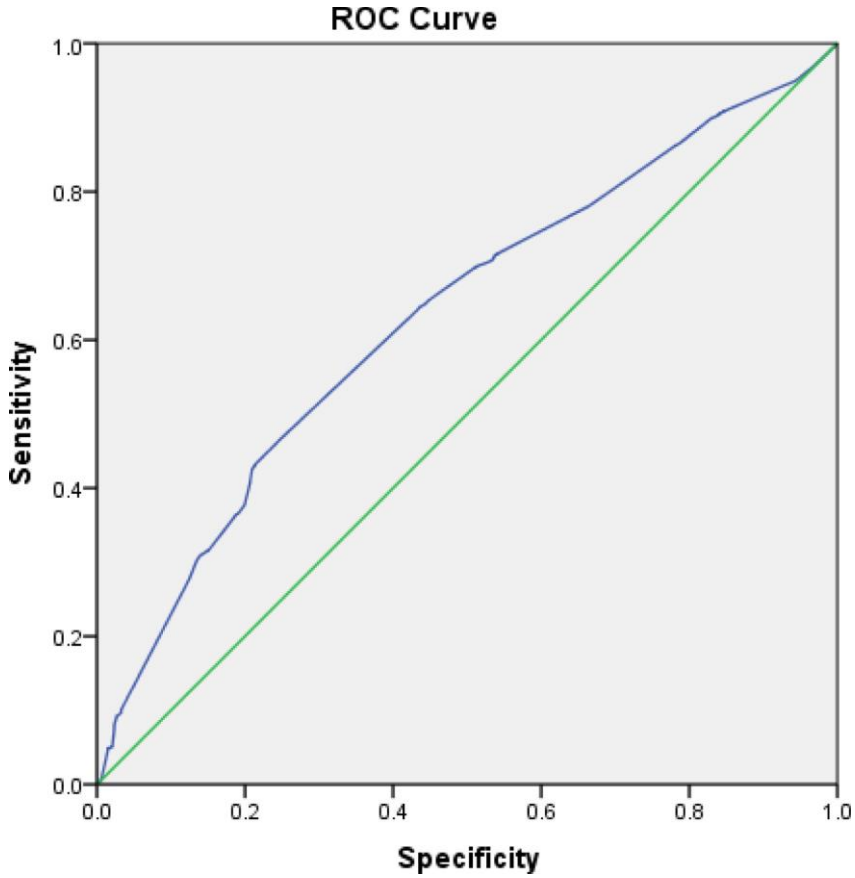


Figure 2.5. Receiver operator characteristic curve. ROC curve is showing the true positive rate (sensitivity) vs. false positive rate (100-specificity) for the discriminant function for reactive and non-reactive HCV. The area under the curve (blue) i.e. the accuracy of the model is computed to be 0.634.

2.4. DISCUSSION

Many epidemiological studies on HCV infections have been conducted in Pakistan in recent years. However, these studies show very inconsistent findings possibly because most of these studies are conducted by targeting small geographical region or by including only a specific population with very small sample size [137-139, 141, 143]. In the present study major geographical regions of the most populated province of Pakistan that accommodates 53% of the total Pakistani population, were covered incorporating a large sample size of population from 80 different cities and towns with diverse socio-demographic backgrounds involving all age groups, sex and genders, and association of these socio-demographic variables with HCV seroprevalence was analyzed.

In terms of geographical distribution, in some areas anti-HCV antibodies based serological response was observed in significantly high percentage of the regional population. In this regard five districts including Khanewal, Nankana Sahib, Sheikhupura, Okara and Faisalabad were identified with over 25% HCV seroprevalence. The analysis outcome in three of these regions, Khanewal, Nankana Sahib and Sheikhupura where number of participating individuals was very small (SI, Table 1), may not represent real situation due to a small sample size. Nevertheless, HCV prevalence in Okara and Faisalabad districts was significantly high where 2705 and 5734 individuals participated in the study, respectively and over 25% showed positive HCV serological response. The prevalence also varied with respect to age but in sex and gender specific way. The age group with highest HCV serological response was 51–60 years in females, 61–70 years in males and 51–60 years in transgenders. In previous studies male sex has been reported with higher HCV prevalence as compared to females in Pakistan [137]. In this population- based study, significantly higher HCV prevalence was observed in females as compared to males. However, contributing factors in this regard remain to be determined. A remarkable variation was observed in HCV prevalence among different occupational groups. A significantly high HCV prevalence was observed among farmers (~44%). The major factor contributing to the high HCV prevalence among farmers could be the sharing of shaving razors among multiple individuals. In countryside and rural areas of Pakistan there has been a culture of one barber for a village under a contract of providing service for obtaining crops and grains from farmers. These barbers lacked any system of hygiene and generally used a special non-replaceable razor for possibly hundreds of times for different customers significantly

contributing to the spread of diseases. It is also likely that these people are being given injections at the clinics of their primary healthcare without changing needles. Other participating groups with high HCV prevalence included house residents (house wives, jobless and retired individuals), laborer and transporters. Around 1/3rd of the total participating individuals were truck/bus/taxi drivers and associated helpers and over 26% of these were positive for anti-HCV antibody response. Two occupational groups including students and staff of educational institutions, and businessmen showed a significantly low HCV seroprevalence (<3%) suggesting the awareness about the virus transmitting factors is possibly the main factor to prevent HCV infection. Interestingly the sex workers, which were considered as a high-risk group, did not show high prevalence as hepatitis C has not been declared as sexually transmitted disease. In this study, a special emphasis was given to include socially underprivileged transgender community, to determine HCV prevalence through the specific efforts of the staff of screening camps. Access to proper health care for a large proportion of the Pakistani population is limited and this situation is further exacerbated for transgenders. Transgender communities in South Asia are socially excluded and face disparities in healthcare, education, career opportunities and in acquisition of proper status in the society [144]. As a result a vast majority of these individuals become commercial sex workers or beggars to make its financial needs [145]. In Pakistan incident rates of HIV infections among transgenders are very high where 17.5% of the total reported HIV infected cases are transgenders [146]. In order to find if similar situation exists for HCV infections, available data of 877 transgenders from the screening camps were analyzed and found HCV prevalence in these individuals is not higher than their counterpart cis genders. High rate of HIV infections among transgenders is attributed to sexual transmissions, as majority of the transgenders works as sex workers. Exposure to HCV risk factors for transgenders is possibly the same as that for general population. Another faction of the society, that faces health disparities in Pakistan are IDUs. Most of the IDUs belong to lower middle class families and most of these people are jobless. An effective rehabilitation program does not exist for such individuals.

In this study over 80% of the tested IDUs were found positive for anti-HCV antibodies. Although the sample size was not large enough, only 43 participants, to draw a conclusion, yet high percentage of the prevalence among tested individuals is indicative of seriousness of the

situation and urges to perform a large scale study. World Health Organization and some other studies report HCV incident rates as 4.5% in Pakistan [147-149] while many recent studies report up to 15% HCV prevalence [137, 150] corroborating our current analysis that suggests HCV seroprevalence as 17% in the major part of Pakistan. The current study is based on the presence of anti-HCV antibody in the serum, which does not necessarily represent the situation analysis of active viraemia. However, this study represents the retrospective analysis of HCV infections irrespective of the presence of active viraemia or treatment-induced or spontaneous clearance of the virus and highlights that certain socio-demographic groups have significantly high exposure to HCV infections [151-153]. This high exposure may not be directly related to the specific demographic background such as being farmer or truck driver or laborer, but behavior, such as using injectable drugs, and lack of awareness common in certain socio-demographic background may be the direct risk factors. Moreover, in these identified socio-demographic groups detailed investigation on risk factors such as unnecessary clinical use of injections, unsafe practices of medical equipment by dentists, unsafe blood transfusion, reuse of shaving razors and unhygienic state of instrumentation at barber salons needs to be done in the follow up studies. Despite the limitations regarding availability of data on all selected socio-demographic variables from all participants resulted from unwillingness of a proportion of the participants to provide complete demographic information, the current study identifies certain socio-demographic groups with high HCV prevalence. In conclusion, we performed retrospective study of HCV infections by analyzing the prevalence of anti-HCV antibodies involving over 60,000 participants from all major cities of the Punjab and the key findings are highlighted as follow: socio-demographic variables such as age, sex and gender, and occupation were associated with HCV seroprevalence. Females demonstrated significantly higher HCV prevalence as compared to males and transgenders. Farmers were identified with the highest HCV prevalence among all occupational groups, though jobless people, transporters and laborers also demonstrated significantly high prevalence.

In terms of geographical regions, HCV prevalence in two of the districts of Punjab, Faisalabad and Okara was found significantly high in general population that urges detailed analysis of risk factors in those areas and proper measures to control these infections. Moreover, considering the magnitude of hepatitis C as public health problem in Pakistan, health authorities are urged to

enhance their focus on following measures: (i) develop prevention campaigns, especially targeted at the most affected groups, (ii) intervene in establishments that are known to contribute to maintaining the high prevalence of Hepatitis C in order to guide and monitor safe procedures and penalize those establishments that do not follow such guidelines, (iii) provide access and monitor adherence to treatment indicated in these cases, (iv) develop and implement an official government information system for reporting hepatitis C positive cases diagnosed in routine health services, making this disease as compulsory notification, and (v) periodically prepare epidemiological reports on the disease in order to follow the behavior of this epidemic, in addition to seeking to improve the quality and completeness of the data in order to have a better characterization of the disease and regional differences observed.

Project 3

Characterization of different FcγR activation by HBsAg specific antibodies in Hepatitis B vaccinated and self-resolved individuals

3.1. INTRODUCTION

3.3.1. Prevalence of Hepatitis B

Currently 3.5% of the world population is infected by hepatitis B virus (HBV) [154]. According to the WHO factsheet approximately 240 million people are chronic HBV carrier [155]. Only 10.5% of the infected people are aware of their infection status and among them only 16% have an access to treatment [156]. Geographically over 6.0% of the adult population of Western Pacific and African regions is HBV infected while the prevalence is 3.3%, 2% and 1.6% in Eastern Mediterranean Region, South-East Asia Region and European Region, respectively. In America 0.7% of the population is infected [157]. African countries are considered to be the most endemic for HBV where around 65 million individuals (over 8% of the population) are infected. Among them 25% deaths occur due to liver failure [158]. The infection is more prevalent in rural areas and in men population. Alarmingly, 12% of the HBV infected people have HIV-1 co-infections. Transition to chronic infection is higher in infants (90%) and children (25-50%) than adults (1-5%).

