MOLECULAR EPIDEMIOLOGY AND GENETIC CHARACTERIZATION OF HEPATITIS C VIRUS IN PATIENTS FROM SUBURBAN RAWALPINDI, PAKISTAN

A Dissertation Submitted in the Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in BIOLOGICAL SCIENCES.

BY

NAZISH BOSTAN

FACULTY OF BIOLOGICAL SCIENCES,
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN.
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DECLARATION

This is to certify that this dissertation entitled **"Molecular Epidemiology and Genetic Characterization of Hepatitis C Virus in Patients from Suburban Rawalpindi, Pakistan"** submitted by **Nazish Bostan** is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of Ph.D. in BIOLOGICAL SCIENCES.

Supervisor:

Dr. Tariq Mahmood
Assistant Professor, Faculty of Biological Sciences
Quaid-i-Azam University, Islamabad

Co-Supervisor:

Dr. Anwer Ali Siddiqui
Professor of Biochemistry and Director Research Office
The Aga Khan Health University, Karachi

External Examiner:

Prof. Dr. S. M. Saqlan Naqvi
Chairman, Department of Biochemistry,
PMAS University of Arid Agriculture, Rawalpindi

External Examiner:

Dr. Muhammad Zeeshan Hyder
Assistant Professor, Department of Biosciences
COMSATS Institute of Information Technology, Islamabad

Chairperson:

Prof. Dr. Asghari Bano
Chairperson, Faculty of Biological Sciences
Quaid-i-Azam University, Islamabad

Date: 27/1/2011

……."for whosoever fears Allah, He ever prepares a way out (from difficulty) and He provides for him from (sources) he never could imagine. Allah is sufficient for a person who puts his trust in Him".

Al Quran 65:2,3(29:60,62)33:3,48.

Hepatitis C does not discriminate. It affects
people of all ages, gender, and sexual orientations. . .. It affects people
from all walks of life, in every state, in every country. . ..I wish All humans must Understand the risk that this disease poses.
We must help all in the fight against this disease,
both here and around the World.

Dedicated To

My Loving Parents, Dearest Aapa g

&

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HEV	Hepatitis E virus
HGV	Hepatitis G virus
HIV	Human immunodeficiency virus
HPVR	Hyper variable region
HSV	Herpes simplex Virus
HSV-2	Herpes simplex Virus-2
HVR	Hypervariable region
IBA	Immunoblot assay
ID	Identity
IDU	Injection drug user
Ig	Immunoglobulin
IgG	Immunoglobulin-G
IRES	Internal ribosomal entry site
IV	Intra venous
IVDA	Intravenous drug abuse
IVDU	Intravenous drug user
KPK	Khyber Pukhtun Khwa
K2EDTA	Di-Potassium Ethylene diamine tetra acetic acid
LDL	Low density lipoprotein
LIPA	Line probe assay
MEGA	Molecular evolutionary genetics analysis
MHC	Major hisotcompatibility complex
µl	Microlitre
ml	Millilitre
MRCA	Most recent common ancestor
NC	Negative control
NCBI	National centre for biotechnology information
NCR	Non coding region
NIH	National institute of Health
NHANES	National center for Health and Nutrition Examination Survey
NJ	Neighbour joining

NS	Non structural
NS5a	Non-structural 5a protein
NS5b	Non-structural 5b protein
OD	Optical density
OPD	Out patient department
OR	Odd ratios
ORF	Open reading frame
%	Percentage
P	Probability value
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PIMS	Pakistan Institute of Medical Sciences
PTB	Poly pyrimidine tract binding protein
RdRp	Ribonucleic acid dependant ribonucleic acid polymerase
RFLP	Random fragment length polymorphism
RNA	Ribonucleic acid
Rs	Pakistani Rupees
RT-PCR	Reverse transcriptase polymerase chain reaction
SR	Sex ratio
STD	Sexually transmitted disease
STI	Sexually transmitted infections
SVR	Sustained virological response
TAE	Tris acetate EDTA
TE	Tris EDTA
Th-1	Th-1 Stimulating cytokines
TMA	Transmission mediated amplification
TTV	Transfusion transmitted virus
U/L	Units per liter
UK	United Kingdom
UNDP	United Nation development program
USA	United States of America

UTR	Untranslated region
VR	Variable region
WHO	World Health Organization
χ^2	Chi- Square analysis

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ABSTRACT

Hepatitis C virus is a devastating virus, known to mankind since 1989, but circulating in the human blood for centuries. It has affected millions of people from all ethnic origins, all walks of life and all socioeconomic statuses. Viral prevalence and incidence rates vary from country to country. Chronicity of the disease causes liver damage, functional impairment, liver shrinkage and hepatocellular carcinoma. Virus disperses via blood and blood products. This study was basically conducted for identification of potential risk factors, their correlation with particular genotypes, their role in HCV transmission and evolutionary relationships of the genotypes. In order to accomplish the task 1200 patients from ethnic origins (residentials) of Gujar Khan, suburban Rawalpindi, Pakistan were randomly selected from two hospitals i.e. Fauji Foundation Hospital Rawalpindi and Pakistan Institute of Medical Sciences Islamabad, by convenient sampling method, for epidemiological studies. Further, plasma of 400 out of 1200 randomly selected studied participants was taken for RNA extraction. To find out the circulating genotypes in this population molecular reverse transcription PCR (RT-PCR), nested PCR and Restriction Fragment Length Polymorphism (RFLP) based analysis was performed. The genotypes were assigned to the samples by RFLP analysis and unresolved samples by RFLP were taken to sequencing level and 5'UTR was the target site for the sequencing. Sequenced samples were then cleaned, aligned and submitted to GenBank. Neighbour joining (NJ) method was used for phylogenetic analysis and rooted dendrograms were dragged to study the association between different viral types and subtypes. It was observed that majority of the studied participants, were chronic HCV carriers and married individuals are more likely to acquire the disease than unmarried individuals. There were high numbers of females; with major affected age group 40-49 years for both genders. The disease was related to the low socioeconomic status of the patients and low literacy levels in general; people from all profession were at equal risk of disease acquisition, with no additional risk for health care providers. Mostly reported risk factors in studied cohort were dental procedures, blood transfusions and therapeutic injections. There was no significant association between genotypes and any specific risk factor. In other words it will be more appropriate to say that all risk factors were equally contributing in the spread of HCV. Moreover, it was found that major

genotype was 3a followed by 3b; second most prevalent genotype was 1a and 1b, while there were a few cases of genotype 4 as well. The most important finding in the present study was the presence of subtypes 6v, 1d and 2k, which is the first ever report from Pakistan and second from all over the world. Further, from the analysis of Dendrograms, it was concluded that evolutionary history of HCV endemicity in Pakistan is more ancient than the neighboring countries and, and perhaps this was the area from which genotype 3 has evolved and spread to the neighbouring countries. Overall, on the basis of these facts it can be concluded that awareness at public level is required to check the spread of HCV and a comprehensive screening of all genotypes in Pakistani population is necessary to determine the treatment responses of the genotypes. More efficient and better treatment regimes will be needed in future due to changing distribution pattern of HCV genotypes and subtypes. It is the responsibility of health policy makers to design the health policy by keeping in mind that the viral heterogeneity is changing very fast and there are chances that those viral strains will be more dangerous in coming years.

Chapter 1

INTRODUCTION

1.1 HISTORICAL BACKGROUND

As early as 752, Pope Zacharias wrote in a letter to St. Boniface, Bishop of Mainz (Germany), about, "Jaundice of a contagious nature" where effectees were isolated. Hippocrates described it as a highly life threatening disease mainly of young people and is accompanied by jaundice (Kuntz and Kuntz 2006, 413). Infectious jaundice, now called hepatitis presents the most imposing medical representation in hepatology and has gained attention of physicians for more than 2,500 years. It had been one of the major causes of epidemics and pandemics around the world. This fact engaged workers to a number of activities leading to theories and controversies. Treatments sometimes included ridiculous acts, i.e., live sheep lice were taken orally (Kuntz and Kuntz 2008, 419). Acute viral hepatitis is a systemic infection that predominantly affects the liver. There are five viral agents reported causing acute hepatitis namely hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis B virus related delta agent or hepatitis D virus (HDV) and hepatitis E virus (HEV). Other transfusion transmitted agents belonging to this category of viruses that do not cause hepatitis are hepatitis G virus (HGV) and TTV (Transfusion Transmitted Virus) (Jules et al. 2001).

HCV is an important human pathogen in terms of its high prevalence and potentially serious complications of persistent HCV infection (Alter 2007) that include cirrhosis, hepatocellular carcinoma (HCC) and end-stage liver disease. It had been suggested that incident rates of all these complications may rise in future (Kim 2002).

1.2 STRUCTURE OF HCV

Structure of HCV is similar to other viruses bearing lipid cover enclosing shell of protein around genetic material (RNA). Storage of genetic information in RNA has imparted viron its remarkable ability to mutate (Fleury et al. 2006 and Kuntz and Kuntz 2006, 413). Moreover, RNA is a very reactive molecule that can react with itself under

suitable conditions. Frequent mistakes are observed during RNA copying with an average of one mistake per 10,000 nucleotides per copy, making RNA poorly suited for storage of genetic information (Horwitz 1996 and Ong et al. 2006). Hence, RNA is ideal for storage of viral genetic information (Wesley and Alter 2000 and Anonymous 2009).

1.3 DIFFERENCES IN TREATMENT SUSCEPTIBILITY

Host body produces antibodies against infecting virus, which then no longer reproduce in it. Hence, it has to mutate for escaping the immune system of host (Wesley and Alter 2000 and Anonymous 2009). Although the reasons for the differences in response to treatment is not fully understood but it had been proposed that genotype differences play a critical role in this context (Shoukry et al. 2004) that may be attributed partly in the interaction of certain HCV protein with intracellular biochemical pathway such as nonstructural 5A protein coding region (NS5A), Envelop 2 (E2) and intracellular pathways that mediate the effects of interferon (Shoukry et al. 2004). A consistent association has been found between HCV genotype 3 infections and liver steatosis, suggesting that HCV genotype 3 may interrupt intracellular metabolism of lipids (Lonardo et al. 2004). Another study suggested a link of sequence diversity of the viral NS5A to interferon responsiveness (Yuan et al. 2009).

There are stable regions in HCV genome such as non structural 5B (NS5B) that may be a target for epidemiological reconstruction. On the other hand hyper variable regions (HVRs) of E2 and NS5A are highly variable regions (McGarvey and Houghtom 2005, 381). These variable regions may have been formed due to specific selection, i.e., natural selection for survival of fittest operate virus associated with immune escape such as the HVR-I in E2 may be a target for neutralizing antibody. In this case viral persistence might require continuous mutations to avoid B-cell responses (Farci et al. 2000 and Kantzanou et al. 2003).

Comparison studies on baseline sequences showed substitutions in variable region 3 (V3) and flanking regions in the patients with relapse but in the patients with sustained

virological response this trend was not observed (Pawlotsky et al. 1998). High numbers of substitutions in NS5A for both patients groups, with relapse and with sustained virological response were found to emphasize on selective pressure association with viral response to therapy. Amino acid substitutions within the NS5A coding region are an example of viral adaptation owing to selective pressure (Yuan et al. 2009). However, viral diversification was observed to be due to adaptive selection resulting in viral escape from immune system. HVR-1 in E2 region of virus genome has been reported as dominant neutralization epitope and its carboxy-terminal also possess epitopes for T-helper cells and other cytotoxic response (Brown et al. 2007).

The presence of immune pressures may also help to distinguish maternal viral clones passed to child and this may also control the fruition of the viruses in both the mother and child over time. Other factors involved might be the mode of transmission and potentially different quasi species populations (Henry et al. 2004).

1.4 PATHOGENESIS OF HCV

As an RNA virus, HCV has a powerful reproductive strategy. Its genetic information is stored in "sense" strand of RNA that is read by host cell's translation machinery just like cell's own RNA (Brown 1995). Thus, virus depends on its host cell's translation machinery to produce everything it needs, to takeover the cellular processes and also for its reproduction. Since only small portion of RNA encodes core information and rest of it is available for genetic variation to occur (Zeuzem et al. 1996). There are fewer common and conserved characteristics that can be readily identified by host's immune system and may be exploited by scientists working to find treatments (Farci et al. 2000).

Pathogenesis of hepatitis C depends on viral factors that pose their effects either directly through cell injury associated with accumulation of intact virus or viral proteins, or indirectly, through a differential immune response associated with one viral strain but not with other. Severe liver dysfunction has been reported in immuno-compromised transplant recipients who demonstrate only moderate inflammation in liver biopsy

specimens. Direct cytopathic effect of HCV has been observed to be related to high levels of HCV RNA in patient's serum (Davis et al. 2003, Rotman and Liang 2009 and Shi and Lai 2001).

Chronic HCV in many patients show only a serene form of liver disease and has been suggested to possess highly heterogeneous nature. Damage to the liver parenchyma is mediated by inflammatory cytokines. Persistent inflammatory mediators activate steallate cells of liver parenchyma resulting in various degrees of hepatic fibrosis e.g. progressive fibrosis leading to cirrhosis are observed in some patients (Ramaiah and Jaeschke 2007). Other reasons for development of disease have also been reported like homosexual relationships for males, age at start of infection, alcoholism, coinfections and liver steatosis (Alberti et al. 2004 and Pawlotsky 2004). Moreover, a study demonstrated that around 50% of HCV carriers tested had normal Alanine Aminotransferases (ALT) level whereas two-third of same study group showed mild lesions in liver tissue (Silini et al. 2005 and Zein 2000). Thus, patients with chronic HCV and eminent ALT levels showing no contraindications should be directed for antiviral therapy (Alberti et al. 2004).

1.5 PHYLOGENETICS OF HCV

The evolution of HCV is a highly dynamic process that is proposed to occur not only through adaptive selection but also through genetic drift (Budowle et al. 2005). Diversification of HCV had been observed to occur and at different rates during the course of evolution. Evolution of HCV in patients who acquired disease from a common source emphasize that host immune response are pronounced in determining quasispecies transmission (Henderson 1997). Despite its ability to mutate rapidly, the evolution of HCV appeared to be conservative. Fundamental changes in HCV genotype relationship to its host are obvious and differences in treatment response between genotypes are clinically important e.g., their ability to flourish during long periods of evolution thereby successfully filling specific ecological niche in human populations (Araujo et al. 2008).

HCV transmission in HIV-positive homosexual males was revealed through phylogenetic analysis of HCV genome core region (Aral et al. 2007). It was observed that larger proportion of European homosexual males taking antiretroviral therapy (74%) were HCV positive for viral strain co-circulating in different European countries (Laar et al. 2009).

Further, characterization of 3'-X-tail element (Drexler et al. 2009) suggested it being highly conserved for all genotypes and may be targeted for molecular detection and quantification to circumvent the limitation posed by 5'NCR (Elbeik et al. 2004, Caliendo et al. 2006 and Tuaillon et al. 2007). In order to understand pathogenesis and treatment development strategies, host parasite relationship needs more attention (Simmonds 2004).

1.6 HCV IN PAKISTAN

HCV is one of the major health issues for Pakistan with incidence rate between 6 to 10 percent. It is a matter of global health concern as no active immunization is available (*Daily Times* 2005). It has been proposed that the family members of HCV patient are at risk on coming into close contact with patient's blood and saliva (Hamid et al. 2004). Other factors involved include blood and blood product transfusion, surgical procedures, dental procedures, hemodialysis, razor sharing, percutaneous and sexual transmission, reuse of syringes, drug administration, body piercing and travelling (Pasha et al. 1999, Nasir 2004, Luby et al. 2005, Masood et al. 2005, Highlyman 2009, Jaureguiberry et al. 2005, Bollepalli et al. 2006, Nafees et al. 2008 and Raja and Janjua 2008). According to WHO report, 8 to 9 injections per person per year are used for drug administration in Pakistan raising the threat of infection (WHO 1999a, WHO 1999b and Raja and Janjua 2008).

It had already been reported that seroprevelance of HCV positive is 30.98 percent in subjects from Lahore, Pakistan. Although many non-sexual modes of transmission are still not recognized and needs attention (Akhter and Moatter 2007), proper management

of HCV may offer response rate upto 80 percent (Akhter et al. 2002, Bari et al. 2001 and Aslam and Syed 2005).

1.7 OBJECTIVES

HCV is becoming one of the major reasons of many deaths in Pakistan without knowing the cause. The virus can remain latent for 10-15 years (Zein 2000) and kills its host silently. People in Pakistan lack awareness in this context and need preaching to lead a healthy and long life. The main objectives of present study were

- Identification and prevalence of HCV genotypes in sub urban Rawalpindi.
- Identification of prevalent risk factors, their correlation with particular genotypes and role in HCV transmission in the specific area.
- Examining the role of demographic factors gender, age and socioeconomic status with reference to transmission of HCV.
- Assessment of the viral evolution in studied HCV sequences and a comparative study from neighbouring countries as well as all over the world.

Chapter 2

LITERATURE REVIEW

2.1 HEPATITIS C VIRUS FROM LABORATORY TO CLINIC/HCV HISTORY

HCV infection has become a global health issue, affecting almost 200-500 million individuals worldwide (Ghany et al. 2009). Over 70% patients may develop chronic hepatitis, liver cirrhosis, hepatic failure or HCC. HCV belongs to the family Flaviviridae, genera *hepacivirus*, sharing a number of structural and virological characteristics. The virus is enclosed in a lipid bilayer where two or more envelop proteins (E) are anchored, surrounding nucleocapsid composed of multiple copies of a small basic protein (core protein or C protein). The HCV genome is 9.6 kb single stranded sense RNA, with one ORF encoding a poly protein of 3000 amino acids and diameter of the particle is 50 nm. It is found to be pretty unstable to storage at room temperature and frequent freezing and thawing, and is inactivated by organic solvents and detergents, formaldehyde, β-propriolactone and ultra violet irradiation (WHO 2001a, b).

HCV was introduced in 1970's as inflammatory disease of liver not attributable HAV and HBV and was termed as non-A non-B hepatitis (Alter et al. 1975). Later in 1970s similar infection was observed to be transmitted from man to chimpanzee (Purcell 1994). He et al. (1978) reported the size of non-A non-B hepatitis agent was less than 80nm and therefore proposed it to be a virus. It was observed to be an enveloped virus owing to its chloroform sensitivity followed by its characterization by the amino acid sequencing and analysis of RNA properties (CDC 1991).

Molecular cloning technique was used to identify the virus in detail (Choo et al. 1989), later in continuation to the work of Choo et al. (1989), overlapping clones were isolated and used for first generation enzyme linked immunosorbent assay (ELISA). This response was further used to define infection with a new virus, HCV (Sherman et al. 1989).

2.2 GENOTYPE ORIGINS

Although it is difficult to estimate HCV in human population, the endemic nature of virus may be proposed on the basis of prevalence of particular genotypes in particular areas of world, for example diversity of variants within genotypes 1, 2 and 4 in Sub-Saharan Africa and genotypes 3 and 6 in South-East Asia. Diversity among the subtypes was observed to be neutral during evolution and it is now possible to study the times of divergence, i.e., splitting of subtypes (Okamoto et al. 1992 and Smith et al. 1997). As an example time period of divergence for West African genotype 2 was calculated to be approximately 200-250 years ago and for other genotypes it is about 100 years. From this analysis it was suggested that the origin of these genotypes is about 1000 years ago. But it was later found to be even older than thought as for major types of HCV genotypes (1-6) earlier divergence was suggested to have occurred at least 500-2000 years ago whereas for genotype 1b, time of divergence was found out to be 70-80 years ago (Smith et al. 1997 and Pybus et al. 2009).

2.3 STRUCTURE AND GENOMIC ORGANIZATION

Untranslated regions at 5' end (341 nucleotides) and 3' end (230 nucleotides) bear highly conserved RNA structures essential for polyprotein translation and genome translation. An ORF flank these UTRs and is translated into protein of approximately 3000 amino acids (Penin et al. 2004a and Balvey. 2009). The processing of this polyprotein is observed by cellular and viral proteases in endoplasmic reticulum of host cell yielding 10 mature proteins (Lindenbach and Rice 2001, 991). Structural proteins are found in viral nucleocapsid and in envelope where it exists as glycoprotein, i.e., E1 and E2. Structural proteins are distinguished from non-structural (NS) proteins on the basis of short membrane peptide p7, which is assumed to be a viroporin. The role of non-structural proteins NS2 and NS5B is observed to be in polyprotein processing and viral replication. The processing reaction of NS proteins is specific and requires distinct proteinase, i.e., Zinc dependant metalloproteinases for NS2-NS3 and NS3 serine proteinases. This NS3 proteinase property is contained in NS3 N-terminal region and utilizes cofactor NS 4A for release of remaining NS proteins. NS5A is

polyphosphorylated protein of unknown function whereas NS5B is RNA dependant RNA polymerase (RdRp) (Penin et al. 2004). It was suggested that an HCV protein is potentially synthesized by ribosomal frame shift (Walewski et al. 2002) owing to four highly conserved structural domains in 5'UTR and stem loop structure and internal polypyrimidine tract/poly Uridine in 3' UTR (Higgs et al. 2010).

2.4 LIFE CYCLE OF HCV

Blight and Gowans (1995) proposed HCV positive cells in infected liver tissue range from less than 5% to 100%, corresponding to virion replication rate of 50 particles per hepatocyte per day. It can also replicate in peripheral blood mononuclear cells (Neumann et al. 1998).

The virus attaches to the host cell by a specific and very strong interaction with CD81 (a B-cell surface receptor) receptor on the host cell surface and a viral protein E2 as well as with viral particles when studied *in vitro* (Pileri et al. 1998). In addition HCV can also enter the cell by binding to low density lipoprotein (LDL) receptors. E1 is involved in membrane fusion while E2 acts as a Chaperon for E1, which in the absence of E2 forms misfolded aggregates (Michalak et al. 1997 and Flint et al. 1999). Virus binding to receptor forms a complex that triggers receptor-mediated endocytosis. Unlike flavivirus translation that is cap-dependent (5' UTR-m7 Gppp Amp), HCV 5'UTR is not capped and folds into a complex secondary RNA (Brinton and Dispoto 1988). The HCV internal ribosomal entry site (IRES) has potential to form pre-initiation complex by binding directly to 40S ribosomal subunit (Spahn et al. 2000 and Otto et al. 2002).

NS5B RdRp catalyses the synthesis of minus and plus strand of RNA. Its 3' end attains a special conformation that can be used for elongation of the product (Al et al. 1998). NS5B can synthesize RNA Primer by utilizing high amount of cellular energy in the form of ATP/GTP (Luo et al. 2000) and can copy full length HCV genome *In vitro* (Lohman et al. 1997). Helicase activity to unwind template thereby facilitating replication is performed by NS3 whereas NS5A regulates the whole process. In addition cellular components Poly pyrimidine tract-binding protein (PTB) interacts with the sequences at

the 3' UTR and Glyceraldehyde-3-phosphate dehydrogenase, binding to the poly (U)-sequence in the 3' UTR and finally, cellular proteins called p87 and p130 (Ito and Lai, 1997, Tsuchihara et al. 1997, Chung and Kaplan 1999 and Petrik et al. 1999). Thus, HCV genome is translated into precursor polyprotein, which is processed by the internal signal sequence and by host signal peptidase to yield immature form of the protein. Since HCV E is located in ER membranes of host cell supporting the view that nucleocapsid attains its envelope in ER. The presence of N-linked glycans on viral surface further support the export of particle through golgi via constitutive secretory pathway (Sato et al. 1993).

2.5 IMMUNOPATHOGENESIS OF HCV

Viral proteins are transported to antibody producing B cells by macrophages and dendritic cells thus clearing circulating virus and protect host cell from reinfection. These viral proteins are recognized by helper T cells, which phagocytose and breakdown these proteins in close vicinity of class II Major Histocompatibility complex (MHC). This leads to the activation of T-cell receptors for activation of B-cells followed by initiation and stimulation of virus specific T-cells (Ridge et al. 1998). Most of these effects are mediated by different sets of immunoregulatory Thi-stimulating cytokines called Th1 and Th2. CD8-positive cytotoxic T cells in the proximity of class I MHC identify HCV peptides produced in infected cells and may lead to lysis of virus infected cells.

HCV can cause persistent infection even when hypervariable region 1 is stable (Bassett et al. 1999). Several HCV proteins, such as core, E2 and NS5A, hamper immune response. The liver has been proposed as the major site where activated T Cells are destroyed (Large et al. 1999, Tailor et al. 1999 and Gale et al. 1999).

2.6 DIAGNOSTIC METHODS FOR THE DETECTION OF HCV

Diagnostic tests for HCV infection are divided into two categories, i.e., serological assays for antibody detection and molecular tests for viral particles. The most important screening test is the detection of anti immunoglobulin G (IgG) in the serum

using Enzyme Immuno assay (EIA) (McHutchinson et al. 1992 and Lauer and Walker 2001). A few patients have been reported for gradual antibody level decline leading to sudden loss of infection (Takaki et al. 2000).

2.6.1 Serological Assays

Serological assays include first generation assays, second generation assays, third generation assays, enzyme immuno assay (EIA), enzyme linked immunosorbent assays (ELISA), line probe assay (LiPA), immunoblot assay (IBA), hepatitis C virus branched DNA-probe assay (bDNA-probe), HCV amplicor monitor assay (amplicor monitor), HCV core protein assay (core protein) and HCV competitive polymerase chain reaction (competitive PCR) respectively (Kobayashi et al. 1999 and Schroter et al. 2001). The sensitivity of the above mentioned serological assays for the detection of recent infections is good enough, but the drawback of these indirect tests is their inability to differentiate between a recent infection and a past infection, resulting in misclassification of recognized infections (Guy et al. 2009).

2.6.2 Molecular techniques

Shortcomings of serological assays for HCV antibodies led to the discovery of direct detection of HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR) (Germer and Zain 2001). Unstability of viral RNA suggests careful handling of samples to reduce the chances of false negative results. Hence, samples should be kept isolated and frozen within 2-3 hours of phlebotomy (Busch et al. 1992). This is a qualitative test that can detect infection at even less than 100 copies of HCV RNA per millilitre of blood (Beld et al 2000) and having main pro of timely detection of infection even in the patients showing no symptoms (Germer and Zein 2001 and Lauer and Walker 2001). Amplicor test for HCV using RT-PCR targeting 5'UTR was introduced by Roche Diagnostics (Branchburg, NJ) (Nolte et al. 1995). It was soon followed by the introduction of semi automated COBAS Amplicor HCV test 1.0 (Albadalejo et al. 1998). It was then subjected to a number of modifications to increase its sensitivity and a newer version was invented (Doglio et al. 1999). A more advanced technique employing transcription mediated amplification (TMA) found to be more reliable, robust and

sensitive has now become commercially available by Bayer Diagnostics, Emeryville, California (Sarrazin et al. 2000).

Measurement of ALT level is inexpensive, easily available but a non specific test for identification of HCV (NIH 1997a,b). However, it may be best option for monitoring HCV infection and observing the efficacy of therapy. But ALT levels do not promise success of antiviral therapy. Physicians still rely on liver biopsy and suggest it the most reliable source for diagnosis (Niederau et al. 1998) and disease progression (Yan et al. 1996). It may also help to identify other causes of liver disease and is therefore, recommended for the initial assessment of persons with chronic HCV infection (NIH 1997a, NIH 1997b and EASL 1999).

Overall, these quantitative assays have been developed not only for management of infection but also for accurate quantification of virus in serum or plasma even at low levels of viremia (Germer and Zain 2001). Blood virus level has been shown related to the success of antiviral therapy (McHuschitson et al. 1999). Three commercial tests are currently available to quantify HCV infection, i.e., a branched chain DNA assay (Quantiplex HCV RNA, Version 2.0) and two assays involving RT PCR (COBAS Amplicor HCV Monitor, version 2.0 and HCV Superquant) (Lauer and Walker 2001). All systems deliver reliable but do not provide comparable results (Martinot-Peignoux et al. 2000).

2.7 SEQUENCE DIVERGENCE OF HCV

HCV has been classified on the basis of nucleotide sequence comparison that appeared equivalent in different parts of genome. Six main groups of variants found in the NS5 gene (Simmonds et al. 1993) are paralleled by equivalent groupings in EI (Bukh et al. I993), core (Bukh et al. 1994) and NS4 (Bhattacherjee et al. 1995), and by comparison of available complete genomic sequences (types Ia, Ib, Ic, 2a, 2b, 3a, 3b) (Sakamoto et al. 1994). So far, no HCV recombinants of different genotypes have bee reported (Smith et al. 1995). It was also observed that length of the sequence matters in viral classification, ie., longer sequences reveal more information and are therefore may

be used even to identify genotypes by sequence comparison of relatively short subgenomic regions of HCV (Bukh 1995). Novel genotypes are identified on the basis of at least two parts of genome for which 5' UTR being highly conserved is useful (Simmonds et al. 1994a). Distinct sequences in this region are observed in almost all types and some sub types of HCV (Smith et al. 1995, Tokita et al. 1995 and Mellor et al. 1996). Classification may also be done by phylogenetic analysis by pairwise sequence similarity and this has become primary mean to identify and classify new genotypesfor example a number of HCV variants have been found in Vietnam, Thailand, Burma and Indonesia (Simmonds et al. 1993, Doi et al. 1996, Mellor et al.1996 and Tokita et al.1996). These variants were classified on the basis of previously established ranges for type and subtypes in NS5 and core/E1 and were proposed to be classified as types 6a, 6b, 7a, 7b, 7c, 7d, 8a, 8b, 9a, 9b, 9c, 10a and 11a (Tokita et al. 1996).

2.7.1 Genotype 1

Da Silva et al. (2007) investigated the prevalence and distribution of HCV genotypes in Brazil using 5'UTR. Genotypes 1, 2, 3 and 4 were found in the decreasing order of prevalence i.e. 63%, 43%, 31.3%, and 0.3% and a few mixed infections. In a previous study performed by (Krug et al. 1996), genotypes 1, 2, and 3 were found at frequencies of 55%, 8%, and 37%, respectively. Data showed similarity in genotypes frequencies to those previously found, indicating the absence of substantial changes during the last decade. Studies carried out in Sao Paulo (Moreira et al. 2003 and Perez et al. 2003), Tocantins (Souza et al. 2003) and Recife (Albuquerque et al. 2005) in Goias State, Central Brazil, (Espirito-Santo et al. 2007) showed that HCV genotype 1 (subtype 1a) is predominant in haemodialysis patients in Central Brazil. However, the genotyping methods employed in those studies, which were based on 5'UTR region analyses, did not permit the correct identification of HCV subtypes (Halfon et al. 2001, Chen and Weck 2002b, Dussaix 2002, Germer et al. 2003, Nolte et al. 2003, Roque-Afonso et al. 2002b, Othman et al. 2004 and Laperche et al. 2005). Subtype 1a is more likely to disseminate in the haemodialysis environment or could be more adapted to the immunosuppression of these patients (Perez et al. 2003).

In Tunisia, previous studies reported prevalences of HCV infection ranging 0.4-0.7% in the general population, with a large predominance of subtype 1b (79%) and circulation at lower levels of subtypes 1a, 2, 3 and 4 (Djebbi et al. 2003). Higher prevalences were found in neighbour African countries: 7.7% in Morocco, 6.9% in Libya and more than 10% in Egypt (Kamel et al. 1992, Saleh et al. 1994, Waked et al. 1995 and Cacoub et al. 2000) except for Algeria where very low anti HCV prevalence (0.18%) was reported (Ayed et al. 1995).

The predominant genotype in Turkey is 1b (67%-94%) and next to it is 1a (6%-19%) (Bozdayi et al. 2004). Current genotypic map may be changed by risk factors as migration and travel (Ross et al. 2003). Genotype 1a is observed to be predominant among intravenous drug users (Turhan et al. 2005) where genotype 1b level was 17.4% whereas among non-intravenous drug users it was 100%. In some European countries genotype 1a and 3a are found to be increasing with decrease in genotype 2a, 2c and 1b especially in young patients (Ross et al. 2003). Genotype 1 is reported as predominant in patients suffering from post-transfusion hepatitis (Pujol and Loureiro 2007).

In West and North Iran major prevalent HCV genotype is 1a in 55.8%. Second most prevalent is genotype 3 with 28.8% occurrence, 1.3% with genotype 4, and 0.6% had mixed infection of genotypes 1 and 3 in complete absence of genotype 2. Subtype 1a is more frequently seen in cases with history of hospitalization, major surgery, dental surgery, transfusion, alcohol consumption and minor surgery (Zali et al. 2000, Samimi-Rad et al. 2004 and Kabir et al. 2006). Among drug users, 40% and 37.8% patients with IVDA had genotype 1a and 3a respectively. Lifestyles of young addicts seem to have influenced molecular epidemiology of HCV by the introduction of subtype 1a and 3a from USA and Southeast Asia (Dal Molin et al. 2002 and Kabir et al. 2006). Genotype 1 to be the predominant one in Luxembourg population (53.4%) followed by genotype 3, 4, 2 and 5. Slightly different pattern was observed in prison inmates with genotype 3, followed by genotype 1, 4 and 2 (Roman et al. 2008).

The frequency of genotype 1 is reported to be increasing in Pakistan with no effect in the frequency of genotype 3. It is proposed that in coming 15-20 years, current

prevalent genotype 3a will be replaced by less common genotype 1 (a or b). If this actually happens in Pakistan, it will complicate the current situation of HCV in country (Idrees 2001 and Idrees and Riazuddin 2008). One such trend of changing genotype prevalence has been reported from Venezuela where HCV genotype 1b was replaced by genotype 2 in only ten years (Flor et al. 2007).

Dominant genotype of western Iran is genotype 1. HCV genotype distribution varies with age in both male and female patients had been reported (Benani et al. 1997). Subtypes 1a and 1b were found most prevalent among older patients, whereas subtype 2a/2b and 3a/3b were mainly found among younger ones. Since border is shared between Iran and Balochistan region of Pakistan, this genotype may be introduced by people entering Pakistan for different reasons (Kabir et al. 2006).

2.7.2 Genotype 2

In Sub-Saharan Africa, HCV prevalence has been reported to be less than 1% in Southern African countries (Tucker et al. 1997 and Vardas et al. 1999) and range between 1.7 and 27.5% in Central Africa (Ndjomou et al. 2002) and between 1.4 and 7% in West and East Africa (Combe et al. 2001and Sarkodie et al. 2001). A study reported presence and high prevalence of HCV genotype 2 in Africa. An overall 6.7% anti HCV prevalence was found in a sero-epidemiological study from town of Conakry (Candotti et al. 2003). High level subtype heterogeneity was observed from the genotype 2 of Guinea HCV isolates whereas four of the subtypes were newly discovered i.e. Nigerian (genotype 1), Gambian (Genotype 2), South East Asia specifically India, Pakistan and Bengladesh (genotype 3) and Genotype 4 from Middle East (Mellor et al. 1995).

However, for HCV genotype distribution in Ghana, the subtype distribution was observed to be more complex. Ghanaian HCV strains (genotype 1 or 2), exhibited considerable genetic diversity not only between themselves but also to reference strains representating subtypes 1a, 1b, 1c, 2a, 2b, or 2c (Candotti et al. 2003). A similar subtype of wide distribution was obtained from the neighbouring countries Benin, Burkina Faso, and Guinea (Jeannel et al. 1998). On analysing these sequences phylogenetically with Ghanaian NS5B sequences, genetic distribution observed was similar to West African

strains type 2. None of the African sequences clustered with subtypes 2a to 2c, irrespective of their geographical origin. Moreover, commonly reported subtypes were subtype 2a from East Asia, subtype 2b in Northern America, and subtype 2c in Europe. None of these subtypes have been clearly identified in West Africa (Larghi et al. 2002).

2.7.3 Genotype 3

A relatively higher proportion of genotype 3 has been reported from India (53.69%) in comparison to genotype 1 (38.25%), genotype 4 (6.04%) and genotype 6 (0.671%). It was also observed that type 1 and type 3 are found in southern India in almost equal proportions (36.11 and 38.88%, respectively) and in western India (53.52 and 43.66%, respectively) thereby suggesting more active flourishing of type 3 variants than type 1 around the country (Pal et al. 2003). But the trend is changed on moving towards northern and eastern regions of India where genotype 3 shows greater occurance than genotype 1 (Kavita et al. 2003). Moreover, mutated forms of genotype 3 are prevalent in adjoining countries from northern and eastern regions of India that includes Bangladesh, Pakistan, Thailand, Nepal, Indonesia, and Vietnam (Shah et al. 1997, Simmonds 1995, Tokita et al. 1994a, Tokita et al. 1994b, Tokita et al. 1995 and Tokita et al. 1996). This percentage is may be due to the geographical proximity to neighbouring countries and a long history of travel, trade, and communication among the people of these areas (Lole et al. 2003). However, the spread of HCV genotypes and subtypes in Pakistan and many of the risk factors associated with mode of transmission are not known and therefore attention is required in order to observe the frequency of different HCV genotypes from the region (Idrees and Riazuddin 2008). Only a few reports are available in this context and this data is based on pilot studies (Shah et al. 1997, Abdulkarim and Zein 1998 and Idrees 2001).

In near past a very successful attempt in this direction revealed six genotypes for the first time from Pakistan, i.e., 1c, 2c, 3c, 4, 5a and 6a (Idrees and Riazuddin 2008). Genotype 4 is believed to be absent from Pakistan (Shah et al. 1997) whereas predominant genotype of this region was suggested to be genotype 3 providing 18% untypable results in the same study (Khokhar et al. 2003a, b). These untypable sera were also observed from other areas of the country in lower proportions (Yu et al. 2000)

whereas higher percentage was diagnosed for alcohol users. Moreover, antiviral treatment response against genotype 3 is found to be better than for other types (Mujeeb 2002).

The reasons for untypable serotypes may be various e.g. antibodies not produced against NS4 protein, mixed infection (by different viruses at a time) due to super infection in certain patients may also result in cross-reactivity and untypability (Van Doorn et al. 1996 and Toyoda et al. 1999). Observed rise Serotype 3a levels among young addicts suggest the effects of social behaviour on viral epidemiology (Kovalev et al. 2003).

Some studies found no correlation between steatosis and necroinflammation or fibrosis in HCV genotype 3 patients having low cholesterol levels belonging young age group. Although (injection drug users) IDU serve as a major risk factor for genotype 3 patients with moderate to severe steatosis but is not an independent cause (Samimi-Rad and Shahbaz 2007). Alteration have been observed in trend of prevalence of different HCV genotypes in european countries for example, with the decrease in occurrence of HCV 1b, HCV 1a and 3a prevalence has increased (Ross et al. 2000b, Bourlière et al. 2002, Dal Molin et al. 2002 and Roudot-Thoroval 2002).

2.7.4 Genotype 4

HCV 4 subtypes are distributed varyingly with geographical origin of transmission. Sustained virological response (SVR) after antiviral therapy for HCV 4a and 4d is observed in French patients using injections for drug intake (Roulot et al. 2007). Subtype 4a was found to be predominant in Egyptian patients (93%) who got infected probably due to parenteral treatment for schistosomiasis. More than seven different genotypes and four subtypes were found among patients from Sub-Saharan Africa, central Africa and Middle East, for which route of infection is unknown (Xu et al. 1994, Fretz et al. 1995, Angelico et al. 1997, Shemer-Avni et al. 1998, Shobokshi et al. 1999 and Ray et al. 2000).

Like HCV 1, HCV 4 is observed to be resistant to therapy and its pathogenecity is still not known. HCV 4a and 4d among French drug users were found to possess

extended subclades in phylogenetic studies and this high diversity served in contrary to previous findings (Castelain et al. 1997 and Martinot-Peignoux et al. 1999). It may be reasoned due to the presence of variety of ethnic groups especiall from South Africa (19%) owing to historical relationships between France and Africa (Iles et al. 2004). In Italy genotype 4, was determined at a level of 13%. Again the connection of Italy with northern Africa and probably the southern Mediterranean basin with nearby Sardinia, favoured that changes would have occured in the HCV subtype pattern (Coppola et al. 2000).

Genotype 4 is also found to be common in Yemen, Kuwait, Iraq, and Saudi Arabia (Simmonds et al. 1993). It has been suggested to be exclusively found in Middle East and Western countries (Mellor et al. 1999), uncommon in Iran. The causes for infection have been suggested as dialysis, minor surgery, piercing or hejamat providing highest risk for haemodialysis patients from Tehran. The infection has not been found related to transfusion, intravenous drug abuse (IVDA) or sexual contacts (Samimi-Rad et al. 2004). However, Kabir et al. (2006) could not obviate any definite conclusion on genotype 4 transmissions with small sample size.

Martinez et al. (2005), reported high infection rate for HCV Genotype 4 from Galicia, Spain. Epidemiological and medical characteristics found in this series differed from those for African and Middle Eastern countries in terms of i.e., all patients were white and the predominant mode of acquisition of the infection in two third population was IVDU like observed from other Western countries. In contrast, a high proportion of unknown source HCV was observed in Egypt and in Kuwait (Hasan et al. 2004).

The few publications on HCV genotype determination from Saudi Arabia indicated principal genotypes were either type 1 or 4, (Al Faleh and Ramia 1997 and Martinez et al. 2005). HCV genotype 4 is prevalent in the Middle East and Central Africa and generally observed to be related to cirrhosis and a poor response to interferon (Xu et al. 1994 and El-Zayadi et al. 1996). It is rarely found in the United Sates like Pakistan. The published data regarding response to therapy in patients with HCV genotype 4 infections in Pakistan and United States is not enough and needs attention (Lyra Andre et

al. 2004). Some rare genotypes have recently emerged in the western world thereby Genotype 4 remains leading genotype in France, in the Northern Parisian suburbs (Morice et al. 2001), in the south of Spain (Sanchez-Quijano et al. 1997), and in Italy (Matera et al. 2002).

2.7.5 Genotype 5

Comprehensive information on natural history of the HCV genotype 5 in terms of chronicity and response to the treatment is lacking and needs attention (Henquell et al. 2004). Characterization of various type 5 infections and corresponding strains will contribute to progress in the fields of medical practices and treatment. As genotypes 4 to 6 have been found mostly in developing countries including India, countries in Southeast Asia and the Middle East, genotype 5 is observed to be limited to South Africa (Forns and Bukh 1998 and Zein 2000). Only two studies from Saudi Arabia had recorded the presence of genotype 5 in the Kingdom (Shobokshi et al. 1997 and Mahgoub et al. 1998). In 2001, an unusually high dominance of genotype 5 was reported for the region of Alicante in Spain (Jover et al. 2001 and Henquell et al. 2004).

2.7.6 Genotype 6

Raghuraman et al. (2005), reported the case history of two patients from eastern India, suffering from HCV genotype 6 which was previously been reported only from Hong Kong and Southeast Asia. This proved that with increasing globalization the geographical isolation of certain HCV genotypes may become obsolete. HCV is found to be highly flourished and endemic for long times in Africa and south east Asia (Smith et al. 1997 and Pybus et al. 2001). Recent reports provided important new epidemiological data for example; spontaneous recombinant isolate was reported in 2002 from St. Petersburg, Russia (Kalinina et al. 2002).

2.8 SOCIODEMOGRAPHY OF HCV

The age of those infected with HCV varies around the world. The infection rate in Egypt increases steadily with age, with a 10% HCV rate among those 50 and older and a 5% infection rate in those ages 30 to 39. The infection rate is nearly zero in children

under age 9 years and younger (Karmochkine et al. 2006). The reason of this age curve of infection was mass inoculation with reused, contaminated syringes that took place in Egypt from 1960 to 1987, in an attempt to stop Schistosomiasis. It was in 1982, when oral treatment to prevent the schistosomiasis was available (Frank et al. 2000 and Roberts and Yeung 2002).

The practice of reusing syringes in medical settings has played a significant role in transmitting blood borne viruses in many countries including Romania, Moldova, and Pakistan (Kermode 2004). In Japan there is a demographic disease curve similar to that in Egypt. According to national health and nutrition examination survey (NHANES), a survey conducted by CDC's National Center for Health Statistics, the highest HCV infection prevalence in United States is among the 30 to 39 years age group i.e. about 5%. Illegal injection drug use is believed to cause most infections in this age group (Moriya et al. 1999). The infection rate decreases to 1% in those 50 and older. HCV infection in young children in developing countries results primarily from improperly sterilized syringes and medical equipment and vertical transmission (Quer and Mur 2007).

Henquell et al. (2004) studied that HCV was more common among male patients (53.5%) older than 50 years than in those younger than 49. Type 5 infection was found to be predominant for 50+ patients from general population without discriminating gender. A significant increase in rate of prevalence by age for genotype 2 variants in west central Africa from 4.6% and 7% to 12% among the age groups 16-25, 26-35 and 36- 45, respectively (Ruggieri et al. 1996). Turkish youngsters were observed to be more infected because of intravenous drug abuse, having spent time in prison, multiple sexual partners and tattoos. However, ethnic differences are of less concern in genotype distribution than geographical area and routes of transmission of infection (Turhan et al. 2005).

2.9 BIOCHEMICAL FINDINGS IN DIFFERENT HCV STRAINS

HCV had been reported as a highly heterogeneous virus, a leading cause of chronic liver disease, cirrhosis and HCC. In Western countries 50% of HCV carriers have

continually normal ALT levels and two-third show mild histological liver lesions (Alberti et al. 2004). It was observed on studying natural history of initially mild chronic disease that the short term outcome is always benign. However, it may lead to development of liver fibrosis at long term (>5-7 years) follow-up, particularly in patients showing elevated and/or fluctuating transaminase levels. All genotypes are capable of developing HCC (Mangia et al. 1997). Cirrhosis or severe fibrosis may develop from 10 to 15 years in HCV carriers having persistently normal ALT, around 5 to 10% in patients with elevated ALT and F0 (no fibrosis) in the initial biopsy and more than 30-40% in chronic carriers with elevated ALT and F1 (portal fibrosis) in the initial biopsy (Alberti et al. 2004). Infection is observed to progress under the effects of cofactors like age at infection, alcohol, coinfections and liver steatosis. Hence, initially mild chronic cases having elevated ALT levels are recommended with suitable antiviral therapy even when contraindications are lacking (Sobesky et al. 2008). Although no correlation was observed for disease severity and duration of infection to genotype distribution, however, for genotype 1b effect of age is strongly related to disease progression (Mangia et al. 1997).

The risk for getting infected with HCV is similar irrespective of gender and was found to be minimal for age group of 40-49 years and doubled for age group 50-59. Other factors include blood transfusion history, jaundice and increasing age (Kim et al. 1998a, b, c). Similarly, HCV was suggested cause of liver cirrhosis in 40.1% cases (Carrao et al. 1998). Another similar work reported the reactivity of anti HCV antibodies to be 58.9% in chronic liver disease (CLD) from subjects belonging to Chicago (Jose et al. 1990). Studies carried in Pakistan show shifting results for example, in liver biopsy diagnosed hepatitis, 77.7% were seropositive for HCV antibodies. This trend observed was in contrast to previously reported results, was twice as common in females than in males from Lahore, Pakistan (Abdullah et al. 1992). In Rawalpindi, Pakistan HCV seropositivity was detected in 60% of chronic active hepatitis cases 16.6% cirrhosis and only 6.6% in HCC (Tariq et al. 1999).

Seropositivity for anti HCV antibodies in chronic liver disease has shown similar drift for Hazara region to other areas of Pakistan with almost equal numbers of male and

female patients (Zuberi 1995). However, only few studies reported HCV in Pakistani blood donors where higher infected population is contributed from southern Pakistan. This trend is similar to that for other countries of the region but higher than that for western countries. Hence, ALT level is not a useful marker for HCV detection in the region (Kakepoto et al. 1996). On the contrary, ALT being available in higher concentrations in the cytosol of hepatocyte than other parts of liver, was thought to be more specific for hepatic injury. Although some scientists suggest that annual screening of healthy, asymptomatic patients for liver disease using ALT and Aspartate amino transferases (AST) levels is not useful (Yano et al. 2001) but their level less than five times upper limit of normal, i.e., upto 250 U/L and are common for primary care medicine. However, the range of possible etiologies at this level is broader thereby making tests less specific (Davern and Scharschmidt 2002, 1227 and Pratt and Kaplan 2000). ALT and AST levels may rise as high as 2000 U/L as in cases of hepatic injury, necrosis related drugs, toxins, ischemia and hepatitis. Moreover, it is also obvious that patients having normal ALT and AST levels can have significant liver disease with chronic hepatocyte injury (e.g., cirrhosis, HCV).

2.10 ASSOCIATION OF GENOTYPES WITH SPECIFIC RISK FACTORS

In France and in Bahia, genotypes 1b and 3a have been reported, i.e., HCV 1b is found among patients having a previous blood transfusion (Nousbaum et al. 1995) and HCV 3a is mainly associated IVDUs. Thus, understanding to the effects of genotypes on course of the disease, as well as their peculiarities at regional level is of utmost importance (Pawlotsky et al. 1996 and Codes et al. 2005).

Different routes of transmission have been suggested to be related to different types of virus and more than one route of transmission had already been reported in majority of HCV patients (Idrees and Riazuddin 2008). Moreover, for the spread of genotype 3a, use of non-disposable syringes for multiple patients by doctors and vaccinators before 1990s is main culprit. Although such practices have been eradicated from most parts of the country but it still exists in rural areas of the country. It has been estimated that more than 86% of HCV patients in Pakistan have received multiple

injections. For other genotypes of the country, i.e., 1a and 1b, major routes of transmission were found to be surgeries and dental procedures.

Another exemplary study suggested genotype 1 patients of hungry had been hospitalised for major/minor surgery, dental procedures and shaving by barbers. It was further observed that among genotype 1 infected patients, 95% contained subtype 1b and had blood transfusion and had received blood products (Jarvis et al. 1996a, b). On the other hand, no apparent cause of spread was reported for genotype 2a. Similarly for untypable genotypes, means of diffusion is still not clear (Idrees and Riazuddin 2008).

It was estimated that in Pakistan use of injection is very frequent and more than nine injections are used per person per year. This is one of the highest numbers in the world and many patients (upto 49%) are injected at their very first outpatient visit (Khan 2000 and Jafri et al. 2001). In addition, the matter of concern is not only the redundant use of injections but also the insecure injecting procedures. Similarly, for vaccination safe practices are compromised in many areas including the use of already used syringes (Idrees et al. 2008). A survey conducted by Ministry of Health, Pakistan, it had been argued that for more than 72% therapeutic injections and 50% immunization injections in public health-care facilities are unsafe and potentially dangerous (Ministry of Health Pakistan 2002). Furthermore, use of multiple-dose vials is regular practice in various government and private sector hospitals of Pakistan providing major threat spread of HCV infection particularly genotype 3a (Khan 2000, Jafri et al. 2001 and Ministry of Health Pakistan 2002).

In Venezuela, infection of HCV genotype 1b is associated with transfusion than other types from area. HCV genotype 2 is observed to have replaced the existing subtype 1b in a fairly short period, with no increase in ocuurance of genotype 3. Intravenous drug usage is relatively less common practice in the area supporting low frequency of genotype 3 (Flor and Carmen 2007). In Kabul, the prevalence of HCV and high-risk behaviour are frighteningly high. The reasons suggested for this increase include political instability, poverty, mobility, and low literacy that eventually lead to susceptibility of IDUs to HIV and other blood borne or sexually transmitted infections (Hankins et al. 2002).

Intra-household spread of HCV infection, in Pakistan was studied by Akhtar and Moatter, (2007). The prevalence of antibodies to hepatitis C virus (anti- HCV) in young healthy Pakistani adults in recent studies carried out in cross section of population has ranged from 2.3 to 5.3%. (Zakaria et al. 2003, Khokhar et al. 2004a, Khokhar et al. 2004b and Farooq et al. 2005). Population group belonging to Karachi, Southern Punjab and Central Punjab had frequency of anti HCV in the range of 2.2 to 3.3%, whereas in the north, it was 5.3% (Zakaria et al. 2003, Khokhar et al. 2004a, Khokhar et al. 2004b, Farooq et al. 2005 and Mirza et al. 2007).

2.11 EPIDEMIOLOGICAL CHARACTERISTICS OF HCV

2.11.1 Prevalence of HCV

Prevalence of HCV varies from area to area globally. The lowest prevalence is reported from Northern European countries and highest prevalence is in Egypt.

2.11.1.1 Africa: The overall prevalence of HCV infection in Africa is 5.3%. However, within Africa the infection varies from Rawanda's 17% rate, followed by Burundi at 11.1%, Guinea at 10.7%, and Zimbabwe at 7.7%. On the low range Ghana and Ethopia has a low infection rate of 2.8% and 0.8% respectively (Frommel et al. 1993, WHO 1999c and Madhava et al. 2002).

2.11.1.2 South and Central America and Mexico: Bolivia has the highest HCV infection of 11.2%, Suriname is at 5.5% and Trinidad and Tobago are at 4.9%. On the lower range, the Dominican Republic has a rate of 2.4%, Peru is at 1.6% and the Mexico has a low 0.7% prevalence (Hyams et al. 1992, Islas et al. 1994 and WHO 1999c).

2.11.1.3 Middle East and Southern Asia: In the Eastern Mediterranean region, the overall prevalence is 4.6%, second only to Africa for regional infection rates. Egypt has the region's highest rate, with 18.1% of the population infected with HCV. Kuwait has a 3.3% occurence, Saudi Arabia 1.8% and in morocco HCV reported prevalence rate is 1.1% (Al-Naseer 1992, Al-Faleh et al. 1997, Medhat et al. 2002 and Denis et al. 2005).

2.11.1.4 Europe: Europe has an overall 1.03% prevalence of HCV. On the high end, in Romania the percentage is 4.5% while Russia has a 2% rate and Turkey and Greece both report a prevalence of 1.5%. The UK has a 0.02% rate and does Finland and Denmark, Germany 0.6% (Shepard et al. 2005), France 1.1% (Desenclos 2000), Italy 2.2% (Puro et al. 1995).

2.11.1.5 Asia and South Pacific: South East Asia has an over all prevalence rate of 2.15%. Thailand's rate of HCV infection is 5.6%, Vietnam 6.1%, Cambodia 4.1%, and Nepal at 0.6%. Mongolia has the highest prevalence rate in Asia with 10.7% prevalence. Japan has a 2.3% prevalence rate (Ito et al. 1991, Hayashi et al. 1994 and Kao and Chen 2000). China describes one fifth of the world population, has a reported seroprevalence of 3.2-4% (Shepard et al. 2005). In addition, India where one fifth of the world's population lives, one community based survey reported an overall HCV infection rate of 1.8% (Medhat et al. 2002). Data available for seroprevalence in Pakistan, reports rates range between 2.4% and 6.5% (Mujeeb et al. 2000, Sultana et al. 2000 and Khattak et al. 2002).

2.11.1.6 Australia and Newzealand: lowest prevalence There are reports that Australia and New Zealand has HCV infection rate of 1.1% and 0.3% respectively (Law et al. 2003). HCV transmission in those countries occurs primarily from the use of improperly sterilized needles and syringes and IDU.

2.11.1.7 North America: The incidence of chronic HCV infection in the United States is 1.8% (Alter et al. 1999) while Canada's rate of infection is estimated at 0.1%-0.8% (Zou et al. 2000; PKID's online 2003).

2.11.2 Incidents and Trends in HCV Infection

National survey was conducted by CDC, USA from 1988 to 1994 to provide national representative seroprevalence estimates for HCV (Alter et al. 1990 and Alter et al. 1999). It was observed that before 1965 incidence rates were low (0-44 per 100,000) followed by the era of increasing rates from 1965 to 1980. The period of high occurance started in 1980's when 100-200 cases on average per 100,000 were reported (Armstrong et al. 2000). A model to estimate HCV load in France used death rates from HCC and

cross-sectional seroprevalence studies. A similar trend of increasing incidence through the 1980's was observed (Deuffic et al. 1999). Another attempt recorded to model disease burden in Australia showed a steady boost in new HCV infections from 1961 to 2001 (Law et al. 2001). Conversely, a sharp and steady drop in HCV incidence was observed in USA through the 1990's (Alter 1997). Similar trend was observed for fresh HCV infections in Italy in 1990's (Shepard et al. 2005). In 2004, it was stated by Scotland's health minister that "HCV is one of the most serious and significant public health risks of our generations". The number of HCV diagnosed cases admitted to hospital with very first appearance of cirrhosis in Scotland augmented from 171 (during 1996-1998) to 209 (during 1999-2001). It indicates increase in HCV related diseases lumber on health care resources (Hutchinson et al. 2006). Differences in the 1990's incident trends not withstanding, all published models predict rise in incidents of HCV related Sequelae respective countries in near future even in hyper endemic areas (Alter 2007).

2.12 COMMON MODES OF TRANSMISSION OF HCV

HCV mainly transmits by three different routs, namely, percutaneous, non-percutaneous and sporadic. The non-percutaneous transmission may represent occult percutaneous exposure (Murphy et al. 2000). Non-transfusion related HCV infection now is of increasing importance and injection drug use is now the identified risk factor in more then 60% of cases (Alter 2002). It has been observed that 10% to 40% infected individuals have no identifiable risk factors (WHO 2001a, WHO 2001b and WHO 2001c).

2.12.1 Percutanious Transmission

Percutanious mode of transmission involves the following major routes of HCV dispersal i.e. blood transfusion, injection drug use, chronic haemodialysis and needle stick injuries/ unsafe therapeutic injections.

2.12.1.1 Blood Transfusion: The universal rates among blood donors diagnosed with HCV range between 0.4 to 19.2 % providing an indication towards high prevalence (46-90%) of HCV for Haemophilics (Memon and Memon 2002). In Japan, United States,

England, Italy and Lebanon, this rate was observed to drop after having a transfusion during 1989-2002 (Japanese red cross non-A, non-B hepatitis research group 1991, Donahue et al. 1992, Irani-Hakime et al. 2001, La Torre et al. 2002 and Theodore and Mazen 2006). It was reported from Brazil that in decade extending 1991-2001, there was considerable decrease in acquiring the infection after blood transfusion. However it was still ten times higher than that of developed countries, i.e., lowest risk was found to be in ratio of 1:13721 (Kupek 2001). In India transfusion related HCV rates vary from area to area, (Gupta and Kaur 2002 and Thakral et al. 2006).

Similarly, wide spread risk factor for HCV was blood transfusion (54%) when compared to another noteworthy factor, IVDU which was calculated only 5% (Soza et al. 2004). Further it has been reported that HCV prevalence in healthy blood donors from Peshawar, Pakistan is 2.2%. In other transfusion centres the higher rates 2.57% for anti HCV Antibody (Ahmad et al. 2004a, Ahmad et al. 2004b and Fan et al. 2004). In the areas of the world, where HCV test for blood donors is a regular practice, the infection is supposed to have eradicated (Busch et al. 2005). However, it is still an addressable issue with respect to HCV infection in other parts of the world. Use of commercial donors by some countries for blood supply is not considered safe particularly when not screened. (Hladik et al. 2006). People with transfusion-associated HCV infection are more likely to develop liver decompensation than those infected in other ways (even after controlling duration of infection and age at infection) (Gordon et al. 1998 and Kiyosawa et al. 1990).

2.12.1.2 Injection Drug Use: The predominant mode of drug administration during past forty years in USA and Australia is through injection and is now observed in many countries of Europe (Alter 2007). Although rate of infection was observed to be declining in first 2-3 years of injecting from 80% to 30% in the decade extending late 1980s to late 1990s but annual incidence rate ranges from 15% to more than 30% for new abusers (Des Jarlais et al. 2003). Sharing is observed to be more pronounced and direct reason for HCV spread than for other blood borne viruses. Other indirect practices include back loading, sharing cotton, cooker and rinse water (Thorpe et al. 2002 and Murray et al. 2003). HCV in Chienes heroine users was 72% (Garten et al. 2004). Iran showed that 31.5% IVDA and 20.1% non IVDA had a high prevalence of blood borne disease in

Iranian prisoners (Alizadeh et al. 2005). In Taiwan 2004 to 2005 anti HCV positivity among injecting heroin abusers was 89.9% at a male prison (Liao et al. 2006).

The frequency of HCV in IDUs is 48% to 90%. Although the risk factors for HBV infection and HIV coinfection overlap with those for HCV, the prevalence of HCV is higher (Nyamathi et al. 2002). The risk has become alarming for those who are away from their houses or don't have one. They apply unhygienic procedures for meeting their demand of addiction as for example sharing injections, use of injections non-sterile open places, excessive needle usage etc (Lovell 2002). On the other hand, positivity of HCV test made these patients to change their mode of life and shift towards comparably safe practices. Also it was observed that homeless people delegate duty for preventing HCV spread to their peers, especially when using syringes with others (Elizabeth et al. 2009). Over all, It is encouraging to know that awareness among common people about spread of infection upon sharing the equipment have made them vigilant. Thus it has become an important duty to emphasize on health promotion issue in this context (Wright et al. 2004).

2.12.1.3 Chronic Haemodialysis: It had been associated with cases of occasional sporadic outbreaks of HCV infection. The frequency of anti HCV in patients on haemodialysis ranges from 10% to 20% (Murphy et al. 2000). A correlation has been found between increasing years on dialysis and anti-HCV positivity, suggesting that HCV is transmitted in dialysis units by inadequate infection control procedures (Alter 2002).

2.12.1.4 Needle Stick Injuries and Unsafe Therapeutic Injections: According to WHO report (1999a, b), annually 10 million injections are used around the world, of which 5% are used for vaccinations and rest for curative purposes. Besides the use of sterile needle and syringes (Drucker et al. 2001), an estimate suggests that due to such practices, every year 0.6 million HIV, 4.7 million HBV and 16 million HCV infections are acquired (Simonsen et al. 1999). This fact relates the major contribution in HCV progression is by unsafe therapeutic injection practiced not only by health care professional but also non professionals and this accounts for upto 2 million (40%) infections world wide (Hauri et

al. 2004 and Frank et al. 2000). Moreover, lack of sterile conditions for equipment used for dental and other surgeries in hospitals may also be a source of infection transmission. It was reported by Todd (2007) that in Afghanistan, HCV patients were found to obtain injections from non-medical suppliers and this fact was linked to high rate of HCV occurance in Pakistan. HCV prevalence and its associated risks were found to be frighteningly high and reasons proposed for this include political instability, poverty, mobility, and low literacy rates (Khan et al. 2000; Hankin et al. 2002).

2.12.2. Non Percutaneous Transmission

Transmission of HCV via non percutaneous routes involves transmission between sexual partners/ spouses, transmission between monogamous couples, transmission among homosexual gay men, sporadic HCV infection, homosexual transmission and heterosexual transmission and intrafamilial/ other than sexual transmission.

