

Hepatitis



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Hepatitis

6. Hepatitis

6.1 Development of candidate vaccine for hepatitis E (46/02)

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Hepatitis E is an important public health problem in India with several epidemics being reported all over the country causing morbidity and mortality (particularly in pregnant women). In sporadic settings, fulminant hepatitis E has been observed in men and non-pregnant women. Travellers to endemic areas, military personnel, sewage workers are at a high-risk of HEV infection. Hepatitis E in HBV and HCV carriers may have serious complications. Therefore there is a need for hepatitis E vaccine.

Objectives

- To develop Recombinant protein-based, DNA-based and Prime and boost approach based hepatitis E vaccine candidates.

Work Done

Neutralizing Epitope Region (NE) of HEV

As described in previous annual report, the region 458 aa. 607 aa. within HEV (type 1) ORF2 protein has been reported to have neutralizing epitopes. The partial gene corresponding to this region was cloned in baculovirus and bacterial expression systems. To further assess the utility of NE-DNA as vaccine, this region was cloned in pcDNA3.1 and the plasmid construct was delivered to laboratory animals as DNA vaccine.

Experiments in mice

Two groups of 6-8 weeks old female Swiss albino mice (n=6/group) were immunized with 1µg/ dose of NE DNA alone or with mouse GM-CSF DNA. Total 3 doses were given at 0, 4 & 8 weeks. The mice were bled at 4 weeks post dose 1 and weekly thereafter. The mice were monitored for humoral response employing both recombinant ORF2 protein (rORF2p) and recombinant NE protein (rNEp) in ELISA. Mice showed good antibody response with NE DNA. No difference in immunoreactivity was noted with GM-CSF in mice indicating that use of genetic adjuvant does not have additional benefit.

Immunogenicity using aluminum phosphate / hydroxide as adjuvant

Swiss albino mice were immunized with 3 doses of 2µg/injection purified rNEp adsorbed to Al₂(SO₄)₃ or 2µg/injection purified rORF2p adsorbed to Al(OH)₃. Both proteins gave good anti-HEV response.

DNA Prime Protein Boost approach

Swiss albino mice (n=8/group) were primed with 1µg/ dose of ORF2 DNA & boosted with 2µg/injection of rORF2p with CFA/IFA in DNA prime protein boost approach. Total 3 doses were given at 0, 4 & 8 weeks and 0, 4 & 20 weeks interval. Two groups in each type of dose schedule were given two different dose regimens:

- First DNA dose by gene gun followed by two protein doses (DPP) and
- Two DNA doses by gene gun followed by a single protein dose (DDP).

The mice were bled at 4 weeks post dose 1 and weekly thereafter. The mice were monitored for humoral response and subsequently sacrificed for assessment of cell-mediated immune response. Results are briefly depicted in Table1 and 2.

Table 1: Anti-HEV response in mice immunized employing DNA-prime-protein-boost approach

Dose schedule	Dose type	Sero conversion		Titer at the time of sacrifice
		4th week post dose 1	1st week post dose 2	
0,4 & 8 weeks	DDP	8/8	8/8	1:100 to >1:1000
	DPP	8/8	8/8	1:100 to 1:1000
0,4 & 24 weeks	DDP	8/8	8/8	on going
	DPP	7/8	6/8	on going

Table 2: Anti-HEV response in mice immunized employing DNA-prime-protein-boost approach

Group	Responders/Non-responders	
	rORF2p	RNEp
DDP	2/7	2/7
DPP	2/6	2/6

Conclusion

- Neutralizing epitope region within ORF2 of HEV (type 1) may replace as the shortest region in any kind of experimental vaccine approach for hepatitis E.
- A two-dose schedule (at 0 & 4 weeks interval) of two DNA doses OR first DNA followed by protein dose seems to be effective, which needs further evaluation.
- The rNEp + Al.PO4 and rORF2p + Al.(OH)3 combinations are immunogenic in mice.
- DNA by Gene gun proved better than the only protein approach in mice.

6.2 Development of Combined DNA Vaccine for Hepatitis B and E viruses

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To explore the possibility of developing combined DNA vaccine for HBV and HCV, immunogenicity of candidate combination vaccine was tested in mice.