Symptoms

The main symptoms of HBV infection include dark yellow urine, yellow eyes and skin, abdominal pain with nausea, vomiting and consistent fatigue. However, a proportion of infected people don't develop any symptom. The untreated infections can lead to liver failure, followed by liver cirrhosis and liver cancer that in many cases can result in death[157].

3.1.2. About HBV

HBV belongs to Hepadnaviridae family and is a DNA virus having genome size of 3.2 kb (Figure 3.1). It is spherical enveloped virus having a diameter of 42 nm with capsid enclosing a polymerase and covalently closed circular DNA (cccDNA) that replicates via RNA intermediate. Lack of proofreading ability during transcription is the cause of high mutation rate of 2 x 10^4 base substitutions/site/year. Its genome exhibits four open reading frames (ORFs) encoding different proteins including DNA *polymerase, surface protein (HBsAg), core (HBcAg) and the X protein [159].*

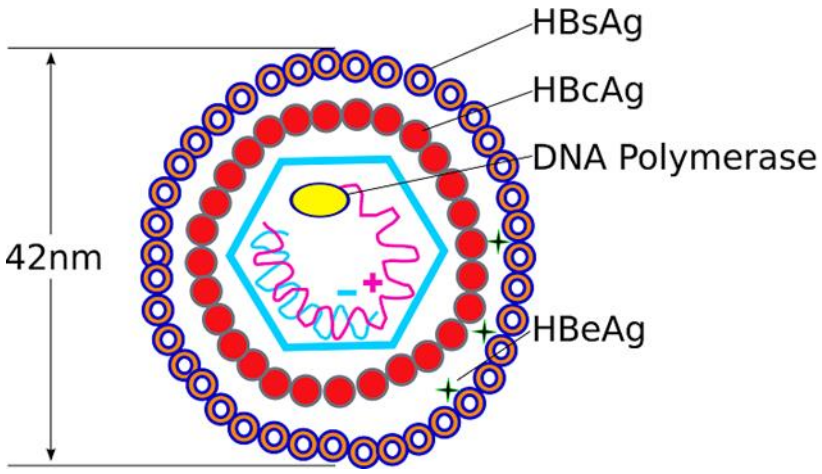


Figure 3.1. Structure of hepatitis B virus. Hepatitis B virus is an enveloped DNA virus of 42 nm. Envelope is formed by HBs antigen. Hbc protein forms viral core and encloses viral DNA and DNA polymerase [160]

3.1.3. Life cycle of HBV

*HBV has an i*ncubation period of 30-180 days but on average it is about 70 days and can be detected within 1-2 months of infection. Virus interacts with cell surface receptors, sodium taurocholate co-transporting polypeptide (NTCP) on hepatocytes and enters into the primary hepatocytes with the help of preS1 region of L-HBsAg through receptor-mediated endocytosis (RME). After entry, the virus is imported in the nucleus and the relaxed circular DNA (rcDNA) is converted to covalently closed circular DNA (cccDNA). After transcription, cccDNA is transcribed to RNAs encoding different viral proteins and synthesizes large, medium and small surface proteins, core protein, DNA polymerase and HBx protein. Pregenomic RNA (pgRNA), which is transcribed to negative sense stranded rcDNA and encapsidated with P-protein (polymerase protein) to form nucleocapsid. This nucleocapsid has two fates, either enveloped by endoplasmic reticulum and transported out of the cell or re-imported to nucleus for cccDNA synthesis [159] (Figure 3.2).

3.1.4. Genotypes

HBV has been divided into 10 Genotypes (GTs) (A-J) on the basis of difference in genome sequence. The individual genotypes of HBV exhibit 8% difference in the nucleotide sequence while its 40 sub-genotypes are different in the genome sequence by 4% [129]. HBV genotypes

show highly specific geographical distribution. Genotype A1, A2, E, and *B & C* are the most prevalent genotypes in Asia, Europe, central and west Africa, and *China, respectively. In Pakistan, genotype D is the most common genotype of HBV where its sub-genotypes D1 and D3 exist [161, 162]*.

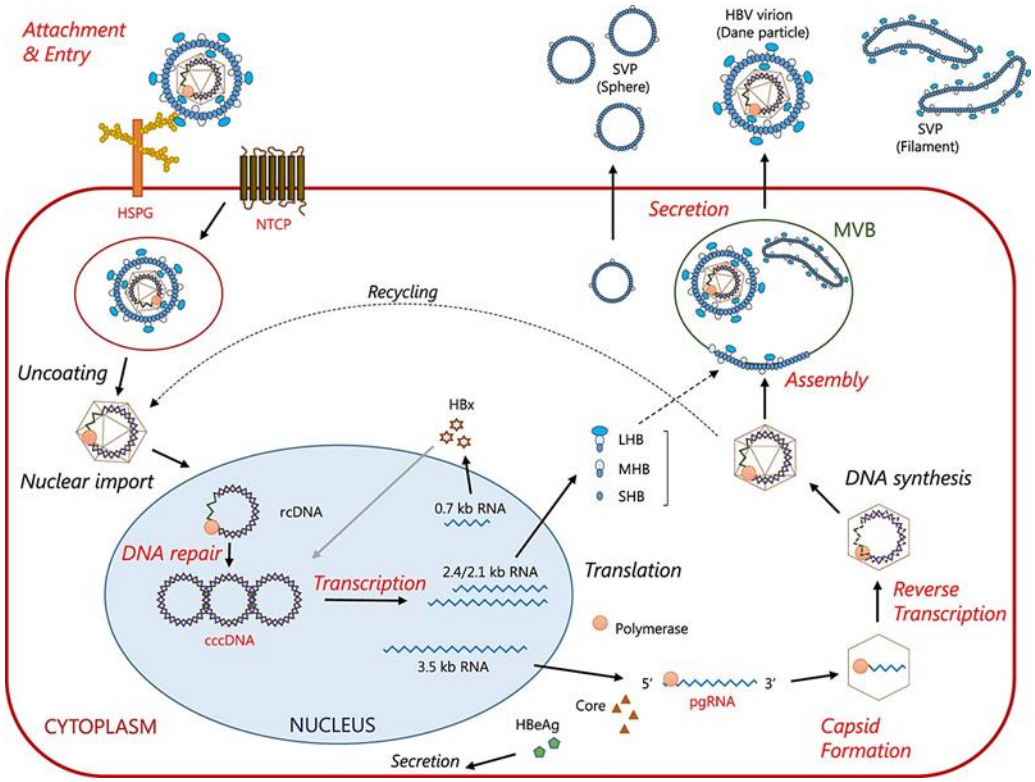


Figure 3.2. Schematic presentation of HBV replication cycle showing different steps [163].

3.1.5. Transmission

In highly endemic areas, perinatal transmission is the most common route for vertical spread while contaminated blood is responsible for horizontal transmission especially among children under 5 years of age. In 95% of the HBV positive neonates, infection is likely to become chronic. Like other blood borne infections, it also spreads from body fluids, skin piercing, tattooing, and seminal and vaginal fluids. Homosexual men and individuals with multiple sex partners are also responsible to spread the disease. Intravenous drug users (IDUs) and health care workers using

reusable needles, syringes, unsterilized medical and surgical instruments, contaminated razors and brushes also contributes to the spread of the disease.

3.1.6. High-risk groups

IDUs, prisoners, individuals having received blood/blood products, persons in close contact with chronic HBV patients, individuals having sexual contacts with HBV individuals, individuals with multiple sex partners, health care workers, non-vaccinated infants and travelers moving to HBV endemic countries are considered as high risk groups of getting HBV[154].

3.1.7. Diagnosis

Presence of HBV in the serum is detected during 60 days of incubation period. Diagnosis is based on the presence of Hepatitis B surface Antigen (HBsAg) in the blood using DiaSorin LIAISON® XL Murex kit. Plasma samples having ≥0.05 IU/ml of HBsAg are considered positive. Reactive sample are counter confirmed by one step RT PCR to detect viral DNA in blood. Another HBV small polypeptide, Hepatitis B e-antigen (HBe antigen) can also be detected in the serum. In addition, presence of IgM antibodies against HBV core antigen is also tested. It had a direct correlation with the number of infectious virion and indicates active HBV infection. Presence of HBsAg for more than six months is an indication of chronic infection.

3.1.8. Hepatitis B surface antigen (HBsAg)

HBsAg is a surface protein of HBV and is considered an important target from diagnostic, preventive and therapeutic perspective. This protein exists in three different antigenic forms, large, medium and small antigen. Large HBs antigen had PreS1 region consisting of 108 amino acids, the PreS2 region consisting of 55 amino acids and the S region consisting of 227 amino acids (Figure 3.3). Medium HBs antigen lacks the PreS1 region while small HBs antigen lacks both the PreS1 and PreS2 regions. Most of the small HBs antigens are secreted in the blood in the form of non-infectious spherical or filamentous sub viral particles (SVPs).

Subtypes of HBs

This protein is encoded by the *S* gene and has a special antigenic structure known as ‘a’ determinant which has further sub types, w/r and d/y. These two sub types are mutually

exclusive and responsible for the four HBs antigens subtypes i.e. adw, adr, ayw and ayr [164-166].

