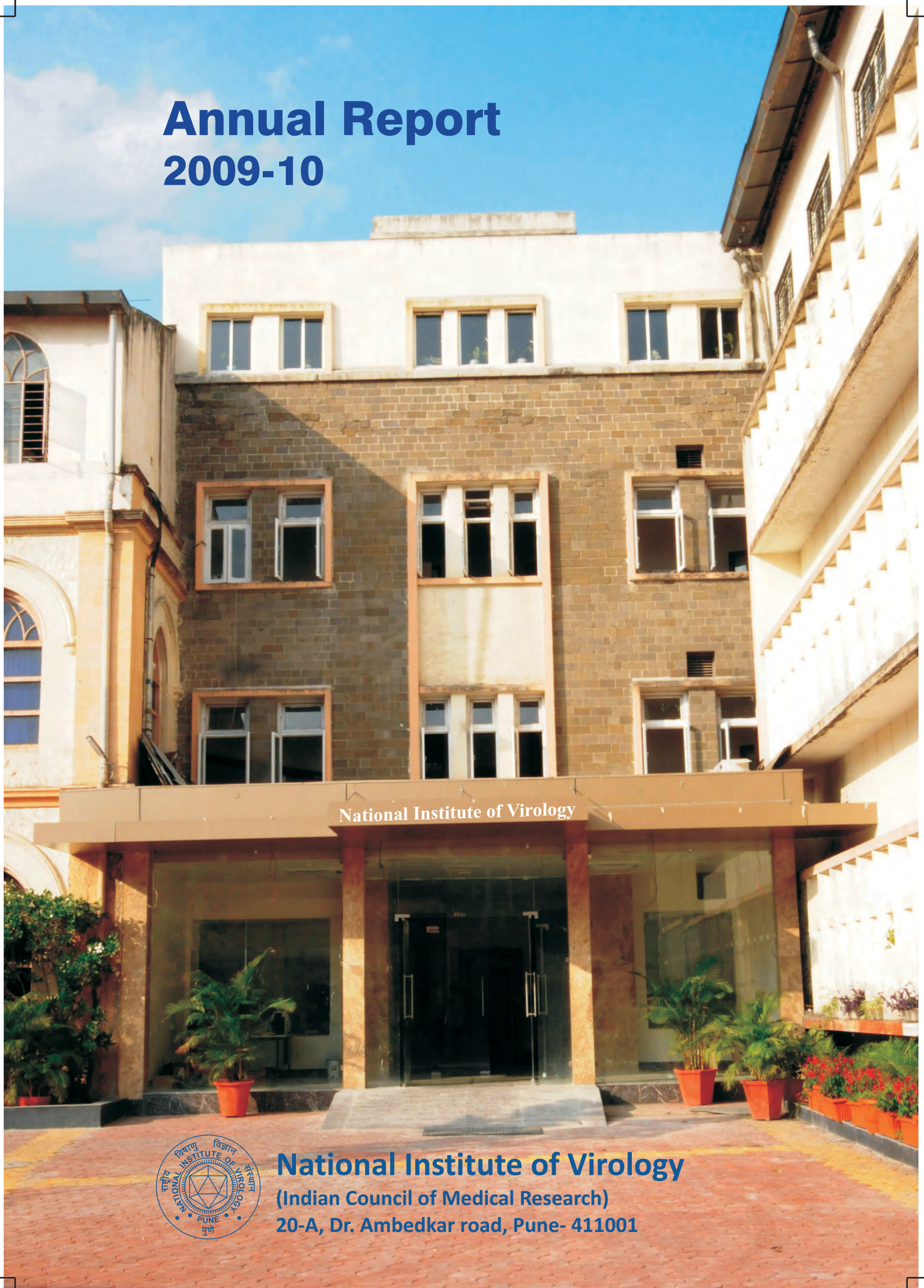
Annual Report
2009-10



National Institute of Virology
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Shri. Gulam Nabi Azad,
Honorable Health Minister of India,
addressing NIV staff

Director's Preface

During the reporting year, the highly pathogenic strain of pandemic influenza H1N1 (pH1N1) virus posed a serious threat to the global public health. The virus was first detected in India in May 2009 following which there were many outbreaks of the virus in other parts of India, with several fatal cases. With the rapid global spread of the pH1N1 virus, there was a need for development of effective vaccines for mass vaccination. The NIV fully supported the Serum Institute, Pune in the development of an inactivated intranasal vaccine, by conducting the immunogenic and protective efficacy tests in BALB/c mice as a pre-clinical animal model, resulting in the introduction of the intranasal vaccine in the market. As in the previous year the current year was also was eventful, with a number of accomplishments in outbreak investigations, vaccine development for different viruses, diagnostics, infrastructure developments, teaching, training and manpower development. Active participation in the National polio and measles virus surveillance and characterization programs was continued with high performance scores.

In continuation of our intensive studies on other human influenza viruses, a project entitled "Multisite epidemiological and virological monitoring of human influenza, Surveillance Network in India, Phase II" was initiated wherein it was shown that pH1N1 and seasonal, predominantly Influenza A (H3N2) co-circulated. During May 2009 to March 2010, 25488 clinical samples were received and assayed by real time PCR for laboratory diagnosis. Overall, 5345 (21%) samples were found to be positive for pH1N1 and 2077 (8.1%) for seasonal Influenza cases. Genetic analyses of whole genome and HA gene sequences of 13 Indian isolates, showed that clade 7 was the dominant pH1N1 lineage in India (PLOS One, 2010; 5(3): e9693). A D222G mutation in the HA receptor binding domain was found in two of the Indian isolates both from fatal cases. All the isolates were sensitive to Oseltamivir drug. A novel avian influenza (H11N1) virus was isolated during routine surveillance from a Eurasian spoonbill.

At Gorakhpur Unit, a total of 7209 samples consisting of 1704 CSF and 2494 acute serum samples were collected along with 2387 rectal swabs. 375 cases were laboratory confirmed JEV infection by standard IgM serology tests and in 75 CSF samples JEV genome could be detected by PCR. Highlight of the

study was detection of Genotype I JE virus in the CSF samples for the first time in India, in addition to currently prevalent Genotype 3 strain. Enterovirus was detected in CSF of 13 out of 693 CSF tested. A total of 214 out of 2038 serum samples were tested positive for presence of IgM antibodies against Hepatitis A virus. Development of an indigenous ELISA based detection kit is in process. Complete genome sequence of a JEV isolated from Gorakhpur confirmed its close genetic relationship with the Japanese swine Sw/Mie/41/2002 isolate with 97.60% sequence similarities

Characterization of rotavirus VP7 and VP4 genes have shown G10P [nontypeable] specificities in bovine and G2P[8], G4P[6], G3P[6] P[8] and G1G3P[6], G9P[19], G12P[4], G4P[NT] and GNT P[4] specificities in porcine species indicating human-bovine / porcine rotavirus reassortment. As part of vaccine studies formulations of candidate vaccine for hepatitis E and combination vaccine for Hepatitis E and B (in collaboration with industry partner) were made with five different adjuvants and tested in mice and further evaluation done in rhesus monkeys. Immunogenicity levels of the neutralizing epitope region (NE) from ORF2 of genotype 1 hepatitis E virus (HEV) and corresponding region in genotype 4 HEV were compared and genotype 4 NE was found to be less efficient. With the view of the vaccination of domestic poultry as a tool to combat AI H5N1, we received two bottles of the H5N2 vaccine (M/s Intervet India Pvt. Ltd., Batch No. M10622708/01-2010 on 6/3/2009) from the Animal Quarantine and Certification Services (NR), Government of India, New Delhi for evaluation of vaccine efficacy.

Our studies on arboviruses included mainly Dengue, Chikungunya, JE, and Chandipura. Dengue disease burden was studied using Pune as a micro-model for urban dengue spread. The study has shown that the severity of disease was lower than our previous years' observations, with 7% being categorized as DHF compared to 25-30% DHF cases being recorded in the last four years. All four serotypes were circulating. DENV-4 appeared for the first time in Pune city in the last 6 years and showed 92% similarity to the DENV-4 from Thailand. Genomic variation studies based on domain III of the E gene revealed that inter outbreak variation was higher in DENV-1 compared to DENV-2 and 3. Studies on dengue morphogenesis revealed that the DENV-2 envelope protein utilized for retrograde microtubule dependent trafficking. Monoclonal antibody against Chandipura virus was developed as a part of development of diagnosis kit.

Bioinformatics analysis of Avian Influenza H5N1 viruses of the 2008-09 outbreaks in West Bengal and north-east India, revealed that the viruses belonged to clade 2.2 and were introduced in phases into the country. The evolutionary dynamics of Dengue-2 viruses (1963-2005) was investigated to determine the evolutionary rates and ancestral timescales of these viruses and to determine the extent of selection pressure existing in the Envelope gene.

Homology- based modeling of the Hepaptis A Virus capsid proteins was carried out for antigenic mapping. Sequence and structure-based analysis of the proteins of the Chandipura virus was done to get insight into the possible markers of pathogenicity.

Our outbreak response group investigated mainly five outbreaks: pH1N1 (1), Hepatitis E (2), Chikungunya (1) and acute diarrheal disease (1). The pH1N1 outbreak due to local transmission was confirmed in India for the first time among the students of a residential school in Panchgani. Mathematical modeling and simulations were carried out to study the transmission dynamics of the disease in the residential school setting.

Laboratory diagnosis of referred samples of unusual cases were received from NIV field unit Kerala (40), Rajkot (2), Pune (2) and Mumbai (1) and were processed for determining Ross River Virus, Murray Valley Encephalitis, Sindbis Virus and Hantaan. However, all were found negative. To create a specialized maximum containment facility to handle the pathogens of risk group-4, standard operational methodology and protocols were developed. The BSL 4 project activities were carried out successfully on schedule and is expected to be operational during 2011.

Large scale development and supply of JE, Dengue and Chikungunya MAC ELISA kits were made possible and provided to Sentinel Surveillance Hospitals (SSHs) and other Apex labs through the National Programme. These kits were evaluated by WHO at CDC and demonstrated good reproducibility and quality.

We are indeed grateful to Dr V. M. Katoch, Director General of I.C.M.R. for all the generous and excellent support and encouragement provided during different phases of our activities, especially in difficult times. We are also thankful to our team of NIV scientists, and various colleagues in other organizations, without whose assistance our accomplishments would not have been possible.

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Shri. Gulam Nabi Azad,
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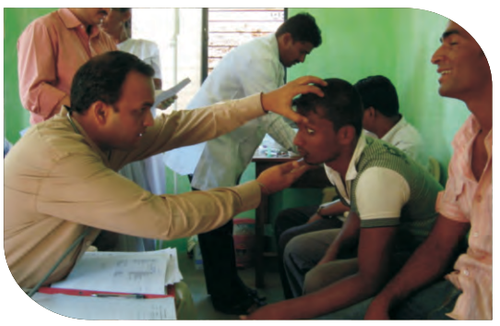

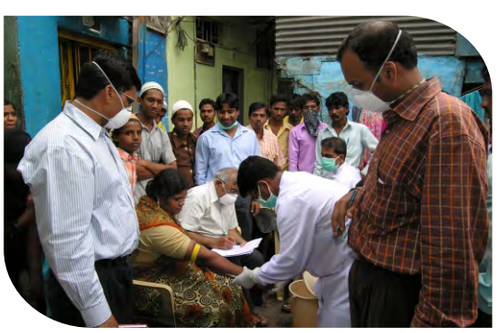

Outbreaks

- Pandemic influenza A (H1N1) 2009 outbreak in a residential school, Panchgani, Satara district, Maharashtra
- Pandemic influenza A (H1N1) 2009 exploratory serosurvey, Mumbai, Maharashtra
- Hepatitis E outbreak, Tasgaon, Sangli district, Maharashtra
- Hepatitis E outbreak, Roha, Raigad district, Maharashtra
- Chikungunya viral fever outbreak, Kozhikode district, Kerala
- Acute diarrheal disease (ADD) outbreak, Alappuzha, Kerala
- Investigation of fatal case of septicemia with renal failure for viral etiology

Projects

- Study of the seroprevalence of Japanese B Encephalitis in the State of Goa
- Post-licensure efficacy of a single dose of live attenuated SA 14-14-2 vaccine against Japanese encephalitis in India
- Sentinel community surveillance for viral diseases/syndromes in Pune, Maharashtra

Pandemic Influenza A (H1N1) 2009 outbreak in a residential school, Panchgani, Satara district, Maharashtra

Y. K. Gurav, S. D. Pawar, M. S. Chadha, V. A. Potdar, S. S. Koratkar

An outbreak of influenza like illness (ILI) was reported in a boys' residential school in Panchgani, Satara district, Maharashtra on 22nd June 2009. The outbreak was investigated from July 23-29, 2009.

Objectives

- To determine the etiology and describe clinico-epidemiological features.
- To estimate the extent of infection among school children, staff and community.

Work done

Attack rate for influenza-like illness among 415 school population was 71.1%. Throat swabs were collected from 82 ILI cases to determine the influenza types (A or B) and sub-types. Real time reverse transcriptase polymerase chain reaction (RT-PCR) was performed on throat swabs. Pandemic influenza A (H1N1) 2009 virus was detected in 15 (18.3%) of 82 cases, seasonal influenza type A in six cases, and influenza type B in one case.

Local transmission was confirmed for the first time in India among students in a residential school at Panchgani, Maharashtra. The public health authorities started screening other residential schools and local community. The finding from this study led to the change in policy for throat swab collection from community even if the cases had no foreign travel history or close contact with a confirmed case. Following these investigations, public health authorities proactively began to administer Oseltamivir to suspected cases and contacts.

Serological survey in the school was undertaken in the last week of July and again in the first week of November 2009. Serosurvey in community was done in August 2009. Haemagglutination inhibition (HI) assay was performed on sera to detect antibodies against pandemic influenza A (H1N1) 2009. Antibody titres ≥10 for pandemic influenza A (H1N1) 2009 and ≥20 for seasonal influenza A and B were considered as positive. In July, among 415 school population, HI antibodies against pandemic influenza A (H1N1) 2009 virus were detected in 216 (52%) subjects. Among these 216 subjects, 165 (76.4%) reported ILI. In a repeat survey of 472 subjects from the same school in the first week of November, seropositivity was 71.5%. In the community survey in August, 9% of the 245 subjects were seropositive.

