2.0 Review of Literature
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Hepatitis A virus</td>
<td>3</td>
</tr>
<tr>
<td>2.2 Studies on Molecular epidemiology</td>
<td>18</td>
</tr>
<tr>
<td>2.3 Studies on hepatitis A in India</td>
<td>21</td>
</tr>
</tbody>
</table>
2.0 Review of Literature

2.1 Hepatitis A virus

HAV is a nonenveloped virus 27 to 32 nm in diameter that is morphologically indistinguishable from other picorna viruses (Figure 1) (Feinstone et al. 1973:1026-1028; Siegl 1982:13-20). By electron microscopy "full" and "empty" particles appear to be spherical and featureless but fine structural analysis has demonstrated icosahedral symmetry (Feinstone et al. 1973:1026-1028; Siegl 1982:13-20). Recently medium resolution images of the HAV particle have been obtained by cryo-electron microscopy, which are suggesting the significant differences in structure of HAV as compared to other picornaviruses. (R.H. Cheng, unpublished data).

Figure 1: Electron Micrograph of Hepatitis A virus particles.

The buoyant density of the mature particle is between 1.32-1.34 g/cm3 in CsCl solutions and the sedimentation coefficient is 156-160S in neutral sucrose solutions. However, additional populations of virus with different buoyant density and sedimentation coefficient have been isolated from human feces and cell cultures (Siegl, 1982:13-20; Lemon et al. 1985:78-85). Empty particles (lacking RNA) have been detected at 1.20 and 1.29 to 1.31 g/cm3 with sedimentation coefficient ranging from 50S to 90S (Bradley 1977:219-226).

HAV was provisionally classified as enterovirus type 72 due to its similarities with enteroviruses and cardioviruses in biophysical/biochemical characteristics (Gust et al. 1983:1-7). Later studies demonstrated several characteristics that distinguish HAV from other picornviruses. These included nucleotide and amino acid sequences, difficulties in adaptation to cell culture, resistance to temperature, pH and drugs, which inactivate other picornviruses and occurrence of only one serotype. These features differentiated HAV as a unique member of the family picornaviridae resulting in its classification into new genus hepatovirus (Palmenberg 1989:211-241; Minor 1991:320-326).

The well characterized HAV strains include HM 175 from Australia, CR 326 from Costa Rica, MS-1 from New York, SD 11 and LA from California, HAS15 from Arizona,

2.1.1 Genomic organization

The infectious HAV particle consists of capsid proteins and the RNA genome. The genome is a linear, positive stranded RNA of approximately 7.5 kb with a single open reading frame (ORF) for a large polyprotein of about 250 kd which is translated into a polyprotein of 2225 - 2228 amino acids (Baroudy et al. 1985: 2142-2147; Cohen et al. 1987:50-59; Endo et al. 2007:116-127). HAV RNA is enclosed in an icosahedral protein capsid. The RNA genome shares common characteristics with other picornavirus strains; however, the nucleotide or amino acid sequences are different (Ticehurst et al. 1983:5885-5889). HAV genome can be divided into three parts as 5' noncoding region (NCR), ORF and 3'NCR (Figure 2). 5'NCR that comprises approximately 10% of the genome is covalently linked at the 5' terminus to viral protein Vpg. Translation occurs in a cap independent fashion under control of an internal ribosome entry site located within the 5' NCR. ORF that appears to encode all of the viral proteins, with regions designated as P1 for capsid proteins and P2 and P3 for nonstructural proteins (Figure 2). The translation terminator sequence, the short 3' NCR, which terminates into poly A tail of 40-80 nucleotides (Ticehurst et al. 1983:5885-5889).

2.1.2 Proteins

HAV virion contains a capsid comprising of 60 copies of each of 3 major structural proteins (Figure 2) which have been detected by polyacrylamide gel electrophoresis include, VP1 (or 1D, molecular weight (MW) 30-33 kd), VP2 (or 1B, MW:24-30 kd) and VP3(or 1C, MW:21-28 kd). (Coulepis et al. 1978:24-31; Siegl 1981:331-334; Tratschin et al. 1981:151-156; Hughes and Stanton 1983:395-401; Siegl 1984:9-32; Wheeler et al. 1986:434-440; Gauss-Muller et al. 1986:732-735). The existence of a fourth, small protein VP4 (or 1A) has not been confirmed in HAV (Updike et al. 1991:411-418; Bishop and Anderson 1993:616-623). Some HAV studies have
detected a protein of 7-14 kd, however, it has not been identified as VP4 by immunologic reactivity or by sequence analysis (Tratschin et al. 1981:151-156; Coulepis et al. 1980:572-574). Evidence for additional proteins VPg (or 3B, MW: 2.5 kd) covalently linked to the 5' genome terminus, precursor polypeptides VPO (VP4-VP2) (MW: 27-31 kd) and PX (VP1-2A) (MW: 38-40 kd) has also been obtained (Weltz et al. 1986:124-130; Kusov et al. 1992:220-227; Bishop & Anderson 1993:616-623). Indirect evidence for the synthesis of P2 and P3 proteins has been provided by presence of anti P2 and P3 antibodies in HAV infected primates (Jia et al. 1992:273-280; Robertson et al. 1993:76-82).

**Figure 2: Genomic Organization of Hepatitis A Virus**
Like other picornaviruses, HAV proteins are cleaved from a large polyprotein, but the processing and some products are quite different. The primary polyprotein cleavage event occurs at the 2A/2B junction and differs from other well-studied picornaviruses in that it is mediated by the 3C protease (Martin et al. 1995:213-222). The resulting P1-2A structural precursor is further cleaved by the viral protease to generate capsid protein precursors, VP0 (VP4-VP2) and PX (VP1-2A) (Anderson & Ross 1990:5284-5289) as well as the mature VP3 capsid protein. VP1-2A is a critical structural intermediate in virion morphogenesis (Cohen et al. 2002:7495-7505). Cleavage at the VP1/2A junction occurs late in the process of virion morphogenesis and results from the action of an unknown cellular protease (Martin et al. 1999:6220-6227; Graff et al. 1999:6015-6023). However, the mature 2A protein has never been identified directly in infected cells. The non-structural 2A protein segment is not required for RNA synthesis, however essential for capsid assembly (Cohen et al. 2002:7495-7505). It remains unclear how the VP2-VP4 cleavage occurs. The role of the small VP4 polypeptide in virion morphogenesis is unknown. Most other picornaviruses have 4 polypeptides within their capsid, including a small VP4 protein located at the amino terminus of the polyprotein. Whereas the polyprotein of HAV appears to possess a very short VP4 polypeptide segment at its amino terminus, this putative VP4 moiety has never been demonstrated directly in purified virus preparations. N terminal myristoylation of other picornavirus VP4 proteins is important for virion morphogenesis. The HAV VP4 sequence does not contain a similarly placed myristoylation signal (Tesar et al. 1993:616-626). Some data suggest that VP4 is necessary for assembly of pentamers into empty capsids (Probst et al. 1999:4527-4531). However, empty capsids can be synthesized in the absence of VP4 (Benichou et al. unpublished data). Thus two small proteins VP4 and 2A has mounted considerable controversy on their role in the assembly of virus particles.

