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4.0 Materials and Methods

4.1 Virus source

Stool and serum specimens were collected from acute hepatitis patients for studies on molecular epidemiology of HAV strains from Pune, western India in the years 1992-2004 (Chitambar et al. 2006:1011-1014; Chitambar et al. 2007:85-93). Indigenously developed anti HAV IgM capture ELISA and anti HEV IgM ELISA were used for the serological diagnosis (Chitambar et al. 1994:243-251; Tsarev et al. 1993:369-378) Commercially available ELISA kit was used for detection of HBsAg (Surase B-96, General Biologicals Corp., Taiwan). Three patients investigated in this study were positive only for anti HAV IgM indicating acute hepatitis A infection. Two patients CP and PN recovered completely within 4 weeks of illness without any aid of hospitalization.

The third patient, a 17-year-old male with a 25-day history of febrile illness was admitted to the Sassoon General Hospital (SGH) from Pune in June, 2003. The patient was a resident of a village from district Satara, western India. Prior to admission, in the 2nd week of illness patient complained of nausea, vomiting, tingling of both hands and feet. The patient was diagnosed to have jaundice and was treated locally with ayurvedic medicine. In the following week of illness he developed progressive weakness initially in both lower limbs and subsequently in upper limbs. Simultaneously respiratory distress developed. He was admitted to Satara Civil Hospital and was on ventilator for a period of one week. On account of ventilatory failure, he was brought to SGH, Pune. Physical examination on arrival at hospital revealed blood pressure (BP) 120/80, mild icterus, flaccid quadriplegia, and weakness of neck muscle, generalized hypotonia and areflexia. Liver function tests showed abnormalities with total bilirubin 2mg% (direct 1.2mg% and indirect 0.8mg%). SGPT 95IU/ml and alkaline phosphatase 148IU/L. Prothrombin time was 17/15. Examination of central nervous system (CNS) indicated ascending paralysis, normal sensory system, no cranial nerve palsy, no cerebellar signs, normal higher mental functions and single breath count 8. Power was grade III in left upper limb and grade I in right upper limb. Lower limbs proximally and distally had power of grade I and III, respectively. Electrodiagnostic examination revealed severe motor neuropathy of axonal degeneration type. The patient was classified as a case of Guillain-Barre Syndrome subtype acute motor axonal neuropathy (AMAN) (Chitambar et al. 2006:1011-1014). The patient was treated with ventilatory support, fluids, nutrition, chest and limb physiotherapy and antibiotics. Liver function tests improved completely in a weeks time after admission to SGH. Neurologically he improved over three weeks time and was weaned off ventilator. After 3 months, power in both upper and lower limbs
returned to grade V proximally and distally. The patient was asymptomatic and fully ambulatory.

A CSF sample collected on 29th day after onset and urine samples collected on post onset days 34, 55, 77, 92, and 126 were positive for anti HAV antibodies. Serum anti HAV IgM declined gradually (1:256,000 to 1:64,000), anti HAV IgA persisted in a cyclic manner (1:128000 → 1:32000 → 1:128000) and anti-HAV IgG persisted at remarkably high level (1:1024000 → 1:16384000 → 1:8192000) through out the period of study (~19 weeks).

4.2 Preparation of 10% (w/v) stool suspension

The fecal samples collected from the patients were processed to prepare 10% (w/v) suspensions in sterilized phosphate buffered saline (PBS) (0.05M, pH 7.2) by sonication (Vibracell, Sonics) at 70% duty cycle for 3 minutes and clarified by centrifugation at 2000g for 20min (Beckman, USA) at 4°C. The supernatants were stored at -70°C until tested.

4.3 Immune Electron Microscopy (IEM)

**Principle:** Electron microscopy is a useful tool to examine virus morphology. A negative staining technique uses heavy metal salts to enhance the contrast between the background and the virion’s image. This is a very simple and direct technique.

The supernatants of the stool suspensions were subjected to IEM as described earlier (Kapikian et. al 1972:1075-1081). Briefly, 900μl supernatant of 10% W/V clarified stool suspension was allowed to react with 100μl of acute phase serum at 1:20 dilution of a hepatitis A patient at 37°C for 1hr. The antigen antibody mixture was centrifuged at 25,000 X g for 1hr and was observed in a Transmission electron microscope at 100kv after negative staining with 2%(W/V) phosphotungstic acid (pH6.0).