Initially, 'S' gene of HBV was cloned in pVAX1 vector followed by "preS1+preS2" gene cloning at the C-terminal end of 'S' gene. PreS1 and preS2 genes were also cloned in pET15b vector for protein expression. HEV ORF2 and S gene of HBV were cloned separately in pVAX1 vector and together in a single vector pcDNA3.1, along with IRES (Internal ribosome entry site) region. For anti-HBs detection, ELISA was standardized employing recombinant HBsAg procured from Serum Institute of India, Pune.

Swiss Albino mice were immunized with 3 doses of 1µg/dose pVaxI+ORF2 DNA vaccine using Gene Gun method at 0, 4 wks and 8 wks. At 5th wk (1st wk after 2nd dose) 8 out of 10 mice showed anti-HEV antibodies in ELISA.

Assessment of cell mediated immune response

In a pilot experiment, two weeks after the last dose of immunization, spleenocytes were harvested and Lymphocyte Proliferation Assay (LPA) was carried out using rHBsAg as the recall antigen. Two out of 4 inbred mice and 2/2 out bred mice immunized with HBsAg showed memory T cell response having SI>3. None of the control mice responded to rHBsAg. This preliminary data shows promising cell mediated response in mice.

6.3 Hepatitis A component of combined Hepatitis (A+ B+E) vaccine

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As hepatitis A will be a component of combined vaccine(s) and genotype IIIA is the predominant circulating strain, attempts are being made to adapt several HAV isolates to MRC-5, vero-E6 and BGMK cell lines.

Work done

For the isolation of HAV in tissue culture, the inoculums used were HAV RNA positive, 10% stool suspension from IgM anti-HAV positive patients. As HAV does not produce cytopathic effect and is fastidious with long incubation period, the inoculated virus was passaged in MRC-5, vero-E6 and in BGMK cell lines at weekly, 2-weekly, 3-weekly and 4-weekly time points. Cells were maintained in closed cultures at 35°C. So far cultures did not yield HAV Genotype IIIA isolate. Details of the passages are depicted in table 3.

Table 3: Passage details of HAV isolates in different cell-lines

Cell line	Inoculum	Weekly Passage	2 weekly Passage	3 weekly passage	4 weekly passage
MRC-5	1*	39	28	20	20
MRC-5	2**	42	30	28	14
MRC-5	3***	33	22	18	16

In vero-E6 cell line, * 12 passages, *** 5 passages
In BGMK cell line, * 19 passages, ** 8 passages

6.4 Generation of infectious cDNA clones for swine and human HEV and chimeric swine-human HEV clones (48-02)

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Understanding of basic biology of HEV, mechanism of its pathogenesis, virus replication strategies have suffered on account of lack of efficient cell culture system and practical animal model. We have taken the approach of development of full-length infectious cDNA clones for both type-1 and 4 Indian viruses, construction of chimeras in different combinations and assessment of all such constructs for infectivity in pigs. Infectivity of genotype-1 cDNA clone in rhesus monkeys also needs to be documented.

Objectives

- To develop HEV genotype-1 and genotype-4 full-length infectious cDNA clones
- To assess the infectivity of HEV genotype-1 and genotype-4 full-length infectious cDNA clones in rhesus monkeys and pigs respectively
- To evaluate the replication competence of the above clones in different cell-lines
- To develop chimeric type-1/ type-4 HEV infectious cDNA clones with combination of nonstructural and structural genes from the two genotypes of HEV
- Assessment of the different chimeric cDNA clones for infectivity in pigs and cell-lines

Work done

This project was submitted to DBT for funding and was sanctioned in November 2005. HEV genotype 1 full-length infectious clone is being reconstituted from overlapping TA-clones using restriction enzymes.

6.5 Determination of occupational risk of Hepatitis E Virus in animal handlers

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Objectives

- Determination of occupational risk of Hepatitis E Virus in animal handler
- To look for different HEV genotypes in acute cases of hepatitis among animal handlers

Achievements

Animal handlers residing in Kolhapur were interviewed and their blood samples were taken to screen for IgG-anti HEV. 27/32 (84.4%) large animal handlers, 11/23 (47.8%) small animal handlers, 5/6 (83.3%) poultry handlers and 7/11(63.6%) staff of the veterinary polyclinic were positive indicating past exposure to HEV.