2.1.3 Antigen and antigenic structure

All current immunoassays indicate that there is only one known serotype of HAV (Lemon and Binn 1983:418-420; Zahn et al. 1984:9-17; Lemon et al. 1987:735-742; Siegl 1988:3-7). Reactivity with monoclonal antibodies suggests that there are limited numbers of antigenic epitopes, which are closely grouped at the surface of virus (Stapleton et al. 1987:491-498; Ping et al. 1988:8281-8285; Ping & Lemon 1992:2208-2216). Analysis of neutralization escape mutants isolated in vitro has provided evidence for the existence of an immunodominant antigenic site on the capsid surface that is conformationally determined and involves residues contributed by capsid protein VP3 and probably by VP1 (Ping et al. 1988:8281-8285). Convalescent phase sera obtained from hepatitis A patients were shown to be reactive primarily to VP1 and to a lesser
extent to VP0 and VP3 (Wang et al. 1996:707-713). Recombinant VP1 fusion protein expressed in *Escherichia coli* reacted with rabbit anti HAV serum (Ostermayr et al. 1987:3645-3647). However, generation of anti HAV neutralizing antibody using individual recombinant HAV proteins and synthetic peptides has been unsuccessful or generated low titre antibody (Ostermayr et al. 1987:3645-3647; Johnston et al. 1988:1203-1210; Rosen et al. 1993:706-712). Chimpanzees immunized with this recombinant protein produced antibodies that reacted with VP1 of only denatured and not intact virus and the animals were not protected after challenge with wild type HAV (CDC, unpublished data). Accurately processed and assembled recombinant HAV polyprotein has been produced, which was able to elicit neutralizing antibodies (Stapleton et al. 1993:1080-1085; Xia et al. 1997:208-211; LaBrecque et al. 1998:2014-2018). Overall, the antigenic structure of the virus is reported to have a restricted number of overlapping epitopes combining to form a single dominant antigenic site that interacts with virus neutralizing antibodies. Antigens of the intact virion appear to be conformation dependent and generally different from those of individual HAV proteins. Empty particles appear to be antigenically indistinguishable from infectious, RNA containing virions, suggesting that antigenicity may depend on assembly of the major capsid proteins or smaller capsid precursors (Nainan et al. 2006:63-79).

The known antigenic variants of HAV were identified in strains collected from old world monkeys (Tsarev et al. 1991:1677-1683; Nainan et al. 1991:1685-1689). Recently, human HAV strains with capsid amino acid substitutions and deletions in the immunodominant antigenic site were reported (Costa-Mattioli et al. 2002:9516-9525; Sanchez et al. 2002:4148-4155). However, it is not clear if capsid antigenicity or virus neutralization was affected by these changes.

### 2.1.4 Stability

HAV is stable to acid and at 60°C at neutral pH for at least 60 min and is only partially inactivated after 10-12 hr. (Provost et al. 1975:532-539; Parry and Mortimer 1984:277-283; Siegl et al. 1984: 9-32). It is inactivated by autoclaving, ultraviolet radiation, formaline, β propiolactone, potassium permanganate, iodine, chlorine (Havens 1945:202-203; Neefe et al. 1946:3-22; Provost et al. 1975:532-539; Scheid et al. 1982:627-628; Siegl 1982:13-20; Peterson et al. 1983:223-227). Some of these methods are regularly used for treatment of drinking water. HAV is not inactivated by oxidizing agent, such as chloramine T, or perchloracetic acid (Coulepis et al. 1987:129-169). Infectivity can be preserved for at least 1 month after drying and storage at 25°C with 42% humidity or for years at −20°C or lower (Mc Caustland et al. 1982:957-958). It was also found to survive for days to months and for longer periods than poliovirus in

2.1.5 Replication in cell culture

HAV is propagated, in vitro in a variety of cell cultures of human and non-human primate origin. These cultures include primary marmoset liver cultures, primary and low passage monkey kidney cell cultures (fetal and adult), several transformed monkey kidney cell cultures, human hepatoma cell line, human embryonic kidney cells, human diploid embryonic lung fibroblasts and transformed human amnion cells (Provost and Hilleman 1979:213-221; Frosner et al. 1979:303-305; Flehmig 1980:239-248; McCollum and Zuckerman 1981: 1-29; Gauss Muller et al. 1981:233-239). Replication of HAV in cell culture is reported to be very slow. Attachment and penetration occur rapidly but release of viral RNA is delayed due to high affinity of capsid and RNA. The virion RNA is transcribed and the polyprotein is processed efficiently yielding capsid precursors and replicative proteins. Few of the positive strands are used for transcription of negative strands. Due to high affinity of capsid proteins and RNA, encapsidation of the majority of positive strand RNA occurs thus depleting the pool of RNA available for replication and the final yield of HAV (Ross et al. 1991:209-253). A putative receptor for HAV, an integral membrane mucin like glycoprotein of unknown function, has been identified in African Green Monkey Kidney cells (Feigelstock et al. 1998:6621-6628).

The cell culture mutants of HAV spontaneously arise during persistent infection without selective pressure from antibody, a neutralization escape phenotype with changes at some amino acid positions associated with immunodominant site. It was suggested that a long period of persistent infection could reduce the pressure for maintaining structural features necessary for infecting new cells (Lemon et al. 1991:2056-2065). Some mutations in 5'NCR 2B, 2C and 3A proteins are identified to enhance viral replication or to induce cytopathogenic effects in the viruses adapted to cell cultures (Emerson et al. 1993:475-480, Beneduce et al. 1995:299-309; Graff et al. 1997:1841-1849).