4.4 Antigen detection by blocking ELISA

**Principle:** Human anti HAV IgG antibody coated on the wells captures HAV antigen (if present in the fecal specimens) added to the wells in duplicate. To check the specificity of the captured antigen, known anti HAV negative and positive sera were added in identified wells. The captured antigen reacts with anti-HAV-IgG HRP conjugate in the presence of anti-HAV negative serum and therefore presence of color indicates HAV antigen positivity. The well with addition of known anti-HAV IgG positive serum binds to the captured antigen (Figure 4). HAV is thus blocked by the specific antibody and is not available for binding to anti-HAV HRP conjugate. Therefore, absence of color
after addition of substrate indicates the specificity of the HAV antigen in the fecal sample. Thus inclusion of known negative and known positive serum samples in the test helps detection of HAV antigen and its specificity. In case of specimen negative for HAV antigen no color is expected in both wells.

Flow Diagram

Coating of Anti HAV IgG on solid phase

Addition of fecal specimen from Hepatitis-A patient

Addition of known anti HAV Positive serum

Addition of known anti HAV Negative serum

Addition of anti HAV IgG HRP conjugate

Addition of substrate

No Color

Development of orange color

Y = Anti HAV antibody (coating)
Y = Antibody from Positive serum
Y = Antibody from Negative serum
Y = HAV antigen from fecal specimen
A = Anti HAV IgG HRP conjugate

Test principle of antigen detection by blocking ELISA
Protocol

- 120 \mu l of human anti HAV-IgG at 1:300 dilution suspended in 0.05 M carbonate bicarbonate buffer, pH 9.5 (Appendix 8.1.1) was added in polystyrene immuno wells (Nunc, maxisorb, Denmark). Wells were incubated overnight at RT (25°C). Unbound material was aspirated from the wells. The wells were post coated with 1% Bovine Serum Albumin (BSA) in PBS (0.05M, pH 7.4). The modules were incubated at RT for 30 min.
- The wells were washed (x6) with wash buffer (Appendix 8.1.3) and dried by tapping on tissue paper.
- 100 \mu l test specimens and known HAV antigen positive and negative fecal specimens were added in duplicate in identified wells and incubated at 37°C for 1 hr.
- The wells were washed (x6) with wash buffer (Appendix 8.1.3) and dried by tapping on tissue paper.
- After addition of 50 \mu l PBS into each well, 50\mu l each of known anti-HAV negative and positive serum samples were added on each test specimens and controls and mixed carefully into the bottom of the identified wells. The wells were incubated at 37°C for 1 hr.
- The wells were washed (x6) with wash buffer and dried by tapping on tissue paper.
- 100 \mu l of working anti-HAV IgG HRP conjugate was added into each well (Appendix 8.1.5) and incubated at 37°C for 1 hour.
- The wells were washed (x6) with wash buffer and dried by tapping on tissue paper.
- 200 \mu l working substrate solution (Appendix 8.1.6) was added to each well along with blank and incubated in dark at RT for 10 minutes. Control wells were checked for development of yellow orange color.
- 100 \mu l sulphuric acid (4N) (Appendix 8.1.7) was added into each well to stop the reaction. The optical density was read at 492 nm in ELISA Plate Reader (Multiskan plus, Labsystems).

Calculations for Cut Off value

\[
\text{Optical density of test antigen with Negative control serum (N)}\frac{1}{\text{Optical density of test antigen with Positive control serum (P)}}
\]

Interpretation of Results

Specimens with N/P ratio >2 are considered positive.
Specimens with N/P ratio <2 are considered negative.
4.5 Tissue culture isolation
Following Cell lines were used in attempts for HAV isolation

- Buffalo Green Monkey Kidney (BGMK) cell line at passage level 101 procured from National Institute of Health, USA, certified free of mycoplasma and Simian Virus-5.
- Vero E6 cell line procured from American Type Culture Collection (ATCC) at passage level 23 certified free of mycoplasma and Simian Virus-5.
- BSC1 cell line procured from National Center for Cell Sciences, Pune, India at passage level 80, certified free of mycoplasma and Simian Virus-5.