2.12.2.1 Transmission between Sexual Partners/ Spouses: Arif et al. (1996) investigated the intrafamilial transmission of HCV and its associated risk factors for Saudi population, in house hold and in blood donors. Although this has not been found related to transmission of HCV in the Saudi community and it was strongly agued in favour of sexual transmission. It was revealed in case control studies during 1979s an 1980s, in the United States that heterosexual activities may also serve as an independent source of HCV spread (Alter et al. 1989). From that time onwards, of all acute cases reported, 15-20% are infected due to sexual practices. However, another study where monogamous relationships whether homosexual or heterosexual, were maintained for long time with a partner having chronic HCV did not provide strong support for sexual exposure being a risk factor for spread of HCV (CDC 1998a and Terrault 2002). One possible justification for this contrary could be the requirement of sexual intercourse for the successful transmission of infection provided the infected partner is in early phase of acute infection, i.e., antibodies are not produced in sufficient amounts to complex the viron yet. Such early studies were conducted when infection was at its height and a great number of adults had multiple sex partners (Alter et al. 1999). Thus there was a probability to have sex with an infected partner and in turn getting infection. This also describe unequal amount of HCV burden posed owing to sexual activity and suggest

inefficiency of this mode of transmission. Another report on similar activity from Italy favours the discussion above (Mele et al. 1999), which is further supported by the works of European scientist in this direction particularly acute HCV among HIV infected male homosexuals (Browne et al. 2004, Gambotti et al. 2005, Gotz et al. 2005 and Rauch et al. 2006).

It was also observed that the risk of female partner getting HCV infection increases 6% in cases where male partner is co infected with HIV (Hisada et al. 2003 and Yasmeen et al. 2009). However, the chances for a man acquiring infection from HCV infected female partner is similar if this was a case of homosexual relationship with one partner being acquiring infection from other. Thus it agrees that HCV transmission by sexual activities would be a rare incident (Thomas et al. 1995). Thus, in this case the chances for a female to get infected from her male partner are more than for a man getting infection from female partner (Browne et al. 2004), i.e., a study conducted for 10-15 years reported none of 94 males acquired HCV infection from 86 female HCV positive partners (Meisel et al. 1995). Other studies report that there is 0.2% risk for male partners becoming HCV positive in 17 years that suggest 1 main 10,000 infection per year (Guiliani et al. 1997). Thus sexual transmission of HCV may occur and sexual partners are at risk of getting infected. It may be due to low amounts of HCV RNA in semen and vaginal secretions of HCV patients (Liou et al. 1992).

Available evidence indicates that in contrast to percutaneous modes of transmission, transmission by non-percutaneous routs is inefficient (Marco 2000). In addition, only 10% of patients with acute HCV had sexual exposure to HCV infected partner. Most sero-epidemiological studies demonstrated small number of infected people having sexual contacts possessed anti HCV (Alter 2007). More than 3% females having haemophiliac male partners contained anti HCV in their serum (Itskowitz 2007). Partners of HCV positive homosexual males and heterosexual individuals have increased tendency to develop infection (Bernard 2002). Overall, sexual partners of the index patients with anti-HCV have rates of HCV infection ranging from 0% to 7%. Sexual partners of low risk HCV anti HCV positive persons without liver disease and without high risk behaviour e.g. injection drug use or sexual activity with multiple partners, have a

frequency of HCV ranging from 0% to 7% (Sciacca et al. 2001). By contrast sexual partners of the persons with liver disease or with high risk sexual behaviour (or both) and whose partners may themselves participate in high risk behaviour-have anti HCV frequency rates of 11% to 27% (Terrault 2002).

Needed studies include evaluation of long term steady partners as well as testing of persons with high-risk sexual practices, including those with sexually transmitted diseases that might promote HCV transmission through increased level of viremia or breakdown of mucosal barriers (CDC 1998b). Although the effectiveness of HCV spread via sexual contact remains controversial, HCV infected persons commonly are counselled to advise current spouse for their infection status (Alvarado-Ramy et al. 2001). Although patients are normally informed that the risk of sexual transmission is low and precautions in stable relationship should be encouraged. Such precautionary measures are generally advised for polygamous relationship (McGee et al. 2008).

A few studies observed that sexual transmission of HCV occurs occasionally among spouses as reported from Egypt, i.e., infection attained from wife to husband was 34% and from husband to wife transmission was estimated at 3%, and an overall HCV transmission rate between sexual partners was 6% (Magder et al. 2005). In Italy the same genotype 1b was found, in 8 of 13 couples, with both partners HCV RNA positive (Tahan et al. 2005 and Stroffolini et al. 2001). Vandelli et al. (2004) followed selected couples for a 10 years period prospectively. These spouses became anti-HCV positive after 7, 8 and 9 years respectively.

In urban Chennai, Southern India, women with genital ulcer were prone to HCV infection. For men infected with Herpes simplex virus 2 (HSV-2), or homosexual had increased risk of becoming HCV positive (Marx et al. 2003). In Brazil 1992 to 1996, of studied blood donors with HCV infection, one third had regular partners for at-least 6 months (Tengen et al. 2001). HCV associated antibodies and HCV RNA were detected in only 1.88% of the spouses in Bangkok, Thailand, (Boonyarad et al. 2003). The interspousal transmission of HCV infection was estimated 0.23% per year, suggesting its low incidence (Kao et al. 2000).

2.12.2.2 Transmission among Monogamous Couples: It has been observed from various studies that risk of acquiring HCV infection is very low (0-3%) for those having heterosexual relationship for long (Highleyman 2006). When partner or spouse is HIV negative, the risk of becoming HCV positive ranges from 1 in 1000 to 1 in 10,000. Antibodies were found in 27% of patient's spouses and 18% were found to be HCV positive (Akahane et al. 1994). Acute HCV was transmitted most probably by sexual intercourse from a 72 years old man to 63 years old wife after a 42 year marriage in 2001. HCV genotype 1b was concordant with the husband (Yagura et al. 2002).

In a study from Islamabad, Pakistan, suggested the risk of sexual transmission to be low (Irfan and Arfeen 2004). In one study in which 24 couples were diagnosed with anti HCV, 22 had matching viral subtypes. This support the hypothesis of transmission between these couples (Cavalheiro 2004). Since HCV was transmitted from a chronic carrier to his female partner, the phylogenetic tree analysis 97% homology is strongly in favour with viral transmission during sexual intercourse (Halfon et al. 2001).

2.12.2.3 Transmission among HIV Positive Gay Men: A study reported more than 200 cases of HCV for homosexual males from London and Brighton, from October 2002 to August 2005. The men who contracted HCV were polygamous having three times more sex partners (30 vs. 10) than men who remained HCV negative (Carter 2009). Other important risk factors included sex abuse, unprotected receptive and insertive anal intercourse, fisting, use of sex toys, group sex, and sexual activity under the influence of recreational drugs (92% vs. 62%). These factors appeared to in individuals practicing three or four of these practices in group sex settings and had 23 times high chance to get infected (Rooney and Gilson 1998). In addition, most such partners (92%) had simultaneous sexually transmitted diseases (STDs) were found in sex clubs, bathhouses and through internet. High-risk and mucosal traumatic sexual factors are significantly associated with the recent transmission of HCV. Both fisting and STD causing genital ulcers was found to be associated with HCV. Moreover, HIV infection and extreme sexual techniques may smooth the progress of sexual transmission of HCV (Schmidt 2009).

2.12.2.4 Vertical Transmission: Although the rate of vertical transmission is increased for HIV positive mothers, studies suggest that for women in whom HIV disease is controlled with use of high activity antiretroviral therapy (HAART). In such cases, risk of HCV transmission to the newborn is analogous to that for HIV negative women. Data regarding the risk associated with vaginal delivery as opposed to caesarean section cannot be recommended in HCV infected women. There are negligible chances of HCV transmission to infant from breastfeeding (Murphy et al. 2000, Conte et al. 2000 and Alter 2002). However, more work is required in this direction to reveal related information for example time of prenatal infection (*in utero*), significance of breast feeding transmission in new born, and history of infection in offspring (PKID's online 2003, Zanetti and Tanzi 1995, Romano et al. 1994, Kuroki et al. 1991 and Weintrub et al. 1991). Risk of HCV transmission is increased when mother has active infection and the rate of infection and maternal viremia are found to be correlated (Kuroki et al. 1991 and Ohto et al. 1994).

HCV infections acquired at birth or in early childhood usually progress slowly, but these infections are not always mild (Casiraghi 2004, Guido 2003 and Jara 2003). Mother to child transmission is a low efficiency means of the spread of virus, occurring in <5% of the babies born to HCV infected mothers. A study from Taiwan showed that risk of transmission of infection is less in electric caesarean sections as compared spontaneous delivery in emergency caesarean sections (Boucher et al. 2000). Similarly, spread of HCV by breast milk has been recommended but certainly not proven (Memon and Memon 2002). Parker et al. (1999) estimated the seroprevalence of HCV antibody in the dried blood spot samples in Lahore, Pakistan. In women of child bearing age, seroprevalence of HCV antibodies was to be 6.7%, and in children, to be atleast 1.3%. The diffusion of infection from mother to child is estimated between 0% and 10% and is suggested very low (Terrault 2002).

Ades et al. (2000) estimated the prevalence of HCV to be 0.1% each year in pregnant women in UK. The infection is found to occur only when HCV RNA is obvious in maternal serum at the time of delivery with the rate being 4% to 7% per pregnancy.

The frequency of anti HCV positivity in pregnant women ranges from 0.7% to 4.4% (Murphy et al. 2000, Alter 2002, Terrault 2002 and Conte et al. 2000), with a rate of HCV RNA detectability in these HCV positive women of 65% to 72%. Although the reports on whether virus concentration is an important parameter are in conflict to eachother, HCV spread is related to elevated level, i.e., above 10^6 copies per ml (Roberts and Yeung 2002). Symptoms associated with perinatal infections include prolonged labour after membrane rupture and internal foetal monitoring (Mast et al. 2005). Rate of HCV transmission increases 4 to 5 times in case of HIV co infection but no such link has been observed with vaginal delivery, caesarean section or breastfeeding.

2.12.3. Sporadic HCV Infection

Source of infection for sporadic transmission is not known and is transmitted by means other than blood or blood products, reported to occur in about 10% of acute HCV cases, and 30% in chronic cases. No identifiable risk factors could be recognized but in 50% cases contaminated blood was observed and are known as sporadic, or community acquired infections (Balasekaran et al. 1999). These infections may occur through introduction of virus to injury like cuts, wounds, or by medical procedures (Moradpour et al. 2001). It was observed that 18.1% of the Egyptian residents from rural area and 22.1% of army recruits were anti HCV positive. In this case sporadic transmission was found among a great number of patients and the trend was observed to be increasing with age (Memon and Memon 2002).

The rate of sporadic transmission of HCV infection was observed to be very high in developing countries of Asia and Africa than other parts of world, owing to the lack of facilities (Kao et al. 1994). However, the potency of disease related to sporadic infection when compared to parenteral route is not known. It was further argued that severity of disease is higher for patients having transfusion history than sporadically infected people showing slow progression (Kiyosawa et al. 1990 and Alter et al. 1992). But accurate detection of time of infection is not yet possible and may serve as an important parameter to find severity of the disease (Myron 1995).

2.12.3.1 Homosexual Transmission: The incidence for positive anti-HCV was observed to be high (18.9%) among healthy homosexuals from Italian gay clubs and this accounts for 15 times more incidence rates for the whole population from same region provided exclusion of intra venous drug usage. Statistically high degree correlation was found for intercourse and prevelance of infection (Melbye et al. 1990 and Mele et al. 1999). Similar trend was observed for homosexual males from Australia with incident rate of 34.15% (Shepard et al. 2005).

2.12.3.2 Heterosexual Transmission: It has already been mentioned that HCV is transmitted between sexual partners and the rate of incidence is found to be higher for women seeking infection from their anti HCV positive spouse (58%) than men of HCV positive women (44%). A number of studies also favour spouse-to-spouse infection rate on average at 20%. However, there are number of studies that favour female-male transmission s while others do not (Zein 2000). Thus, relationships (homosexual or heterosexual) may serve as an important risk factor for HCV spread (Shepard et al. 2005).

2.12.3.3 Intrafamilial, other than Heterosexual Transmission: The non sexual household transmission of HCV has also been observed due to sharing of tooth brushes, razor and nails cutter and files (Caporaso et al. 1998). Although no correlation was found for intrafamilial transmission, risk raised 7.5 folds when marital relationship were observed for more than 20 years (Guadagnino et al. 1998). Sharing hygienic utensils has a much higher correlation of possible route of transmission, better explained by the sequence homology data than by the other associated risk factors which is difficult to infer (Cavalheiro 2004). The calculated risk for transmission among relatives and children is found to be 2.1% and 2.3% respectively. The risk among household contacts was not as high as expected (1.33%) (Minola et al. 2006 and Hajiani et al. 2006). Similarly, occurrence of HCV in thalesemia patients (20.5%) from Pakistan was found to be higher than expected (16%) where HCV prevelance in general population was 6.5% (Pasha et al. 1999 and Akhtar et al. 2002). Mastromatteo et al. (1996) confirms a threat of HCV infection between relatives of the index cases, in particular spouses (5.7%). HCV RNA was detected in saliva in serum PCR negative patients. Later non sexual

intrafamilial transmission of HCV was argued as non significant mean of spread and sexual transmission was set responsible (Hajiani et al. 2006).

Infection was found to be transmitted directly from mother to newborn and in such cases children are found to have high levels of anti HCV in their blood (Jafri et al. 2006 and Newman 1996). It is not recommended by CDC to change a long term relationship with one sexual partner. Higher risk has been observed for people with acute illness and those having more than one sex partner. Precautions like use of condoms are encouraged to avoid HCV and other STDs (WHO 2001a). Similarly, sex should be avoided during menstruation, with HCV positive immuno-compromised partner and with those partners having other infections (WHO 2001b).

2.12.3.4 Transmission of HCV via Tears, Saliva, Urine and other Body Fluids: Fried et al. (1999), analysed the presence of HCV antibody in saliva and semen of chronic HCV patients and reported the HCV RNA in these body fluids, suggested the rare multiple exposure of HCV to chronic carriers of disease and may justify occasional spread of disease by sexual or close physical contact. However, sporadic transmission of HCV is higher than parenteral origin thereby providing saliva as possible biological source but not a route for infection (Fleruy et al. 1993).

Hadeed et al. (1992) confirmed the presence of HCV RNA in saliva of half of the patients with acute and chronic infections. No such evidence is available for breast feeding related spread but infected mothers should avoid breast-feeding particularly when breast nipples are cracked or bleeding. The occurrence of HCV in saliva has already been investigated but it is still a controversial issue (Takamatsu et al. 1990, Fried et al. 1992, Hadeed et al. 1992 and Young et al. 1993).

In a study, 61% of cases were positive for HCV RNA from saliva and six HCV genotypes from saliva were later reported, i.e., genotype 1, 1a, 1b, 2a, 3 and 4. Chiron assay was suggested to be the best optimum technique for HCV identification in saliva. Besides, some patients immuned with HCV showed complete absence of viremia and RNA from saliva (Mariette et al. 1995). It is still not known that whether HCV infects

salivary epithelial cells but it may cause lymphocytic sialadenitis in some patients. Oropharynx may not be the site of virus replication or latency as high level of viremia is prerequisite for detecting the infection from saliva (Alter 2007). In fact, the only possible explanation to this could be mixing of plasma with saliva providing a role in horizontal spread of virus for which mechanism is still not known (Xavier et al. 1995).

The finding of HCV RNA in human secretions like saliva, seminal fluid, urine and bile has been described this suggested easily obtainable fecal specimens to be analysed (Hsu et al. 1991, Liou et al. 1992, Couzigou et al. 1993, Chen et al. 1995, Kuan et al. 1997 and Boom et al. 2000). Beld et al. (2000) reported presence and quantitation of HCV RNA in feces of chronically cases. It was later found that feces of chronic patients contain relatively large amounts of HCV RNA suggesting high rate for RNA recovery and that the inhibition of reverse transcription and amplification was not present. The significance in this context is to be revealed yet and several mechanisms could attribute to the detection of HCV RNA in feces. Blood in feces may be obvious and can be tested with freshly obtained fecal specimens by tests for occult blood. Extrahepatocellular HCV replication in lymphocytes was described (Lerat et al. 1993) and may add to availability of HCV RNA in feaces. Leakage of virions in body fluids by liver damage may also occur, and detection of HCV RNA in biliary epithelial cells (Aria et al. 1993) and the presence of HCV RNA in bile from 80% seropositive patients (Kuan et al. 1997) have recently been described. Therefore, bile and feces are human secretions in which the virus is possibly excreted. Whether HCV was excreted in an infectious form remains to be addressed.

The presence of seminal HCV RNA has been reported from 1/3rd of HCV positive men (Leruez-Ville et al. 2000). These low and positive levels explain incidence for infection in sexual partners of patients with HCV is scarce. Since there are no animal models for HCV studies except human and chimpanzees, sexual intercourse cannot be suggested main cause of transmission (Chayama et al. 1995). Thus, possiblility that hepatitis C virus may spread via sexual interaction, the rate of transmission is low due to l lower amounts of virus in semen. HCV has also been reported from cervical smears (Manavi et al. 1999). Potential role for tears in HCV spread was studied and it was found

that the presence is independent of severity of disease. Different genotypes were observed from tears and serum but in other patient HCV was present in tear while absent from serum suggesting the role of tears in transmission. However, not much is known in this regard (Mendel et al. 1997).

2.12.3.5 Unknown Way of Transmission: For about 10-30% of the cases source of infection is not reported. Such "sporadic" HCV infection probably results from occult to unidentified percutaneous routes. Similarly how infection was acquired is not obvious for 40% chronic HCV patients. In order to find out the reason behind the context, various social practices like personal equipment sharing and sexual practices are under investigation (Ward et al. 2000).

2.12.3.6 Intranasal Coccaine Use: Another possible route for HCV transmission could be snorting, i.e., intranasal coccaine usage (Murphy et al. 2000 and Alter 2002). Although coccain itself is not an approved cause of HCV but the social behaviours of users are observed to make them more susceptible to infection as for example, sharing of snorting straw.Health issues of female sex labour of London were found linked to growing use of "crack" cocaine (Ward et al. 2000). The crack has been found to be less challenging for medical management of disease and its ever growing usage is also found to be related to HCV, sexually transmitted infections (STI), and spontaneous abortions. In addition, prostitution and drug abuse by injection may also be reasoned for spread of infection (Faruque et al. 1996 and Green et al. 2000).

2.12.3.7 Tattooing: HCV infection probably acquired from tattooing and body piercing. Transmission by these practices was more common in the past, when less attention was given to the aseptic techniques. Although tattooing was proposed as a risk factor for HCV infection in epidemiological studies, not enough eveidence is available in this regard (Abildgaard and Peterslund 1991, Thompso et al. 1996, Sun et al. 1996, Murphy et al. 2000 and Alter 2002). The human behaviors involving blood to blood contact and correctional facilities are involved in HCV spread (Thompson et al. 1996 and Haber et al. 1999). Seroprevalence of HCV among those imprisoned is observed high but not sufficient reports are available in this direction (Dolan et al. 1998, Vlahov et al. 1993,

Rosen 1997 and Rosen 2000). Jeffrey et al. (2001) found removal of acute HCV infection in a prisoner after tattooing. However, tattoo equipment sharing is common practice in jails and was therefore subjected to consideration and later categorized under risk factors for HCV spread (Thompson et al. 1996). Tsang et al. (2001) showed that a patient received a tattoo in mid June in 1998 and became anti HCV positive in August 1998 in a prison.

2.12.3.8 Iatrogenic Transmission of HCV: The rate of HCV occurrence is a variable parameter in various areas of the world and even in different dialysis centres of the same country. In Brazil 82% prevalence was reported being the highest in the world whereas 4% prevalence was reported from Europe as lowest figure (Memon and Memon 2002). In Saudi Arabia, HCV among haemodialysis patients was reported higher (9.24%) than blood donors (0.30%) (Qadi et al. 2004). Similarly, anti HCV positivity was found to be was 6.7% for Saudis, again more than for Mexican population (1.2%) (Mendez-Sanchez et al. 2004). Moreover, quite sound rates (1.38 to 1.9% per year) for disease occurrence have been reported in haemodialysis patients when no other risk factor taken into account (Halfon et al. 1998 and Fabrizi et al. 1999).

The prevalence of HCV was observed to decrease considerably in Belgian cohort from 1999 to 2000, i.e., 13.5% to 6.8% respectively. Similar results were observed for another cohort comprising of haemodialysis patients with reduction rated as in France 42% to 30%, in Sweden 19% to 16%, in Italy 28% to 16%, in UK 7% to 3% and in Hungary 26% to 15%. However reverse was observed when similar study was conducted in Germany (7-6%), Spain (5-12%) and Poland (42-45%) (Jadoul et al. 2004). Haemodialysis patients (55.9%) and thalassemia sufferers (63.8%) were found more susceptible to HCV infection in Rasht, North Iran, as compared to 0.5% prevalence among blood donors (Ansar and Kooloobandi 2002).

Iatrogenic transmission of HCV is well documented in a variety of circumstances, including delivery of anti schistosomal treatment with reusable syringes in Egypt (Frank et al. 2000). Mini or single donor unit blood transfusion to multiple infant recipients, used in many countries in the past has been implicated in the transmission of HCV (Casiraghi

et al. 2004). Often recipients are unaware of the history of transfusion when, decades later, they are asked about HCV exposure. It is also likely that until the implementation of universal precautions in medical practices, HCV was transmitted by inadequately sterilized multiuse instruments and multi-dose vials of vaccines and topical anesthetics. The frequent lack of symptoms in patients with acute HCV infection confounded the identification of these occult parenteral modes of acquiring HCV infection.

From epidemiological data, a huge assortment of human activities and other addressable means of transmission of HCV infection were identified (Alter 2007 and Thompson et al. 2008). For example cosmetic procedures like tattooing and body-piercing, drug snorting, religious rituals and cultural practices. However not much is available to designate involvement of these risk factors in spreading of disease (Alter 2002 and Hwang et al. 2006). During the period 1978-1979, 2533 females reported for being infected due to reception of virus-contaminated anti-D immunoglobulin. It is still an important risk for intrauterine or perinatal transmission of HCV (Helga and Angela 1995).

2.12.3.9 Occupationally Acquired HCV: Under this category, health care professionals who employ carelessness during medical practices were grouped. The sero conversion from HCV positive source was averaged at 1.8% provided transmission of infection was linked to the use of hollow bore needles and deep injuries (Yazdanpanah et al. 2005). Transfer of infection via mucosal membrane to blood was considered a rare event and no such transfer was observed from intact skin areas (Beltrami et al. 2003). The health care professionals at higher risk of gaining infection include orthopaedics, surgeons and dental practitioners. However, transfer of infection from practitioner to patient has been observed very low (0-5% on average) (Zuckerman et al. 1994).

2.12.3.9.1 Transfer from patient to practitioner: Hepatitis is attributed a blood borne disease that is communicable easily providing risk not only to related professional but also to other patients around. Primary route for direct percutaneous spread of infection among health car professionals and patients is evidenced in literature (David 2003, Ray 2002 and Thomas and Seeff 2005). Blood is referred to as a main route for transmission

as for other body fluids calculated HCV titre is significantly low (Astagneau et al. 2002). Longitudinal studies on health care staff having contaminated needle prick suggest anti HCV seroconversion rates of 0% to 4%, which is in coincidence to what was observed for general population (Alter 1997 and Murphy et al. 2000).

2.12.3.9.2 Patient to Patient Transmission: Infection outbreaks during transfusion received by renal failure patients have raised the risk of patient to patient spread. There are cases not supporting this mode of transmission (Tokars et al. 2002). In some of these cases cross contamination by non sterile equipment usage and lack of care, was taken into account in part for possible mean of gaining infection (Astagneau et al. 2002 and Sivapalasingam et al. 2002). Patient to patient spread has been suggested to be due to environmental contamination, contaminated dialysis machines, insufficient infection management measures in the dialysis unit and understaffing of the dialysis unit (Krause et al. 2003).

2.12.3.9.3 Provider to Patient Transmission: Provider to patient transmission was initially reported from England in 1995 (Public Health Laboratory Service 1995). The obstetrician-gynaecologist had been shown to transfer HCV infections to a patient during caesarean section (Ross et al. 2002a,b,c). In another study the United States Veteran Affairs Medical Centres have reported a higher occurrence (35%) of HCV among iatrogenic groups than the general population (Cheung 2000) thereby supporting transfer of infection from health care workers to patient (Alter 1997). Another group of health care takers was also determined that was involved in drug abuse to him/herself and then reusing the needle for patient. An anaesthesiologist was found to infect 171 patients that were identified on the basis of presence of identical HCV strain in their sera (Bosch 1998 and 2000). Likewise, the countries having HCV as an endemic problem of general population, hospitalization and invasive procedures are reported as noteworthy risk factors (Di Stefano et al. 2002, Mele et al. 2001, Sun et al. 1999 and Sun et al. 2001).

2.12.3.9.4 Nosocomial HCV: Larke et al. (2002) reported acute nosocomial HCV infection from regular blood donors. On analysing quasispecies of HCV at molecular levels and sequencing the genome, HPVR 1 of HCV isolates from donor blood and

putative source patient were found highly related as compared to controls. Moreover, viruses from two individual sources were observed genetically very close to each other. These studies are strongly in support and therefore evidence patient-to-patient transmission of HCV in an intravenous (IV) clinic (Bendinelli et al. 1999, 95). Nosocomial infection is observed previliged than previouslt though as acute HCV infection is often observed at sub-clinical levels (Alter 1997).

2.12.3.10 Shaving and Barber Shears: The traditional barbershop shave being considered male luxury could be risky. Anonymous (1995) studied Sicilian barbers relying on contaminated and non disposable apparatus showed increased risk of HCV infection and this was not limited to shaving but also to hair cut. It was also observed that the fluid used for sterilizing the equipment could not annihilate the viral RNA and may be a possible godown for infection (Gitlin et al. 1997 and Highleyman 2009).

2.13 DEMOGRAPHIC FACTORS RELATED TO HCV

Racial differences are involved in the treatment response to HCV infection. Black Americans are at higher risk than white American owing to lower response rate to therapies e.g., greater transcriptional response to interferon, high frequency of genotype 1 infection, high histological activity index 17 scores, increased weight, and increased iron stores. However, the natural course of HCV in black patients has not been defined (McHutchison et al. 2000, Lindenbach and Rice 2001, 991, Ioannou et al. 2003, Wiley et al. 2002, Crosse 2004 and He et al. 2006). Nevertheless combination of therapies may eliminate this difference to some extent (McHutchison et al. 2001). From some other recent studies it may be concluded that Whites offer better response to peg interferon alfa-2b and ribavirin (Muir et al. 2004, Jeffers et al. 2004, Daniel 2005 and Conjeevaram et al. 2006). These differences in the treatment may be due to racial bias, clinical factors and sociodemographic factors (Conigliaro 2002, Kressin et al. 2002 and Rousseau et al. 2008).

Al Naseer (1992), studied age-specific prevalence of antibody to HCV Saudis from Al Baha region, South-West Saudi Arabia. There was a gradual exposure to HCV

early in life with a gradual increase with age, reaching a peak of 5.3% in the 30-40 years age group. The overall prevalence in males and females was 3.6% and 3.1%, respectively. The route of HCV transmission among the Saudi population has been observed to be intrafamilial. Anti-HCV positivity in family members of HCV positive index cases was statistically significant compared with HCV index cases (Cantilena et al. 1992).

Hajiani et al. (2006), studied HCV communication and associated cause within families of HCV patients from Southern Iran, Khuzestan. It was observed that both Intrafamilial and sexual transmission of HCV are not concerned areas for HCV transfer within a family. Roman et al. (2008) found that HCV positivity sex ratio (SR) was largely males. Patients of age less than 40 years make up 49.6% of all patients provided their time of acquiring the infection could be supposed. Males represent higher proportion of genotype 4 than females where genotype 2 and 5 are predominant. However, genotype 1 and 4 were observed independent of gender and age. Moreover, among the patients aged less than 40 years genotype 3 was obvious and genotype 2 was predominantly assocated for patients aged more than 40 years (Idrees and Riazuddin 2008).

Further more, three discrete sets of transmission patterns were recognized by using age specific prevalence data. First pattern is obvious in countries like United States, Pakistan and Australia, suggest increased risk of HCV infections during past 10-30 years and predominant among young individuals of 30-49 years of age. The predominant risk factor was found to be drug intake by injections (Wasley and Alter 2000 and Alavian et al. 2008). Secondly, countries like Japan and Italy, constitute second pattern, identified by old age infections during distant past (Kiyosawa et al. 1994 and Guadagnino et al. 1997). Finally, third pattern countries including Egypt show infection distributed among all age groups, indicating an in progress threat for acquiring HCV infection. The associated risk factors were observed for second and third patterns include unsafe injections and contaminated equipment usage in healthcare-related procedures (Frank et al. 2000, Bari et al. 2001, Medhat et al. 2002 and Alter 2007). Further studies could be done in this direction to uncover important information regarding the extent to which different risk factors contribute in transmission and establishing the approaches that could

be applied for hindering spread. Assessment could also be done in untouched developing areas and prevention programs can be designed (Zein 2000).

MATERIALS AND METHODS

HCV is an important virus and is causative agent of liver infection (inflammation, dysfunction, shrinkage and HCC) among millions of humans. Till yet no immunization techniques are available for its control and only preventive measures can be applied to avoid the disease. Therefore in present circumstances epidemiological and phylogentic studies are very important to gather maximum useful information about HCV to tackle this devastating virus. In this regard the present study was designed to collect the epidemiological data from ELISA positive HCV patients attending the liver Out Patient Departments (OPDs) of the Pakistan Institute of Medical Sciences (PIMS), Islamabad and Fauji Foundation Hospital (FFH), Rawalpindi. The blood samples of the patients were taken and further analysis was carried out for the characterization of HCV. All patients were residentials as well as from the ethnic origin of Gujar Khan, a town of Rawalpindi city. Convenient sampling method was applied (A method that involves the collection of data and materials from hospitals) for data and sample collection.

3.1 OVERVIEW OF GUJAR KHAN

Gujar Khan is one of the towns of district Rawalpindi and it is the largest tehsil (administrative subdivision of district) of Punjab province (Population census organization 2010). From the capital city of Islamabad, it is approximately 55 kilometers towards Southeast. The town has an estimated population of nearly 78,309 individuals (Helders 2010). Gujar Khan depicts the historically rich culture of Potohar, the upper Punjab. Important natural resources of tehsil Gujar Khan are organic compound in the form of methane gas and petroleum reserves (Fig. 3.1).

3.2 PROFORMA DESIGNING

A proforma was designed for data collection with the help of Dr. Tashfeen Adam (Consultant hepatologist, Professor of medicine, Pakistan Institute of Medical

Sciences) and Dr. Asghar Aurungzeib Durrani (Consultant hepatologist, Professor of medicine, Fauji Foundation Hospital Rawalpindi). The Proforma was used to gather the data on demographics and self reported risk behaviors and prior consents were taken from all the studied participants. In the proforma demographics (age, sex, educational levels, occupations), daily work activity (sedentary or non sedentary), and health behaviors (smoking and alcohol consumption) etc, were taken into consideration (Annexure I). The details of the included features for proforma are as follows.

3.2.1 Age

Patients were randomly selected from those visiting the OPDs of selected hospitals and were grouped between age 12 to 65, moreover patients of all ages were considered in order to relate the prevalence of HCV with age. Ages were further grouped as follows (for male as well as female patients) 10-19, 20-29, 30-39, 40-49, 50-59 and 60 to 65 years.

3.2.2 Gender

Patients were randomly selected from male as well as female gender, but the hospital visiting patients were mainly females (as females being housewives find it convenient to visit the hospitals) therefore data mainly focuses on females than males. The number of female patients was 1048 and the number of male patients was 162.

3.2.3 Occupations

The occupation was considered in order to find out the mode of acquisition of disease, as well as daily activity index of each patient. Another purpose of this factor was to access the income sources and on the basis of that the socioeconomic factors of this disease can be estimated. Male patients were mainly government sector employee while a large number of them were jobless. The remaining males were mainly from all fields of life including cooks, drivers, labors, landowners, policemen, health care practitioners, private jobs, vendors and shopkeepers, students, teachers and tailors. Females were mainly housewives and a few of them were working women including teachers and midwives.

3.2.4 Marital Status

Marital status of every patient was noted in order to find out the chances of exposure to disease. With reference to marital status, married females are more likely to acquire hospital based HCV infection than unmarried females because of their increased exposure due to maternity related issues, as they are more exposed to different factors such as caesarian sections.

3.2.5 Rural and Urban Residence of Patients

Living setup of each patient was considered in order to find the prevalence rate of hepatitis in selected group of patients. It is important to note that living practices are very much different in rural areas than urban ones.

3.2.6 Educational Levels

The educational levels of the patients were divided into illiterates, school level, college levels and universities in order to access the self understanding and awareness about disease condition. Primary purpose of this activity was to find out the response rate about disease treatment among different educational levels.

3.2.7 Other Family Member Affected

In the questionnaire it was asked from all interviewed affecties that if they had some other close relative affected from hepatitis C in order to understand the mode of acquisition of disease.

3.2.8 Duration of Present Illness

Natural history of the virus is very important to make treatment decisions. It clearly depicts the chronicity or acute phases of hepatitis C, therefore natural history was considered for the selected cases. Chronicity leads to the development of disease complications and progression of disease to cirrhosis, necrosis and HCC.

3.2.9 Possible Mode of Acquiring the Disease

In order to find the network of disease spread, understanding of the possible modes of acquiring the disease is crucial owing to its role in disease control. During interviews the patients were asked weather one had a surgery, blood transfusion or dental

procedures, as it indicates the nosocomial infections. Other aspects were unsafe injection use and injection drug usage, accidental exposure to human blood (normally in the case of health care practioners), barber shears, shared razors, tooth brushes and tattoos. These factors reflect the life style of an individual. There are certain other factors as well for example, the females are more likely to visit hospital (during pregnancy, child births and postpartum) than males. Therefore the females have more chances to acquire the infection.

3.2.10 Mode of Presentation

There are two modes of presenting the disease, incidental and symptomatic. Hepatitis C is the disease of inflammation of hepatocytes. In certain cases the disease remains asymptomatic. Patient does not feel any specific symptom related to this disease. Incidental cases are normally diagnosed accidentally like during medical checkup prior to job, during blood tests in pregnancy, while diagnosing some other problem. There are many cases which are diagnosed on the basis of symptoms which patients feel, like nausea, myalgia, artilagia, anorexia, ascites, jaundice, pallor etc. The aim of including these factors in proforma was to find out the proportion of symptomatic and asymptomatic cases and also if the diagnosis is on the basis of symptoms then what are the most prevalent symptoms in these cases. To some extent these symptoms indicate the chronicity level of disease and also the degree of liver damage as for example ascites is related to later stages of disease progression.

3.2.11 Treatment Affordability

Source of expenditures for treatment of disease was digged out as treatment is very important to stop the disease progression. Three categories of patients were made on the basis of income levels. For this purpose patients who can afford the treatment by themselves, were divided into two groups (high level income and moderate level income) and a single group was for the patients who would take treatment on behalf of government of Pakistan Zakwat and Bait-ul-Mall department (Islamic treasury) or Prime Minister's Program for the control of hepatitis C. It is very important to estimate the affordability of the patients as in the cases where the patients are non-affording and are dependent on government and in case they are unable to get treatment they will proceed

to advanced liver disease and development of HCC in the coming years. It will increase the disease burden in Pakistan and hence globally.

3.2.12 Personal History

An Inclusion criterion for personal history was smoking, alcohol consumption, any other documented addiction and marital status. Although till yet it is not known that weather smoking has some effect on enhancing the disease or not but to some extent it has been proved that it has some additive effects on raised ALT levels in hepatitis C sufferers. While co-relation of alcohol addiction with disease severity is well known and well established, but in Pakistan, due to religious factors it is hard to find any patient involved in alcohol addiction. Even those involved in such activities, hide the truth due to social values.

3.2.13 Monthly Income of the House Hold

In the designed questionnaire it was asked that how much was the monthly income of the house hold. Furthermore three categories were made according to monthly income i.e., less than 5,000, 5,000-10,000, and above 10,000 Rs. The purpose of this activity was to find out, how many of the patients are capable of affording the costs involved in the treatment by themselves, the involvement of socioeconomic issues in the management of HCV, exploring the relationship of disease in some specific class of people, i.e lower class, middle class and upper class.

3.2.14 History of Traveling Abroad

It was asked from the interviewed patients if they had traveled abroad or not. In case of positive answer they were further inquired for duration of stay, place to be visited any illness or accident during stay, surgery, blood transfusion, dental treatment etc. The question was particularly important with reference to mode of acquisition of disease and genotyping (as genotypes are different in different areas).

3.3 STATISTICAL ANALYSIS FOR EPIDEMIOLOGY

Binary logistic regression analysis is a statistical analysis used to build a relationship between polytomous outcome variables and a given set of explanatory

variables. In other words it is applied when several outcome variables are expected. Binary logistic regression analysis was performed for the risk factor assessment. Pearson's chi-square analysis is the test applied to make discrimination between the test statistics and its distribution. The test was applied to find the significance of association between risk factors and genotypes, for the given patients. Statistical correlation was also applied on the given set of data. The scope of applying this test was to find out if the risk factors and genotypes were related. The probability of the specific genotype involvement with particular risk factors was found out. All tests were applied using Minitab version 11 (Zgonc 1996, 792) and E-Views version 3.1 software (Hill et al. 2001, 192).

3.4 INCLUSION CRITERIA

Selection of patients was made very carefully by considering HCV third generation ELISA positive index cases of ages between 12-65 years.

3.5 EXCLUSION CRITERIA

There were certain factors (if present in someone), was not selected as a study participant. If some one was co-infected with HIV or HBV, though positive By third generation ELISA for HCV was not considered. Female patients were selected but all cases of pregnancies were rejected, like wise children of ages less than 12 years and elderly people above 65 years, the patients currently on interferon treatment and those suffering from HCC were not entertained, as immune responses of these cases differ from those selected by inclusion criteria.

3.6 BLOOD SAMPLE COLLECTION

Out of 980 considered patients 400 were randomly selected for blood sample collection, followed by venipuncture for HCV antibody testing and molecular analysis. For sample collection 5ml of the blood was taken from the patient, and was stored in a Lavender top vacutainer supplied with K_2EDTA. The samples were then centrifuged at 5000 rpm for 5 minutes to draw the plasma. Plasma is the portion of blood that mainly

constitutes of water, essential proteins, clotting factors and fibrinogen etc. With the help of a graduated pasture pipette the plasma was shifted to a 1.5ml eppendorf, and was stored at -80°C for further analysis (Greiner Bio-One 2006).

3.7 RNA EXTRACTION

RNA was extracted using the modified protocol of Lau (1998) (Annexue, II) in a Biosafety level III laboratory of The Aga Khan Health University, Karachi, Pakistan using Class II Type B 1 safety cabinet (Baker Company, Stanford Maine).

3.8 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR is a commonly used in studying the genomes of viruses whose genomes are composed of RNA (Mackay et al. 2002a, 2002b). Primers were designed from 5'UTR of HCV using software primer 3. Primer sequences were as follows

SP1:	5'	CTGTGAGGAACTACTGTCTT	3'
ASP1:	5'	ATACTCGAGGTGCACGGTCTACGAGACCT	3'
SP2:	5'	TTCACGCAGAAAGCGTCTAG	3'
ASP2:	5'	CACTCTCGAGCACCCTATCAGGCAGT	3'

cDNA was made from extracted RNA as HCV is a single stranded virus and its further molecular analysis demands it to be double stranded. The details of reaction conditions and PCR conditions are mentioned in the (annexure III). After completion of reaction the PCR tubes were immediately removed from the thermocycler (Gene Amp PCR 9700, Applied Biosystems) and were stored at -20°C till further use.

3.9 NESTED PCR

Nested PCR is advisable for viruses like HCV and it was carried by following the guideline of Mc Omish et al. 1994 by using the above mentioned primers. This is a

sensitive and accurate technique for the amplification of this fastidious virus from blood. Two negative and two positive controls were run every time to ensure the reliability of the results. These Nested PCR amplified products were run on 1.5% Agarose Gel Electrophoresis (Annexure IV).

3.10 RFLP ANALYSIS

RFLP is considered to be one of the best methods to analyze polymorphism in the sequence and is able to trace even point mutations (Hummel 2003). Three sets of restriction enzymes were used for this purpose. Restriction sites of the enzymes are given in Annexure V. Restriction endonucleases were used in combination of *RSa*I and *Hinf*I, *MVa*I and *Hae*III, and any of the *Bme*1390I (*Scr*fI) for subtyping of genotype 3 or Bsh1236I (*Fun*DII) for subtyping of genotype 1 and 2 (Davidson et al. 1995 and McOmish et al. 1994). Restriction digestion was performed by storing the restriction product at 37°C for 16h and the resulting product was then run on gel.

3.11 PAGE (POLYACRYLAMIDE GEL ELECTROPHORESIS)

16% PAGE was used for the restriction analysis as the minimum expected band size of the restricted product was 12bp, using vertical gel assembly (Bio Rad Mini Protein Tetra Cell). A 50bp molecular weight marker was used (Fermentas # SM0373) for the expected band size estimation of restriction products. Applied voltage across the electrodes was 120 volts. The results obtained by RFLP were visualized by ethidium bromide staining (5µg/100ml) after running the PAGE (Annexure VI).

3.12 DNA PURIFICATION

DNA was purified using Jet Quick (PCR Product purification spin Kit by Genomed) by following the instructions given by the manufacturer (Annexur VII).

3.13 SEQUENCING

GenomeLab Dye Terminator cycle sequencing with Quick Start Kit was used for DNA Sequencing. It involved a four step process involving Preparation of the DNA sequencing reaction, ethanol precipitation, precipitation in the sample plates and sample preparation for loading into the sequencer (Annexure VIII). The sequenced samples were cleaned using software BioEdit version 7.0.5 (Hall 2005). Samples were sequenced using first SP2 primer and then using ASP2 primer from 5'UTR of the HCV genome. Then basic local alignment tool (BLAST) was applied to the sequenced samples to resolve their status. Fasta format was used for the sequenced samples to be taken to the Clustal W and MUSCLE (Multiple Sequene Comparison by Log Expectation) software (Mackey et al. 2002a, 2002b) for alignment (Altschul et al. 1990, 1997). For all sequenced samples both of the sequences were aligned, overlapped using Clustal W software and were further processed for their submissions to Genbank (Larkin et al. 2007). All of the untypable sequences by the RFLP analysis were submitted to GenBank for the allotments of Accession numbers.

3.14 PHYLOGENETIC ANALYSIS

Statistical selection pairing, by applying Tajima's test (Tajima 1989) and Neighbour Joining method (NJ) (Saitou and Nei 1987) methods were used for phylogenetic reconstruction and the NJ *p-distance* model was used for distance analysis (Nei and Kumar 2000). Base statistical robustness was performed by using 500 bootstrap repeats and the whole process was developed by MEGA 5 Beta # 7 software (Kumar et al.2004).

3.14.1 Comparative Phylogenetic Analysis of Studied Sequences with other Sequences from Pakistan

For the comparison of the studied sequences with the reference accessions already reported from Pakistan, an exhaustive search was made in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and European Molecular Biology Laboratory (EMBL) (http://www.ensembl.org/index.html) databases for partial 5'UTR sequence

comparative study are 32 with accession numbers from AM228866 to AM228898 (Yasmeen et al. 2009). The purpose of selecting these particular sequences was to get the representative genotypes from the whole country (Idrees and Riazuddin (2008) had reported these sequences from whole Pakistan).

3.14.2 Comparative Phylogenetic Analysis of Studied Sequences with Sequences from Pakistan's Neighboring Countries

Neighboring countries of Pakistan are Afghanistan situated towards west, China situated in the north east of Pakistan, India towards east and Iran towards south west of Pakistan (Fig. 3.2). Samples from India were selected on the basis of targeting specifically 5' UTR region of viral genome (Chaudhuri et al. 2005 and Valliammai et al 1995). Reference accessions from China were retrieved form available 5'UTR sequences of HCV genome. Representatives of all Chinese genotypes were taken. There were only 16 reference accessions available on NCBI from Iran and all were retrieved for the comperative study (Amini et al. 2006). After extensive search not even a single accession of HCV was found from Afghanistan. Therefore despite of the fact that Afghanistan being most important with reference to a comparative study, it was not considered. The absence of any study related to HCV from Afghanistan is obvious as the country is suffering from war and facing political instability.

3.14.3 Comparative Phylogenetic Analysis of Studied Sequences with Other Sequences from Countries World over

Accessions numbers of world over reference strains were selected randomly by retrieving the 5'UTR sequences of HCV from NCBI. Representative sequences were selected from United States of America, United Kingdom, Australia, Japan, Maldives, Hong Kong, New Zealand, Vietnam and Turkey and were aligned with the studied sequences to find the evolutionary and phylogenetic relationships of different HCV genotypes and subtypes.

Fig. 3.2: Map of Pakistan with her neighboring countries Afghanistan, China, India and Iran. The country shares her largest boarder with Afghanistan and then with India (Source: Magellan 1997).

RESULTS AND DISCUSSION

4.1 EPIDEMIOLOGY STUDY

The data was collected on HCV from PIMS, Islamabad and FFH Rawalpindi, from the patients visiting the liver OPDs with ethnic origins from Gujar Khan a town 54Km away from Rawalpindi city. Initially 1200 patients participated in the study, but later on due to incomplete data of the 75 patients, they were excluded from the epidemiological analysis. So, 1125 patients were studied, constituting of 962 (86%) females and 163 (14%) males (Fig. 4.1.1).

4.1.1 MALE TO FEMALE RATIO OF THE STUDIED COHORT

Results of present study regarding the genders of the studied participants, do not agree with majority of the previous studies reported all over the world including Pakistan, where the numbers of male patients were greater than female patients (Kim et al. 1998a, 1998b, 1998c, Khan et al. 2003 and Khan and Zarif 2006). In different studies (Bain et al. 2004, Mendez-Sanchez et al. 2005, Chen and Morgan 2006 and Hajiani et al. 2006) the male to female ratio was almost 2: 1. However, in a report, no gender difference was observed in the prevalence of disease (Stroffolini et al. 2001). Further, various reports from Saudi Arabia, Myanmar and United States of America (Fakeh and Zaki 1999, Lwin et al. 2007 and Armstrong et al. 2006) were in accordance with the present study where 61%, 53% and 51% of the participants were females and only 39%, 47% and 49% were the males. From the previous studies in Pakistan, one conducted by Khan and co-workers provided a different pattern of gender distribution, with 33% females and 67% males (Khan et al. 2000). The results were also in accordance with the ones reported from Republic of Guinea-Bissau, where female patients were twice the male participants (Plamondon et al. 2007).

The reason of this trend might be that males particularly from suburban Rawalpindi don't have health seeking behaviour to visit the hospitals as they have to

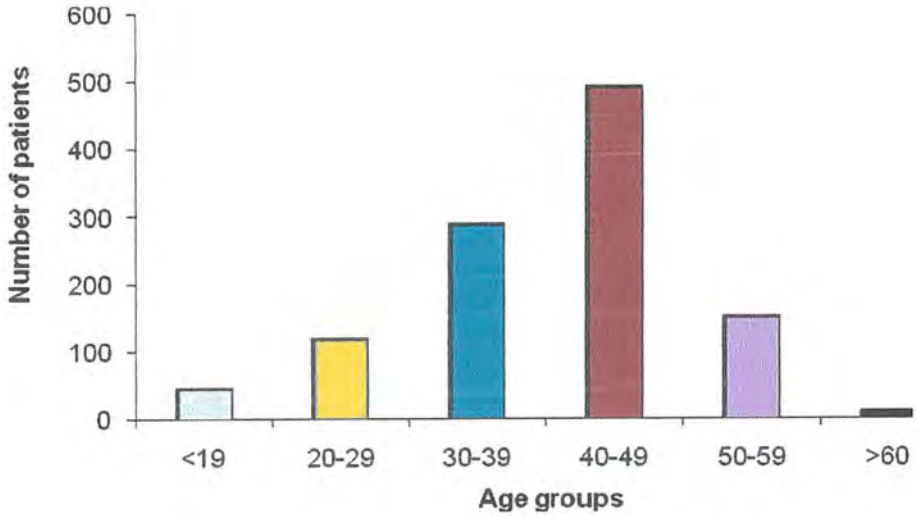


Fig 4.1.1: Age group wise distribution of the study participants.

financially and socially, support the whole family. Due to different financial and social pressures, they are working for whole day. While majority of the female patients being housewives, find it comparatively easier to visit the health care practitioners (Fig. 4.1.1, 4.1.2).

4.1.2 NATURAL HISTORY OF THE HCV FOR THE STUDIED COHORT

HCV infection has two broad categories, acute and chronic HCV infection. The term acute is used for the infection that persists for six months or less time period, while chronic HCV infection is marked by the persistence of the viral RNA in the blood and hepatocytes for at least six months after the onset of infection (Chen and Morgan 2006). The selected cohort of patients from suburban Rawalpindi constituted of 93% chronic cases of HCV and only 7% acute infection was observed (Fig. 4.1.3). The results were in agreement with the ones previously reported by Al-Moslih and Al-Huraibi (2001) from Yamen, where the majority of the reported cases were chronic and the number of acute cases was just 19.4%. A report from United States has estimated 1.8% of the population infected with HCV, and 75% of those are the chronic cases (NIH 2002).

Major reason supposed to be involved in this chronicity pattern is that the HCV infection is asymptomatic in 75-80% of the cases. It has been studied that only 20-25% of the patients report to the hospitals with some presenting complaints (Herrine 2002). There were no apparent gender differences in the rate of chronicity in hepatitis C infection. Majority of the considered cases weather male or females were equally suffering from the chronicity of the disease (Fig. 4.1.3). The same observations were reported from other studies around the world, such as the chronicity was related to age and the estimated rate was 30% and 76% in subject's $\leq$ 20 and $\geq$ 20 years of age, respectively (Alter et al. 1999 and Bellentani and Tiribelli 2001).

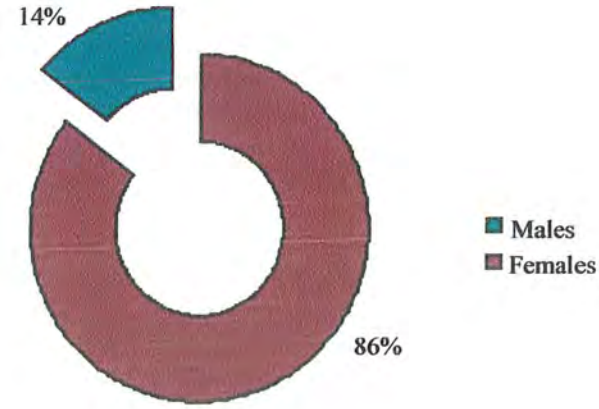


Fig. 4.1.2: Total percentage representation of the studied cohort.

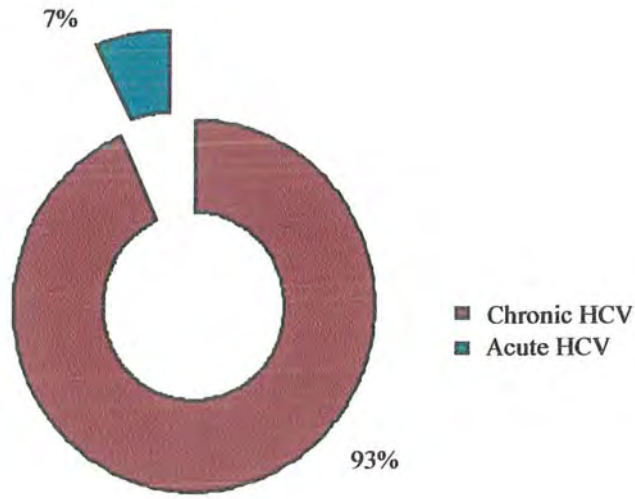


Fig. 4.1.3: Distribution of patients on the basis of chronicity of disease.

4.1.3 MARITAL STATUSES OF THE HCV PATIENTS

In total, 87% of the studied patients were married, while 13% were unmarried (Fig. 4.1.4) and majority of the patients belonged to the age groups between 30 to 50 years. This is an indication that the rate of disease prevalence was higher in the married individuals. The results were in accordance with the already available report by Bari et al. (2001), who got the same result with mean ages of patients 43 years and 88% of them were married. Similar trend was observed in the Egypt where larger numbers of reported cases were married and there was a difference in the mode of acquisition of disease in married and unmarried studied groups (Nafeh et al. 2000).

The rate of chronicity is directly related to the age of an individual at the onset of disease and that was in turn proportional to the marital status of the index cases. Married studied participants were more likely to be the chronic carriers of the HCV. In the conducted study, there was an aggravating trend of chronicity with advancing age (Fig. 4.1.3). All cases of acute HCV infection were representatives of the age groups ≤ 20 years and a few of age groups 20-29. The results are concordant to the already available reports from US population and from Italian population, where the rate of chronicity was 30% and 56% simultaneously in those with ages ≥ 25 and 70% and 87% respectively, in cases where ages were above 25 (Alter et al. 1999 and Bellentani and Tiribelli 2001)

4.1.4 AGE GROUP WISE DISTRIBUTION OF THE STUDIED COHORT

The studied index cases were divided into six main groups (Fig. 4.1.1 and 4.1.5). Forty five (4%) cases were reported in the age group ≥12-19 year age group. The number of patients in 20-29 years age group was 120 (10.7%), while 30-39 years age group was the number was 289 (25.7%). However, most of the patients belonged to the age group 40-49 years i.e., 493 (43.8%). Further, there were 151 (13.4%) patients in 50-59 years age group while age group ≥60, was with least number of patients i.e.10 (0.9%). Similar results have been reported from Karachi Pakistan, by Akhter et al. (2002) and Irfan and Arfeen (2004).

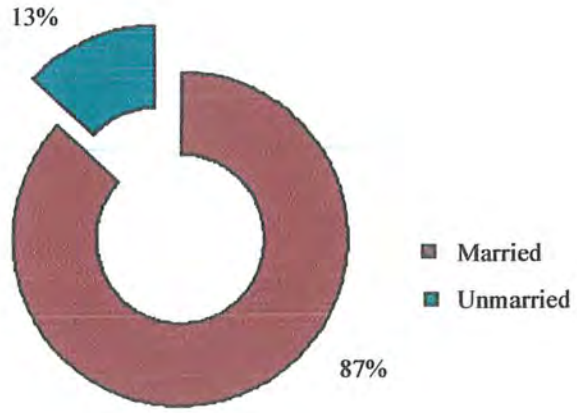


Fig. 4.1.4: Marital status of the studied cohort of the patients.

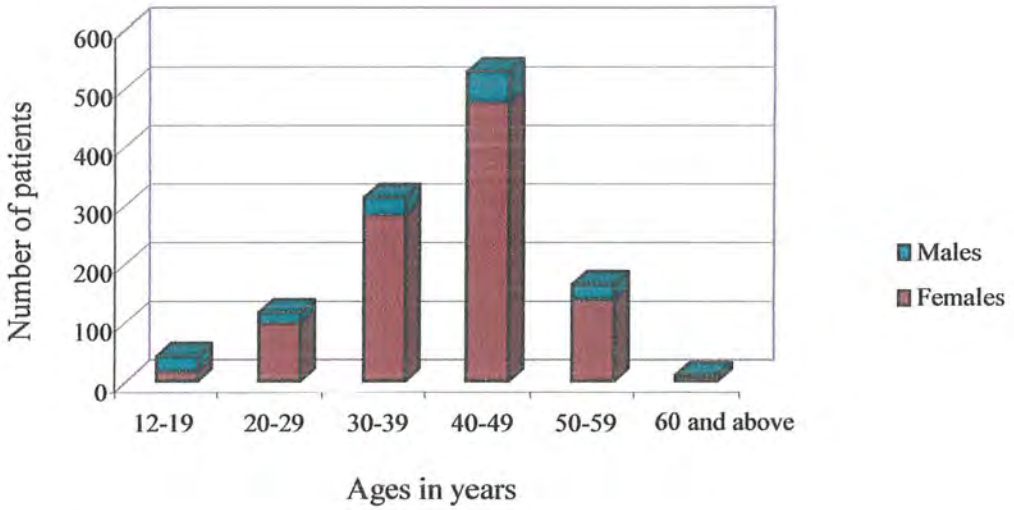


Fig. 4.1.5: Total number of patients in each age group.

Concurrent results have been reported from Brazil and Italy, where the patient's distribution was similar to the present study i.e. 14-19 years age group had 0.5% and 5.2% patients, 20-39 years age group had 27.5% and 28.8% prevalence and 40-59 years age group was the most dominant one with 58% and 38.6% representation, while ≥60 age group had a prevalence of 14% and 27.4 % (Stroffolini et al. 2001 and Focaccia et al. 2004). The results were also in complete conformity with the reports from Puerto Rico and Myanmar, where HCV prevalence was highest in the age group 30-49 years (Perez et al. 2005).

Pakistan lies in the first group as majority of the reported cases belonged to age groups between 30-49 according to age distribution patterns of HCV by Wasley and Alter (2000). These results are in agreement with the studies reported from Korea where most of the patients were in the fiftieth decade of their lives. Another report by Armstrong et al. (2006) mentioning high prevalence in the age range of 40-49 years is available in the literature to further strengthen the results of present study. Moreover, there are still a large number of reported cases in age groups 20-29, indicating a surge in the HCV incidence in Pakistan (Wasley and Alter 2000 and *Daily Times* 2005).

On contrary, the results were in a bit disagreement with the a report by Siddiqui et al. (2008), where the majority of the cases were in the fifth decade of their life and the second most representative group was that in sixth decade of life. Basically, it indicates the time period in which the acquisition of infection was at its peak, in a particular population. In a study reported from Saudia Arabia, the prevalence of HCV infection showed an increase in prevalence with age, showing the maximum incidence above the age of 45 years while the (P, 0.001) lowest prevalence ranged in the ages of 15 and 24 years. The chi-square test showed significant differences among the ages (Fakeeh and Zaki 1999).

4.1.5 ADDICTIONS AS LIFE HABITS IN THE STUDIED COHORT

Majority of the considered patients were non-addicts as 85% of the interviewed persons did not report any sort of addiction. Only 15% of the patients provided the details

of their addiction habits. Out of these 15% cases, 61% patients were the smokers while 33% of the addicts were tobacco snuff consumers and only 6% reported the use of alcohol. Tobacco snuff is the specific sort of addiction that is confounded particularly in the Khyber Pukhtun Khwa (KPK) province, rural and suburban areas of Pakistan and in Afghanistan. Tobacco snuffing is more devastating than smoking as it involves the oral intake of tobacco, where its extracts penetrate in the body from mouth as well as through digestive tract (Fig 4.1.6, Table 4.1.1).

The results were similar to a report from India, where the 90% of the participants were non-addicts and 10% were the addicts (Ahmed et al. 2000). In HCV positive patients, increase in ALT level is independently associated with smoking and alcohol consumption, that leads to liver dysfunction, which can be reduce by avoiding alcohol consuming and smoking (Wang et al. 2002).

4.1.6 MAJOR INCOME GROUPS OF THE STUDY PARTICIPANTS

The patients were divided into three income groups. Group one was of patients with monthly income around 5000 Rs. Group two was of those patients who had monthly incomes between 5000-10000 Rs, and the third group was of the patients with incomes more than 10,000 Rs per month (Fig 4.1.7, Table 4.1.2).

It was observed that on the basis of income 72% of the studied cases belonged to group one. Similarly, 21% belonged to second group while the least representative was the third group in which the total percentage was 7%. Pakistan is a developing country, where 65.67% of the population is below poverty level (i.e they earn less than 2 US$/ day) UNDP 2007, and they are unable to live up to a standard life. Secondly, the patients were even unable to afford the expenditures involved in the treatment. Majority of the index cases were planning to seek dependency on the prime minister's program for the control and prevention of HCV. A few of them were hopeful to use the facility provided by Islamic Treasury (bait-ul-mall, a department of government of Pakistan) where the source of money is alms (Zakwat) that is sufficient only for a few of the reported cases.

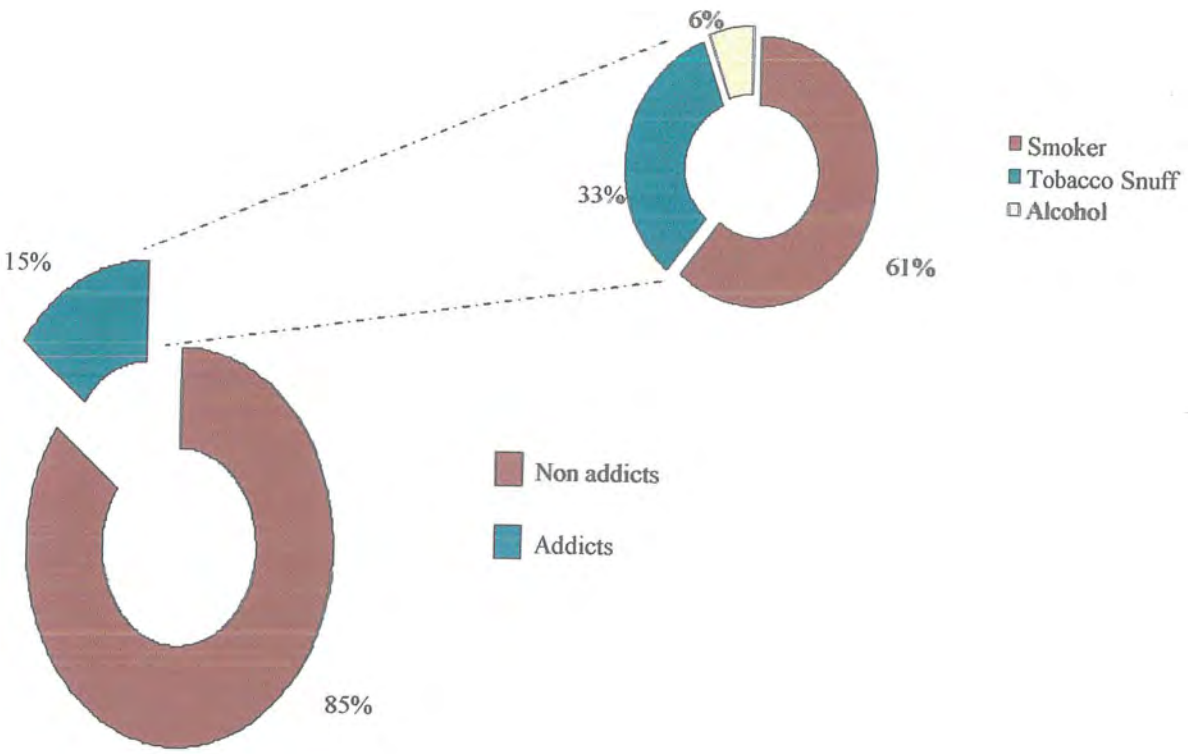


Fig. 4.1.6: **Life habits of the index cases on the basis of addictions.**

Table 4.1.1: Distribution of common demographic factors and presenting complaints, in HCV positive studied cohort with age specificity.