Pandemic influenza A (H1N1) 2009 serosurvey, Pune, Maharashtra

B. V. Tandale, Y. K. Gurav, S. D. Pawar

Community transmission was established in July 2009. The serosurveys were undertaken for knowing the extent of infection in population.

Objective

- To estimate the seroprevalence of Pandemic influenza A (H1N1) 2009 virus infection

Work done

Serosurveys were undertaken in Pune city from August 15 to December 11, 2009. Hospital staffs, general practitioners (GPs), school children and school staff, workplace adults and general populations were surveyed. Haemagglutination-inhibition (HI) antibody assays were performed employing standard protocols. A titre of ≥1:10 was considered positive.

In the last week of August, 8.7% of the 495 hospital staffs were seropositive. Seropositivity was 7.6% (40/524) in October and 20.0% (77/385) in November. Among 104 seronegative subjects, 12 (11.5%) became positive after 9 weeks.

In the fourth week of August, 16.1% of the 385 general practitioners were seropositive. The seropositivity increased to 27.8% (67/278) in November and 35.6% (80/225) in December. Among 43 seronegative subjects, 15 (34.9%) became seropositive after 13 weeks.

Seropositivity among 348 school staff was 6.6% on 15th August 2009 and it increased to 26% (46/177) by the end of September. In the schools with the reports of PCR-confirmed cases, 33.3% school staffs were seropositive as compared to 4.2% in a school without PCR-confirmed case. Among 96 seronegative subjects, 18 (18.8%) became seropositive after 5 weeks. Influenza-like illness was reported by 4 of these 18 seropositive subjects.

In September, the overall seropositivity among 2527 school children (31.7%) and among 177 school staff (26.0%) was similar. The 15-19 years age group showed the highest seropositivity (55.4%), followed by 10-14 years age group (34.1%). Among 846 seropositive subjects, 92 (10.9%) reported the recent history of influenza-like illness. The highest incidence of influenza-like illness (20%) was recorded in the students from 15-19 years age group.

In September, seropositivity was similar among railway commuters (27/225, 12%), office-staffs (25/233, 10.7%) and slum-dwellers (67/651, 10.3%). Influenza-like illness was reported by 7 (10.4%) seropositive subjects from the slums.

In a community survey of 2520 subjects in October, the overall seropositivity was 9.6%. The seropositivity was similar in higher (10.7%), middle (8.9%) and lower (10.1%) social strata. Males and females had similar seropositivity. Seropositivity among children (116/877, 13.2%) was significantly higher than the adults (126/1643, 7.7%). The highest seropositivity of 28.4% was observed in 15-19 years followed by 19.7% in 20-29 years and 13.2% in 30-39 years.

Among 195 household contacts of 74 PCR-confirmed cases, 70 (35.9%) were seropositive. Among these, 10 (14.3%) reported influenza-like illness within 2-7 days of the onset of illness in the index case. The age-specific seropositivity was the highest in 5-19 years age group (57.1%), followed by 20-39 years age group (42.6%).

The extent of infection was widespread in all the sections of community. Most infections were asymptomatic or mild. This helped alleviate panic in general community.

Pandemic influenza A (H1N1) 2009 exploratory serosurvey, Mumbai, Maharashtra

Y. K. Gurav, B. V. Tandale, S. D. Pawar

An exploratory serosurvey was done among hospital staff and school children in Mumbai from February 15 26, 2010.

Objective

- To determine the extent of infection among the hospital staff and school children

Work done

Sera were collected from 203 hospital staff and 254 school children and staff after written consent. HI antibody assays were done and titres ≥10 were considered as positive. Seropositivity among hospital staff was 25.6%. Seropositivity among school population was 56.7%, 62.2% among 201 students and 35.8% among 53 staff.

Hepatitis E outbreak, Tasgaon, Sangli district, Maharashtra

Y.K. Gurav, V.A. Arankalle

An outbreak of viral Hepatitis was reported in Tasgaon town in Sangli district, Maharashtra in June 2009. Ad hoc investigations were done from June 23 to September 8, 2009.

Objectives

- Understand the magnitude of outbreak.
- To characterize the outbreak in terms of time, place and person distribution
- To identify the source of contamination and suggest the preventive and control measures

Work done

Operational case definition of acute jaundice syndrome was used. Blood and stool samples were collected from the cases. Clinico-epidemiological investigations and sanitary surveys were also done. Two hundred and forty five cases were line listed from May 25 to June 28, 2009. Cases were reported from all 19 municipal wards. Male to female ratio was 2:1. The most commonly affected age group was 20-49 years (72.3%). The overall attack rate was 0.7%.

The clinical symptoms recorded in 245 cases were - dark urine (97.5%), jaundice (93.5%), fatigue (35.9%), abdominal pain (32.6%), anorexia (29.4%), vomiting (26.5%), fever (22.8%), giddiness (14.3%), diarrhea (12.6%) and arthralgia (3.7%). An antenatal case confirmed as

Hepatitis E recovered completely. A death of 32 year male confirmed as Hepatitis E had cirrhosis of liver with esophageal varices.

Sera were collected from 162 cases. Anti HEV IgM antibodies were detected in 45.7% cases. Anti HAV IgM antibodies were not detected in sera of 85 cases. Anti HEV IgM antibody was detected in 2 of 72 asymptomatic pregnant women. Also, sequential sera (n=174) from 72 confirmed hepatitis E cases were collected weekly till 4th week and again in 8th week for assaying liver enzyme levels and IgM/IgG antibodies.

Sanitary survey revealed that water pipelines were led in close proximity of sewerage system and water posts were without taps. Among 17 water samples, 5 were found unfit for drinking purpose as per the routine bacteriological testing conducted in State Public Health Laboratory, Pune. This re-emphasizes the need for safe water supply and sewage disposal.

Hepatitis E outbreak, Roha, Raigad district, Maharashtra

Y. K. Gurav, V. A. Arankalle

Following the detection of IgM antibodies in the referred sera of representative patients, field investigations were initiated on March 23, 2010.

Objectives

- Understand the magnitude of outbreak.
- To characterize the outbreak in terms of time, place and person distribution
- To find the source of contamination and suggest preventive and control measures.

Work done

Three hundred and thirty three cases were line-listed till 31st March 2010. Sera were collected from 53 cases. Anti HEV IgM antibodies were detected in 34 (64.1%) of 53 cases. Anti HEV IgM antibody was detected in 4 of 15 sera from pregnant women. Clinical symptoms recorded in 53 cases were- dark urine (70.3%), jaundice (67.2%), fatigue (34.4%), abdominal pain (51.6%), anorexia (43.8%), vomiting (29.7%), nausea (29.7%), diarrhea (7.8%) fever (17.2%) and giddiness (4.7%).

Sanitary survey revealed water pipelines led in close proximity of sewerage system. This re-emphasized the need for safe water supply systems to the community.

Chikungunya viral fever outbreak, Kozhikode district, Kerala

B. V. Tandale, G. P. Jacob, V. A. Arankalle

An outbreak of viral fever was reported in Kozhikode district in Kerala in June-July 2009. Investigations were done from August 2-6, 2009.

Objective

- To identify the etiology and describe the clinico-epidemiological features of the outbreak

Work done

Most of the patients presented with acute onset of fever and joint manifestations. Fever and entomological surveys were carried out by house-to-house visits for attack rates and vector density. Collection of larvae and adult mosquito vectors was done. Fever surveys in 9 rural localities indicated attack rate of 62.5%. Four of 9 areas were having Breteau index (BI) >50. *Aedes albopictus* mosquitoes were abundant in rural areas. In two urban areas, very high vector indices were noted with *Aedes aegypti* as the predominant vector. Anti-CHIKV IgM antibodies were detected in 38 (35.8%) of 106 sera tested by Chikungunya group.

Acute diarrheal disease (ADD) outbreak, Alappuzha, Kerala

B. V. Tandale, S. D. Chitambar, G. P. Jacob

Acute diarrheal disease (ADD) outbreak was reported in Alappuzha, Kerala in May-June 2009. The first case was hospitalized on 14th May 2009. Until 8th June 2009, 65 cases were line-listed. The investigations by the central team had identified Cholera etiology in 15 cases.

Objective

- To investigate the role of enteric viruses in acute diarrheal disease outbreak

Work done

Investigations were done from June 8-11, 2009 with the technical help from NIV Alappuzha unit. The staff of the general hospital, Alappuzha was contacted for identification, investigation, sampling and reporting of ADD cases. Medical Superintendent of Medical College Hospital was contacted for seeking collaboration. Twenty two stool specimens were collected from General hospital. The results of stool specimen testing are reported by Enteric virus group.

Investigation of a fatal case of septicemia with renal failure for viral etiology

B. V. Tandale, S. D. Chitambar, M. S. Chadha

A 63 year old female having hypertension, diabetes mellitus and hypothyroidism, was admitted in Niramay Hospital, Chinchwad on 17th January 2010. Presenting complaints included fever with macolopapular/purpuric rash, itching all over body and giddiness since 3 days. On admission, there was history of fever for two days and breathlessness. Total leukocyte count was high with lymphopenia and normal platelet count. Serum creatinine was high. Blood urea was also raised. ECG showed evidence of myocarditis. On 19th January, chest X-ray showed

haziness all over left lobe of lung indicating pneumonitis. Serum lactate was also raised. Malaria, EBV, CMV, Widal test and blood culture were negative. Paul Bunnell test was negative. Dengue IgM was found negative by private laboratory. The cause of death was septicemia with acute renal failure. No illness was reported by 2 family contacts and 25 hospital contacts.

Objective

- To investigate viral etiology in a fatal case of septicemia with acute renal failure

Work done

Serum collected on 18th and 19th January 2010 were negative for anti Dengue IgM antibodies. Nasal, throat and rectal swabs were negative for pandemic influenza and enteroviruses. Puncture biopsies of liver, lung and heart were negative for enterovirus. Histopathological investigations done by Electron Microscopy group were inconclusive.

Study of the seroprevalence of Japanese B Encephalitis in the State of Goa

B. V. Tandale, M. M. Gore

An age-stratified serosurvey was undertaken prior to vaccination campaign by employing 30-cluster methodology in collaboration with the Medical College and state health department.

Objective

- To undertake serological survey for estimating seroprevalence of JE virus infection

Work done

Field activity was completed in the month of June 2009. A total of 1015 subjects were sampled with sera from 34 clusters with 30 subjects from each cluster. Sera were tested for neutralizing antibodies by JE group. Overall seroprevalence of neutralizing antibodies against JE virus was 41.2%. Seropositivity increased with age. The lowest seropositivity of 12.9% was noted in 1-4 years and the highest of 63.6% in 30-39 years. There were no gender differences. Seropositivity was similar in rural and urban areas.

Post-licensure efficacy of a single dose of live attenuated SA 14-14-2 vaccine against Japanese encephalitis in India

B. V. Tandale, M. M. Gore

A field efficacy of SA 14-14-2 live attenuated JE vaccine was recommended by national technical advisory group on immunisation.

Objective

- To determine the efficacy of a single dose of live attenuated SA 14-14-2 vaccine against Japanese encephalitis in India.

Work done

Protocol documents including questionnaires and consent forms were revised/ amended following the revisions in the study protocol. Ethical reviews and approvals were sought. Field activities were initiated following finalization of field guides and training of project staff.

A draft report was prepared for review by expert group.

Sentinel community surveillance for viral diseases/syndromes in Pune, Maharashtra.

Y. K. Gurav, B. V. Tandale

During the pandemic of Influenza A (H1N1) 2009, it became important to generate community-based parameters of influenza transmission. Also, Dengue is an important public health problem in urban areas in Pune and has a potential cause of major outbreak in communities. It is extremely essential to understand the behavior of viral diseases in community.

Objectives

- To identify cases / clusters of influenza like illness (ILI), severe acute respiratory illness (SARI), dengue like illness (DLI) and dengue shock syndrome (DSS) and investigate them for identifying etiology and describing clinico-epidemiological features.
- To monitor the disease incidence prospectively by surveillance of syndromes in community
- To identify the host factors, seasonality patterns, and spatial distribution

Work done

A project on influenza like illness and dengue like illness syndromic surveillance in Janata Vasahat slum in Pune city was prepared on the backdrop of continuing pandemic. The project was approved by the SAC 2009. The project document was finalized after meetings with community representatives, health officials and workers. Study protocol and study documents were prepared and submitted for review by Ethics Committee.

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Hepatitis Group

Projects

- Development of candidate vaccine for hepatitis E and development of combination vaccine for hepatitis B and E viruses
- Innate and adaptive immune response in Hepatitis E (HE) infection
- Construction of genotype 1 (human)/ genotype 4 (swine) hepatitis E virus chimeras and their characterization *in-vivo* and *in-vitro*
- Study of processing and characterization of ORF1 encoded protein/s of hepatitis E virus
- Fulminant hepatitis E: Association with cytokine polymorphisms and viral sequence variations
- Toll like receptors in Hepatitis E virus infection.
- A multicentric randomized controlled clinical trial of Adefovir, Adefovir + Lamivudin and combination of Adefovir and Glycyrrhizin in HBV related decompensated cirrhosis
- Epidemic of Hepatitis B with high mortality in India: Association of fulminant disease with lack of CCL4 and Natural Killer T cells

Continued.....

Hepatitis Group

Projects

- Evaluation of immunogenicity of recombinant hypervariable region and non-structural region 3 of hepatitis C virus as vaccine candidates
- Evaluation of non-structural regions of Hepatitis C virus genotype 3 as immunogens
- Development of Hepatitis C virus replicons (genotype 3 based) for testing antiviral compounds
- Development of Chikungunya vaccine
- Study of IgG subclass profiles of anti-CHIKV in individuals with different Chikungunya virus infection profiles
- Studies on the roles of host factors in Chandipura virus infection
- Development of liposome encapsulated recombinant DNA/ protein vaccine(s) for H5N1 and other influenza viruses

Hepatitis E:

Development of candidate vaccine for hepatitis E

Development of combination vaccine for hepatitis B and E viruses

V. A. Arankalle, K. S. Lole, T. M. Deshmukh, M. Kulkarni, S. Srivastava, A. S. Tripathy

Hepatitis E is an important public health problem in India. Several epidemics have been reported all over the country causing morbidity and mortality in pregnant women. In sporadic settings, fulminant hepatitis E has been observed in men and non-pregnant women. Travelers to endemic areas, military personnel, elderly individuals, sewage workers etc. are at a high-risk of HEV infection. Therefore there is a need for development of hepatitis E vaccine. Further aim is to develop a combined vaccine for HEV and HBV. For that, combinations of HEV 'NE' and 'S' proteins of HBV were tested in mice with formulations made using different adjuvants.