4.5.1 Cultivation, Subculture and maintenance
Confluent cell monolayers maintained in T-25 cm$^2$ flask (Corning Inc. USA) containing MEM (Appendix 8.2.1) with 2% FCS were trypsinized using 4 ml TPVG (Appendix 8.2.2). The TPVG was discarded after 2-3 min when the monolayer became opaque. The flasks were incubated at 37°C till the cells detached from the flask surface. The cells were suspended in 15 ml MEM containing 10% Fetal Calf Serum (FCS-GIBCO, Invitrogen, USA) and distributed in three T-25 cm$^2$ flasks. Thus a split ratio of 1:3 was maintained. All the flasks were incubated at 37°C with 5% CO$_2$. Confluent monolayers appeared after 72-96hrs. The medium was changed at weekly intervals. The cells were subcultured at weekly interval.

4.5.2 Processing of stool specimens for inoculation in cell cultures
Fecal specimens of HAV strains CP-IND, PN-IND and GBS-IND were subjected to ultra centrifugation at 25,000g at 4°C for 2 hrs. The pellets obtained were suspended in 500μl of sterile PBS (Appendix 8.1.2) containing antibiotics (penicillin 200μg/ml, streptomycin 200 units/ml), kept at RT for 30min and inoculated on monolayer of normal cells (negative for mycoplasma and HAV RNA by PCR) in duplicate. At each passage level, normal cell cultures were included as 'Controls'. One set of infected cultures including controls was maintained and harvested after 4 weeks at each passage level. Passaging of other set of cultures was carried out weekly upto passage 10. Cell lysates were suspended in PBS (1 ml for T-25cm$^2$ flask) and tested for HAV antigen and RNA by ELISA and RT-PCR respectively.

4.6 Antigen detection ELISA
Principle: Anti HAV IgG antibody coated on the wells captures HAV if present in the test sample. Anti-HAV HRP conjugate added to the wells as probe binds to HAV. Appearance of colour after addition of substrate indicates the positivity of the sample for
HAV. If the sample is negative for HAV, the HRP conjugate added to the wells remains unbound and is washed away. Hence no colour develops on addition of substrate.

**Protocol**

- 120 µl of human anti HAV-IgG at 1:300 dilution suspended in 0.05 M carbonate bicarbonate buffer, pH 9.5 (Appendix 8.1.1) was added in polystyrene immuno wells (Nunc, maxisorb, Denmark). Wells were incubated overnight at RT (25°C). Unbound material was aspirated from the wells. The wells were post coated with 1% BSA in PBS (0.05M, pH 7.4) and were incubated at RT for 30 min.
- The wells were washed (x6) with wash buffer (Appendix 8.1.3) and dried by tapping on tissue paper.
- 100 µl of test sample that included normal and HAV infected cell lysates and known HAV negative control (duplicate) and positive control were added in identified wells and incubated at 37°C for 1hr.
- The wells were washed (x6) with wash buffer (Appendix 8.1.3) and dried by tapping on tissue paper.
- 100 µl of working anti-HAV IgG HRP conjugate was added into each well (Appendix 8.1.5) and incubated at 37°C for 1 hour.
- The wells were washed (x6) with wash buffer and dried by tapping on tissue paper.
- 200 µl working substrate solution (Appendix 8.1.6) was added to each well along with blank and incubated in dark at RT for 10 minutes. Control wells were checked for development of yellow orange color.
- 100 µl sulphuric acid (4N) (Appendix 8.1.7) was added into each well to stop the reaction. The optical density was read at 492 nm in ELISA Plate Reader (Multiskan plus, Labsystems).

**Calculations for Cut Off value**

(i) Calculation of Mean Negative Control Absorbance:
   \[ NC = \frac{\text{Total Absorbance of 2 Negative Control wells}}{2} \]

(ii) Calculation of the Cut Off Absorbance value:
   \[ \text{Cut Off value} = NC \times 2.1 \]

**Interpretation of Results**

- Specimens with absorbance values greater than the cut off value are considered positive.
- Specimens with absorbance values less than the cut off value are considered negative.
4.7 Designing of Primers

In most PCR applications, it is the sequence and the concentration of the primers that determine the overall assay success. Several primer pairs were designed to amplify and sequence DNA in both the directions by using the nucleotide sequences of the strains, HMH (AY644337) from Germany and Nor21 (AJ299464) from Norway of genotype IIIA. Primers were also designed and synthesized by using nucleotide sequences obtained in this study (Table 1). Primers were designed manually by following the guidelines described in PCR applications manual (2nd edition, Roche molecular biochemicals). General characteristics of the primers were as follows.