HBs incorporates several important neutralizing epitopes. The PreS1 region includes an important epitope encompassing aa21-47. This epitope is part of sodium taurocholate co-transporting polypeptide (NTCP) receptor binding site. N-terminal of PreS1 is myristoylated. This myristic acid changes the topology of large HBs antigen in such a way that that this epitope becomes inaccessible for some antibodies. Different monoclonal antibodies (mAbs) have been identified against this epitope including potently neutralizing mAbs MA18/7, 4D11 and 7H11 [167] [168]. Another important epitope is a part of the PreS2 region spanning aa33-aa52 and is present in the translocation (TLM) motif [169]. S-region exhibits three epitope clusters. One named as 'a' determinant, which is a conformational epitope with two loops spanning 124-aa137 and aa139-aa147 [170]. This "a" determinant is responsible for early contact between the virus and heparin sulfate proteoglycans therefore antibodies against this epitope are of high significance [171, 172]. Two other linear epitopes adjacent to the 'a' determinant in surface exposed major hydrophilic region (MHR) are in the form of two loops, the first loop spans aa119-aa125 [173] and the second loop spans aa139-aa147 (Figure 2). First loop is a part of disulphide isomerase related proteins, and includes CXXC motif, which is conserved among different genotypes. [174]. A group of antibodies binding to the N-terminal of the first loop are designated as sA group of antibodies while those targeting the second loop are designated as sE. The sA group of mAbs are more efficacious than the sE specific antibodies. *In vitro* neutralization of PreS1 RBD specific mAb doesn't correlate with viral suppression, decline in HBsAg level or HBV DNA. mAbs specific to the 'a' determinant or to the second loop have more therapeutic effects *in vivo* but these are transient. The sE group is highly sensitive to HBV escape mutants[175-177]. Mutations within MHR (aa99-161) are responsible for antiviral resistance and vaccine escape [178].

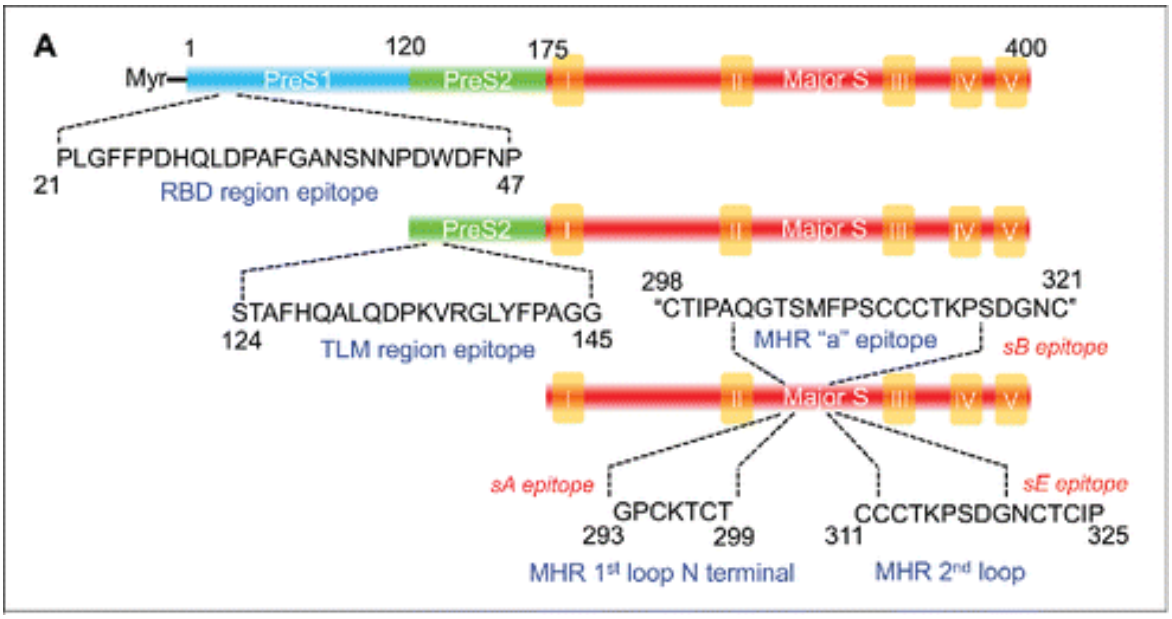


Figure 3.3. Schematic presentation of different forms of HBs antigens. HBs antigen exists in three forms, HBs small antigen, HBs medium antigen and HBS large antigen. Three regions preS1, preS2 and major S region constitute viral HBs large antigen. The HBs medium antigen does not include preS1 while the HBs small antigen is devoid of preS1 and preS2. Receptor binding domain (RBD) is present in the PreS1 region. Translocation motif (TLM) is present in the PreS2 region. First and second loop of major hydrophilic region (MHR) are present in small HBs antigen [172].

3.1.9. Phases of HBV infection

HBV infection for a period of or less than six months is considered as the acute phase of infection. But if the infection progresses beyond six months, it is characterized as chronic phase of infection and may persist for 1-2 decades. The infection can also resolve spontaneously in 8-15 % of the cases. HBV infection is divided into four different phases including the immune tolerant phase, the immune clearance phase, the inactive carrier phase and the reactivation phase. Initially immune tolerance is developed and is characterized by high levels of HBV DNA (10^7–10^{11} copies/mL) with normal ALT levels and diminished histological activity in the liver. HBeAg, which is a truncated product of HBcAg is produced at higher levels in blood and can tolerate HBV specific T cell response. This phase lasts from 2-4 weeks to lifelong. This is followed by immune clearance phase, characterized by less HBV DNA, increased ALT levels and higher level of HBcAg in the infected hepatocytes followed by immune mediated killing of

these hepatocytes. This contributes to excessive liver damage therefore the patient develops cirrhosis and fibrosis. As a result of excessive killing to infected hepatocytes, viral replication level goes down and patient is seroconverted to anti-HBe. This is followed by a third phase referred to as residual or carrier phase characterized by low levels of HBV DNA in the serum and the low ALT levels. The persistent infection becomes inactive because HBV cccDNA enters in the hepatocytes and integrates into host DNA as mini-chromosome. Around 2% patients are able to clear HBsAg from blood and seroconvert to HBs antibodies. In 30% of the cases, fourth phase characterized by reactivation of viral infection occurs but some patients still have active infection after seroconverting to HBs antibodies and have higher ALT levels [179].

3.1.10 Animal models for study

HBV transgenic mice have terminally redundant 1.3 fold HBV genome insertion and produce high levels of HBsAg and HBe antigen comparable to the chronic HBV infection [180]. Although the HBsAg produced does not enter in the hepatocytes but these are good animal models for *in vivo* studies to investigate antibody mediated viral clearance. The other transgenic model is FRG mice. These are human liver chimeric mice that support HBV replication *in vivo* [181].

3.1.11. Immune response against HBV

Both innate and adaptive immune responses play a role in the clearance of HBV. Innate immune response at an initial stage of the infection produces interferon alpha (IFN-α), interferon beta (IFN-β) and interleukin-2 (IL-2). In addition, NK cells are activated, MHC-I molecules are down regulated, and IFN-α and TNF-α are secreted. Liver inflammation is caused by inflammatory cytokines produced by macrophages including interleukin IL-1B, IL-6, tumor necrosis factor (TNF), and C-X-C motif chemokine ligand 10 (CXCL10). T cell mediated immune response serves two purposes: firstly, $CD8^{+}$T cells kill the infected hepatocytes by apoptosis and secondly, they secrete cytokines, which lead to non-cytolytic clearance of the virus [182]. During chronic phase, the host expresses excessive HBsAg that is secreted in the blood in the form of spherical of filamentous sub viral particles. Excessive antigenic HBs protein in the blood triggers the production of PD1 protein. PD1 protein production indicates that T cells are exhausted. At this point, T cell response has an inverse correlation with HBV DNA levels. IFNα production by

TLR9 from plasmacytoid dendritic cells is also disrupted. HBV specific and nonspecific T cells accumulate in the intrahepatic compartment and leads to liver inflammation [183, 184].

For B cell response, antibodies play a critical role for clearance of extracellular HBsAg and interrupt viral entry in the cells. Antibodies directed against HBsAg bind to HBV and perform a dual purpose. First they inhibit viral entry in the hepatocytes and secondly they opsonize the viral particles circulating in blood by phagocytes. But for intracellular virus, MHC1 mediated T cell function is required. Antibodies targeted against NTCP receptors are more potent. Anti-Hbc and anti-HBe antibodies are also detectable in the serum. Presence of anti-HBe antibodies leads to HBe negative HBV mutant [183, 184]. Anti HBV antibodies can prevent reinfection in liver grafts [184].

3.1.12. Treatment regimens for HBV infections

For acute infection, no specific treatment is recommended. Primary focus is to replace the water loss due to vomiting and diarrhea. Medication like acetaminophen has been recommended to avoid. For chronic cases, only a proportion of patients require treatment. Treatment primarily focuses on to halt the disease progression and improves long-term survival. As immune tolerance is developed because of the presence of high titer of HBsAg hence primary target is to restore the exhaustive immune system. The restored immune system then will be in a good capacity to tame the viral infection. Tenofovir, a first line drug against HIV-1 is also effective against HBV [157]. Entecavir is off patent drug has also been recommended. In most of the cases, treatment suppresses viral replication [157]. Although, interferon and nucleoside/nucleotide analogues have also been considered as potential candidates for HBV treatment but their efficacy have not been proved to be sufficient for viral clearance. Nucleoside/nucleotide analogue resistant mutants have also been reported to arise due to mutation in the *pol* gene [185]. In addition, these antiviral strategies cannot restore the exhausted immune system [186, 187]. An attractive approach is to target cccDNA but it is also challenging because of the lack of knowledge about cccDNA maintenance and regulation [188-190].

3.1.13. Vaccination

HBV is better prevented than cure because HBV vaccine is more effective than HBV treatments. HBV vaccine is produced in two ways; one comprises HBsAg prepared from the serum of chronic HBV patients. The second one consists of recombinant subviral particles (SVPs) expressed from a plasmid containing the *S* gene [191]. The vaccine is administered in three doses, first dose is monovalent and administered immediately after birth while second and third doses are administered with diphtheria, pertussis, tetanus (DPT).

The vaccine is included in routine vaccine schedules and has an excellent efficacy record (95%). Vaccine induced neutralizing antibodies targeted against the 'a' determinant of HBsAg. In 5-10% of the cases, immunized infants capture HBV infection even after vaccination. This is because of a G145R substitution mutation in the 'a' determinant that changes the loop conformation in amino acid 139-147. This conformational change affects the binding of vaccine-induced antibodies and let the mutant to replicate and establish HBV infection. The antibody based protection lasts for 20 years or lifelong. But the efficacy is compromised in individuals over 40 years of age and in immune-compromised subjects [192]. After 2015, global HBV prevalence in children under 5 years of age has decreased from 8-15% to 1.3% and is attributed to HBV vaccination program [193].