Gender	Age groups	Number of patients	Marital status		Family history		Addictions		Presenting complaints	
			Single	Double	Positive	Negative	Positive	Negative	Incidental	Symptomatic
Males	10-19	27	26	1	4	23	1	26	10	17
	20-29	21	15	6	2	19	6	15	10	11
	30-39	31	6	25	5	26	1	30	14	17
	40-49	51	1	50	10	41	26	25	14	37
	50-59	27	0	27	7	20	13	14	12	15
	60 & <	4	0	4	0	4	4	0	3	1
Females	10-19	17	16	1	7	10	0	17	4	13
	20-29	99	62	37	28	71	3	96	34	65
	30-39	285	16	269	52	233	32	253	60	225
	40-49	479	6	473	120	359	44	435	122	357
	50-59	141	2	139	31	110	19	122	49	92
	60 & <	6	0	6	1	5	1	5	3	3

Total number of considered cases was 1188 for the above mentioned demographic factors.

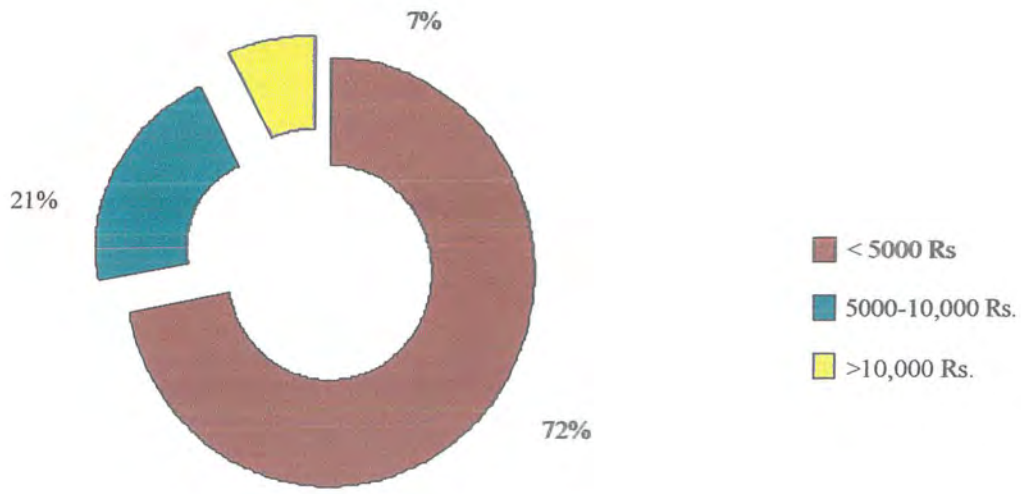


Fig. 4.1.7: **Monthly incomes of the index cases in Pakistani rupees.**

Table 4.1.2: Sociodemographic factors including monthly incomes, literacy levels and occupations of the studied patients.

Demographic Factors	% (Numbers)	X^2	d.f.
Annual Income (US$)			
<723(<5000 Rs)	72.1(290)		
723-1446 (5000-10000 Rs)	20.6(83)	283.30***	2
>1446 (>10000 Rs)	7.21(29)		
Literacy levels			
Illiterate	77.6 (312)		
School	12.2 (49)	601.48***	3
College	8 (32)		
University	2.2 (9)		
Occupations			
Cook	0.5 (2)		
Government servant	5.0 (20)		
Jobless	7.0 (28)		
Labourer	1.5 (6)		
Landowner	0.7 (3)		
Police	0.7 (3)	2261.27***	13
Private Jobs	2.5 (10)		
Students	3.7 (15)		
Shopkeepers	2.7 (11)		
Tailors	1.0 (4)		
Teachers	3.0 (12)		
Healthcare Practioners	3.7 (15)		
House Wives	68 (273)		

*** $p < 0.001$

In a report by Jafri and colleagues from Pakistan, they have provided the poverty related data where a two room accommodation was shared by an average of 7 households (Jafri et al. 2006). Similar study from India provided the approximately same pattern of income groups (40% from lower class, 37% from lower-middle class and 23% from middle class) (Ahmed 2009). In another study reported from Australia, 60% of the study participants were from low socio-economic level (Gifford et al. 2004). It can be observed clearly, that the hygienic conditions of lower class are poor and this might be one of the reasons of high prevalence of HCV infection in this socio-economic level (Bacher 1998 and Lopes et al. 2006). Similar studies were carried out by Khan et al. (2008), where she concluded that 44% of the studied patients were having monthly incomes less than 3000 Rs and 56% of them had monthly incomes more than 3000 Rs.

4.1.7 EDUCATIONAL STATUS OF THE STUDIED PARTICIPANTS

Educational status of the patients was divided into two broad categories i.e., illiterate and literate (Fig 4.1.8, Table 4.1.1). The literacy was further subdivided into three levels i.e. schooling, college level, and university level education. It was observed that 78% of the study participants were illiterates, while just 12% had 5-10 years of education. Further, 8% had 12-14 years education and only 2% participants were found to complete a 16 year education. The results resemble to the study reported from upper Egypt in which the majority of the patients did not received any formal education and only 14.4 % were formally educated (Nafeh et al. 2000). A slightly dissimilar literacy pattern was observed in the patients from Karachi where 55% of the studied participants had attended the schools for formal education while 23% completed the 11-14 years of education and 22% participants had no formal education from any institute (Akhter et al. 2002). In another related study conducted by Khan et al. (2000), from peri urban areas of Pakistan, indicated that 75% of females and 54% of the males were illiterate and were unable to read and write.

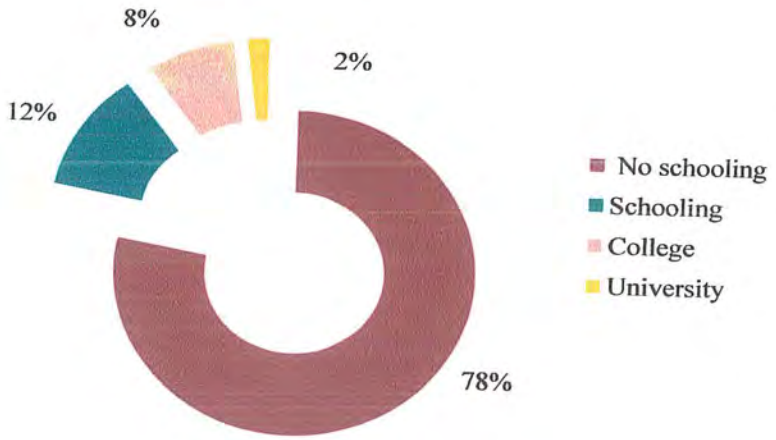


Fig. 4.1.8: Literacy levels of the HCV positive patients.

Recently, Khan et al. (2008) reported a study of female HCV positive patients from Karachi, where 50% participants had no formal schooling, 19% had a primary level education, 24% had 6-10 years of formal education and 7% had an education upto 12 academic years. In another related study from Brazil, it was observed that patients with less than and equivalent to 8 years of study were 5.6% of the total representatives, 12 years of study category had 9.8% patients and greater than 12 academic years educated patients were 13.8%, while the remaining 70.2 % patients reported either no formal education or upto eight academic years. So, it is quite obvious that low socio-economic and literacy rate is directly associated with the prevalence of infectious diseases (Jafri et al. 2006 and Lopes et al. 2006).

4.1.8 OCCUPATIONS OF THE STUDIED COHORT

It was identified that 68% of the participants were housewives. The majority of the male subjects 7% were jobless, while 4% were government employees followed by 3.7% students (Fig. 4.1. 9, Table 4.1.2). Further, it was observed that 3. 7% of all the reported cases were health care providers. Their total number was 15 (including 13 males and 2 females) and 6 of them reported the incidental needle stick injuries as a cause of acquiring HCV. The results were concordant with a study in which the health care providers were less likely to be infected with HCV (Nafeh et al. 2000).

The studied cohort of the patients consisted of majority of female patients and almost all of them were housewives and the male patients were from almost all walks of life. There appeared no specific risk of disease accusation due to some particular profession, as it is normally assumed that the health care practitioners might also at a greater risk of acquiring the disease. Among males the highest prevalence of disease was observed in jobless individuals i.e., 7% . Earlier, Bari et al . (2001), reported form Rawalpindi and Islamabad, Pakistan that a total 30% of the studied cohort of patients consisted of service providers, while 28% of them were government servants, there were 19% businessmen and 16% of professionals, while 7% of the considered cases were jobless individuals. The results were similar to that of the control group,

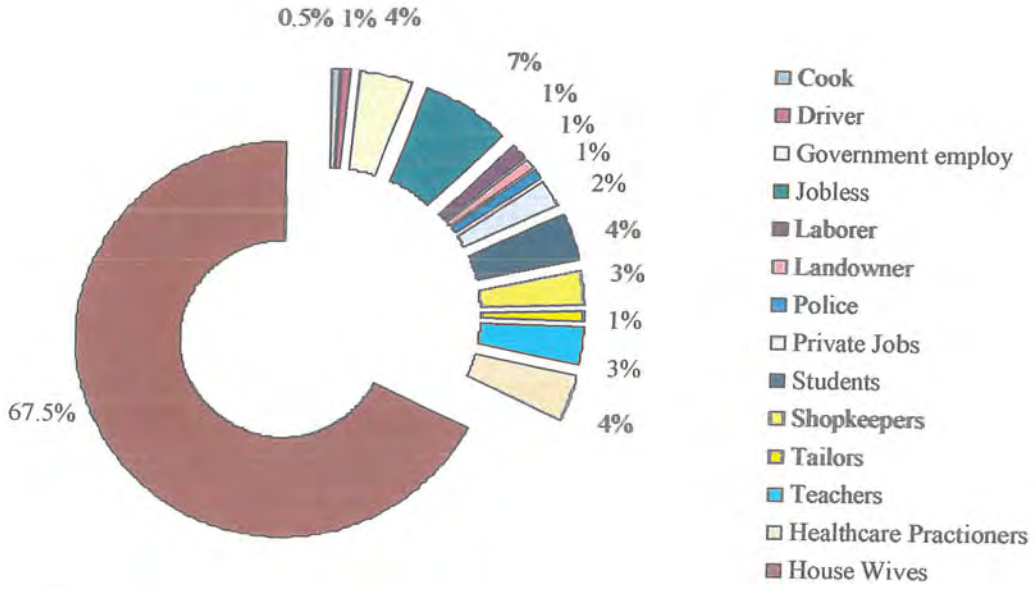


Fig. 4.1.9: **Occupations of the selected patients.**

indicating that no substantial risk of HCV transmission from infected patients to the various professional groups, although there were reported cases of anti-HCV antibody among the physicians and paramedical staff (Thomas et al. 1993 and Montella et al. 2005).

4.1.9 RISK FACTORS FOR HCV TRANSMISSION IN THE STUDIED PARTICIPANTS

It was observed that approximately 13% males rated injections as a main source of acquiring the infection while 91% of the females claimed the surgery to be the cause of infection (Table 4.1.3). Any source of contaminated blood or blood products appeared to be capable of carrying the virus even though the source was indirect e.g. sharing tooth brushes, household commodities like body swapping jewellery, cosmetics, towels, nail cutters and trimmers, pedicure and manicure tools etc. These were exclusive of female patients, while none of male patients responded to these categories. A significant frequency difference was observed towards, blood transfusion, dental procedures, child birth, barber shears and tattoos with chi-square value range 5.91-192.9 (significance level from 0.05-0.001).

There was a non-significant difference on therapeutic injection, needle sticks, shared towels and toothbrushes. The above given figures indicate the high rate (45.1%) of use of therapeutic injections. Unsafe injection practices are common in suburban areas where private practioners and quacks use either non-disposable syringes, injecting equipments are not properly sterilized, or the reuse of the used disposable syringe. The injections were reported by 12.8% males and 87.2% females to be the possible cause of catching the infection. The relationship between unsafe injections and HCV has been observed elsewhere in Pakistan (Khan et al. 2000), to be the highest among other risk factors (Table 4.1.4).

Unsafe therapeutic injections and syringes might have been a known source of HBV and HCV infection (Kahn et al. 2000). As majority of the patients were from

Table 4.1.3: Most common potential risk factors observed in the studied cohort of patients.

Risk factors	Overall percentage (numbers)	Percentage (numbers)		χ^2
		Females	Males	
Therapeutic injections	45.1(507)	87.2 (442)	12.8 (65)	1.89
Surgical procedures	43.6(490)	91 (446)	9 (44)	2.27
Dental procedure	43.4 (488)	88.7 (433)	11.3 (55)	7.26**
Blood transfusion	25.9(291)	93.5 (272)	6.5 (19)	20.07***
Sporadic	17.3(195)	83.1 (162)	16.9 (33)	1.13
Child birth	5.2 (59)	100 (59)	0 (0)	10.55**
Barbers shears/ Beauticians	3 (34)	2.9 (1)	97.1 (33)	192.93***
Shared towels/Tooth Brushes/Razors	1.2 (13)	100 (13)	0 (0)	2.23
Needle stick injuries	1.0 (12)	83.33 (10)	16.66 (2)	1.73
Tattoos	0.4 (5)	60 (3)	40 (2)	8.39**

d.f=1; **p<0.01; ***P<0.001

Table 4.1.4: Age groups wise distribution of different risk factors.

Age groups	Risk factors									
	Therapeutic injections	Surgical procedures	Dental procedures	Blood transfusion	Sporadic	Child birth	Barber shears/ Beauticians	Shared towels/tooth brushes/Razors	Needle stick injuries	Tattoos
19 <	4.2 (21)	1.0 (5)	1.0 (5)	2.1(6)	7.3 (14)	0 (0)	14.7 (5)	7.7 (1)	16.66 (2)	20 (1)
20-29	12.2 (61)	7.6 (37)	6.5 (31)	5.2 (15)	13.0 (25)	10.2 (6)	14.7 (5)	7.7 (1)	8.33 (1)	40 (2)
30-39	21.7 (108)	26.5 (129)	23 (110)	26.4 (76)	29.0 (56)	39.0 (23)	29.4 (10)	38.5 (5)	8.33 (1)	20 (1)
40-49	49.2 (245)	51.5 (251)	52.7 (252)	52.8 (152)	36.3 (70)	42.4 (25)	29.4 (10)	30.8 (4)	66.66 (8)	0 (0)
50-59	12.0 (60)	12.5 (61)	15.3 (73)	12.5 (36)	14.5 (28)	8.5 (5)	11.8 (4)	15.4 (2)	0 (0)	20 (0)
60>	0.6 (3)	0.8 (4)	1.5 (7)	1.0 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
χ^2	15.24	38.68	53.73	21.57	13.40	8.37	12.62	2.02	7.02	9.59

Total number of considered study participants was 1108 for the above mentioned risk factors.

low socio-economic level and suburban area; they were unable to visit the authorized health care physicians. It might be due to their visits to untrained and unauthorized physicians and street doctors having no practice licences. Moreover, Pakistan is a country where washed and repacked syringes are being frequently sold illegally with labelling them as new syringes and injections (Ahmed 2004b). These results were similar to the study on unsafe injections in low-income country by Kermode (2004). At government level, ministry of health have conducted a nationwide survey and concluded that 72% therapeutic injections and 50% of immunization injections were consider to be unsafe and potentially dangerous (Ahmed 2004a, 2004b). Further, it was reported that 73% hepatitis C infections are due to newly idetified risk factors with unexplained modes of transmission (Raja and Janjua 2008).

HCV surviveillance may be improved by the recognition of multiple risk factors infection (Karmochkine et al. 2006). Siddiqui et al. (2002) reported that almost 48% of private health practitioners and 22% of public health providers used atleast one injectable drug in their prescription ($p<0.0001$). The studied participant, both males and females were unaware of the importance of blood as a powerful media for the transmission of HCV, disease consequences, and outcomes of the chronic HCV infection. Knowledge regarding the severity of this silent killer and potentiality to transmit this virus to healthy individuals was scarce.

Lack of screening of blood before 1990, has caused a tremendous spread of the disease and a continuous threat in the form of increasing disease burden in coming years (Zein 2000). As majority of the cases in present study are in the age groups ranged 30-59 years, it indicated that they might have caught the infection in 1980s and early 1990s. The lack of serological screening in the hospitals prior to surgery might have been one of the factors that increased the chances of disease transmission as a major risk factor in the blood transfusions 25.9 % collectively (Table 4. 1.3). Blood transfusion recipients were mainly the females (93.5%) than the males (6.5%), $p<0.001$.

Pakistan is a country still facing the problems related to transfusable-screened blood for the patients. Reasons might be the poorly equipped resources, weak

infrastructure, untrained staff, increasing power breakdowns and ineffective screening of blood donors (Akhtar and Moatter 2004a, Akhtar and Moatter 2004b and Aslam and Syed 2005). Masood et al. (2005) reported almost same results from other areas of Pakistan where the blood transfusions were the apparently major cause of disease spread. Blood transfusions reported along with multiple risk factors like surgery, dental procedures and therapeutic injections, was the highest in ages 40-49 ($p<0.001$).

Collectively the surgery, as a risk factor, was 9% in males and 91% in females, $p<0.001$ (Table 4.1.3). When surgery was viewed as a multiple risk factor, it was significantly high in both genders ($p<0.001$) and in the married participants, ($p<0.001$) (Table 4.1.5). Almost similar results were reported by Chaudhari et al. (2005), where 11% of the surgery patients had HCV antibodies present in the blood and it was observed that majority of the cases (62.50%) were female patients. Their ages ranged 50-60 years and this high rate of HCV reported in female patients was attributed to the births conducted in unhygienic conditions, by traditional birth attendants, making the females vulnerable to the virus, unfortunately, in Pakistan, about 80% of the deliveries are performed in this way. Studies performed by (Chaudhary et al. 2007a, b), were in some disagreement with the present study where the prevalence of HCV antibody in patients being operated was 8%, but there were more male patients than females.

In patients, high prevalence of HCV was probably due to nosocomial transmission and the patients are at high risk of exposure to blood borne pathogen transmission in surgery. In total, fifty nine females (5.2%) $p< 0.01$ reported childbirth including the caesarean sections as a highest possible risk factor. Similar risk was also observed in dental patients, as haemorrhaging is a common phenomenon during treatment of these patients. As road side dentist are common in rural and suburban areas where there is no concept of sterilization of the equipments used in the treatment. Most of the HCV positive patients are treated as usual patients unknowingly. The association of HCV with dental procedures as a risk factor was significant, ($p <0.01$) 11.3% in males and 88.7% in females (Table 4.1.3). Married individuals 93.4% were probably at a higher risk of acquiring HCV, $p<0.001$ (Table 4.1.5) and peaked at 40-49 years of age by 2.7% ($p <0.001$). A similar trend of strong relationship between dental procedures and the spread

Table 4.1.5: Frequency distribution of risk factors according to marital statuses of patients.

Risk factors	Percentage (numbers)		χ^2
	Married	Unmarried	
Therapeutic injections	85.6 (434)	14.4 (73)	1.05
Surgical procedures	93.5 (458)	6.5 (32)	34.06***
Dental procedure	93.4 (456)	6.6 (32)	33.66***
Blood transfusion	93.1 (271)	6.9 (20)	13.87***
Sporadic	78.5 (153)	21.5 (42)	14.12***
Child birth	100 (59)	0 (0)	9.51**
Barbers shears	79.4 (27)	20.6 (7)	1.65
Shared towels/Tooth Brushes/Razors	84.6 (11)	15.4 (2)	0.05
Needle stick injuries	75.0 (9)	25.0 (3)	2.12
Tattoos	60.0 (3)	40.0 (2)	3.13

d.f=1; **p<0.01; ***P<0.001

of HCV was found in another study (La Torre et al. 2003).

There might be some other biologically modes of HCV transmission including cosmetic procedures and cultural practices. Barbers as a main source of catching the infection were reported by 97.1% of the males ($p<0.001$), the results were concordant with the studies by Bari et al. (2001), where exposure to barbers had a strong relationship with HCV acquisition. Barbers in the third world countries are usually unaware of the concept of personal hygiene and the concept of transmission of blood born pathogens. Therefore they do not either use separate tools like razors, blades and scissors, nor do they properly sterilize them. At times microtrauma on the skin causes the natural barriers to be ruptured and eventually provides a space for virus to enter (Janjua and Nizami 2004 and Raja and Janjua 2008).

Majority of these exposed patients were married (79%), the results were statistically non-significant. Another factor tattooing (Table 4.1.3) was the least reported risk factor, reported by 3 males and 2 females only, (0.4%) $p<0.01$. It is also reported by Liao et al. (2006) that in chi square test ($p=0.225$), there was no strong association between tattooing and HCV infection. The results reported by Perez from Puerto Rico, do not agree with the present study as 34.2% of the participants in that study reported the history of tattooing. The reason might be the difference in religious and cultural practices (Perez et al. 2005). Sharing the toothbrushes was reported only by 13 female patients and none of the male study participant, but the results were non significant statistically and mounted no relationship of tooth brush sharing with the spread of disease. On the other hand in Pakistan, several others risk factors have also been reported for the transmission of HCV infection like ear piercing and dialysis, tattooing, circumcision and other surgical and dental procedures (Muhammad and Jan 2005, Butt et al. 2003 and khokhar et al. 2003a, 2003b).

Earlier, a study by Lock et al. (2006) showed the contamination with HCV RNA detected in a large number of toothbrushes used by HCV patients. In another report, Al-Naseer et al. (1992) stated that toothbrush sharing is a common practice in the families of low socio economic status in South East Asia including Pakistan. The results are also

supported by the previous study by Jafri et al., (2006). It is difficult to estimate the percentage risk factors ascribe to viral infections as the number of cases was small and none achieved even the univariable significance level. The results were also in accordance with the findings of Lwin et al. (2007), where no clear rout of acquisition of the infection was established. There was very little evidence of the tattooing association with HCV, and the family history. On the other hand a few reports from Pakistan are not in agreement with the given study, as they report cultural practices of ear piercing and tattooing, and other factors like sharing of contaminated needles in IDUs and sporadic sources to be involved in the transmission of infection (Sheikh et al. 2003, Janjua and Nizami 2004, Hamid et al. 2004, Luby et al. 2005 and Muhammad and Jan 2005).

4.1.10 INTRAFAMILIAL HCV IN THE STUDIED COHORT OF HCV POSITIVE INDEX CASES

In order to find the pattern of distribution of HCV infection, and the intrafamilial contacts in the house holds of the index cases, the data was taken from the patients. The first perspective in this study was the HCV infected parents of the index cases (Table 4.1.6). The number of infected mothers of both male and female patients was higher than the number of their infected fathers. More female patients had, in turn; a higher number of HCV infected mothers than the male patients.

The results were contradicted with the ones reported by Akhter et al. (2002), where the seroprevalence of HCV among the fathers and mothers of male index cases was substantially higher than the female participants. Married patients (n=40) had a higher ratio of HCV positive parents than the unmarried subjects (n=25) (Table 4.1.6). Just like the results of the study of the patients with the infected parents in which a trend in female patients having more affected mothers was found, married and unmarried subjects with infected parents showed that the number of mothers of both married and unmarried individuals was higher than their infected fathers, (46 ratio19) as $p<0.001$ (Table 4.1.6). The data showed that the female patients had a much higher number of infected offspring as compared to their counterparts. On the same note, all the HCV

Table 4.1.6: Demographic characteristics and the relationships of family members affected in studied patients.

	Relationship of the affected individuals with affected relatives											
Demographics	**Father**	**Mother**	**Son**	**Daughter**	**Brother**	**Sister**	**Husband**	**Wife**	**In-laws**	**Uncle /Aunts**	**Cousins**	**Others**
Gender												
Male	4	5	1	0	4	4	0	11	1	1	1	1
Female	15	41	7	8	22	28	94	0	17	12	23	22
χ^2	2.22	0.0	0.02	1.005	0.61	0.10	0.0	0.0	0.56	0.14	1.23	1.11
Age in years												
< 19	2	7	0	0	0	0	1	0	0	1	0	1
20-29	5	11	0	0	2	4	3	1	0	6	4	3
30-39	6	10	0	1	4	6	23	1	7	2	7	4
40-49	4	16	6	3	15	16	54	4	6	4	9	12
50-59	2	2	2	4	4	5	13	5	5	0	4	3
> 60	0	0	0	0	0	1	0	0	0	0	0	0
χ^2	10.41	31.28***	5.06	9.27	2.87	8.48	13.93	1.72	8.99	18.15	3.19	0.53
Marital Status												
Married	10	30	-	-	24	28	94	11	18	7	22	22
Single	9	16	-	-	2	4	0	0	0	6	2	1
X^2	21.88***	24.23***	-	-	0.69	0.004	0.0	0.0	0.0	13.58	0.48	1.64

***p<.001

positive daughters were of female subjects, and in turn, outnumbered the infected sons of both the male and female patients (Table 4.1.1, 4.1.6). The prevalence of the HCV in the siblings of the subjects showed the female subjects had more infected siblings than the male subjects, and that the number of infected sisters was higher than the number of infected brothers. The subjects with the higher number of infected siblings were aged 40-49 years and majority of them were married (Table 4.1.6). Kim et al. (1998) reported from Korea, that the prevalence of HCV infection was very low in other family members of patients where familial clustering did not appear to occur.

The prevalence of HCV infection in married couples indicated that more female patients had infected spouses than the male subjects. Taking the ages of married subjects into account, the highest number of patients, both males and females with infected spouses, fell into the 40-49 years age group ($p< 0.05$). In total, 246 patients recorded 315 family members to be infected by HCV. The overall HCV prevalence among household contacts was 28%, which was higher than the findings of other studies that reported HCV seroprevalence to be 16% (Pasha et al. 1999), and 20% (Akhtar et al. 2002). The HCV prevalence in parents was 5.7%, siblings 5.1%, offspring 1.4% and spouses was 8.7% respectively, which showed a higher prevalence among spouses. The results correspond to the studies by Minola et al. (2006) and Guadagnino et al. (1998) that reported the highest prevalence among spouses was high compared to other household contacts i.e., 13.8% and 11.3% respectively. Spousal transmission was low 2% in a study by Tahan et al. (1999). So, the sexual route in the transmission of the virus is still controversial.

Study of the possible risk factors showed that for the subject with the highest percentage of infected family members (Table 4.1.6, 4.1.7), mostly are mothers and sisters, but no convincing evidence for the interspousal transmission, however, risk of disease acquisition might be the household contacts. Earlier, it has been reported in a study (Boonyarad et al. 2003) from Thailand that interspousal transmission of HCV infection seemed to be very rare. The index cases, with a high number of infected immediate family members, were unaware of their own risk factors of infection. The results strongly indicated that it was parenteral exposures rather than interfamilial contact which was a possible risk factor, because not a single patient had reported belief that

Table 4.1.7: Chi-square analysis of the association between the risk factors and HCV history in the families of affected individuals.

Relation with index cases	Risk factors involved																		
	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9	RF10	RF11	RF12	RF13	RF14	RF15	RF16	RF17	RF18	χ^2
Parents																			
Father	4	1	2	1	0	5	1	1	0	0	0	0	0	2	1	0	0	0	11.07
Mother	5	1	1	0	1	7	3	1	2	2	0	1	0	3	5	0	3	4	24.91
Offsprings																			
Son	1	0	0	0	0	3	0	0	0	0	1	0	0	0	0	1	0	1	17.91
Daughter	0	3	1	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	33.12*
Siblings																			
Brothers	1	0	2	0	1	2	1	1	0	3	1	0	1	2	1	1	1	1	13.44
Sisters	2	1	5	0	1	6	0	4	0	4	0	1	0	1	0	0	1	3	16.01
Spouses																			
Husband	10	2	3	3	0	16	3	11	2	3	1	0	3	7	7	4	0	10	31.77
Wife	1	0	0	1	0	2	0	0	0	2	0	0	1	0	1	0	0	2	19.79
In-laws	1	2	3	0	0	0	2	1	1	2	1	0	0	2	0	0	0	1	19.33
Uncle/Aunt	0	0	4	0	1	2	1	1	0	1	0	0	0	1	0	0	0	0	14.33
Cousins	1	0	3	0	1	6	0	0	0	3	0	0	0	2	1	0	1	1	14.33
Others	1	2	7	2	1	0	1	0	0	1	0	1	0	0	0	0	0	0	43.74

d.f= 17; *p< 0.05

RF1 Injections
RF2 Surgery
RF3 Dental
RF4 Blood Transfusion
RF5 Barber Shears
RF6 Unknown
RF7 Injection, Surgery
RF8 Injection, Dental
RF9 Injection, Blood Transfusion
RF10 Surgery, Dental
RF11 Surgery, Blood Transfusion
RF12 Surgery, Child Birth
RF13 Dental, Blood Transfusion
RF14 Injection, Surgery, Dental
RF15 Injection, Surgery, Blood Transfusion
RF16 Injection, Dental, Blood Transfusion
RF17 Surgery, Dental, Blood Transfusion
RF18 Injection, Surgery, Dental, Blood Transfusion

he/she could have been infected by sharing personal articles at home or by documented blood exposure. The majority of subjects (19%) with infected spouses did not know that what the possible risk factors of their own infection are. These results were backed by a study that 30-50% of HCV patients and their infected spouses could not find a single risk factor for their infection (Irfan and Arfeen 2004). Injections, as the main parenteral exposure, were recorded the highest known risk factors among patients with infected spouses. Similarly, the majority of patients with infected relatives recorded dental procedures as a single highest risk factor, followed by a combination of surgery and dental procedures as well as injections, and dental procedures (dichotomous factors) to be the most probable rout of disease acquisition. The results were not in accordance with the ones reported by Akhter et al. (2002), who reported no significant difference in the HCV seropositive proportions of house hold contacts by age, educational level and the type of relationship with index cases.

It has been found that the patients with infected family members were dominated by 8.7% patients with infected spouses. The data on the interfamilial spread of HCV may have been underestimated but it would be wrong to assume that the major cause of infection was sexual transmission just on the basis of this data. Sexual transmission is difficult to prove since interfamilial transmission might cause lesions and HCV transmission between spouses (Tengan et al. 2001). Caporoso et al. (1998) found that spouses of HCV positive cases were more infected than other family members; and rate of infection was directly proportional to the increasing age. While large family size was not supposed to be associated with the increased risk of infection. It is important to note that interfamilial spread of HCV might be caused by biological fluids. The existence of HCV infected saliva in infected relatives has been found by Mastromatteo et al. (1996), which suggested infected saliva to be one of the causes of interfamilial spread of HCV. Many researchers, however, have pointed out that parenteral exposure is the most common risk factor (Memon and Memon 2002), which coincides with the results of present study.

4.1.11 GENOTYPE DISTRIBUTION OF HCV IN PATIENTS FROM SUBURBAN RAWALPINDI

Genotypes distribution had almost the same patterns in males and female participants with genotype 3 as a most commonly observed genotype of the study. The pattern of distribution of genotypes in different age groups is given in the figure 4.1.10. Age group12-19, showed maximum reported cases with genotype 3, while a significant number of the patients were untypable in this age group by RFLP method. Genotype 4 was found first time in suburban Rawalpindi District of Pakistan, however there was no reported case of genotype 1 or 2 in age group 12-19. In age group 20-29 most prevalent genotype in males was genotype 3 and the second most common was the genotype 1.

Whereas the incidence of genotype 4 and untypable genotypes was the same in 20-29 years age group. Same pattern was observed in age groups 30-49, where genotype 3 was followed by genotype 1 and number of untypable cases was more than the cases of genotype 4. In age group 50-60, only two genotypes were observed namely genotype 3 and 1. However, no case of genotype 2, 4 or untypable genotypes was reported. The results of the study by Lwin et al. (2007), were strongly in accordance with the present study. Most of the HCV positive patients were with 20-39 years and 40-59 years age groups i.e., but the prominent genotype there was 3b rather than 3a. There was a difference of genotype distribution in males and females where genotype 1b and 3a were the dominant ones in males and 6n in females (Nafeh et al. 2000).

The female participants were, though in larger numbers, but had almost the same genotypes distribution patterns. The age group 12-19 in case of female participants was carrying just two representative genotypes namely genotype 3 and untypable genotypes (Fig. 4.1.10). In age group 20-29 the most prominent genotype was 3 and second most reported genotype was genotype 1. A few of the participants had untypable genotypes as well while there was no reported case of either genotype 2 or 4 in this age group. The largest group was age group 40-49 and the second largest group was age group 30-39. The genotype distribution pattern was similar in age groups 30-39, 40-49 and 50-59, where a large number of the patients were suffering from genotype 3. The second most

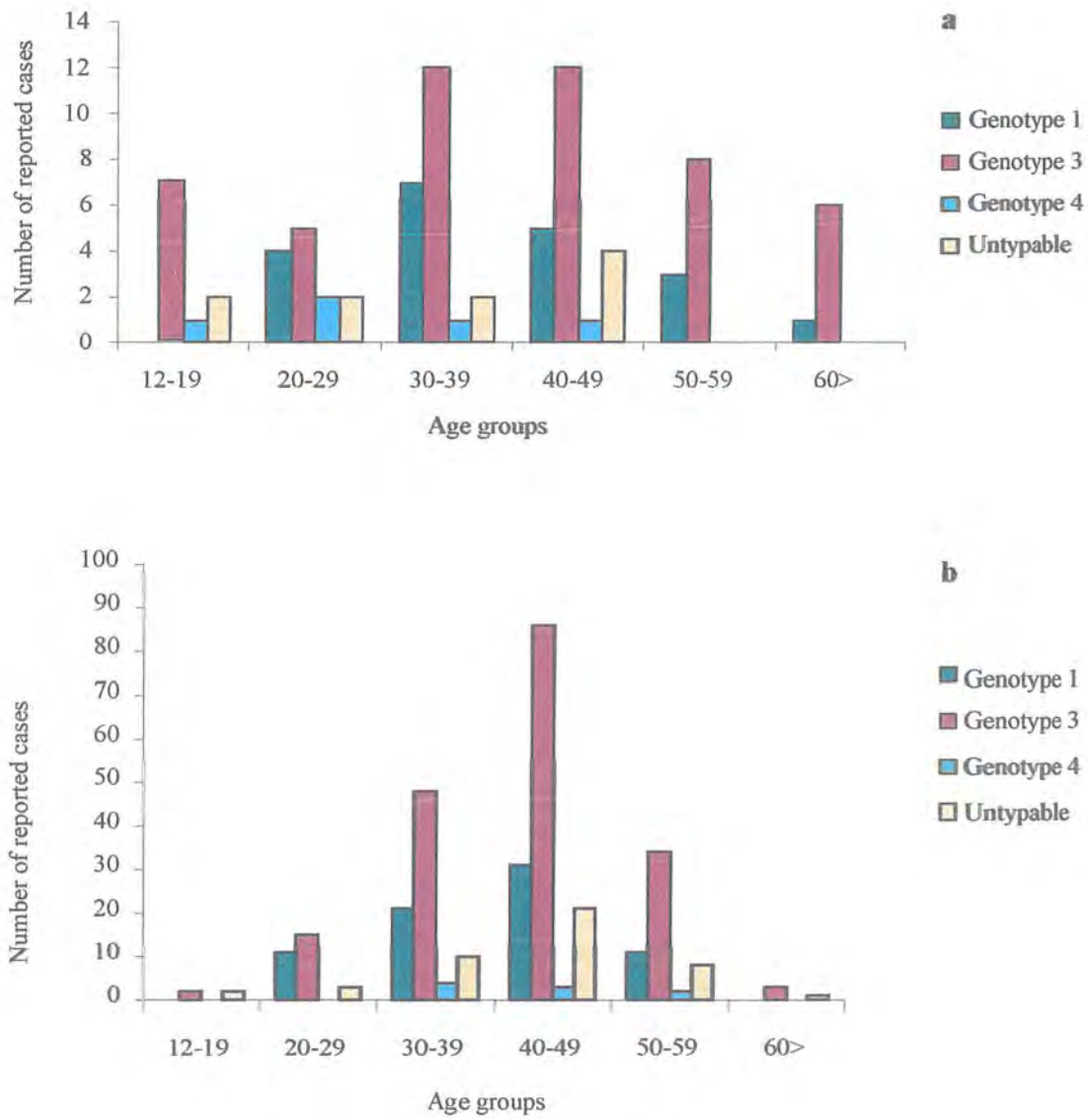


Fig 4.1.10: Genotype distribution pattern in different age groups of males (a) and females (b).

prevalent genotype was the genotype 1, and a considerably large number of untypable cases. The least represented age group with ages 60 years and above had either genotype 3 or untypable genotype cases.

There was a little bit difference in the prevalence of the genotypes in different age groups. The reason for the difference might be the presentation of the group as a whole i.e. the overall more representative groups i.e. in age ranges 30-50 were the most diverse groups with respect to genotype diversity. Another study also enlightened the facts with dynamic distribution of genotypes in age groups 30-50 years (Ayesha et al. 2009). According to a recent study, in Pakistan the observed genotypic distribution were 3a (49.05%), 3b (17.66%), 1a (8.35%), 2a (7.52%), 1b (3.01%), 4 (1.49%), 3c (0.75%), 2b (0.80%), 5a (0.18%), 1c (0.15%), 6a (0.12%), 2c (0.09%) and mixed infection (4.80%) (Idrees and Riazuddin 2008). It has been confirmed by Idrees (2008), Idrees et al. (2008) and Sarwat et al. (2008) that the most prevalent genotype in Pakistan is 3a with the rate of 50% followed by genotype 3b and 1a, respectively.

Among neighbouring countries of Pakistan different kinds of genotypes are available. In China, 1b is the most prevalent HCV genotype and other multiple genotypes are co-prevalent specially genotype 1b, 2a, 3b and subtypes of genotype 6 (Lu et al. 2005). However, in India genotype 3 is a predominant circulating genotype in its population (Kavita et al. 2003 and Chaudhuri et al. 2005). The results of present study somer elatedness with other countries as well. Thailand has almost same genotype distribution as reported from Pakistan from this study, where subtype 3a is the most common genotype followed by subtype 1b and 1a (Kanistanon et al. 1997 and Kyi et al. 2002), though the country is not bordering Pakistan (Apichartpiyakul et al. 1994, Greene et al. 1995, Mellor et al.1995, Doi et al. 1996 and Chinchai et al. 2003). Bangladesh, a country of South-East Asia has also the genotype 3 with subtype b as a major subtype (Ohno et al. 1997).

4.1.12 BINARY LOGISTIC REGRESSION ANALYSIS OF OCCURING GENOTYPES IN THE STUDIED PATIENTS

Binary logistic regression analysis of specific risk factors for genotypes was applied to find statistical significance in terms of odd ratios (OR) and confidence interval (CI), where genotype 1a showed statistically no significance between risk factors and the genotypes (Table 4.1.8, 4.1.9) . Genotype 1b, when analyzed using binary logistic regression analysis for few prominent and specific risk factors, showed statistically no significant association except for blood transfusion and dental procedures (OR=3.46, p value 0.054) . Further, binary logistic regression analysis of specific risk factors for genotype 3a showed no statistically significant association between risk factors and the genotypes (Table 4.1.10).

The statistical analysis of Genotype 3b, by binary logistic regression analysis for prominent and specific risk factors, showed a statistically significant association between risk factors and the genotypes (Table 4.1.11) where surgery and dental procedure had a significant association with the spread of genotype 3b (OR=4.99, 95% CI= 1.62-15.34, p value 0.005) . Surgical procedures in wards and theatres are important modes of transmission of HCV. Contaminated needles, surgical equipments, surgical disposables, blood transfusions, and self pricks during the procedure can be the cause of transmission of HCV from patient to patient and even to the doctors and para medical staff and nurses (Chaudhuri et al. 2005 and Chaudhary et al. 2007a, 2007b). Analysis of specific risk factors for genotype 4 and untypable genotypes showed no statistically significant association between risk factors and the genotypes (Table 4.1.12, 4.1.13).

4.1.13 RELATIONSHIP BETWEEN THE GENOTYPES AND RISK FACTORS

Probabilities were calculated in terms of percentages, for the 400 patients in which the genotypes were determined. The probability of occurrence of genotype 1a due to some unknown risk factors in the given study was 4% , whereas the chances of occurrence of genotype 3a and 3b due to unknown risk factors were 10% and 2% simultaneously while untypability caused by the unknown risk factors was also reported

Table 4.1.8: Binary logistic regression analysis of the specific risk factors for HCV infection from genotype 1a.

Risk factor	Odd ratios	95% CI	P values
Unknown	0.85	0.28-2.59	0.774
Dental procedure	1.01	0.43-2.35	0.988
Barber shears/ Beauticians	0.90	0.34-2.33	0.820
Injections	0.64	0.28-1.48	0.298
Child birth	0.84	0.14-5.08	0.851
Surgery and dental procedure	0.77	0.20-2.90	0.695
Blood transfusion and dental procedure	0.49	0.15-1.55	0.225

Table 4.1.9: Binary logistic analysis of the specific risk factors for HCV infection from genotype 1b.

Risk factor	Odd ratios	95% CI	P values
Unknown	1.76	0.28-11.11	0.548
Dental procedure	0.90	0.26-3.15	0.875
Barber shears/ Beauticians	1.40	0.39-4.98	0.606
Injections	3.12	0.82-11.86	0.695
Child birth	0.00	0.00	0.999
Surgery and dental procedure	0.00	0.00	0.998
Blood transfusion and dental procedure	3.46	12.20	0.054*

*p< 0.05

Table 4.1.10: Binary logistic analysis of the specific risk factors for HCV infection from genotype 3a.

Risk factor	Odd ratios	95% CI	P values
Unknown	0.86	0.38-1.93	0.715
Dental procedure	0.90	0.49-1.65	0.741
Barber shears/ Beauticians	0.75	0.38-1.49	0.412
Injections	0.85	0.47-1.54	0.594
Child birth	0.37	0.08-1.63	0.190
Surgery and dental procedure	0.55	0.21-1.45	0.226
Blood transfusion and dental procedure	1.63	0.79-3.37	0.186

Table 4.1.11: Binary logistic analysis of the specific risk factors for HCV infection from genotype 3b.

Risk factor	Odd ratios	95% CI	P values
Unknown	1.63	0.49-5.40	0.423
Dental procedure	1.62	0.69-3.80	0.270
Barber shears/ Beauticians	1.73	0.69-4.33	0.241
Injections	1.73	0.75-4.00	0.199
Child birth	4.21	0.81-21.82	0.086
Surgery and dental procedure	4.99	1.62-15.34	0.005*
Blood transfusion and dental procedure	0.24	0.05-1.09	0.064

*p<.005

Table 4.1.12: Binary logistic analysis of the specific risk factors for HCV infection from genotype 4.

Risk factor	Odd ratios	95% CI	P values
Unknown	0.65	0.06-7.13	0.727
Dental procedure	0.40	0.06-2.86	0.361
Barber shears/ Beauticians	0.78	0.07-8.61	0.839
Injections	0.41	0.06-2.90	0.372
Child birth	0.00	0.00	0.999
Surgery and dental procedure	1.50	0.14-16.09	0.739
Blood transfusion and dental procedure	1.01	0.09-11.57	0.995

Table 4.1.13: Binary logistic analysis of the specific risk factors for HCV infection from untypable genotype.

Risk factor	Odd ratios	95% CI	P values
Unknown	0.76	0.24-2.46	0.650
Dental procedure	0.85	0.36-2.00	0.703
Barber shears/ Beauticians	0.86	0.32-2.34	0.772
Injections	0.87	0.36-2.08	0.754
Child birth	1.75	0.34-8.98	0.505
Surgery and dental procedure	0.40	0.08-2.09	0.280
Blood transfusion and dental procedure	0.79	0.27-2.33	0.664

in 3% of the cases. Surgery was associated risk factor in 4% of the cases with genotype 3a and 1% in genotype 1a and untypable cases. Blood transfusion has a negligible effect on the transmission of genotype 3b and 1a and only 1% probability for the occurrence of genotype 3a with reference to blood transfusion exists. Similarly, blood transfusion is negligibly associated with the transmission of genotype 1a, 1b, 3b, 4 and untypable genotypes (Table 4.1.14).

Dental procedures have an 11% association with spread of genotype 3a, while the genotype 3b, untypable genotypes and 1a has a 3% association with dental procedures. There was only 1% chance of the occurrence of genotype 3a when more than a single risk factors were apparently involved in the occurrence of specific genotype, while all other genotypes were statistically non significantly associated with surgery as well as blood transfusion, dental procedures and child birth. Barber shears/ Beauticians, and child birth as independent risk factors had no specific association in the spread of any particular genotype. However, the combined effect of the 2 risk factors i.e., blood transfusion and dental procedure, and dental procedures and therapeutic injections had negligible associations with any of the reported genotypes (Table 4.1.15).

Genotype 3a had only 1% association with a multiple risk factor combination surgery, dental procedure and child birth and 2% probability of occurrence for genotype 3a (surgery and child birth). Surgery and blood transfusion as a combined single risk factor had 1% association with the occurrence of genotypes 3a and 3b and all other genotypes with this combined single risk factor had no significant associations. A risk factor with multi options (surgery, blood transfusion and dental procedure) had only 1% probability for genotype 3a and untypable genotypes while all other genotypes in relation to this particular risk factor had no association at all. Surgery when considered in combination with dental procedure, as reported by many of the index cases had only 1% association with genotype 1a, 3% with genotype 3a and 1% with untypable genotypes. Similarly, surgery in a combination with blood transfusion is also a potential risk factor in the spread of a particular genotype has only 1% association with for genotype 3a as

Table 4.1.14: Probability table for specific risk factors with reference to reported genotypes.

Genotypes	Sporadic	Surgery	Blood transfusion	Dental procedure	Barber shears/ Beauticians	Therapeutic injections	Child birth
1a	0.044 (4%)	0.015 (1%)	0.009 (0%)	0.031 (3%)	0.006 (0%)	0.009 (0%)	0 (0%)
1b	0.009 (0%)	0.003 (0%)	0 (0%)	0.015 (1%)	0 (0%)	0 (0%)	0 (0%)
3a	0.103 (10%)	0.044 (4%)	0.012 (1%)	0.113 (11%)	0.009 (0%)	0.025 (2%)	0.009 (0%)
3b	0.028 (2%)	0.009 (0%)	0.003 (0%)	0.037 (3%)	0.009 (0%)	0.015 (1%)	0 (0%)
4	0.009 (0%)	0 (0%)	0 (0%)	0.009 (0%)	0 (0%)	0.006 (0%)	0 (0%)
U	0.031(3%)	0.015 (1%)	0.003 (0%)	0.031 (3%)	0.009 (0%)	0.003 (0%)	0.009 (0%)

Table 4.1.15: Probability table for multiple risk factors with reference to reported genotypes.

Genotypes	Surgery and blood transfusion	Surgery and dental procedure	Blood transfusion and dental procedure	Dental procedure and therapeutic injections	Surgery and child birth	Surgery blood transfusion and dental procedure	Surgery, dental procedure and child birth	Surgery, blood transfusion, dental procedure and child birth
1a	0.006 (0%)	0.018 (1%)	0.003 (0%)	0.003 (0%)	0.009 (0%)	0 (0%)	0 (0%)	0.003 (0%)
1b	0.003 (0%)	0.006 (0%)	0.003 (0%)	0 (0%)	0.003 (0%)	0 (0%)	0.006 (0%)	0.006 (0%)
3a	0.015 (1%)	0.031 (3%)	0.009 (0%)	0.003 (0%)	0.028 (2%)	0.012 (1%)	0.025 (2%)	0.012 (1%)
3b	0.015 (1%)	0.009 (0%)	0 (0%)	0.009 (0%)	0 (0%)	0.009 (0%)	0.006 (0%)	0 (0%)
4	0.003 (0%)	0 (0%)	0 (0%)	0 (0%)	0.003 (0%)	0 (0%)	0 (0%)	0 (0%)
U	0 (0%)	0.012 (1%)	0.003 (0%)	0.012 (1%)	0.006 (0%)	0.012 (1%)	0 (0%)	0 (0%)

well as 3b. In the neighbouring countries trade connections and travelling might be a cause of distribution of similar genotype patterns (Lwin et al. 2007).

4.1.14 CHI SQUARE ANALYSIS FOR ASSOCIATION BETWEEN GENOTYPES AND RISK FACTORS

Chi square analysis performed for the association between the genotypes and the potential risk factors showed no remarkable association between the particular genotypes and any of the risk factors. It was observed that all genotypes are independently associated with the risk factors (Table 4.1.16). This non-significant association indicates that in Pakistan, from this study, it is not possible to conclude any genotype specific risk factor involvement in the spread of disease. Almost all of the risk factors had an equal association with all of the reported genotypes.

The similarity of results was found in the present study with the previous reports from Pakistan and its neighbouring countries like Iran and India where there is a trend of prevalence of genotype 3 and its subtypes while the genotype 2 is very rare. Genotype 1 is the prevalent genotype in Pakistan, India and Iran (Khokhar et al. 2003b, Chowdhury et al. 2003 and Kabir et al. 2006). Some of the studies from Iran fully support the results obtained in this study as there is no report of any case related to genotype 2 in Iran as well (Zali et al. 2000, Elahi et al. 2003 and Samimi-Rad et al. 2004). The reason of this relatedness between these two countries might be the mass migrations between these countries, but further research is needed to conclude anything. When roots of HCV transmission were linked with specific genotypes the results of present study were observed to be in accordance with the already reported studies that most of the patients seeded to have multiple routs of contamination which limits the conclusion on relationship between root of contamination and genotype (Kabir et al. 2006). Genotype 3 is generally correlated with intravenous drug use and genotype 4 with patients undergoing haemodialysis or commercial barbers. But, the present study did not find any specific root of transmission for any genotype. Likewise, there was no difference in genotypes in terms of age and the sex of the patients. The results were in disagreement with the studies by Dal Molin et al. (2002) with respect to zero frequency of genotype 2,

Table 4.1.16: Chi-square analysis of the association between the risk factors and prevalent genotypes.

Risk Factors	Genotypes					
	1A	1B	3A	3B	4	U*
Sporadic	14	3	33	9	3	10
χ^2	0.521	0.284	0.001	0.395	0.239	0.003
Surgery	5	1	14	3	0	5
χ^2	0.058	0.216	0.119	0.400	0.881	0.272
Blood transfusion	3	0	4	1	0	1
χ^2	1.679	0.509	0.003	0.108	0.283	0.059
Dental procedure	10	5	36	12	3	10
χ^2	0.393	0.113	0.052	0.007	0.156	0.053
Barber shears/ Beauticians	2	0	3	3	0	3
χ^2	0.032	0.623	0.810	1.005	0.346	1.338
Injections	3	0	8	5	2	1
χ^2	0.001	1.075	0.051	1.467	3.292	1.061
Child Birth	0	0	3	0	0	3
χ^2	0.962	0.340	0.026	0.925	0.189	5.449
Surgery, blood transfusion	2	1	5	5	1	0
χ^2	0.027	0.054	0.300	3.746	0.712	1.981
Surgery, dental procedure	6	2	10	3	0	4
χ^2	0.988	0.242	0.172	0.189	0.786	0.060
Surgery, child birth	3	1	9	0	1	2
χ^2	0.073	0.010	0.398	2.465	0.491	0.031
Blood transfusion, Dental	1	1	3	0	0	1
χ^2	0.001	1.284	0.026	0.925	0.189	0.027
Dental procedure, Injection	1	0	1	3	0	1
χ^2	0.001	0.340	1.101	4.659	0.189	0.027
Surgery, blood transfusion, dental	0	0	4	3	0	4
χ^2	1.764	0.623	0.206	1.005	0.346	3.835
Surgery, blood T, Dental, Child birth	1	2	4	0	0	0
χ^2	0.013	6.491	0.205	1.079	0.220	0.991
Surgery, Dental procedure, Child birth	0	2	8	2	0	0
χ^2	1.925	2.568	1.168	0.012	0.377	1.698

* Untypable

the data obtained was different from those published in USA, Europe and Asia, which shows the different prevalence of genotype 2 (Takada et al. 1993, Pistello et al. 1994, Mellor et al. 1995 and Davis 1999). Nasir (2004) reported from Faisalabad, Pakistan that genotype 3 was the predominant genotype of the area, and there were a considerably large number of untypable genotypes as well and these results are in accordance with the observations of the present study.

4.2 MOLECULAR CHARACTERIZATION AND PHYLOGENY

Like other reported members of family flaviviridae, HCV has certain conserved regions (5'UTR and 3'UTR) and actively mutating HVRs (core, envelop, non structural proteins). The highly conserved region of 5'UTR is almost exclusively used for routine reverse transcription (RT)-PCR detection of HCV. This method is currently the most sensitive and reliable for establishing ongoing infection. In most of the studies the 5'UTR of the HCV genome was targeted for the viral characterization, as it is considered a gold standard for the genotyping and diagnostic purposes (Davidson et al. 1995 and Mc Omish et al. 1994). By nested PCR of 5'UTR it is possible to efficiently predict a comprehensive range of possible electropherotypes with the restriction enzymes and to reliably differentiate these genotypes from each other (Chaudhuri et al. 2005). The 5'UTR also exhibits specific polymorphisms between types and subtypes, allowing classification into six genotypes of HCV genotype 1, 2, 3, 4, 5 and 6 (Smith et al. 1995). Genotyping assays, which have used 5'UTR PCR product include, direct sequencing (Holland et al. 1998), RFLP analysis (Brien et al. 1997 and Mc Omish et al. 1994), and the use of genotype-specific probes (Stuyver et al. 1996).

4.2.1 STUDY DESIGN

A total of 400 randomly selected plasma samples were collected from HCV positive index cases and RNA was extracted and amplified for genotype analysis. The purpose was to characterize the viral populations circulating in the suburban Rawalpindi. Initial characterization was performed using RFLP analysis and advanced characterization by sequencing of the samples that were not resolved (untypable samples) by the RFLP procedure.

4.2.2 RNA EXTRACTION

Viral RNA extraction is a demanding task in terms of expertise in handling HCV and care required while conducting the extraction procedure. Careful handling of HCV was maintained for better yield of HCV RNA and for avoiding any health related risk.

RNA was extracted by modified procedure of Lau (1998, 9). High yield and good quality of RNA are important to ensure the success of further experimentation. The RNA quality and quantity was confirmed by using the spectrophotometry of the extracted RNA. The optical densities (OD) value range of 1.8-2 is considered ideal for an RNA sample (Tataurov et al. 2008). The samples that did not meet these levels were repeated till the required concentration of RNA was yielded.

4.2.3 RT-PCR, NESTED PCR AND AGAROSE GEL ELECTROPHORESIS

HCV being single stranded sense virus is unable to be taken for molecular amplification without making a cDNA. The cDNA synthesis was done by using external forward primer under controlled conditions, for all the samples and stored at -147 °C to prevent its degradation. The cDNA was further processed by two independent rounds of PCR. The results of nested PCR were run on 1.5% agarose, made in 1X TAE buffer and the gel was stained by using ethidium bromide. Positive and negative controls were used whenever the amplification was performed, in order to avoid false positive and false negative results. The results of agarose gel electrophoresis revealed a 256bp fragment as per expectation (Fig. 4.2.1). 100µl of the HCV RNA were amplified for further analysis.

4.2.4 RFLP AND PAGE

RFLP revealed two different sets of patterns for restriction with two different sets of enzymes i.e., *Rsa*I and *Hae*III, *Hin*fI and *Mva*I (Mc Omish et al. 1994 and Davidson et al. 1995). High quality HCV RNA was used for this purpose and a 16 hours digestion with restriction enzymes provided the results.

4.2.5 RFLP USING *Rsa*I AND *Hae*III

RFLP combination of restriction endonucleases *Rsa*I and *Hae*III, when run on 16% PAGE and stained with ethidium bromide, revealed the variation band pattern for different genotypes (Fig. 4.2.2). However, this restriction combination is unable to discriminate between genotype 1 and 5. This can be further resolved by using a

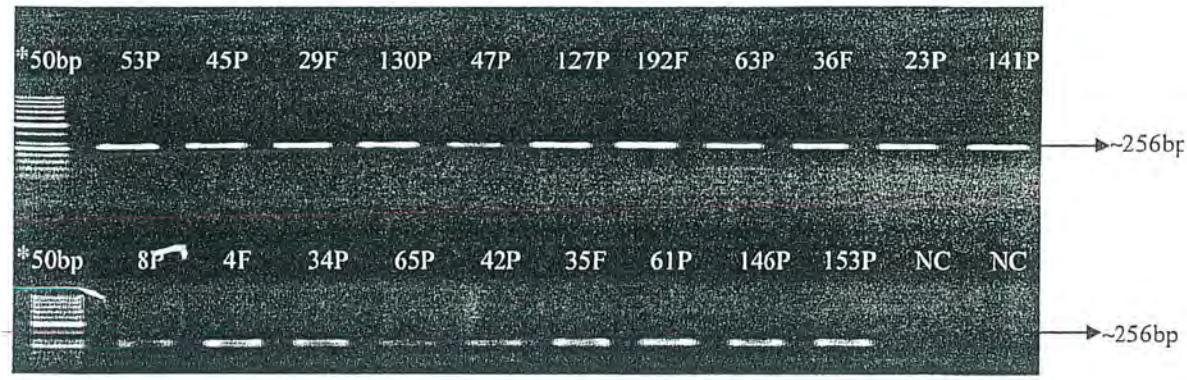


Fig. 4.2.1: Agarose gel electrophoresis of the representative samples of HCV from suburban Rawalpindi, Pakistan. NC stands for negative control of HCV. *(50 bp DNA Ladder; Fermentas)
1.5% agarose gel electrophoresis in 1X TAE.

combination of *Mva*I and *Hin*fI that gives a series of 41bp, 53bp, 63bp and 94bp fragments for genotype 1 and 53bp and 198bp fragment for genotype 5.

4.2.6 RFLP USING *Scr*FI AND *Hin*fI

The second set of restriction enzyme *Scr*F1 and *Hin*fI was able to discriminate five most prevalent genotype i.e., genotype 1, 2, 3, 4, and 6, but this restriction enzyme again was unable to resolve and discriminate genotype 5. Restriction patterns obtained by this set of restriction enzymes are shown in the figure (Fig. 4.2.3). There is only a slight difference in band sizes for genotype 1 and 6 as an insertion of three bases in genotype 6 provides it a slightly different appearance from genotype 1 on electropherotypes (Davidson et al. 1995). Results of RFLP for genotypes of 400 HCV positive patients showed that genotype 3 is the most prevalent genotype of suburban Rawalpindi, Pakistan as already discussed in (Fig 4.1.10).

From analysis of HCV samples, it was found that 60.5% of the samples were the representatives of genotype 3 whereas genotype 1 was 22.5%. Only 2.75% samples were the genotype 4, the genotype reported for the first time from suburban areas of Rawalpindi. Genotype 4 is the most important and prevalent strain of Egypt (Genovese et al. 2005). Only couple of previous studies from Pakistan supported the presence of genotype 4 in 3% and 2.48% respectively of the tested blood samples, in Pakistani population (Idrees et al. 2008 and Iqbal et al. 2007). Among the neighboring countries of Pakistan this genotype 4 was also detected in 7.2% of the Indian patients in a study (Sukanya et al. 2004). Similar changing pattern of genotype 4 distributions in Europe has been reported (Bruijne et al. 2009). One possible reason for the existence of this genotype in Pakistan might be the neighboring country Iran where genotype 4 is a prevalent genotype, which may be due to its geographical location near to Europe and Middle East (Mellor et al. 1995 and Kabir et al. 2006). Pakistan has a centuries old history of religious relationships with Iran, which is the land of spiritual importance as far as Pakistanies are concerned. There is a mass level trade between two countries and this appears to be a major reason for the presence of this genotype in Pakistani population. Though the numbers of reported cases are less but the treatment responses of genotype 4 are

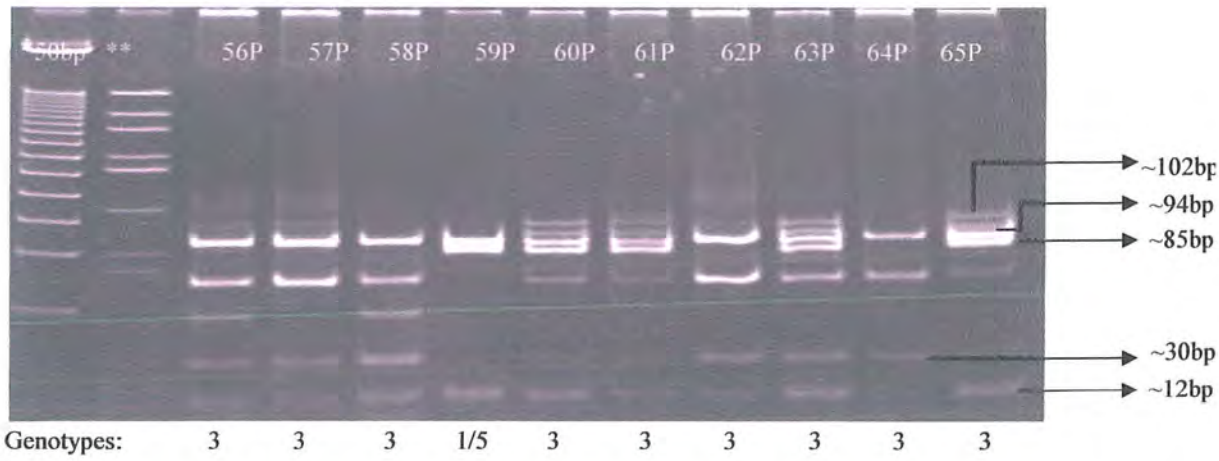


Fig 4.2.2a: RFLP of HCV samples using restriction enzymes *Rsa*I and *Hae*III.
*(50 bp Ladder; D3812 by Sigma Aldrich)
**(Puc 18/HeaIII digest, product No. D 6293 Sigma Aldirch)
16% polyacrylamide gel.

Genotypes	*Hae*III and *Rsa*I band sizes in base pairs												
	9bp*	12bp	23bp	26bp	33bp	44bp	46bp	56bp	58bp	69bp	102bp	114bp	117bp
1/5	■			■		■			■			■	
2	■	■		■		■	■	■	■				
2	■	■		■		■	■					■	
2	■			■			■	■				■	
3	■			■	■					■		■	
3	■		■	■	■		■					■	
4	■			■							■	■	
6	■	■		■		■	■						■

Fig. 4.2.2b: Restriction patterns obtained by restriction enzymes *Hae*III and *Rsa*I. The band patterns obtained for different genotypes are depicted with blue coloured box and the white coloured box shows the absence of bands.
*The figure has resolved upto 12bp bands while 9bp band was monomorphic and was over run for the better resolution of the gel.