Objectives

- To develop recombinant protein and/ or DNA based vaccine for hepatitis E
- To develop HEV and HBV combination vaccine

Work done

Evaluation of different adjuvants

A) Testing of MW adjuvant: Dose optimization of NE and HBV protein

Earlier experiments done using MW formulations with 1µg of each protein required three doses of the candidate vaccine to achieve 100% seroconversion in mice. These new set of experiments were done with MW formulations containing 5 and 10µg of each protein. Swiss albino mice (6-8 weeks old) were immunized with different formulations of HEV (NEp) and HBV protein (HBsAg) along with MW adjuvant with and without $Al.PO_4$.

Dose: 5µg and 10µg of protein (S and/or NE protein)

Dose schedule: 0, 4 and 8 weeks

Route of administration: Intramuscular (i.m.)

Mice: 10/ group

Antibody response was assessed by IgG ELISA against specific viral antigen of HBV and HEV. With 5 and 10µg of NE protein formulations, 90-100% seropositivity was obtained after three doses. While, 100% anti-HBsAg seropositivity was observed in mice immunized with 5 and 10µg HBsAg after two doses.

- When compared with HBsAg, NE protein (both 5 and 10µg formulations) showed delayed seroconversion.
- $Al.PO_4$ did not show any additional advantage for both antigens. Figure 1a and 1b depict results for NE and HBsAg respectively.

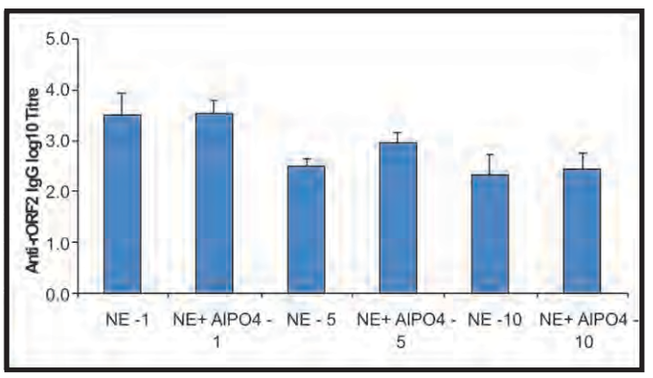

Figure 1a: Anti-HEV IgG titres in mice immunized with 1, 5 and 10µg NE formulations

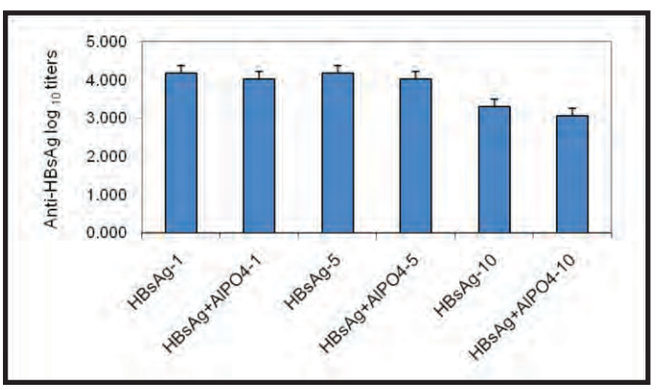

Figure 1b: Anti-HBV IgG titres in mice immunized with 1, 5 and 10µg HBsAg formulations

B) Evaluation of MW adjuvant with lower concentrations of NE protein

Dose optimization

Since MW formulations made with high amounts (5 and 10µg) of NE protein showed negative effect in terms of antibody titres, concentrations lower than 1µg were tested.

Dose: 1000, 500, 250 and 10 ng of NE protein along with 100µl of MW adjuvant

Schedule: 0, 4, 8 weeks

Mice: 10/ group

Route of administration: i.m.

Table 1: Week wise seroconversion of mice immunized with MW formulations of NE

Dose variation of NE protein	% seroconversion		
	4 weeks	8 weeks	10 weeks
MW + NEp (1000ng)	10	20	100
MW + NEp (500ng)	10	20	100
MW + NEp (250ng)	0	0	100
MW + NEp (10ng)	0	0	30

1. The IgG titres decreased significantly as NE protein concentration decreased from 1000ng to 10ng. Antibody titres and % seroconversion rates for 1000 and 500ng were found to be similar (table 1, fig.2).
2. Isotype analysis showed predominance of IgG1 antibodies as compared to IgG2a in mice immunized with 1000ng NE protein, while those immunized with 500 and 250ng NE protein showed balanced IgG1/IgG2a titres.
3. Cytokine analysis showed higher levels of INF-γ and TNF-α in 1000ng NE protein group, while 500 and 250ng groups showed elevated levels of TNF-α alone.
4. NE protein at concentration of 1000 and 500ng showed optimal level of antibody titres and cytokine levels.

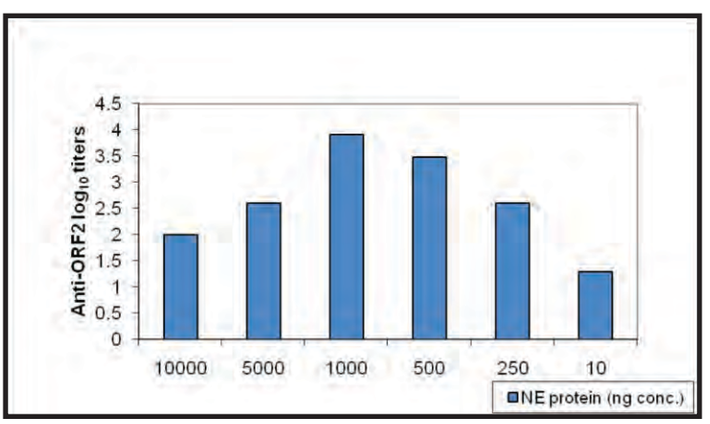

Figure 2: Anti-HEV (ORF2) IgG titres in mice immunized with different concentrations of NE with MW

C) Evaluation of other adjuvants: Cad-B, Cad-G5 and Cad-G6:

Three other adjuvants- Cad-B, Cad-G5, Cad-G6 were used to make different formulations containing NE DNA, NE protein (DP) and HBs DNA, HBsAg protein (DP). Swiss albino mice were immunized with these formulations and monitored.

Route of immunizations: Both i.m. and s.c. for Cad-B and i.m. for Cad-G5 and Cad-G6.

No. of Mice per group: six mice per group.

Dose scheduled: Rapid, 0, 2, 4 weeks.

1. Among the 3 adjuvants evaluated, Cad-B and Cad-G5 were found to give better percent seroconversion rates (table 2) as compared to Cad-G6.
2. Adjuvant Cad B showed good antibody titres for NEp group which were significantly enhanced with NEp +NE DNA (DP) combination (fig.3).
3. Adjuvant Cad-B also showed high anti-HBsAg antibody titres for HBsAg group and combo DP group (fig.4).
4. Adjuvant Cad-G5 showed good antibody titres for NEp group but comparatively lower titres for NEp +NE DNA combination (fig. 3).
5. Both Cad-B and Cad-G5 adjuvants showed low anti-ORF2 antibody titres in NEp + HBsAg group and combo DP group (NEp +NE DNA+HBsAg + HBV DNA) while comparatively high titres were recorded for NEp +NE DNA combination (fig. 3, 4).
6. Adjuvant Cad-G5 showed high anti-HBs antibody titres for HBsAg protein group and HBsAg protein + S DNA group as compared to combo DP group.
7. Cad-B showed predominance of IgG1 type of antibodies indicating Th2 type of immune response in NE protein as well as in NE DNA+ protein groups (fig. 5).
8. Cad-B adjuvant showed predominance of IgG1 type of antibodies indicating Th2 type of immune response against NE protein in all the four groups. For HBsAg antigen, Cad-B showed predominance of IgG1 and 2b of antibodies indicating Th2 type of immune response while addition of DNA with protein, in the combination group a balanced Th1/ Th2 response was noted.

Table 2: Seroconversion of mice immunized with formulations made with Cad-B, Cad-G5 and Cad-G6 adjuvants

Group: (NE antigen)	2weeks	4 weeks	6 weeks
Cad-B+rNEp (i.m.)	83.33	100	100
Cad-B+rNEp (s.c.)	66.66	83.33	83.33
Cad-B+ NEDP (i.m.)	66.66	100	100
Cad-B+ NEDP (s.c.)	33.33	83.33	83.33
Cad-B rNEp+HBsAg (i.m.)	33.33	50	100
Cad-B rNEp+HBsAg (s.c.)	50	50	100
Cad-B combo DP (i.m.)	50	50	16.66
Cad-B combo DP (i.m.)	50	50	16.66
Cad-B combo DP (s.c.)	0	0	0
Cad-B NE DNA (i.m.)	83.33	66.6	0
Cad-B NE DNA (s.c.)	0	16.6	16.6
Cad-G5 rNEP (i.m.)	100	100	100
Cad-G5 NEDP (i.m.)	50	100	100
Cad-G5 rNEp+HBsAg (i.m.)	50	83.3	100
Cad-G5 Combo DP (i.m.)	33.33	83.3	100
Cad-G5 NE DNA (i.m.)	66.66	33.33	0
Cad-G6 rNEp (i.m.)	83.3	100	100
Cad-G6 rNEp+HbsAg (i.m.)	0	16.6	33.3
Group: (HBsAg antigen)	2wk	4 wk	6 wk
Cad-B-HBsAg(i.m.)	33.33	100	100
Cad-B-HBsAg(s.c.)	33.33	100	100
Cad-B S-DP(i.m.)	50	100	100
Cad-B S-DP(s.c.)	16.6	83.33	100
Cad-B-rNEp+HBsAg(i.m.)	50	100	100
Cad-B-rNEp+HBsAg(s.c.)	50	100	100
Cad-B Combo DP(i.m.)	33.33	100	100
Cad-B Combo DP(s.c.)	16.6	100	100
Cad-G5 HBsAg(i.m.)	0	100	100
Cad-G5 S-DP(i.m.)	0	100	100
Cad-G5-NEp+HBsAg(i.m.)	0	100	100
Cad-G5 Combo DP(i.m.)	0	83.33	100
Cad-G6 HBsAg(i.m.)	0	100	100
Cad-G6-NEp+HBsAg(i.m.)	16.6	83.33	83.33

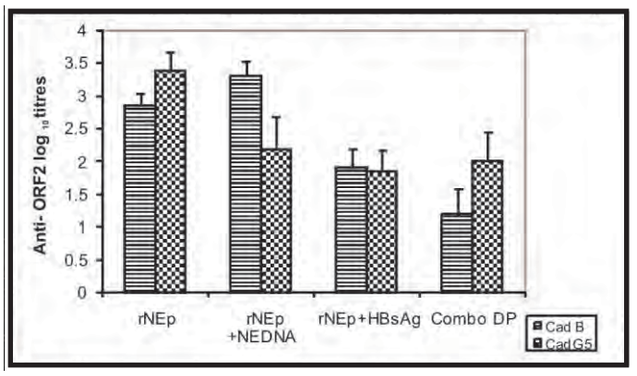

Figure 3: Anti ORF2 titres in mice immunized with Cad-B or Cad-G5

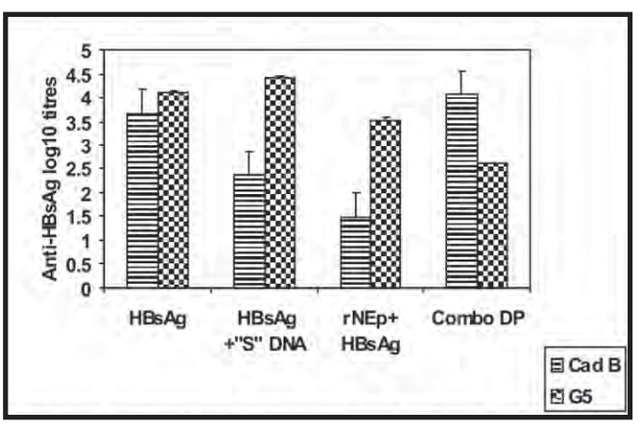

Figure 4: Anti HBsAg titres against HBsAg protein with Cad-B and Cad-G5

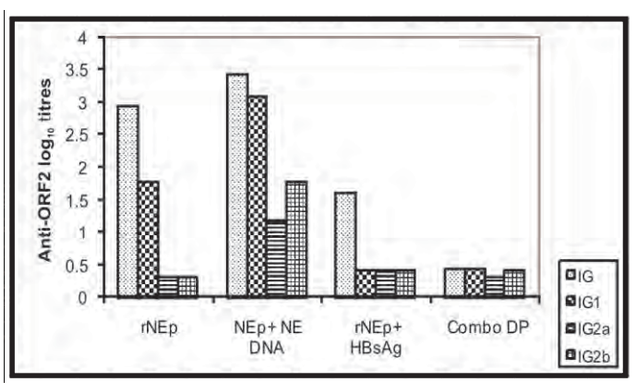

Figure 5: Anti-HEV (ORF2) IgG isotype analysis in mice immunized with Cad-B formulations

D) Evaluation of immunogenicity of different partial protein constructs of HEV genotype 4 ORF2

It was earlier observed by us that HEV genotype 4 (swine) neutralizing epitope region (NE) of ORF2 was less immunogenic in mice when compared to of genotype 1 (human) NE. On contrary, complete ORF2 proteins of genotype 1 (human) and genotype 4 (swine) HEV were found to be equally efficient in detecting anti-HEV antibodies of both type 1 or type 4 infections in ELISA. To identify the immunogenic region within the type 4 ORF2; eight subgenic genotype 4 ORF2 constructs were made in pET15b, proteins were expressed, purified and tested in Swiss albino mice for immunogenicity (fig. 6).

i. Constructs:

I) NE region of swine ORF2 (450 bp) (1F7R), ii) NE+15 a.a at N-terminal (2F7R), iii) NE+30 a.a at N-terminal (3F7R), iv) NE+45 a.a at N-terminal (4F7R), v) NE+60 a.a at N-terminal (5F7R), vi) NE+75 a.a at N-terminal (6F7R), vii) NE+75 a.a at N-terminal +15 a.a at C-terminal (6F8R) & viii) NE+75aa at N-terminal+30 aa at C-terminal (6F9R).