Even though a large proportion of the newly replicated virus remains cell associated, cytopathic variants of HAV have been observed in selected cell culture systems. These cytopathic viruses produce an acute rather than persistent infection (Cromeans et al 1987:45-56; Nasser and Metcalf 1987:2967-2971). The replication cycle of these variants is shorter (2 to 3 days) than that observed for noncytopathic HAV, and they produce a much higher viral yield (Cromeans et al 1987:45-56). In
polarized, human colonic epithelial cell cultures release of virions occurs exclusively through apical cellular membranes (Blank et al. 2000:6476-6484).

2.1.6 Host Range

HAV is known to produce disease in humans, chimpanzees, owl monkeys, stump tailed monkeys, several species of south American marmoset (tamarin) most notably Saguinus mystax and S. labiatus (Cohen et al. 1989:887-890; Dienstag et al. 1975:532-545; Lemon et al. 1982:25-36; Mao et al. 1981:55-60; Purcell and Dienstag 1978:3-12). Disease in non-human primates resembles that in humans but is usually milder (Burke et al. 1981:928-933; Burke et al. 1984:940-944). Absence of anti-HAV prevalence noted in horse, cow, pig, sheep, dog and cat suggested that HAV is unlikely to circulate in vertebrates other than primates (Gust 1980; Savinskaya et al. 1983:118-120). Anti-HAV was found in 9 out of 64 coprophagic dogs and horse (Gust, 1982:241-351; Savinskaya et al. 1983:118-120). However, recently presence of neutralizing anti-HAV antibodies have been detected in cattle, goats and dogs in India, the region known to be endemic for hepatitis A (Chitambar et al. unpublished data).

2.1.7 Pathogenesis and immune response

Natural infection with HAV usually follows ingestion of material contaminated with feces containing HAV and is associated with extensive shedding of the virus in the feces during incubation period (3 to 6 weeks) and extending into the early days of the illness (Figure 3). Exceptional stability of HAV at ambient temperatures and low pH explains the ability of it to survive in the environment and transmission by contaminated foods and drinking water. Resistance to acidic pH and detergents also accounts for its ability to survive in the stomach, and exit the host via the biliary tract. These are the features that contribute significantly to the pathogenesis of hepatitis A.

The pathogenetic events that occur during HAV infection have been determined in experimental infection of nonhuman primates and natural infection of humans (Figure 3). The incubation period in common source exposures is nearly one month. The sequence of events with entry via the gastrointestinal tract resulting into hepatitis is unresolved. Hepatocytes are the primary site of HAV replication and intestinal replication has not been identified using highly sensitive detection methods (Krawczynski et al. 1981:698-706; Taylor et al. 1992:642-648). Detection of HAV in tonsils and saliva, shortly after viremia suggests that an early replicative event may occur in the oropharynx or salivary glands (Purcell et al. 1984:9-22; Cohen et al. 1989:887-890). However, some data suggest that HAV may undergo initial replication within crypt cells of the small intestine before reaching the liver (Asher et al. 1995:260-
HAV antigen is found primarily in the cytoplasm of hepatocytes, but can also be found in liver macrophages (kupffer cells). HAV appears in hepatocytes prior to detection in feces and prior to the onset of liver enzyme elevations. HAV is excreted via the biliary system into the feces where it appears in high concentrations from 1 to 2 weeks prior to onset of clinical illness. The mechanism of viral release and secretion is unknown but clearly is not dependent on cell destruction, since high viral titres are present in stool before there is any evidence of hepatocyte necrosis (Coulepis et al. 1980:151-156; Ticehurst et al. 1987:1822-1829).

HAV particles have been shown in feces, maximum at the end of incubation period of the disease and during rising levels of serum alanine aminotransferase (ALT) by IEM (Dienstag et al. 1975:765-767; Rakela and Mosley 1977:933-938). With the availability of radioimmunoassay, HAV antigen was detected up to a period of 3 weeks even after the onset of jaundice (Carl et al. 1982:125-129). Polymerase chain reaction (PCR) employed to monitor the presence of HAV RNA in the feces and serum indicate prolonged fecal shedding of HAV as well as viremia (up to 90 days) in natural and experimental HAV infections (Fujiwara et al. 1997:1634-1639; Bower et al. 2000:12-17; Chitambar et al. 2001:237-246).

Figure 3: Virological, immunological and biochemical status during the course of hepatitis A
(Figure adopted from Martin and Lemon, 2006: s164-s172). The sequence of events includes HAV viremia (yellow), shedding of infectious HAV in feces (blue), followed by increases in serum alanine aminotransferase (ALT) activity (red line), and the appearance of IgM and IgG anti-HAV antibody responses (blue lines).
The profile of immune response in hepatitis A includes appearance of IgM anti-HAV, immune complexes, and reduced levels of serum complement (Baer and Walker, 1977:1-7; Stapleton et al.1995:s9-s14; Inman et al. 1986:700-703; Thomas et al.1978:150-157; Margolis et al.1988:315-326). Analysis of the immune complexes has revealed presence of IgM and IgG, HAV capsid proteins, and the C3d component of complement (Margolis et al. 1988:315-326). The early antibody response is composed of IgM, although IgG may also be present shortly after the onset of symptoms (Figure 3). Anti HAV IgG persists for life and confers protection against reinfection. Both fecal and serum anti HAV IgA have been described (Locarnini et al. 1980:710-716; Joshi et al. 2002:840-845) but the role of secretory immunity in protection against HAV infection appears to be very limited (Stapleton et al. 1991:7-11). Patients with hepatitis A appear to lack complement dependent cytotoxic antibodies (Gabriel et al. 1986:23-31).

Although the mechanisms responsible for hepatocellular injury in hepatitis A are not clearly understood it is suggested that it involves immunopathologic response rather than a direct cytopathic effect of the virus. HLA restricted, virus specific, cytotoxic, CD8+ T cells have been identified in the liver in acute hepatitis A (Fleischer et al. 1990:14-19). Such cells have been shown to secret gamma interferon that subsequently activates the recruitment of additional, nonspecific inflammatory cells at the site of virus replication probably leading to viral clearance and in the production of liver injury (Vallbracht et al. 1986:1308-1314; Fleischer et al. 1990:14-19; Tripathy et al. 2005:283-284).