6.6 Role of viral and host factors in the pathogenesis of fulminant hepatitis E

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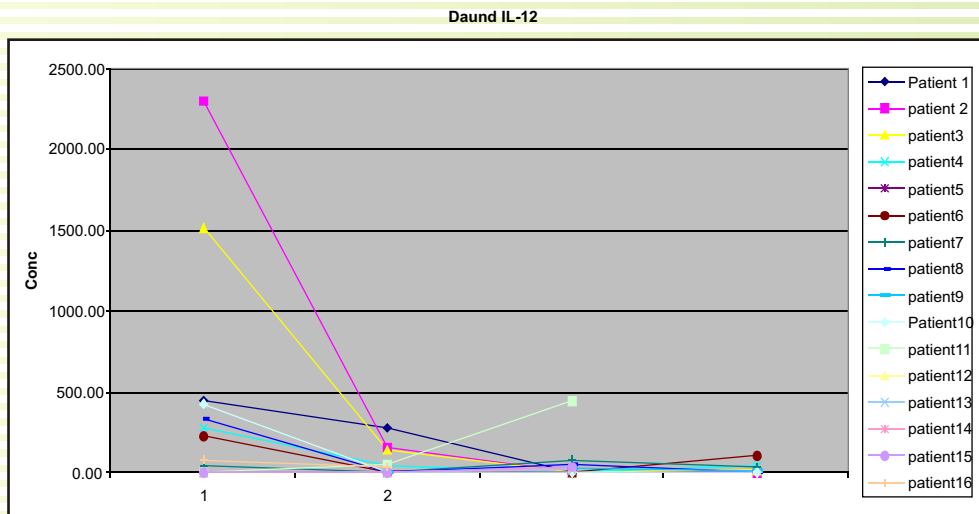
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During the current year, 12 fulminant hepatic failure (FHF) cases were screened for HEV etiology; 5 were positive for anti-HEV IgM (FHF-E group), 3 were positive for HBsAg (anti-HBc IgM to be tested), 1 patient was diagnosed as alcohol induced FHF and the rest 4 patients were categorized as non A-E cases. Sequential samples were obtained from 5 FHF-E cases. In FHF-E group, 3 patients expired (1 male, 2 females). Serum sample obtained from husband of an FHF-E patient was also positive for anti-HEV IgM and was diagnosed as acute-self resolving HEV. Liver autopsy was obtained from 2 fatal cases. To assess the host factors, cytokine and proliferation assays (rORF2 and mitogen specific) were done for one acute and all FHF cases. Culture supernatants were stored at -70° C for further processing.

IL-4 and IL-12 levels in acute hepatitis E cases

ELISAs were carried out for the quantitation of IL-4 and IL-12 cytokines in 57 acute-recovering hepatitis E patients (including all the acute samples from the previous year) in 5 healthy controls, and in sequential samples of 16 acute resolving patients. IL-4 levels remained within normal limits in all samples. Based on analysis of follow-up samples, higher IL-12 levels were recorded during the initial stage of disease.(Figure 1)

Figure 1:IL-12 levels in sequential samples of Hepatitis E patients, Daund epidemic



IL-4 and IL-12 levels in FHF-E cases

Among FHF cases, IL-4 levels remained within normal limits. After recovering from encephalopathy, increased IL-12 levels were observed.

HLA typing by PCR-SSP

In order to find out the role of human leucocyte antigen (HLA) as a host factor towards the different clinical manifestation in HEV infection, 12 FHF-E patients with different clinical outcomes, 44 acute-E resolving patients and 67 racially matched controls were analysed for their HLA Class I alleles by PCR-SSP method. Results are as follows: In FHF-E cases, frequency of HLA alleles (a) A*32 (AF: 29.17 vs 0, OR=13.2, P=0), (b) A*26 (AF: 25 vs 0.74, OR=44.3, P<0.001) were increased when compared with controls. In acute E resolving patients, frequency of alleles (a) A*03 (AF: 21.59 vs 5.22, OR=5, P<0.001), (b) A*26 (AF: 14.77 vs 0.74, OR=23.05, P<0.001), (c) Cw*01 (AF: 17.05 vs 1.49, OR=13.56, P<0.001) were increased, where as allele Cw*06 (AF: 3.41 vs 19.4, OR=0.15, P<0.001) was decreased in acute patients compared to controls.