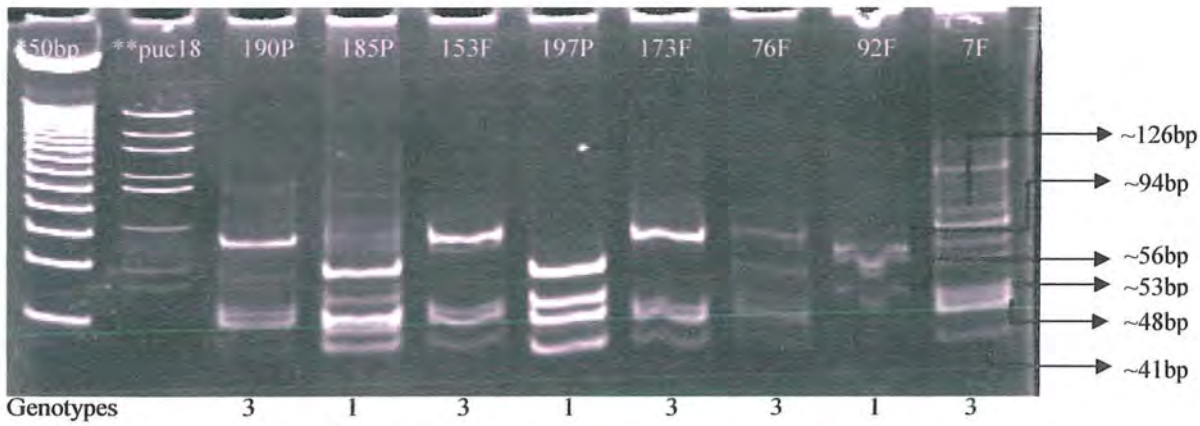


Fig 4.2.3a: RFLP of HCV samples using restriction enzymes *Mva*I and *Hin*fI
*(50 bp Ladder; D3812 by Sigma Aldrich)
**(Puc 18/HealII digest, product No. D 6293 Sigma Aldirch)
16% polyacrylamide gel.

Geno-types	*Mva*I and *Hin*fI Band sizes in base pairs													
	7bp	9bp*	15bp*	16bp*	32bp*	35b9	41bp	48bp	53bp	57bp	94bp	126bp	135bp	183bp
1/5		■	■		■			■	■		■			
1/5			■		■				■	■	■			
2			■				■	■	■		■			
2			■			■								■
2			■					■	■				■	
3			■	■			■		■			■		
4	■	■	■		■		■		■		■			
6		■	■			■		■	■		■			

Fig. 4.2.3b: Restriction patterns obtained by restriction enzymes *Mva*I and *Hin*fI. The band patterns obtained for different genotypes are depicted with blue coloured box and the white coloured box shows the absence of bands.
*9bp, 15bp, 16bp and 32bp fragments were over runned for the better resolution of high molecular weight bands.

controversial as a few studies report that genotype 4 is as less friendly to interferon treatment as genotype 1 (Zein 2000) but another study suggests that the treatment response is as good as any of the treatment friendly genotypes with 24 weeks interferon treatment (El-Zayadi et al. 2005). The samples resulting in the genotype 1 and 3 were further considered for the sub typing as they were further considered for characterization as subtypes a and b. Considerably large numbers of samples (14.25%) observed as untypable and were taken to sequencing for their further characterization.

4.2.7 SUBTYPING OF GENOTYPE 3 USING *Scr*FI RESTRICTION ENZYME

Previous reports from Pakistan had evaluated the HCV genotype 3 (3a and 3b) to be the most prevalent (Akbar et al. 2009). The representative samples of genotype 3 confirmed by restriction digestion with "*Hae*III-*Rsa*I" and "*Mva*I-*Hin*fI" was subtyped using restriction enzyme *Scr*F1 by targeting 5'UTR region of HVC genome. Results obtained after restriction digestion and PAGE indicated that 76.6% samples were genotype 3a and 23.4% were found to be genotype 3b. In previous reports from Pakistan the number of HCV genotype 3 patients was less than the present study 73.85% and 59% (38% 3a and 21% 3b genotype) and 50% respectively (Idrees and Riazuddin 2008, Idrees 2008, Idrees et al. 2008, Iqbal et al. 2007 and Pawlotsky 2009). This high percentage of HCV genotype 3 in the present studied cohort is considered better in terms of response to the treatment regimes or in other words it will be considered a blessing for Pakistan for disease management (being an under developed country with low socioeconomic population status) (Fig. 4.2.4).

In south east Asia, a similar endemicity pattern of genotype 3 was seen, where genotypes 3 and 1, are most prevalent in Bangladesh, India, Iran, Nepal, Thailand, Indonesia and Vietnam (Kavita et al. 2003, Shah et al. 1997, Simmonds 1995 and Tokita et al. 1996, 1995, 1994a, 1994b). The high percentage of genotype 3 in the Gujar Khan area may be attributed on one hand to endemicity of genotype 3 and on other hand to its location in the upper Punjab, Pakistan from where the neighboring countries (Afghanistan, China, India and Iran), are in close geographical proximity. Moreover,

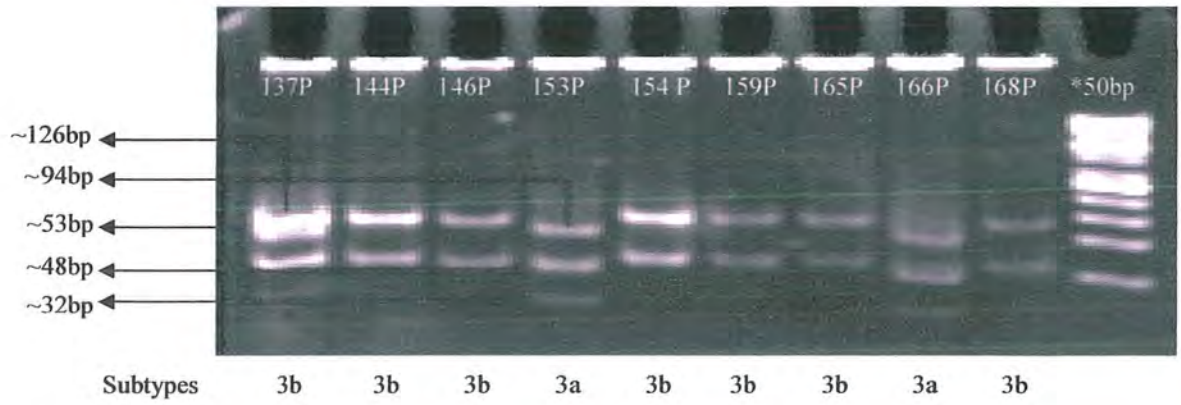


Fig 4.2.4a: RFLP of HCV samples using restriction enzymes *Bme*1390I (*Scr*fI).
*(50 bp Ladder; SM0373, Fermentas).
16% polyacrylamide gel.

Genotypes	*Scrf*I							
	Band sizes in base pairs							
	*9bp	*15bp	32bp	48bp	53bp	57bp	94bp	126bp
3a								
3b								

Fig. 4.2.4b: Restriction patterns obtained by restriction enzyme *Scrf*I. The band patterns obtained for different subtypes are depicted with blue coloured box and the white coloured box shows the absence of bands.
*9bp and 15bp bands were dim and are not resolved in the electropherogram.

there exists a centuries old legendry history of trade and travelling between Pakistan, India, Afghanistan, Iran and Bangladesh as far as general population is concerned (Idrees et al. 2008).

4.2.8 SUBTYPING OF GENOTYPE 1 USING *Fund*III RESTRICTION ENZYME

Subtyping of confirmed genotype 1 samples by two sets of restriction endonucleases *Hae*III and *Rsa*I" and "*Mva*I and *Hin*fI" was carried with the help of using restriction enzyme *Fund*III (Fig. 4.2.5). Of the considered samples, 57.14% were subtyped to be genotype 1a and 42.86% were subtyped as 1b. The subtype distribution percentage in another related study was observed as 7% for 1a and 5% for 1b by Idrees et al (2008). According to the previous reports from Pakistan it is evident that genotype 1 is rated second most prevalent genotype of the country (Akber et al. 2009 and Sarwat et al. 2008). Genotype 1b has a poor interferon response and is not considered a good subtype, as far as its treatment response is concerned. Earlier, it has been reported from Lahore that, only 2.78% samples have genotype 1a and 2.06% were of genotype 1b (Iqbal et al. 2007). Some of the reports revealed that 5'UTR may be less sensitive to subtyping of genotype 1 (subtypes of genotype 1 mix together, make grouping of subtype 1 a difficult task). Other genotypes could be grouped precisely into subgroups and variants using 5'UTR (Salemi and Vandamme 2002 and Son and Van 2010).

The existence of HCV variants containing an adenine at position 235 may affect cluster determination by RFLP. Other authors have demonstrated that, as it is the most conserved region in HCV genome, the 5'UTR may also be useful to identify many of different genotypes by phylogenetic analysis (Alfonso et al. 2001). For genotype assignment purposes, mutated isolates may be classified as genotype 1. It should be considered that the untypable strains disclosed a subtype a/c associated pattern of bands when digested with *Bst*UI. Further phylogenetic analysis did not indicate an association between them and the prototypic genotype 1 subtype a and c strains. Thus, it may be advisable to classify the new HCV isolates showing this abnormal restriction pattern only

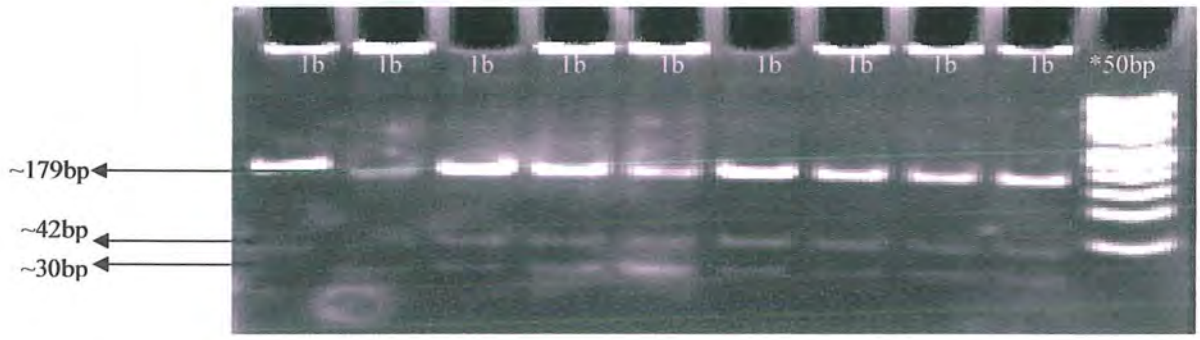


Fig 4.2.5a RFLP of HCV samples using restriction enzymes *Bsh*1236I (*Fund*II)
*(50 bp Ladder; Fermentas).

Genotypes	*Bst*UI			
	Band sizes in base pairs			
	30bp	42bp	179bp	209bp
1a				
1b				

Fig. 4.2.5b: Restriction patterns obtained for different subtypes of genotype 1 by using *Bst*UI. Blue coloured boxes depict the presence of band. While the white coloured boxes show the absence of bands.

as genotype 1, without a subtype label, until this novel genetic lineage is completely characterized (Gismondi et al. 2004).

4.2.9 UNTYPABLE SAMPLES

The facts given in already discussed sections may be involved partly in the untypability of some samples. Untypability of the genotypes is caused by the mutations (Mc Omish et al. 1994 and Davidson et al. 1995) and these mutations may be either point mutations (Transitions and transversions) or they may be insertions, inversions, or deletions and translocations etc. Further, variability may exist naturally in 5'UTR region of HCV. The changes existing or induced with time, in the sequence of this genomic region might induce alteration in the secondary structure of HCV, in turn affecting the functions associated with 5'UTR. The inability of HCV to perform proof reading and its high mutation rate both have made it genetically successful according to Darwinian theory of natural selection (Stumpf and Pybus 2002). The present study rated 14.13% of the studied samples to be either novel variants of the existing genotypes or they might be the representatives of the recombinant forms of mixed genotype. In previous recent report from Lahore, Pakistan there were 14% novel genotypes (Idrees et al. 2008). Therefore, on the basis of these facts, from whole countrywide it can be concluded that genotype distribution is not even in all areas of Pakistan. The rate of distribution of genotypes and their genetic make up varies at sub population levels of the same area.

4.2.10 SEQUENCING OF HCV 5'UTR

Sequencing procedures are very important as they provide direct glimpse as far as viral genotypes are concerned secondly sequencing can be the basis for molecular phylogeny. It is a very important tool exploided in recent years to find the routs of transmission of HCV, treatment responses, mutation sites, secondary protein structures etc. During the present study sequencing was performed for the 52 samples that remained unresolved by RFLP and one representative sample of each genotype 24P (1a), 101P(1b), 172P (3a), 35F (3b) and 17F (4) (Fig 4.2.6).

4.2.11 BLAST ANALYSIS

Blast analysis was performed for all the sequences. The task was to verify the results of sequenced samples that were remained untypable by RFLP analysis. The results of blast analysis provided high sequence similarity scores between the analyzed sequences and already reported sequences of 5'UTR. Percentage similarity of untypable sequenced sample with already reported sequences from blast is mentioned in table (Table 4.2.1). Different web sites used for blast are NCBI http://blast.ncbi./ nlm.nih./gov/Blast.cgi/ and EMBL http://www.ebi./ ac.uk/Tools// blast2// nucleotide.html.

Out of 52 untypable sequences, blast analysis resolved 24.56% genotype 3 descendents, 5.26% and 21.05% subtype 1a and 1b respectively, 14% diversified genotype 4, 3.5% subtype 2k, 1.75% each of 1d and 6v and 19.29% were unresolved novel variants (as they pooled them in a single Cluster), while 8.77% of the samples showed no significant similarity (formed exclusively independent branches on the phylogenetic tree). In Pakistan subtypes 2k, 1d and 6v were identified for the first time in present study. A couple of studies from Pakistan also rated a few of the uncommon genotypes (with reference to Pakistan) including 1c, 2c, 3c, 3k, 4, 5a and 6a (Idrees and Riazuddin 2008 and Khan et al. 2009). In a study from Punjab, Pakistan there was a reported predominance of genotype 3a (81.4%), followed by 9.3% cases of sub type 3b while subtypes 1a, 1c, 1b and 2a were reported between 1-2% ranges (Khan et al. 2009). Every sample was repeated three times for sequencing to eliminate the chances of errors or false results.

4.2.12 SAMPLE SUBMISSION TO GENBANK

All sequenced samples were submitted to GenBank and their accession numbers were allotted. The details are mentioned in the Table 4.2.2.

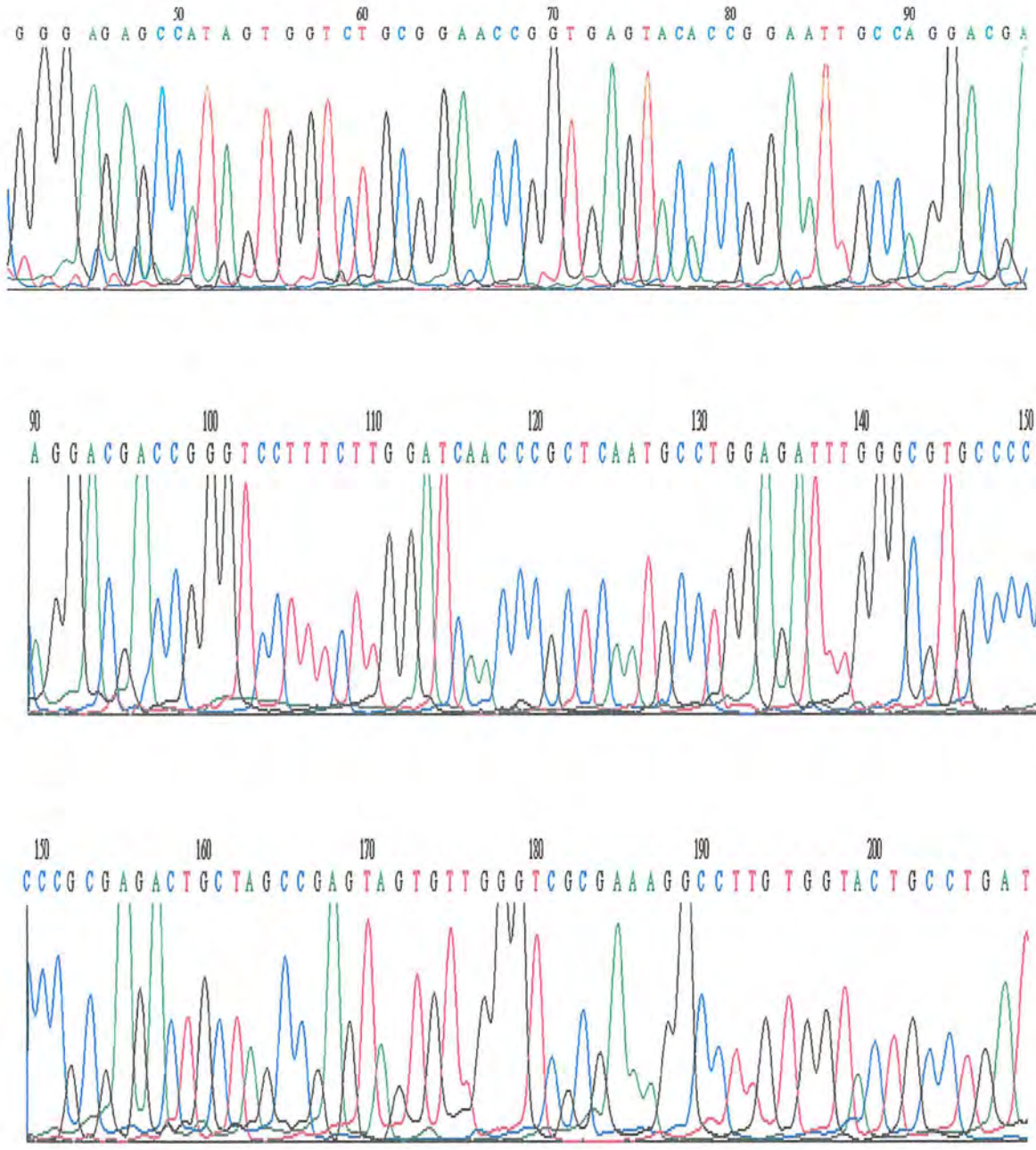


Fig. 4.2.6: Results of sequencing in the form of sharp peaks of Pak-12P, Genotype 4 (Accession number GU208785).

Table 4.2.1: The results of blast analysis representing percentage similarities with already reported sequences and the resolved genotypes.

Sample ID	Reference Accessions	Similarity %	Expected genotype
46F	AB442220	91	1b
53F	EU527916	92	Untypable
154F	AB442222	95	Untypable
155F	EU155333	78	1b
163F	D37839	98	Untypable
167F	DQ67704/DQ67705	91	India (2k/1d)
168F	DQ284931	98	Untypable
169F	EF025301	98	3a
173F	EF025301	88	3
190F	AJ006325	85	4
12P	AJ006325	93	4
20P	AF207762	95	1b
23P	AR027783	99	Untypable
33P	AB492183	98	1b
34P*	AM502673	87	3
37P	EU781830	95	1a/1b
65P	NS		
74P	Ab442222	99	1b
94P	FJ78453	99	1b
98P	AJ006325	96	4
101P*	FJ390396	95	1b
133P	AJ006324	99	4
139P	AM502654	89	3
141P	AF06866	98	3a
145P	AJ006321	95	1b
151P	AJ006325	97	4
156P	DQ284962	90	Untypable
172P*	AB444575	96	3a
180P	AM502673	88	3
181P	AJ006325	87	4
187P	Ab44221	98	1b
194P	DQ674704	83	1d
200P	AJ006318	96	3a
185P	EU781830	96	1b
197F	AF046866	95	3a
65F	NS		
138F	NS		
39P	EU678715	88	Untypable
42P	NS		
130P	AM228894	71	3a
136P	NS		
155P	FJ407092	93	India (3a)
1F	DQ67705	97	India (2k)
4F	DQ67704	98	Untypable

17F*	AJ29158	97	China (4)
35F	AM502650	87	3b
56F	AB444582	98	3a
181F	Z84279	100	Untypable
5P	GQ418245	95	1
17P	AF207772	98	1b
24P*	GQ18254	96	1a
59P	GQ418211	95	1
71P	GQ418238	96	4
126P	GQ418265	97	3
152P	DQ140383	100	Untypable
183P	EU798761	98	6v
190P	AY145944	73	Untypable

NS stands for no significant similarity.
F is sample ID: samples from Fauji Foundation Hospital Rawalpindi
P is sample ID: samples from Pakistan Institute of Medical Sciences, Islamabad.
* Samples with known genotypes 24P(1a), 101P(1b), 172P (3a), 35F (3b) and 17F (4)

Table 4.2.2: Details of submitted sequences and their accession numbers.

Sr. No.	Sequence size in base pairs	Accession number	Sample ID	Collection date
1	244	GU208775	46F	14-Oct-06
2	238	GU208776	53F	14-Oct-06
3	225	GU208777	154F	18-Nov-06
4	188	GU208778	155F	18-Nov-06
5	215	GU208779	163F	22-Nov-06
6	284	GU208780	167F	22-Nov-06
7	216	GU208781	168F	22-Nov-06
8	214	GU208782	169F	22-Nov-06
9	281	GU208783	173F	22-Nov-06
10	219	GU208784	190F	29-Nov-06
11	256	GU208785	12P	15-Jun-06
12	223	GU208786	20P	17-Jun-06
13	216	GU208787	23P	17-Jun-06
14	250	GU208788	33P	20-Jun-06
15	226	GU208789	34P	20-Jun-06
16	223	GU208790	37P	20-Jun-06
17	276	GU208791	65P	24-Jun-06
18	218	GU208792	74P	1-Jul-06
19	225	GU208793	94P	7-Jul-07
20	255	GU208794	98P	10-Jul-06
21	250	GU208795	101P	10-Jul-06
22	218	GU208796	133P	18-Jul-06
23	206	GU208797	139P	21-Jul-06
24	223	GU208798	141P	21-Jul-06
25	226	GU208799	145P	22-Jul-06
26	220	GU208800	151P	22-Jul-06
27	226	GU208801	156P	25-Jul-06
28	222	GU208802	172P	1-Aug-06
29	242	GU208803	180P	7-Aug-06
30	237	GU208804	181P	8-Aug-06
31	226	GU208805	187P	8-Aug-06
32	259	GU208806	194P	15-Aug-06
33	222	GU208807	200P	21-Aug-06
34	254	GU208808	185P	8-Aug-06
35	251	GU208809	197F	6-Dec-06
36	274	GU208810	65F	14-Oct-06
37	261	GU208811	138F	11-Nov-06
38	263	GU208812	39P	21-Jul-06
39	242	GU208813	42P	20-Jun-06
40	275	GU208814	130P	18-Jul-06
41	247	GU208815	136P	21-Jul-06
42	231	GU208816	155P	25-Jul-06
43	218	GU197378	1F	4-Oct-06
44	249	GU197379	4F	4-Oct-06
45	213	GU197380	17F	4-Oct-06

Sr. No.	Sequence size in base pairs	Accession number	Sample ID	Collection date
46	213	GU324081	35F	11-Oct-06
47	245	GU324082	56F	25-Nov-06
48	278	GU324083	181F	25-Nov-06
49	237	GU324084	5P	13-Jun-06
50	230	GU324085	17P	17-Jun-06
51	235	GU324086	24P	17-Jun-06
52	232	GU324087	59P	24-Jun-06
53	236	GU324088	71P	1-Jul-06
54	233	GU324089	126P	17-Jul-06
55	243	GU324090	152P	24-Jul-06
56	214	GU324091	183P	8-Aug-06
57	285	GU324092	190P	15-Aug-06

F is sample ID: samples from Fauji Foundation Hospital Rawalpindi
P is sample ID: samples from Pakistan Institute of Medical Sciences, Islamabad.

4.3 SEQUENCE ANALYSIS

4.3.1 ALIGNMENT USING CLUSTALW SOFTWARE AND PHYLOGENETIC ANALYSIS

The samples were successfully aligned by using online software of Clustal W. Aligned sequences were further used for phylogenetic tree construction using the software MEGA 5 Beta # 7. Phylogenetic trees and dendrograms of sequenced accessions were constructed to find out the evolutionary relationships among the sequences, novel genotypes, sub types and variants and to study the punctuated equilibrium for the studied samples. It is very obvious that both viral and host factors play an ambient role in determining tree topologies. Circular trees with *p-distances* were dragged to discuss the branch topologies and phylogenetic trees were dragged independently with *p-distances* and with 500 repeats boot strap analysis, one of the most commonly used tests of the reliability of an inferred tree Felsenstein's (1985) bootstrap test, evaluation of which is made by Efron's (1982) technique of bootstrap resampling. If for assumption there are "m" sequences, and nucleotides in each are "n", some tree building method will be used to reconstruct a phylogenetic tree. Out of every sequence, with replacement "n" nucleotides are randomly chosen, that gives rise to "m" rows constituting each of "n" columns. In this way a new set of sequences is formed and on the basis of these sequences a tree is then reconstructed by using the same tree building method. Tree topologies of both the trees are compared. Then the differences in interior branch of the computed and bootstrap tree is given a score 0 and the remaining interior branches are computed value 1. This procedure of resampling is repeated both 500 or 1000 times and it is known as the bootstrap value. As a general rule, if the bootstrap value for a given interior branch is 95% or higher, then the branch topology is considered accurate while 75% is considered reasonably good (Nei and Kumar 2000, 260). Bootstrapping was performed to validate the results with bootstrap values above 75 were considered significant. Further dendrograms analysis was also performed to find out the relationship between the sequenced samples.

4.3.2 MERITS OF PHYLOGENETIC ANALYSIS

Phylogenetic analyses are important as they are used to conclude the outcomes when an infection emerges, like an epidemic (Yasmeen et al. 2009) e.g. one such out break in case of HCV was reported, in 1995 in Ireland where all affected female patients received a contaminated anti D immunoglobulin. Sequence similarity of viral genome and viral phylogeny resolved the breakthrough source (Helga and Angela 1995). More over by the help of such analyses one can detect the single source infections and transmission networks as e.g. transmission between the sexual partners (Cavalheiro 2004, Halfon et al. 2001, Irfan and Arfeen 2004,Stroffolini et al. 2001, Tahan et al. 2005 and Yagura et al. 2002), transmission of HCV by vertical source (Jafri et al. 2006 and Newman 1996) transmission from patients to providers and vice versa can be digged out by genotype similarity and strengthened further by sequence similarity (Alter 1997, Ross et al. 2002a and Ross et al. 2002b).

Phylogenetic analysis can also be very useful in classification of the sequenced samples on the bases of their positioning in particular clades. If the lineages position themselves in already known clades, it is an indication of them being a part of that clade. But if they do not position themselves in existing clades and form independent clades they may be classified on those bases as independent genotypes, sub genotypes, variants or quasiaspecies (four levels of genetic variability of HCV). Phylogentic analyses are important because they provide useful information regarding origin of evolutionary process. In case of HCV these trees are important to provide the ancestry and the details of with time emergence of new variants from the existing genotypes and also the endemicity of the particular genotypes in specific areas (Stumpf and Pybus 2002).

4.3.3 PHYLOGENETIC TREES AND DENDROGRAMS OF THE STUDIED SEQUENCES

HCV genotypes and subtypes exhibit complex epidemiological patterns with respect to geographical distribution, prevalence, response to treatment and transmission modes. The recognition of factors responsible for this complex epidemiology is difficult,

but will undoubtedly contribute to a better comprehension of HCV genetic dynamics required to establish a preventive strategy of disease control (Jimenez-Hernandez et al. 2007). The association and evolutionary relationship of the species can be traced by construction of phylogenetic trees (Fig. 4.3.1-4.3.4).

Normally adorn evolutionary trees have data only at the tips and nodes of their branches; the rest is imputation (Gould 1977a). Species are generally stable, changing little for millions of years. This smooth phase is "punctuated" by a rapid burst of change resulting in a new form of life and is called punctuated equilibrium (Gould and Niles 1972, 82) and its merits are, connecting the missing links of evolutionary process. Gould (1977b) emphasized that biological evolution takes place in terms of intermittent burst of activity separating relatively long periods of quiescence, rather than in gradual manner. Extinctions are indeed episodic at all scales and fitness landscape represents the ability of species to survive as a function of their genetic code (Raup 1986). In order to understand the phenomenon of punctuated equilibrium in HCV an unrooted phylogenetic tree of the untypable accessions from suburban areas of Rawalpindi was divided into eight clusters revealing at least three distinct patterns of evolution. Pattern 1 (Fig. 4.3.1) depicted that the accessions GU208815 Pak-136P (Cluster II) and GU208786 Pak-20P (Cluster III) had evolved very earlier during the course of evolution showing stable and smooth pattern of tree topology. GU208799 Pak-145P and GU208793 Pak-94P (Cluster III) GU208805 Pak-187P and GU208794 Pak-98P (Cluster IV), GU324086 Pak-24P and GU324088 Pak-71P (Cluster VI) had a comparatively smooth transition patterns with spurts of relatively rapid changes followed by the longer periods of stasis. The speed of evolution in the species will depend on the pressure of natural selection.

In pattern 2 the twists in the branches for GU197380 Pak-17F, GU197378 Pak-1F, GU208803 Pak-180P, GU208783 Pak-173P and GU208807 Pak-200P (Cluster I), GU208790 Pak-37P, GU208797 Pak-139P and GU324081 Pak-35F (Cluster I) GU324087 Pak-59P, GU324089 Pak-126P, GU208795 Pak-101P, GU208788 Pak-33P, GU208780 Pak-167F and GU208808 Pak-185P (Cluster II), GU208776 Pak 53F, GU208806 Pak-194P, GU324082 Pak-56F, GU208811 Pak 138F

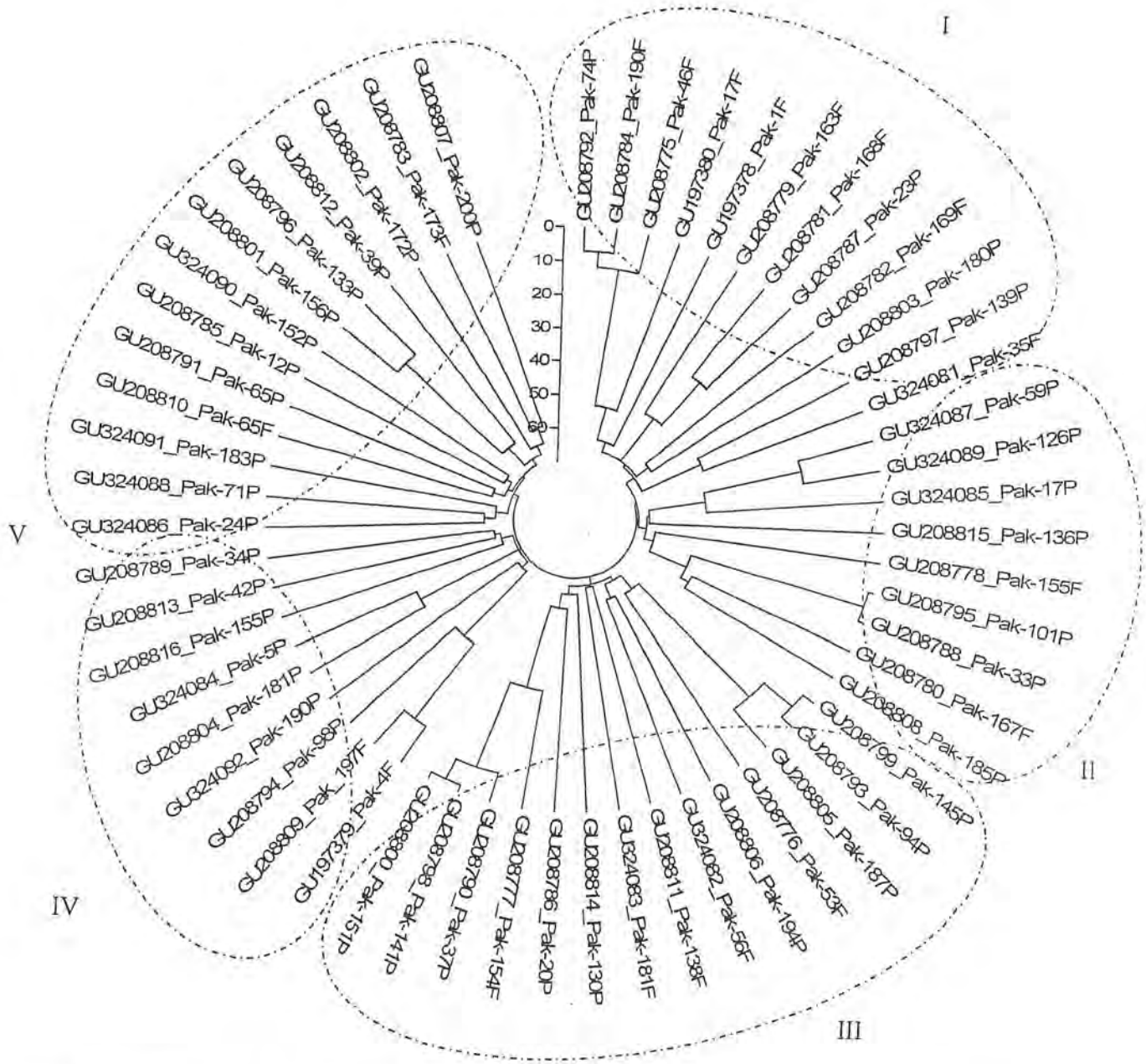
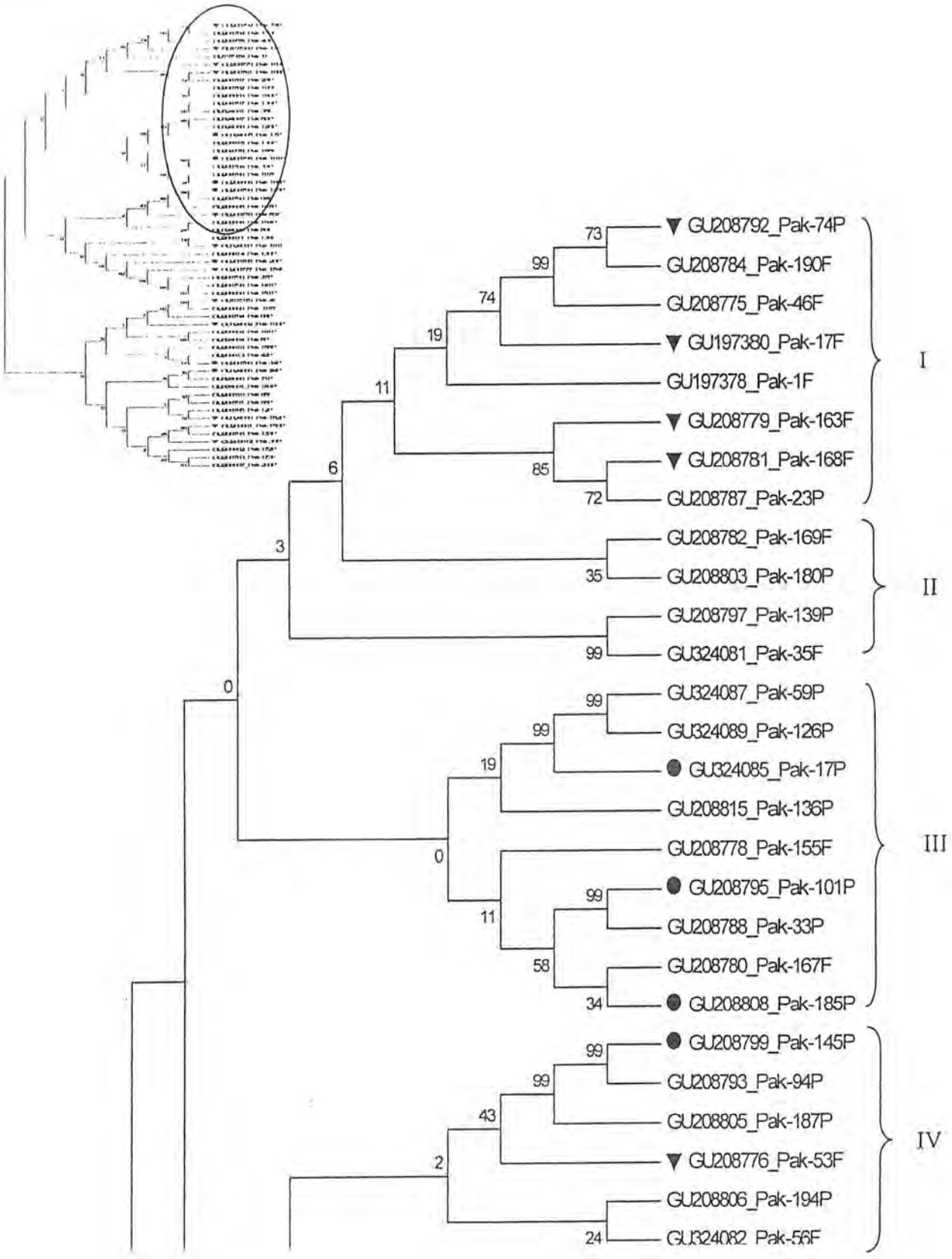


Fig. 4.3.1: Unrooted NJ phylogenetic tree of untypable sequences of HCV 5' UTR from sub urban Rawalpindi, Pakistan showing punctuated equilibrium.


GU208792_Pak-74P
GU208784_Pak-190F
GU208775_Pak-46F
GU197380_Pak-17F
GU197378_Pak-1F
GU208779_Pak-163F
GU208781_Pak-168F
GU208787_Pak-23P
GU208782_Pak-169F
GU208803_Pak-180P
GU208797_Pak-139P
GU324081_Pak-35F
GU324087_Pak-59P
GU324089_Pak-126P
GU324085_Pak-17P
GU208815_Pak-136P
GU208778_Pak-155F
GU208795_Pak-101P
GU208788_Pak-33P
GU208780_Pak-167F
GU208808_Pak-185P
GU208799_Pak-145P
GU208793_Pak-94P
GU208805_Pak-187P
GU208776_Pak-53F
GU208806_Pak-194P
GU324082_Pak-56F
I
II
III
IV

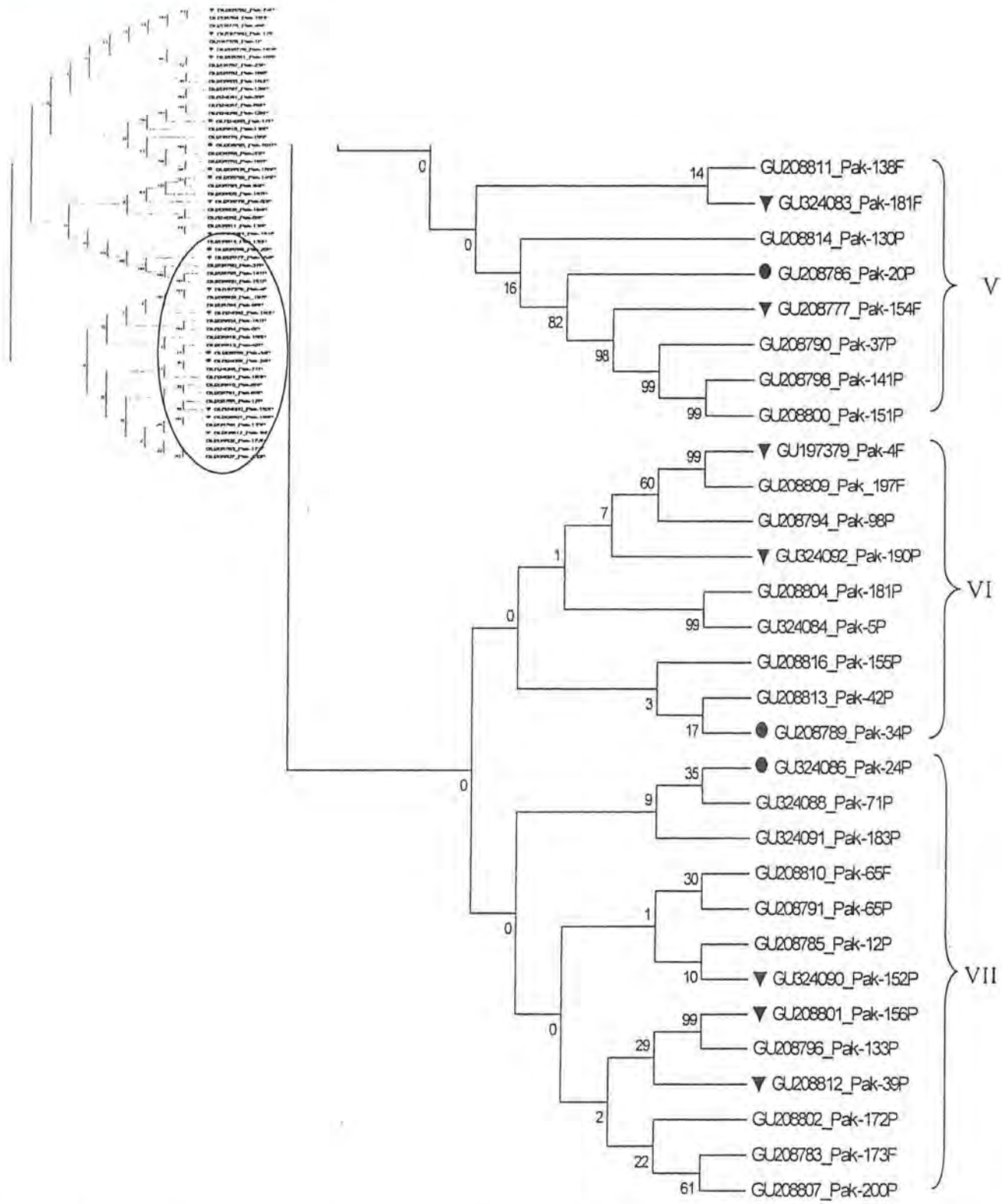
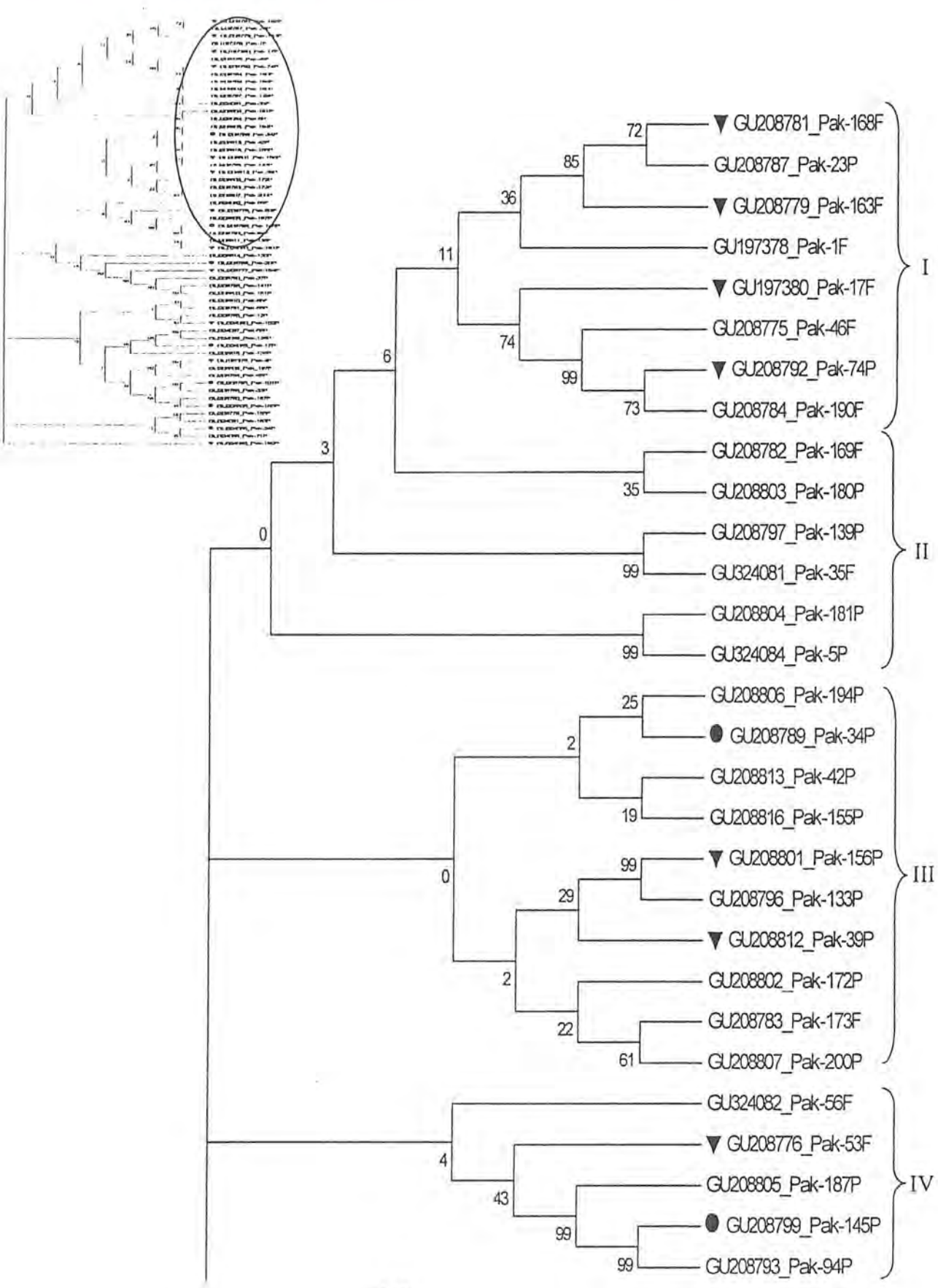


Fig. 4.3.2: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan with *p*-Distances. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208781_Pak-168F
GU208787_Pak-23P
GU208779_Pak-163F
GU197378_Pak-1F
GU197380_Pak-17F
GU208775_Pak-46F
GU208792_Pak-74P
GU208784_Pak-190F
GU208782_Pak-169F
GU208803_Pak-180P
GU208797_Pak-139P
GU324081_Pak-35F
GU208804_Pak-181P
GU324084_Pak-5P
GU208806_Pak-194P
GU208789_Pak-34P
GU208813_Pak-42P
GU208816_Pak-155P
GU208801_Pak-156P
GU208796_Pak-133P
GU208812_Pak-39P
GU208802_Pak-172P
GU208783_Pak-173F
GU208807_Pak-200P
GU324082_Pak-56F
GU208776_Pak-53F
GU208805_Pak-187P
GU208799_Pak-145P
GU208793_Pak-94P
I
II
III
IV

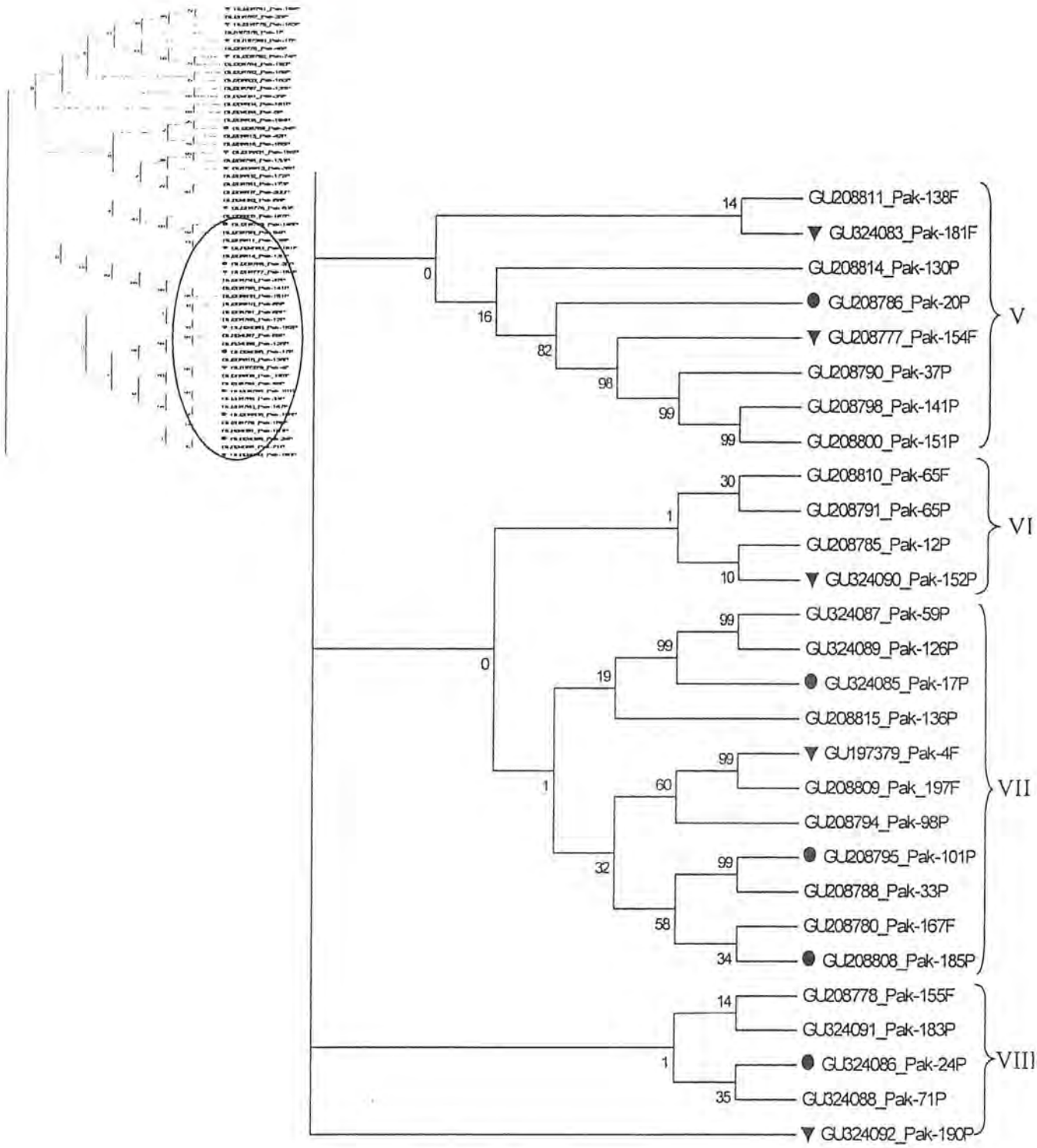
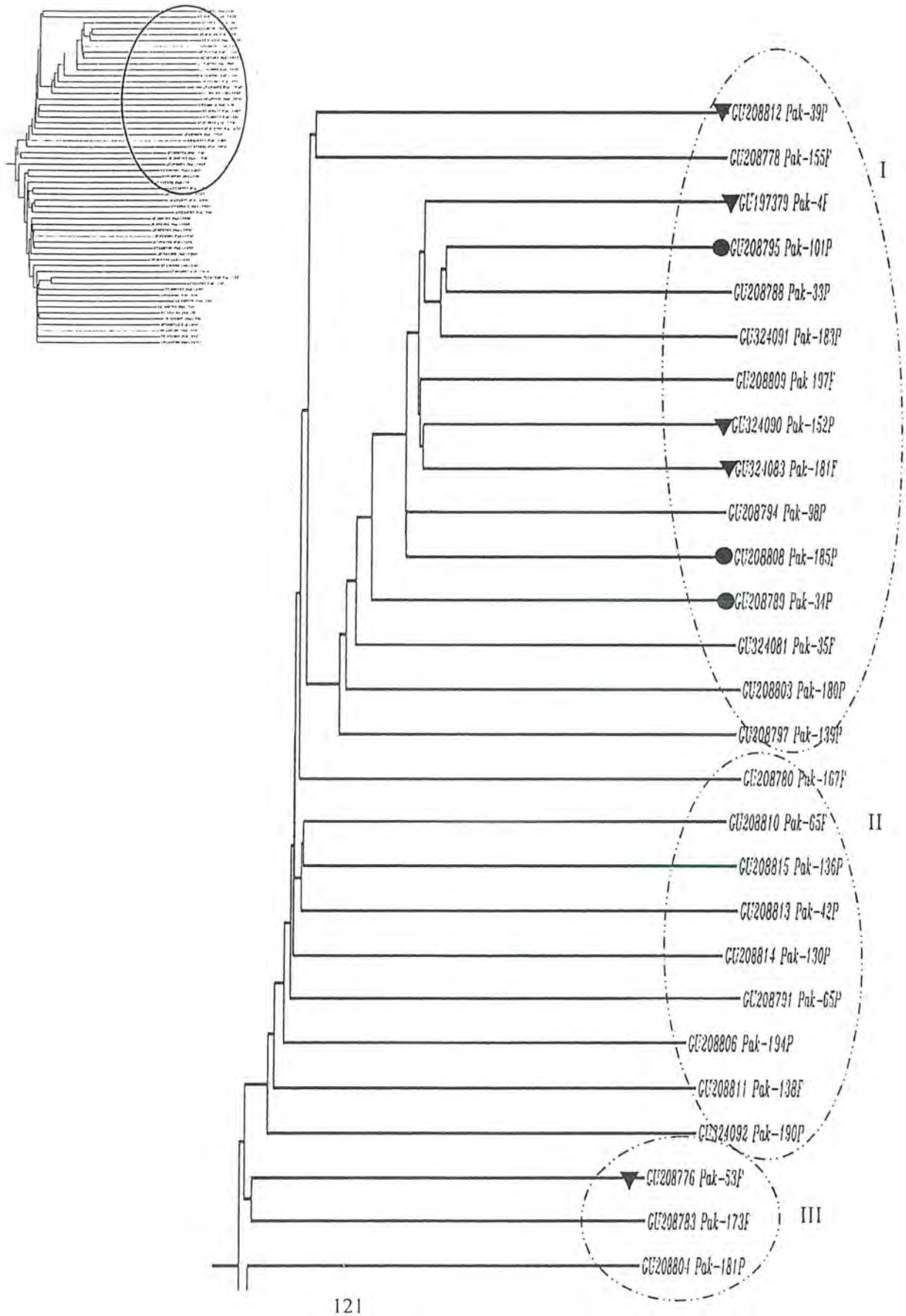


Fig. 4.3.3: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan with Bootstrap values. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208812 Pak-39P
GU208778 Pak-155F
GU197379 Pak-4F
GU208795 Pak-101P
GU208788 Pak-33P
GU324091 Pak-183P
GU208809 Pak 197F
GU324090 Pak-152P
GU324083 Pak-181F
GU208794 Pak-98P
GU208808 Pak-185P
GU208789 Pak-34P
GU324081 Pak-35F
GU208803 Pak-180P
GU208797 Pak-139P
GU208780 Pak-167P
GU208810 Pak-65P
GU208815 Pak-136P
GU208813 Pak-42P
GU208814 Pak-130P
GU208791 Pak-65P
GU208806 Pak-194P
GU208811 Pak-138F
GU324092 Pak-190P
GU208776 Pak-53F
GU208783 Pak-173F
GU208804 Pak-181P
I
II
III

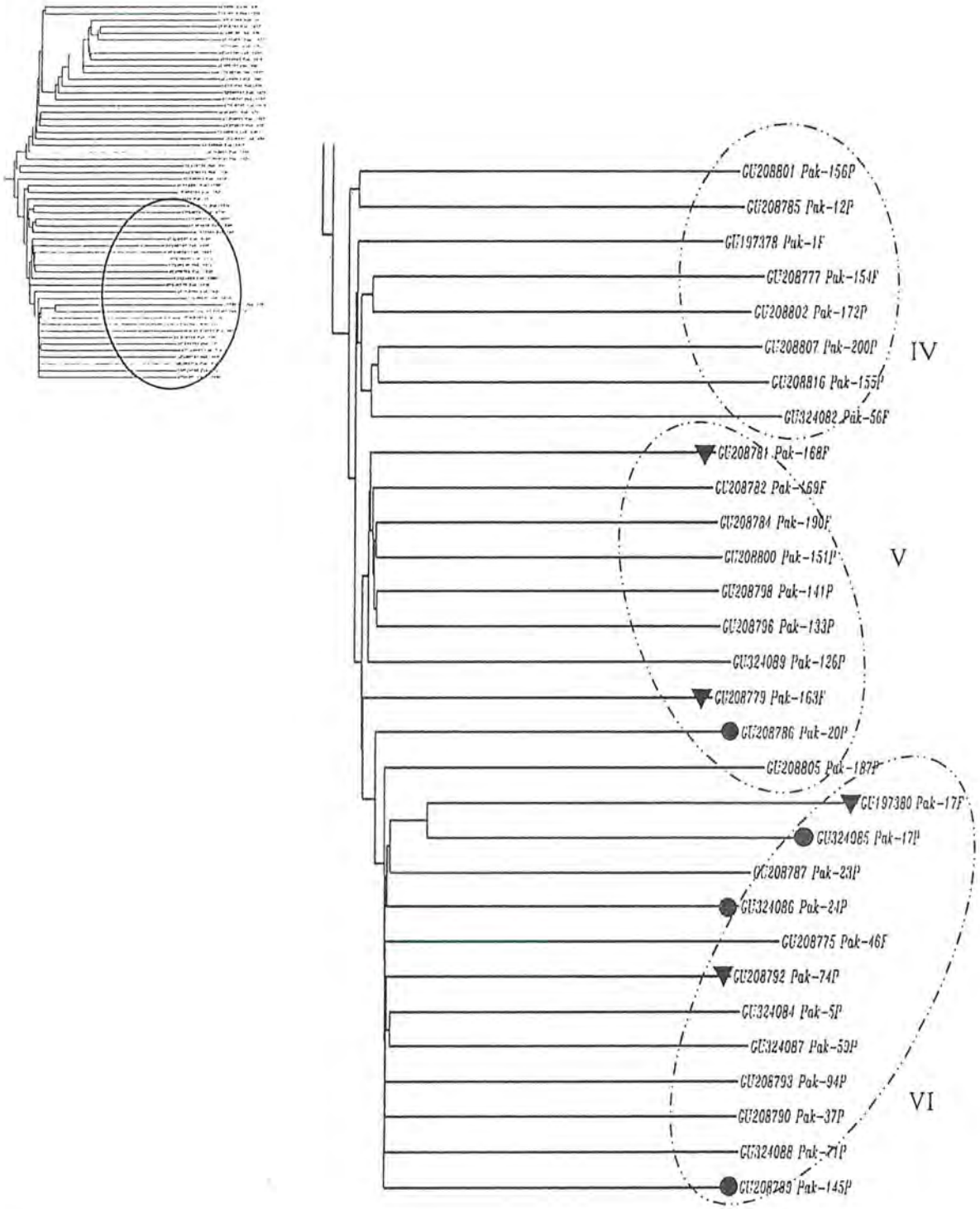


Fig 4.3.4: Dendrogram of the untypable sequences of HCV 5'UTR from suburban Rawalpindi, Pakistan with Branch lengths. Circle stands for known representative genotypes and triangle is for unresolved samples.

(Cluster III). GU208804 Pak-181P, GU324084 Pak-5P, GU208816 Pak-155P and GU208813 Pak42P (Cluster IV), GU20881 Pak-65F, GU208791 Pak-65P, GU208785 Pak-12P and GU324090 Pak-152P (Cluster V), GU208802 Pak-172P, GU208783 Pak-173F and GU208807 Pak-200P were remarkably prominent. It appears from the tree topology that these variants had been under extensive pressure of natural selection as thick twists in their evolutionary branches indicate the rapid changes followed by short periods of stasis (Fig. 4.3.1).

The evolutionary pattern 3 was observed for the following accessions, GU208782 Pak -169F, GU208781 Pak-168F (Cluster I), GU324085 Pak-17P, GU208778 Pak-155F (Cluster II), GU208814 Pak-130P (Cluster III), GU208794 Pak-98P, GU324092 Pak-92P (Cluster IV), GU324086 Pak-24P, GU324088 Pak-71P and GU208812 Pak-39P (Cluster V), GU208796 Pak-133P and GU208801 Pak-156P (Cluster VI). Branch topology was observed to be intermediate between the patterns, one and two (Fig. 4.3.1). However, all six clusters had a blend of viral populations under different punctuation equilibrium patterns. The grossly unequal rates of change within this single species imply an underlying mechanism at odds with the prevailing notion that neutral changes are the dominating feature of molecular evolution. This is also a demonstration of punctuated equilibrium at the molecular level but a novel configuration is accepted if it increases the fitness of the population representatives, i.e., species evolves to a local fitness maximum with a fast adaptive motion (a surge of evolution from an original ancestral form as new forms fan out, adapting over time to new niches).

Further, evolution takes place only if non-beneficial moves are accepted with low probability. Thus, the species are almost always at local fitness maxima (Bak and Sneppen 1993) as can be observed in the circular tree with *p-distances* that the species under positive immune pressure of the host were evolving actively (GU208783 Pak-173F, GU208778 Pak-155F, GU208784 Pak-190F and GU324082 Pak-56F etc) and on the other hand the species that were under less immune pressure were comparatively more stable and articulated (GU208815 Pak-155P, GU324086 Pak-24P, GU208805 Pak-187P and GU208794 Pak-98P) (Fig. 4.3.1). Almost all of the studied sequences of HCV were competent of genetic adaptation and were the representatives of the chronicity

causers, hence evolved to optimize their fitness according to immune responses of their hosts and this whole process is a summation of negative and positive selection in the form of functional constraint on replication and immune recognition. The results were in agreement with the ones reported from Ireland (Ray et al. 2005). The extremely enhanced capability of HCV evolution is attributed to NS5B RNA polymerase's lack of proof reading ability that causes 10^{12} virions to production each day through an error-prone mechanism (Neumann et al. 1992 and Martell et al. 1992).

NJ Phylogenetic tree of the untypable sequences of 5'UTR from suburban areas of Rawalpindi showed divergent evolution for existing two major clades (Fig. 4.3.2) because representatives of HCV that are capable of causing chronic infection (as in present study), have a property of mutability and over time divergence (Ray et al. 2005). Rooted tree indicated that all the descendants had some most recent common ancestor (MRCA). Clade I was divided into five main clusters and clade II was divided into two main clusters. Also the tree was an ambient example of bifurcation at each internode level that divided the decedents into two branches after every major evolutionary change. As all the variants were the representatives of the untypable 5'UTR sequences. The numbers on the branches indicate the number of times the partition of the species into the two sets which are separated by that branch occurred among trees, out of 100.00 trees. In clade 1, cluster II, III, IV and V showed active mutation of viruses with p-distance 99 (with 99% bootstrap support) for sister texa/ sister groups. Bootstrap replicates are created randomly; therefore results for same analysis can slightly differ. It is thus more meaningful to perform the analysis with 500 replicates.

Sample ID GU208808 Pak-185P was known representatives of the genotype 1a; on the other hand GU208789 Pak-34P was taken as known representatives of the genotype 1b. Sample ID GU208795 Pak-101P was the representatives of subtype 3a. For 3b sample ID GU324085 Pak-17P and GU208786 Pak-20P were selected and sequenced. While genotype 4 was assigned the representative samples GU208799 Pak-145P and GU324086 Pak-24P (Fig 4.3.2 and Fig 4.3.3). As indicated by the phylogenetic tree sample ID GU208786 Pak-20P was positioned in cluster II, GU208789 Pak-34P in cluster III, GU324086 Pak-24P in cluster V, GU208799 Pak-145P in cluster VI and

GU208795 Pak-101P, GU208808 Pak-185P and IDGU324085 Pak-17P in cluster VII. It is surprising to note that sample ID GU208786 Pak-20P rather than showing homology with ID GU324085 Pak-17P (17P had a 99% homology with its sister texa with 99% bootstrap support), and known representative of genotype 3 GU208789 Pak-34P with GU208780 Pak-167F and known representative of genotype 1b GU208808 Pak-185P, had shown non significant homologies with their sister groups.