2.1.8 Clinical and biochemical features

The clinical course of acute viral hepatitis A is similar to other types and has been divided into four clinical phases. Incubation/preclinical phase ranges from 10-50 days, with a median of approximately 1 month, (Paul et al.1945: 911-915; Neefe et al.1946:3-11). During this period the patient remains asymptomatic despite active replication of the virus and with greatest possibility of transmitting the virus. Prodromal/preicteric phase, varying between days and week, precedes the onset of jaundice. This is characterized by anorexia, fever, fatigue, malaise, myalgia, nausea, and vomiting. Icteric phase of acute viral hepatitis is ushered in by the appearance of dark, golden brown urine due to bilirubinuria, followed by one or several days later by pale stools and yellowish discolouration of the mucosal membranes, conjunctivae sclerae and skin. This icteric phase begins within 10 days of the initial symptoms in over 85% of HAV cases (Molner and Meyer 1940:509-515; Zuckerman 1965:340-344). Approximately 5-15% patients have splenomegaly. The mortality rate is considerably higher among patients with chronic hepatitis B or underlying liver disease that are super
infected with HAV (Hadler 1991:14-20; Keeffe 1995:201-205; Yao 1991:76-78). During convalescent phase, liver function tests return to baseline. Anti-HAV IgM persists at least for six months. Anti-HAV IgG persists for life long time.

Hepatitis A is known to exist in mild to severe forms. Severe forms include fulminant hepatic failure, persistent infection, renal failure, late recovery, clinical relapse, cholestatic hepatitis and chronic hepatitis (Chio and Bakir 1992:413-416; Inoue et al. 1996:322-324; Bendre et al. 1999:1107-1112; Saunders et al. 1979:549-584; Glikson et al. 1992:14-23, Schiff 1992:s18-s20; McDonald et al. 1989:223-228).

2.1.9 Fulminant Hepatitis

Occasionally, severe impairment of hepatic synthetic processes, excretory functions and detoxifying mechanisms occurs, which is identified as fulminant hepatitis. It occurs during the first 6 to 8 weeks of illness, and is characterized by the sudden onset of high fever, marked abdominal pain, vomiting and jaundice followed by the development of hepatic encephalopathy associated with deep coma and seizures (Adams and Foley 1953:198-237; Saunders et al. 1979:569-584; Trey et al. 1966:473-481). Fulminant disease occurs more frequently in adults than children (Willner et al. 1998:111-114) but can occur in childhood (Debray et al. 1997:1018-1022). Fulminant hepatitis A was diagnosed in 20 of 295 patients in a retrospective study of acute hepatic failure in the United States (Schiodt et al. 1999:29-34). Between 1983 and 1987, CDC reported 381 deaths due to hepatitis A (Lemon 1994:1363-1364). In the largest epidemic, in Shanghai -1988, fatality rate of 0.01% was recorded (32 deaths) among 292,301 cases (Halliday et al. 1991:852-859). In India, 37.5%-50% fulminant hepatitis cases among children are caused due to HAV alone or in combination with other hepatitis agents (Arora et al. 1996:215-221; Bendre et al. 1999:1107-1112). In recent reports, rate of acute liver failure was 1% among 1932 acute viral hepatitis patients (Hussain et al. 2006:689-693). The spontaneous recovery rate for patients with fulminant acute hepatitis A was 35% in the retrospective U.S. study, whereas it was 39% in a French study (Debray et al. 1997:1018-1022). Occasionally, hepatitis A infection recurs following liver transplantation (Fagan et al. 1990:131-136, Gane et al. 1995:35-39), while others may survive without transplantation (Debray et al. 1997:1018-1022; Schiodt et al. 1999:29-34).

2.1.10 Other atypical manifestations

Atypical manifestations of hepatitis A include the development of cholestasis, relapsing hepatitis, extra hepatic manifestations and the possible induction of type 1 autoimmune chronic hepatitis (Schiff 1992:s18-s20). Relapsing hepatitis has been
reported to occur in 3% to 20% of the cases of acute hepatitis A (Sjogren et al. 1987:221-226; Jelic et al. 1990:565-576; Glikson et al. 1992:14-23). Extrahepatic manifestations of hepatitis A are unusual, although transient rashes are observed infrequently such as arthralgia. Clinically apparent arthritis and leukocytoclastic vasculitis have been reported and mild membranoproliferative glomerulonephritis associated with immune complex deposits has been seen in a postmortem specimen (Hollinger et al. 1983:2313-2317). HAV also has been associated with gall bladder wall thickening (Toppet et al.1990:249-257) and cholecystitis on rare occasions (Mourani et al.1994:398-400). Neurological syndromes such as myelitis, peripheral neuropathy, myeloradiculopathy, mononeuritis, exacerbation of multiple sclerosis and Guillain Barre' Syndrome (GBS) have been reported in association with hepatitis A (Adams and Asbury 1980:2097-3030; Owen et al.1980:2307-2309; Bosch et al.1983:685-687; Pelletier et al. 1985:53-56; Chitambar et al. 2006:1011-1014).

Clinical diagnosis of acute viral hepatitis is made by biochemical assessment of liver function. Initial laboratory tests include testing of urine for bile salts, bile pigments and urobilinogen and testing of serum for total and direct (conjugated) bilirubin. Total serum bilirubin level is usually elevated in acute HAV infection (normal range 0.3-1mg/dl) (Swift et al. 1950:614-622). Serum ALT and aspartate transaminase (AST) concentrations are raised (normal range upto 40 IU/lit) and are highly sensitive indicators of hepatocellular damage. The alkaline phosphatase level (normal range 110-310 IU/lit) is also raised and usually determined to assess the degree of cholestasis. Since serum proteins such as albumin, prothrombin, fibrinogen and vitamin K-dependent coagulation factors are synthesized exclusively or predominantly by the liver, the values of prothrombin time, partial thromboplastin time and albumin concentration are deranged. As it is difficult to determine the etiology of acute hepatitis on the basis of clinical examination and biochemical tests, specific seroimmunological tests are required for diagnosis of hepatitis A.