ii. Groups of mice were immunized with the above 8 proteins. Type 1 NE, Type 1 ORF2 and Type 4 ORF2 proteins were also included for comparison

iii. Two different adjuvants (Cad-B and Cad-G5) were tested separately, for each set of proteins

Dose: 1 µg of each purified protein + Cad-B/ G5 as adjuvant

Route of administration: Intramuscular

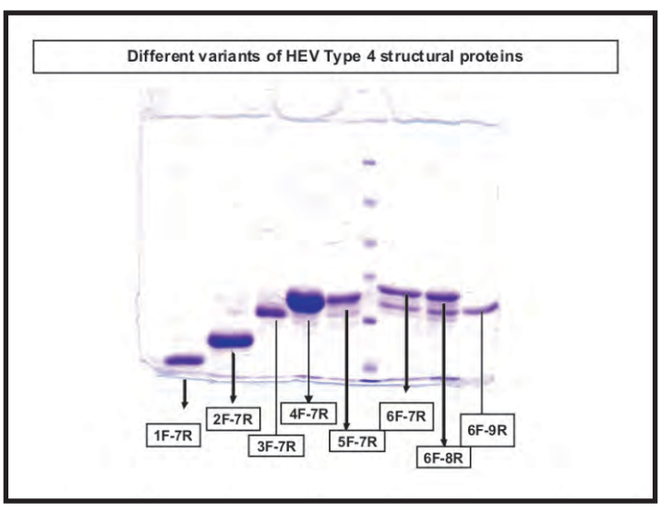

Figure 6: SDS-PAGE analysis of purified proteins

Table 3: Seroconversion of mice immunized with partial type 4 ORF2 proteins along with Cad-G5 adjuvant

Different ORF2 proteins +	No.of mice per group	% seroconversion 8th week	% seroconversion 10th week
1F-7R	8	62.5	62.5
2F-7R	8	37.5	50
3F-7R	8	50	75
4F-7R	8	37.5	100
5F-7R	8	75	75
6F-7R	8	0	37.5
6F-8R	8	50	50
6F-9R	8	25	75
Type 1 NE	8	50	100
Type 1 ORF2	8	75	100

Table 4: Seroconversion of mice immunized with partial type 4 ORF2 proteins along with Cad-B adjuvant

Different ORF2 proteins +	No.of mice per group	% seroconversion 8th week	% seroconversion 10th week
1F-7R	8	87.5	62.5
2F-7R	8	87.5	62.5
3F-7R	8	75	62.5
4F-7R	8	100	100
5F-7R	8	87.5	50
6F-7R	8	87.5	25
6F-8R	8	100	87.5
6F-9R	8	100	62.5
Type 1 NE	8	87.5	100
Type 1 ORF2	8	87.5	87.5

Amongst all the 8 tested proteins, 45N-NE (4F-7R) protein showed better seroconversion rates along with high antibody titres as compared to other type 4 structural protein constructs. Further characterization is in progress.

E) Immunogenicity of NE region of genotype 1 (HEV) and the corresponding region in genotype 4 HEV

V. A. Arankalle, T. M. Deshmukh, P. B. Devhare, K. S. Lole

Earlier, we assessed vaccine candidates based on genotype 1 HEV (complete or truncated ORF2 DNA and/or protein) in mice and Rhesus monkeys employing different approaches. We now compared the immunogenicity of the neutralizing epitopes region (458-607 a.a.) within the ORF2 (660 a.a.) of genotype 1 HEV (T1NE) and the corresponding region in genotype 4 HEV (T4NE) as vaccine candidates employing DNA prime protein boost and liposome based approaches.

Work done

Complete and truncated ORF2 proteins of HEV genotypes 1 and 4 (T1ORF2, T4ORF2, T1NE and T4NE) were expressed in baculovirus and bacterial systems respectively. The T1NE and T4NE genes were cloned in pVAX1 vector. The binding/neutralizing capacity of anti-HEV antibodies in mice sera with genotype 1 and 4 HEV was assessed in an in vitro T1ORF2, T4ORF2, T1NE and T4NE ELISA-based assay. The IgG isotype profile for each group of mice was determined using T1ORF2 ELISA.

a) DNA prime protein boost approach

Mice used: 6-8 weeks old female Swiss albino mice (n=120)

Dose schedule: 0, 4, 8 weeks; total 3 doses

Dose: 1µg DNA/ protein per dose

Adjuvant: Complete/ incomplete Freund's adjuvant (CFA/ IFA)

Route/ Method of administration: DNA by gene gun and protein by i.m. injection

Groups (n=10/ group):

1= pVAX1

2= PBS+CFA/ IFA

3= pVAX1 & T1NE protein

4= pVAX1 & T4NE protein

5= T1NE DNA

6= T1NE DNA & T1NE protein

7= T1NE DNA & T4NE protein

8= T4NE DNA

9= T4NE DNA & T4NE protein

10= T4NE DNA & T1NE protein

11= T1NE protein

12= T4NE protein

b) Liposome based approach:

Mice used: 6-8 weeks old female Swiss albino mice (n=30)

Dose schedule: 0 and 4 weeks; total 2 doses

Dose: 1µg each of DNA and protein co-entrapped in liposomes per dose

Route/ Method of administration: s.c.

1= T1NE DNA+T4NE protein

2= T4NE DNA+T4NE protein

3= T4NE DNA+T1NE protein

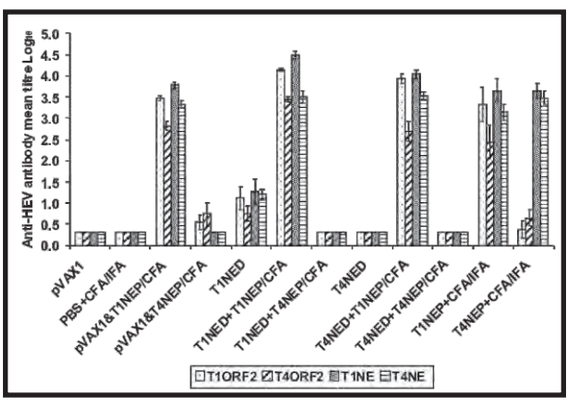

Figure 7: Anti-HEV antibody titres in the sera of immunized mice in T1ORF2, T4ORF2, T1NE and T4NE ELISAs (DNA prime protein boost approach)

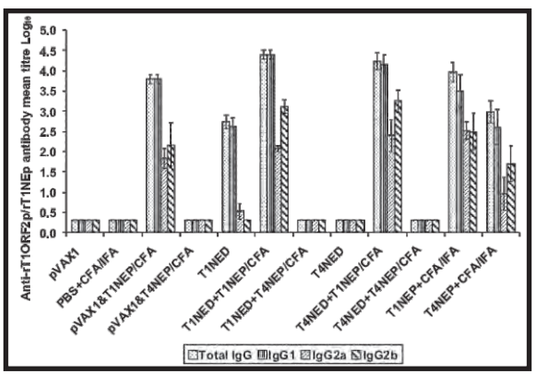

Figure 8: Anti-HEV IgG isotype profiles in immunized mice (DNA prime protein boost approach)

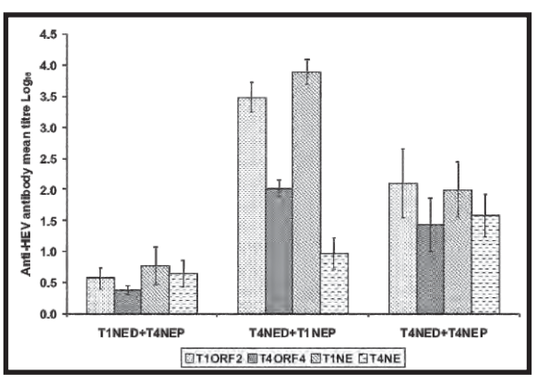

Figure 9: Anti-HEV antibody titres in immunized mice in T1ORF2, T4ORF2, T1NE and T4NE ELISAs (Liposome based approach)

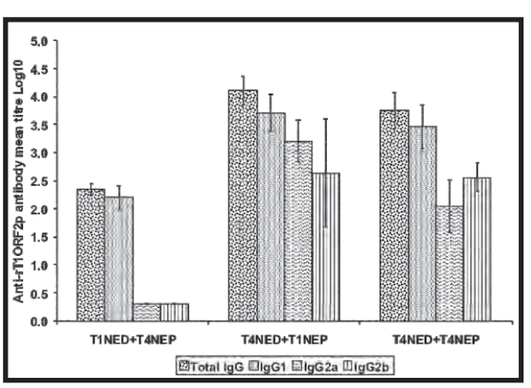

Figure 10: Anti-HEV IgG isotype profiles in immunized mice (Liposome based approach)

ELISA based in vitro HEV binding/ neutralization assay

In the absence of availability of an in vitro or in vivo neutralization assay for the detection/ titration of anti-HEV neutralizing antibodies in the immunized mice, an ELISA based HEV binding/ neutralization assay was done. A 10% (w/v) stool suspension containing 1.3×10^{11} and 2.9×10^{7} RNA copies of HEV/ ml were used as the virus sources from HEV patient (genotype 1 HEV) and inoculated pig (genotype 4 HEV) respectively. In T1ORF2 based ELISA, the mice pre-immune were scored negative. The post-immunization mice sera representing each group were diluted 2-folds serially, and equal volumes of the diluted sera were allowed to react with the virus positive stool suspension at 37°C for 1 hour. HEV specific antibodies in mice sera could bind/ neutralize genotype 1 HEV in an in vitro T1ORF2 ELISA based assay, whereas a partial binding/ neutralization of genotype 4 HEV with HEV specific antibodies in mice sera was noted.

Innate and adaptive immune response in Hepatitis E (HE) infection

A. S. Tripathy, R. Das, S. Rathod, V. A. Arankalle

Introduction

Hepatitis E virus causes self-limiting disease of varying severity from anicteric hepatitis to fulminant hepatitis (FH). Reports on characteristic features of fulminant hepatitis in Indian population suggest involvement of the host factors towards differential outcomes in FH. The role of humoral immune responses in protection has led to development of a candidate vaccine against hepatitis E. Very few studies have documented host cellular immune responses associated with HEV infection in humans. Both adaptive and innate immune responses are likely to be involved in the immunopathogenesis of liver disease. Preliminary work carried out on host viral factors in HEV infection suggests major involvement of these factors towards disease pathogenesis that need further exploration. Hence, it is essential to understand the role of host immune responses generated in patients during infection and recovery.

Work done

Total 154 individuals were included in the study (41 with acute hepatitis E infection, 16 during convalescent phase, 18 recovered cases and 79 healthy adults). All hepatitis E cases were from the hepatitis E outbreak in Tasgaon, Maharashtra. IFN-γ specific Elispot suggests probable involvement of virus specific CTLs in recovery. Significantly high levels of NKT cells were observed in the acute hepatitis E patients compared to controls, returning to normal during the recovery phase. We determined HLA class II frequencies for 73 hepatitis E patients and 31 ethnically matched controls. The frequencies of DQB1*05 and *06 were high in both patients and the controls.

Construction of genotype 1 (human)/ genotype 4 (swine) hepatitis E virus chimeras and their characterization *in-vivo* and *in-vitro*

K. S. Lole, V. A. Arankalle

In order to know viral genes responsible for species specificity and pathogenicity of HEV we are developing genotype 1 (human) and genotype 4 (swine) chimeric viruses. Infectious clone for type 1 and type 4 HEV have already been established earlier in the lab. When tested in hepatoma cell lines- HepG2 and Huh-7 both type 1 and type 4 infectious clones showed very low IFA positivity.

Work done

To increase the infectivity of the type 1 clone, two point mutations in the RdRp encoding region were removed by stepwise site directed mutagenesis. The new clone was then used for generation of subgenomic clone by replacing ORF2 with GFP gene. On transfection of different hepatoma cell lines HepG2 cells showed 3-5% GFP positivity indicating replication. Attempts are underway to separate these GFP positive cells by cell sorting.

Study of processing and characterization of ORF1 encoded protein/s of hepatitis E virus

K. S. Lole, Y. A. Karpe

Open reading frame one (ORF1) protein of HEV encodes nonstructural polyprotein with putative domains for methyltransferase, cysteine protease, helicase and RNA dependent RNA polymerase. It is not yet known whether ORF1 functions as a single protein with multiple domains or is processed to form separate functional units.

Objectives

- To understand the role of non-structural proteins encoded by open reading frame 1 (ORF1) of hepatitis E virus in its replication
- To study posttranslational processing of the ORF1 protein
- To characterize helicase domain of virus

Work done

Helicase is one of the most important and multifunctional proteins in viral life cycle. NTPase/ helicase domain of HEV was cloned and expressed in E. coli and purified. Enzyme hydrolyzed all rNTPs efficiently, dATP and dCTP with moderate efficiency while it showed less hydrolysis of dGTP and dTTP (fig.11 and 12). It showed unwinding of RNA duplexes with 5' overhangs showing 5' to 3' polarity (fig. 13). HEV helicase mutant I (Hel mut I), with substitution in the nucleotide-binding motif I; GKS to GAS showed 30% ATPase activity.Helicase mutant II with substitutions in the Mg^{2+} binding motif II (Hel mut II); DEAP to

AAAP, showed 50% ATPase activity. Both mutants completely lost ability to unwind RNA duplexes with 5' overhangs. In vitro assays are being carried out to test effect of different known inhibitors on HEV helicase activity.