For sample ID GU208799 Pak-145P sequence homology with GU208793 Pak-94P and GU208805 Pak-187P was 99% (with 99% bootstrap support). All other accessions were considered according to these representative sequences and the tree topology revealed multiple genetic duplication events supported by high bootstrap values. The results were in agreement with the results obtained by Escobar and Castano (2009). Dendrograms of the untypable sequences of HCV were dragged to determine inter and intra clusters distances. Among these distances, most important are the distances between sequential vertical lines. Sample ID GU208804-Pak181P has a longer and stable branch that has evolved earlier than all other sister texas of the cluster.

All the dragged dendrograms (Fig 4.3.4) were the rooted trees; with tree topologies indicated the bifurcating internodes with asymmetrical branching structures. Dendrogram of the studied accessions was divided into VI clusters. Cluster VI had three inter nodes and it evolved at the same time with cluster III, IV and V. Cluster I and II had evolved later during evolution; while in cluster III, IV, V and VI the branch topologies indicated a slow and stable evolution; one possible reason might be the suppressed immune responses of the host. All sister subgroups of cluster II were distantly related to the members of cluster I. In cluster II the branches formed asymmetrical structure indicating that these strains have evolved later during the evolutionary process and it is supported by the high mutational rate of HCV. Topologies of sample ID GU208795 Pak-101P, GU208788 Pak-33P, GU324091 Pak-183P and GU197379 Pak-4F indicate very recent emergence of these variants and how stable they will remain during the later evolutionary era will be premature to sugggest.

In cluster three at the level of fourth internode a mini sub cluster was formed with 97% P-distance among accessions GU208812, GU208796 and GU208801 (Fig. 4.3.2) forming a mini cluster of genotype 4 variants (with 99% Bootstrap support). Like wise, when bootstrap was applied to sequenced accessions, unresolved GU208776 showed homology with a resolved variant of genotype 4 (with 90% bootstrap support). Further GU197379 showed homology with a variant of 3a resolved sequence GU208809 (with 99% bootstrap support). It indicates the fact that genotype 4 variants from Pakistan are more close to genotype 3 variants (Fig. 4.3.3). At the level ninth of substitution of nucleotide, it was revealed from the tree topology that GU197379 had evolved from MRCA with GU324090, but the evolution of GU197379 was recent than GU324090 and both sister subgroups were a part of genotype 3 variants sharing the same node.

In second cluster unresolved sample GU208777 showed 99% homology at 99% with multiple genotypes and this trend was also noted in other clusters as well e.g. in cluster VIII, GU324092 showed non-significant homology with genotype 4. Although, both the sequences are an example of divergent evolution, but might be a novel subtype. One of the unique finding of study was the presence of subtype 6v in three samples, this subtype has never been reported from Pakistan and first time it was observed in china (Wang et al. 2009) and genotype 6 is most prevalent HCV genotype in China and Thailand (Chen et al. 2002 and Doi et al. 1996). Though HCV genotype 6 is very rich in subtypes and till yet, it had been expanded from 6a to 6u and still new variants are continuously being reported from different regions of the world (Wang at al. 2009b). Apparently the presence of HCV subtype 6v variants in Pakistan has been attributed to china. Pakistan and China have very deep rooted and enduring relationships at state and public level and both the countries had signed open trade, education and tourism treaties.

Present study also revealed the presence of some other new variants in cluster one. This cluster had three sister subgroups that remained unresolved and they had maintained their independent existence, as evident from the tree topologies. These accessions namely GU208781, GU208787 and GU208779 had 87% sequence homologies (with reasonably good bootstrap support 85%) (Fig. 4.3.3) evolved from MRCA having sister cluster of subtype 2k (with non significant bootstrap support). These

might be some novel strains of HCV and their further characterization is required, in the form of full genome sequencing, in order to place them some where in existing classification. Bootstrap analysis revealed GU324092 as an out-group due to its non-significant homology with existing clades. Present study also identified subtype 1d (GU208806), in one of the resolved sequences, as a new finding with reference to Pakistan.

Dendrograms analyzed tree topology indicates that two unresolved sister subgroups had evolved at nucleotide substitution level six from MRCA and GU208781 shares ancestry with reported 2k (GU197378) and other with 3a but 3a (GU208782) has evolved earlier than unresolved GU208781. A general trend in the dendrograms indicates that actively mutated and newly evolved sequences were mainly unresolved. The reason of them being unresolved is the high mutation rate of HCV (Khan et al. 2009 and Zein 2000) different geographical and epidemiological patterns determine the genotypes and subtypes of HCV (Carrington et al. 2005, Simmonds 2004 and smith et al. 1997).

4.3.4 COMPARATIVE PHYLOGENETIC ANALYSIS OF THE STUDIED SEQUENCES WITH REFERENCE ACCESSIONS FROM PAKISTAN

A comparative Phylogenetic analysis of reported sequences was made with reference accessions from Pakistan (Fig. 4.3.5-4.3.8). For this purpose two very recent reports from Pakistan were followed. These reports have representative sequences from all over the Pakistan (Idrees et al. 2008 and Yasmeen et al. 2009). The reference accessions were retrieved from the web site of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The accessions already reported from Pakistan used for this comparison are M62321 Pak 1a, D90208 Pak 1b, D00944 Pak 2a, D10988 Pak 2b, D10075 Pak 2c, D14307 Pak 3a, D11443 Pak 3b, D16612 Pak 3c, M84848 Pak 4a, M84845 Pak 4b, M84862 Pak 4c, M84832 Pak 4d, M84828 Pak 4e, M84829 Pak 4f, M84860 Pak 5a, M84827 Pak 6a, AM228881 Pak 3a, AM228882 Pak 3a, AM228883 Pak 3a, AM228884 Pak 3a, AM228885 Pak 3b, AM228886 Pak 3a, AM228887 Pak 3b, AM228888 Pak 3b AM228889 Pak 3a, AM228890

Pak 3a, AM228891 Pak 3a, AM228892 Pak 3a, AM228893 Pak 3a, AM228894 Pak 3a, AM228895 Pak 3a, AM228896 Pak 3a, AM228897 Pak 3a, AM228898 Pak 3a, AM228866 Pak 3b, AM228867 Pak 3a, AM228868 Pak 3a, AM228869 Pak 3a, AM228870 Pak 3a, AM228871 Pak 3b, AM228872 Pak 3a, AM228873 Pak 3a, AM228874 Pak 3a, AM228875 Pak 3a, AM228876 Pak 3a, AM228877 Pak 3a, AM228878 Pak 3a, AM228879 Pak 3a, AM228880 Pak 3a and JK049 Pak 3.

NJ Phylogenetic tree was dragged for all the accessions. Tree topology indicated four major clades of the studied samples, where cluster II in clade one showed a trend of comparatively stable strains, and cluster one and three showed actively mutating variants of genotype 3. Majority of genotype 3 accessions, reported by Yasmeen et al (2009), maintained their independent identity and just a couple of unresolved sequences and a resolved sequence 3a (GU208785) from the study, showed homology with reference accessions from Yasmeen et al (2009). Tree topology (Fig. 4.3.5) revealed that subgroup of 6v subtype was in cluster nine. This subgroup maintained its individuality and did not rearrange itself with any of the reference accessions from Pakistan. Tree topologies suggest that the accessions share a MRCA with a resolved sequence of genotype 4 (Fig. 4.3.6). Tree topologies showed non-significant validation by boot strap values less than 75% (Fig. 4.3.7).

When the phylogeny of the studied strains was compared with reference strains from Pakistan (Fig 4.2.7), the tree topology showed multiple independent clusters where all reference strains placed them in distinct clusters showing negligible homologies with any of the studied sequences. Only reference sequences AM228895 Pak-3a in cluster IV, D90208 Pak 1b, in cluster V1, M62321 Pak-1a and D10988 Pak 2b in clusterVIII, D00944 Pak 2a, D10075 Pak 2c in cluster IX, showed homologies with the studied sequences. While 12P in cluster I, 190P, 56P, 190P and 34P in cluster 4 were more homologous to the reference strains. It is apparent from the tree topology that during the course of evolution large number of the study accessions have evolved late during era of evolution and remained stable for very long time periods and during the late evolutionary period they had mutated and evolved actively. Sample IDs 33P, 101P, 167F and 185P were clustered up forming sister texas when they were pooled with reference sequences

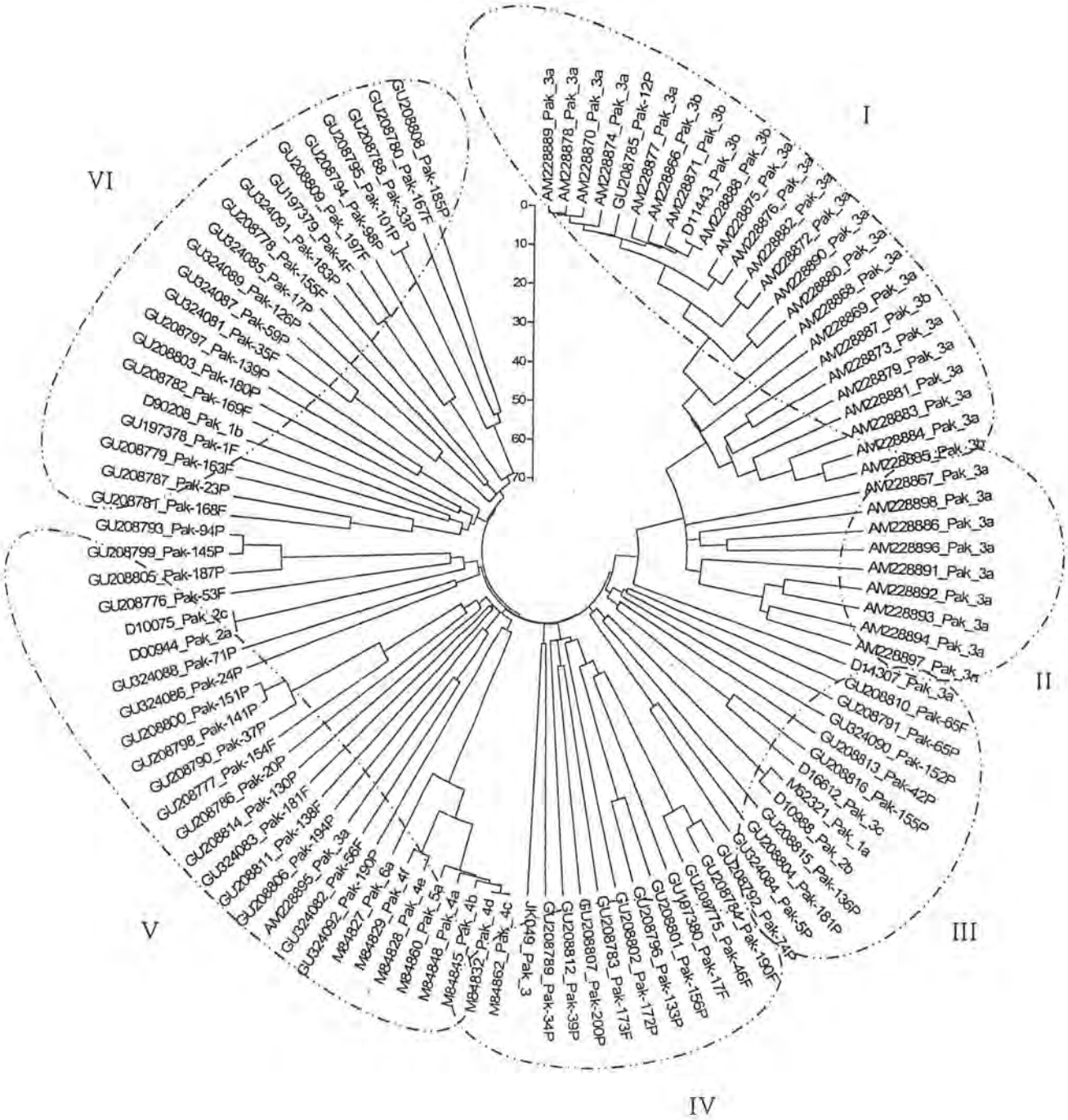
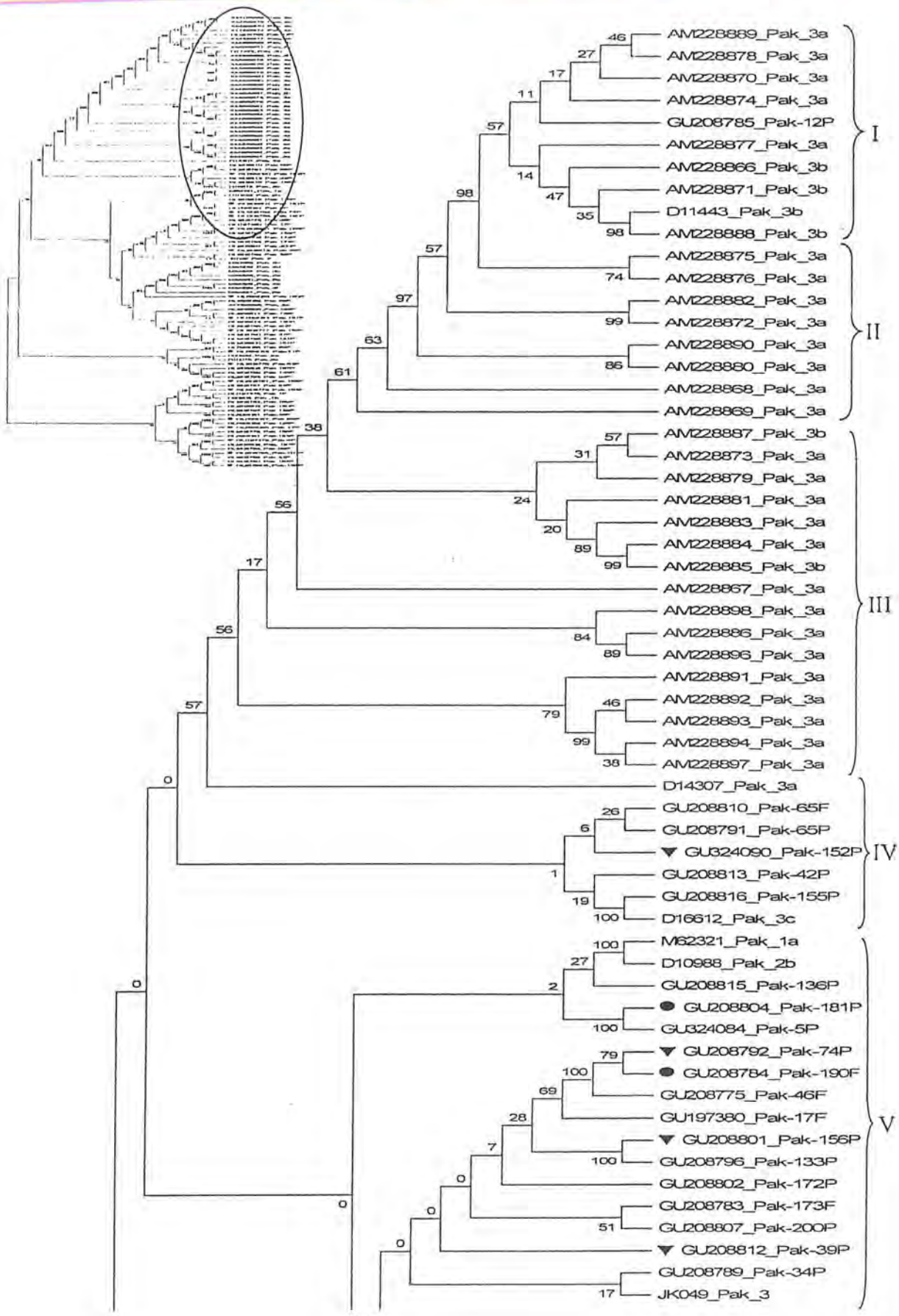


Fig. 4.3.5: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan with reference Pakistani accessions showing punctuated equilibrium.


AM228889_Pak_3a
AM228878_Pak_3a
AM228870_Pak_3a
AM228874_Pak_3a
GU208785_Pak-12P
AM228877_Pak_3a
AM228866_Pak_3b
AM228871_Pak_3b
D11443_Pak_3b
AM228888_Pak_3b
I
AM228875_Pak_3a
AM228876_Pak_3a
AM228882_Pak_3a
AM228872_Pak_3a
AM228890_Pak_3a
AM228880_Pak_3a
AM228868_Pak_3a
AM228869_Pak_3a
II
AM228887_Pak_3b
AM228873_Pak_3a
AM228879_Pak_3a
AM228881_Pak_3a
AM228883_Pak_3a
AM228884_Pak_3a
AM228885_Pak_3b
AM228867_Pak_3a
AM228898_Pak_3a
AM228886_Pak_3a
AM228896_Pak_3a
AM228891_Pak_3a
AM228892_Pak_3a
AM228893_Pak_3a
AM228894_Pak_3a
AM228897_Pak_3a
III
D14307_Pak_3a
GU208810_Pak-65F
GU208791_Pak-65P
GU324090_Pak-152P
GU208813_Pak-42P
GU208816_Pak-155P
D16612_Pak_3c
IV
M62321_Pak_1a
D10988_Pak_2b
GU208815_Pak-136P
GU208804_Pak-181P
GU324084_Pak-5P
GU208792_Pak-74P
GU208784_Pak-190F
GU208775_Pak-46F
GU197380_Pak-17F
GU208801_Pak-156P
GU208796_Pak-133P
GU208802_Pak-172P
GU208783_Pak-173F
GU208807_Pak-200P
GU208812_Pak-39P
GU208789_Pak-34P
JK049_Pak_3
V

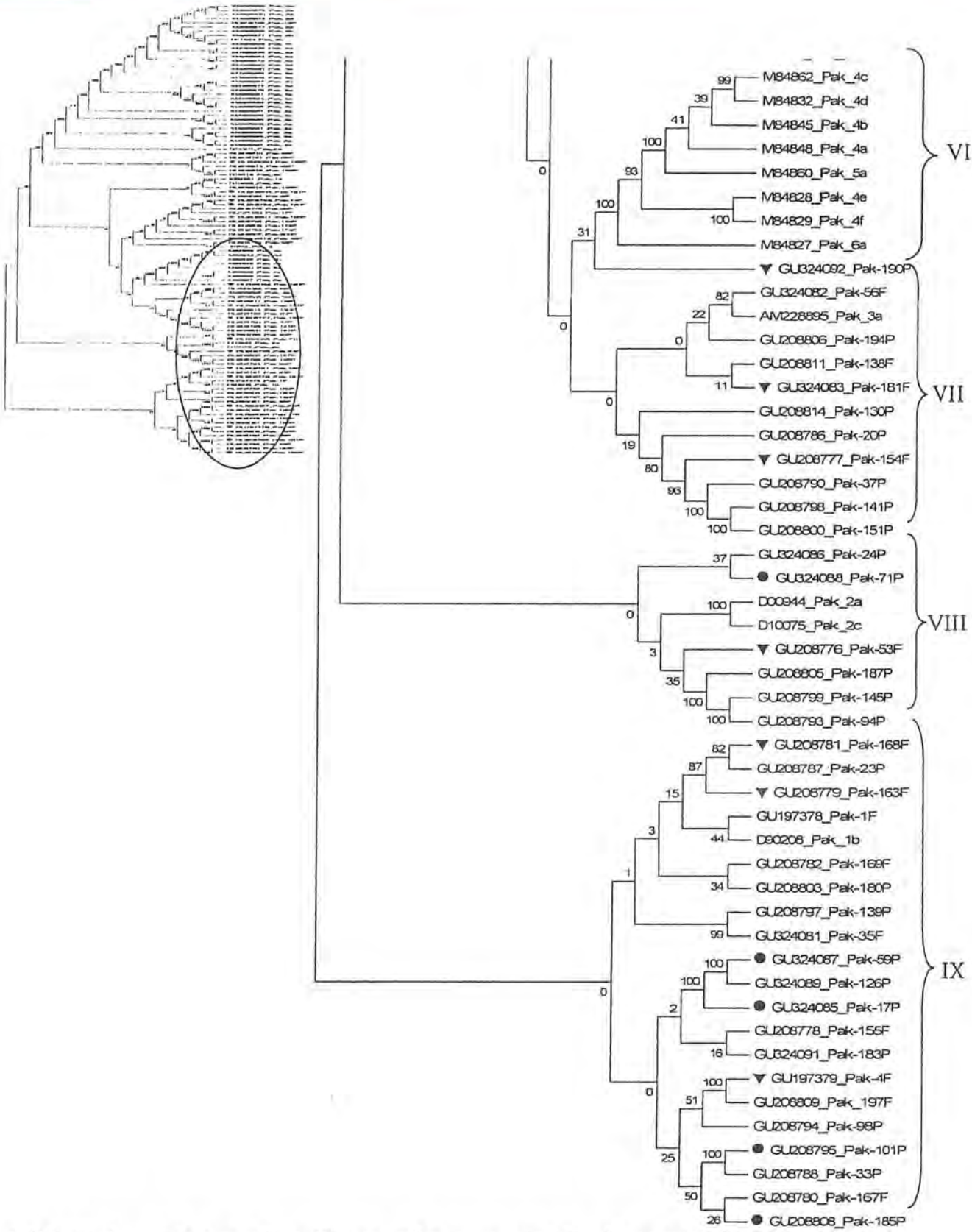
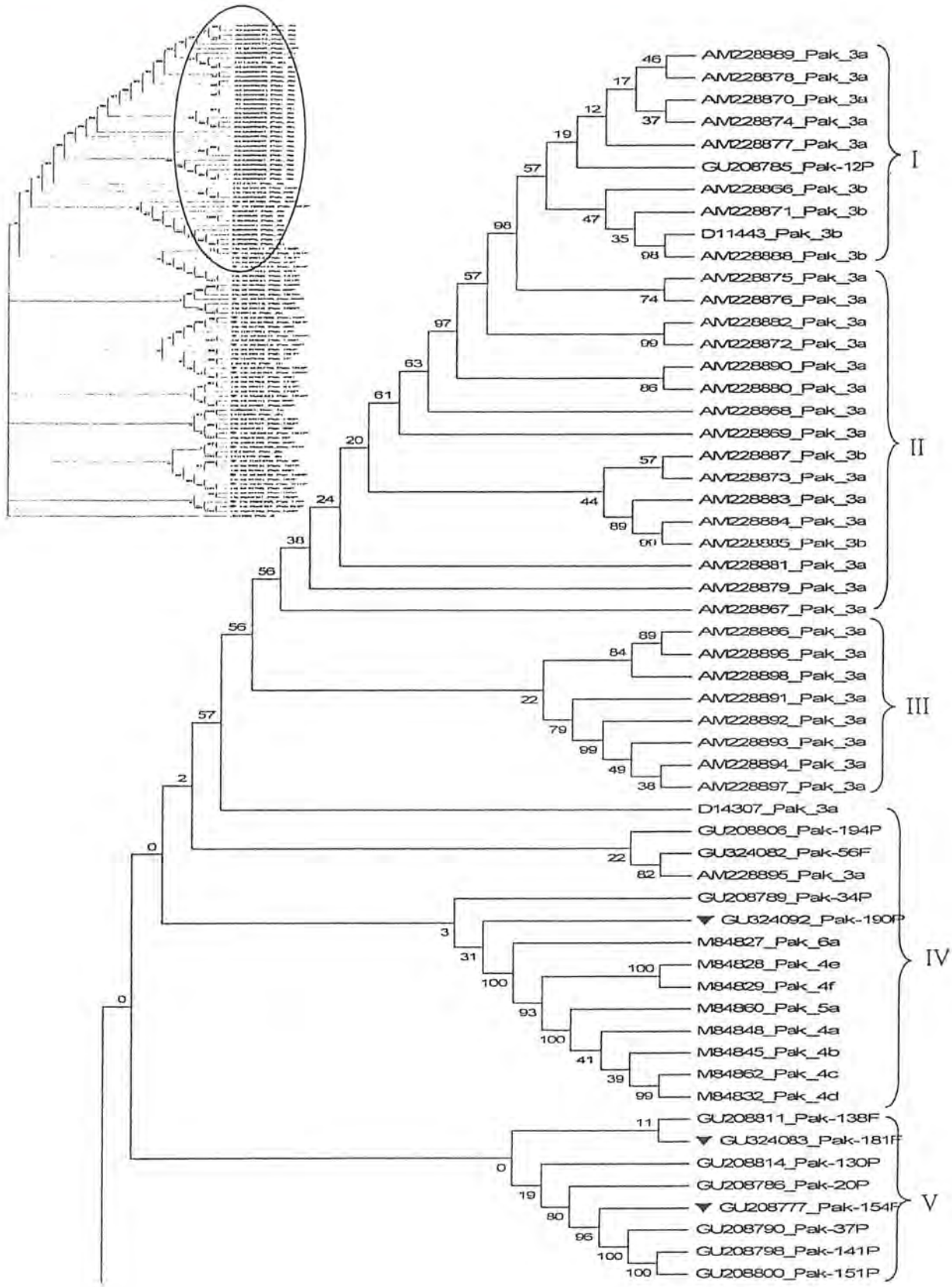


Fig. 4.3.6: Phylogenetic tree with *p*-Distances for accessions already reported from Pakistan. Circle stands for known representative genotypes and triangle is for unresolved samples.


AM228889_Pak_3a
AM228878_Pak_3a
AM228870_Pak_3a
AM228874_Pak_3a
AM228877_Pak_3a
GU208785_Pak-12P
AM228866_Pak_3b
AM228871_Pak_3b
D11443_Pak_3b
AM228888_Pak_3b
I
AM228875_Pak_3a
AM228876_Pak_3a
AM228882_Pak_3a
AM228872_Pak_3a
AM228890_Pak_3a
AM228880_Pak_3a
AM228868_Pak_3a
AM228869_Pak_3a
AM228887_Pak_3b
AM228873_Pak_3a
AM228883_Pak_3a
AM228884_Pak_3a
AM228885_Pak_3b
AM228881_Pak_3a
AM228879_Pak_3a
AM228867_Pak_3a
II
AM228886_Pak_3a
AM228896_Pak_3a
AM228898_Pak_3a
AM228891_Pak_3a
AM228892_Pak_3a
AM228893_Pak_3a
AM228894_Pak_3a
AM228897_Pak_3a
III
D14307_Pak_3a
GU208806_Pak-194P
GU324082_Pak-56F
AM228895_Pak_3a
GU208789_Pak-34P
GU324092_Pak-190P
M84827_Pak_6a
M84828_Pak_4e
M84829_Pak_4f
M84860_Pak_5a
M84848_Pak_4a
M84845_Pak_4b
M84862_Pak_4c
M84832_Pak_4d
IV
GU208811_Pak-138F
GU324083_Pak-181P
GU208814_Pak-130P
GU208786_Pak-20P
GU208777_Pak-154P
GU208790_Pak-37P
GU208798_Pak-141P
GU208800_Pak-151P
V

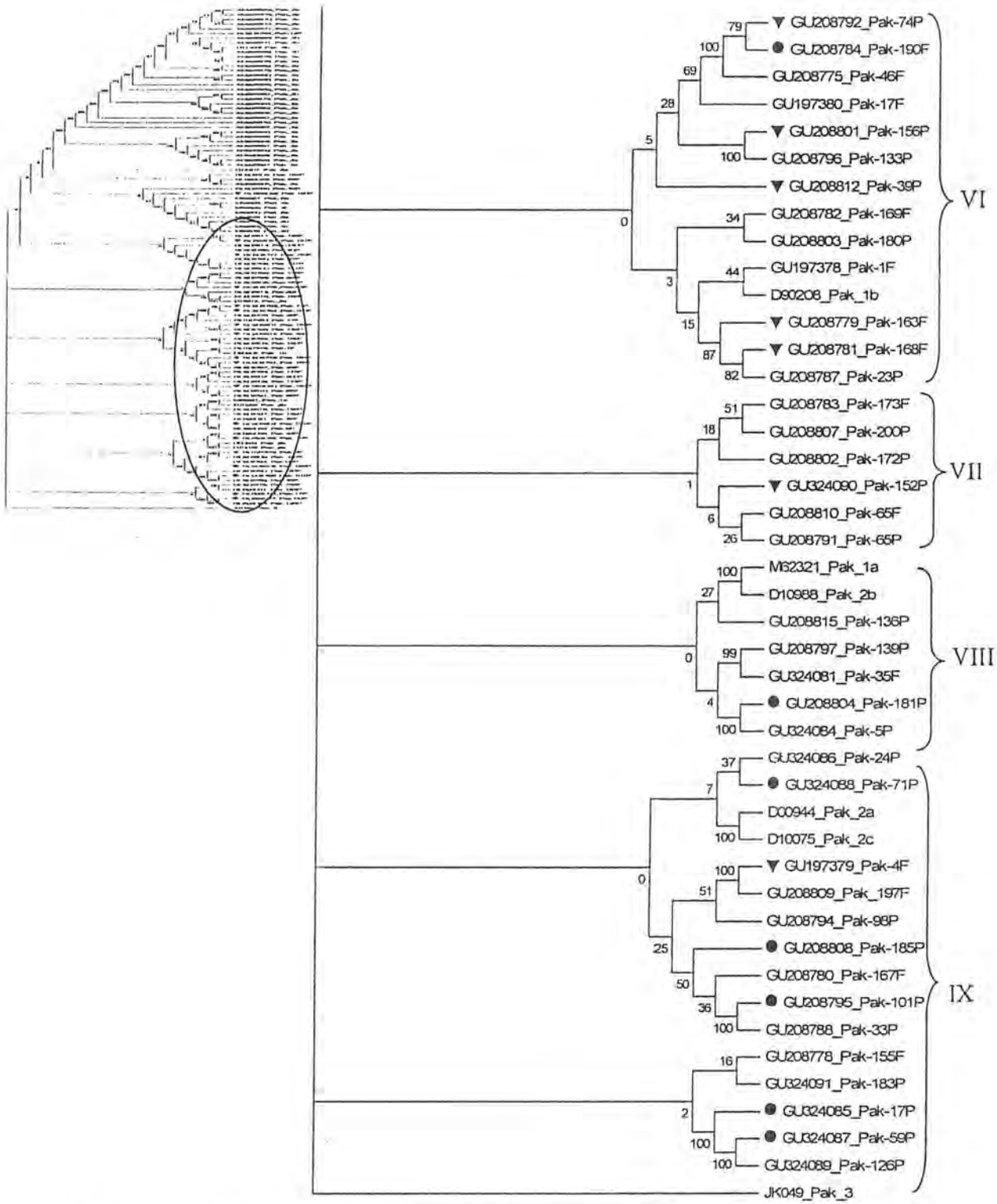
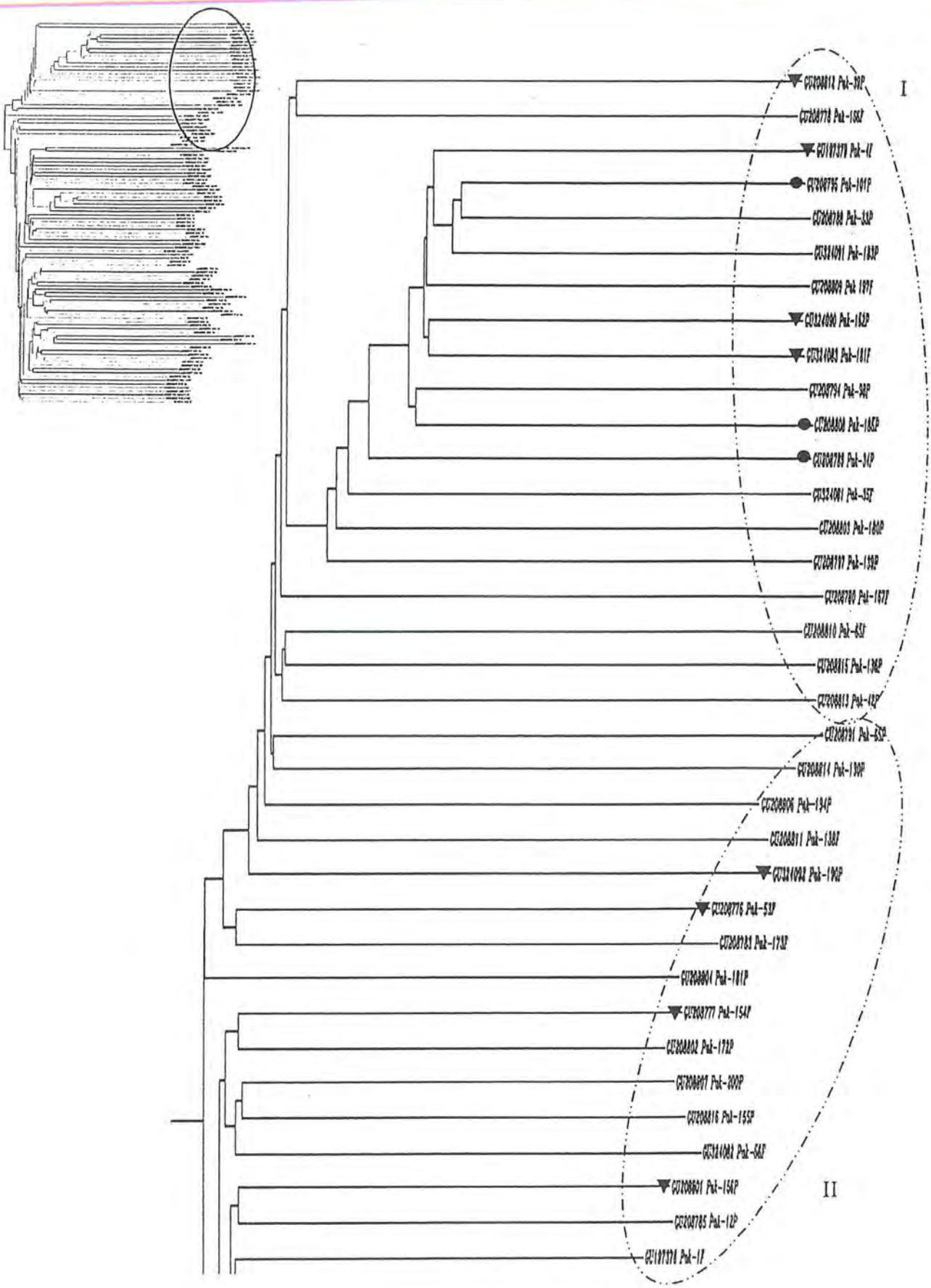
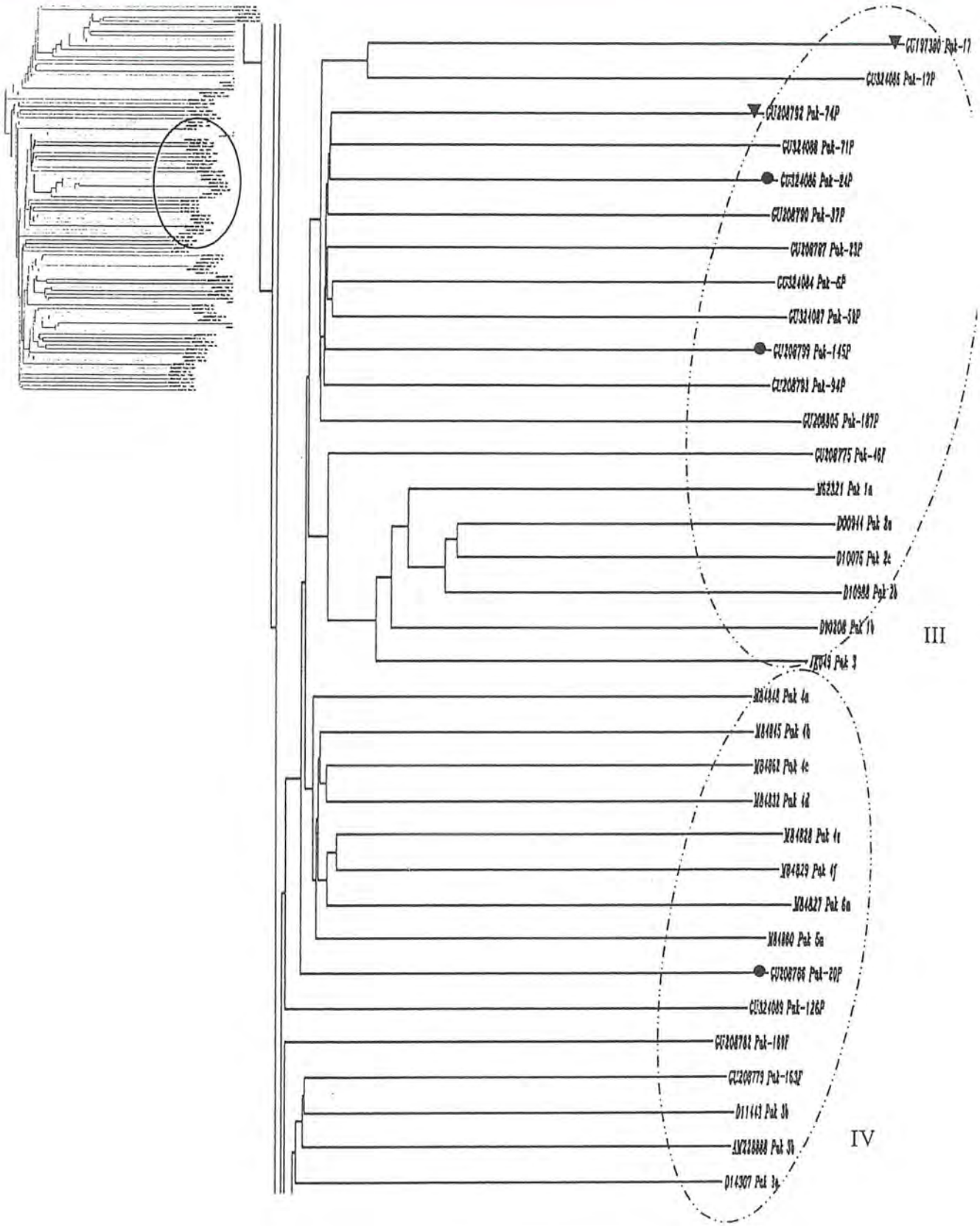


Fig. 4.3.7: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi with reference accessions already reported from Pakistan showing Bootstrap values. Circle stands for known representative genotypes and triangle is for unresolved samples.


I
II


GU197380 Puk-17
GU324086 Puk-17P
GU208792 Puk-74P
GU324088 Puk-71P
GU324086 Puk-24P
GU208790 Puk-37P
GU208787 Puk-23P
GU324084 Puk-6P
GU321087 Puk-51P
GU208799 Puk-145P
GU208781 Puk-94P
GU208805 Puk-187P
GU208775 Puk-46P
M62321 Puk 1a
D00941 Puk 2a
D10075 Puk 2c
D10588 Puk 2b
D90208 Puk 1b
III
JX049 Puk 3
M84848 Puk 4a
M84845 Puk 4b
M84862 Puk 4c
M84832 Puk 4d
M84838 Puk 4e
M84829 Puk 4f
M84827 Puk 6a
M84860 Puk 5a
GU208786 Puk-20P
GU324089 Puk-126P
GU208782 Puk-189P
GU208776 Puk-163P
D11443 Puk 3b
AY228668 Puk 3b
IV
D14307 Puk 3a

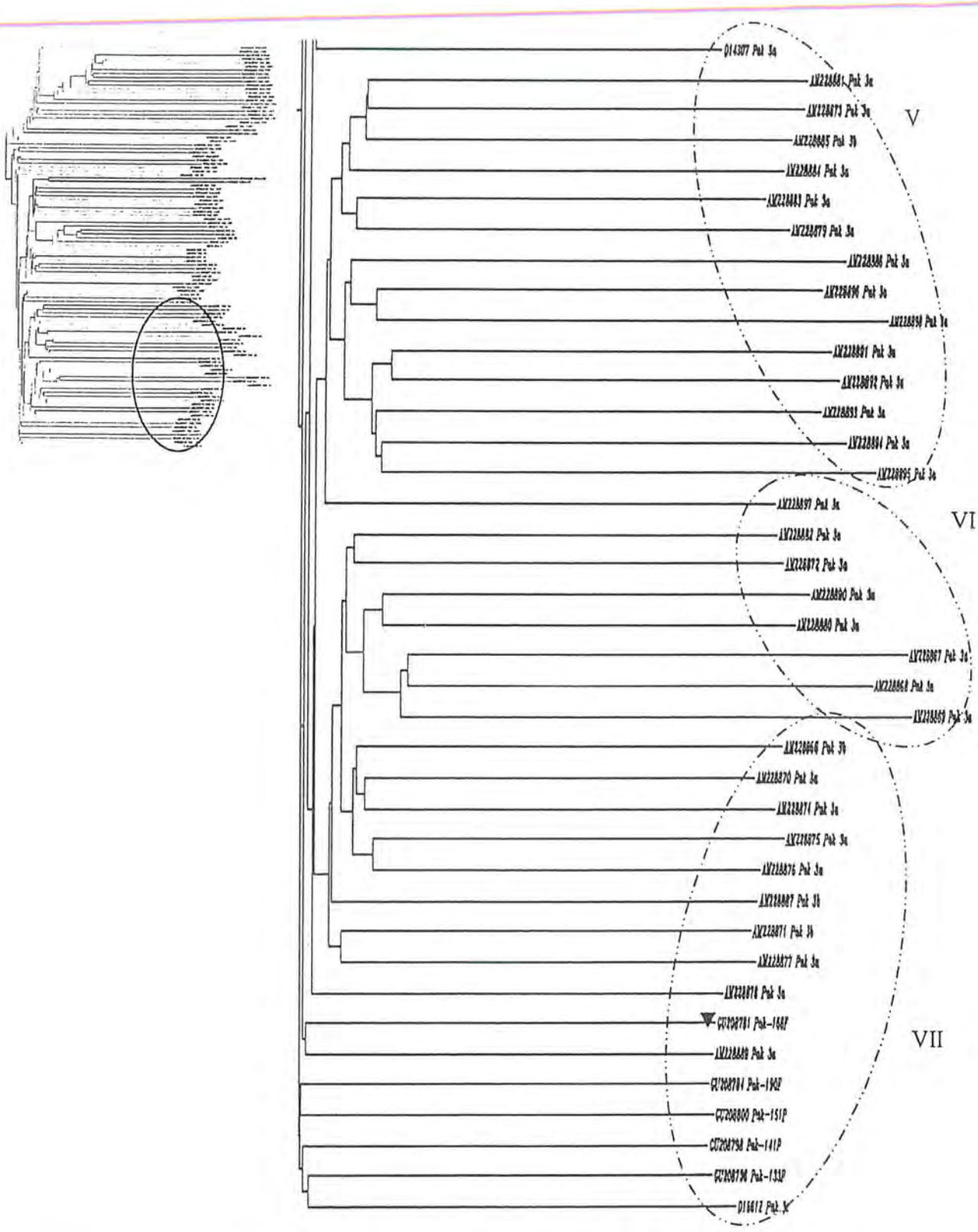


Fig. 4.3.8: Dendrogram of the untypable sequences of HCV 5'UTR from suburban Rawalpindi with reference already reported accessions from Pakistan showing Branch lengths.

from Pakistan where 101P was 100% homologous to 33P (with100% bootstrap support). On the other hand its homology with 185P was not supported by bootstrap while further, sample ID 74P showed homology with sister subgroups 190F and 46F at 6th inter-node level in cluster VI (with 100% bootstrap support). Sample ID 34P was though grouped with 190P accession but did not show significant homology with its sister groups (evident by non-significant bootstrap support). On the other hand, 17P showed homology with its sister groups at all internodes, ranging between 90-100% and the entire sister groups were representatives of the studied accessions (with 100% bootstrap support).

In cluster six unresolved accession GU208801 showed 99% homology with resolved genotype 4 sample GU208796 (homology was supported with 100% bootstrap value). It is note worthy that cluster four five and eight shares the heterogeneous sequences consisting of variants of genotype 1a, 1b, 3a and 4. Sample ID JK049 Pak-3 showed no significant sequence similarity with any of the accessions and it remained independent, as evident by its branch topology (Fig. 4.3.8).

4.3.5 COMPARATIVE PHYLOGENETIC ANALYSIS OF THE STUDIED SEQUENCES WITH REFERENCE ACCESSIONS FROM CHINA

China is situated towards the north east of Pakistan and on the basis of it being a neighbouring country reference accessions from China were retrieved randomly from NCBI web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by particularly targeting the partial sequences of 5'UTR at random. The purpose was a comparison of the present study with sequences reported from neighbouring countries of Pakistan (Fig. 4.3.9-4.3.12). Retrieved Chinese accessions were DQ480524 Chi 6a, DQ480521Chi 6a, AY859526 Chi 6a, EF612777 Chi 3a, EF612776 Chi 3b, EF612775 Chi 3b, EF612774 Chi 3b, EF616481 Chi 3b, DQ777778 Chi 2a, DQ777780 Chi 6k, DQ777787 Chi 1b, DQ777794 Chi 1b, DQ777807 Chi 1b, DQ777806 Chi 1b, DQ777804 Chi 3b, DQ777798 Chi 6n, DQ777797 Chi 6n, DQ777789 Chi 1b, DQ777784 Chi 3b, DQ777781 Chi 3b, DQ777776 Chi 2a, DQ777775 Chi 2a, DQ087250 Chi 1a, DQ087249 Chi 1a, DQ087248 Chi 1a, DQ087247 Chi 1a, DQ087246 Chi 1a, DQ087245 Chi 1a,

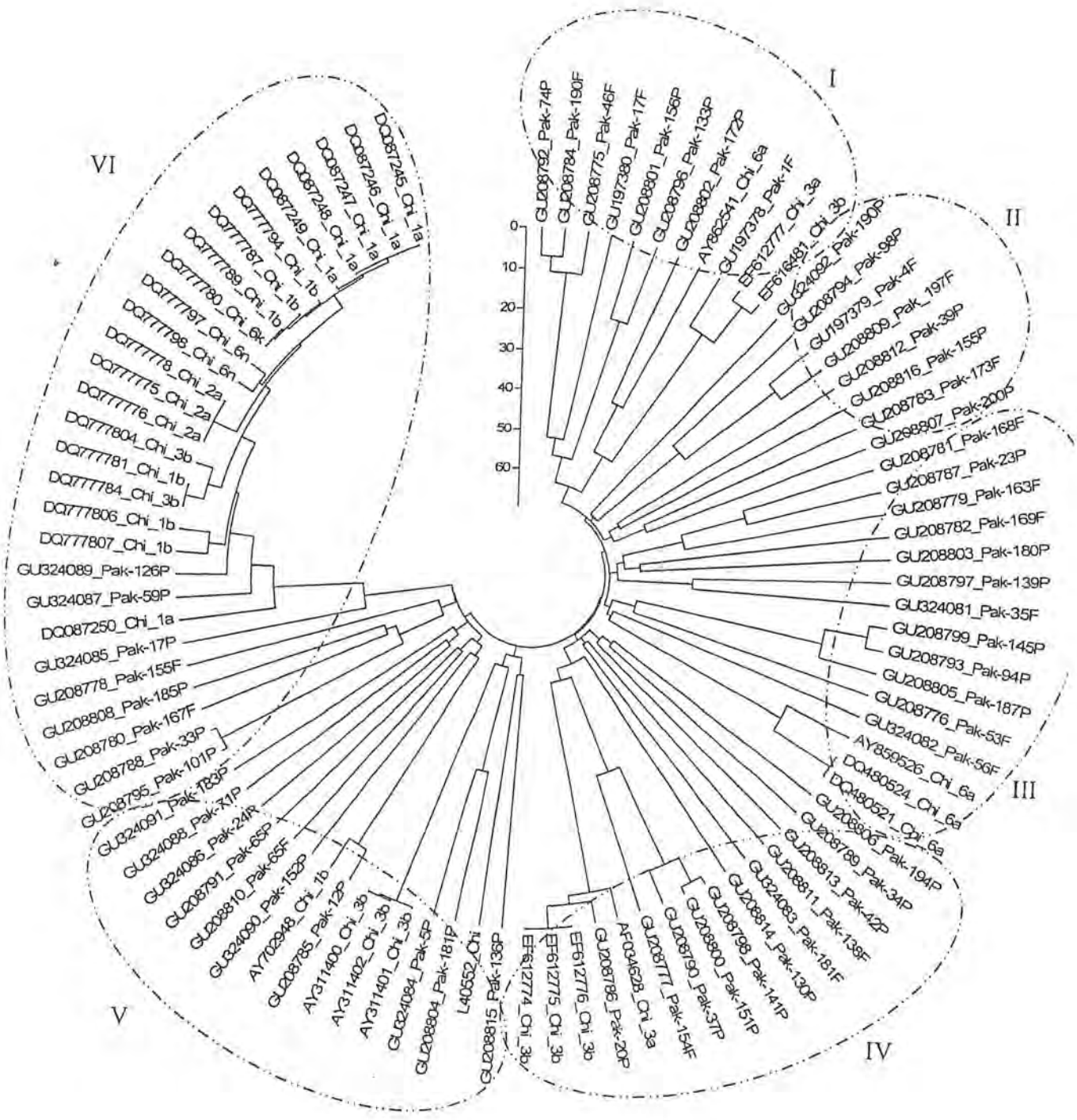


Fig. 4.3.9: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective samples from China.

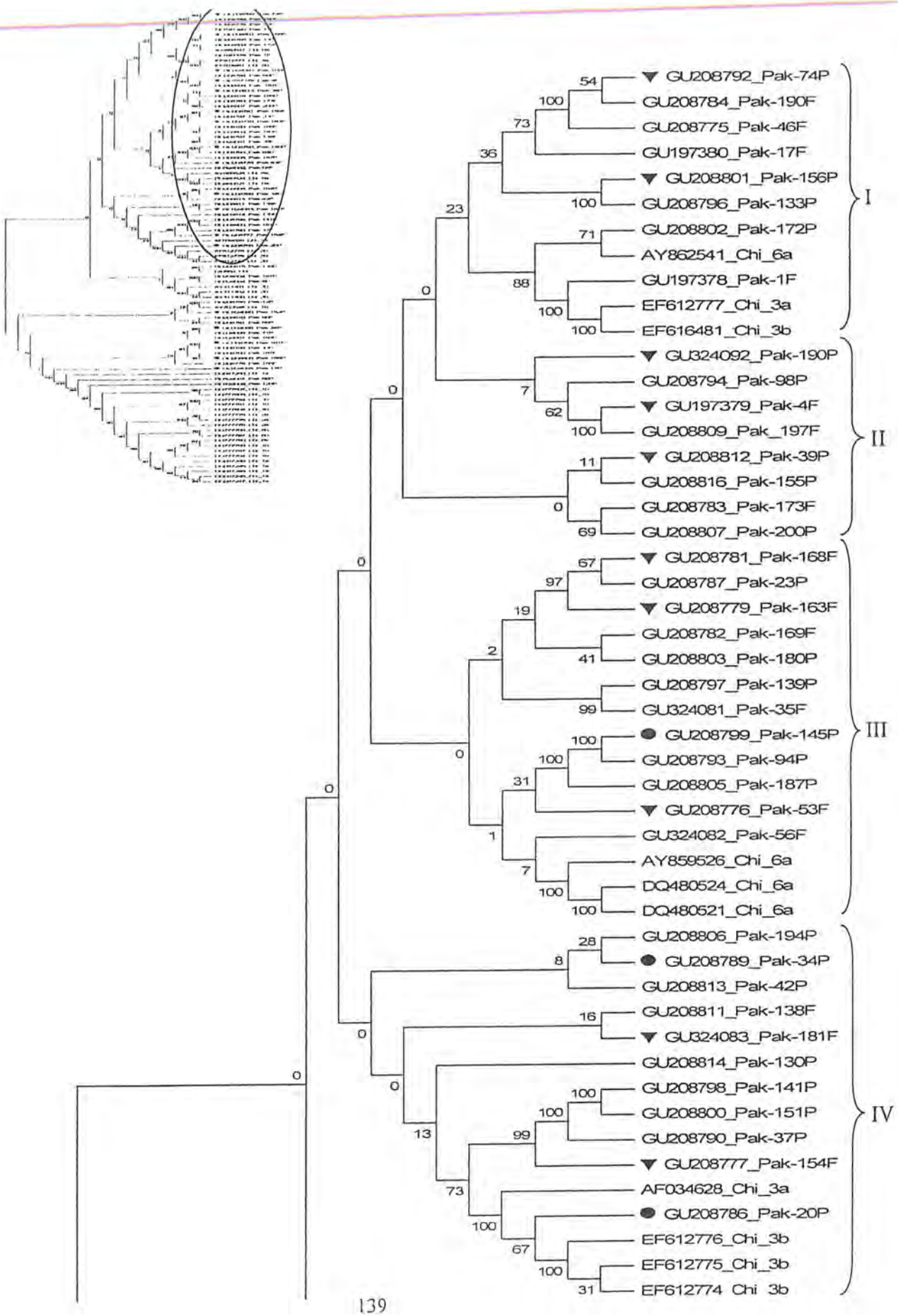
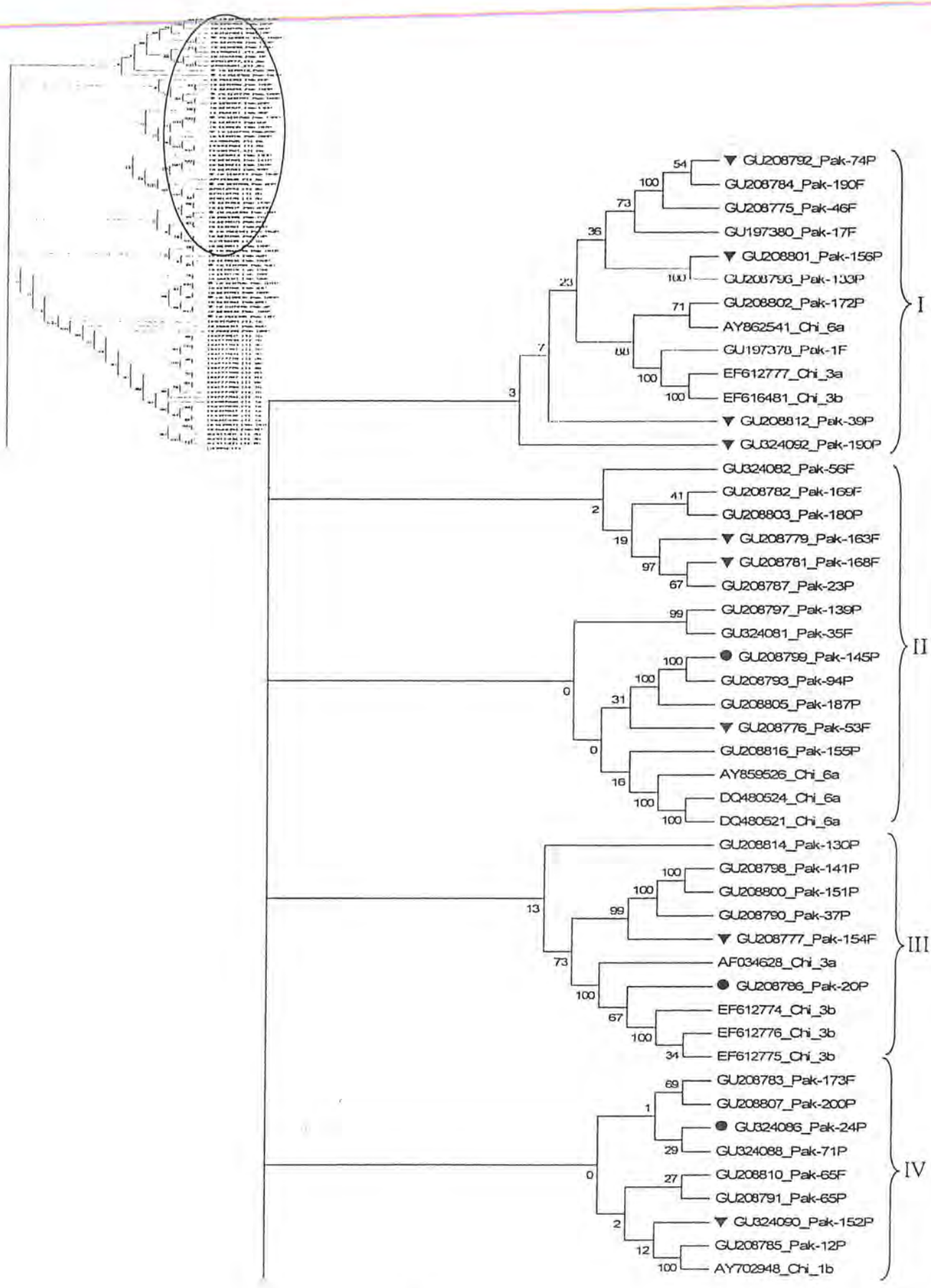

GU208792_Pak-74P
GU208784_Pak-190F
GU208775_Pak-46F
GU197380_Pak-17F
GU208801_Pak-156P
GU208796_Pak-133P
GU208802_Pak-172P
AY862541_Chi_6a
GU197378_Pak-1F
EF612777_Chi_3a
EF616481_Chi_3b
I
GU324092_Pak-190P
GU208794_Pak-98P
GU197379_Pak-4F
GU208809_Pak_197F
GU208812_Pak-39P
GU208816_Pak-155P
GU208783_Pak-173F
GU208807_Pak-200P
II
GU208781_Pak-168F
GU208787_Pak-23P
GU208779_Pak-163F
GU208782_Pak-169F
GU208803_Pak-180P
GU208797_Pak-139P
GU324081_Pak-35F
GU208799_Pak-145P
GU208793_Pak-94P
GU208805_Pak-187P
GU208776_Pak-53F
GU324082_Pak-56F
AY859526_Chi_6a
DQ480524_Chi_6a
DQ480521_Chi_6a
III
GU208806_Pak-194P
GU208789_Pak-34P
GU208813_Pak-42P
GU208811_Pak-138F
GU324083_Pak-181F
GU208814_Pak-130P
GU208798_Pak-141P
GU208800_Pak-151P
GU208790_Pak-37P
GU208777_Pak-154F
AF034628_Chi_3a
GU208786_Pak-20P
EF612776_Chi_3b
EF612775_Chi_3b
EF612774 Chi 3b
IV

Fig. 4.3.10: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective samples from China with *p*-distances. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208792_Pak-74P
GU208784_Pak-190F
GU208775_Pak-46F
GU197380_Pak-17F
GU208801_Pak-156P
GU208796_Pak-133P
GU208802_Pak-172P
AY862541_Chi_6a
GU197378_Pak-1F
EF612777_Chi_3a
EF616481_Chi_3b
GU208812_Pak-39P
GU324092_Pak-190P
I
GU324082_Pak-56F
GU208782_Pak-169F
GU208803_Pak-180P
GU208779_Pak-163F
GU208781_Pak-168F
GU208787_Pak-23P
GU208797_Pak-139P
GU324081_Pak-35F
GU208799_Pak-145P
GU208793_Pak-94P
GU208805_Pak-187P
GU208776_Pak-53F
GU208816_Pak-155P
AY859526_Chi_6a
DQ480524_Chi_6a
DQ480521_Chi_6a
II
GU208814_Pak-130P
GU208798_Pak-141P
GU208800_Pak-151P
GU208790_Pak-37P
GU208777_Pak-154F
AF034628_Chi_3a
GU208786_Pak-20P
EF612774_Chi_3b
EF612776_Chi_3b
EF612775_Chi_3b
III
GU208783_Pak-173F
GU208807_Pak-200P
GU324086_Pak-24P
GU324088_Pak-71P
GU208810_Pak-65F
GU208791_Pak-65P
GU324090_Pak-152P
GU208785_Pak-12P
AY702948_Chi_1b
IV