- Length 18-27 bases
- Absence of internal secondary structure
- G/C Content 40-60%
- Balanced distribution of G/C and A/T rich domains
- Forward & Reverse primers were not complementary to each other at the 3' ends (to avoid primer dimer formation)
- 5' end and 3' end within a primer were not complementary to each other
- Melting temperature (Tm) that allows annealing at 50-55°C
- Approximately similar Tm for both primers (Pedro's Biomolecular Research Tool)

Primers were tested for their specificity by using NCBI BLAST program with three different options- virus, homosapians and none and those indicating homology only with hepatitis A virus and not with homosapians and other species were selected. List of primers used in the study is included in "Results section 5.2". In addition to this 5'RACE CDS primer A, SMART IIA oligonucleotide, 3' RACE CDS primer A, 10X universal primer mix (UPM) and nested universal primer (NUPM) from SMART RACE kit (BD Clonetech) were used for amplification of extreme 5' and 3' ends.

4.8 RNA extraction

Principle: Successful RT-PCR requires a high quality, intact RNA template. TRIZOL LS reagent (Invitrogen, Life Technologies, USA) was used for RNA extraction. It is a ready to use reagent for the isolation of total RNA from liquid samples. It is a mono phasic solution of phenol and guanidium isothiocynate. During sample homogenization or lysis, TRIZOL LS reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the
RNA is recovered by precipitation with isopropyl alcohol and pelleted. Pelleted RNA is washed with 70% ethanol.

Procedure

- Viral RNA was extracted from 250 μl of 10% fecal suspension using TRIZOL LS reagent (Invitrogen, Life Technologies, USA) according to manufacturer’s instructions.
- 250μl aliquot of the 10% fecal suspension stored at −70°C was thawed on ice.
- 750 μl TRIZOL was added to the sample, mixed thoroughly by inverting the tube for 40-60 times and incubated at RT for 20 minutes.
- 200 μl of chloroform was added, mixed vigorously for 20 seconds and incubated at RT for 15 minutes.
- The mixture was centrifuged at 12,000 X g (Plasto craft, India) for 5 minutes at +4°C.
- The top aqueous phase was transferred to a newly labeled Eppendorf tube. 500 μl of isopropyl alcohol was added and mixed by inverting for about 50 times. It was then covered with the aluminum foil and incubated at RT for 30 minutes.
- The tube was centrifuged at 12,000 X g for 20 minutes at +4°C and the supernatant was removed.
- The pellet was washed with 1ml cold ethanol (80%). The ethanol was poured out after centrifugation at 7,500 X g at +4°C for 20 minutes.
- The pellet was again centrifuged at 7,500 X g for 5 minutes at +4°C and traces of ethanol were completely removed.
- The RNA pellet was dried in an incubator (37°C) for 10 minutes and used for RT-PCR.

4.9 Reverse Transcription

Principle: Superscript™ II RNase H" Reverse transcriptase (Invitrogen, Life Technologies, USA) was used for cDNA synthesis. It is purified to near homogeneity from E.coli containing the polymerase gene of Moloney Murine Leukemia virus. It has been genetically modified to remove Ribonuclease H (RNase H) activity. RNase H is an endoribonuclease that specifically degrades the RNA strand of an RNA-DNA hybrid to produce single stranded DNA and thus eliminates the potential source of PCR inhibition. However, in presence of RNase H chances of self-annealing or loop formations are
more especially during long cDNA synthesis, therefore enzymes with RNase H activity was used.