HEV RNA quantification

Acute-recovering cases: A total of 31 acute-phase plasma samples and sequential plasma samples from 16 IgM-anti-HEV positive patients were assessed for the quantitation of HEV RNA by real-time PCR. Higher viral load was present during the initial period of the disease i.e. 1-10 days post-onset of the disease (POD) (63713 ± 54851). HEV RNA was detected up to 47 days after onset of disease; not detected after 50 POD.

FHF-E cases

This year, plasma viral load was determined for 13 FHF-E patients using real-time PCR..

Full Genome sequencing of HEV

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Employing 15 sets of primers, full genomes were amplified and sequenced from three FHF E cases and were compared with other cases belonging to Type-1, Type-3 and Type-4 genotypes. We noticed that sequences of all FHF cases resembled with Type 1 isolates and were showing major difference with Type 3 and Type 4 isolates.

6.7 Molecular evaluation of water samples and water treatment protocols with special reference to Hepatitis A & E viruses

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Standardization of Methodologies

- A multiplex nested RT-PCR for the simultaneous detection of HAV (342 bp), HEV (416 bp) and enterovirus (200 bp) viruses was standardized.
- For the standardization of Real time PCR for HEV (Type 1), new set of primers were designed to avoid any mismatch from the HEV type 1 strain prevalent in the region (Previous primers had mismatches). A 293 bp target region of HEV type 1 strain was cloned, in vitro transcribed and quantified to determine number of RNA copies (Standard RNA). Primer and Probe concentrations were optimized to give best amplification. The sensitivity of this assay is 10 copies of HEV type I RNA / reaction. The standard curve showed good correlation ($r=0.99$) and the results were reproducible. (Figure 2a, 2b)

- Efficiency of membrane filter based virus concentration system was evaluated employing Real Time PCR. Number of HAV RNA copies was determined for a stool sample collected from a hepatitis patient. Known amount of virus was added to 5 liters of distilled water and concentrated to 3 ml. Number of HAV RNA copies was determined in this concentrated water sample by Real Time PCR. Recovery of HAV was noted to be > 90%, attesting the utility of the concentration system.
- For optimization of membrane filter based virus concentration system, thickness of spirally wound membrane filter cartridge was increased to increase the flow rate thereby reducing the time for the concentration leading to reduced possibility of viruses being adsorbed to the positively charged membrane surface. Position of 'O' rings in the spiral filter cartridge was adjusted to obtain minimal residual volume. By applying above two modifications the residual volume after first round of concentration was reduced to ~250ml as compared to ~600 ml obtained by prior settings.

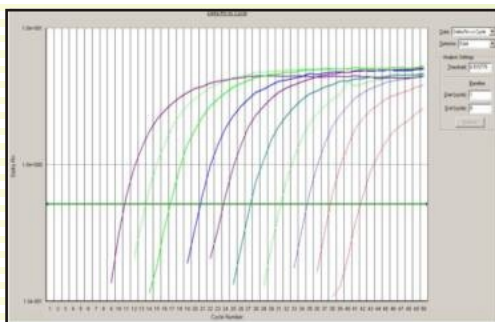


Figure 2a : Amplification curves for 10 fold dilution of standard RNA ((1010-101 copies)

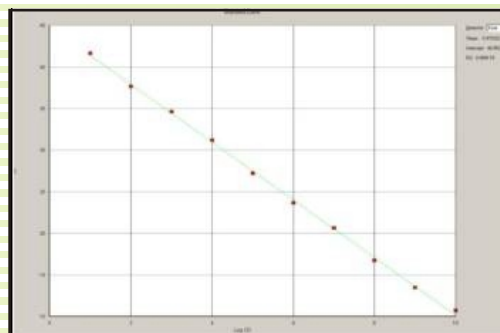


Figure 2b : Standard curve for 10 fold dilution of HEV type I RNA.