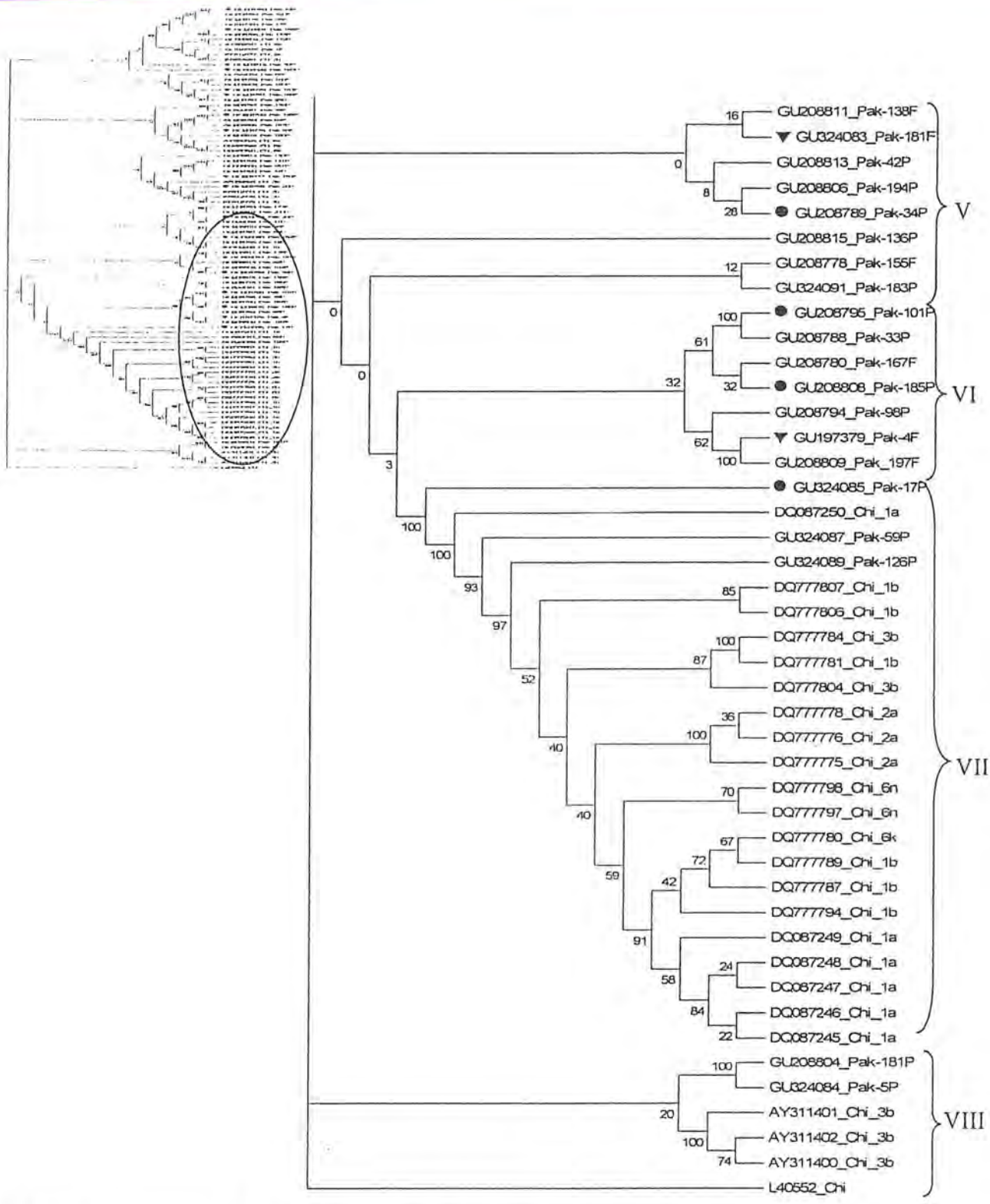
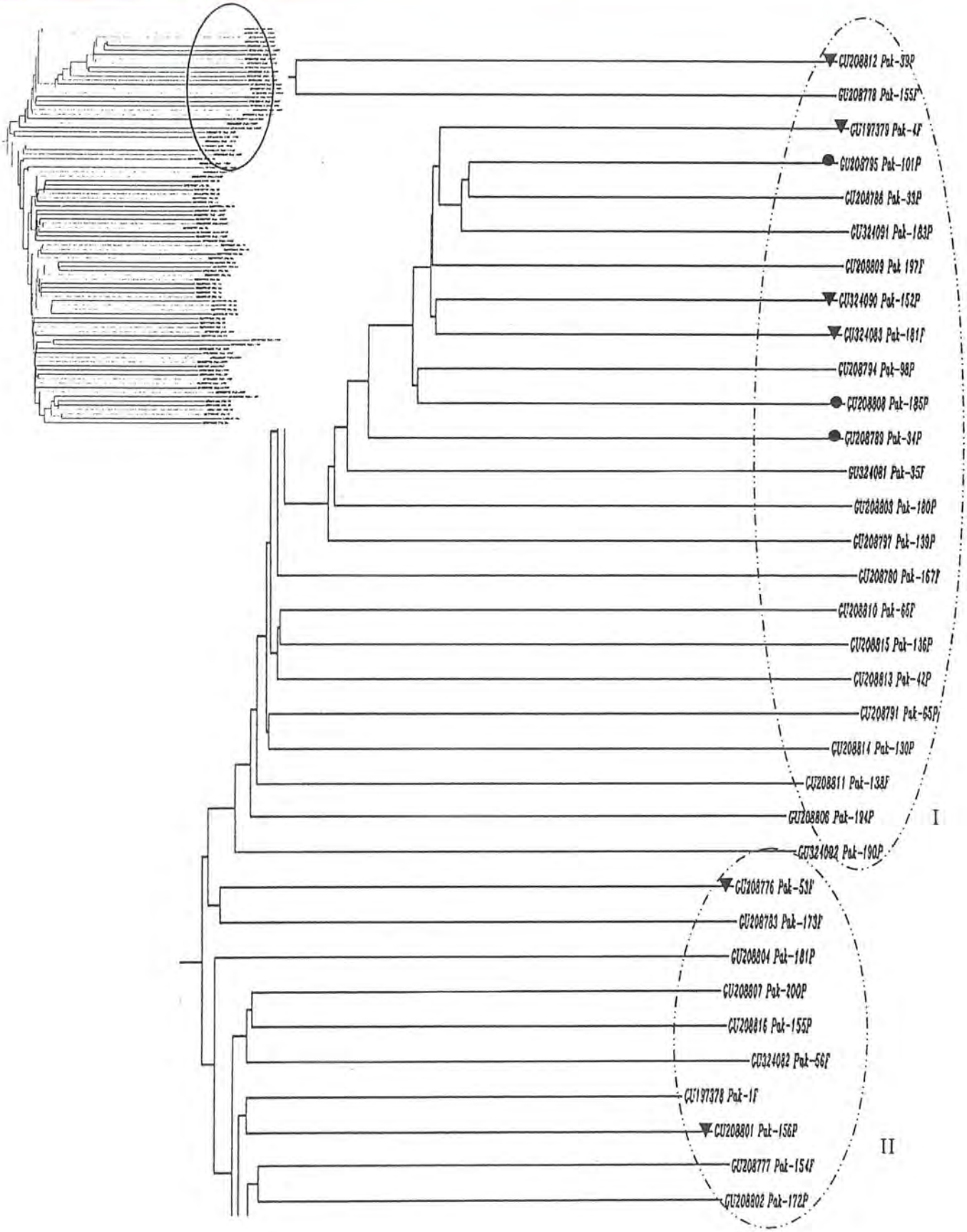


Fig. 4.3.11: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and with reference accessions from china showing bootstrap values. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208812 Pak-33P
GU208778 Pak-155F
GU197379 Pak-4F
GU208795 Pak-101P
GU208788 Pak-33P
GU324091 Pak-183P
GU208809 Pak 197F
GU324090 Pak-152P
GU324083 Pak-181F
GU208794 Pak-98P
GU208808 Pak-185P
GU208789 Pak-34P
GU324081 Pak-35F
GU208803 Pak-180P
GU208797 Pak-139P
GU208780 Pak-167F
GU208810 Pak-66F
GU208815 Pak-136P
GU208813 Pak-42P
GU208791 Pak-65P
GU208814 Pak-130P
GU208811 Pak-138F
GU208806 Pak-194P
GU324092 Pak-190P
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GU208783 Pak-173F
GU208804 Pak-181P
GU208807 Pak-200P
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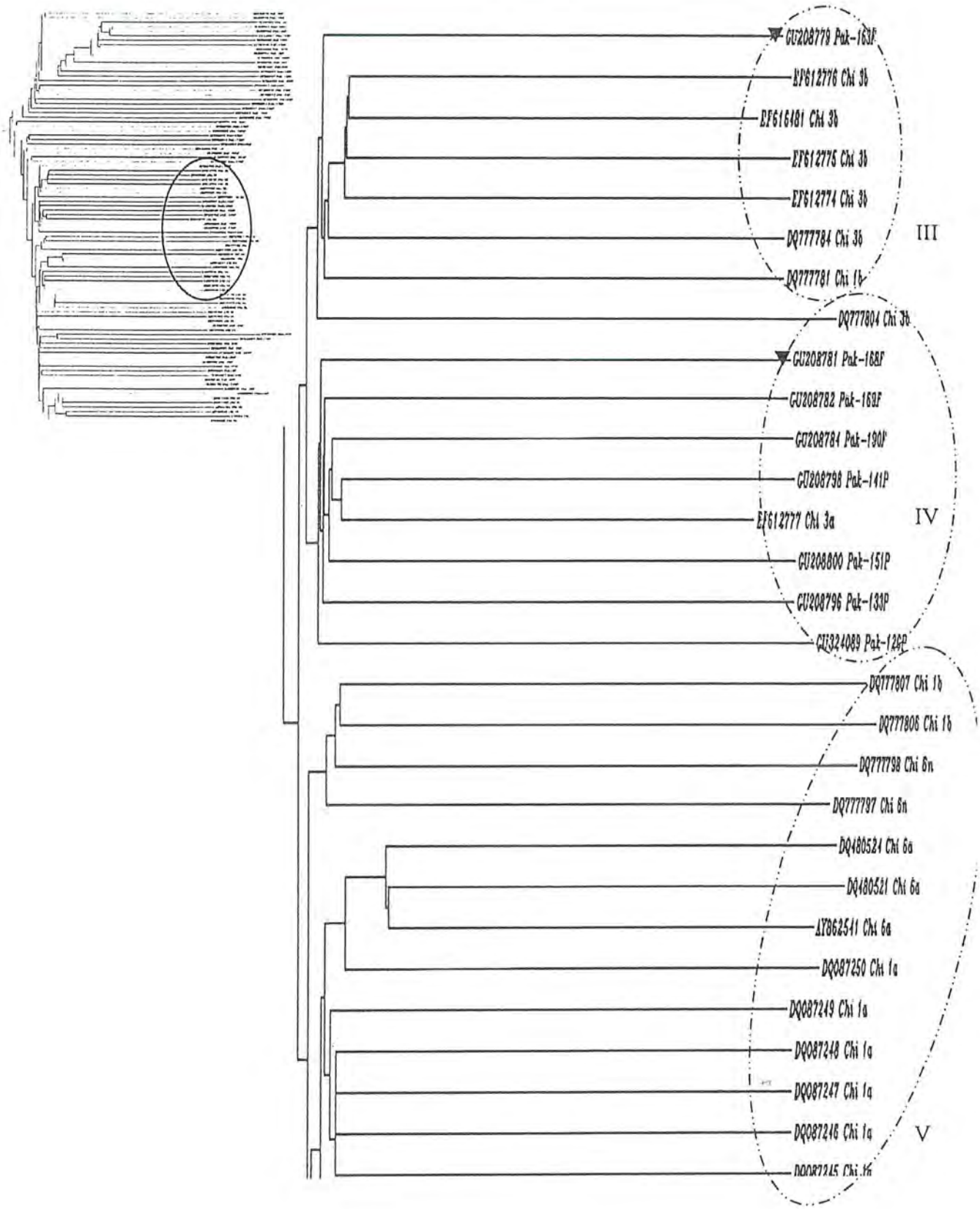
GU208801 Pak-156P
GU208777 Pak-154F
GU208802 Pak-172P
I
II


GU208779 Pak-163F
EF612776 Chi 3b
EF616481 Chi 3b
EF612775 Chi 3b
EF612774 Chi 3b
DQ777784 Chi 3b
DQ777781 Chi 1b
III
DQ777804 Chi 3b
GU208781 Pak-168F
GU208782 Pak-169F
GU208784 Pak-190F
GU208798 Pak-141P
EF612777 Chi 3a
IV
GU208800 Pak-151P
GU208796 Pak-133P
GU324089 Pak-126P
DQ777807 Chi 1b
DQ777806 Chi 1b
DQ777798 Chi 8n
DQ777797 Chi 8n
DQ480524 Chi 6a
DQ480521 Chi 6a
AY862541 Chi 6a
DQ087250 Chi 1a
DQ087249 Chi 1a
DQ087248 Chi 1a
DQ087247 Chi 1a
DQ087246 Chi 1a
V

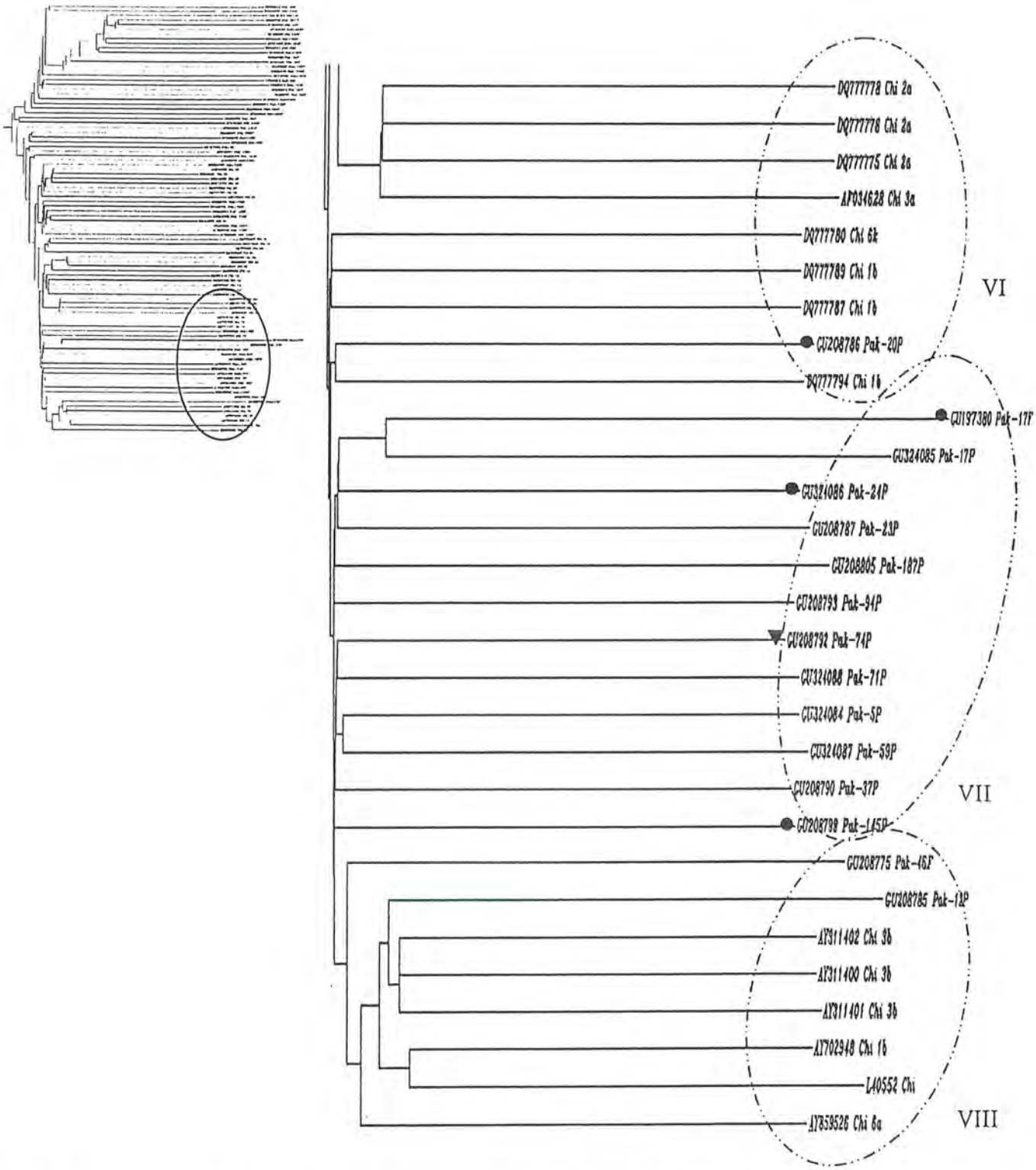


Fig. 4.3.12: Dendrogram of the untypable sequences of 5'UTR from suburban Rawalpindi with reference accessions from China showing branch lengths. Circle stands for known representative genotypes and triangle is for unresolved samples.

AY862541 Chi 6a, AY702948 Chi 1b, AY311402 Chi 3b, AY311401 Chi 3b, AY311400 Chi 3b, AF034628 Chi 3a and L40552 Chi.

NJ Phylogenetic tree of the comparative study between 57 sequenced samples of the present study and Chinese accessions (Fig. 4.3.9) revealed some slightly different pattern from comparative study with reference Pakistani accessions. Chinese accessions pooled themselves in two placement patterns, of that cluster two was a big cluster with very actively diverging genotypes and that cluster was further subdivided into sub clusters one and two. Subcluster one had all the representative accessions of subtype 1a and 1b, and subcluster two consisted of actively mutating subtypes 6n, 2a, 2b and 1b variants.

Two of studied accessions GU324089 and GU324087 (resolved genotype 3 and 1), showed 64% homologies between them (with 98% boot strap support) and provided a proof of right placement of these accessions with Chinese accessions. Tree topology (Fig. 4.3.9) clearly identified the presence of divergent variants in the same clade one. Both Chinese and studied variants were significantly homologous (with non significant bootstrap support) for sister subgroups of Pakistani strains. Tree topology revealed three major subclusters, where subcluster 1 was the representative of the subtypes of genotype 1a, 1b, 6n, 6k, 2a, 2b and 3b etc and had branches of Chinese as well as studied accessions (Fig 4.2.9). While in clade 2 there were 4 clusters, representing majority of the studied sequences. In cluster 1 of clade 2 only AY702948 Chi 1B has placed itself as sister texa with 100% homology (with 100% boot strap support), L40552 Chi was an outlier of cluster VIII, showed homology with non of the representative sequences.

Sample IDs101P, 185P and 4F were the sister subgroups of the same cluster. Sample ID 17P showed 100% homology with Chinese 1a (DQ087250) like wise the homology between 101P and 185P was 61%. Sample ID 34P clustered with 194P and 42P but showed a non-significant homology from boot strap validation point of view, at terminal node level. Sequence homology at 69% level was observed for sample ID 200P for its subgroup texa 173F that assorted itself in an independent cluster. Bootstrapping of

sample ID 20P showed a significant support with Chinese accession AF034628 Chi 3a (with 100% bootstrap support) while Sample 185P had non-significant homology with its sister groups in cluster VI. When the bootstrap tree was computed at a value of 500 replicates, the results revealed a changing trend in the branch topology of 185P, which showed a non-significant homology with its sister texas. Sample ID 17P showed strong homology with Chinese accession DQ087250 1a (with 100% bootstrap support).

In cluster four (Fig. 4.3.9), all resolved studies accessions grouped themselves with Chinese accession 1b (AY702948). Cluster five is very important as it contains representatives of subtype 6v for this subgroup there was a non-significant sequence similarity among the sequences, and they pooled themselves as a subgroup with non significant homologies to any accessions from Pakistan and China. Three of unresolved accessions (GU208781, GU208787 and GU208779) expressed 99% sequence homology (with 74% bootstrap support) by forming a subgroup in cluster II, and did not rearrange themselves with any of the Chinese accessions, clearly indicated that the results were in support of the previously discussed comparison between study samples and Pakistani accessions. Thus it can be concluded that unresolved cluster, constituting of three accessions might be a subtype of genotype 3, on the basis of their homologies with the variants of genotype 3. Over all by segregating pattern of Chinese accessions, from studied sequences it can be concluded that though all the variants have evolved from a single common ancestor but the genetically homogenous samples clustered themselves independently on the basis of their geographical origin and there exists, a regional difference in HCV genotype distribution (Fig. 4.3.11).

When dendrograms analysis of the studied accessions was performed with Chinese accessions using Clustal W software, an evident trend of active mutations and changing genetic make up of HCV was observed (Fig. 4.3.12). Branch lengths of 6v isolates indicate their recent emergence. While majority of other unresolved sequences pooled themselves in first and second cluster (Fig. 4.3.12). A representative of genotype 3 (GU208783) was the most ancient accession of all studied accessions. The results reconfirmed the endemicity of genotype 3 in Gujar Khan, Pakistan and are in agreement with studies reported by (Hamid et al. 2004, Jafri et al. 2006 and Khokhar 2005).

4.3.6 COMPARATIVE PHYLOGENETIC ANALYSIS OF THE STUDIED SEQUENCES WITH REFERENCE ACCESSIONS FROM IRAN

Iran is one of the neighbouring countries of Pakistan and is situated in the Middle East and forms a bridge between Southeast Asia, Europe and Arabian Peninsula (Alavian et al. 2004a, Alavian et al. 2004b and Alavian et al. 2005), with a very low prevalence of disease in the region (Moghadam et al. 2003). Only 1% prevalence of HCV has been reported from Iran (Alavian et al. 2002 and Hosseini et al. 2004). A phylogenetic analysis was performed, in order to find the influence of Irani HCV strains on the distribution of genotypes and subtypes in Pakistan (Fig. 4.3.13-4.3.16). Reference accessions were retrieved from one of the recent Irani research paper (Amini et al. 2009), being relevant to 5'UTR region of HCV. Accessions numbers were taken from this study DQ202322 Irn 3, DQ835671 Irn, DQ835670 Irn, AY545677 Irn 1a, AY545676 Irn 3a, AY545675 Irn 4, AY545674 Irn 1b, AY545673 Irn 4, AY545672 Irn 1a, AY545671 Irn 1a, AY545678 Irn 3a, AY523466 Irn 4, AY523465 Irn 3a, AY523464 Irn 1b, AY523463 Irn 1a and AY515300 Irn 2a respectively. Thirteen of a total sixteen Irani accessions made an independent clade with main representatives of the studied accessions in upper extreme side of the tree (Cluster I) (Fig. 4.3.13). Nine of the study accessions showed non-significant homology with their respective sister subgroups in clade I. Sample resolved as 6v though had evolved from a common ancestor was in close association with Irani accessions Cluster V (Fig. 4.3.14 and Fig. 4.3.15) (non significant bootstrap support) but had arranged themselves as an independent group on the cluster. On the other hand three of Irani accessions (DQ835670, DQ835671 and DQ202322) have placed themselves with present studied sequences. The association was non significant both by *p-distances* and bootstrap analysis. Sample IDs GU197379 Pak-4F showed strong homology with GU208809 Pak-197F and GU208794 Pak-98P (with 99% boot strap support) (Fig. 4.3.14).

Topology of the NJ phylogenetic tree revealed a trend of divergent evolution. Sample GU20886 Pak-20P showed 99% homology with sister groups (With 99% bootstrap support), while a resolved sample of genotype 3 GU208789 Pak-34P was 74% homologous to sample ID 181F but the association was non-significant (Fig 4.4.3.14).

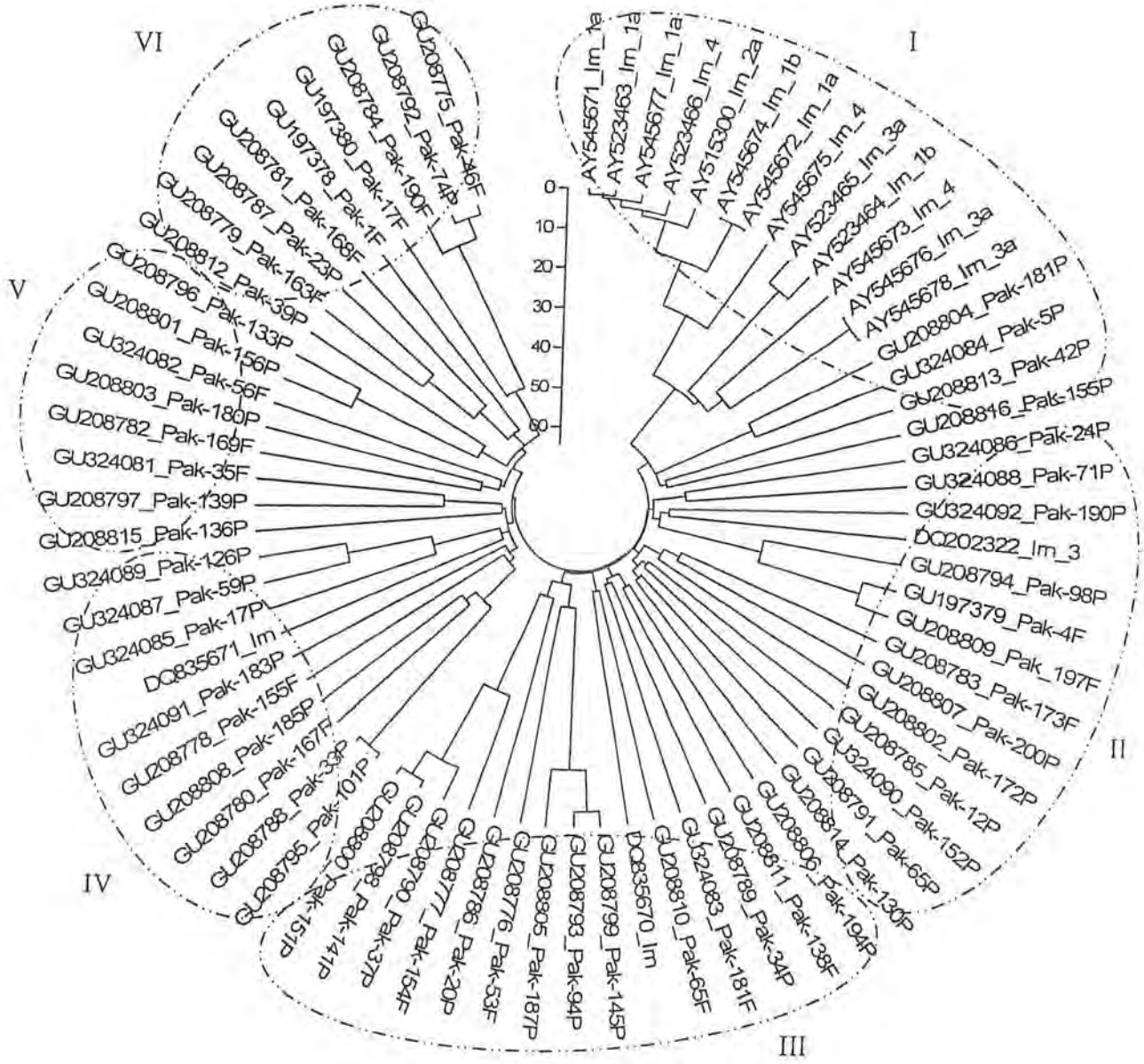
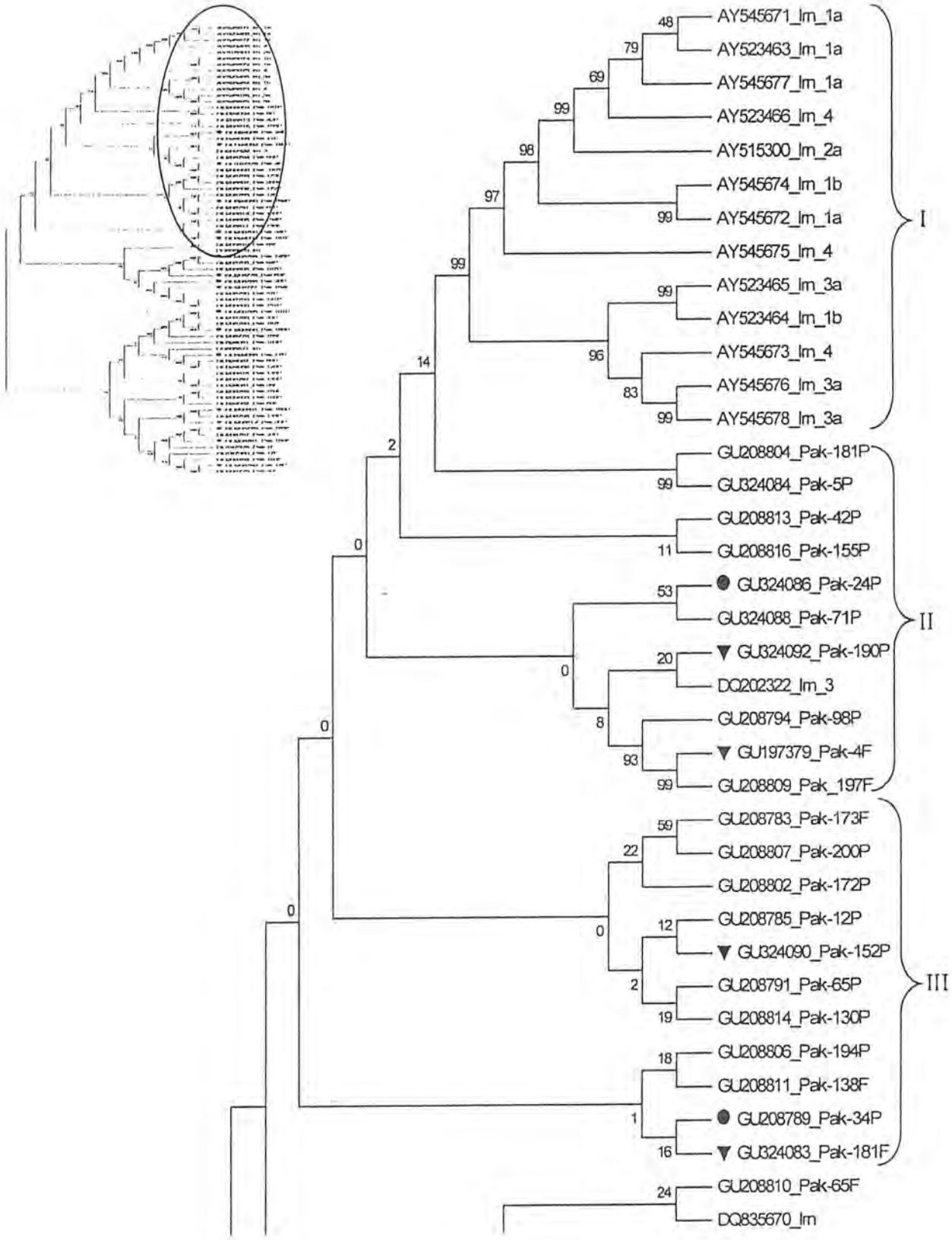


Fig. 4.3.13: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective samples from Iran.


AY545671_lm_1a
AY523463_lm_1a
AY545677_lm_1a
AY523466_lm_4
AY515300_lm_2a
AY545674_lm_1b
AY545672_lm_1a
AY545675_lm_4
AY523465_lm_3a
AY523464_lm_1b
AY545673_lm_4
AY545676_lm_3a
AY545678_lm_3a
I
GU208804_Pak-181P
GU324084_Pak-5P
GU208813_Pak-42P
GU208816_Pak-155P
GU324086_Pak-24P
GU324088_Pak-71P
GU324092_Pak-190P
DQ202322_lm_3
GU208794_Pak-98P
GU197379_Pak-4F
GU208809_Pak_197F
II
GU208783_Pak-173F
GU208807_Pak-200P
GU208802_Pak-172P
GU208785_Pak-12P
GU324090_Pak-152P
GU208791_Pak-65P
GU208814_Pak-130P
GU208806_Pak-194P
GU208811_Pak-138F
GU208789_Pak-34P
GU324083_Pak-181F
III
GU208810_Pak-65F
DQ835670_lm

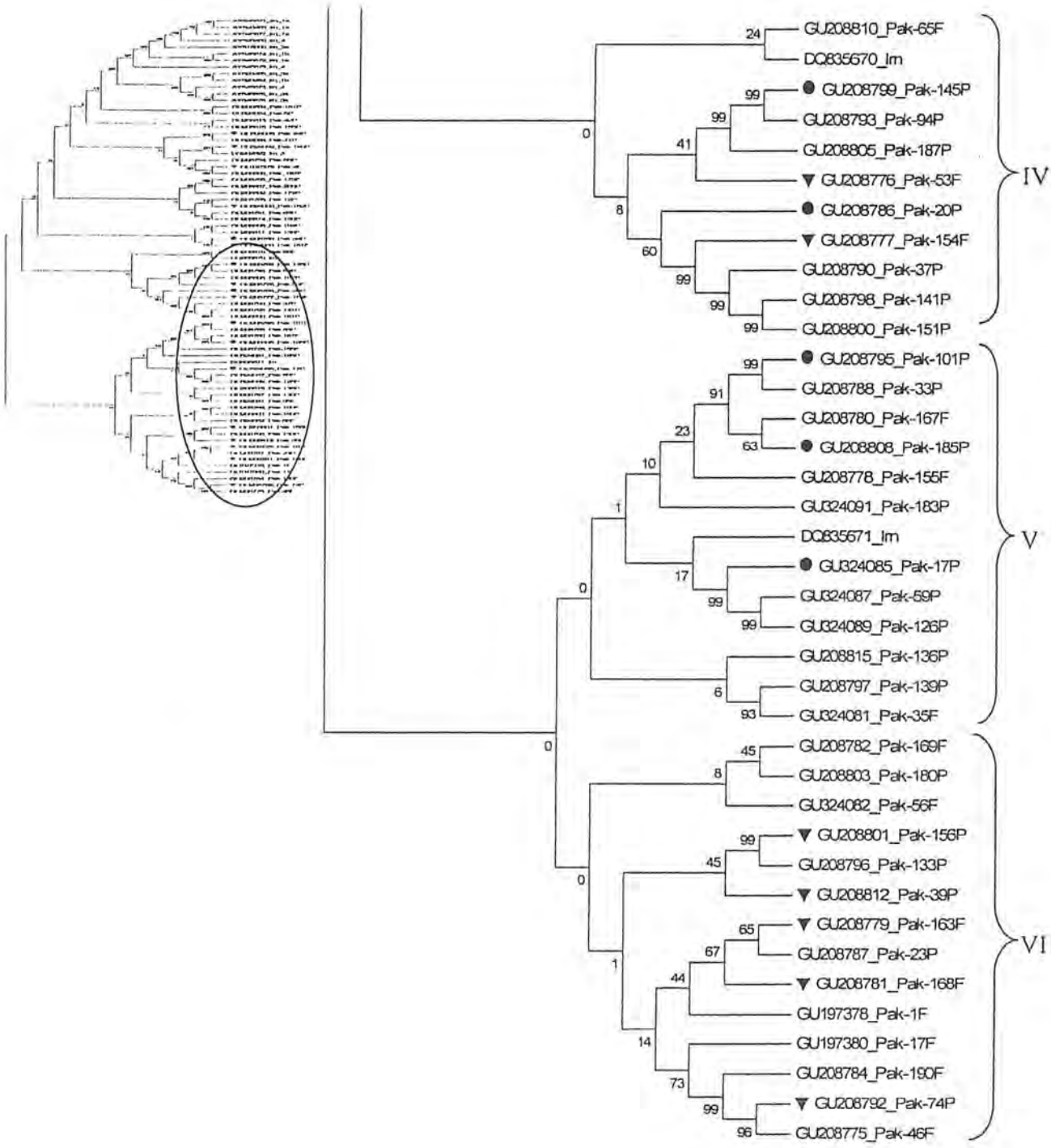
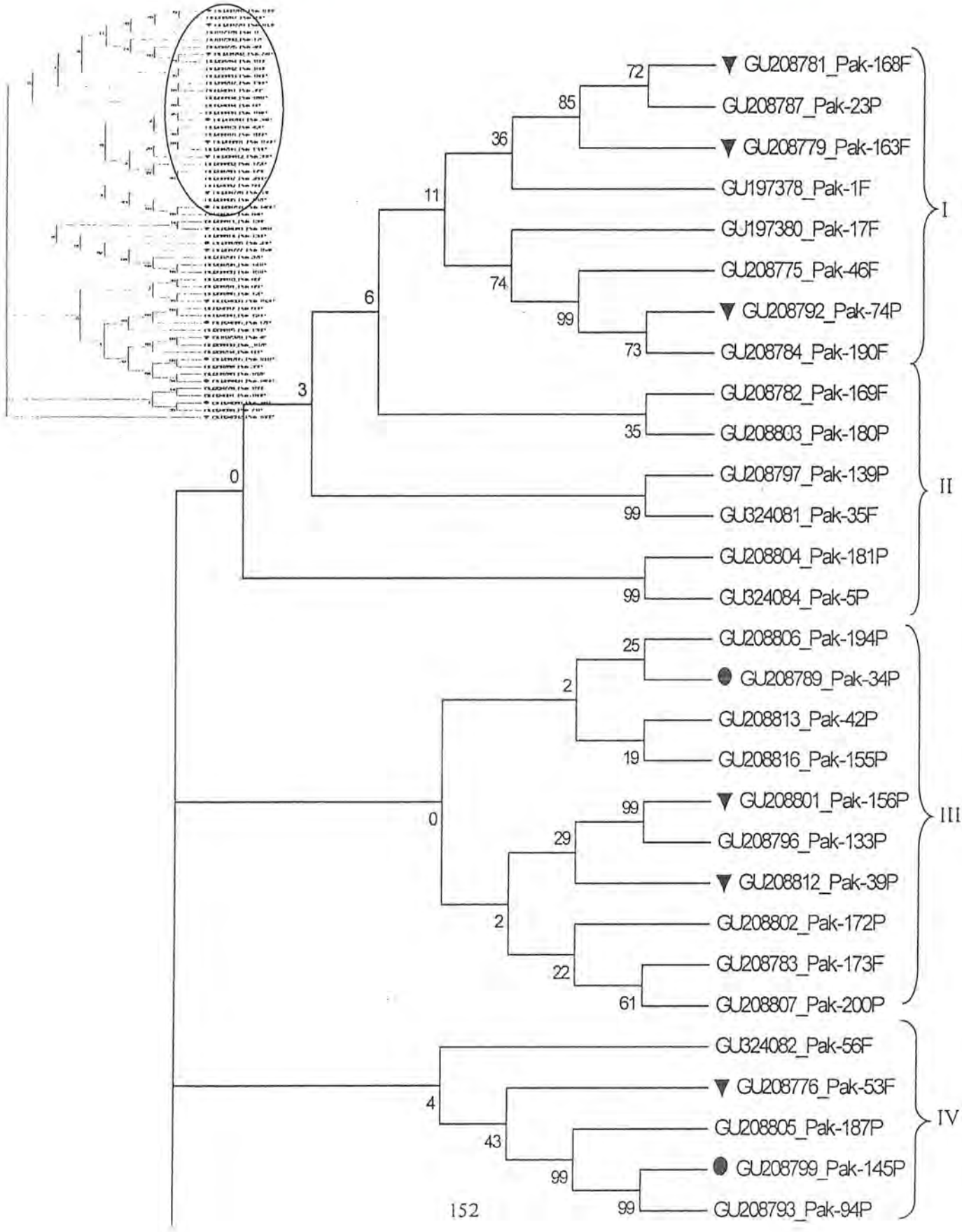


Fig. 4.3.14: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective samples from Iran with *p*-distances. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208781_Pak-168F
GU208787_Pak-23P
GU208779_Pak-163F
GU197378_Pak-1F
GU197380_Pak-17F
GU208775_Pak-46F
GU208792_Pak-74P
GU208784_Pak-190F
GU208782_Pak-169F
GU208803_Pak-180P
GU208797_Pak-139P
GU324081_Pak-35F
GU208804_Pak-181P
GU324084_Pak-5P
GU208806_Pak-194P
GU208789_Pak-34P
GU208813_Pak-42P
GU208816_Pak-155P
GU208801_Pak-156P
GU208796_Pak-133P
GU208812_Pak-39P
GU208802_Pak-172P
GU208783_Pak-173F
GU208807_Pak-200P
GU324082_Pak-56F
GU208776_Pak-53F
GU208805_Pak-187P
GU208799_Pak-145P
GU208793_Pak-94P
72
85
36
11
74
99
73
6
3
35
0
99
99
25
2
19
0
99
29
2
22
61
4
43
99
99
I
II
III
IV

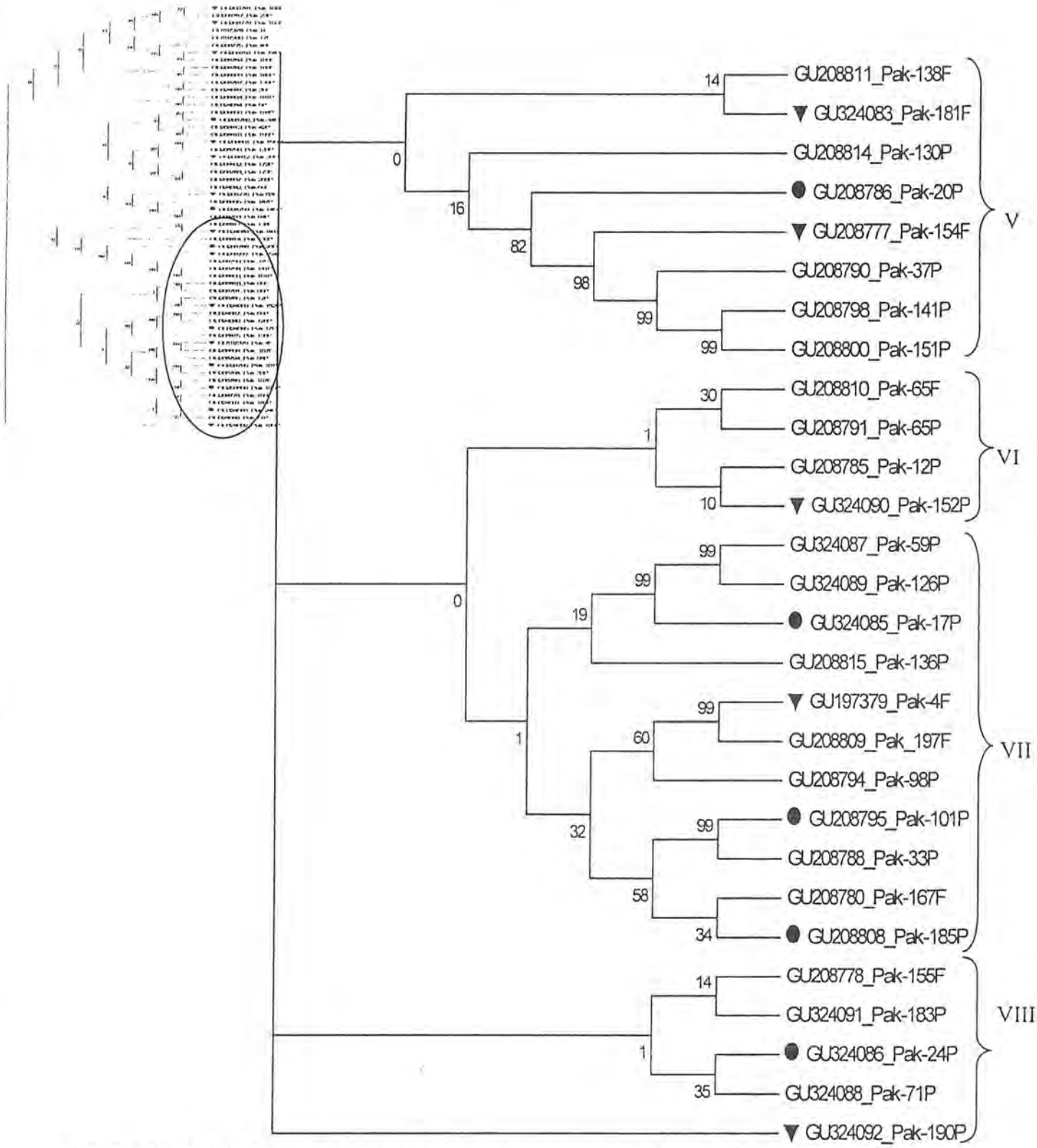
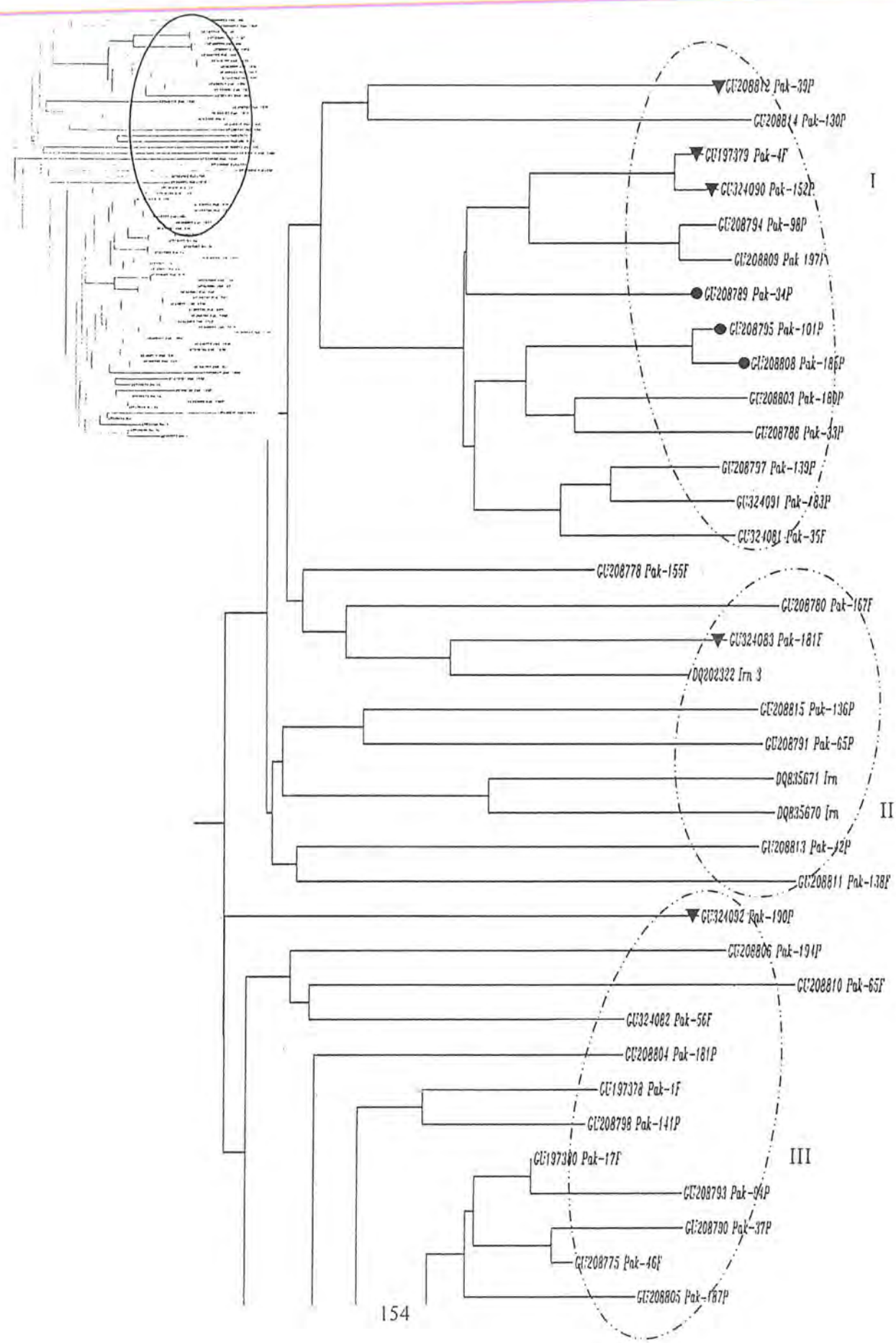


Fig. 4.3.15: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and with reference accessions from Iran showing bootstrap values. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208812 Pak-39P
GU208814 Pak-130P
GU197379 Pak-4F
GU324090 Pak-152P
GU208794 Pak-98P
GU208809 Pak 197P
GU208789 Pak-34P
GU208795 Pak-101P
GU208808 Pak-186P
GU208803 Pak-180P
GU208788 Pak-33P
GU208797 Pak-139P
GU324091 Pak-183P
GU324081 Pak-35F
I
GU208778 Pak-155F
GU208780 Pak-167F
GU324083 Pak-181F
DQ202322 Irn 3
GU208815 Pak-136P
GU208791 Pak-65P
DQ835671 Irn
DQ835670 Irn
II
GU208813 Pak-12P
GU208811 Pak-138F
GU324092 Pak-190P
GU208806 Pak-194P
GU208810 Pak-65F
GU324082 Pak-56F
GU208804 Pak-181P
GU197378 Pak-1F
GU208798 Pak-141P
GU197380 Pak-17F
III
GU208793 Pak-94P
GU208790 Pak-37P
GU208775 Pak-46F
GU208805 Pak-187P

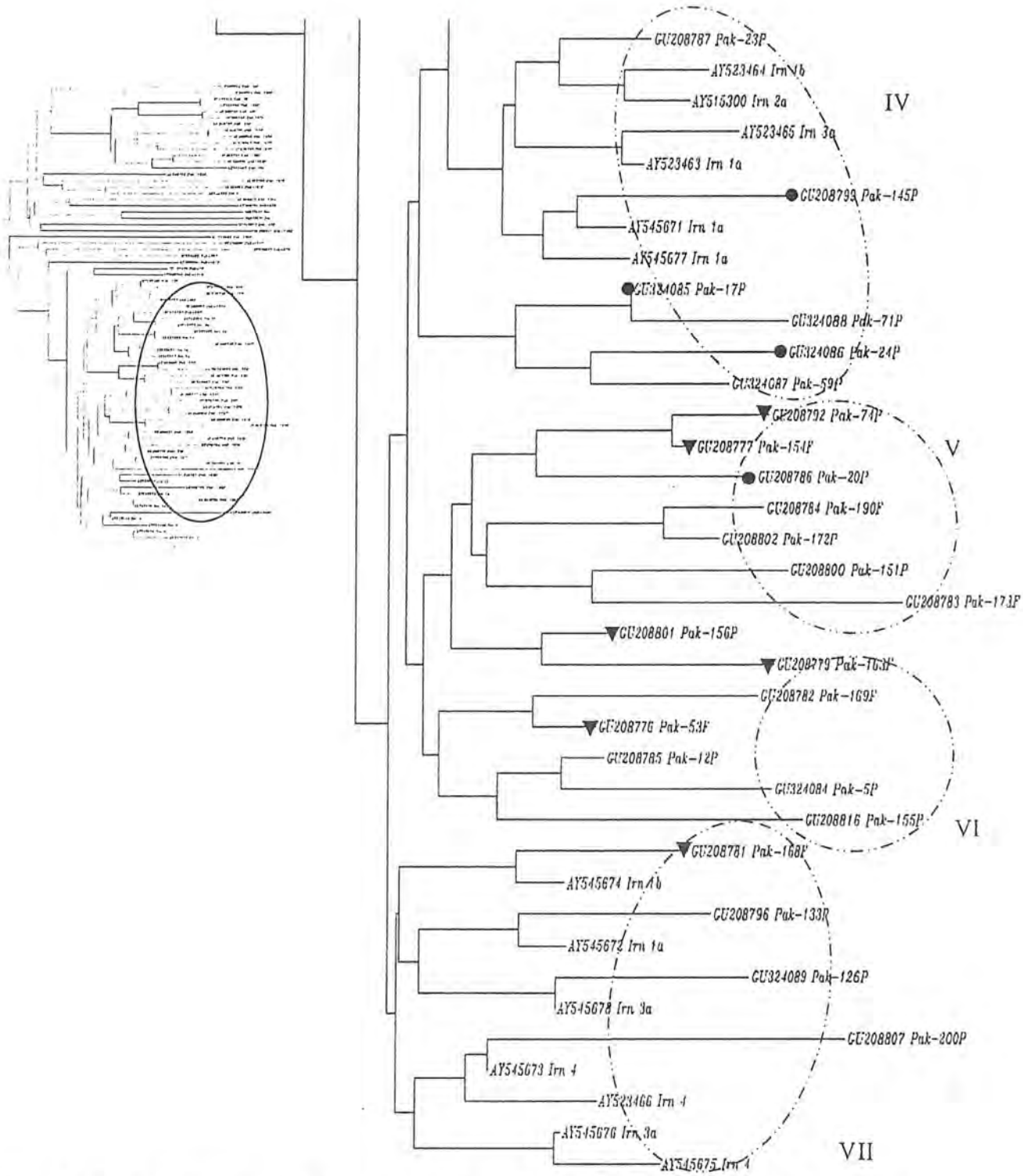


Fig. 4.3.16: Dendrogram of the untypable sequences of HCV 5'UTR from suburban Rawalpindi, Pakistan with reference accessions from Iran showing branch lengths. Circle stands for known representative genotypes and triangle is for unresolved samples.

The similarity of 24P with its sister texa 71P was 53% (non significant bootstrap support). Sample IDs 145P and 101P had sequence similarity 99% for their sister subgroups, 94P and 33P. Sample ID 185P though had 67% homology for 167F but the bootstrap values did not support the tree topologies. From all of the Irani accessions only DQ202322 Irn 3, DQ835670 Irn, DQ835671 had no significant homologies with studied samples. Remaining accessions from Iran have shown an independent cluster I (Fig 4.3.15). Dendrograms topology revealed a trend in which studied samples had evolved earlier than the reference Irani accessions indicating far more ancient endemicity history of HCV in subcontinent and particularly Pakistan (Fig. 4.3.16). The negligibly low homology of studied sample with Irani accessions indicated that the HCV genotypes of Pakistan were not overlapping with the genotypes from Iran, though all of the representative HCV strains were the descendents of the same ancestor but all had evolved independently. These findings are in strong disagreement with the results reported by Alavian et al (2005) from Iran, where he attributed the geographical position of Iran, mass migration of the refugees from Afghanistan to Iran, legal and illegal immigration to Europe, illegal network of drug trafficking among Iran, Afghanistan and Pakistan, as the key factors affecting the HCV genotype distribution patterns in all these countries. In general, it can be concluded that though there might be an affect of neighbouring countries on the distribution of Pakistani HCV genotypes but these affects are negligible in majority of instances.

4.3.7 COMPARATIVE PHYLOGENETIC ANALYSIS OF THE STUDIED SEQUENCES WITH REFERENCE ACCESSIONS FROM INDIA

India is the world's second largest populous country with an estimated population to be 1.1 billion (Census of India 2001). Reference accessions from India were retrieved from two studies by Chaudhuri et al (2004) and Valliammai et al. (1995). A Phylogenetic analysis was performed, in order to find the influence of Indian HCV strains on the distribution of genotypes and subtypes in Pakistan (Fig. 4.3.17-4.3.20). The reason of selection of these particular research papers was their selection of 5'UTR region of HCV for sequencing. The retrieved accessions were FJ407092 Ind 3i, AY231582 Ind 1a, AY231583 Ind 6b,

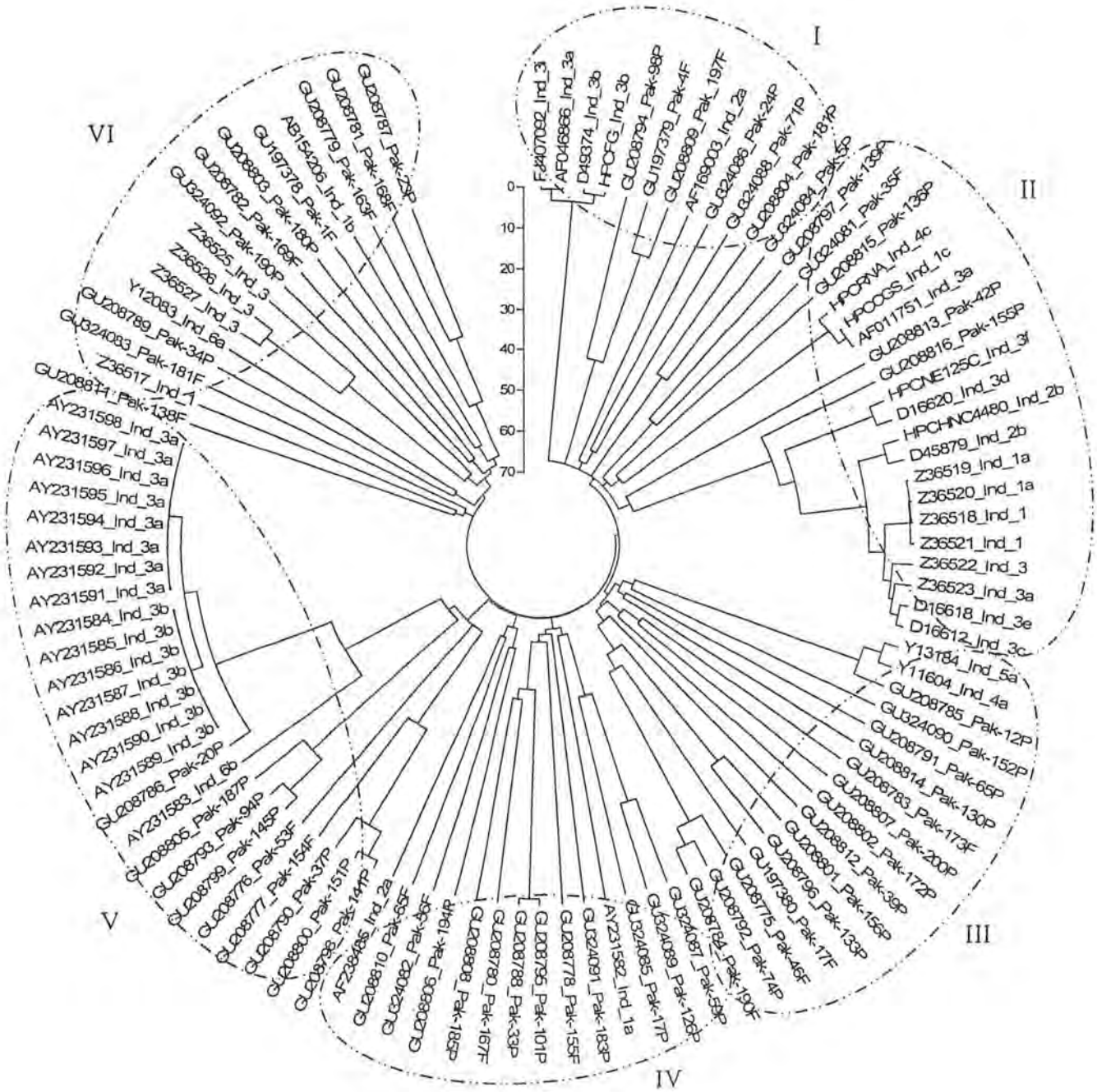
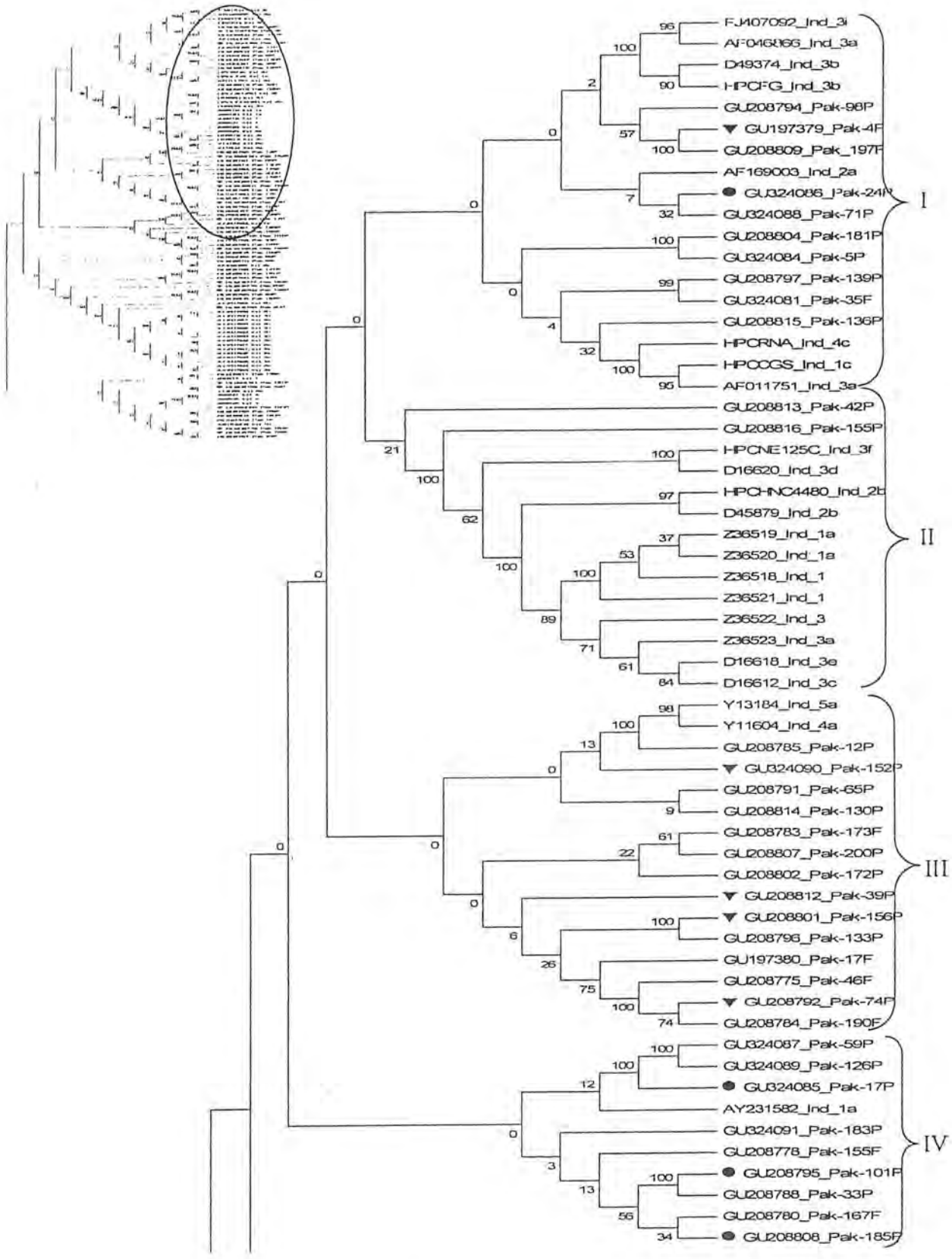


Fig. 4.3.17: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective samples from India.