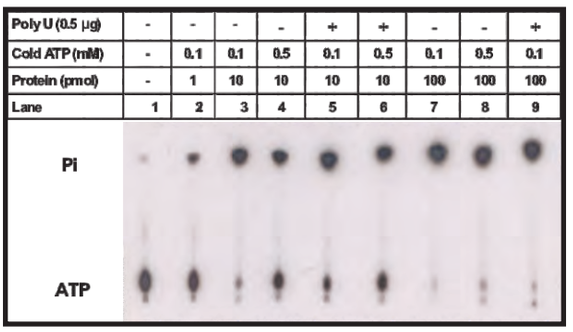

Figure 11: ATPase activity of recombinant HEV Hel protein

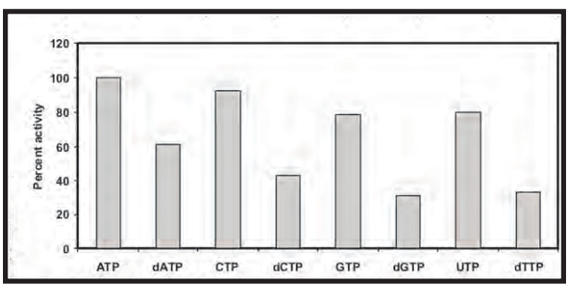

Figure 12: NTP hydrolysis

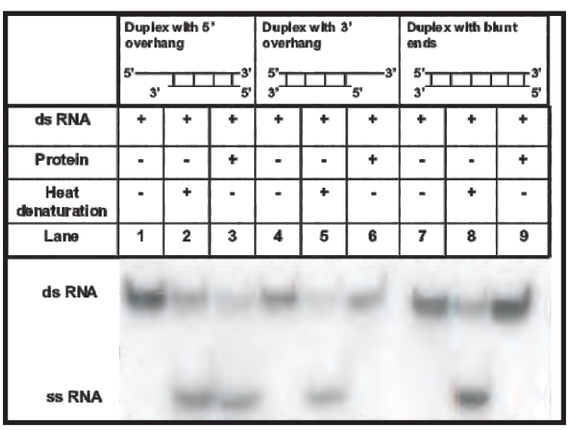

Figure 13: Strand displacement activity of HEV Helicase with RNA substrates

Fulminant hepatitis E: Association with cytokine polymorphisms and viral sequence variations

V. A. Arankalle, N. Mishra

Hepatitis E virus (HEV) is the predominant cause of acute viral hepatitis (AVH-E) and fulminant hepatic failure (FHF-E) among adults from developing countries. Pathogenesis of Hepatitis E is poorly understood. Earlier, we showed association of elevated serum levels of TNF-α, IFN-γ and IL-12 with FHF-E. This study investigates the role of TNF-α and IFN-γ gene polymorphisms with disease severity.

Work done

The study population included 374 healthy controls and 353 AVH-E, 136 subclinical hepatitis E, 25 FHF-E and 46 alcoholic liver disease patients presenting with FHF (FHF-ALD). Polymorphisms at promoter regions of TNF-α (-1031 and -308) and IFN-γ (-874) were investigated employing allelic discrimination/SNaPshot™ methods.

Table 5 describes the details of the study subjects. Table 6 describes the genotype frequencies in the TNF-α -308 G>A promoter region among different categories. When compared to healthy individuals, genotype -308-AA frequency was significantly higher among HEV-infected individuals presenting with sub-clinical, acute-recovering or FHF (p=0.007, 0.0001, 0.0107 respectively) with corresponding high OR values (0.08, 0.06 and 0.06). No significant difference was observed among the AVH-E/FHF-E and FHF-E/FHF-ALD categories. As history of alcohol intake was not available for the controls, no comparisons were made with FHF-ALD group. In addition, genotype -308 GG was significantly lower (p=0.017) in AVH than the control group.

Comparisons with respect to the genotypes on -1031 TNF-α locus (TT, TC or CC) is presented in Table 7. Genotype -1031CC was significantly higher among both AVH and FHF patients while TC was significantly lower among AVH group. No significant difference in FHF category may probably be due to small sample size. Thus, irrespective of outcome, the CC genotype favored clinical hepatitis E while genotype TC was associated with self-limiting disease. Similar pattern was noted when subclinical category was compared with AVH or FHF groups. However, no difference was recorded among AVH/FHF and FHF-E/FHF-ALD groups.

Table 8 presents the genotype frequencies of the three possible genotypes on IFN- +874 T>A locus (TT, TA or AA).Genotype +874TA was more frequent (p=0.005) in subclinical infection while AA frequency was lower in the AVH category (p=0.0032) when compared to the controls. FHF-E patients did not differ from the controls. Comparisons of subclinical cases with AVH or FHF-E categories revealed a significant difference in the frequencies of TT or TA genotypes (p=0.003, 0.025 and 0.026, 0.027 respectively), associating these genotypes with clinical disease. No significant difference was observed among the AVH-E and FHF-E groups. Genotype +874 TT was significantly higher in FHF-E than FHF-ALD patients.

Frequencies of the polymorphic allele for all the three positions are shown in table 9. At TNF-α-308, the A allele was significantly higher in all HEV-infected individuals (for FHF, p=0.058) when compared to controls, no difference emerging for other group comparisons. No difference was recorded at TNF-α -1031 position. At IFN-γ +874, significant difference was noted between controls/AVH, subclinical/AVH and FHF-E/FHF-ALD groups.

At TNF-α -308, carrier frequency (figure 14) was significantly higher in AVH group when compared to healthy controls (p=.0171). No difference was observed at TNF-α-1031 position. For IFN-γ +874 T>A locus, significantly increased carrier frequency was recorded in subclinical cases than AVH and FHF-E categories. Carrier frequency was significantly increased in FHF-ALD category in comparison to FHF-E (p=0.0145).

We further compared FHF-E recovered and FHF-E fatal patients with each other, though the numbers were small (Table 10). Except for the lower frequency of the GG genotype among FHF-F patients at -308 position (p=0.07), no significance difference emerged. Importantly, -308A allele frequency was higher among FHF fatal patients than the recovered category (p=.024). No significant difference was noted when the carrier frequencies at all the three positions were compared among both groups (figure 15).

The data reveals association of TNF-α -308 genotype with susceptibility to HEV and that of TNF-α -1031 and IFN-γ +874 with clinical disease, irrespective of the outcome. Higher -308A allele frequency was associated with susceptibility to HEV and fatal outcome of FHF.

Table 5: Characteristics of study groups

Parameters	Controls (a)	Subclinical(b)	AVH (c)	FHF-E (d)	FHF-ALD (e)	p-value
Number	374	136	353	25	46	
Age	31.96±15.83	37.48±18.18	38.74±16.22	25.00+3.39	39.93+16.84	
Sex (M : F)	251:123	8O : 56	196:157	16:09	46 : O	
ALT (IU/L)		44.69 ± 58.92	117.65 ± 125.48	814.0 ± 640.7	199.25 ± 98.26	<.00001 (b-c) , <.00001 (b-d), <.0001 (b-e), <.00001 (c-d), 0.015 (c-e), .0002 (d-e)
AST (IU/L)				465.83±303.7	269.55±394.77	0.069
D-bil (mg/dl)				6.69±4.20	3.33±3.32	0.0006
I-bil (mg/dl)				4.12±3.35	2.79±3.73	0.22
T-bil (mg/dl)				10.91±6.73	6.13±6.76	0.94

AVH- Acute Viral Hepatitis, FHF-E Fulminant Hepatic Failure Hepatitis E, FHF-ALD Fulminant Hepatic Failure Alcoholic Liver Disease, ALT- alanine aminotransferase, T-bil -Total Bilirubin, D-bil-Direct Bilirubin, I-bil -Indirect Bilirubin, AST- Aspartate aminotransferase

Table 6: The relationship of clinical presentations of Hepatitis E and frequencies of Polymorphisms in the TNF-α promoter region at -308 G>A Locus

TNF-α -308	Controls (n=374)	Subclinical (n=136)	OR (95 % CI)	p-value
GG	334(89.30%)	115(84.56%)	1.52(0.86-2.69)	0.144
GA	39 (10.43%)	17 (12.50%)	0.81(0.44-1.49)	0.508
AA	1 (0.27%)	4 (2.94%)	0.08 (0.009-0.79)	0.007
	Controls (n=374)	AVH (n=345)		
GG	334(89.30%)	287(83.19%)	1.68(1.09-2.60)	0.017
GA	39 (10.43%)	43 (12.46%)	0.81(0.51-1.29)	0.390
AA	1 (0.27%)	15 (4.35%)	0.06(0.007-0.44)	.0001
	Controls (n=374)	FHF-E (n= 25)		
GG	334(89.30%)	20(80.00%)	2.08(0.74-5.86)	0.155
GA	39 (10.43%)	4 (16.00%)	0.61(0.19-1.87)	0.385
AA	1 (0.27%)	1 (4.00%)	0.06(0.003-1.06)	0.0107
	Subclinical (n=136)	AVH (n=345)		
GG	115(84.56%)	287(83.19%)	1.10(0.64-1.90)	0.715
GA	17 (12.50%)	43 (12.46%)	1.00(0.55-1.82)	0.991
AA	4 (2.94%)	15 (4.35%)	0.66(0.21-2.04)	0.476
	Subclinical (n=136)	FHF-E (n= 25)		
GG	115(84.56%)	20(80.00%)	1.36(0.46-4.05)	0.569
GA	17 (12.50%)	4 (16.00%)	0.75(0.22-2.45)	0.633
AA	4 (2.94%)	1 (4.00%)	0.72(0.07-6.79)	0.779
	AVH (n=345)	FHF-E (n= 25)		
GG	287(83.19%)	20(80.00%)	1.23(0.44-3.42)	0.682
GA	43 (11.08%)	4 (16.00%)	0.74(0.24-2.28)	0.608
AA	15 (4.35%)	1 (4.00%)	1.09(0.13-8.61)	0.934
	FHF-ALD (n=46)	FHF-E (n= 25)		
GG	38(82.61%)	20(80.00%)	0.95(0.25-3.54)	0.939
GA	6 (13.04%)	4 (16.00%)	0.78(0.19-3.10)	0.680
AA	2 (4.35%)	1 (4.00%)	1.09(0.093-12.6)	0.945

Table 7: The relationship of clinical presentations of Hepatitis E and frequencies of Polymorphisms in the TNF-α promoter region at -1031 T>C Locus

TNF-α -1031	Controls (n=374)	Subclinical (n=136)	OR (95 % CI)	p-value
TT	227(60.70%)	78(57.35%)	0.87(0.58-1.29)	0.49
TC	136(36.36%)	51(37.50%)	1.04(0.69-1.57)	0.81
CC	11(2.94%)	7(5.15%)	1.79(0.67-4.71)	0.23
	Controls (n=374)	AVH (n=345)		
TT	227(60.70%)	217(62.90%)	1.09(0.81-1.48)	0.54
TC	136(36.36%)	90(26.09%)	0.61(0.44-0.85)	0.003
CC	11(2.94%)	38(11.01%)	4.08(2.05-8.12)	0.0001
	Controls (n=374)	FHF- E (n= 24)		
TT	227(60.70%)	13(54.17%)	0.76(0.33-1.75)	0.52
TC	136(36.36%)	7 (29.17%)	0.72(0.29-1.78)	0.47
CC	11(2.94%)	4 (16.67%)	6.59(1.92-22.57)	0.0008
	Subclinical (n=136)	AVH (n=345)		
TT	78(57.35%)	217(62.90%)	0.79(0.52-1.11)	0.26
TC	51(37.50%)	90(26.09%)	1.70(1.11-2.59)	0.013
CC	7(5.15%)	38(11.01%)	0.43(0.19-1.00)	0.046
	Subclinical (n=136)	FHF- E (n= 24)		
TT	78(57.35%)	13(54.17%)	1.13(0.47-2.72)	0.77
TC	51(37.50%)	7 (29.17%)	1.45 (0.56-3.75)	0.43
CC	7(5.15%)	4 (16.67%)	0.27(0.07-1.01)	0.039
	AVH (n=345)	FHF- E (n= 24)	OR (95 % CI)	
TT	217(62.90%)	13(54.17%)	1.43(0.62-3.29)	0.39
TC	90(26.09%)	7 (29.17%)	0.85(0.34-2.13)	0.74
CC	38(11.01%)	4 (16.67%)	0.61(0.20-1.90)	0.39
	FHF-ALD (n=46)	FHF- E (n= 24)		
TT	24(52.17%)	13(54.17%)	0.92(0.34-2.48)	0.87
TC	12(26.09%)	7 (29.17%)	0.85 (0.28-2.57)	0.78
CC	10(21.74%)	4 (16.67%)	1.38(0.38-5.00)	0.61

Table 8: The relationship of clinical presentations of Hepatitis E and frequencies of Polymorphisms in the TNF-α promoter region at +874 T>A Locus

IFN-γ +874	Controls (n=374)	Subclinical (n=136)	OR (95 % CI)	p-value
TT	106 (28.34 %)	28 (20.59 %)	1.52(0.95-2.44)	0.079
TA	178 (47.59 %)	84 (61.67 %)	0.56(0.37-0.83)	0.005
AA	90 (24.06%)	24 (17.65 %)	0.56(0.37-0.83)	0.124
	Controls (n=374)	AVH (n=353)		
TT	106 (28.34 %)	121(34.28%)	0.75(0.55-1.03)	0.085
TA	178 (47.59 %)	178(49.86%)	0.89(0.66-1.19)	0.445
AA	90 (24.06%)	54(15.21%)	1.75(1.20-2.55)	0.0032
	Controls (n=374)	FHF- E (n= 24)		
TT	106 (28.34 %)	10(41.67%)	0.55(0.23-1.28)	0.163
TA	178 (47.59 %)	9(37.50%)	1.51(0.64-3.54)	0.336
AA	90 (24.06%)	5(20.83%)	1.20(0.43-3.31)	0.718
	Subclinical (n=136)	AVH (n=353)		
TT	28 (20.59 %)	121 (34.28 %)	0.49(0.31-0.79)	0.003
TA	84 (61.67 %)	178(50.42%)	1.58(1.06-2.37)	0.025
AA	24 (17.65 %)	54 (15.21%)	1.18 (0.70-2.01)	0.524
	Subclinical (n=136)	FHF- E (n= 24)		
TT	28 (20.59 %)	10(41.67%)	0.36(0.14-0.90)	0.026
TA	84 (61.67 %)	9(37.50%)	2.69(1.09-6.59)	0.027
AA	24 (17.65 %)	5(20.83%)	0.81(0.27-2.39)	0.709
	AVH (n=353)	FHF- E (n= 24)		
TT	121 (34.28 %)	10(41.67%)	0.73(0.31-1.69)	0.462
TA	178(50.42%)	9(37.50%)	1.69(0.72-3.97)	0.221
AA	54 (15.21%)	5(20.83%)	0.68(0.24-1.91)	0.470
	FHF-ALD (n=46)	FHF- E (n= 24)		
TT	7(15.22%)	10(41.67%)	0.25(0.08-0.78)	0.015
TA	20(43.48%)	9(37.50%)	1.28(0.46-3.52)	0.630
AA	19(41.30%)	5(20.83%)	2.67(0.84-8.41)	0.087