3.1.14. Fcγ receptor based antibodies and their potential antiviral response

Immunoglobulin plays an important role in viral clearance. Different antibodies are produced against different epitopes of the virus. Some of these antibodies have a potency to neutralize the virus while others are non-neutralizing. Viral clearance is mediated by a range of activities performed either by antigen binding fragment (FAB) or the Fc region of antibodies. FAB of an antibody interacts directly with viral proteins and inhibits viral entry to the cell. These antibodies are designated as neutralizing antibodies. Many neutralizing antibodies play a prominent role in controlling primary and recurrent infections as observed in case of Rubiola virus, the herpes simplex virus and the lymphocytic choriomeningitis mammarena virus [194-196]. A number of non-neutralizing antibodies are also produced in response to an infection that cannot neutralize the virus owing to low antibody avidity or affinity. However these non-neutralizing antibodies are not useless. Many of these non-neutralizing and neutralizing antibodies utilize their Fc portions to mediate certain antiviral effector functions by activating Fcγ receptors (FcγR). These

FcγR present on different cells of immune system including monocytes, macrophages, neutrophils, dendritic cells and NK cells that lead to antibody dependent cellular cytotoxicity (ADCC) or antibody dependent cell mediated viral inhibition (ADCVI). Hence divergent antibody responses can be elucidated by selective engagement of FcγR present on different cells that can lead to different downstream 0immunological signaling pathways. So combined effect of FAB and Fc parts augments the antiviral immune response and mediates an effective antiviral response [197, 198].

Five main types of FcγRs are FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb. FcγRI, FcγRIIa and FcγRIIIa mediate activating function because of the associated cytoplasmic motif named as Immuno tyrosine based activation motif (ITAM). FcγRIIIb is present on neutrophils and eosinophil and is inhibitory in nature because of its association with Immunoreceptor tyrosine-based inhibitory motif (ITIM)[199] .

There are different subclasses of immunoglobulin-G (IgG), IgG1 and IgG3 binds to all classes of FcγRs. IgG2 binds to FcγRIIa and FcγRIIIa, IgG4 binds to FcγRI, FcγRIIa, IIb and IIc and FcγRIIIa. The inhibitory FcγR binds to IgG1, IgG2, and IgG3 but with a lower affinity than other FcγRs [200]. Inhibitory FcγRs set up the threshold for an effective immune response against pathogen while preventing self-destructing capacity of immune cells by helping them to differentiate between self and non self.

The Fc portion of immunoglobulin either binds independently to FcγR or depends on some conformational changes induced by Fab-antigen complex that favors Fc-FcγR complex formation. So Fc dependent antiviral response depends on the type of antibody and specific ADCC inducing epitopes. For example in influenza virus, HA head-specific antibodies are Fc independent while antibodies against the stalk region mediate Fc dependent effector cell antiviral response [201]. This specific response is possibly an adaptation of non-neutralizing antibodies to elicit a response. For example, in case of Influenza virus, head specific antibodies had capacity to inhibit viral entry and fusion while stalk specific antibodies don’t provide stearic hindrance for viral entry process so they adapted the other mechanism (i.e. FcγR dependent) for viral inhibition. Fcγ dependent response is much faster than NAb based response and enhance the

antiviral activity of phagocytic cells to many folds [202]. In addition, serum with less antibody titer mediate FcγR dependent response while the same serum with higher titer leads to NAb dependent viral inhibition [203, 204]. HIV vaccine induced antibodies also depends upon FcγR [195, 205].

Many HIV [196, 202, 206-208] and influenza [201, 203] specific antibodies activate FcγR and remove virus infected cells. HIV specific antibodies following Fcγ receptor activation based mechanism are more potent *in vivo* [198]. In HIV positive individuals, antibodies are produced that are directed against the immune-dominant epitope gp41 and exhibits FcγR mediated inhibition, which shows that some special features are associated with the FcγR-mediated functional activity.

Rituximab, an antibody to treat autoimmune disease binds to inhibitory FcγR while Drozitumab, the antibody against the DR5 receptor on cancer cells, binds to both activating and inhibitory FcγRs and mediates FcγR mediated apoptosis of cancerous cells [209]. Examples of other FcγR activating antibodies include HIV neutralizing antibodies, 2F5, 4E10, 2G12, b12 and 447-D [210]. Anti HBV antibody, E6F6 that targets a unique epitope on HBsAg, can provides a therapeutic approach for restoration of immune tolerance. It follows Fc receptor mediated suppression that leads to phagocytosis of the virus and durably suppress the virus and HBV DNA. This antibody not only activates Fcγ receptor but also restores exhausted immune response. Fcγ receptor mediated clearance of virus has multiple downstream effects on adaptive immune response including modulation of antigen processing, presentation and regulation of T and B cell immunological pathways. It suppresses initial viral infection and subsequent viral spread to other hepatocytes possibly because of the inhibition of viral entry process [172, 177]. This implies that FcγR dependent mechanism is a more robust and effective to direct antiviral response.

Antibodies having capacity to activate different classes of FcRs can mediate different responses like antibody dependent cellular cytotoxicity (ADCC) to induce viral clearance. In the current study two patients groups, HBV vaccinated individuals and HBV self-resolved individuals who

had resolved the HBV infection without any treatment, were investigated to delineate the role of HBs specific antibody mediated viral clearance involving Fcγ receptor mediated responses.

3.2. MATERIALS and METHODS

3.2.1. Evaluation of BW5147 cells for FcγR activation using purified antibodies

Materials

1. BW5147 cells (T lymphocytes who have stable expression of different classes of Fcγ receptors)
2. cRPMI medium
   10% FCS, 1% P/S, 1% Sod pyruvate, β-Mercaptoethanol 500ul/500ml medium
3. PFT
   PBS+10%FCS+0.05% Tween 20
4. EBB (ELISA binding buffer)
5. Sodium bicarbonate buffer pH 9.5
6. Blocking buffer
   PBS+10% FCS
7. Washing buffer I
   PBS+Tween20 0.05%
8. Washing buffer II
   cRPMI medium w/o B-Me

Methods

For the control experiment, 96 well plate was coated with a purified FAB antigen at 1:500 dilution in EBB buffer (50 µl/well) for overnight at 4^{o}C. Extra amount of the FAB fragment was removed and plate was blocked with blocking buffer (100 µl/well) for 0.5 hr at 37^{o}C followed by washing with washing buffer I. Primary antibody specific for the Fab fragment was diluted in PFT in an appropriate ratio and incubated for 1hr at 37^{o}C. Three primary antibodies with specificity of their Fc domains for CD16 (BD purified MαH CD16 Cat No 555404), CD32 (AT10 sc-13527 mouse monoclonal IgG) and CD64 (Ancell Anti CD64 216-020 1 mg/ml) Fc receptors were used. Primary antibodies were removed after 1 hr followed by washing thrice with washing buffer I. BW5147 cells (2X10^{4} cells/well in a total volume of 150 µl/well) expressing the CD16, CD32a or CD64 receptors were diluted in washing medium II, added in 96 well plate containing respective antibodies and incubated overnight at 37^{o}C. Cells with activated receptors produced interleukin 2 (IL-2) in the cells. To screen for activated BW5147 cells, IL2 ELISA was performed. PFT (100 µl/well) was added in BW5147 cells and incubated at -80^{o}C for 1 hour followed by incubation at 37^{o}C for 1 hour.

3.2.2. IL2 ELISA

ELISA plate (greener bio-one micro plate 96well PS flat bottom clear, Ref: 655101) was coated with RαM IL2 (BD purified RαM IL2 Cat No.: 554424 0.5 mg/ml) at 1:500 dilutions in PBS and incubated at 4^{o}C for overnight. Plate was blocked with blocking buffer (100 µl/well) for 0.5 hr at 37^{o}C. Blocking buffer was removed and plate was washed three times with washing buffer I. Supernatant from the lysed cells (BW5147 plate) was transferred to ELISA plate and incubated at 37^{o}C for 1hr. Plate was washed the same way as mentioned above and incubated with Biotin-Anti IL2 antibody (BD Biotin RαMIL2 Cat. No 55442 g 0.5 mg/ml) diluted to 1:500 in PFT for 1hr at 37^{o}C. The anti-IL2 antibody was washed away just as in the last step and incubated with streptavidin (1:1000 in PFT) for 15 min at room temperature. Unbound streptavidin was washed away with washing buffer I (Five times) and incubated with substrate (1-step ultra TMB-ELISA Ref: 34028 Thermo) for 1 min. Substrate was neutralized with 1 M H_2SO_4 and reading was taken with microplate reader (Tecan infinite mplex tecande 01805) at 450 nm. Data was plotted using graph pad prism7 and analyzed. Area under the curve (AUC) was calculated and normalized with positive serum as a reference to get AUC index.

3.2.3. Evaluation of FcγRs activation by HBV sera using purified HBsAg

Purified HBsAg (Cat No. 6Z11 Cosmo Bio Co Ltd) was diluted to 500 fold in EBB buffer and coated on the surface of 96-well plate for overnight at 4^{o}C. Antigen was removed and blocking buffer (100 µl/well) was added for 0.5 hr at 37^{o}C. Blocking buffer was removed and plate was washed three times with washing buffer I. Test serum was diluted in PFT making different dilutions as 1:5, 1:10, 1:20, 1:40 and 1:80 and incubated for 1hr at 37^{o}C. Pooled serum from 10 HBV positive patients was considered as positive control while negative pool corresponds to serum from 10 HBV negative sera. Same dilutions of positive and negative pool were used as that of the test sera. Following washing, BW5147 cells (2X10^{4} cells/well in a total volume of 150 µl/well) expressing CD16, CD32a or CD64 receptors were diluted in washing medium II, added in 96 well plate and incubated overnight at 37^{o}C. IL2 was produced in cells. These cells were lyzed by adding PFT (100 µl/well) followed by freeze thaw cycle to release IL2 in the medium. IL2 production was quantified by performing IL2 ELISA as described previously. Graph for each sera having different dilutions was drawn using graph pad software. Area under the curve (AUC) was calculated. AUC index was determined considering AUC of positive pool as a

reference. Linear regression analysis was applied to determine correlation between HBV antibody titer and AUC index.

3.2.3. Evaluation of FcγRs activation by HBV sera using HepG2 cells expressing HBsAg

HepG2 cells are human hepatoma cells and have adherent nature. They are transduced with HBV DNA in pTetOFF plasmid with tetracycline inducible promoter. These cells constitutively express HBsAg and HBeAg and labeled as Tet ON system while on induction with doxycycline they produce HBsAg only and referred as Tet OFF system[211]. HBsAg and HBe antigen expression was determined using Liason Diasorin Kit. These were cultured in DMEM medium.