FJ407092_Ind_3i
AF046866_Ind_3a
D49374_Ind_3b
HPCFG_Ind_3b
GU208794_Pak-98P
GU197379_Pak-4F
GU208809_Pak_197F
AF169003_Ind_2a
GU324086_Pak-24P
GU324088_Pak-71P
GU208804_Pak-181P
GU324084_Pak-5P
GU208797_Pak-139P
GU324081_Pak-35F
GU208815_Pak-136P
HPCRNA_Ind_4c
HPCCGS_Ind_1c
AF011751_Ind_3a
GU208813_Pak-42P
GU208816_Pak-155P
HPCNE125C_Ind_3f
D16620_Ind_3d
HPCHNC4480_Ind_2b
D45879_Ind_2b
Z36519_Ind_1a
Z36520_Ind_1a
Z36518_Ind_1
Z36521_Ind_1
Z36522_Ind_3
Z36523_Ind_3a
D16618_Ind_3e
D16612_Ind_3c
Y13184_Ind_5a
Y11604_Ind_4a
GU208785_Pak-12P
GU324090_Pak-152P
GU208791_Pak-65P
GU208814_Pak-130P
GU208783_Pak-173F
GU208807_Pak-200P
GU208802_Pak-172P
GU208812_Pak-39P
GU208801_Pak-156P
GU208796_Pak-133P
GU197380_Pak-17F
GU208775_Pak-46F
GU208792_Pak-74P
GU208784_Pak-190F
GU324087_Pak-59P
GU324089_Pak-126P
GU324085_Pak-17P
AY231582_Ind_1a
GU324091_Pak-183P
GU208778_Pak-155F
GU208795_Pak-101P
GU208788_Pak-33P
GU208780_Pak-167F
GU208808_Pak-185P
I
II
III
IV

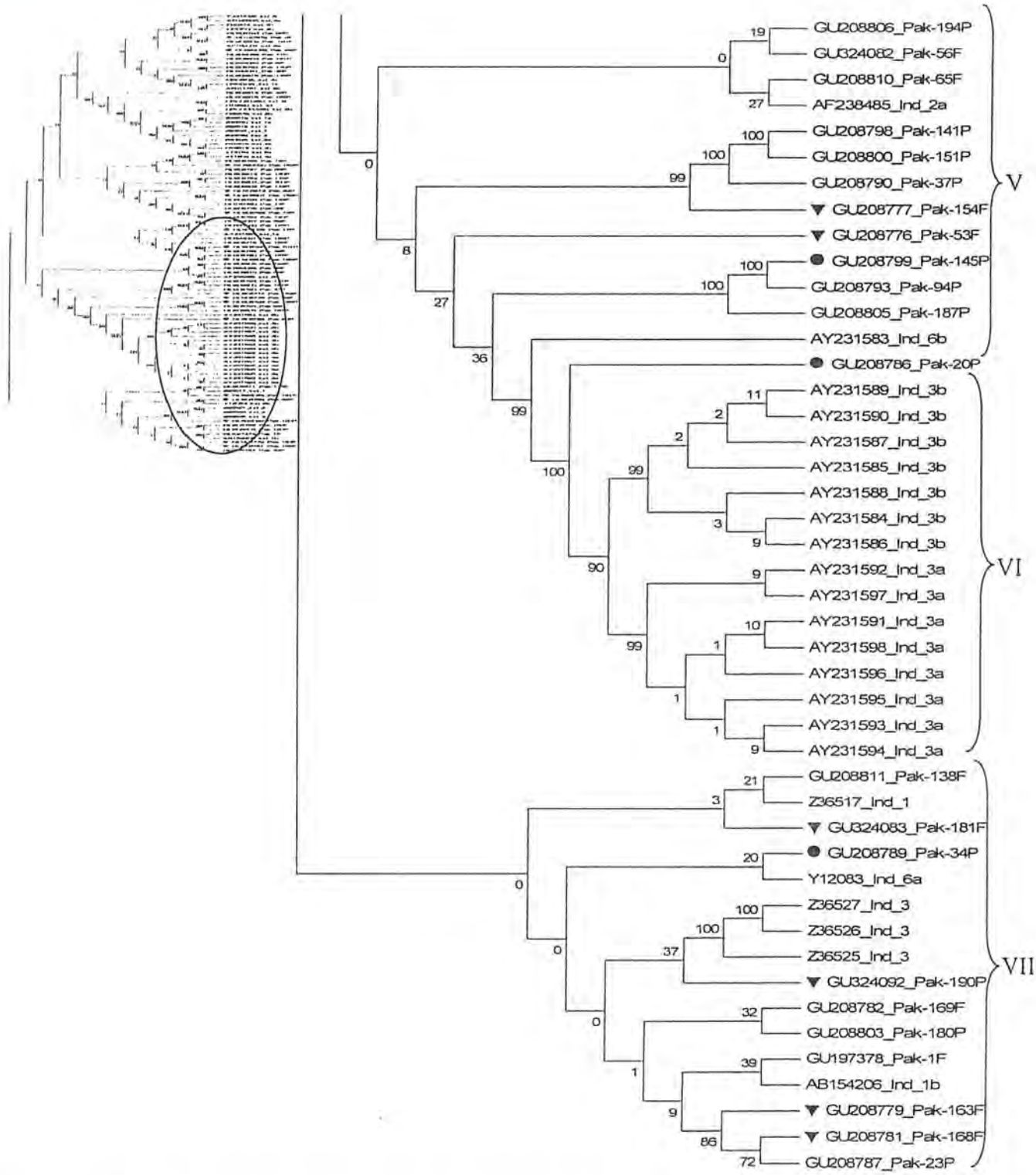
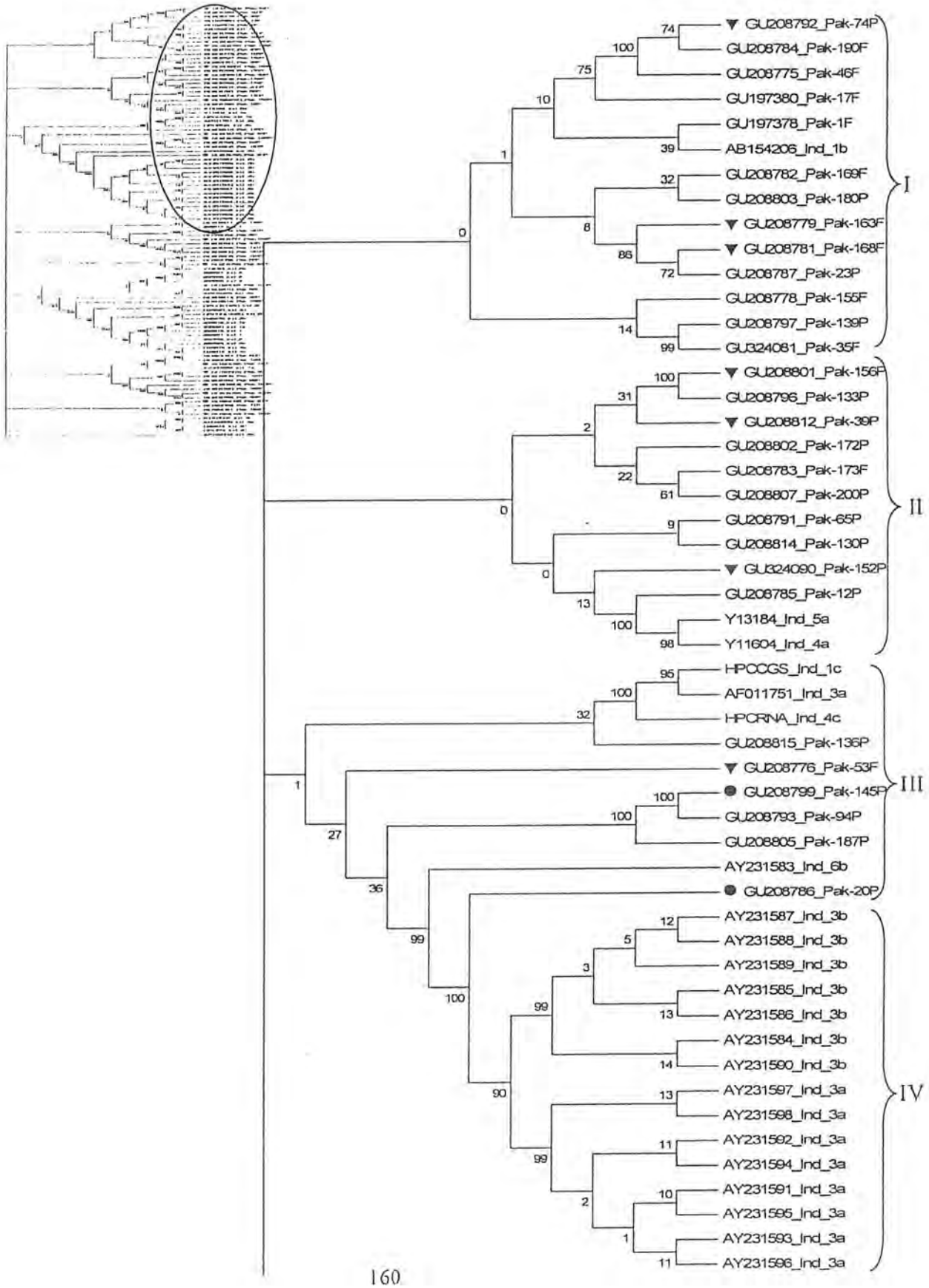


Fig. 4.3.18: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective accessions from India with *p*-distances. Circle stands for known representative genotypes and triangle is for unresolved samples.


GU208792_Pak-74P
GU208784_Pak-190F
GU208775_Pak-46F
GU197380_Pak-17F
GU197378_Pak-1F
AB154206_Ind_1b
GU208782_Pak-169F
GU208803_Pak-180P
GU208779_Pak-163F
GU208781_Pak-168F
GU208787_Pak-23P
GU208778_Pak-155F
GU208797_Pak-139P
GU324081_Pak-35F
I
GU208801_Pak-156P
GU208796_Pak-133P
GU208812_Pak-39P
GU208802_Pak-172P
GU208783_Pak-173F
GU208807_Pak-200P
GU208791_Pak-65P
GU208814_Pak-130P
GU324090_Pak-152P
GU208785_Pak-12P
Y13184_Ind_5a
Y11604_Ind_4a
II
HPCCGS_Ind_1c
AF011751_Ind_3a
HPCRNA_Ind_4c
GU208815_Pak-136P
GU208776_Pak-53F
GU208799_Pak-145P
GU208793_Pak-94P
GU208805_Pak-187P
AY231583_Ind_6b
GU208786_Pak-20P
III
AY231587_Ind_3b
AY231588_Ind_3b
AY231589_Ind_3b
AY231585_Ind_3b
AY231586_Ind_3b
AY231584_Ind_3b
AY231590_Ind_3b
AY231597_Ind_3a
AY231598_Ind_3a
AY231592_Ind_3a
AY231594_Ind_3a
AY231591_Ind_3a
AY231595_Ind_3a
AY231593_Ind_3a
AY231596_Ind_3a
IV

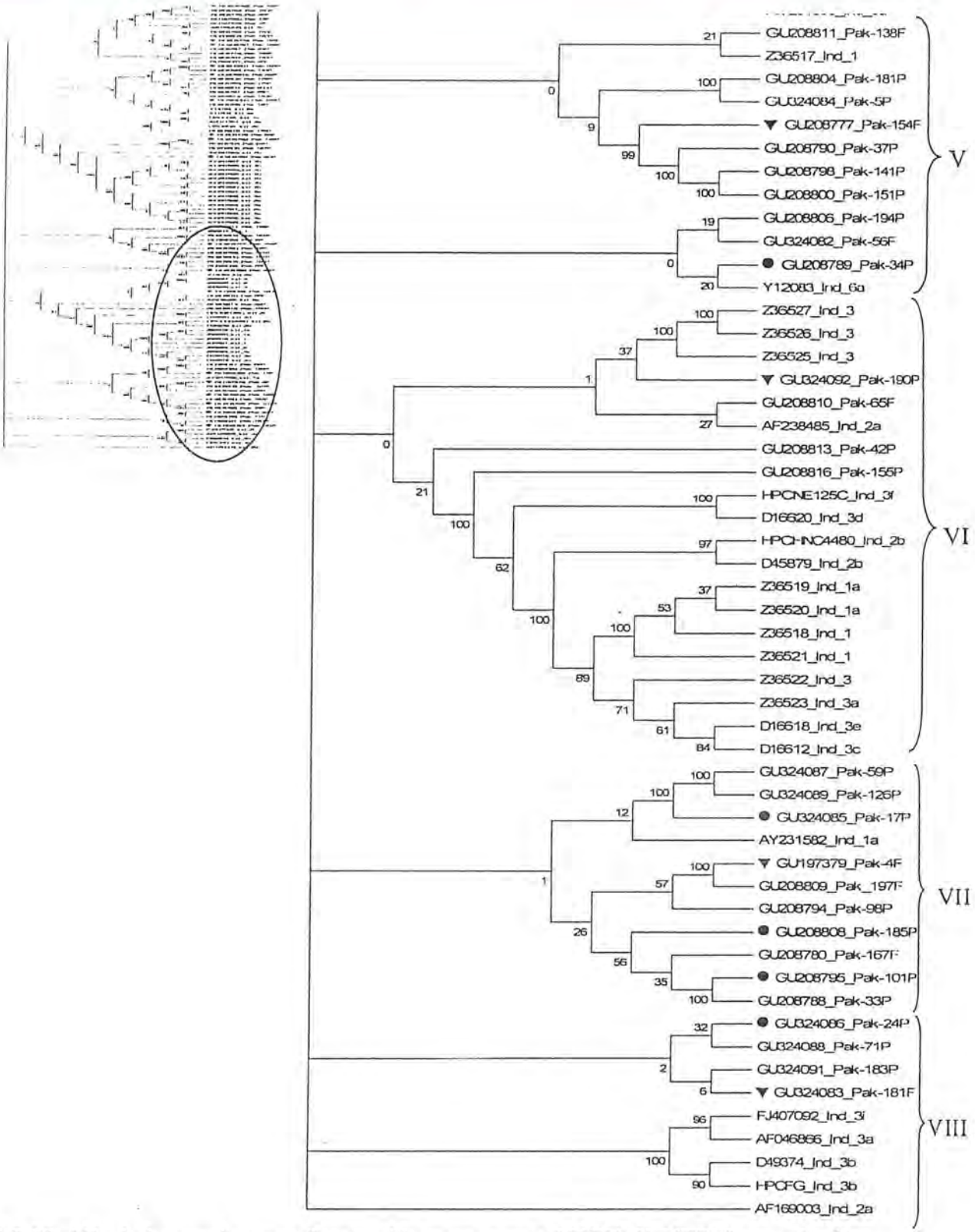
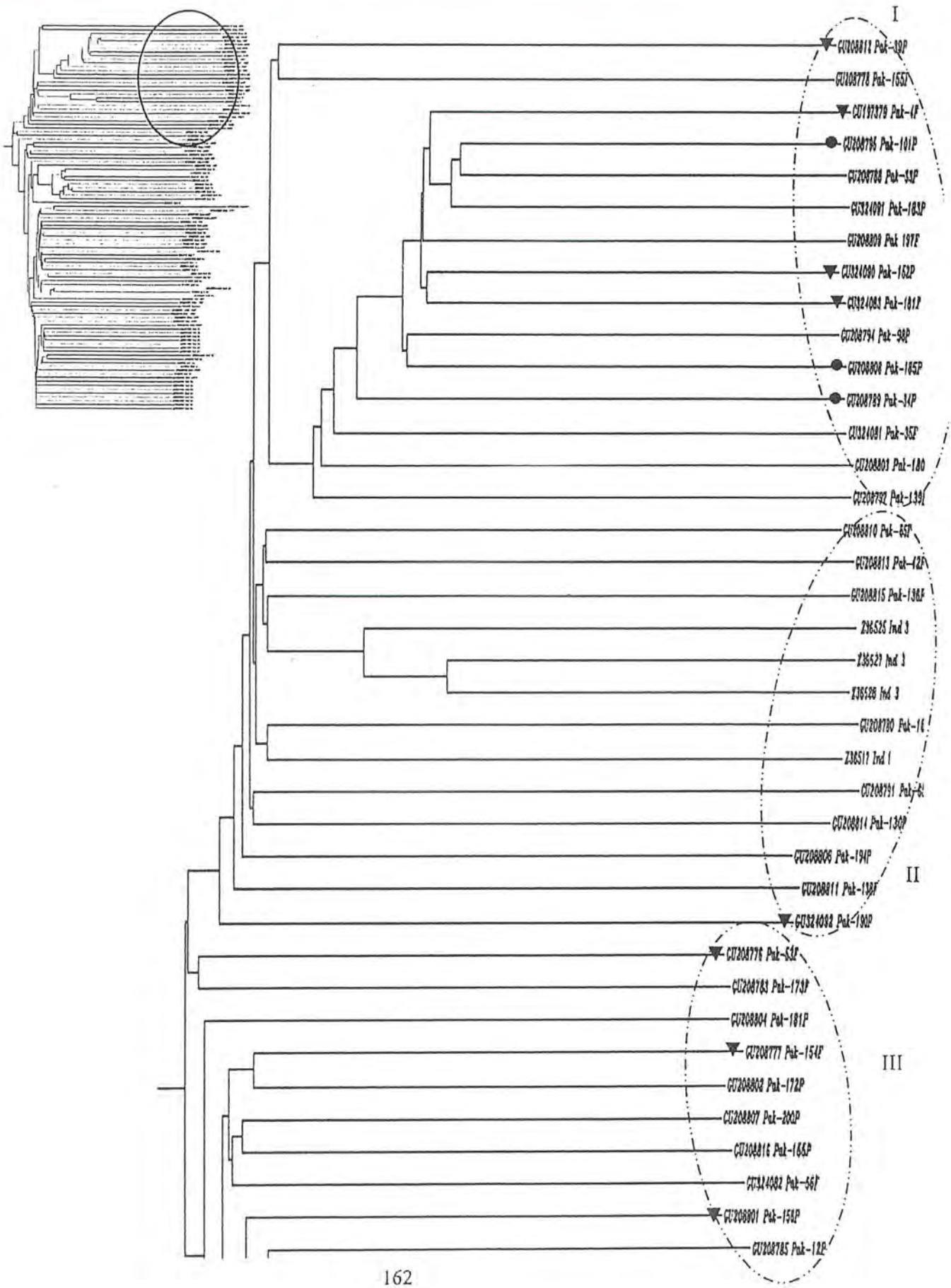
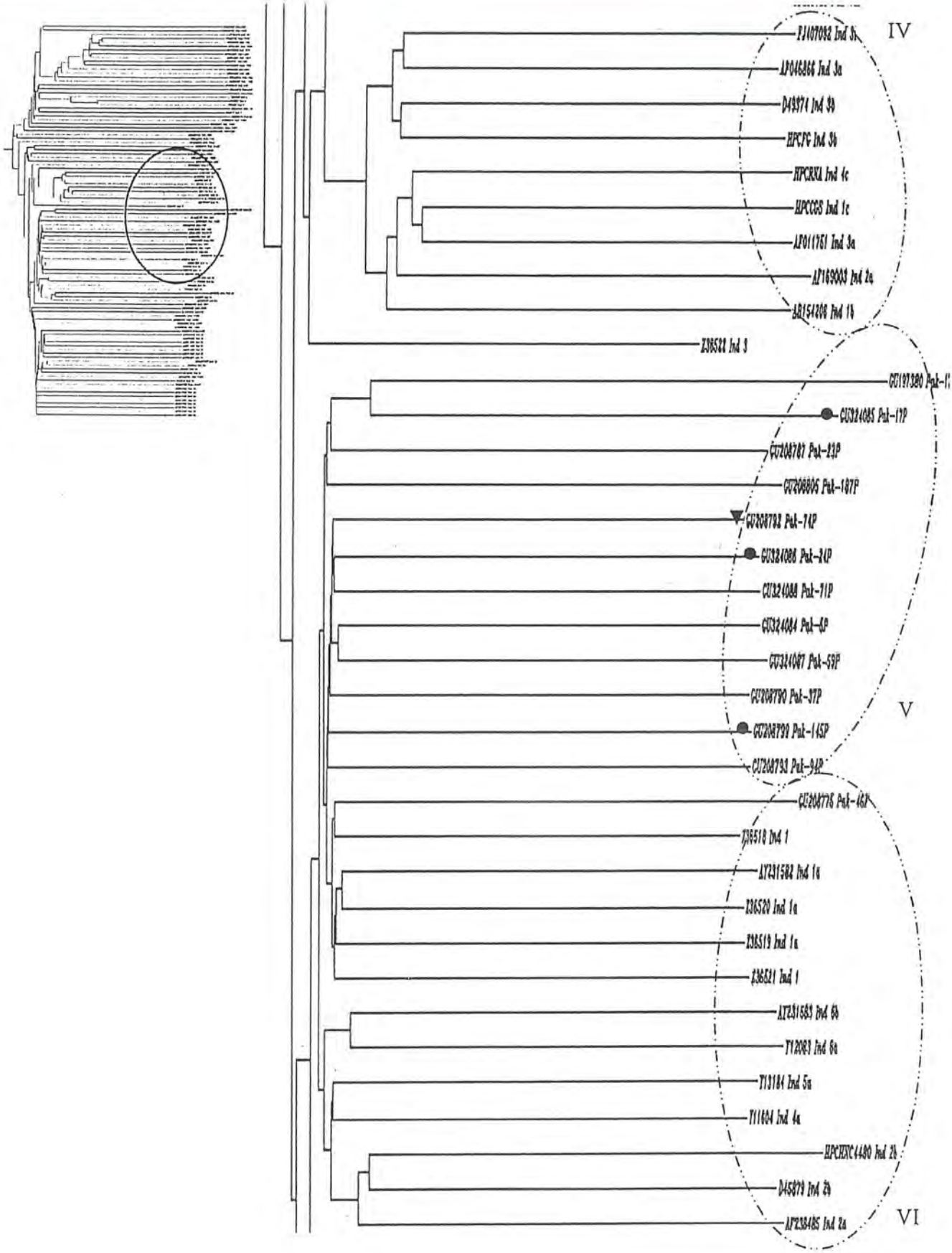


Fig. 4.3.19: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and with reference accessions from India showing bootstrap values. Circle stands for known representative genotypes and triangle is for unresolved samples.


I
II
III
GU208812 Pak-19P
GU208778 Pak-155P
GU197879 Pak-1F
GU208795 Pak-101P
GU208788 Pak-93P
GU324091 Pak-183P
GU208809 Pak 197P
GU324090 Pak-162P
GU324083 Pak-181P
GU208794 Pak-98P
GU208808 Pak-185P
GU208789 Pak-34P
GU324081 Pak-35P
GU208803 Pak-180
GU208792 Pak-139P
GU208810 Pak-65P
GU208813 Pak-12P
GU208815 Pak-136P
Z36525 Ind 3
Z36527 Ind 3
Z36528 Ind 3
GU208780 Pak-16
Z36517 Ind 1
GU208791 Pak-6
GU208814 Pak-130P
GU208806 Pak-194P
GU208811 Pak-138P
GU324092 Pak-190P
GU208776 Pak-53P
GU208783 Pak-173P
GU208804 Pak-181P
GU208777 Pak-154P
GU208802 Pak-172P
GU208807 Pak-200P
GU208816 Pak-165P
GU324082 Pak-56P
GU208801 Pak-158P
GU208785 Pak-12P


IV
V
VI

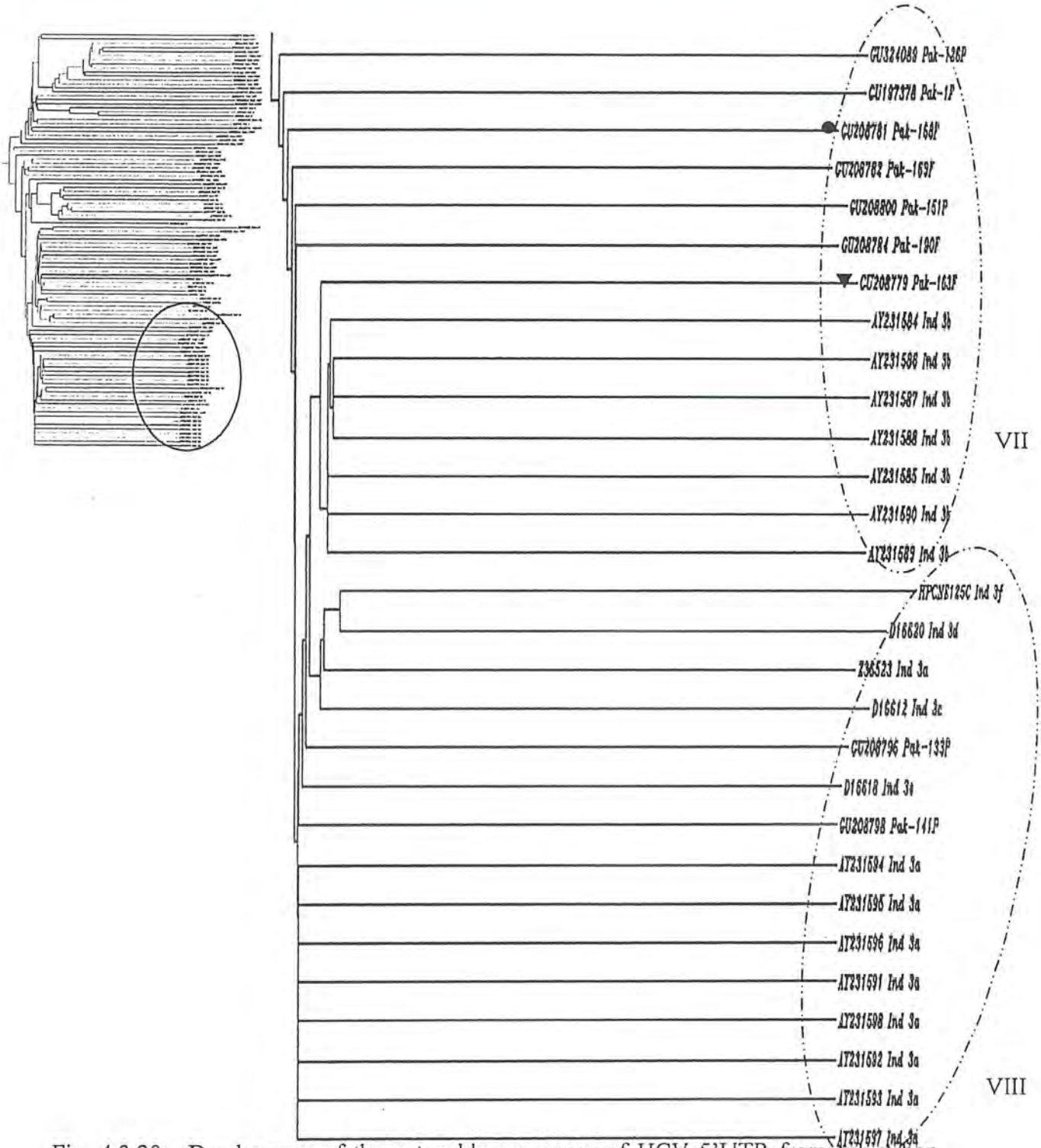


Fig. 4.3.20: Dendrogram of the untypable sequences of HCV 5'UTR from sub urban Rawalpindi, Pakistan with reference accessions from India showing branch lengths. Circle stands for known representative genotypes and triangle is for unresolved samples.

AY231584 Ind 3b, AY231585 Ind 3b, AY231586 Ind 3b, AY231587 Ind 3b, AY231588 Ind 3b, AY231589 Ind 3b, AY231590 Ind 3b, AY231591 Ind 3a, AY231592 Ind 3a, AY231593 Ind 3a, AY231594 Ind 3a, AY231595 Ind 3a, AY231596 Ind 3a, AY231597 Ind 3a, AY231598 Ind 3a, Y12083 Ind 6a, Y13184 Ind 5a, HPCRNA Ind 4c, Y11604 Ind 4a, AF046866 Ind 3a, HPCHNC448O Ind 2b, Z36519 Ind 1a, Z36520 Ind 1a, Z36521 Ind 1, Z36522 Ind 3, D49374 Ind 3b, HPCFG Ind 3b, AF238485 Ind 2a, HPCCGS Ind 1c, AB154206 Ind 1b, HPCNE125C Ind 3f, D16618 Ind 3e, D16620 Ind 3d, D16612 Ind 3c, D45879 Ind 2b, AF011751 Ind 3a, Z36523 Ind 3, Z36525 Ind 3, Z36527 Ind 3, Z36517 Ind 1, Z36526 Ind 3, Z36518 Ind 1 and AF169003 Ind 2a.

When these retrieved accessions were compared by phylogenetic analysis with the studied samples, to find the temporal trends of HCV genotypes in Pakistan and India, the resulting tree topology revealed that cluster I and IV formed independent Sub groups constituting of Indian accessions only (Fig. 4.3.17). Though many of Indian HCV accessions were homologous to study samples but the homologies were non significant. In cluster VII there was 99% homology between resolved genotype 4 of studied sample (GU208793) resolved studied sample ID GU208799 and GU208805 (with a 100% bootstrap support) while in cluster IV a mini group of studied sample IDs GU324091 Pak-183P, GU324090 Pak-152P and GU324083 Pak-181F (resolved 6V) remained in the form of an independent divergent group (cluster III) with 99% homologies and 100% bootstrap support, further strengthened the divergence of these samples from the already known subtypes of Pakistan and India. As far as unresolved sequences were concerned, 163F (GU208779) showed 99% homology with unresolved sample IDs GU208781 and GU208787 (with 85% bootstrap support), this observation was similar to previous comparisons of the study samples with reference accessions from Pakistan and Iran. In cluster VII novel accession 2k showed 85% homology with Indian 1b (non significant 39% bootstrap support). Similarly in the same cluster GU208806 (1d) was 82% homologous to resolved sequence of 3a (GU324082) (bootstrap support non significant). When bootstrapped Phylogenetic trees were dragged Indian accession formed independent group (cluster I & V) and remained isolated even if present in same cluster (cluster II, III, V and VII) (Fig4.3.19).

Phylogeny of the NJ tree proposed the tree to be rooted with divergent evolution (Fig. 4.3.17, Fig. 4.3.18, Fig. 4.3.19 and Fig. 4.3.20). Resolved representative sample of subtype 1b, Sample ID 20P, branched itself on the basis of 100% similarity with Indian 3b accessions (Significant 100% bootstrap support). Sample IDs 34P and 24P showed 59% and 78% homology between them but the bootstrap values did not validate their tree topologies, while sample ID145P and 101P as observed previously had 99% homologies with their sister subgroups, 94P (100% bootstrap support) and 101P (53% bootstrap support). Sample ID 17P showed 100% homology with its sister subgroups (sample IDs 126P and 59P), the results were validated with 99% bootstrap support.

When dendrogram was dragged to find out the association between the studied sequences and retrieved sequences from India, it was found that studied accessions in general, had evolved earlier than the Indian accession and was stable and less mutated. While the Indian accessions had evolved later during the process of evolution, actively mutating and are in the phase of fast and rapid evolution or in other words it can be said that the accessions are under positive immune pressure. Besides this it seemed from the tree topology that Pakistan might be more endemic place for HCV evolution and it might be that, ancestor virus had evolved from Pakistan and might be history of endemicity of HCV in Pakistan is more ancient than that of India (Fig. 4.3.20).

4.3.8 COMPARATIVE PHYLOGENETIC ANALYSIS OF THE STUDIED SEQUENCES WITH REFERENCE ACCESSIONS FROM ALL NEIGHBOURING COUNTRIES

Every geographical area has its own HCV genotypes and subtypes, even if there are a few universal genotypes e.g. genotype 3 and 1, still there appear differences in their nucleotide composition because of active involvement of viral and host factors. Pakistan and its neighbouring countries had different prevalent HCV genotypes e.g. subtype 1b is most common and the most prevalent subtype of China and the second most prevalent is subtype (2a) Lu et al. (2005) and Zhang et al. (1995). In Pakistan subtype 3a is most commonly found and is followed by genotype 1a (Akber et al. 2009, Raja and Junjua 2008). In India the subtype 3b is predominant followed by 3a and Iran has most dominant

genotype 1b followed by 1a and 3a (Amini et al. 2009). No reports from Afghanistan has been included because the data is not available due to the political and social instability of Afghanistan therefore despite of the fact that Pakistan shares its large border with Afghanistan, no comparison was possible for Pakistani HCV samples with accessions from Afghanistan (Fig. 4.3.21-4.3.24).

A combined rooted NJ tree was dragged, for sequences of the present study with retrieved accessions from China, India and Iran to study the evolutionary relationships and associations between HCV variants from these countries. Tree topology revealed the probability of the existence of MRCA, where viral subtype GU208786 Pak-20P a resolved sample of subtype (1b) had 88% homology with Chinese 3a, (Fig 4.3.21) but bootstrap computed data imparted the relationship to be non significant likewise sample ID GU208789 Pak-34P (resolved 3a subtype) had sequence similarity with unresolved GU324083 Pak-181F, but the results were non-significant when validated by bootstrapping. Sample ID 24P though, was clustered with sister texas but its homology with them was non significant. Sample IDs 94P, 145P and 187P depicted 99% homologies with their respective sister groups (with 99% and 98% bootstrap support). On the other hand sample ID185P though had 99% homology with Sample ID167F but bootstrap values did not support this tree topology to be valid. The only significant homology 99% was observed in case of sample GU324085 Pak-17P (resolved 1b sequence) with Chinese accessions 1b, 1a, 3b, 2a, 6n, 6k, 1b and 1k (99% bootstrap support).

Tree topologies divulged that in cluster I (Fig. 4.3.21) GU197378 (resolved subtype 2k) showed 99% similarity with Chinese accession 3A (with 99% bootstrap support) (Fig. 4.3.21). The study sample ID GU208806 Pak-194P (resolved subtype 1d) mounted affinity with accession GU208814 Pak-130P, (but the bootstrapping did not support the results) on the other hand there was a 99% sequence homology between sequenced sample GU208816 (4) and Indian 3d and 3f (99% bootstrap) (Fig. 4.3.23). From cluster 5 it was ambient that subtype 6V samples did not cluster them with any of neighbouring country's accessions except Chinese 3b and the p-distance value for this association was extremely non significant (Fig. 4.3.22). As before, in cluster II a

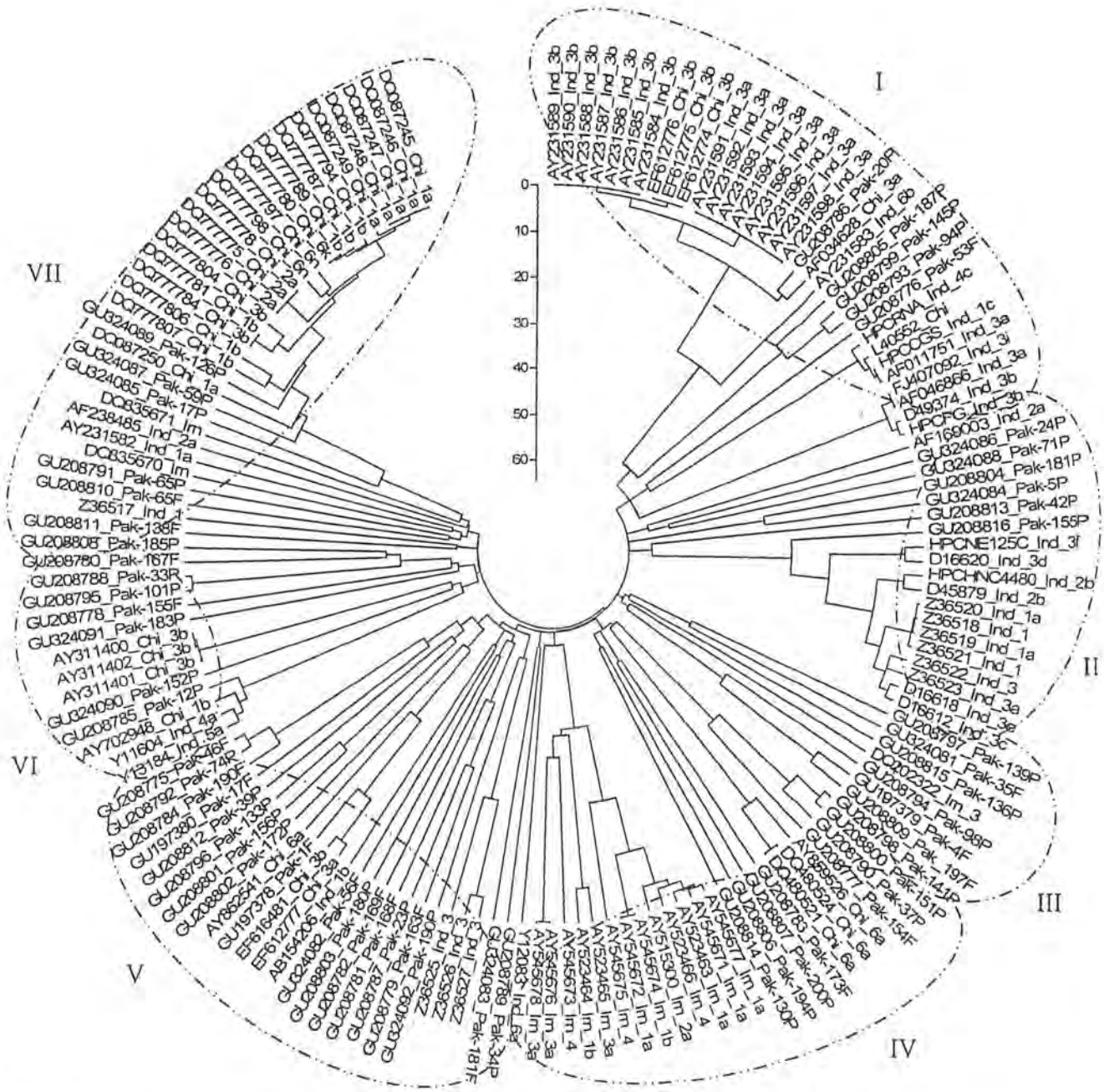


Fig. 4.3.21: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective samples from neighboring countries.


AY231585_Ind_3b
AY231587_Ind_3b
AY231589_Ind_3b
AY231590_Ind_3b
AY231584_Ind_3b
AY231586_Ind_3b
AY231588_Ind_3b
EF612774_Chi_3b
EF612776_Chi_3b
EF612775_Chi_3b
AY231596_Ind_3a
AY231593_Ind_3a
AY231594_Ind_3a
AY231591_Ind_3a
AY231592_Ind_3a
AY231595_Ind_3a
AY231597_Ind_3a
AY231598_Ind_3a
GU208786_Pak-20P
AF034628_Chi_3a
AY231583_Ind_6b
GU208805_Pak-187P
GU208799_Pak-145P
GU208793_Pak-94P
GU208776_Pak-53F
HPCRNA_Ind_4c
L40552_Chi
HPCCGS_Ind_1c
AF011751_Ind_3a
FJ407092_Ind_3i
AF046866_Ind_3a
D49374_Ind_3b
HPCFG_Ind_3b
AF169003_Ind_2a
GU324086_Pak-24P
GU324088_Pak-71P
GU208804_Pak-181P
GU324084_Pak-5P
GU208813_Pak-42P
GU208816_Pak-155P
HPCNE125C_Ind_3f
D16620_Ind_3d
HPCHNC4480_Ind_2b
D45879_Ind_2b
Z36520_Ind_1a
Z36518_Ind_1
Z36519_Ind_1a
Z36521_Ind_1
Z36522_Ind_3
Z36523_Ind_3a
D16618_Ind_3e
D16612_Ind_3c
GU208797_Pak-139P
GU324081_Pak-35F
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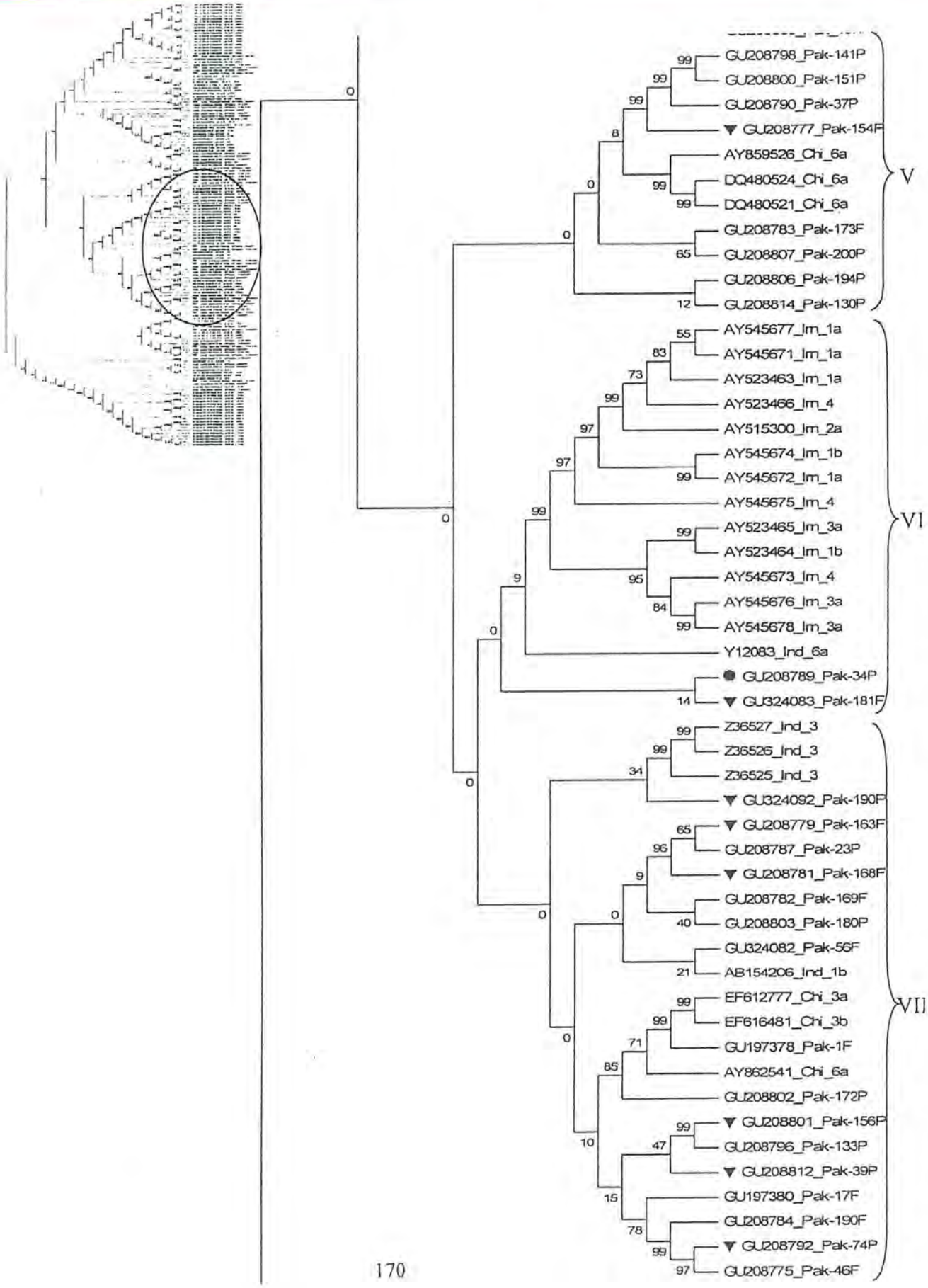
GU208809_Pak_197F
I
II
III
IV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GU208777_Pak-154F
AY859526_Chi_6a
DQ480524_Chi_6a
DQ480521_Chi_6a
GU208783_Pak-173F
GU208807_Pak-200P
GU208806_Pak-194P
GU208814_Pak-130P
V
AY545677_Im_1a
AY545671_Im_1a
AY523463_Im_1a
AY523466_Im_4
AY515300_Im_2a
AY545674_Im_1b
AY545672_Im_1a
AY545675_Im_4
AY523465_Im_3a
AY523464_Im_1b
AY545673_Im_4
AY545676_Im_3a
AY545678_Im_3a
Y12083_Ind_6a
GU208789_Pak-34P
GU324083_Pak-181F
VI
Z36527_Ind_3
Z36526_Ind_3
Z36525_Ind_3
GU324092_Pak-190P
GU208779_Pak-163F
GU208787_Pak-23P
GU208781_Pak-168F
GU208782_Pak-169F
GU208803_Pak-180P
GU324082_Pak-56F
AB154206_Ind_1b
EF612777_Chi_3a
EF616481_Chi_3b
GU197378_Pak-1F
AY862541_Chi_6a
GU208802_Pak-172P
GU208801_Pak-156P
GU208796_Pak-133P
GU208812_Pak-39P
GU197380_Pak-17F
GU208784_Pak-190F
GU208792_Pak-74P
GU208775_Pak-46F
VII

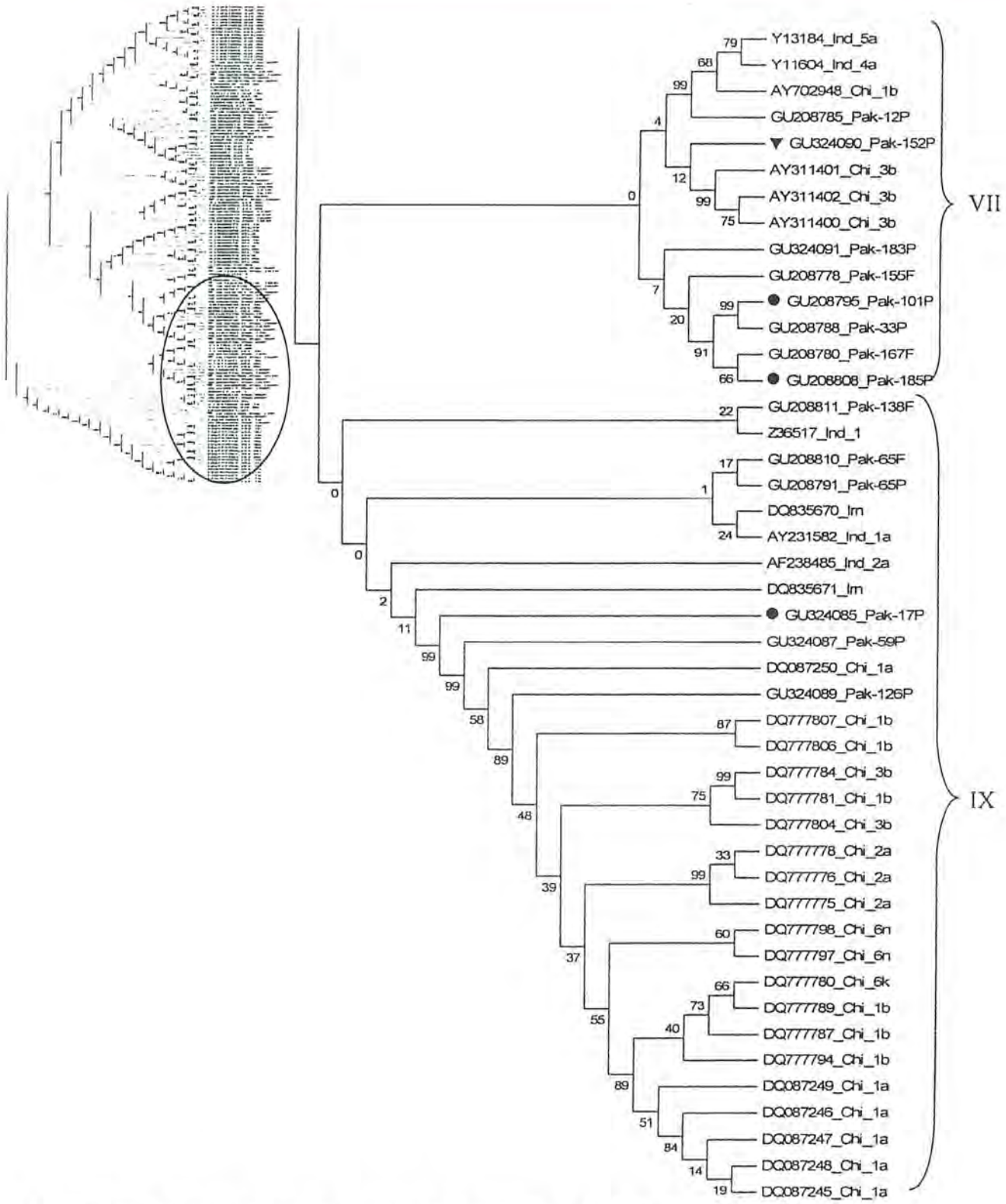
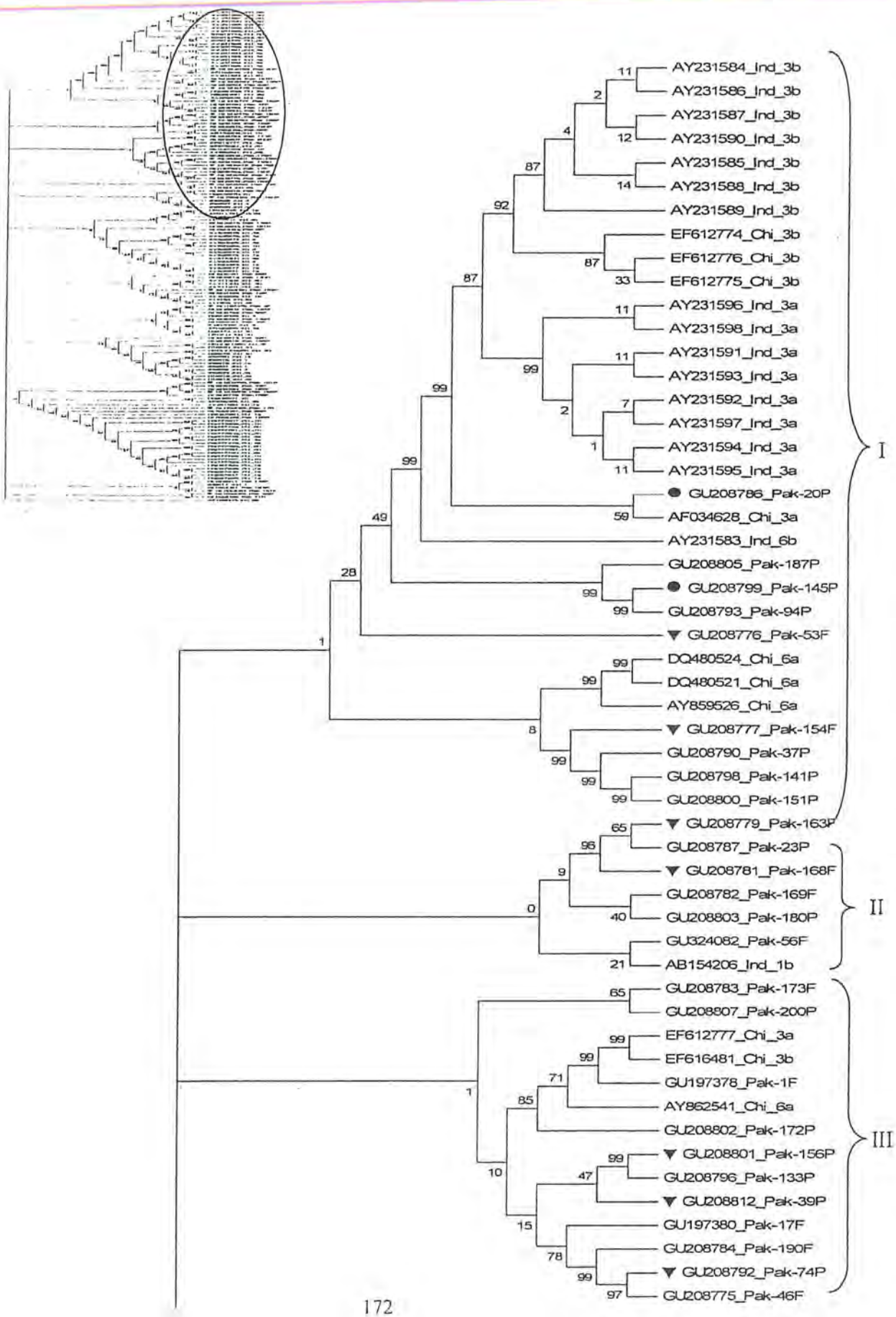


Fig. 4.3.22: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective accessions from neighboring countries with *p*-distances. Circle stands for known representative genotypes and triangle is for unresolved samples.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AY231587_Ind_3b
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EF612774_Chi_3b
EF612776_Chi_3b
EF612775_Chi_3b
AY231596_Ind_3a
AY231598_Ind_3a
AY231591_Ind_3a
AY231593_Ind_3a
AY231592_Ind_3a
AY231597_Ind_3a
AY231594_Ind_3a
AY231595_Ind_3a
GU208786_Pak-20P
AF034628_Chi_3a
AY231583_Ind_6b
GU208805_Pak-187P
GU208799_Pak-145P
GU208793_Pak-94P
GU208776_Pak-53F
DQ480524_Chi_6a
DQ480521_Chi_6a
AY859526_Chi_6a
GU208777_Pak-154F
GU208790_Pak-37P
GU208798_Pak-141P
GU208800_Pak-151P
GU208779_Pak-163P
GU208787_Pak-23P
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GU208782_Pak-169F
GU208803_Pak-180P
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GU208807_Pak-200P
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EF616481_Chi_3b
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AY862541_Chi_6a
GU208802_Pak-172P
GU208801_Pak-156P
GU208796_Pak-133P
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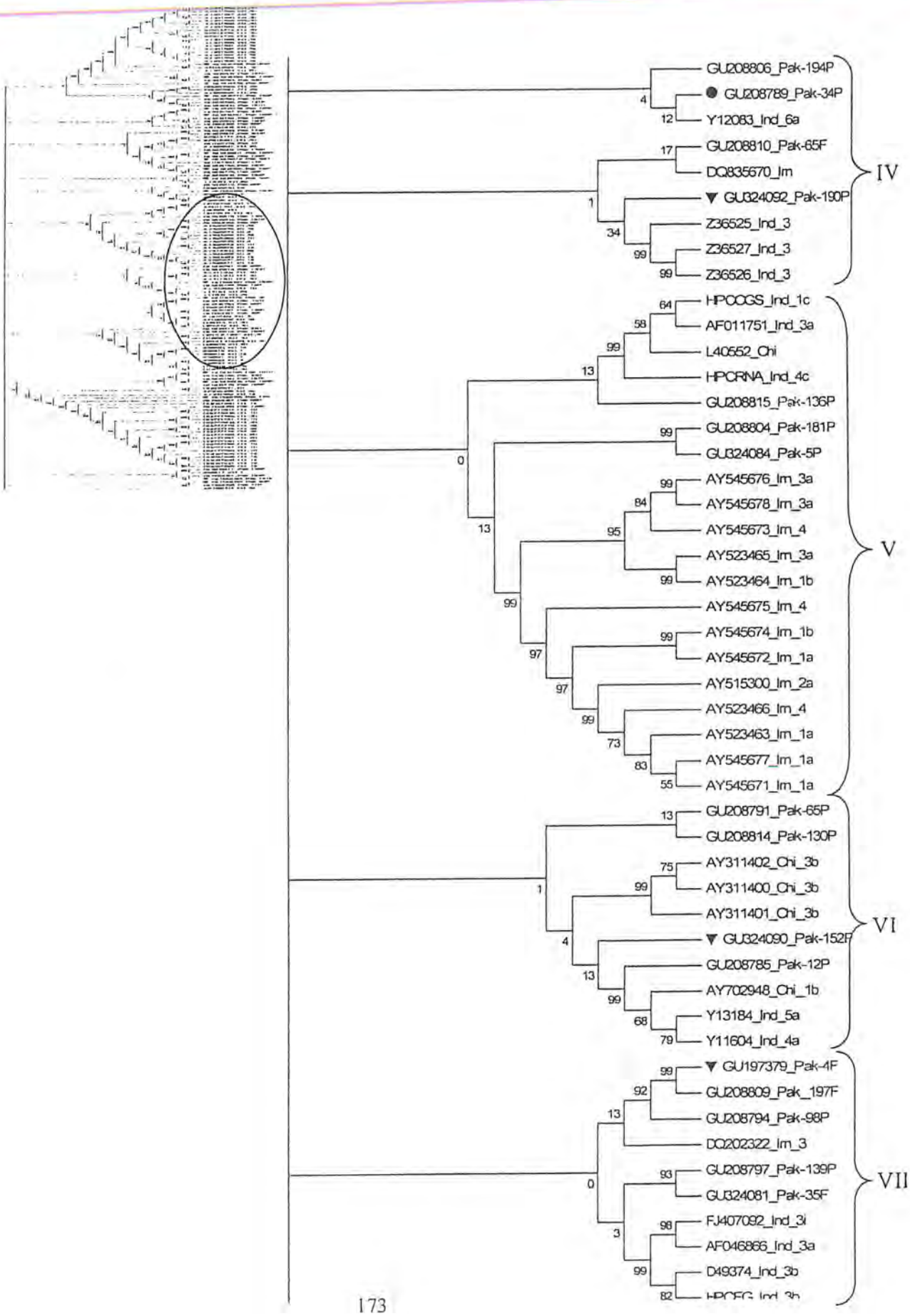
II
III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GU324092_Pak-190P
Z36525_Ind_3
Z36527_Ind_3
Z36526_Ind_3
IV
HPCOGS_Ind_1c
AF011751_Ind_3a
L40552_Chi
HPCRNA_Ind_4c
GU208815_Pak-136P
GU208804_Pak-181P
GU324084_Pak-5P
AY545676_Im_3a
AY545678_Im_3a
AY545673_Im_4
AY523465_Im_3a
AY523464_Im_1b
AY545675_Im_4
AY545674_Im_1b
AY545672_Im_1a
AY515300_Im_2a
AY523466_Im_4
AY523463_Im_1a
AY545677_Im_1a
AY545671_Im_1a
V
GU208791_Pak-65P
GU208814_Pak-130P
AY311402_Chi_3b
AY311400_Chi_3b
AY311401_Chi_3b
GU324090_Pak-152P
GU208785_Pak-12P
AY702948_Chi_1b
Y13184_Ind_5a
Y11604_Ind_4a
VI
GU197379_Pak-4F
GU208809_Pak_197F
GU208794_Pak-98P
DQ202322_Im_3
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AF046866_Ind_3a
D49374_Ind_3b
VII

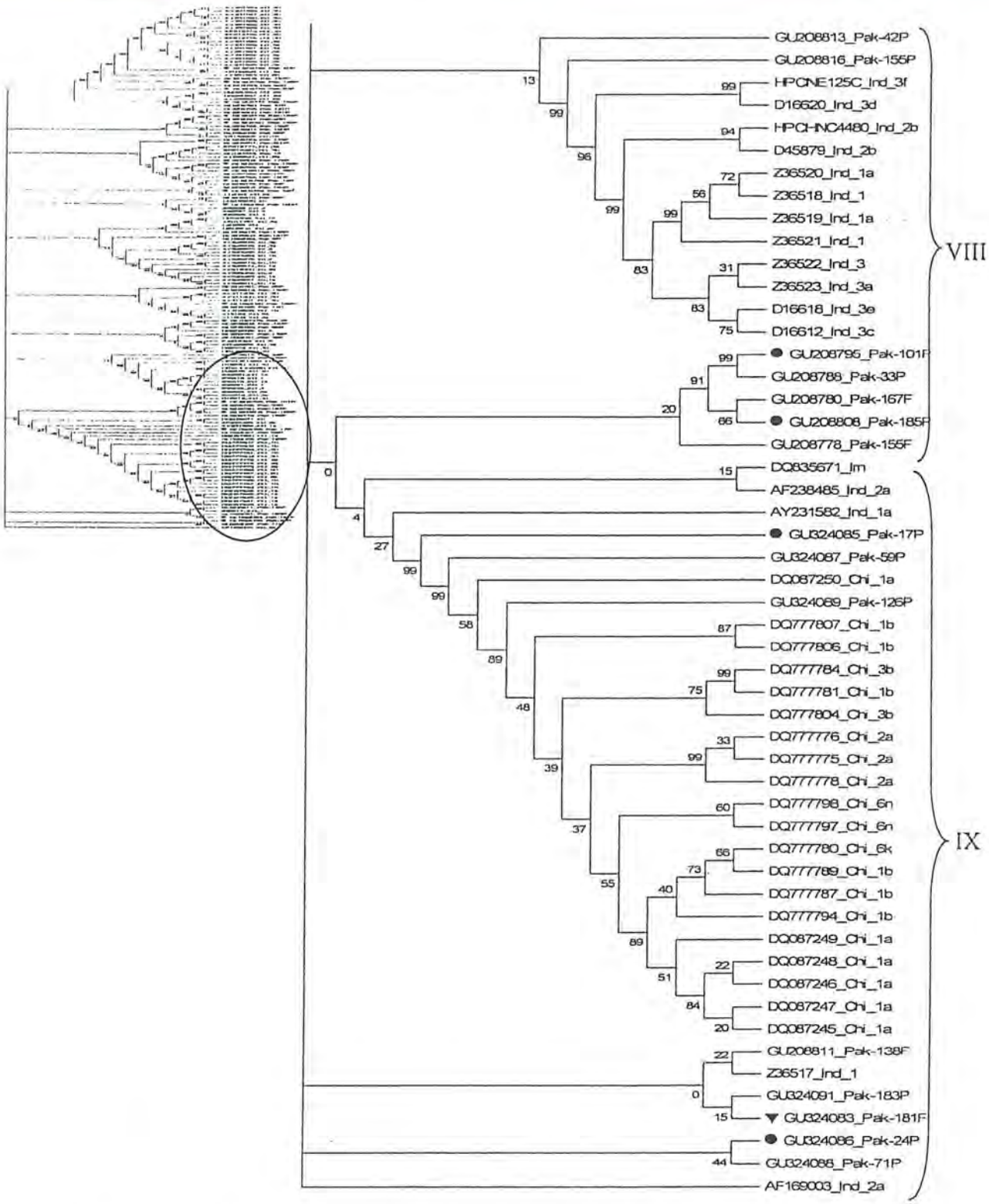
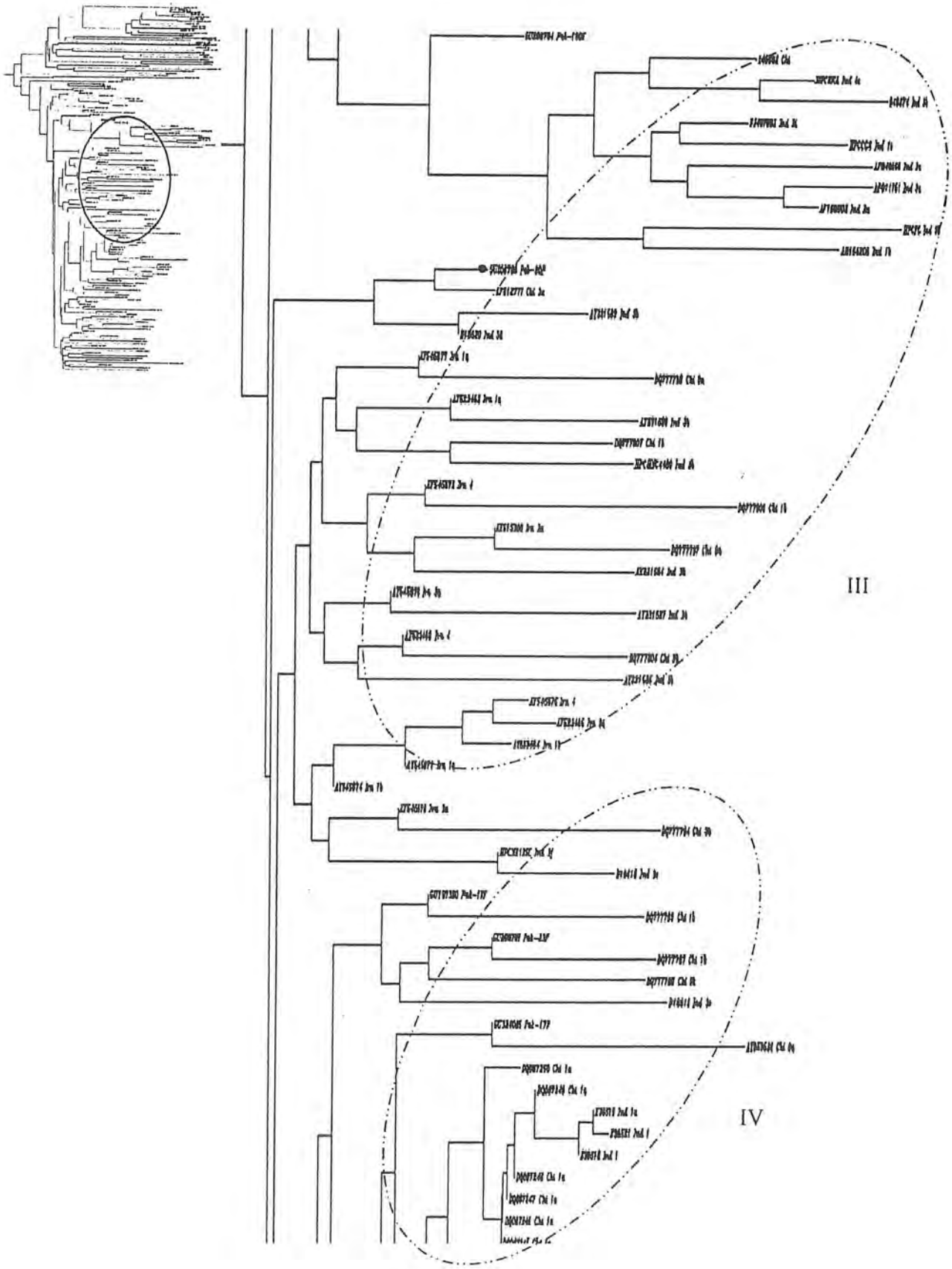


Fig. 4.3.23: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and with samples from neighboring countries showing bootstrap values. Circle stands for known representative genotypes and triangle is for unresolved samples.