Table 9: Allele Frequencies in TNF-α (-308A & -1031C allele) and IFN-γ (+874A) genes in hepatitis E patients presenting with different clinical manifestations

Alleles	Controls (a)	Subclinical (b)	AVH (c)	FHF-E (d)	FHF-ALD (e)	OR (95 % CI)*	p value*
TNF-α -308 G>A	(n=374)	(n=136)	(n=345)	(n=25)	(n=46)	0.57(0.34-0.96)	0.0334(a-b)
G	94.52	90.80	89.42	88.00	89.13	0.49(0.32-0.72)	0.0006(a-c)
A*	5.48	9.20	10.58	12.00	10.87	0.43(0.17-1.05)	0.058(a-d)
						0.86(0.53-1.37)	0.52(b-c)
						0.74(0.28-1.91)	0.53(b-d)
						0.87(0.35-2.10)	0.75(c-d)
						1.12(0.38-3.28)	0.83(d-e)
TNF-α -1031 T>C	(n=374)	(n=136)	(n=345)	75.94	(n=46)	0.85 (0.61-1.18)	0.34(a-b)
(n=24)	T	78.88	76.10	68.75	65.21	0.84 (0.65-1.08)	0.18(a-c)
C*	21.12	23.90	24.06	31.25	34.79	0.65(0.34-1.21)	0.17(a-d)
						0.99(0.71-1.37)	0.95(b-c)
						0.69(0.35-1.35)	0.27(b-d)
						0.70(0.36-1.31)	0.26(c-d)
						0.85(0.40-1.79)	0.67(d-e)
IFN-γ +874 T>A	(n=374)	(n=136)	(n=353)	(n=24)	(n=46)	0.97(0.73-1.28)	0.85(a-b)
T	52.14	51.47	59.49	60.41	36.96	1.35(1.09-1.65)	0.0041(a-c)
A*	47.86	48.53	40.51	39.59	63.04	1.40(0.77-2.54)	0.26(a-d)
						1.38(1.04-1.83)	0.0233(b-c)
						1.44(0.76-2.68)	0.25(b-d)
						1.04(0.57-1.88)	0.89(c-d)
						0.38(0.18-0.78)	0.008(d-e)

*frequency and statistical analysis with respect of polymorphic allele

Table 10: Genotype and allelic frequencies among recovered and fatal FHF-E patients

Genotype frequency	(a)	(b)	(c)	OR (95 % CI)	p value	Allele Frequency (%)	OR (95 % CI)	p value
TNF-α -308	GG	GA	AA			A allele		
FHF-E-recovered (n=18)	16 (88.89%)	2 (11.11%)	0	6.0 (0.73-48.90)	0.07(a)	2(5.56%)	0.16 (0.02-1.35)	0.024
FHF-E-fatal (n=7)	4 (57.14%)	2 (28.57%)	1 (14.29%)	0.31 (0.03-2.82)	0.28(b)	4(28.57%)		
					0.10(c)			
TNF-α -1031	TT	TC	CC			C allele		
FHF-E-recovered (n=18)	10 (55.56%)	5 (27.78%)	3 (16.67%)	1.25 (0.19-7.95)	0.81(a)	11 (30.56%)	0.88 (0.21-3.54)	0.85
FHF-E-fatal (n=6)	3 (50.00%)	2 (33.33%)	1 (16.67%)	0.76 (0.10-5.60)	0.79(b)	4(33.33%)		
				1 (0.08-11.93)	1(c)			
IFN-γ +874	TT	TA	AA		0.63(a)	A allele		
FHF-E-recovered (n=18)	8 (44.44%)	7 (38.89%)	3 (16.67%)	1.60 (0.23-11.08)	0.80(b)	13 (36.11%)	0.56 (0.15-2.11)	0.39
FHF-E-fatal (n=6)	2 (33.33%)	2 (33.33%)	2 (33.33%)	1.27 (0.18-8.89)	0.38(c)	6 (50.0%)		
				0.4 (0.04-3.27)				

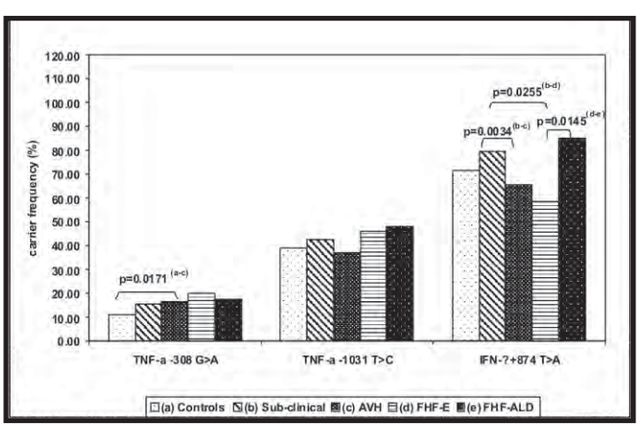

Figure 14: Carrier frequencies of TNF-α polymorphisms (-308A and -1031C) and IFN- γ Polymorphism (+874A) among Controls, Subclinical, AVH, FHF-E and FHF-ALD categories

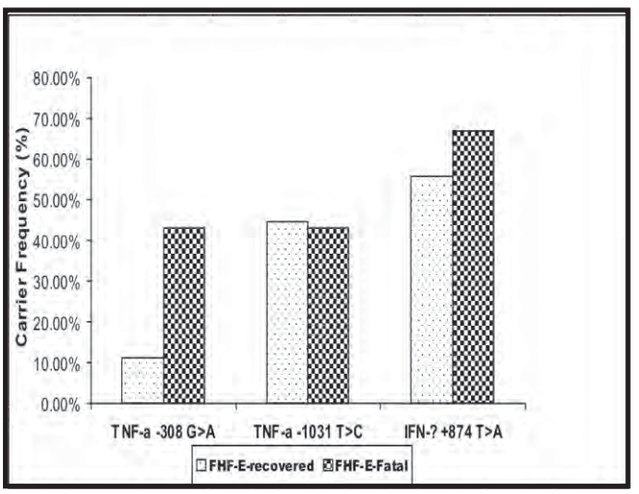

Figure 15: Carrier frequencies of TNF-α polymorphisms (-308A and-1031C) and IFN-γ Polymorphism (+874A) among recovered and fatal FHF-E patients

Toll like receptors in Hepatitis E virus infection

V. A. Arankalle, R. Arya

In vertebrates innate immune response is the first line of defense against invading microorganism. The main players in innate immunity are neutrophils, macrophages and dendritic cells. These cells can discriminate between pathogens and self by utilizing signals from the Toll like receptors (TLRs). Stimulation of TLRs causes an immediately defensive response, including the production of an array of antimicrobial peptides and cytokines. TLRs have been shown to play an important role in the up/ down regulation of genes, in hepatitis A, B and C viruses. In context of HEV, no work has been reported.

Work done

Blood samples were collected from apparently healthy individuals and from Hepatitis E patients. Cell surface and intracellular staining was performed on whole blood using anti-human TLR 2, 4, 3, 7, 8 and 9 antibodies. Stained cells were analyzed by using FACS Calibur flow cytometer. Results were collected for 10,000 cells. Lymphocytes were gated and analyzed by using FACS Diva software and expression was recorded in median fluorescence intensity (MFI).

In Hepatitis E patients, the level of TLR 2, 4, 7 and 8 were significantly increased (p=0.03, 0.00, 0.01, 0.00) while TLR 3 levels were decreased (p=0.00) with respect to healthy control. TLR 9 levels were comparable in the patients and controls.

Hepatitis B

A multicentric randomized controlled clinical trial of Adefovir, Adefovir+ Lamivudin and combination of Adefovir and Glycyrrhizin in HBV related decompensated cirrhosis

V. A. Arankalle

Considering the importance and burden of chronic hepatitis B and cirrhosis in India, ICMR is conducting a multi-centric clinical trial. NIV is responsible for the virology component i.e., to determine HBV DNA positivity, viral load and genotype.

Objectives

- Virological monitoring of the patients enrolled in the clinical trial.

Work done

During the current year, a total of 584 blood samples were received from different collaborative centers. These included 86 samples from day 0, 80 samples from 12 weeks, 84 samples from 24 weeks, 85 samples from 36 weeks, 80 samples from 48 weeks, 82 samples from 60 weeks and 82 samples from 72 weeks therapy. 251/ 584 samples were positive and 333/ 584 were negative for HBV DNA. Among positive samples, 60/ 251 were day 0 samples which were further processed for genotype analysis. Thirty samples belonged to genotype D, 20 to genotype A and 10 were of genotype C.

As the follow up of sizable number of patients is complete/nearing completion, full genomes of responders, non-responders and transient responders are being sequenced to understand the role of viral mutations in determining outcome of different therapies.

Epidemic of Hepatitis B with high mortality in India: Association of fulminant disease with lack of CCL4 and Natural Killer T cells

A. S. Tripathy, R. Das, V. A. Arankalle

Immunological/molecular pathways leading to death/recovery in HBV infection are not well understood. In 2009, an explosive outbreak of Hepatitis B with high mortality, in Modasa, Gujarat was investigated by NIV. An association of pre-core and basal core promoter mutants with fulminant outcome of the disease was shown. This study addresses involvement of some of the host factors.

Objectives

- To study different immunological parameters in acute and fulminant hepatitis B patients from Modasa

Work done

The study population comprised of 22 acute viral hepatitis B (AVH-B), 13 fulminant

hepatitis B (FHF-B) and 54 healthy controls. Samples from these patients were processed for measuring HBsAg induced CTL responses by ELISPOT, cytokine and chemokine quantitation by Bioplex assay and peripheral NK, NKT, CD4 and CD8 T cell frequencies by flow cytometry. The median percentage of NK cells in the lymphocytes of the AVH-B and FHF-B patients were significantly lower as compared to controls. AVH-B and FHF-B patients respectively had significantly high and comparable NKT cells when compared to controls. Importantly, NKT cells were significantly lower in FHF-B than AVH-B patients. Circulating peripheral CD4/CD8 T-cell subsets among the patient categories and controls were comparable. In AVH-B category, a significant increase in IFN-γ release was recorded (ELISPOT) by the unstimulated, antigen stimulated and mitogen stimulated cells when compared to controls. IFN-γ release was significantly less in FHF-B patients than the AVH-B patients. Comparisons of cytokines and chemokines among the disease categories revealed significantly lower levels of CCL4 in FHF-B patients. The results suggest importance of NKT cells and CCL4 in limiting HBV infection and need for further evaluation.

Hepatitis C

Evaluation of immunogenicity of recombinant hypervariable region and non-structural region 3 of Hepatitis C virus as vaccine candidates

V. A. Arankalle, G. M. Gupte.

Hepatitis C virus, a major causative agent of chronic hepatitis affects more than 170 million individuals worldwide, and can evolve towards cirrhosis and hepatocellular carcinoma. Therapeutic treatments consisting of pegylated interferon alpha and ribavirin are effective in 50-80% of cases and are associated with severe side effects. Although several attempts for the development of vaccine have been made, no efficacious vaccine could be developed as yet. Hence, development of an effective vaccine becomes imperative. Genotype 3 is most common in India. Most candidate vaccines are being developed employing genotype 1 strains prevalent in the developed countries. Considering the genetic heterogeneity of HCV between and within genotypes and in the same individual, it is essential to develop genotype 3 based vaccines.

Objectives

- To evaluate immunogenicity of Recombinant Hypervariable Region 1 (HVR1) and Non-structural region-3 (NS3) of genotype 3 Hepatitis C virus as possible candidate vaccines either as DNA or peptide polytope or as a combination of both.

Work done

HCV-HVR (27 amino acids) consensus sequence was obtained after comparing the sequences from 50 HCV positive samples belonging to genotype 3 (20 clones per sample). The

amino acid consensus sequence, along with 3 other variants of the consensus were commercially synthesized and used to perform ELISA. A serum panel consisting of 104 Negative and 106 positive sera (both confirmed with Ortho ELISA, RIBA, and PCR) were tested for the reactivity against 4 HVR variants separately or as a combined pool, in ELISA. None of the negative sera show any reactivity with any of the HVR variants. Reactivity of the positive sera was as shown in Table 11.

Table 11: Testing of reactivity of positive sera with different HVR variants:

	Number of samples negative (False negative)	Number of positive samples (% reactivity)
Genotype 1	3/20	17/20 (85%)
Genotype 3	4/80	75/80 (95%)
Genotype 4	-/5	5/5 (100%)
Genotype 6	-/1	1/1
Total	7/106	98/106 (93.39%)

Cloning and expression of NS3 in BL21 cells: HCV NS3 region was cloned in pVAX (mammalian expression vector) and pET15b (bacterial expression vector with N-terminal His tag). pET15b-NS3 construct was transformed into BL21 codon plus cells and protein was induced with IPTG (at 37C for 2 hours). The pellets were lysed in denaturing condition using guanidine lysis buffer and were allowed to bind to a nickel chelating (Probond) column at room temperature for 2 hr and the protein was eluted in native conditions using imidazole. Eluted protein was checked by western blot analysis (fig.16).

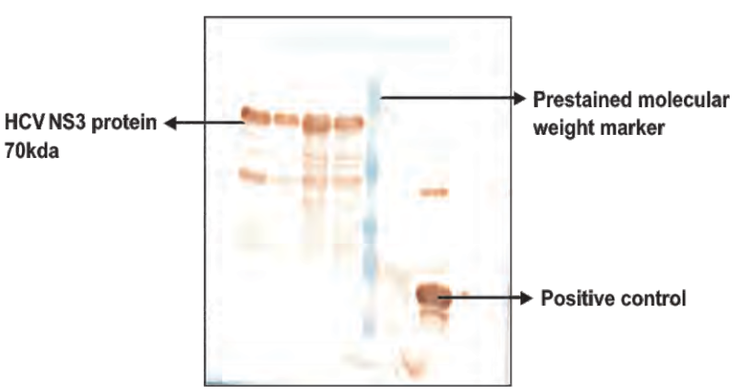

Figure 16: Western blot for HCV-NS3 using anti-histidine antibody

The NS3 protein obtained after partial purification with nickel-chelating column was further subjected to HPLC purification using gel filtration column (Superdex200). Pure protein was obtained as a single band (fig.17).