HepG2 cells expressing HBV viral particles were cultured in DMEM medium for 48 hours [211]. Medium was removed and washed two times with PBS. Cells were trypsinized by adding 2.5 ml of trypsin at 37^{o}C for 6 min followed by tapping and then neutralized with 2.5 ml RPMI medium w/o B-Me. Cells were centrifuged and pellet was dissolved in RPMI wash medium. Plate was coated with 1% collagen in PBS for 10 min at RT in sterilized environment (50 µl/well of 96 well plate or 3 ml in T25 flask). Collagen was removed and cells were added ($5X10^{4}$ cells/well in 150 µl or 0.7 M cells in T25 cells) and incubated for 24-48 hours. After 2-3 days HBsAg and HBeAg titer was determined by Diasorin Liason kit for both cell types. Cells were washed and incubated with different dilutions of serum for 0.5 hr. This was followed by washing and incubated with BW5147 cells for overnight at 37^{o}C. BW5147 cells were lysed by freeze thaw cycle and subjected to IL2 ELISA.

3.3. RESULTS

3.3.1. Serum collection

After the approval of IRB, patient's data available from online directory was collected and screened for HBV self-resolved and HBV vaccinated individuals. HBV vaccinated individuals have anti HBs antibodies in their serum as they are vaccinated with HBsAg while HBV self-resolved individuals have both anti HBs and anti-HBc antibodies in their serum. Serum with HBS IgG titer >1000mIU/ml was selected to proceed with BW assay. In total 77 samples were used in this project, 48 from HBV vaccinated individuals and 23 from HBV resolved individuals. Screened serum samples were collected against their serial numbers from the diagnostics section of Department of Virology, Institute of Medical Microbiology and Hygiene, University of Freiburg, Germany. They were stored in aliquots in -8^{0}C freezer until further use.

3.3.2. Evaluating the FcγRs activation in HBV vaccinated individuals using cell culture and BW assay

Three different types of BW5147 cells, each expressing one of the three, FcγRI (CD64), FcγRII (CD32a) and FcγRIII (CD16) was used [212]. They were cultured in T75 flask containing 20 ml cRPMI medium. After 3 days, cells were transferred to three T175 flasks in fresh medium and incubated for three days at 37^{o}C in a humidified chamber having 5% CO_2. Serum samples from 48 HBV vaccinated individuals of age ranging from 20-60 years, with mean age as 32 years, were collected. HBs antibody titer was in the range from 900-100,000 mIU/ml (Figure 3.4). HBsAg specific antibodies in these sera were allowed to bind with HBsAg that is coated on the surface of 96-well plate. Subsequently HBsAg-antibody complex was incubated with FcγRs expressing cells and let them to activate the receptors. Patient's sera from vaccinated individuals activated all three classes of FcγR (Figure 3.5-3.7). Each individual serum showed variable capacity to activate the receptors that apparently seemed to depend on the antibody titers. Serum with high antibody titers had more activation of the receptors and showed a good correlation with antibody titers and AUC index (Figure 3.8). Comparative analysis showed that vaccinated individuals were more biased towards the activation of FcγR1 (CD64 receptor) than FcγRI1 (CD32a receptor) and FcγR1II (CD16 receptor) while there was no comparison for activation between FcγRI1 (CD32a receptor) and FcγR1II (CD16 receptor) (Figure 3.9).

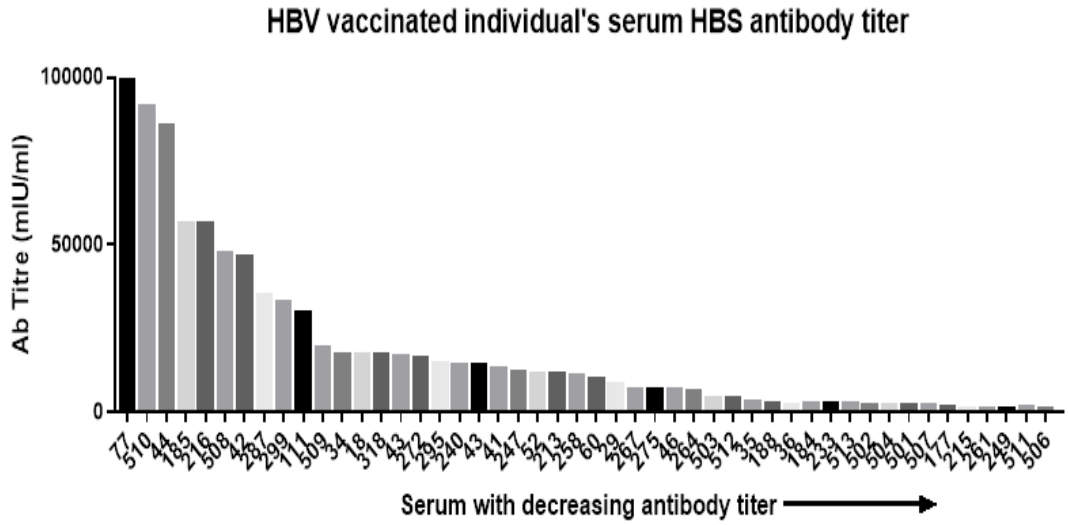


Figure 3.4. Anti HBs antibody titer in HBV vaccinated individuals. Sera are organized with reference to descending order of their antibody titer.

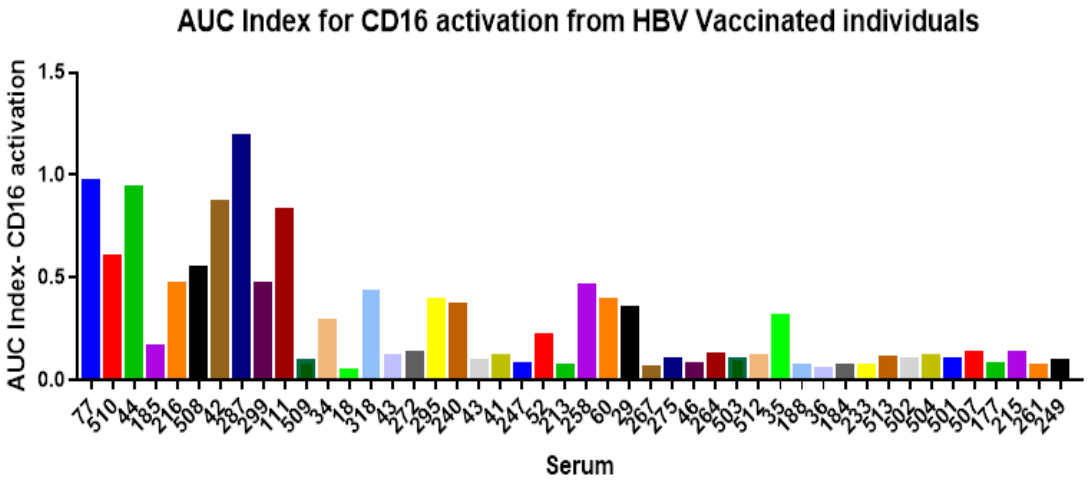


Figure 3.5. AUC index for CD16 activation in HBV vaccinated individuals. HBV vaccinated individuals' sera were incubated and bound with HBsAg in a 96 well plate. This is followed by incubation of BW5147 cells with HBsAg-antibody complex to determine the activation of these receptors. Area under the curve (AUC) for each sera was calculated and used to determine AUC index considering positive pool as reference. Y-axis is representing AUC index of sera from HBV vaccinated individuals. Anti-HBs antibodies from HBV vaccinated individuals can activate FcγRIII (CD16 receptors) present on BW5147 cells.

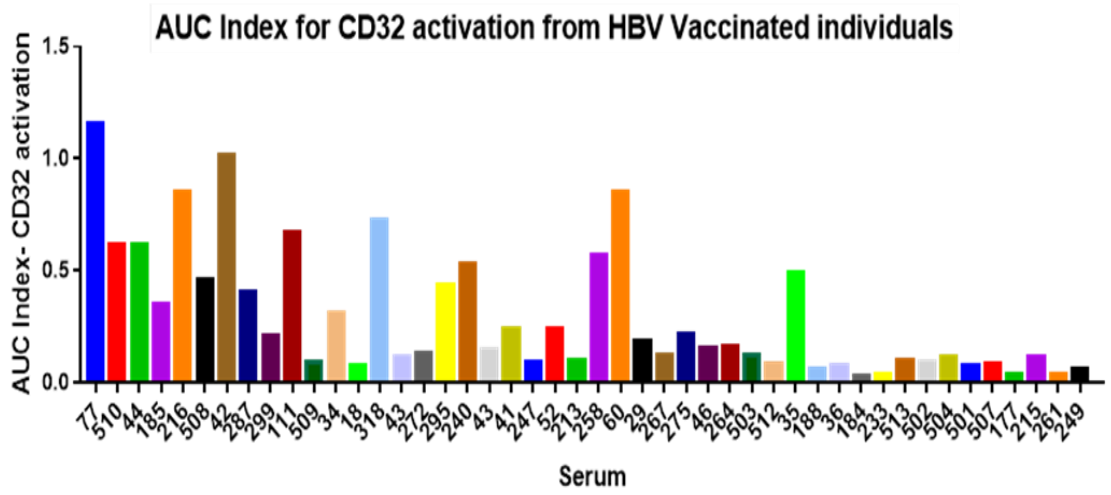


Figure 3.6. AUC index for CD32 activation in HBV vaccinated individuals. HBV vaccinated individuals' sera were incubated and bound with HBsAg in a 96 well plate. This is followed by incubation of BW5147 cells with HBs antigen-antibody complex as per the prescribed protocol to determine the activation of these receptors. Area under the curve (AUC) for each sera was calculated and used to determine AUC index considering positive pool as reference. Y-axis is representing AUC index of sera from HBV vaccinated individuals. Anti-HBs antibodies from HBV vaccinated individuals can activate FcγRII (CD32 receptors) present on BW5147 cells.