**Procedure:** Dried RNA was suspended in 12 μl mixture containing 10 μl nuclease free distilled water (Bangalore Genei, Bangalore, India), 1 μl reverse gene specific primer (M6907, 10μM) and 1 μl dNTP mixture (Deoxynucleotide Triphosphate, Promega, USA, 10mM). The tube was flicked, spun down and incubated at 65°C for 5 minutes followed by quick chill on ice. After brief centrifugation 5X first strand buffer (4μl), 0.1M Dithiothreitol (2μl) and RNAsin (40 units, Promega, USA) were added in the tube. Contents of the tube mixed gently, incubated at 42°C for 2min and then 1μl (200 units) of Superscript™ II RNase H Reverse transcriptase (Invitrogen, Life Technologies, USA) was added. After mixing properly tubes were incubated at 42°C for 50min., heating at 70°C for 15min was carried out to stop the reaction. The tube was immediately kept on ice for 2-3 minutes followed by quick spin. This cDNA was used for PCR amplification. To remove RNA complementary to the cDNA 1μl (2 units) of *E. coli* RNase H was added and incubated the DNA at 37°C for 20 min.

**4.10 Polymerase Chain Reaction**

**Principle:** PCR is an *in vitro* method for enzymatic synthesis of defined sequences of DNA. The reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalyzed by a heat stable DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The ends of the fragment are defined by the 5' ends of the primers.

For DNA amplification BD Advantage 2 PCR kit (BD Clonetech, USA) was used. It is comprised of BD TITANIUM™ Taq DNA polymerase- a nuclease deficient N terminal deletion of Taq DNA polymerase plus BD TaqStart™ antibody to provide automatic hot start PCR and a minor amount of proofreading polymerase.

**Procedure:** The first round PCR was performed by using 2μl of cDNA, 0.2μM of each primer, 0.2μM dNTP mixture, 1μl (50X) BD Advantage 2 polymerase mixture and 5 μl of 10X Advantage 2 PCR buffer containing 40mM of Tricine-KOH pH8.7, 15mM of KOAc, 3.5mM of Mg (OAc)₂, 3.75μg/ml BSA, 0.005% of each of Tween 20 and Nonidet-P40 in 50μl of final reaction mixture. PCR and nested PCR were performed in a 9700 Thermal cycler (Applied Biosystems, CA, USA) using the following conditions: hot start at 94°C for 5 min and 35 cycles of each denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 3 min. Extension time varied between 1 to
4min, depending on the product size. Final extension step was performed at 72°C for
7min. For nested PCR, 45 μl PCR mix was prepared similar to the first round PCR mix.
Internal sets of sense and anti sense primers were used in place of external set of
primers. 5 μl of first round PCR product was added and the amplification was carried out
as described above for the first round PCR using an automated thermal cycler. The
changes were made in annealing temperature according to the Tm of the primers, as
different sets of primers were used to amplify the whole genome.

4.10.1 Amplification of 5'and 3' end

SMART™ RACE cDNA amplification kit (BD Clonetech, USA) was used for the
amplification of extreme 5' and 3' ends of HAV genomes.

Mechanism of SMART cDNA synthesis

First strand synthesis is primed using a modified oligo (dT) primer. After reverse
transcriptase reaches the end of the mRNA template, it adds several dC residues. The
SMART II A oligonucleotide anneals to the tail of the cDNA and serves as an extended
template for PowerScript reverse transcriptase.

Procedure: RNA pellet was dissolved in 3 μl of RNase free distilled water containing 1μl
of each 5'-CDS primer and SMART II A oligo for 5' RACE Ready cDNA. For 3' RACE
Ready cDNA, 4μl of distilled water containing 1 μl of 3'-CDS primer A was added in
RNA pellet. RNA and primer mixture were incubated at 70°C for 2 minutes and then
snap chilled on ice. In each reaction tube 2μl of 5X first strand buffer, 1μl of
dithiothreitol (20mM), 1μl of dNTP mix (10mM) and 1 μl of PowerScript Reverse
Transcriptase were added and the tubes were incubated at 42°C for 1.5hr. First strand
reaction product was diluted with 20μl of Tricine EDTA buffer (10mM Tricine-KOH pH
8.5 and 1mM EDTA) and heated at 72°C to stop the reaction. Using conditions as
mentioned above, PCR and nested PCR were carried out. PCR products were
evaluated by agarose gel electrophoresis.

4.11 Gel electrophoresis

Principle: Gel electrophoresis uses electricity to separate DNA fragments by
size as they migrate through a gel matrix, which is highly purified seaweed. Blue
“tracking “ dye in a loading buffer is mixed with DNA to increase the density of the
mixture, which makes DNA loading easier and DNA migration visible. The phosphate
groups in the DNA backbone carry negatively charged oxygen which gives a DNA
molecule overall negative charge. In an electric current the negatively charged DNA
moves towards the positive pole of the electrophoresis chamber. DNA fragments are separated by size.