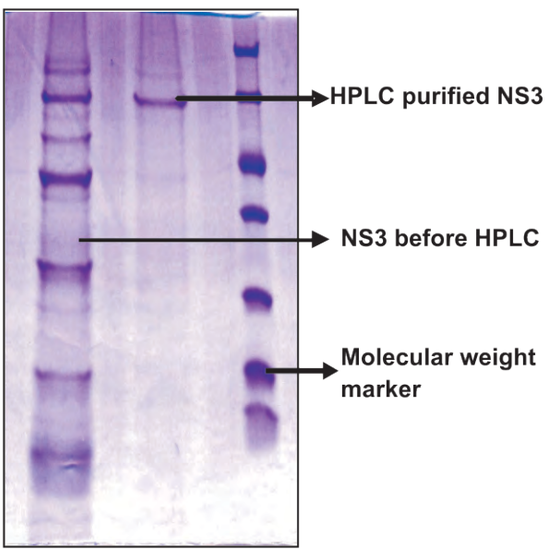

Figure 17: SDS-PAGE showing HPLC purified NS3 protein

Mice immunizations using NS3 protein and HVR peptide variants

4-6 Week old female Balb/c mice were used (8 per group) for the immunization experiments. The peptides (25µg/ dose) were encapsulated in liposomes (Cad-B) either with corresponding DNA or with NS3 protein. The NS3 protein was given along with four adjuvants, namely Cad-B, G5, G6 and MW. NS3 protein was also given in combination with its corresponding DNA along with Cad-B.

Peptide variants 1 and 4 showed a balanced antibody response as detected by serum antibody isotyping. Peptide variants 2 and 3 showed low titres of antibodies and serum isotypes could not be detected, indicating the need for increase in the amount of peptides used for immunization. The peptide pool groups also showed balanced immune response (both Th1 and Th2) based on serum antibody isotyping either when given with their corresponding DNA or when given with NS3 protein.

The antibody response against NS3 protein was relatively lower than the response against peptide groups, indicating the need for increasing the amount of NS3 protein to be given per dose. All the groups showed balanced immune response based on serum antibody isotyping titres. The response was best when NS3 was given along with peptide pool. There

was no significant difference in response against NS3 protein when it was either given alone or with corresponding DNA encapsulated in Cad-B (liposome). The responses against NS3 with addition of the four adjuvants (Cad-G5, Cad-G6, MW and Cad-B) were comparable.

Evaluation of non-structural regions of Hepatitis C virus genotype-3 as immunogens

V. A. Arankalle, A. Y. Ramdasi

Work done

- NS3a and NS4b regions of HCV 3a were cloned in pVAX1 vector.
- NS3+NS4a region was cloned in pGEMT-EASY vector.
- NS5a and NS5b regions were cloned in pET15b vector.

Expression of NS5b protein in E coli RIL Codon plus cells:

Cells were transformed with NS5b gene in pET15b vector. Induction was done with 0.5-1mM IPTG at 25°C, 27°C, 30°C and 37°C. Cells were harvested at different time-points. All the pellets were processed for protein purification using denaturing conditions and protein was checked by running a SDS-PAGE along with the controls. A protein band at expected size of 66kDa was seen for different cell pellets collected. Protein has to be purified further and confirmed by Western blot and ELISA.

Development of Hepatitis C virus replicons (genotype 3 based) for testing antiviral compounds

K. S. Lole, P. Aher

In the absence of efficient cell culture system and small animal model, HCV replicon has been extensively used as a tool for understanding the mechanisms of HCV replication, proliferation and for screening antiviral drugs. Due to the extensive genetic heterogeneity of HCV, it is desirable to develop self-replicating systems separately for each genotype. In the present study attempts will be made to develop genotype 3 based replicons for testing antiviral compounds

Objectives

- Development of infectious cDNA of Hepatitis C virus (Genotype 3 based)
- Testing of antiviral compounds using the infectious cDNA.

Work done

Complete genome of genotype 3a was amplified in 4 overlapping fragments. Since HCV exists as quasispecies within an individual infected with HCV, it was decided to find out the predominant circulating viral species in the selected patient's sample. For that, the amplified fragments were processed for TA cloning. Cloning and sequencing of 3 genomic

fragments (25 clones each) was completed for all the three fragments. Most common clone from each fragment would be finally selected for developing full genome cDNA clone.

Future plans

Cloning and sequencing of remaining region of HCV genome and construction of full genome cDNA clone of HCV.

Chikungunya

Development of Chikungunya vaccine

M. Singh, A.B. Sudeep, V. A. Arankalle

The recent resurgence of Chikungunya in the sub-continent in 2006 and continued activity in India warranted the need for an effective vaccine. Imporatntly, the disease caused by the African genotype was characterized by crippling sequalae of long duration and mortality in certain risk groups.

Objective

- To develop a vaccine against Chikungunya

Work done

Recombinant soluble E2 protein (rsE2) protein was expressed in BL21 codon plus RIL-DE3 cells and was purified using nickel chelating resin. Chikungunya virus was also purified and inactivated using BPL and formalin, which was further used for immunization of mice. Details of the mice experiments are given in the table 12.

Table 12: Mice immunization with different formulations

Groups (n=no of mice)	Type of vaccine candidate	Dose	Route of administration
Group1 (n=6)	rsE2	1µg with Al PO4	s.c.
Group2 (n=6)	rsE2	1 µg with Al PO4	i.m.
Group3 (n=6)	rsE2	2 µg with Al PO4	s.c.
Group4 (n=6)	rsE2	2 µg with Al PO4	i.m.
Group5 (n=6)	rsE2	5 µg with Al PO4	s.c.
Group6 (n=6)	rsE2	5 µg with Al PO4	i.m.
Group7 (n=6)	BPL inactivated CHIKV	100 µl	s.c.
Group8 (n=6)	BPL inactivated CHIKV	100 µl	i.m.
Group9 (n=6)	Formalin inactivated CHIKV	100 µl	s.c.
Group10 (n=6)	Formalin inactivated CHIKV	100 µl	I.m.
Group11 (n=6)	PBS	100 µl	S.c.

s.c.: Subcutaneous i.m.: Intramuscular

There was no seroconversion of immunized mice even after 3 doses of rsE2 whereas 100 % sero conversion was observed in mice immunized with BPL inactivated Chikungunya virus. In the second set of experiments, mice were immunized with rsE2 protein+ different adjuvants and chemically inactivated Chikungunya virus (BPL and Formalin) (table 13 and 14). Total 3 doses were given 4 weeks apart. Mice were bled every 14 days. Sera were collected and stored in −20° C until further use. ELISA and neutralization assays were carried out for the assessment of seroconversion and production of neutralizing antibodies.

Table 13: Comparative neutralizing antibody titres in mice immunized with recombinant E2 protein and inactivated CHIK virus vaccine formulations

Mice gr. (N=10)	Immunogen, (route)	Adjuvant	Neutralizing antibody titres			
			8wk	12wk	16wk	20wk
1	rsE2, 5µg (i.m.)	Cad-G5	7.96± 2.79	25.81± 7.81	11.44 ± 4.54	4.74 ± 3.08
2	rsE2, 5µg (i.m.)	Cad-G6	6.32± 2.85	42.87±8.12	9.28 ± 3.21	6.32 ± 3.18
3	rsE2, 5µg (i.m.)	MW	22.97± 2.93	12.34±4.66	15.89 ± 3.84	8.43 ± 3.08
4	rsE2, 5µg (i.m.)	Cad-B	24.08±4.10	24.08±4.10	15.89 ± 3.84	4.74 ± 3.08
5	BPL inactivated CHIKV , 5µg (i.m.)	-	40± 7.51	25.81±7.81	7.46 ± 3.37	6.32 ± 3.18
6	Formalin inactivated CHIKV, 5µg (i.m.)	-	40 ±6.02	16.28± 7.82	22.08 ± 2.65	6.32 ± 3.18
7	PBS (i.m.)	-	-	-	-	-

Table 14: Neutralizing antibody titres in mice immunized with inactivated vaccine vaccine formulations made with different adjuvants

Mice gr. (n=10)	Immunogen, (route)	Adjuvant	Neutralizing antibody titres **of IgG**	
			4 wk	12wk
1	BPL inactivated CHIKV, 5µg (i.m.)	Cad-G5	42.87 ± 3.79	17.55 ± 7.64
2	BPL inactivated CHIKV, 5µg (i.m.)	Cad-G6	45.95 ± 6.48	22.08 ± 2.65
3	BPL inactivated CHIKV, 5µg (i.m.)	MW	27.66 ± 6.08	10.36 ± 3.07
4	Formalin inactivated CHIKV, 5µg (i.m.)	Cad-G5	40 ± 7.51	29.72 ± 7.79
5	Formalin inactivated CHIKV, 5µg(i.m.)	Cag-G6	31.77 ± 9.31	19.37 ± 5.1
6	Formalin inactivated CHIKV, 5µg (i.m.)	MW	40 ±6.02	48.79 ± 16.66
7	PBS (i.m.)	-	-	-

Based on the results obtained so far, additional experiments in mice for optimizing immune response are underway.

Study subclass profiles of anti-CHIKV in individuals with different Chikungunya virus infection profiles

A.S. Tripathy, R. Rote, A.B. Sudeep, V.A. Arankalle

Infection with Chikungunya has varied clinical presentations in different individuals. Though, substantial evidence indicates that IgG antibodies to CHIKV play a role in protection from Chikungunya, the involvement of biologically different functional differences among the IgG subclasses and their association with the disease status has not been studied. Natural infection with Chikungunya in humans is associated with a Th2 mediated immune response, where as vaccine candidate immunized mice generate a mixed balance of Th1 and Th2 response.

Objectives

- To assess the IgG and IgG subclass responses to Chikungunya virus in Chikungunya infected individuals.

Work done

A total of 176 Chikungunya patients, i.e. 109 classical cases (46.6 ± 12.23 years), and 67 complicated cases (68± 13.46 years) were investigated. IgG and IgG1 titers were 3230 ±3641 and 1560 ±.2183 in the classical cases, 4742± 5372 and 2321 ± 2666 in encephalitis cases and 11083 ± 3763 and 8400±4333 in other systemic involvement (OSI) cases respectively. Seventeen of 36 OSI cases had IgG2 (75 ± 87) subclass of antibodies. Subclass profiles of anti-CHIKV in Chikungunya patients were predominantly of IgG1 isotype irrespective of clinical presentations, age, sex and post onset of days of illness. IgG and IgG1 levels were significantly higher in complicated cases compared to other two categories ($p<0.05$). Further studies are needed to analyze all the immunoglobulin subclasses of anti-E1 and anti-E2 proteins of Chikungunya virus to elucidate the association of the immune response and disease outcome/ progression in Chikungunya infection

Chandipura virus

Studies on the roles of host factors in Chandipura virus infection

P. Gupta Gangrade, A. S. Tripathy

Chandipura virus (CHPV) is a major causative agent of viral encephalitis in children. Till date, no vaccine/ specific therapy is available for it. The tumor necrosis factor (TNF-α) is a pro inflammatory cytokine that plays a key role in viral infections. The recovered individuals from CHPV infection are reported to have higher levels of TNF- α as compared to the

encephalitis cases. The antiviral activity of the same needs further exploration. Apart from cytokines, complement system, the central part of innate immune response, also plays an important role in host defense mechanism. Complement system protects the host from all pathogenic, non-self targets including viruses. Activation of the complement system occurs through classical pathway in Vesiculovirus, a prototype closely related to Chandipura virus. Due to lack of information regarding these aspects in CHPV infection this work was undertaken.

Objectives

- To explore the role of TNF α in immune modulation and viral pathogenesis
- To evaluate the role of complement system in CHPV infection.

Work done

Chandipura virus (AP strain isolate 034627) was propagated in RD cell line. $TCID_{50}$ of the stock virus was determined to be 7 by titration method. 10^6 countable plaques were observed in 10^{-5} dilution of virus by plaque forming assay. To find out the role of complement in CHPV infection, different dilution of CHPV were treated with normal healthy sera (IgG negative for CHPV) and kept at 37°C, followed by virus titration. It was observed that sera up to 1:2 and 1:250 dilutions were able to neutralize 10^{-5} and 10^{-6} titres of Chandipura virus respectively. This was further confirmed with plaque forming assay, where number of plaques were reduced in treated wells. Incubation of the virus at 37°C was found to have no effect on the virus titre. Further *in vitro* studies for assessing the role of complement system are underway.

Influenza virus

Development of liposome encapsulated recombinant DNA/ protein vaccine(s) for H5N1 and other influenza viruses.

V. A. Arankalle, K. S. Lole, S. Kolpe, V. Verma, N. Ingle, R. Virkar

This is a DBT funded project. H5N1 is considered a potential pandemic-causing pathogen. Preparation of killed vaccine using a virus containing HA and NA genes from the virulent strain is time-consuming and warrants alternative approaches to prepare vaccine(s) for virulent influenza virus(es), in case of a pandemic threat. Use of strain-specific HA gene and/or/ protein and liposome as an adjuvant may be one possibility. The present project proposes use of liposome encapsulated DNA and corresponding protein as monovalent, multivalent candidate vaccine(s) for H5N1 and other seasonal influenza viruses. For that initially H5N1 strain-specific haemagglutinin (HA)-based vaccine would be tested. Secondly, in order to prepare a vaccine generating broadly cross-reacting antibodies, conserved nucleoprotein (NP) and Matrix (M) genes from H5N1 (NIV strain) and currently circulating

H3N2 and H1N1 human strains available at NIV will be targeted.

Objectives

- To develop HA gene/protein/liposome based vaccine using H5N1 isolate from NIV representing the recent episode of avian influenza in India.
- To develop monovalent and multivalent vaccine formulations based on conserved NP and M genes using same approach i. e., encapsulation of DNA and corresponding protein components in liposomes.
- Both will be evaluated in mice and chicken for possible use in humans and birds.

Work done

Though H5N1 was the pandemic threat, a swine H1N1 (p-H1N1-09) virus emerged as the pandemic-causing agent. We therefore quickly changed to the development of a vaccine candidate for the pandemic virus employing a strain isolated by the Influenza group. In addition, as only one type of pathogen can be handled in the BSL-3 facility, work with the H5N1 strain had to be suspended.