6.8 Evaluation of the role of HLA factor towards acquisition of HCV infection in Dialysis patients

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis, cirrhosis, hepatocellular carcinoma (HCC) and death. The observation of different clinical presentations despite the same source of infection led to the recognition of the importance of host genetic factors in disease manifestations.

Objectives

- The aim of the present study was to examine the frequencies of human leukocyte antigen (HLA) class I and class II alleles among anti-HCV positive individuals in an Western Indian population and compare them with racially matched healthy controls

Work done

This year, we studied patients undergoing haemodialysis. Sixty-six patients from Pune, were screened for the presence of HCV infection. Eighteen out of 66 (27.5%) were positive for HCV RNA. All the HCV RNA positives had normal liver function tests. Genomic DNA was extracted from PBMCs using Qiagen mini Blood Kit and the frequencies of HLAA, B, C and DQ, DR alleles were examined by PCR-SSP methods. Frequency of alleles namely A*03, A*32, B*15, B55, Cw*18 were significantly increased, frequencies of A*24 and B*40 were increased in these individuals when compared to the racially matched controls.

6.9 HEV diagnostics

A) Detection of anti-HEV antibodies using HEV ORF3 protein in ELISA

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Earlier, HEV ORF2 (capsid) proteins expressed in baculovirus system from genotype 1 (human) and genotype 4 (swine) virus were found equally good for detecting infections with both the genotypes in humans, pigs as well as in experimentally infected animals. On comparison of the results obtained with in-house ELISAs and a commercially available ELISA, our ORF2 based test clearly showed better sensitivity.

Objectives

➤ To extend above comparison to ORF3 proteins from both type-4 and type-1 HEV in detecting HEV infections

Work done

These proteins were expressed in E.coli using vector pET15b. Both proteins formed inclusion bodies in the cells and remained insoluble. Inclusion bodies were purified, dissolved in buffer containing 8M Urea, diluted in 50mM carbonate-bicarbonate buffer (pH9.2) and was directly used for coating micro well plates. Multiple serum samples were tested using these two antigens to assess the utility in anti-HEV antibody detection (IgG as well as IgM). All the samples were also tested with type1 ORF2 antigen (expressed in baculovirus system, HEV type1) in parallel. Serum samples tested were as follows:

- Week wise serum samples of patients from different hepatitis E outbreaks (1-13 weeks, n=114).
- Four serial follow up serum samples from 11 acute hepatitis E patients (n=36)
- Human sero survey samples (n=150)
- Pig sero survey samples (n=80)
- Serial serum samples from 7 experimentally infected (with type-1 HEV) rhesus monkeys (n=28)
- Serial serum samples from experimentally infected pig (with type-4 HEV) (n=15)

Type1 and type 4 ORF3 antigens showed comparable results with sera from both type-1 infections in humans and monkeys and type 4 infections in pigs. There was a good correlation between anti-HEV positivity obtained with ORF2 antigen and both ORF3 antigens in the samples from individuals with recent HEV infections (IgM). On comparison of anti-HEV (IgG) positivity obtained with ORF2 antigen and ORF3 antigens, it was clear that there was comparatively less anti-ORF3. But anti-ORF3 antibodies (IgG) were present in significantly large number of normal human as well as pig sero survey samples.

B) Expression of N-terminal 111 amino acid part of ORF2 protein

The coding frame of ORF2 protein, the capsid protein of HEV is 660 amino acid along with three potential glycosylation sites. Protein expression studies have shown that 112-607 amino acid portion of capsid protein (55kDa) forms virus like particles (VLPs) in vitro as well as in insect cells. Complete ORF2 protein (about 70kD) gets processed in insect cells to yield the 55kDa truncated version. However, the N-terminal 111 amino acid segment harbours multiple B-cell epitopes (both IgM and IgG). We are using recombinant baculo virus based, insect cell expressed ORF2 protein for HEV diagnosis.

Objectives

- To express this N- terminal segment of ORF2 and test for anti-HEV antibody detection for improving the sensitivity of the complete ORF2 protein based assay.