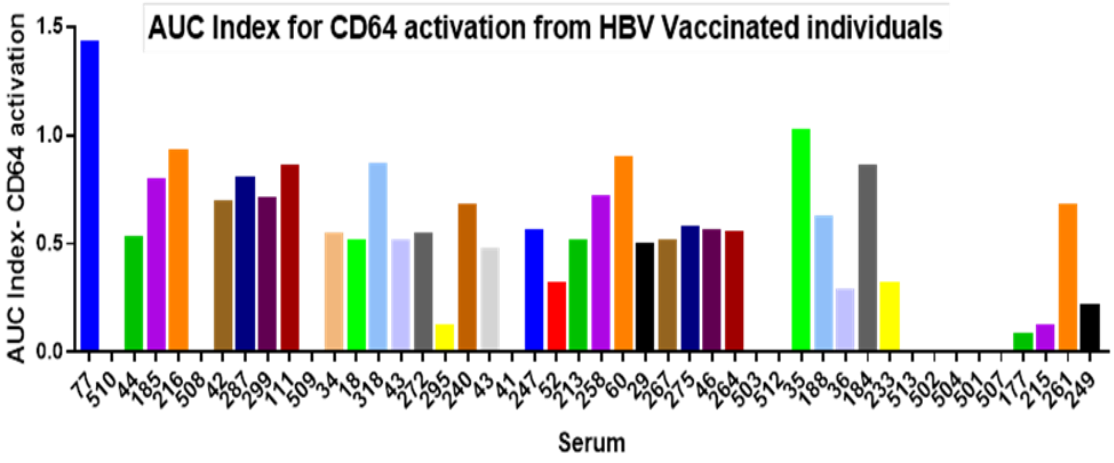


Figure 3.7. **AUC index for CD64 activation in HBV vaccinated individuals**. HBV vaccinated individual's sera were incubated and bound with HBsAg in a 96 well plate. This is followed by

incubation of BW5147 cells with HBsAg-antibody complex as per the prescribed protocol to determine the activation of these receptors. Area under the curve (AUC) for each sera was calculated and used to determine AUC index considering positive pool as reference. Y-axis is representing AUC index of sera from HBV vaccinated individuals. Anti-HBs antibodies from HBV vaccinated individuals can activate FcγRI (CD64 receptors) present on BW5147 cells.

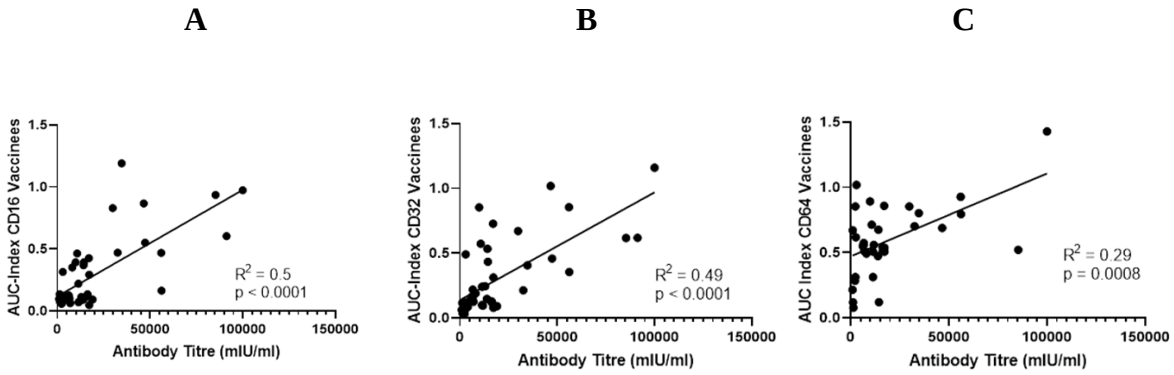


Figure 3.8. **Correlation between HBS antibody titer and different FcγRs activation.** Linear regression analysis is performed to analyze correlation between AUC index and antibody titer. FcγRI, FcγRII and FcγRIII have a good correlation with antibody titer.

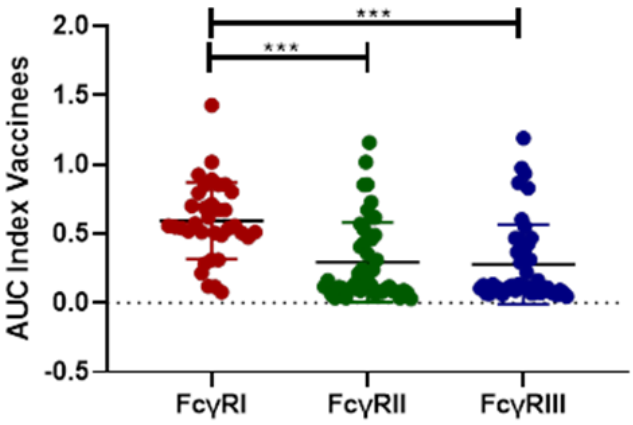


Figure 3.9. Comparative activation of different FcγRs in HBV vaccinated individuals. Activation of FcγRI is higher than, FcγII and FcγIII. AUC index is calculated by dividing the AUC index of each serum with AUC of positive control. Pooled sera from HBsAg positive sera served as positive control. FcγRI showed higher AUC index than FcγRII and FcγRIII.

3.3.4. Evaluating the FcγRs activation in HBV self-resolved individuals using cell culture and BW assay

Serum samples from 28 HBV vaccinated individuals of age ranging from 25-80 years with average age as 47 years, were collected. HBS antibody titer ranges from 50-80,000 mIU/ml (Figure 3.10).

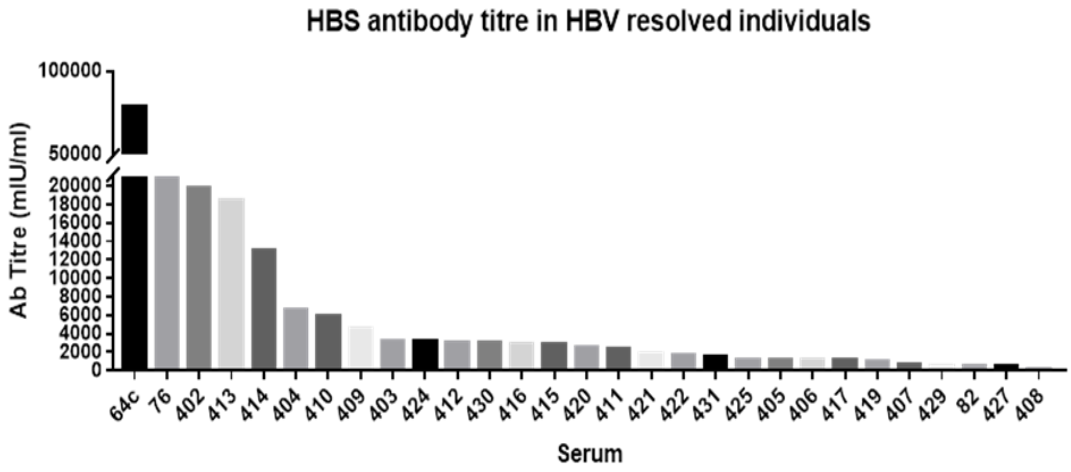


Figure 3.10. Anti HBs antibody titer in HBV self-resolved individuals Sera are organized with reference to descending order of their antibody titer.

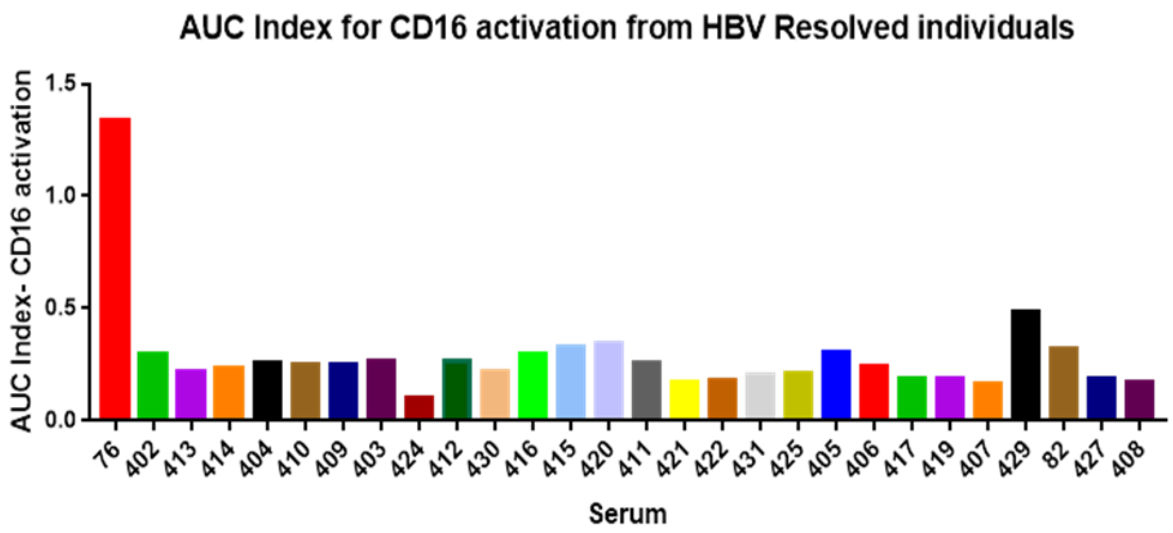
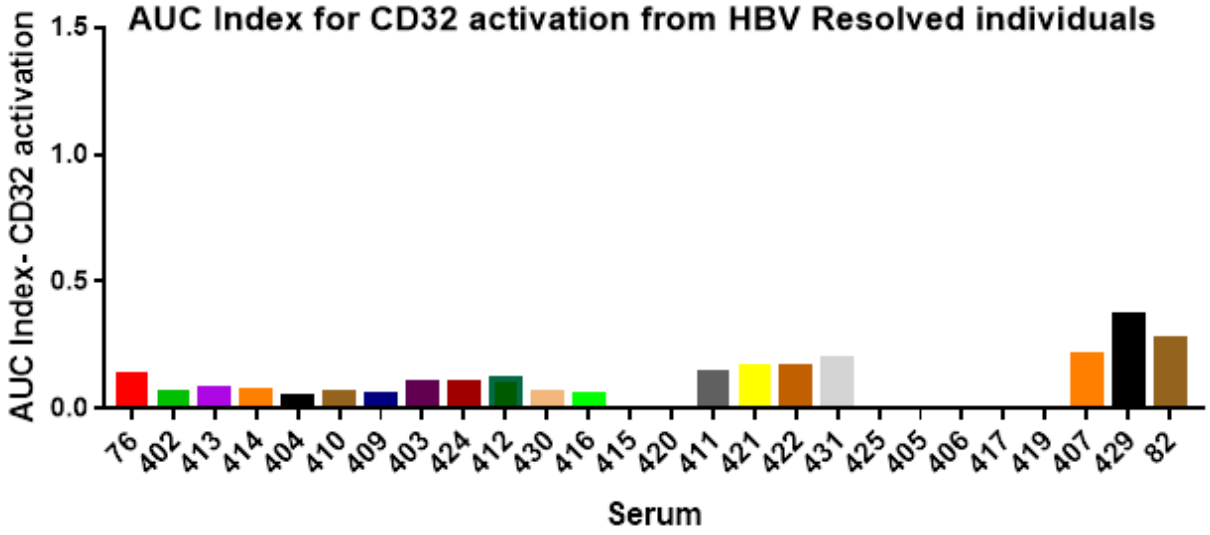