2.1.11 Laboratory Diagnosis

Prior to the development of tests for identification of markers of HAV infection the only method available for diagnosis was the use of experimental animals. Following the discovery of certain species of marmosets that were susceptible to HAV, this model was used extensively to monitor the infectivity of clinical material, to quantitate anti-HAV antibodies and characterize acute and convalescent serum samples by neutralization of known infectious inoculum (Holmes et al. 1973:419-420; Provost et al. 1977:283-286). These in vivo tests, however, were of limited practical value and were supplemented by in vitro methods. First in vitro test to detect HAV antigen and antibody was described by
Feinstone et al. in 1973 during visualization of HAV particles using the technique of IEM. Though IEM was established as the first in vitro serologic technique for virologic, diagnostic and epidemiologic studies, it required specialized expensive equipment, large quantities of reagents and experienced personnel for interpretation of results. Provost et al. (1975:962-969) and Miller et al. (1975:254-261) developed complement fixation (CF) and immune adherence hemagglutination (IAHA) tests for detection of anti-HAV antibodies. Presence of complement fixing antibodies to normal marmoset liver antigens and anti-complement activity in acute phase serum samples as well as need for high concentration of HAV antigen made CF test undesirable (Zachoval 1984:33-44). Application of IAHA test was reported to be limited because of its less specificity and requirement of partially purified antigen preparations (Zachoval 1984:33-44). A simple test of hemagglutination inhibition by HAV was also described for detection of anti-HAV antibodies (Dubois et al. 1990:299-304). A direct immunofluorescence assay for detection of HAV antigen in thin sections of infected marmoset livers and cell cultures was developed (Mathiesen et al. 1977:524-530; Provost and Hilleman 1979:213-221).

All these assays were superseded by highly specific and sensitive radioimmunoassay and enzyme linked immunosorbent assay (Duermeyer and Vander veen 1978:684-685; Safford et al. 1980:25-31). Originally the immunoassays used polyclonal antisera and HAV antigen purified from feces or liver of infected marmosets (Purcell et al. 1976:349-356; Decker et al. 1981:140-147). Subsequently virus grown in various cell cultures and monoclonal antibodies were used (Stapleton et al. 1987:491-498; Chitambar et al. 1994:115-120). Search for sensitive immunoassays was successful for detection of low levels of specific immunoglobulins (Miller et al. 1993:201-204; Chitambar et al. 1996:63-65; Ochnio et al. 1997:98-101). Further, alternative body specimens such as saliva, urine etc. were employed in the assays for detection of specific antibodies (Perry et al. 1992:265-270; Ochnio et al. 1997:98-101; Joshi et al. 2002:840-845). The development of a highly sensitive assay that is specific for IgG anti HAV and can measure antibody in saliva after vaccination is promising (Ochnio et al. 1997:98-101). In practice, antibody titres are not measured routinely after vaccination since the response rate to the vaccine and its effectiveness are so high and therefore a highly sensitive assay has little utility. A search for cheap and robust assay has been suggested which will be useful in different clinical situations prior to vaccination (Cuthbert 2001:38-58). Most assays have used HAV produced in tissue culture as a source of antigen. In the future, recombinant HAV antigen may provide a less costly alternative ((Xia et al. 1997:208-211; LaBrecque et al. 1998:2014-2018).
2.1.12 Epidemiology

Hepatitis A is one of the major public health problems. The level of sanitation or hygiene in the environment markedly influences its worldwide occurrence.

Several patterns of infection have been defined on the basis of age specific anti-HAV prevalence. Areas with a very high endemicity of infection primarily consist of less developed and developing nations of Asia, Africa, South and Central America, the Pacific Islands, and certain population within the United States. In these countries and within ethnically defined populations in the United States, the prevalence of HAV infection in adults reaches 90% or higher. Almost all children become infected by the age of 10 yrs (Dienstag. et al. 1978:328-340; Arankalle et al. 1995:447-450). As infections in early childhood are either asymptomatic or mild, hepatitis A may not appear as a clinical problem. However, in higher socioeconomic status population exposure to HAV is delayed up to adolescents/adult age at which infection with HAV can be clinically severe (Tapia-Conyer et al. 1999:825-829; Ferson et al. 1990:631-636; CDC Report 1990:1-26; Arankalle et al. 2001:293-302).

In more developed countries in Europe and Asia the endemicity of HAV infection is intermediate to low and the prevalence of anti-HAV varies widely (Frosner 1979:63-69; Hadler 1991:14-20). HAV circulation is reduced due to improved hygienic conditions and socioeconomic status. The incidence of symptomatic hepatitis A is higher in adolescents and young adults. Frequent outbreaks of hepatitis A are recognized as serious public health problems (Xu et al. 1992:s67-s68).

In low endemicity areas such as Western Europe and the United States, rare incidence of hepatitis A results in the accumulation of non-immune populations. Susceptible groups include childcare providers, hospital workers or family members with direct patient contact, travelers to endemic areas, drug abusers and homosexuals. In isolated areas like Greenland in the south pacific, epidemics of hepatitis A often result in infection of all the members of community followed by the disappearance of the infection due to hepatitis A immune population in these region (Melnick 1995:s2-s8).

According to recent report on hepatitis A Japan, Australia, New Zealand, Canada, the United States and most European countries have low anti HAV rates while anti HAV rates in most Latin American, Asian and Middle Eastern countries are declining. In Africa, no significant decline in anti HAV prevalence is noted (Jacobsen and Koopman 2004:1005-1022).

Main route of transmission of HAV is fecal oral route. It has been reported to be associated mainly with contaminated food and water. Outbreaks associated with consumption of mussels, clams, contaminated lettuce, ice slush beverages, raw oysters, frozen strawberries, blueberries, raspberries, green onions and other salad items have
been reported (Bosch et al. 2001: 61-65; Calder et al. 2003:745-751; Dentiger et al. 2001:1273-1276, Desenclos et al. 1991:1268-1272, Dienstag et al. 1976: 561-564, Hutin et al. 1999:595-602; Lowry et al. 1986:155-164; Ramsay and Upton 1989:43-44; Rosenblum et al. 1990:1075-1079). The global movement of food items those are not treated for viral inactivation may be a major cause of outbreaks in developed countries in the future. Waterborne outbreaks of hepatitis A appear to be less common than transmission by food. Inadequate sewage treatment is the main reason behind most waterborne outbreaks, which generally involved contaminated groundwater obtained from wells or rivers (Bloch et al. 1990:428-430; De Serres et al. 1999:37-43). The most commonly identified risk factors are personal contact with a hepatitis A patient, association with a day care center, foreign travel linkages with an outbreak and injections of drugs (Shapiro et al. 1992:s59-s62). Blood borne transmission has been considered to be rare (Noble et al. 1984:2711-2715). However, several outbreaks of hepatitis A have been reported among hemophiliacs receiving organic solvent and detergent treated factor VIII (Mannucci et al. 1994:1-7). Transmission among homosexual men has been documented. Whether this occurs through sexual contact or simply by nonsexual intimate contact is not known (CDC report. 1992:161-164; Corey and Holmes 1980:435-438).