The amplified PCR products covering entire genome including 5' and 3' ends were resolved and analyzed using 1.5% agarose gel (Life Technologies, USA) in 1X TAE buffer (Tris Acetate EDTA buffer) (Appendix 8.3.1) containing ethidium bromide (Appendix 8.3.2). 100bp DNA ladder (1μg/μl Fermentas, USA) was used as DNA marker for estimation of size of the PCR products. Bromophenol blue was used as the tracking dye (Appendix 8.3.4). The bands were visualized under UV transilluminator (SYNGENE, UK).

4.12 DNA purification

4.12.1 DNA purification of the PCR Products with single band

PCR products (50μl) with single band were used directly for DNA purification by using Min Elute PCR purification kit (QIAGEN, CA, USA).

Principle: PCR product is mixed with PB solution, which contains chaotrope. In the presence of the chaotrope, the DNA fragment binds selectively to silica membranes of spin columns. The DNA remains bound while a series of rapid “wash and spin” steps removes contaminants like unused primers, ethidium bromide etc. DNA is eluted in elution buffer.

Procedure

- To 50μl of PCR product, 250μl of PB solution was added.
- The mixture was added on the QIAquick column placed on the collection tube and spun at 17,900 X g for 1 minute.
- Flow through was discarded and 750 μl PE buffer was added on the column to wash the DNA. The column was spun for 1 minute.
- Flow through was discarded and column was spun for additional 1 minute to remove PE buffer completely.
- QIAquick column was placed on the new collection tube.
- DNA was eluted in 20μl of elution buffer by spinning the column at 17,900 X g speed for 1 minute.

4.12.2 DNA purification of the PCR products with multiple bands by using QIA quick gel extraction kit method

When multiple bands were observed during agarose gel electrophoresis, the band of the desired size was excised with a sterile blade. The DNA was purified using QIA quick gel extraction kit (QIAGEN, CA, USA).
Principle: A solubilization buffer that contains a chaotropic salt dissolves, the agarose gel slice that contains the PCR product. In the presence of the chaotrope, the DNA fragment binds selectively to silica membranes of spin columns. The DNA remains bound while a series of rapid "wash and spin" steps removes contaminants like unused primers, ethidium bromide, melted agarose etc. DNA is eluted in elution buffer.

Procedure
- To excised DNA fragment from the agarose gel 3 volumes of QG buffer was added to 1 volume of gel (for 100mg of gel slice add 300µl QG buffer).
- The Eppendorf tube was incubated at 50°C until the gel gets completely dissolved and 1 gel volume of isopropanol was added to it and mixed properly.
- The sample was added on the QIAquick column placed on the collection tube and spun at 17,900 X g for 1 minute.
- Flow through was discarded and 500µl of QG buffer was added on the column and centrifuged 17,900 X g for 1 minute.
- Flow through was discarded and 750 µl PE buffer was added on the column and incubated at RT for 2 mins to wash the absorbed DNA. The column was spun for 1 minute.
- Flow through was discarded and column was spun for additional 1 minute to remove PE buffer completely.
- QIAquick column was placed on the new collection tube.
- DNA was eluted in 20 µl of elution buffer by spinning the column at 17,900 X g speed for 1 minute.

4.13 Nucleotide sequencing

Principle: The sequencing method developed by Fred Sanger forms the basis of automated cycle sequencing reactions. It uses chemically altered radiolabeled "dideoxy" bases to terminate newly synthesized fragments at specific bases (Sanger et.al 1977:5463-5467). The 5' carbon of an incoming dNTP is joined to the 3' carbon at the end of the chain. Hydroxyl groups in each position form ester linkages with a central phosphate. In this way the chain elongates. Like a dNTP, a dideoxy dNTP is incorporated into a chain by forming a phosphodiester linkage at its 5' end. However, the dideoxy dNTP lacks 3' hydroxyl group necessary to form the linkage with an incoming nucleotide. So, the addition of a dideoxydNTP halts elongation and incomplete products of various lengths that differ by a single nucleotide are separated based on their size and DNA sequence can be read. Fluorescent dyes are used in place of
radiolabel for labeling of dideoxy dNTPs and laser within automated DNA sequencing machine is used to analyze the DNA fragments produced.