Figure 3.11. **AUC Index for CD16 activation in HBV self-resolved individuals**. HBV SR individual's sera were incubated and bound with HBsAg in a 96 well plate. This is followed by incubation of BW5147 cells with HBs antigen-antibody complex as per the prescribed protocol to determine the activation of these receptors. Area under the curve (AUC) for each serum was calculated and used to determine AUC index considering positive pool as reference. Y-axis is representing AUC index of sera from HBV vaccinated individuals. Anti-HBs antibodies from HBV SR individuals can activate FcγRIII (CD16 receptors) present on BW5147 cells.



Figure

3.12. **AUC Index for CD32 activation in HBV self-resolved individuals:** HBV SR individual's sera were incubated and bound with HBsAg in a 96 well plate. This is followed by incubation of BW5147 cells with HBs antigen-antibody complex as per the prescribed protocol to determine the activation of these receptors. Area under the curve (AUC) for each serum was calculated and used to determine AUC index considering positive pool as reference. Y-axis is representing AUC index of sera from HBV vaccinated individuals. Anti-HBs antibodies from HBV SR individuals cannot activate FcγRII (CD32 receptors) present on BW5147 cells.

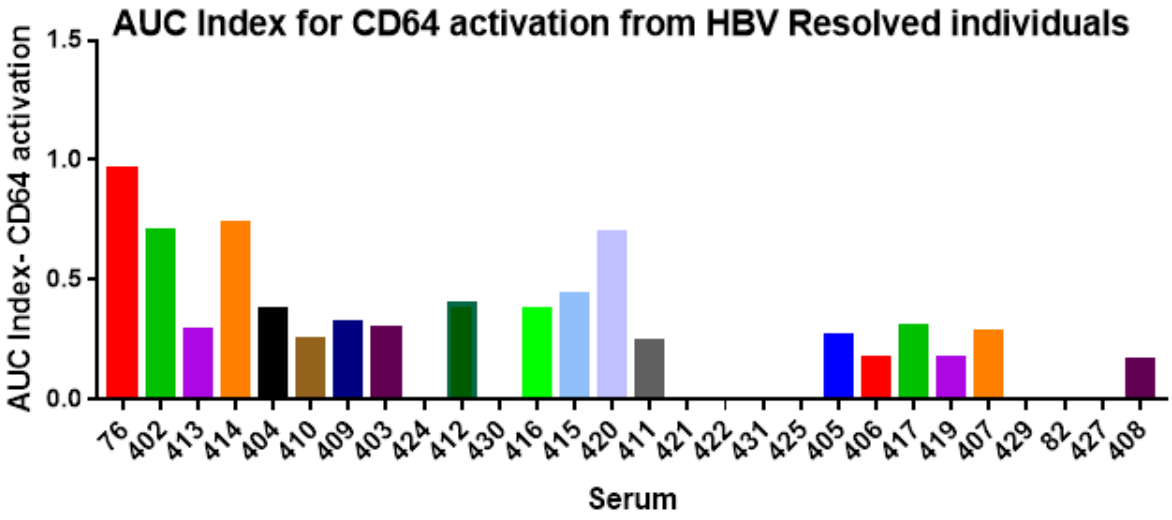
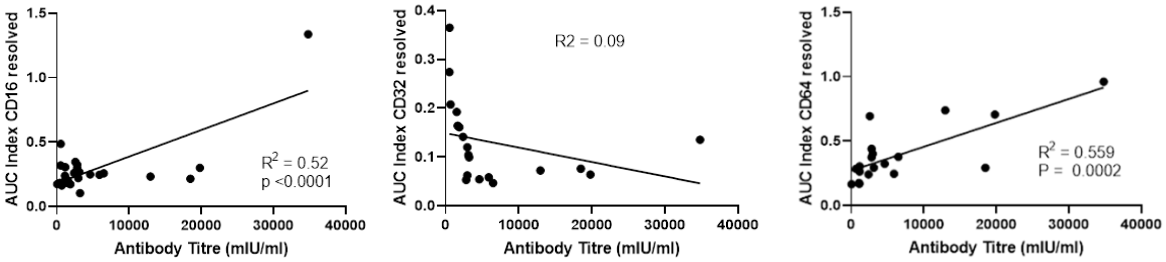


Figure 3.13. **AUC index for CD64 activation in HBV self-resolved individuals**. HBV SR individual's sera were incubated and bound with HBsAg in a 96 well plate. This is followed by incubation of BW5147 cells with HBs antigen-antibody complex as per the prescribed protocol to determine the activation of these receptors. Area under the curve (AUC) for each serum was calculated and used to determine AUC index considering positive pool as reference. Y-axis is representing AUC index of sera from HBV vaccinated individuals. Anti-HBs antibodies from HBV SR individuals can activate FcγRI (CD64 receptors) present on BW5147 cells.



A **B** **C**

Figure 3.14. Correlation between HBS antibody titer and different FcγRs activation potential. Linear regression analysis is performed to analyze correlation between AUC index and antibody titer. FcγRI and FcγRIII have a good correlation with antibody titer while no correlation exists between FcγRII and antibody titer.

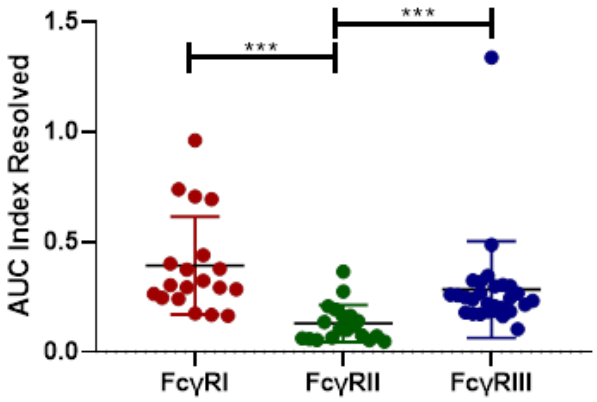


Figure 3.15. Comparative activation potential of different FcγRs in HBV self-resolved individuals. FcγRI have higher AUC index than FcγRII and FcγRIII. AUC index was calculated by dividing the AUC of each serum with AUC of positive control. Pooled sera from HBs positive sera served as positive control.

We observed that anti HBs titer in self-resolved individuals was not significantly high and patient age was biased towards higher age groups as compared to vaccinated group. HBV self-resolved individuals could activate FcγRI (Figure 3.13) and FcγRIII (Figure 3.11) but not FcγRII (Figure 3.12) even with the serum of very high IgG HBs antibody titer. A good correlation existed between antibody titer and AUC index for FcγRI and FcγR1II (Figure 3.14). Just like HBV vaccinated individuals, this group also had a higher activation potential towards CD64 receptor (Figure 3.15).

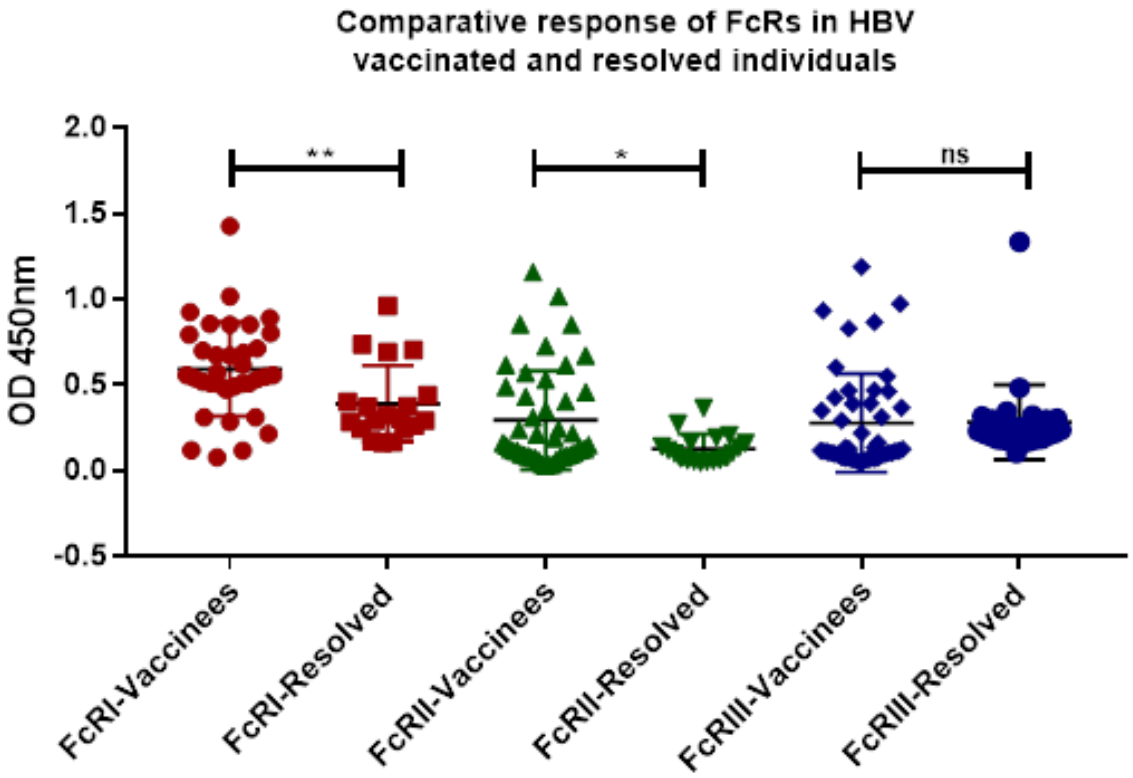


Figure 3.16. **Comparative activation potential of different FcγRs in HBV self-resolved and vaccinated individuals.** In vaccinated individuals titer of antibodies is higher than SR for FcγRI and FcγRII while data is comparable for FcγRIII titer.