2.1.13 Prevention and control

Passive immunization through the administration of immune globulin (IG) has been documented for preventing HAV infection (Havens and Paul 1945:270-272; Hall et al. 1977:72-75). A freeze-dried international reference preparation established by the World Health Organization is known to contain anti-HAV at a protective level (1:500 reciprocal end point titre on RIA) and has been assigned a unitage of 100 lU/ml (Gerety et al. 1983:411-416). Administration of standard anti-HAV IgG either prior to or within 14 days of exposure to HAV is effective in preventing clinical hepatitis A. Current pre-exposure recommendations call for the intramuscular administration of 0.02 ml/kg body weight of IG for short term and 0.06 ml/kg for long term (>3 months) travel to hepatitis A endemic regions. Post exposure prophylaxis demands 0.02 ml/kg body weight IG. Low level neutralizing antibody has been found to persist for at least 55 days in the recipients sera evaluated by a sensitive neutralization assay (Ambrosch et al. 1991:98-100).

Adaptation of HAV to grow in cell culture has allowed the development of inactivated and live attenuated hepatitis A vaccines. Inactivated hepatitis A vaccines have been produced using cell culture derived HAV that has been formalin inactivated in a manner similar to that of polio vaccine (Siegl and Lemon 1990:75-92). This vaccine
has proven to be highly immunogenic and achieved 100% seroconversion rates in children and adults in one, two or three dose schedule (Andre et al. 1992:s160-s168; CDC Report 1996:1-30; Lino et al. 1992:323-328). Antibody following immunization has been shown to persist for at least 12 years as well as studies employing mathematical models predict that following primary vaccination, antibodies will persist for at least 25 years (Van Damme and Van Herck 2007:79-84). Inactivated hepatitis A vaccines have been licensed in most developed and developing countries (Siegl et al. 1990:75-92; Andre 1995:s33-s39; Goilav et al. 1995:287-292; Wiens et al. 1996:235-241). Attenuated vaccine reported from China has been shown to be safe, immunogenic and efficacious (Mao et al. 1989:621-624; Mao et al. 1997:944-947). However, requirement of high inoculum is the main disadvantage of attenuated vaccine. Although hepatitis A vaccines are highly immunogenic two cases of vaccine failure have been reported (Taliani et al. 2003: 4505-4506).

Other approach attempted for preparation of hepatitis A vaccines include live viral vector vaccine: Recombinant vaccinia virus in which genes of HAV encoding VP0, VP3 and the amino portion of VP1 were inserted showed protection against a challenge dose of HAV in monkeys. (Karayiannis et al. 1991:2167-2172; Stapleton et al.1993:1080-1085). However, administration of live vaccinia virus in immunocompromised persons was considered to be potentially hazardous (Cooney et al. 1991:567-571).

Empty capsid particles of HAV without RNA were found to elicit neutralizing antibodies. However, low yield of capsid polyprotein expressed by recombinant vaccinia virus limits its use (Winokur et al. 1991:5029-5036).

The genes that code for the HAV structural peptides (VP0, VP1, VP3) could be transferred into a suitable expression vector, such as E. coli. However, these fusion proteins were unable to elicit good immune response in rabbits (Ostermayr et al. 1987:3645-3647; Gauss-Muller et al. 1990:277-283; Powdrill and Johnston 1991:2686-2690). Because of the complex nature of the neutralizing epitope on the virus, peptide and recombinant vaccines have not been commercially successful.

The Advisory Committee on Immunization Practices issued guidelines for hepatitis A prevention, recommended routine vaccination of travelers to countries with high or intermediate endemicity of infection and of children who were >2 years of age and living in low endemic areas (Anonymous 1996:1-30). In addition vaccination to high-risk groups that include homosexual men, injection drug users, persons with chronic liver disease, and workers with an occupational risk of infection has also been recommended (Gardner et al. 1996:35-40). Different populations have different goals. Governments of each country need to design their immunization policy based on seroprevalence pattern and financial constrains. The seropositivity rates in different
parts of the world vary from 2%-100% (Bottiger et al. 1997:99-102; Bowden et al. 1994:372-373; Bernal et al. 1996:230-234; Darwich et al. 1996:554-558; Morales et al. 1992:194-196). The rise in infection rates due to changing epidemiology and presence of large pool of susceptible individuals will have profound impact on magnitude and severity of the disease (Kanda et al. 2002; 517-522). The cost of HAV infection was calculated to be $332 million to $580 million annually in the United States (Berge et al. 2000:469-473). From the economic perspective, hepatitis A is a costly burden to society that could be mitigated by universal childhood vaccination in areas of high endemicity as a method of disease control. Although universal vaccination against hepatitis A is not recommended due to its cost, selective vaccination of population at high risk based on prior screening for anti-HAV has been suggested as the rational and cost effective approach.

2.2 Studies on Molecular epidemiology

2.2.1 Global status

Weitz & Siegl (1985:53-67) were the first investigators to differentiate the genomes of eight HAV strains from distinct geographical regions such as Europe, North Africa, Middle and North America, Australia and China by using RNase T1 oligonucleotide mapping. Subsequent to this, a number of investigators reported the nucleotide sequences of different strains of HAV isolated from hepatitis outbreaks of diverse origin (Ticehurst et al. 1983:5885-5889; Barudy et al. 1985:2142-2147; Najarian et al. 1985:2627-2631; Ovchinnikov Lu et al. 1985:1014-1018; Divizia et al. 1986:269-278; Cohen et al. 1987a:50-59). Ticehurst et al. (1983; 5885-5889) used HM175 virus for cDNA cloning that was purified from the livers of marmosets with acute hepatitis. Barudy et al. (1985:2143-2147) reported nucleotide sequence corresponding to two large regions of HAV genome, 3274 bases from 5' end and 1590 bases from 3'end of the genome, terminating in a 15 base poly A tract. The study revealed that HAV has genomic organization similar to that of picornaviruses and suggested that the 5' region of HAV codes for capsid protein and that 3'region codes for RNA polymerase region. In the same period, molecular cloning of five HAV strains adapted to cell culture was reported (Najarian et al. 1985:2627-2631; Linmeyer et al. 1985:247-255; Venuti et al. 1985:579-588; Ovchinnikov Lu et al. 1985:1014-1018; Paul et al. 1987:153-171).