Both the strands of purified PCR products were sequenced using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit on ABI 3130XL genetic analyzer.

**Preparation of cycle sequencing reaction**

➢ Two cycle sequencing reactions (20 μl) were prepared for each PCR product in 0.5 ml thin walled Eppendorf tube by adding 8 μl TRRM (Terminator Ready Reaction Mix), 1-5 μl PCR product (50ng), 0.5μl either sense or antisense primer (10μM) and 7.5 – 10.5 μl of nuclease free distilled water.

➢ For bigger fragments multiple reactions were carried out using internal sequencing primers.

➢ The cycle sequencing was performed in a thermal cycler model 9700 (Applied Biosystem, USA). The cycling conditions were 95°C for 5 minutes followed by 25 cycles of 96°C 10 seconds, 50°C 5 seconds and 60°C 4 minutes.

➢ After cycle sequencing the PCR products were purified by using sodium acetate/ethanol method.

**Sodium acetate/ethanol method**

➢ 80 μl distilled water, 10 μl sodium acetate (3M, PH 4.8) (Appendix 8.4.1) and 250 μl ethanol were added to a 20 μl sequencing reaction.

➢ The contents of the tube were mixed properly and kept on ice for 10 minutes and spun for 7,500 X g for 10 minutes at room temperature.

➢ The supernatant was decanted and the DNA pellet was washed twice with 250 μl ethanol by spinning at 7,500 X g for 10 mins at room temperature.

➢ Ethanol was removed completely by air-drying the tube for 30 mins.

➢ Finally 20 μl Template Suppression Reagent (TSR) was added to the purified DNA pellet, incubated at 94°C for 5 min, chilled on ice, spun briefly and loaded on ABI 3130XL genetic analyzer.

**4.14 Alignment and Phylogenetic analyses of HAV genomes**

The nucleotide sequence data obtained on ABI3130XL genetic analyzer was checked for specificity to HAV by using NCBI BLAST program. DNA sequence was checked with both forward and reverse primers and converted in to FASTA format and aligned using Clustal X version 1.83 (Thompson *et al.* 1997:4876-4882). Clustal X is a Windows interface for the Clustal W multiple sequence alignment program. It provides
an integrated environment for performing multiple sequence and profile alignments and analyzing the results. The sequence alignment is displayed in a window on the screen. A specific coloring scheme has been incorporated allowing researcher to highlight conserved features in the alignment. The pull-down menus at the top of the window allow selecting all the options required for traditional multiple sequence and profile alignment. The alignment file is saved after completion. Nucleotide sequences of 25 HAV strains representing all the genotypes (I-VI) available in Genbank database along with Indian IIA strains from this study were aligned.

A phylogenetic tree is a graphical representation of the evolutionary relationship between taxonomic groups. The alignment file using Clustal X was then converted in Molecular Evolutionary Genomic Analyzer software (MEGA) format and saved. This file was opened using MEGA version version 3.1 (Kumar et. al 2004: 150-163). Using MEGA program, aligned sequences were fragmented according to defined regions of HAV genome encoding structural and non-structural proteins, 5'NCR and 3' NCR as described for prototype strain HM175. Phylogenetic analysis was carried out for each fragment of genome.

4.14.1 Pairwise (P) distance and Percent Identity calculations

P distance was calculated by choosing menu option 'compute distance' using P distance and pair wise deletion method in MEGA software. Average similarity within and between groups was obtained between two or more groups as well as two or more individual sequences at nucleotide or amino acid level.

4.14.2 Recombination analysis

To search for recombination events in Indian strains, within or between genotypes, the SimPlot V.5.1 (Lole et al 1999: 52-60) was used. Similarity plotting is a first step to cover, because it is faster and more intuitive than bootscanning. The results don't look as dramatic as bootscanning, but that is why similarity plots are more intuitive. In similarity plots one can see how similar two sequences are, not just how much more related they are to each other than to the other sequences/groups being compared (i.e. absolute versus relative similarity). The basic principle of bootscanning is that mosaicism is suggested when one observes high levels of phylogenetic relatedness between a query sequence and other reference sequence in different genomic regions.