Comparative response of activation potential for all three different classes of FcRs between vaccinees and self resolved individuals have showed that vaccinated individuals have more activation potential for FcγRI (CD64 receptor) than self-resolved ones while no difference was observed for FcγRIII (CD16 receptor) (Figure 3.16).

3.3.5. Evaluating the FcγRIII activation of purified antibodies using HbsAg

Different HBs specific purified antibodies were tested for their activation potential for FcγRIII. 86C antibody binds with two different subtypes of HBs i.e. ad and ay but the specific epitope is not known. M18/7 is another HBs specific monoclonal antibody that binds to N-terminus of pre-S1region of HBs protein. Q19/10 mAb binds to pre-S2 region of HBs antigen. Experiment was performed with both purified HBs antigen coated plate and with HBV producing HepG2 cells.

Both HepG2 ON and OFF cells were cultured in DMEM medium in collagen-coated plate. HepG2 ON cells were producing 10.13 IU/ml of HBs Ag and 14.2 IU/ml HbeAg while HepG2 OFF cells were produding 25.31 IU/ml of HBS Ag and 0.33 IU/ml HbeAg showing no hepatitis B virus production in the induced HepG2 OFF cells. Cells were incubatd with different dilutions of mAbs and subjected to BW assay as described in previous section. It was observed that only 86C and Q19/10 antibodies were able to induce activation of the CD16 receptors. 86C antibody had more activation potential than Q19/10 (Figure 3.17). The activation potential of Q19/10 was diminished when experiment was performed with purified HBs antigen while 86C retained its activation capacity with the purified antigen (Figure 3.18). M18/7 did not activate the receptor niether with purified antigen nor on the surface of hepatoma cells.

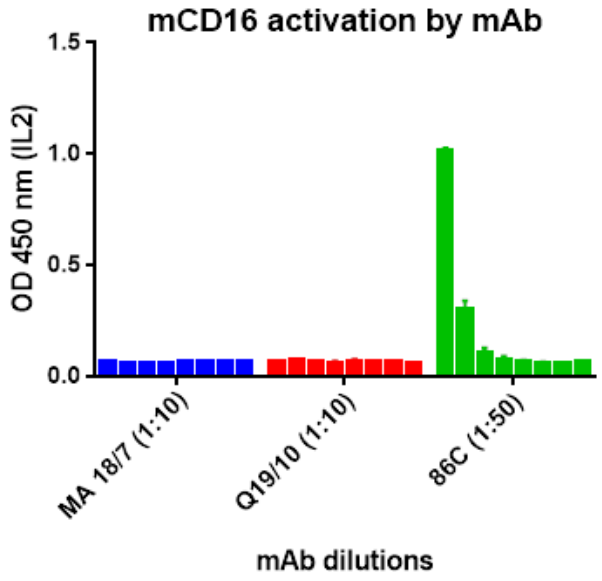


Figure 3.17. Comparative activation of FcγRIII by HBs antigen specific monoclonal antibodies.

3.4. DISCUSSION

Immunoglobulins bind to cell free virions, interact with specific proteins on the viral surface and prevent viral entry to the cell. This specific inhibition is mediated only by neutralizing antibodies (NAb). Non-neutralizing antibodies (NAb) can bind to the virion but don't inhibit its entry. NAb mediated viral entry inhibition occurs primarily at the early stage of infection however these can't prevent spread of the infection to neighboring cells [213-215]. To prevent viral progression, cell mediated immunity is required to eradicate virus-infected cells. Antibodies including non-neutralizing antibodies play their role using their Fc portion to activate different classes of FcγRs present on a diverse range of immune cells such as neutrophils, NK cells, monocytes, macrophages and dendritic cells. FcγR activating antibodies provide an efficient mechanism of antiviral immune response. Antibodies having potential to activate FcγR are also produced in vaccinated individuals and provide effective protection [216, 217].

In the current study, HBV vaccinated and self-resolved individuals were investigated to understand the activation of different classes of FcγR by the antibodies present in their sera. It was observed that sera of HBV vaccinated and self-resolved individuals were able to activate FcγR just like E6F6, an HBV specific IgG1 FcγR activating antibody. E6F6 has been reported to clear HBs antigen from infected serum leading to restoration of the exhausted immune response that was due to higher the HBsAg levels in the serum. In our study we observed that all three types of FcγR were activated by HBV vaccinated sera in antibody titer dependent manner. Activation of FcγRI (CD64) was much higher as compared to the activation of CD16 and CD32 receptors. CD64 requires monomeric IgG for its activation while CD16 and CD32 need multimeric complexes of IgGs or IgGs bound to cells surface [218]. This difference could be the reason of differential activation. In HBV self-resolved individuals, however, CD16 and CD64 were activated but not CD32.

In brief, it was observed that HBV vaccinated and self-resolved sera could activate different classes of FcγRs. However, the two groups exhibit clear differences in their specificity to activate the Fcγ receptors and subsequently augment an antiviral immune response by activating a number of different immune cells. The underlying mechanism of this difference remains to be elucidated in the follow up study.

4. Conclusion and future perspectives

In this study we focused on three different aspects of HCV and HBV infections including understanding the epitope specificity of antibodies present in the sera of chronic HCV patients and spontaneous resolvers of the infection, estimation of HCV prevalence in the Punjab province of Pakistan to illustrate better picture of disease situation particularly considering the impact of asymptomatic nature of the infection in the spread of the infection and characterization of different classes of FcγR activation in Hepatitis B vaccinated and self-resolved individuals.

To illustrate the epitope specificity of antibodies present in the sera of chronic HCV patients and spontaneous resolvers, we used combination of peptide ELISA, pseudo-typed HCV neutralization assay (HCVpp) and peptide competition HCV neutralization assay and estimated the presence of conserved linear epitope specific antibodies in the sera of the two groups of individuals. We observed that both groups of sera showed reactivity for peptides corresponding to different conserved linear epitopes. However, unlike the chronic patients' sera only 46% of the spontaneous resolvers' sera showed reactivity to any of the conserved linear epitopes. In most of these cases this reactivity was primarily for the 434 epitope present in the E2 protein. Most of the chronic patients' and spontaneous resolvers' sera neutralized the virus in HCVpp assay, however the overall ED_{50} values of the chronic patients' sera were much higher as compared to the spontaneous resolvers' sera suggesting higher titer of total neutralizing antibodies in chronic patients. In peptide competition assay the neutralization activity of the chronic patients' sera was hardly affected by the presence of any competing peptide corresponding to linear epitope. However, neutralizing activity of many self-resolvers' sera was significantly decreased by the presence of competing peptide corresponding to the 434 epitope suggesting the viral neutralization by the sera was primarily due to the presence of 434 epitope specific antibodies in those sera. These results suggested the role of antibodies specific for the 434 epitope in viral clearance in self-resolver individuals. Isolation of these viral neutralizing antibodies from these patients and structural characterization of the peptide corresponding to the 434 epitope in the antibody bound conformation could provide a template for immunogen design as potential vaccine. This is highly significant future aspect in the vaccine design perspective.

Moreover, sera from three self-resolvers and six chronic patients showed reactivity for the most conserved linear epitope, the epitope 524. Three of these sera tested in peptide competition viral neutralization assay showed no effect of competing peptide on viral neutralization suggesting the neutralization by the sera was not contributed by the 524 specific antibodies. Interference antibodies directed against an epitope overlapped with the 434 neutralizing epitope have been demonstrated to block the activity of neutralizing antibodies specific for other epitopes. It remains to be elucidated the role of interference antibodies in inhibiting the viral neutralization by the 524 specific antibodies in these individuals in the follow up study. Nevertheless, antibodies directed against the most conserved linear epitope, the 524 epitope are of high significance further characterization towards vaccine design perspective.

Similarly, we also analyzed specificity of antibodies targeted to different epitopes present in the NS3 protein of HCV in the two groups of individuals. We observed antibody response against three epitopes present in the helicase domain of NS3 in significantly higher number of chronic patients and with high titer as compared to self-resolvers. Similarly, a specific peptide containing several conserved amino acid residues of catalytic site of the NS3 protease showed reactivity preferably to self-resolvers sera. This suggests a possible role of NS3 protease domain directed antibodies in spontaneous clearance of the virus. Such clearance could involve antibody dependent cellular cytotoxicity (ADCC) mechanism but not viral neutralization as the NS3 protein is not packaged in the virion. However, its exact mechanism remains to be elucidated in the follow up study.

In the population-based study to perform retrospective analysis of HCV infections in the Punjab province of Pakistan involving over 66,000 participants from all major cities, we observed that 17.3% of the population had contracted the infection. We identified two geographical regions, Faisalabad and Okara districts, and an occupational group, farmers, with significantly high HCV seroprevalence. These socio-demographic groups were identified as the potential focused groups for follow-up studies on factors contributing to the high HCV prevalence in these groups towards orchestrating effective prevention, control and treatment.

In the study to investigate the role of different Fcγ receptors in the viral clearance in HBV vaccinated and self-resolver individuals suggested that vaccinated individuals could activate all three classes of Fcγ receptors including FcγRI, FcγRII and FcγRIII while self-resolvesrs could activate primarily the FcγRI, and FcγRIII receptors. These results describe the role of different Fcγ receptors in spontaneous viral clearance and indicate that the mechanism of spontaneous clearance of the virus could be slightly different than clearance through vaccine induced prophylaxis.

Overall this study identified demographic groups with high HCV prevalence in the most populated province of Pakistan and provided necessary information related the role of particular epitope specific antibodies in spontaneous clearance of HCV and the role of Fcγ receptors in spontaneous clearance of HBV. This information particularly related to HCV could be of high significance in vaccine design perspective.

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