Cohen et al. (1987:50-59) reported the complete nucleotide sequence of wild type HAV HM175 strain. This data was utilized for comparison of sequences with other picornaviruses, cell culture adapted HAV strains and human and non-human strains of HAV using cDNA-RNA hybridization (Cohen et al. 1987:50-59; Lemon et al. 1987:735-742). The nucleotide sequence data obtained in these studies, revealed lack of
homology with other picornaviruses and variations among HAV strains. In 1989, comparison between human and simian HAV strains showed that VP1 is most variable and 5'$NCR is most conserved region in genome (Brown et al. 1989:4932-4937).

Jansen et al. (1990:2867-2871) developed specific and sensitive technique-immunoaffinity linked nucleic acid amplification system (antigen capture PCR) for the detection of viruses in clinical specimens. Thirty-six strains of HAV from clinical specimens were partially amplified using antigen capture PCR, for both VP3 and VP1/2A regions of genome, encoding maximum and minimum conserved domains respectively. Identity at nucleotide level was observed among the amplified regions of the clinical isolates from same geographic region, indicating circulation of one virus strain. Also epidemiological link was established between two geographic regions that is Kansas (USA) and Germany, an identification of similar sequence in virus from the two regions. On the contrary there were two distinct strains of HAV in circulation identified in 1982-83 from two locations of a continent i.e. north and south Europe. Although serologically similar, most of the human HAV strains showed genetic divergence among themselves varying from 1-24% altogether in VP3 and VP1/2A junction region. This divergence could not be related to geographic site, disease expression, or to risk factors in contracting hepatitis A, and hence necessity of further investigations recommended (Jansen et al. 1990:2867-2871).

Then N-terminus of VP1 and VP1-2A junction regions were utilized to study the variability (Robertson et al. 1991:286-292; Robertson et al. 1992:1365-1377). This work was carried out in accordance with that of the poliovirus type I genome, which has been classified using VP1/2A junction region (Rico- Hesse et al. 1987:311-322).

In order to establish the epidemiologic link between the clinical isolates of HAV from different geographic regions, partial VP1 region (247bp) was analyzed from 22 HAV isolates from distinct geographical locations (Robertson et al. 1991:286-292). Three phylogenetic groups were identified differing from each other by approximately 20%, 10% and 5%. In spite of the genetic variation, the derived amino acid sequences revealed limited changes in the VP1 region of the HAV isolates taken for study, except for a couple of isolates. The amino acid changes caused by genetic variations did not result in detectable serologic differences.

Robertson et al. (1992:1365-1377) for the first time carried out genotyping of 152 wild type and cell culture adapted strains of HAV on the basis of partial nucleotide sequence of VP1/2A junction region (168bp). This study showed that HAV strains could be divided into seven genotypes (I-VII). The strains included for the study were representatives of all regions of the world. Majority of strains were from human cases of hepatitis A except a few having simian origin. These seven genotypes differed from one
another by having nucleotide variations of >15% at genotype level and <15% but >7.5% at subgenotype level. Four genotypes (I, II, III and VII) included strains of HAV associated with human diseases whereas remaining genotypes i.e. IV, V and VI included simian HAV strains. The extent of nucleotide variation within the 168bp putative region in these 152 strains revealed measure of relatedness of human strains to each other and to simian HAV strains.

Genetic variability was studied in VP1-N terminal region of the HAV strains isolated during the shellfish outbreak in France (Costa Mattioli et al. 2001:233-240). Analysis of the strains isolated from clinical samples indicated cocirculation of subgenotypes IA, IB and IIIA for the first time in a single outbreak in France. Similarly cocirculation of multiple genotypes or subgenotypes has been reported from different parts of the world (Taylor et al. 1997:273-279; Villar et al. 2004:1779-1789; CDC unpublished data, Nainan et al. 2005:957-963; Hussain et al. 2005:16-24, Chitambar et al. 2007:85-93, Endo et al. 2007:116-127). Dual infections of a single individual with different subgenotypes (de Paula et al. 2003:223-228; Chitambar et al. 2007:85-93; Coppola et al. 2007:73-77) and two recombinant strains namely 9F94 and FG with crossover point within VP1 capsid and 3D region respectively have also been reported (Costa Mattioli et al. 2003:51-59; Endo et al. 2007: 8-17).

The analysis of complete VP1 gene sequence allowed to estimate mode of evolution of HAV and determination of multiple genotypes circulating in outbreaks and also to document the emergence of novel variant with a deletion of 15 amino acids in immunodominant region of VP1, which could not be detected using either VP1 amino terminus or VP1/2A junction region (Costa Mattioli et al. 2002:9516-9525).

Sanchez et al. (2002:4148-4155) reported molecular characterization of HAV isolates from a transcontinental shellfish borne outbreak in Spain. Sequence analysis of VP1/2A junction region showed that HAV isolates belonged to genotype IB. Antigenic variability was observed in these isolates at VP3 and VP1 capsid proteins using MAb K3-4C8, K2-4F2 that showed 81% and 35% reductions in recognition by ELISA compared with the nonmutated HAV control, thus correlating with non-synonymous mutations that appeared in epitope determining regions of VP1 and VP3 respectively. Existence of antigenic variants and genetic variability observed in the region encoding capsid protein pointed towards the inability of genotyping method which placed all the clinical isolates in genotype IB. Reclassification resulted in to only three human (I-III) and three simian (IV-VI) genotypes due to full length genome sequencing of SLF88 and CF-53 strains of genotype VII and II respectively (Ching et al. 2002:53-60; Lu et al. 2004:2943-2952). Requirement of more nucleic acid fragments for comparison was also recommended due to limitations of VP1/2A junction region during classification of DL3.
strain of China (Guo-Dong et al. 2003:499-504). Currently the majority of molecular epidemiologic studies conducted at CDC use a 390bp fragment from the VP1-2B region (Nainan et al. 2005:957-963). However, the possibilities of antigenic variants and recombination events occurring in other genomic regions are ignored making full-length genome sequencing essential.