Work done

N-terminal segment of ORF2 gene from both human (genotype-1) and swine genotype-4 HEV were cloned in vector pET15b in frame with 5'-His tag. After sequence confirmation, recombinant plasmids were put in BL21 (DE3) pLysS bacterial cells for protein expression. Protein expression was monitored by ELISA using a set of anti-HEV positive and negative sera. The optimum expression conditions of IPTG concentration, temperature and time for both the proteins are being standardized. These proteins will be purified on Ni-agarose columns and checked for their utility in ELISA.

6.10 Detection of hepatitis A virus in vegetables obtained from farms being irrigated with treated sewage water

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High stability of hepatitis A virus in the environment increases possibility of its spread through modes like contaminated food, water, vegetables etc. Treated sewage water is generally used to irrigate farms. It has been very well documented that such water sources can contaminate vegetables with HAV. We also have such farms in the suburban areas of Pune city.

Objectives

- To screen vegetables obtained from fields being watered with treated sewage water for hepatitis A virus

Work done

A protocol was developed to detect HAV from vegetables. For that, clean vegetables were soaked in water containing HAV, washed and adsorbed virus was eluted. This elute was concentrated 100 folds using Amicon (Millipore) system and both retentate and filtrate were tested for HAV with PCR. Same protocol was used to screen vegetables obtained from fields being watered with treated sewage water. One sample of spinach was found to be contaminated with HAV. For confirmation, PCR primers from two different regions of HAV were employed and PCR positivity was confirmed in both regions. Sequence analysis of these two regions confirmed the virus as type IIIA that is most commonly circulating in this region of India.

6.11 Immunogenicity of hepatitis A vaccine among children

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One hundred thirty two susceptible children were vaccinated with two pediatric doses of Havrix vaccine after an outbreak of hepatitis A in Daund taluka of Pune district. Blood samples of 58 of the above children were collected one month after administration of the 1st dose, 121 prior to 2nd dose and 95 one-month after the second vaccine dose. These samples were tested for IgG anti HAV. All the children tested showed sero conversion to IgG anti HAV antibodies (Figure 4).

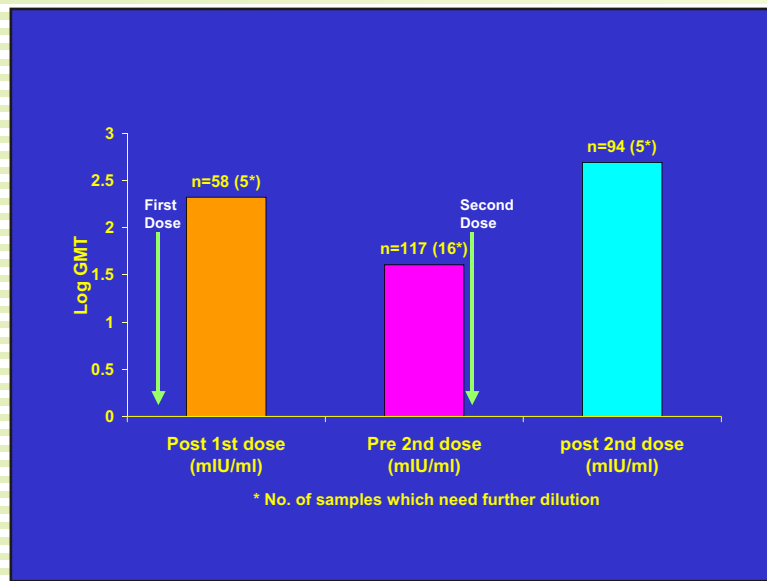


Figure 4: Immunogenicity of hepatitis A Vaccine

6.12 Services provided

Testing for Drug Controller of India

A total of 466 blood products submitted by the drug controller of India were tested for HBsAg and HCV RNA and reports were submitted.

Chronic Hepatitis B and C patients

A total of 46 and 52 patients were tested for the presence of HCV RNA and HBV DNA respectively in PCR.

Sporadic acute viral Hepatitis Patients

526, 447 and 219 serum samples were tested for the detection of anti-HAV-IgM, anti-HEV-IgM and HBsAg respectively.