4.14.3 Phylogenetic trees

For full-length genome sequences (n=28) Neighbour-Joining (NJ) algorithm available in MEGA 3.1 was used for the phylogenetic analyses (Kumar et.al 2004:150-
Kimura 2 parameter distance model was employed for phylogeny of all nucleotide positions, while modified Nei-Gojobori method was used for the phylogeny of synonymous and nonsynonymous nucleotide positions (Nei and Gojobori 1986:418-426). The reliability of various phylogenetic trees was tested by applying bootstrap test with 1000 replications.

In order to confirm the reliability of various genomic regions for classification of HAV strains different phylogenetic methods were used for analyses. NJ and Maximum Parsimony (MP) methods from MEGA 3.1 software and Maximum Likelihood (ML) method from PAUP4b10 and Tree finder 2007 software packages were used.

### 4.14.4 Software Packages

**MEGA 3.1:** From MEGA 3.1 software NJ algorithm with Kimura 2 parameter distance model and MP method by using Close-Neighbor-Interchange (CNI) search method was used. The reliability of the trees was tested by applying bootstrap test with 1000 replications for both the above-mentioned methods.

Bootstrap analysis is a popular method to assess the confidence of inferred relationships. Tree reconstruction is repeated many times with resampled versions of the input data and a majority-rule consensus tree is built from the results. The bootstrap support for any non-trivial split is then the percentage of times it was recovered during the bootstrapping procedure.

**PAUP4b10:** PAUP4b10 software was used for ML method. Different nucleotide substitution models were fitted to the data by using PAUP4b10. The best model was selected by using Akaike Information Criteria (AIC) as implemented in software Model Test 3.7. The ML trees were constructed by using heuristic search algorithm in software PAUP4b10 with the best nucleotide substitution model as given by AIC. The heuristic search was carried out in two steps. In the first step, branch swapping by Nearest-Neighbor Interchange (NNI) was applied on NJ tree as a starting tree, with stepwise addition of trees, random addition of sequences and 10 replicates. The second step was performed on the best tree found in step 1, by using subtree pruning-regrafting method of branch swapping. The trees were midpoint rooted.

**TREEFINDER 2007:** TREEFINDER 2007 software was used for ML method by using HKYG4 model. There are models of both nucleotides and amino acids taking into account the rate heterogeneity among sites. TREEFINDER starts its tree optimization from a NJ tree, which is built from pair-wise maximum-likelihood sequence distances under a simple model. Given whatever greedy tree search algorithm that can find a local likelihood optimum by climbing up from a suitable start tree, the straight forward method to search for the global optimum is redoing the greedy search from a multitude of
different start trees and taking the best of the results. The trees are formed during equidistant random walks of random nearest-neighbor-interchanges (NNI) starting from the center tree.

**TREE-PUZZLE 5.2:** TREE-PUZZLE 5.2 was used for Likelihood mapping analysis. TREE-PUZZLE computes the number and the percentage of resolved, partly resolved and completely unresolved maximum likelihood quartets for the whole dataset as well as each sequence. A partly resolved or completely unresolved quartet is a quartet where the maximum likelihood values are so similar for two or all three, respectively, of the three possible quartet topologies that it is not possible to prefer only one of them (Strimmer *et al.* 1997:210-213). The higher percentage of the unresolved quartets among all possible quartets is an indicator of the unsuitability of the data for phylogenetic analysis.

**4.15 Estimation of surface charge distribution on capsid proteins**

Experimentally derived 3D structure data on HAV is not available. Therefore, an attempt has been made to predict 3D structure of individual VP1, VP2 and VP3 capsid proteins by using knowledge-based homology modeling approach. VP4 capsid protein was excluded due to it's small size (23 aa). Modeling was done by using Modeller 8 V2 (Sali *et al.* 1993:779-815). Among available homologous structures in the Protein Data Bank (PDB) (http://www.rscb.org) Poliovirus (PDB-ID: 1HXS, 2.2 Å resolution), Mengovirus (PDB-ID: 2MEV, 3.0Å°resolution), Human Rhinovirus (PDB-ID: 1RNV, 3.0Å°resolution) and Foot and Mouth Disease (FMD) virus (PDB-ID: 1GGP, 1.9Å°resolution) were used as the template.