Over the years various genomic regions were analyzed to find the genomic changes that may lead to severity of the disease (Fujiwara 2001:112-19; Ching et al. 2002:53-60; Fujiwara et al 2007;871-877). However, further studies are required on this issue.

In the past two decades, the data on entire/nearly entire nucleotide sequences of a total of 33 human and 1 simian genome have been accumulated in GenBank databases for different genotypes of HAV. In human HAV isolates genotype I strains are in majority and very few strains of genotype II and III. Among simian genotypes (IV-VI), complete genome is known only for genotype V (strain AGM27).

2.3 Studies on hepatitis A in India
2.3.1 Epidemiology

Hepatitis A is endemic in India. Hepatitis A is mainly a disease of pediatric population however; cases are being identified in adults (Chadha et al. 2003:11-15; Arankalle et al. 2006:760-769). Hepatitis A usually occurred in sporadic form and epidemics of disease were rarely reported (Joshi et al. 1985:96-101; Chitambar et al. 1996:781-83; Arankalle et al. 2006:760-769). Seroepidemiological surveillance conducted in age stratified lower middle socioeconomic status (LMSS) urban populations indicate widespread HAV infection affecting >90% children aged less than 10 years and generation of anti-HAV antibodies in majority through subclinical infections (Tandon et al. 1984:67-73; Arankalle et al. 1995:447-450). Subsequent serosurvey has noted a decrease in anti-HAV positivity in western Indian children aged 5-10 years (Chitambar et al. 1999:273-276). Recent assessment of hepatitis A epidemiology among age stratified urban high socioeconomic status (HSS), LMSS and rural LMSS populations from western India indicates a clear shift from high to intermediate endemcity only in HSS category and suggests the possibility of hepatitis A outbreaks in HSS populations (Dhawan et al. 1998:16-18; Arankalle et al. 2001:293-303). This change in epidemiology is similar to other parts of the world where in anti-HAV prevalence rate has declined as a result of improvement in the socioeconomical and hygienic conditions (Jacobsen and Koopman 2004:1005-1022).

Analysis of 1612 subjects representing 5 cities from different parts of India (Kolkata, Cochin, Indore, Jaipur and Patna) showed that anti HAV positivity varied from
26.2 to 85.3% (Mall et al. 2001:132-135). In Indian adult chronic hepatitis patients over 97% anti HAV positivity was observed (Acharya et al. 2003:822-827). Gadgil et al. also have shown increase in seronegativity in HSS population and possibility of horizontal transmission of HAV due to voluntary blood donors with subclinical infections (Gadgil et al. 2007:1-4).

According to studies carried out in northwest, north, central and western India on hospitalized cases of acute viral hepatitis the proportion of children suffering due to HAV infection varies from 46%-82%. This proportion in adults is 3.5% -40% (Ghuman and Prabhakar 1995:227-230; Thapa et al.1995:328-329; Kar et al. 1997:43-45). In community studies performed in northwest and central India hepatitis A virus was found to be responsible for 32.1% and 52.6% cases of acute viral hepatitis respectively (Singh et al. 1997:463-468; Singh et al. 1998:105-109). Outbreaks of hepatitis A in day care center and in rural area involving children have been reported (Chitambar et al. 1996:781-783; Chadha et al. unpublished data). Accumulation of susceptible population to hepatitis A resulting in increased incidence of acute hepatitis A among children (from 106%-22%) as well as in adults (from 3.4 % to 12.3%) has been shown during 5 years study period from north India (Hussain et al 2006:689-693). Studies carried out in north and western India highlight HAV as the major causative agent for fulminant hepatic failure in children (Arora et al. 1996:215-221; Bendre et al. 1999:1107-1112). Water borne outbreaks of hepatitis A have been reported from the state of Kerala, southern India (Joshi et al. 1985:96-101; Arankalle et al. 2006:760-769). Vertical transmission of hepatitis A in two neonates has been also described (Renge et al. 2002:535-536).

2.3.2 Diagnostics

Development of Indigenous diagnostic test for detection of recent infection of hepatitis A was reported in 1994 (Chitambar et al. 1994:243-251). Highly specific and sensitive ELISA tests were developed for screening of anti HAV IgG antibodies for pre and post vaccination screening (Chitambar et al. 1996:163-165). Use of filter paper disks has been reported as a suitable choice for hepatitis A surveillance in pre and post immunization programs (Chitambar and Chadha 2000:165-167). Subsequently, urine has been shown to be comparable to serum for diagnosis of recent and past infection with hepatitis A (Joshi et al. 2002:840-845).

2.3.3 Molecular epidemiology

Limited studies have been reported on human HAV genotypes circulating in India (Khanna et al. 1992:118-124; Hussain et al. 2005:16-24; Arankalle et al. 2006:760-769; Chitambar et al. 2007:85-93). Co-circulation of genotype IIIA and IIA was observed
in northern region whereas genotype IIIA and IB were detected in western region (Hussain et al. 2005:16-24; Chitambar et al. 2007:85-93).

2.3.4 Immunology

Hussain et al. reported persistently higher viral load in severe acute hepatitis cases possibly resulting from diminished cellular immunity and hemolysis (Hussain et al. 2006:689-693). Involvement of interferon gamma in the elimination of HAV has been suggested due to presence of universally released interferon gamma in all acute resolving patients (Tripathy et al. 2005:283-284).

2.3.5 Vaccination

In India, selective vaccination of population at high risk especially from high socioeconomic strata with sero-negative status has been recommended (Arankalle et al. 2001:293-303; Hussain et al. 2006:689-693). Based on a study of 499 children aged 3 days – 6yr, appropriate age for vaccination was reported to be 9-10 months (Chadha et al. 1999:11-15). Commercially available hepatitis A vaccine (HAVRIX) has been shown to induce anti HAV antibodies in 100% Indian children. (Chadha and Arankalle, NIV Annual report 2005-2206). Hepatitis A vaccine containing live attenuated H2 strain has been evaluated in Indian children recently. The obvious advantages of a single dose schedule, possible life long immunity, good tolerance and relatively lower costs make it an attractive option (Bhave et al. 2006:983-987).