I
II


III
IV

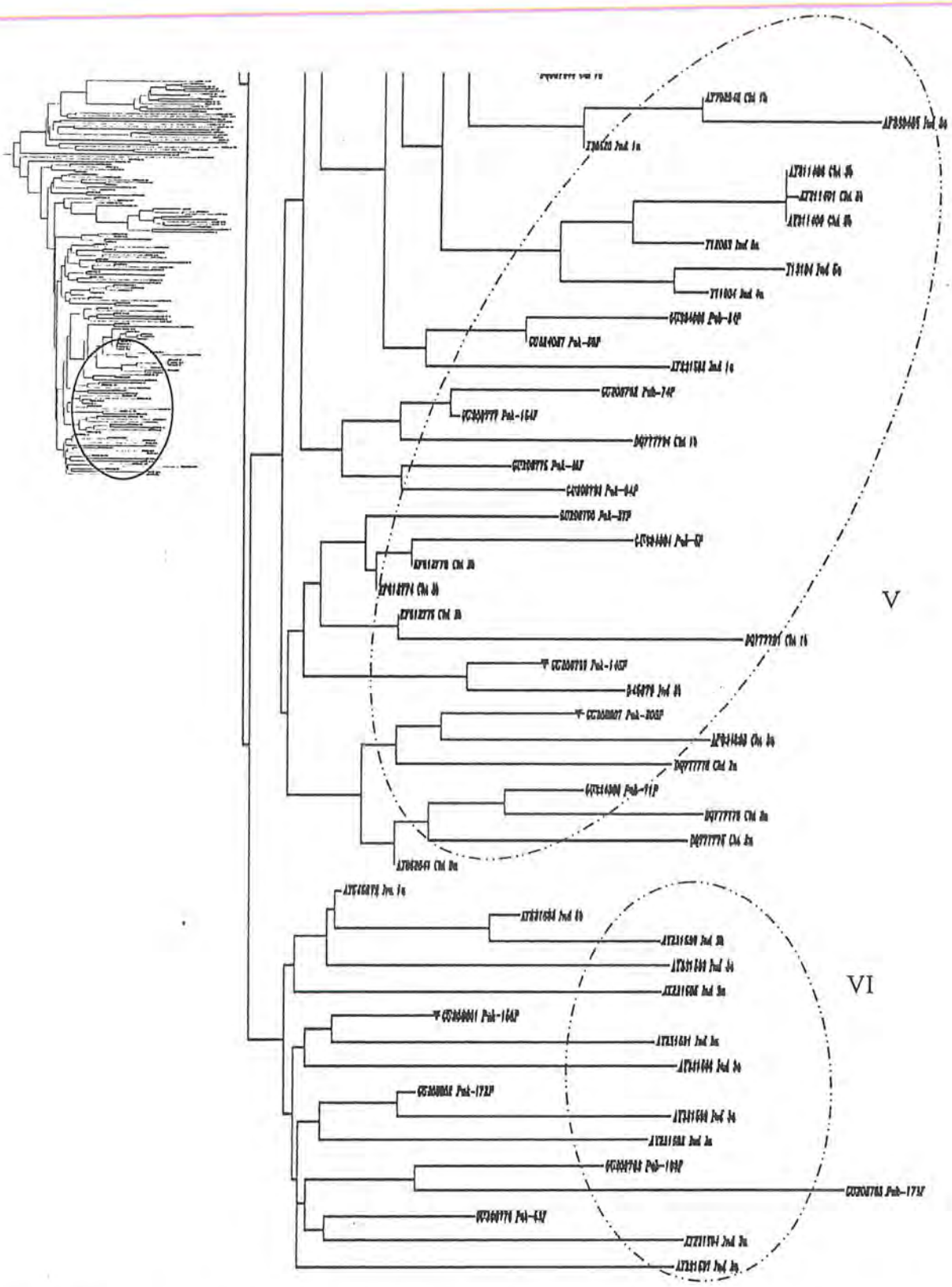


Fig. 4.3.24: Dendrogram of the untypable sequences of HCV 5'UTR from suburban Rawalpindi, Pakistan with reference accessions from neighboring countries showing branch lengths. Circle stands for known representative genotypes and triangle is for unresolved samples.

subgroup of 3 unresolved studied samples retained its individuality with a homology 96% among them GU208781, GU208779, and GU208787 were with 98% bootstrap support. Tree topologies with bootstrap values (Fig. 4.3.23) indicated that, major clusters had country wide distribution of genotypes. In cluster VII and II, unresolved sequences were arranged with 99% bootstrap support. GU208792 and GU208775 were 99% analogies (with 99% bootstrap support), strengthened the findings that as these variants did not showed significant homologies between the sister subgroups, it can be concluded that these are some new variants of HCV.

Dendrograms was dragged to find an association between studied samples with reference accessions from neighbouring countries, it was revealed that in general the studied HCV samples of the present study exhibited long branch lengths, indicating the ancient history of their evolution and their genetically stable genome composition and this might be attributable to the suppressed or compromised immune pressure of the host. Previous reports supported the evidence that the cases where immune response is compromised, there are a less chance of viral clearance (Augenbraun et al. 2003 and Ponamgi et al. 2009). Secondly it was evident that history of evolution of virus is more ancient in Pakistan than her neighbouring countries (Fig. 4.3.22).

When the unrooted phylogenetic trees were dragged for the untypable samples from Rawalpindi, in comparison with other reference accessions from Pakistan (Fig. 4.3.5-4.3.8), China (Fig. 4.3.9-4.3.12), Iran (Fig. 4.3.13-4.3.16), India (Fig. 4.3.17-4.3.20) and the all neighbouring countries (Fig. 4.3.21-4.3.24) and accessions from world over (Fig. 4.3.21-4.3.24), a generalized trend of blended punctuation equilibrium pattern was observed in all clusters. The reasons for some accessions being smooth during the course of evolution and for others being under the extensive pressure is unknown. The reason might be that in immunosupressed and immunocompromised hosts, diminished host immune pressure is more likely to result in less HCV diversification (Mao et al. 2001 and Toyoda et al. 1997).

4.3.9 COMPARATIVE PHYLOGENETIC ANALYSIS OF THE STUDIED SEQUENCES WITH REFERENCE ACCESSIONS FROM WORLD OVER

Randomly selected HCV sequences reported from different countries around the globe were retrieved form NCBI. Representative HCV strains from UK, USA, New Zealand, Indonesia, Japan, Australia, Hong Kong, Maldives, Egypt and Vietnams were retrieved. All of the above mentioned countries have their own HCV genotypes, subtypes, variants and quasiaspecies but the purpose of this activity was to compare the analogies between the studied strains and to find out their evolutionary relationship with the reported accessions around the world (Fig. 4.3.25-4.3.28). The accessions were D11168 Indon 11, AY147810 Turk 1b, AY147811 Turk 1b, EF540344 Turk 3, EF540345 Turk 4, EF540347 Turk 2, EF540348 Turk 2, EF540349 Turk 3, EF540350 USA 3, EF540353 Turk 4, AY859526 Hong Kong 6a, D84262 Jap 6b, D84265 Jap 6h, D00944 Jap 2a, D17763 NZl 3a, D14853 Indon 1c, D63821 Indon 3k, AF009606 USA 1a, AB031663 Vaitm 2k, D10749 Jap HC-Ji, FJ181999 Mald 1a, AB306405 Mald 6i, AB306400 Mald 6e, AY862541 Mald 6a, FJ390398 Mald 1b, DQ295833 Egy 4a, AY766691 UK 4, AY766692 UK 3a, AY766693 UK 3a, AY766689 UK 2a and AY766666 Aus 4 respectively.

The NJ phylogenetic tree dragged for the studied samples with reference accessions form world over, mounted nine main clusters where all representative samples of the present study formed independent clusters, by aligning them with their previously established sister subgroups and showed divergent evolution (Fig. 4.3.25) as no additional variation in tree topologies was observed, in terms of, homology between studied samples and reference accessions of the world. Generally speaking, almost same trend was observed when these samples were compared with the accessions from neighbouring countries (independently and collectively) and even when the accessions were compared with the reference strains from Pakistan. The reason for this independent clustering might be the viral diversity at two levels i.e., genotypes and quasispecies.

When the dendrograms was constructed to find the association among the HCV variants from present study and accessions retrieved from NCBI, it was found that

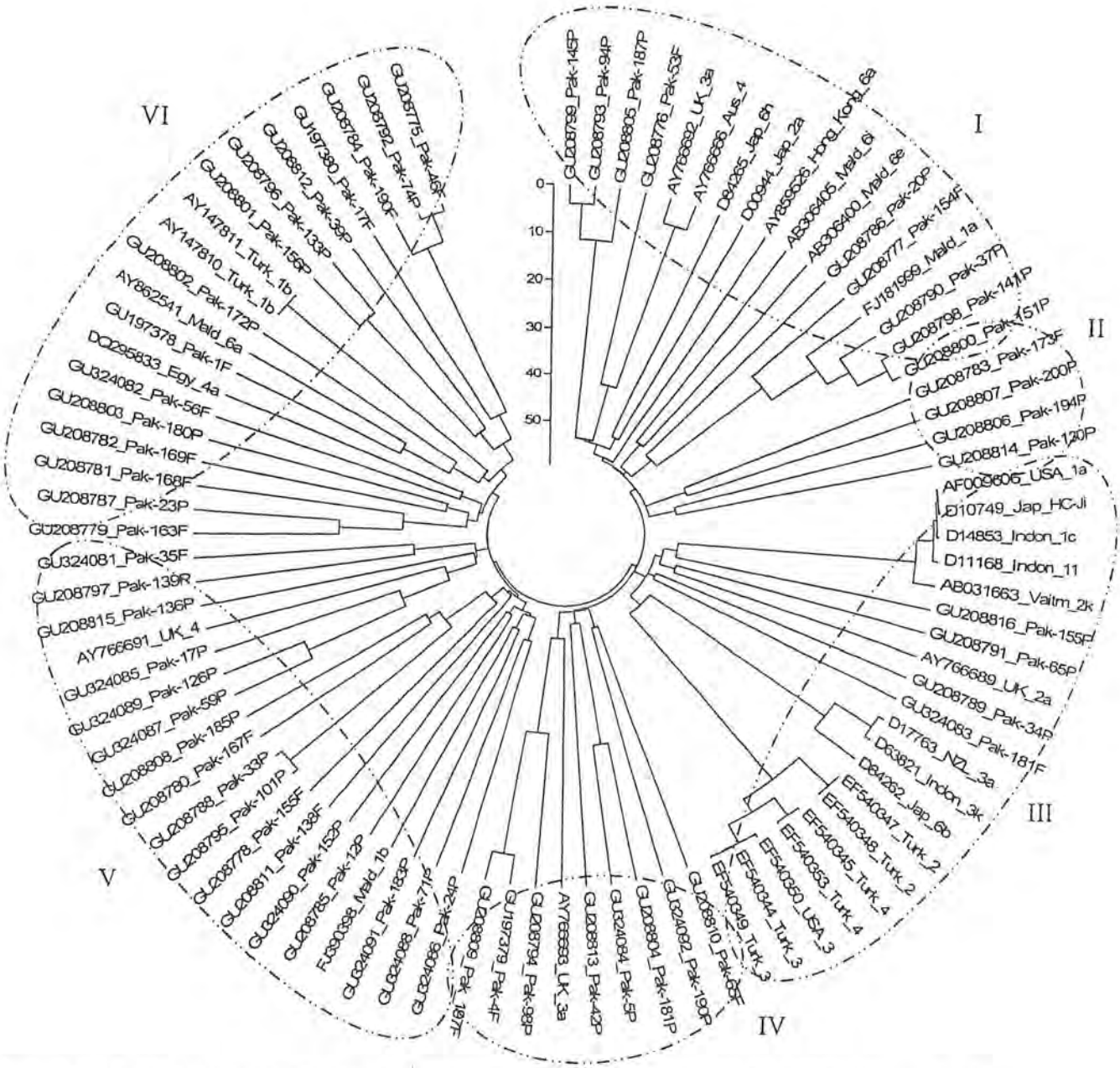
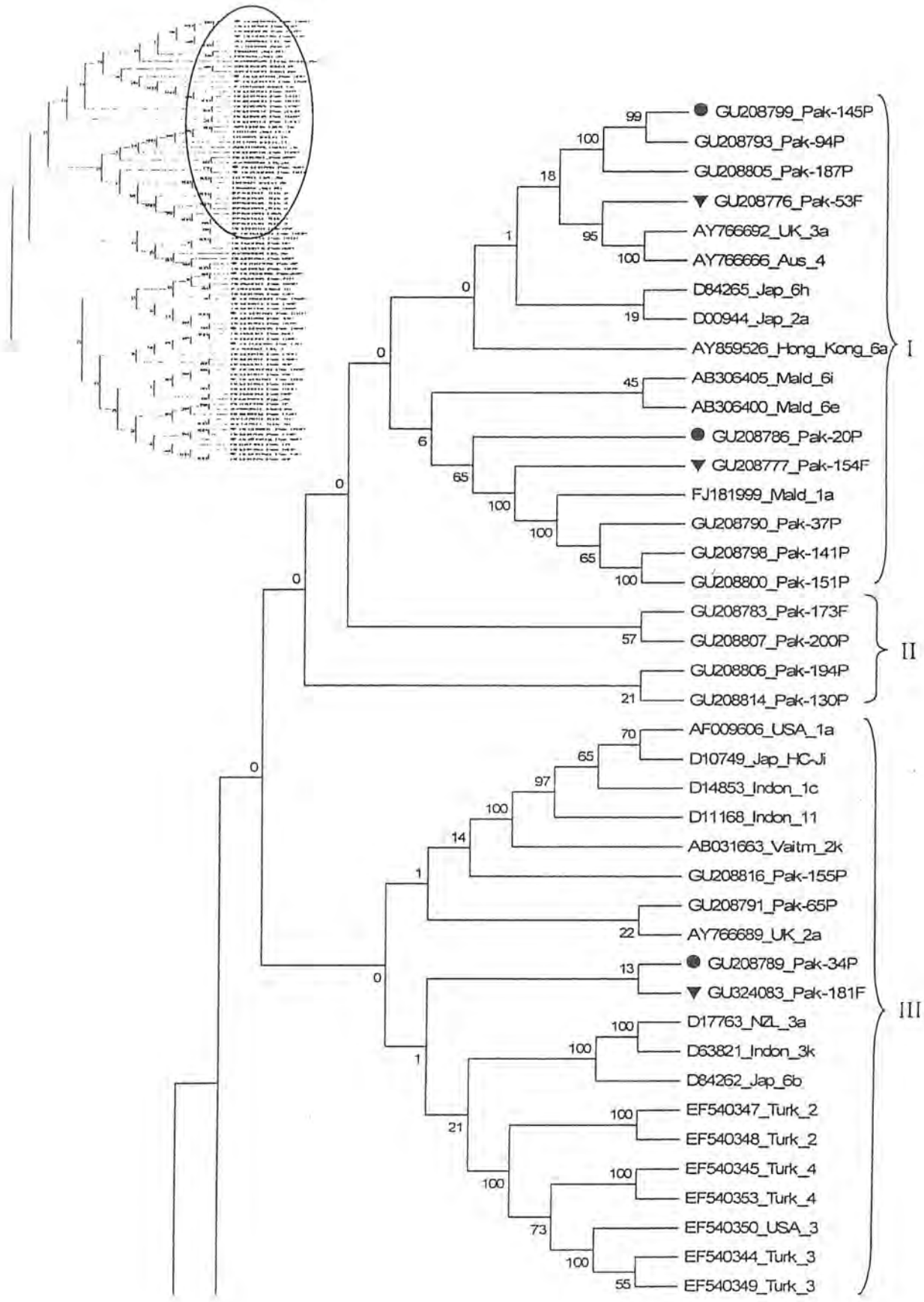


Fig. 4.3.25: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and randomly selected samples from world over.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AY766692_UK_3a
AY766666_Aus_4
D84265_Jap_6h
D00944_Jap_2a
AY859526_Hong_Kong_6a
AB306405_Mald_6i
AB306400_Mald_6e
GU208786_Pak-20P
GU208777_Pak-154F
FJ181999_Mald_1a
GU208790_Pak-37P
GU208798_Pak-141P
GU208800_Pak-151P
I
GU208783_Pak-173F
GU208807_Pak-200P
GU208806_Pak-194P
GU208814_Pak-130P
II
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D14853_Indon_1c
D11168_Indon_11
AB031663_Vaitm_2k
GU208816_Pak-155P
GU208791_Pak-65P
AY766689_UK_2a
GU208789_Pak-34P
GU324083_Pak-181F
D17763_NZL_3a
D63821_Indon_3k
D84262_Jap_6b
EF540347_Turk_2
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EF540349_Turk_3
III

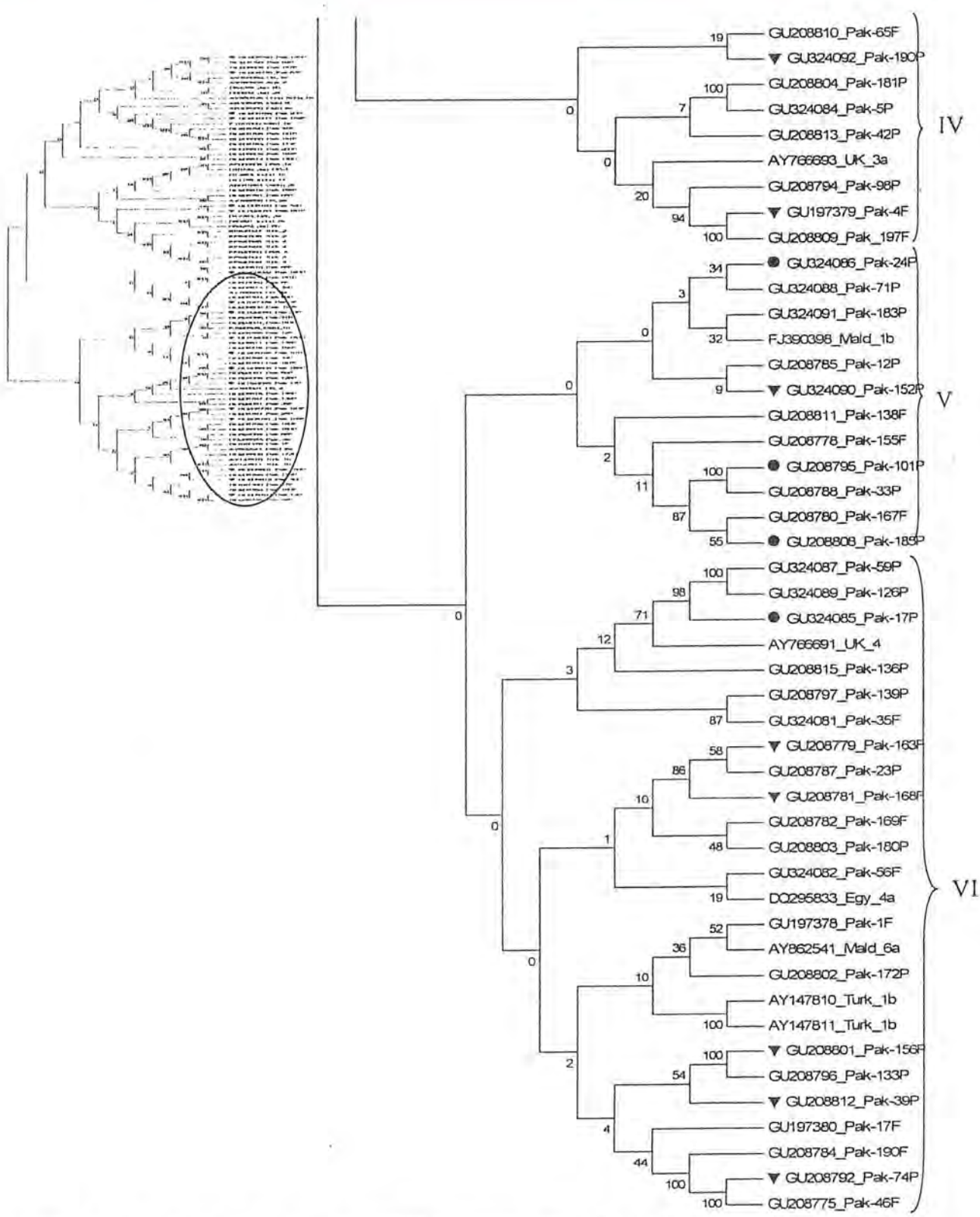
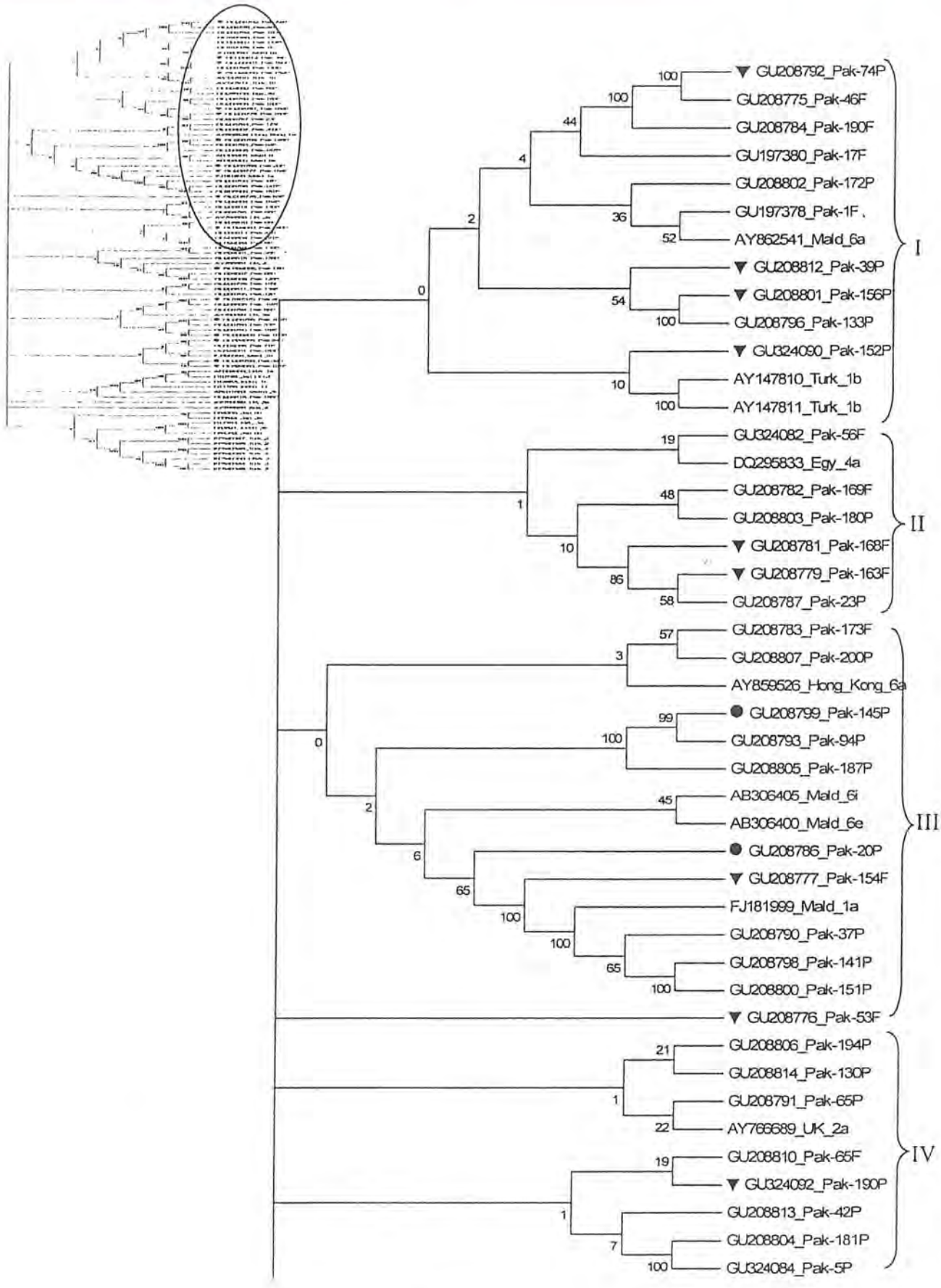


Fig. 4.3.26: NJ phylogenetic tree of untypable sequences of HCV 5' UTR from suburban Rawalpindi, Pakistan and selective accessions from world over with *p*-distances. Circle stands for known representative genotypes and triangle is for unresolved samples.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GU197380_Pak-17F
GU208802_Pak-172P
GU197378_Pak-1F
AY862541_Mald_6a
GU208812_Pak-39P
GU208801_Pak-156P
GU208796_Pak-133P
GU324090_Pak-152P
AY147810_Turk_1b
AY147811_Turk_1b
GU324082_Pak-56F
DQ295833_Egy_4a
GU208782_Pak-169F
GU208803_Pak-180P
GU208781_Pak-168F
GU208779_Pak-163F
GU208787_Pak-23P
GU208783_Pak-173F
GU208807_Pak-200P
AY859526_Hong_Kong_6a
GU208799_Pak-145P
GU208793_Pak-94P
GU208805_Pak-187P
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AB306400_Mald_6e
GU208786_Pak-20P
GU208777_Pak-154F
FJ181999_Mald_1a
GU208790_Pak-37P
GU208798_Pak-141P
GU208800_Pak-151P
GU208776_Pak-53F
GU208806_Pak-194P
GU208814_Pak-130P
GU208791_Pak-65P
AY766689_UK_2a
GU208810_Pak-65F
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I
II
III
IV

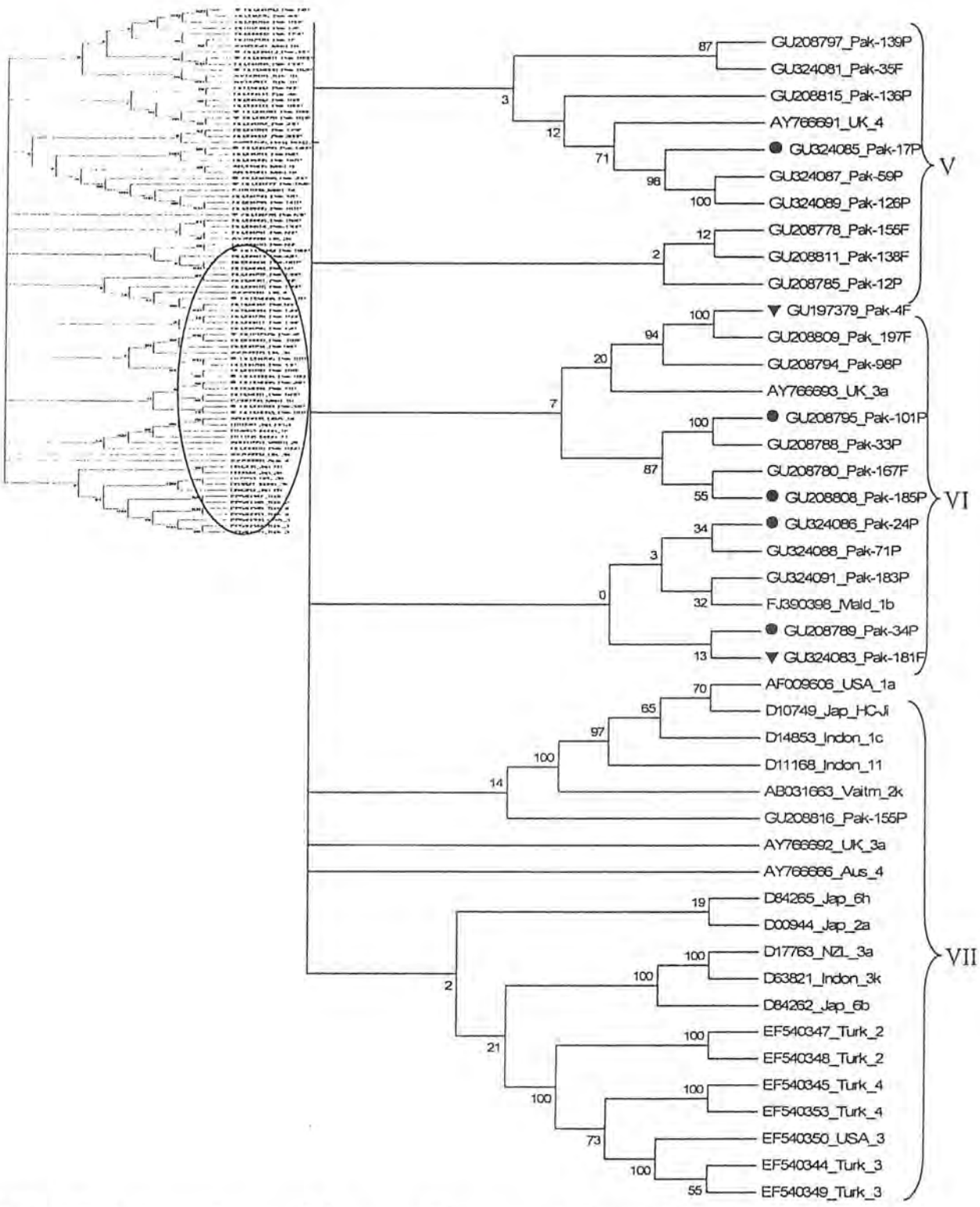
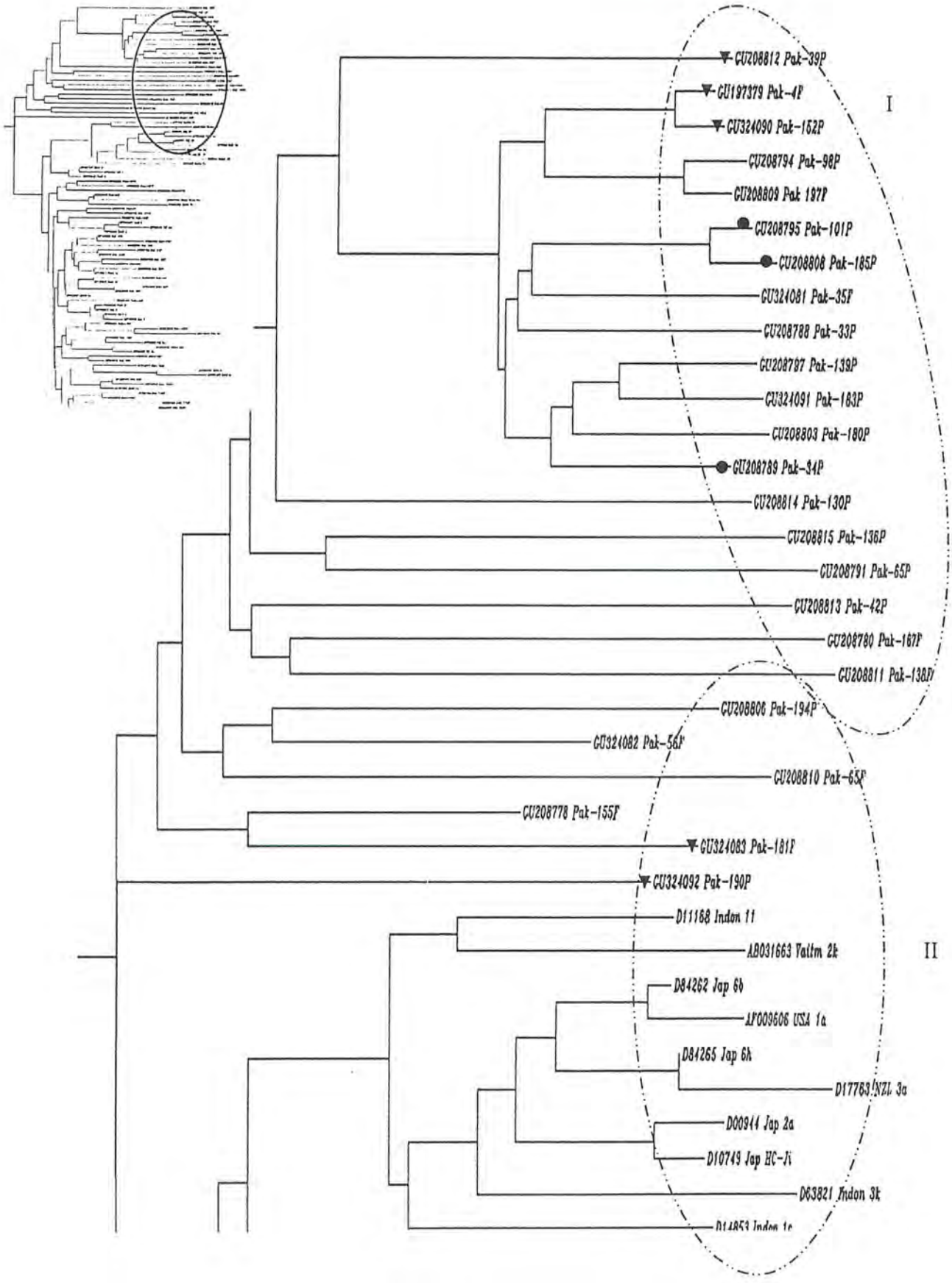


Fig. 4.3.27: NJ phylogenetic tree of the untypable sequences from suburban Rawalpindi, Pakistan with reference accessions from world over showing bootstrap values Circle stands for known representative genotypes and triangle is for unresolved samples.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GU324081 Pak-35F
GU208788 Pak-33P
GU208797 Pak-139P
GU324091 Pak-183P
GU208803 Pak-180P
GU208789 Pak-34P
GU208814 Pak-130P
GU208815 Pak-136P
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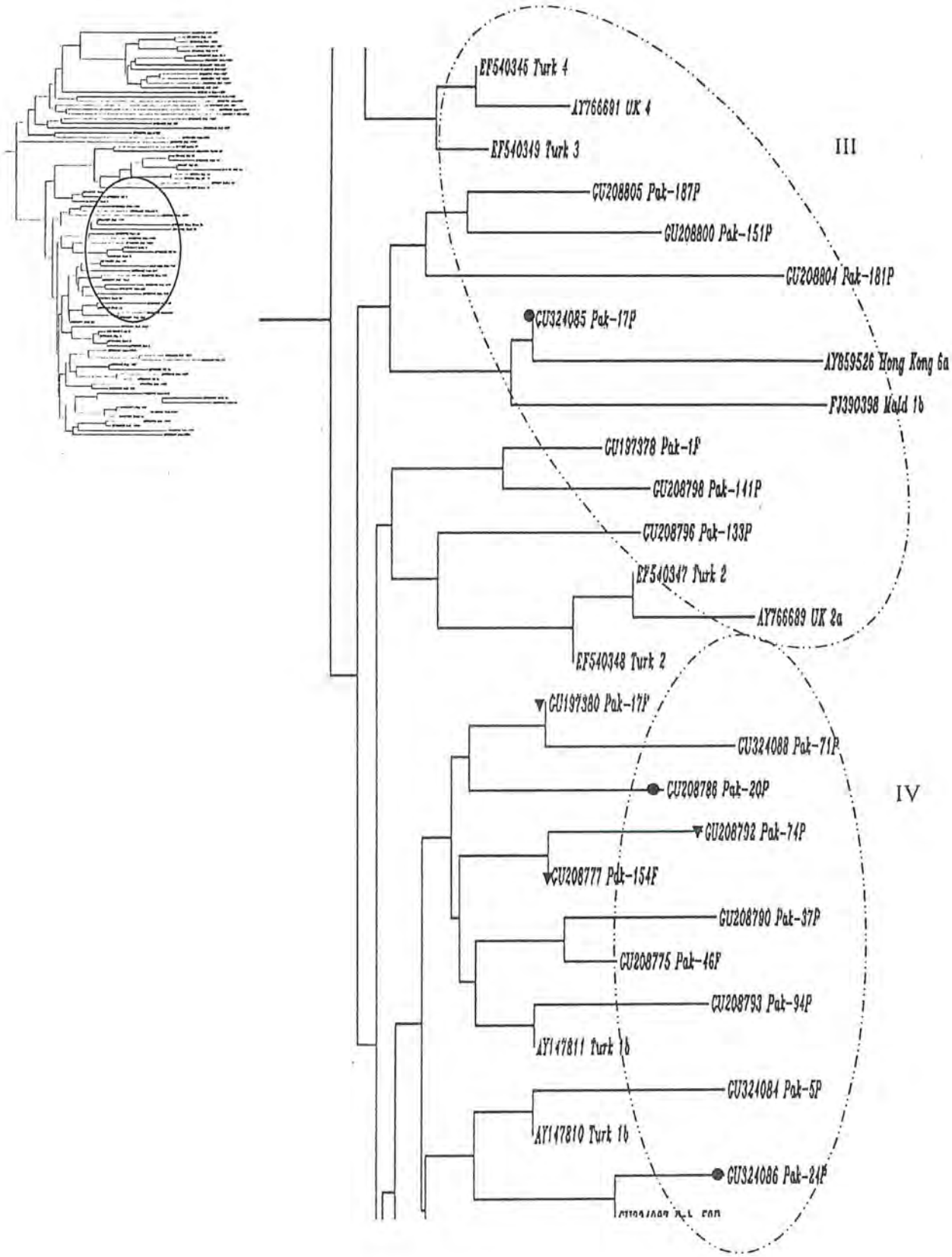
GU208813 Pak-42P
GU208780 Pak-167F
GU208811 Pak-138P
GU208806 Pak-194P
GU324082 Pak-56F
GU208810 Pak-65F
GU208778 Pak-155F
GU324083 Pak-181F
GU324092 Pak-190P
D11168 Indon 1i
AB031663 Yatim 2k
D84262 Jap 6b
AF009606 USA 1a
D84265 Jap 6k
D17763 NZL 3a
D00944 Jap 2a
D10749 Jap HC-Ji
D63821 Indon 3k
I
II


EF540345 Turk 4
AY766691 UK 4
EF540349 Turk 3
III
GU208805 Pak-187P
GU208800 Pak-151P
GU208804 Pak-181P
GU324085 Pak-17P
AY859526 Hong Kong 6a
FJ390398 Mald 1b
GU197378 Pak-1F
GU208798 Pak-141P
GU208796 Pak-133P
EF540347 Turk 2
AY766689 UK 2a
EF540348 Turk 2
GU197380 Pak-17F
GU324088 Pak-71P
GU208788 Pak-20P
IV
GU208792 Pak-74P
GU208777 Pak-154F
GU208790 Pak-97P
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GU208793 Pak-94P
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GU324084 Pak-5P
AY147810 Turk 1b
GU324086 Pak-24P

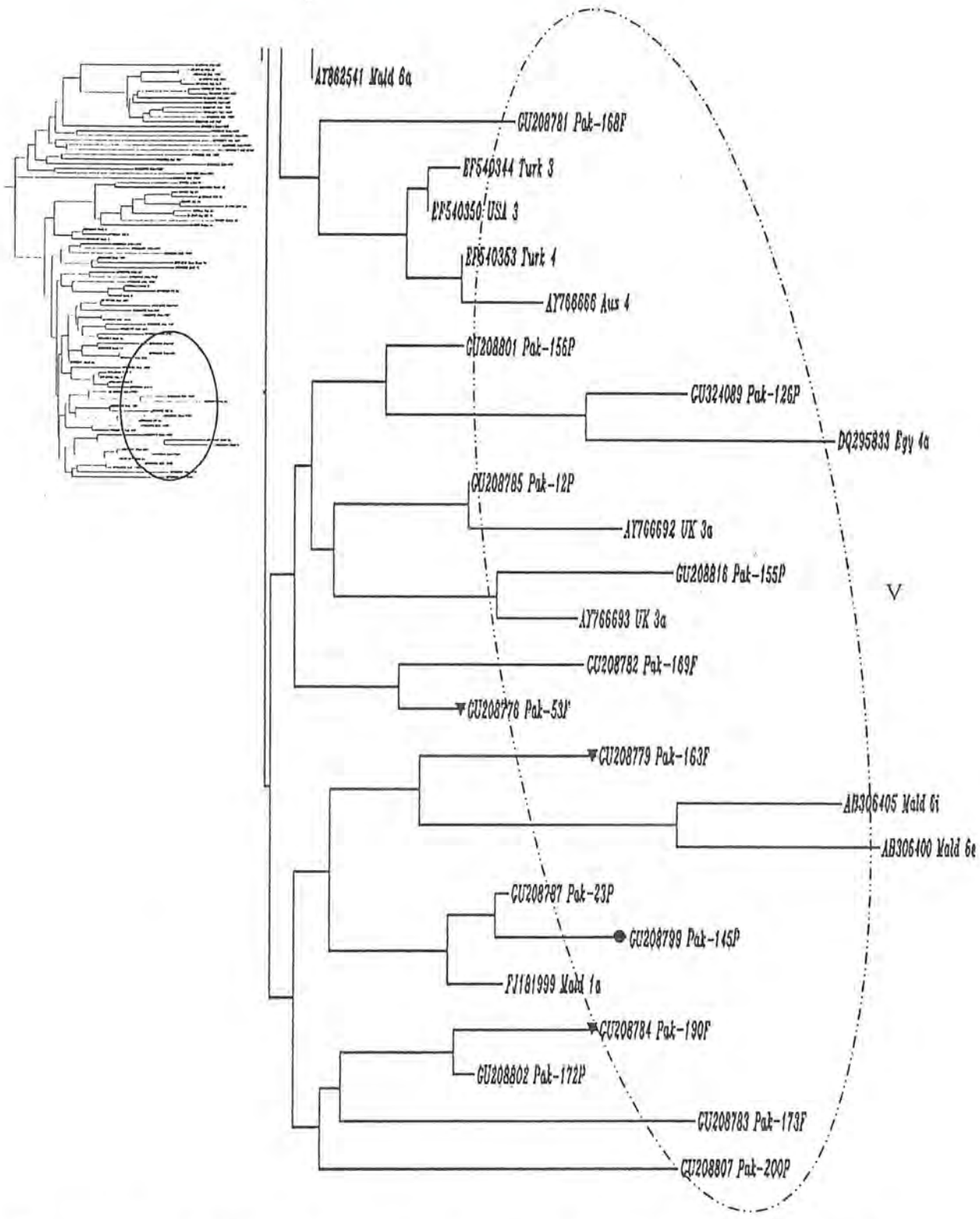

Fig. 4.3.28: Dendrogram of the untypable sequences of HCV 5'UTR from suburban Rawalpindi, Pakistan with reference accessions from world over showing branch lengths. Circle stands for known representative genotypes and triangle is for unresolved samples.

studied samples of present study and reference accessions share some common ancestor, but the studied samples had evolved earlier than the accessions of HCV from other countries. On the other hand the accessions from world over are actively mutating and more divergent, or it can be said that they are still in an active phase of evolution. (Fig. 4.3.28). Moreover, it is evident from the dendrograms that disease is endemic in Pakistan for more time period than reference countries as it is already established that HCV prevalence in Europe is not homologous with reference to the distribution of genotypes (Maio et al. 2000 and Raffaele et al. 2004). Pybus et al (2009) particularly with reference to Asia, explained the origin and maintenance of HCV diversity and reported that Asian model of evolution could be a baseline for investigating HCV spread in other continents.

Tree topologies for comparative studies of Pakistan (Fig. 4.3.5-.4.3.8), India (Fig. 4.3.8-4.3.12), China (Fig. 4.3.13-4.3.16), Iran (Fig. 4.3.17-4.3.20), neighbouring countries (Fig. 4.3.21-4.3.24) and accessions from World over (Fig. 4.3.25-4.3.28) proved that HCV variants were either stable or they were under the high immune pressure and were actively evolving. This high immune pressure of the host works by forming the strain specific neutralizing antibodies and a response to that virus mutates actively to ensure its survival (Farci et al. 1994) plus there is an involvement of both host and viral factors in the steady or actively mutating evolutionary pattern of the virus e.g., host's inability to illicit an active immune response against the invading virus, viral heterogeneity in terms of genotypes, quasispecies and naturally occurring recombinant forms (Bukh et al. 1993, Okamoto et al. 1992, Simmonds et al. 1993 and Zein 2000).

HCV strains 1a, 1b, 2, 3a and 3b are the most cosmopolitan and widely distributed genotypes of virus and they exhibit very limited sequence diversity (Alter 1999), as the accessions that had pooled them in independent clusters might be recombinants. Natural recombination is very important in viral heterogeneity. In case of HCV this recombination may be inter genotypic as well as intra genotypic. A few examples of the naturally occurring recombinant types were observed in past few years (Colina et al. 2004, Kalinina et al. 2002 and Noppornpath et al. 2006). This suggests that viral replicase is not intersepting the HCV chimeric genome. It is already known that there exists an overlap in viral distribution (Zein 2000) that can result in the double

infection in the same host. Because in Pakistan the unsafe therapeutic injection practices are one of the highest reported numbers in the world (Raja and Janjua 2008), therefore the chances of double infection will also increase by repeated exposures to the risk factors. Earlier it has been reported that double infections may increase the chances of intergenotypic recombination and the quasispecies might enhance the chances of intragenotypic recombination (Djebbi et al. 2003). Although many of such chimeric recombinants might be rejected by the processes of natural selection on the basis of viability of these chimeric strains (Simmonds 2004 and viazov et al. 2000). It is not always necessary that all the representatives of a single genotype will form a single cluster. However the genotypes of any particular area may cluster independently (Fig. 4.3.26 and Fig. 4.3.27). The results are in accordance with the ones reported for accessions from Vietnami genotypes (Simmonds 2004). The less diverse a virus is, the more recent is its invasion in a viral population. Likewise the more diversity observed in the actively mutating viral accessions indicate that they had evolved earlier and are circulating in the human population far longer time periods during the course of evolution. These observations are supported by the findings of a study where genetic diversity of HCV was observed on the basis of evolutionary models (Stumpf and Pybus 2002).

4.3.10 TAJIMA'S TEST OF NEUTRALITY

Tajima's test of neutrality collates the number of discriminating sites per site with the nucleotide assortment. A site is considered segregating if in a comparison of m sequences, there are two or more nucleotides at that site; nucleotide diversity is defined as the average number of nucleotide differences per site between two sequences. If all the alleles are selectively neutral, then the product 4Nv (where N is the effective population size and v is the mutation rate per site) can be estimated in two ways, and the difference in the estimate obtained provides an indication of non-neutral evolution (Tajima 1989 and Nei and Kumar 2000, 260). The positive values of Tajima's test obtained in the Table 4.3.1 indicated that the different strain polymorphism found in the 5'UTR of HCV was probably maintained by a balancing selection process, sustained by an evolutionary

method of gene birth and death. Such combination has thus soughed the best polymorphism by host selection (niche explored), according to the viral types.

Test values indicated that the high mutation rate of the HCV might be one of the points of determinant action of the natural selection and thereby cooperated in inducing the divergence of viral species. A similar study was carried out on different members of kingdom Protista where the positive values of the Tajima's neutrality test revealed the same evolutionary behaviour (Escobar and Castano 2009) on the other hand a negative value in this test suggests very low polymorphism frequency, indicating an expansion in the size of viral population or a purifying selection process. At the beginning of HCV infection, there is a reduction in the viral population, i.e., a bottleneck effect demonstrating infection progress in acute infection (Crandall et al. 1999). Same bottleneck effect was observed in the study conducted in Brazil on HCV genotype 1 variants in chronic HCV patients (Araujo et al. 2008). On the contrary, positive values the test indicates high levels of polymorphism and reduced population size thereby mediating a balancing selection process (Kimura and Ota 1971 and Ohta 1992).

Table 4.3.1. Tajima's test of neutrality applied to compute the level of divergence in HCV variants.

Accessions	m	S	Ps	θ	π	D
Thesis (Gujar khan)	57	188	3.298	0.216851	0.730983	8.418773
Pakistan	107	188	1.757	0.190645	0.702624	8.938790
China	93	184	1.978	0.195908	0.696009	8.605906
Iran	73	164	2.246	0.205727	0.723874	8.680081
India	103	188	1.825	0.192046	0.707375	8.963126
Neighbouring countries	154	164	1.064	0.178224	0.706291	9.524572
World over	88	159	1.807	0.198065	0.726093	9.007860

The Tajima test was calculated using MEGA 5 Beta # 7 software. All gaps were eliminated from the data group (complete deletion option). m = Number of sites, S= number of segregation sites, $P_s = S/m$, θ = ps/a1, π = nucleotide diversity. D is the statistical test result.

SUMMARY

One of the most actively mutating virus that has affected the lives of millions of people globally is HCV, causing not only affected individuals to suffer irritably but also the investment of trillions of rupees for the treatment at government and public level. After the tiring and exhaustive work on HCV since its discovery, it has become a challenge for the scientists. Viral epidemiology and its genotyping are two major fields to determine the natural history of the virus, modes of spread of disease and treatment responses in patients. Besides viral phylogenetic studies are equally good tools to determine the network of disease spread in the form of an epidemic, and to explore the disease endemicity etc.

Epidemiological data collected from 1200 patients (later, a total of 986 (163 males and 962 females, were selected on the basis of scarcity of required information), enlightened that most of the studied participants were chronic HCV carriers (93%) because the disease remains asymptomatic till it becomes chronic. The disease was more pronounced in people of low socioeconomic status and in people with no formal education (in studiedcohort); in fact it affected the individuals from all professions. Therefore the need of time is to educate the people of all socioeconomic statuses with reference to its modes of spread and consequences. It is advisable that screening of the blood after every six months should be a routine practice in the country. It was observed that most affected people were married (87%); therefore a blood screening test of the individuals should be performed before marriage. Bounteous age group reported was 40-49 years and most ambient reason for this might be the time before 1990s when there was no blood screening facility available and majority of the people had acquired disease at that time are now in ages between 30-50 years. Another reason of this trend might be that this particular age group was the one, actively visiting the hospitals because of presenting complaints. Overall results of gender and marital status associated mode of HCV transmission were statistically non-significant and showed no association between risk factors and any specific age group.

Prevalent reported risk factors were therapeutic injections, blood transfusions, surgeries and dental procedures, were equally prompt in patients suffering from different genotypes.

Therefore, it is strongly recommended that screening of blood, proper sterilization of the equipments and surgical tools, and safe injection practices should be followed to avoid the further spread of disease. Subtype 1b was significantly associated with the combination of multiple risk factors i.e. blood transfusion and dental procedures, while genotype 3b was significantly related to multiple risk factor, of surgery and dental procedures, on the other hand the remaining genotypes were equally involved in all reported risk factors. From analysis of HCV samples, it was found that 60.5% of the samples were the representatives of genotype 3 (76.6% samples were genotype 3a and 23.4% were found to be genotype 3b), whereas genotype 1 was 22.5% (57.14% were subtyped to be genotype 1a and 42.86% were subtyped as 1b). Only 2.75% samples were the genotype 4 and 14.13% samples were untypable. In earlier reports genotype 1 was not much prevalent. This changing rate of genotype prevalence is alarming, probably an indication of expected genetic drift from genotype 3 to 1.

The multiplicity of the risk factors presented some important off shooting with respect to disease surveillance. Many of the disease examination networks employ a scaling algorithm to determine the routs for HCV acquisition when multiple risk factors are present in an individual. Therefore, health policy formulators should consider the changing tendency in genotype distribution for next 10-20 years health policy designing. Additionally, the presence of unique sequence variability in the 5' UTR of HCV type 3 and 1 isolates from suburban Rawalpindi was observed in the present study. That was caused by mutations found in these unique sequences and led the viruses to mechanism of starting the translation. Further work is required to assign these sequences to new subtype of HCV and determine their clinical impact. Secondly 5'UTR of HCV could completely classify genotype and subtypes, suggesting that HCV 5' UTR genotyping methods give enough information for clinical investigations, reasonable phylogenetic tree analysis and even differentiate the separate cluster of the subtypes among type 1.

Fifty two untypable samples and 5 representative samples of resolved studied genotypes i.e. 24P (1a), 101P (1b), 172P (3a), 35F (3b) and 17F (4) were sequenced, blast analysis resolved 24.56% genotype 3 descendents, 5.263% and 21.05% subtype 1a and 1b respectively, 14% diversified genotype 4, 3.5% subtype 2k, 1.75% each of 1d and 6v and 19.29% were unresolved novel variants belonging to some common genotype/subtype because they branched them as sister subgroups of the single cluster. While 8.77% of the samples mounted no significant

similarities with the existing variants of HCV and full genome sequencing will be the only tool for their further resolution and classification. The emergence of unique subtypes 1d, 2k and 6v (although in a small set of data) had raised many questions with reference to their distribution in general Pakistani population, their treatment responses and their comprehensive epidemiology. It is therefore advisable to further explore these new emergents and to design the treatment plans accordingly. Future research in the field of HCV infection will need to be continued, to evaluate the incidence of virus in both urban and rural areas of developing and under developed countries, as well as the transmissibility of the various genotypes. Awareness of better prevention, screening and treatment methods of HCV infection need to be promoted. Phylogenetic analysis was performed to root out the evolution network of HCV variants and the degree of association among them. Further, comparative analyses were performed for studied sequences with neighbouring countries and with the representatives' sequences from all over world. The analysis unveiled that studied samples assorted them independently during the course of evolution, though they had evolved from some common ancestor. Moreover the history of endemicity of HCV in Pakistan is quite ancient and viral dispersal in subcontinent seems to be associated with Pakistan.

Major achievements so far from the study were a review paper entitled An Overview about Hepatitis C: A devastating Virus, which is in press (Critical reviews in microbiology), one research paper has been submitted and two more research papers are in the process of submission. Further, 57 sequences had been submitted to Genbank.

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Annexure 1: Designed Performa for Data Collection

Reference # _____ Age: _____ Occupation: _____

Sex: Male _____ Female _____

Monthly Income of the Household:

Less than 5000 _____ Between 5000-10000_____ More than 10000 _____

Travelled Abroad: Yes_____ No _____

If yes where: _____ Duration: _____

Any other Family Member Affected: _____

Duration of Present Illness:_____

Treatment: On Zakat and Bait ul Mall_____ Self Payment _____

Affordibility: Affording _____ Non-Affording_____

Mode of presentation: Incidental _____ By Symptoms_____

If by symptoms then what are the symptoms

- Anorexia _____
- Generalized Weakness_____
- Flu Like Symptoms _____
- Jaundice _____
- Fever _____
- Ascites _____
- Gastrointestinal Bleeding_____
- Pain of Muscles and Joints_____

Possible Modes of Disease Acquisition

Exchange of Needle _____

Sexual _____

Blood Transfusion _____

Tattooing_____

Barber Shears _____

Surgery _____

Child Birth _____

Shared razors/Tooth Brushes _____

Documented Blood Exposure_____

Personal History of the Patients

Occupation_____

Literate_____

Smoker_____

Alcohol Consumption _____

Addictions _____

Marital Status_____

Annexure II: Solution Preparationa and RNA Extraction

Following solution was made for the extraction of HCV RNA.

Chomezynski's Solution D stock:

Guanidinium thiocyanate	250g
Distilled water	293ml
0.75M Sodium citrate (pH 4.0)	17.6ml
10% SDS (Sodium Dodesyl Sarcocine)	26.4ml

Dissolved at 65 °C. This solution can be stored at room temperature for three months.

Chomezynski's Solution D working:

Prepared by adding 0.36ml of 2-merceptoethanol in 50ml of stock solution or 0.7µl of 2-merceptoethanol per 1ml of solution D. Vortex for 1 minute (biovortex type V1).

RNA extraction protocol:

1. 0.5µl (10mg/ml) of glycogen was taken in eppandorf then,102µl of sample serum, 309µl of working Chomezynski's Solution D, 64µl of 3 M Sodium acetate $Na(OAc)_2$, 442µl of water saturated phenol and 89µl of Chloroform:Isoamylalcohol (24:1) was also added in that eppendroff.
2. The sample was vortexed for 1 minute and then incubated at –20 °C for 20 minutes. After incubation sample was centrifuged at 10,000 rpm for 10 minutes at 4 °C (model 5417R eppendroff).
3. The aqueous phase was transferred in another eppendroff tube and 555µl of chilled isopropanol was added, and mixed it only by gentle inversion.
4. The sample was incubated at -20 °C for 1 hour and then centrifuged at 14,000 rpm for 15 minutes at 4 °C.
5. The supernatant was discarded carefully with the help of 20-200µl micropipetter.
6. 1000µl of chilled 75% ethanol was added and centrifuged at 4 °C for 9 minutes at 9000 revolutions per minute (rpm).
7. The supernatant was then removed and the pallet was air dried.

8. The pellets were reconstituted in 20µl of Deionized Diethyl Polycarbonate (DEPC) treated water and eppendroff was incubated on the heat block LabLine (no model description available) for 2 min at 45 °C and then stored at –70 °C.

Annexure III: Reaction and PCR Conditions for RT-PCR

Reaction and PCR conditions for cDNA synthesis:

DEPC treated water	6µl
5X RT Buffer	4µl
10mM dNTPs	2µl
RNase inhibitor	40U
Oligo ASP1	1µl
MMuLV reverse transcriptase	200U
Template RNA	5µl

PCR conditions:

37 °C	5 minutes
42 °C	60 minutes
95 °C	5 minutes

Annexure IV: Reaction and PCR Conditions for NESTED PCR

External PCR

Sterowater	14.125µl
10X PCR Buffer	2.5µl
10 mM dNTPs	0.5µl
10 pM/µl SP1	0.625µl
10 pM/µl ASP1	0.625µl
Taq DNA Polymerase	0.125µl
10 mM $MgCl_2$	1.5µl

PCR Conditions:

94 °C	5 minutes	
94 °C	1 minutes	↓ 40 Cycles
56 °C	1 minutes	
72 °C	1 minutes	
72 °C	5 minutes	
4 °C	∞	

Internal PCR

Sterowater	14.125µl
10X PCR Buffer	2.5µl
10 mM dNTPs	0.5µl
10 pM/µl SP2	0.625µl
10 pM/µl ASP2	0.625µl
Taq DNA Polymerase	0.125µl
10 mM $MgCl_2$	1.5µl

PCR Conditions:

94 °C	5 minutes	
94 °C	1 minutes	↓ 40 Cycles
58 °C	1 minutes	
72 °C	1 minutes	
72 °C	5 minutes	
4 °C	∞	

Annexure V: RFLP Protocol

Restriction sites of the restriction enzymes used were for

*RSa*I

5'----GT↓A C-----3'
3'----CA↑T G-----5'

*Hin*fI

5'----G↓ANT C -----3'
3'----C TNA↑G-----5'

*MVa*I

5'----CC↓A/T GG-----3'
3'----GG A /T↑CC-----5'

*Hae*III

5'----GG↓C C-----3'
3'----CC↑G G-----5'

*Bme*1390I(*Scr*fI)

5'----CC↓NGG-----3'
3'----GGN↑CC-----5'

*Bsh*1236I (*Fun*DII)

5'----GG↓C C-----3'
3'----CC↑G G-----5'

Restriction digestion Procedure

PCR Product	10µl
Nuclease free water	18µl
Buffer Tango	2µl
*Rsa*I	10U
*Hae*III	10U

The reaction mixture was incubated in an eppendroff using heat block K-HSYH-11 by Labnet for 16 hours. The sample was run on 16% polyacrylamide gel.

Annexure VI: Protocol for PAGE

Preparation of 10X TBE (Tris Boric acid EDTA) buffer

Tris Base	108g
Boric Acid	55g
0.5 M EDTA, pH 8.0	40ml
Double distilled H_2O	700ml

Volume was brought upto 1 litter and the buffer was then autoclaved (Sambrook and Russell 2001).

Preparation of Acrylamide solution

30% Acrylamide	30.0g Acrylamide
0.8% Bis-Acrylamide	0.8g Bis-Acrylamide
Double distilled H_20	70ml

Volume was brought to 1 litter and filter. Store at 4 °C in the dark (Sambrook and Russell, 2001).

Preparation of 10% ammonium per Sulphate Solution

0.5g ammonium per sulphate (APS) was added in 5ml distilled water to make 10% solution of APS. The solution was stored at -20°C for 1 week however it was preferred to make fresh ammonium per sulphate solution before every use.

Procedure for running 16% PAGE

Glass plates were washed on both sides with soap and rinsed well with distilled water. Each side was doused with 95% ethanol to promote drying. The plates were laid flat and short plates were placed on top with a 0.75mm gap provided by spacer. The top edge of the plates was propped up with a 25ml pipette to provide a shallow slope from top to bottom. An appropriate amount of acrylamide solution was mixed in a 60ml syringe.

For 16% 35ml solution the recipe was:

30% Acrylamide Solution	18.658ml
ddH_2O	12.49ml
10% Ammonium Per Sulphate	350µl
Fresh TEMED (Tetramethyl Ethylene Diamine)	14 µl
10X TBE	1.75ml

The solution was mixed by inversion and quickly applied 3ml across the top of the plates allowed to run between the plates. Continuous provision of acrylamide to the top of the plates was ensured as it ran in. slow running spots were taped as it quickend the flow and avoided bubble formation. After filling comb teeth was inserted and was allowed to polymerised for 3 hours before applying the voltage across the electric field.

Annexure VII: DAN Purification protocol

Sample Preparation for DNA purification

200µl of solution H1 (Guanidine hydrochloride and isopropanol) was added to 40µl of PCR assay and was mixed thoroughly.

Column Loading

The mixture from step 1 was loaded into the Jetquick spin column placed in a 2ml receiver tube followed by spinning for 1 min at 13,000 rpm (using Spectrafuge 24D Labnet). Flow through was discarded.

Column Washing

Reconstituted H2 solution (ethanol, NaCl, EDTA and Tris HCl) was added to spin column inserted in fresh receiver tube. The tube was spined for 1 min at 13,000 rpm. Before using H2 solution sequencing grade ethanol (96-100%) was added to its concentrated buffer. After discarding the flow through, the column was repositioned into the same receiver tube and centrifuged for 1 minute at 13,000 rpm.

DNA Elution

JETQUICK spin column was placed into a fresh 1.5ml microfuge tube and 30µl of Nanopure water was added (or TE buffer or 10mM Tris/HCl, pH 8.0) straight onto the core of silica medium of the JETQUICK spin column. The tube was centrifuged at 13,000 rpm for 2 minute. Elution buffer (preheated to 65-70 °C) was added straight in the middle of the silica matrix of the spin column and was allowed to stand for 1 minute prior to spining. Elution buffer was dispensed directly onto the silica membrane. After centrifugation DNA was stored for further use.

Annexure VIII: DNA sequencing Reaction conditions

Preparation of Sequencing Reaction

The preparation of sequencing reaction was observed in a 0.2ml micro plate well or thin walled tube. Ice cold conditions were maintained for preparation and the reagents were added in the following order.

Sequencing buffer	1.5µl
DNA tamplate	2.0µl
Primer 940 (1.6 pmol/ µl)	4.0µl
DTCS quick start master mix	3.0µl
dH_2O (to make total volume 20µl)	1.5µl

Reaction components were mixed thoroughly and short spinning before sequencing PCR would settle the liquid in the bottom of the tube.

Thermal cycling program was as follows

96 °C	3 minutes	
96 °C	30 seconds	30 Cycles
59 °C	30 seconds	
72 °C	4 minutes	
72 °C	10 minutes	

Ethanol Precipitation in individual tubes

Tubes were labelled and stop solution (2.5 µl per solution) was prepared freshly for each reaction tube as: 1µl of 3 M Sodium Acetate (pH 5.2), 1µl of 100 mM Na_2-EDTA (pH 8) and 500µl of 20mg/ml of glycogen supplied with the kit. The reaction mixture was then transferred into 0.5ml microfuge tube and was mixed carefully. Then 70µl of the cold 95% (V/V) ethanol/dH2O was added and immediately subjected to centrifugation at 14,000rpm for 15 min maintained at 4°C. Using a micropipette, supernatant was removed and the sample was rinsed again by spinning it at 14,000rpm at 4°C for 2 minutes. Sample was allowed to vacuum dry for about 10 minutes and resuspended in 35µl of sample loading solution from kit.

Precipitation in the Samples Plate

Samples were subjected to thermal cycling followed by ethanol precipitation in the plates.

Sample Preparation for loading into the Instrument:

The resuspended samples were transferred to the sample plate wells and each of it was topped with a drop of mineral oil (provided with the kit). The sample plate was placed im the Beckman Coulter CEQ8800 sequencer for sequencing.