Cloning of HA and NP genes from p-H1N1-09 strain:

The HA and NP genes of p-H1N1-09 influenza virus isolated at NIV were PCR amplified and cloned into both pVAX1 and pFASTBac1 vectors. All the clones were confirmed by sequencing and pVAX1 constructs were processed for large scale plasmid preparation. The pFASTBac1 clones were used for transformation of E. coli DH10 MaxBac cells for the generation of recombinant bacmids. The bacmids were confirmed using gene specific PCR and the confirmed bacmids were used for transfection of Sf9 cells. Expression of the proteins was confirmed by western blot analysis and in-house ELISA.

Purification of p-H1N1-09 HA protein:

Sf9 cell pellets, infected with recombinant p-H1N1-09 -HA were used for protein purification using HPLC system. Clarified cell lysate was loaded on to gel filtration column and fractions were collected (Fig. 18). All fractions were subjected to ELISA and HA reactivity was observed in fractions 11, 13 and 18. These fractions were concentrated, analysed on western blot and used for mice immunizations separately.

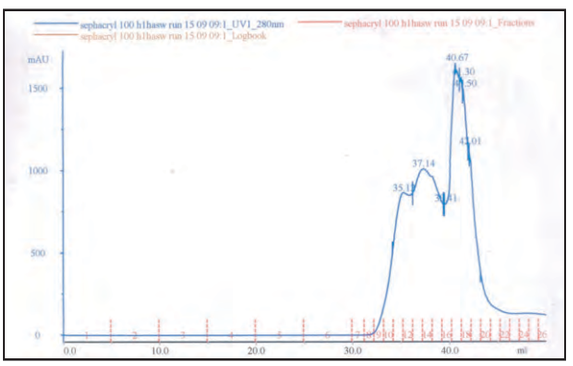

Figure 18: Purification of p-H1N1-09 HA protein by gel filtration chromatography.

Mice immunizations:

Female Balb/c mice (6-8 weeks old) were immunized with two doses of respective vaccine formulations (5 mice/ group) three weeks apart using different adjuvants (table 15). Ten days post 2nd dose, the mice were challenged via intranasal route with p-H1N1-09 virus and daily weights were taken. Pre and post challenge HI titres were also determined.

Table 15: Mice immunized with p-H1N1-2009 HA protein with different adjuvants

Group No.	Dose
1	5 µg Protein (*F 18) + Cad-B
2	5 µg Protein (F 18) + 5 µg HA DNA + Cad-B
3	5 µg Protein (F 18) + 5 µg HA DNA + 5 µg NP DNA + Cad-B
4	5 µg Protein (F 18) + Cad-G5
5	5 µg Protein (F 13) + Cad-G5
6	5 µg Protein (F 11) + Cad-G5
7	5 µg Protein (F 18) + Cad-G6
8	5 µg Protein (F 18) + MW
9	5 µg Protein (F 18) + Al (OH)3
10	2 µg Protein (F 18) + Cad-G5
11	Control (PBS)

*F: indicates fraction number

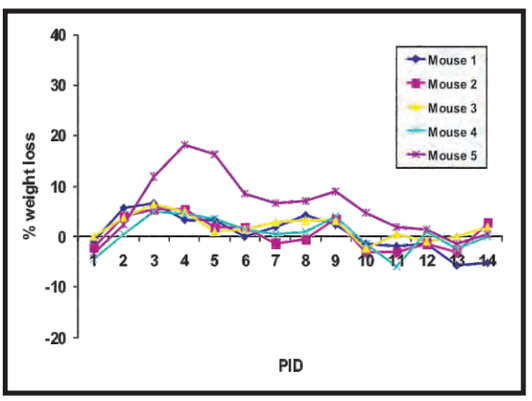

Figure 19a: Weight loss in mice immunized with Cad-B +HA Protein F-18 (5µg) + HA DNA (5µg)

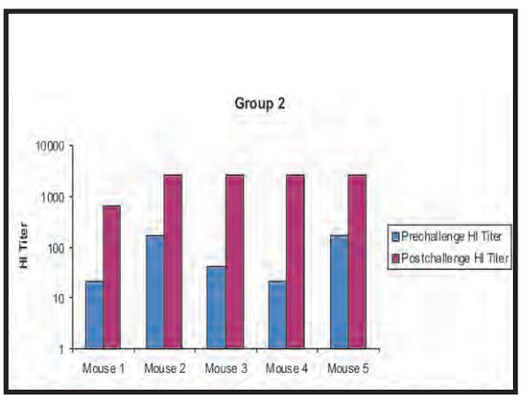

Figure 19b: HI titres in mice immunized with Cad-B+ HA Protein F-18 (5µg) + HA DNA (5µg)

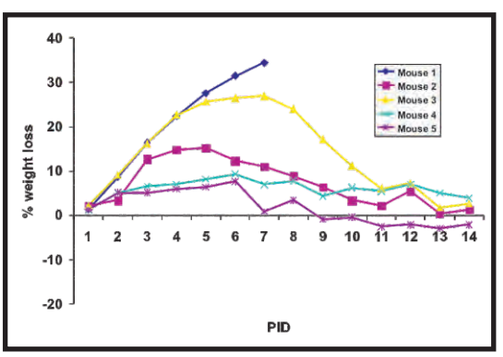

Figure 20a: Weight loss in mice immunized with Cad-G5+ HA protein F-18 (5µg)

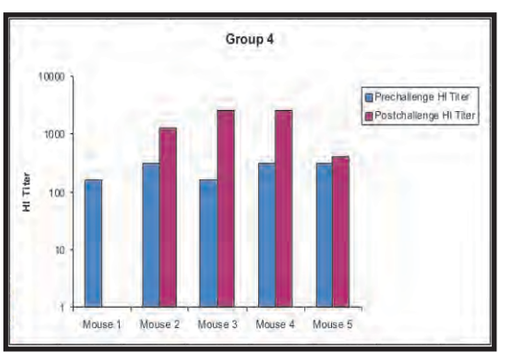

Figure 20b: HI titres in mice immunized with Cad-G5+ HA protein F-18 (5µg)

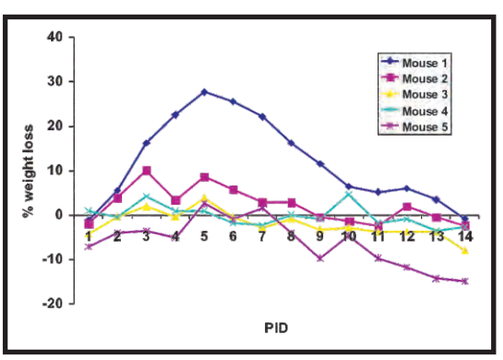

Figure 21a: Weight loss in mice immunized with Cad-G5+ HA protein F-13 (5µg)

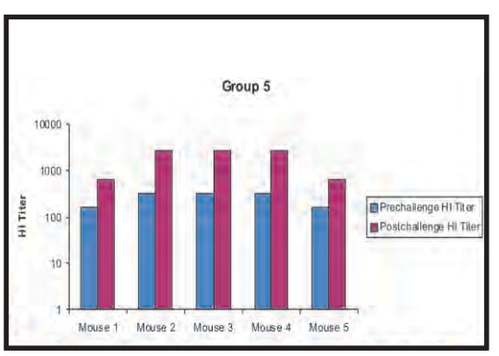

Figure 21b: HI titres in mice immunized with Cad-G5+ HA protein F-13 (5µg)

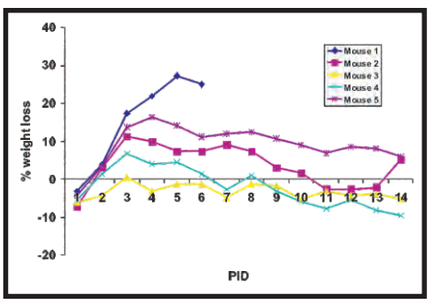

Figure 22a: Weight loss in mice immunized with Cad-G5+ HA protein F-11 (5µg)

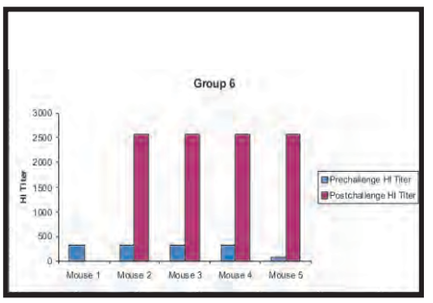

Figure 22b: HI titres in mice immunized with Cad-G5+ HA protein F-11 (5µg)

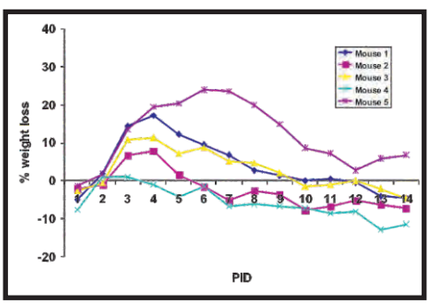

Figure 23a: Weight loss in mice immunized with MW+ HA protein F-18 (5µg)

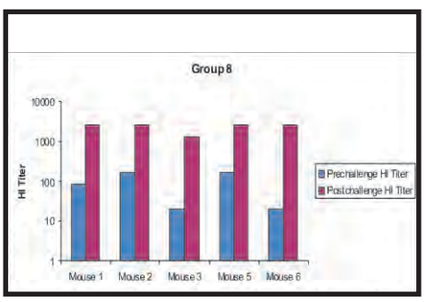

Figure 23b: HI titres in mice immunized with MW+ HA protein F-18 (5µg)

Minimum weight loss was observed in mice immunized with Cad-B+ HA Protein F-18 (5µg) + HA DNA (5µg). Average HI titres of this group were also high as compared other mice groups. To increase the HA protein yield and purity protein purification was also tried by lentil lectin affinity chromatography. This method resulted in better yields and purity of the protein (Fig. 24). Hence for subsequent experiments this HA protein was used.

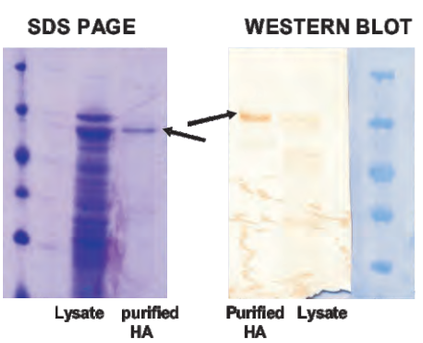

Figure 24: Purification of H1N1 HA protein by lentil lectin chromatography

Table 16: Mice immunized with p-H1N1-2009 HA protein with different adjuvants

Group No.	Dose	No. of mice/gr
1	1 µg Protein + Cad-G5	10
2	1 µg Protein + Cad-G6	10
3	1 µg Protein + MW	8
4	1 µg Protein + Cad-B	10
5	1 µg Protein + Al (OH)3	10
6	5 µg Protein + Cad-G5	10
7	5 µg Protein + Cad-G6	10
8	5 µg Protein + MW	8
9	5 µg Protein + Cad-B	10
10	5 µg Protein + Al (OH)3	10
11	5 µg Protein + 5 µg HA DNA + Cad-B	10
12	5 µg Protein + 5 µg HA DNA + MW	8
13	10 µg Protein + Cad-G5	10
14	10 µg Protein + Cad-G6	10
15	10 µg Protein + MW	8
16	10 µg Protein + Cad-B	10
17	10 µg Protein + Al (OH)3	10
18	5 µg HA DNA + Cad-B	8
19	Control	8

Hundred percent seroconversion was obtained in all groups except groups 18 (HA DNA + Cad B) and 19 (control). Ten days post 2nd dose the mice were challenged via intranasal route with p-H1N1-2009 virus (SF310-64) and daily weight was observed. The figures 34-37 depict weight loss and HI titres following virus the challenge.

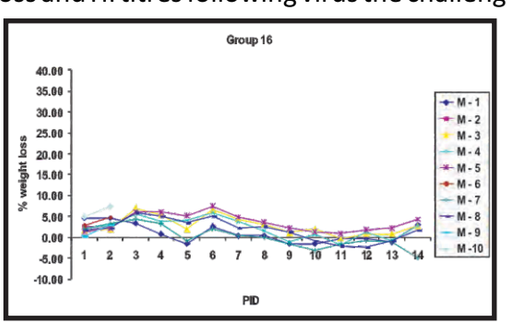

Figure 25a: Weight loss in mice immunized with 10µg HA protein+ Cad-B

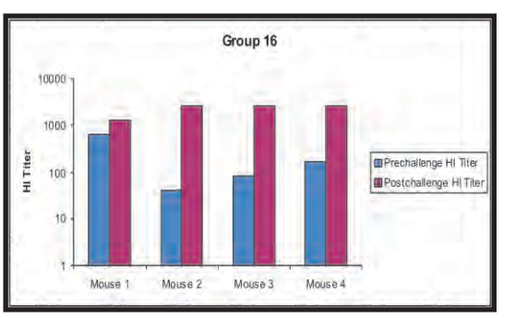

Figure 25b: HI titres in mice immunized with 10µg HA protein+ Cad-B

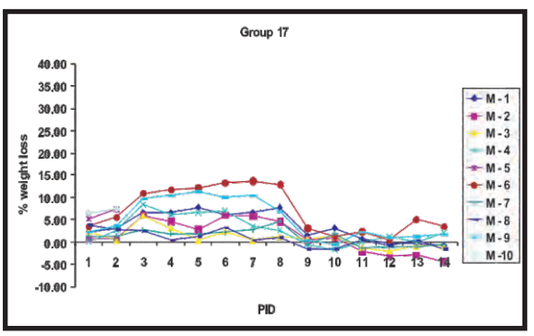

Figure 26a: Weight loss in mice immunized with 10µg HA protein + Al (OH)3

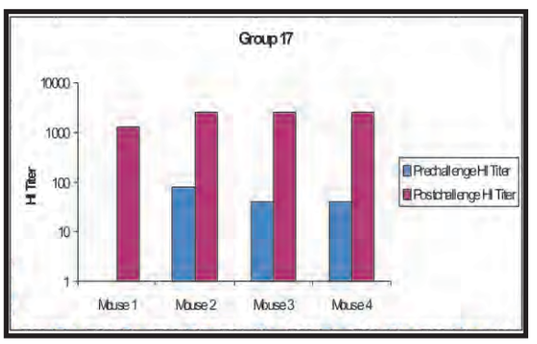

Figure 26b: HI titres in mice immunized with 10µg HA protein + Al (OH)3

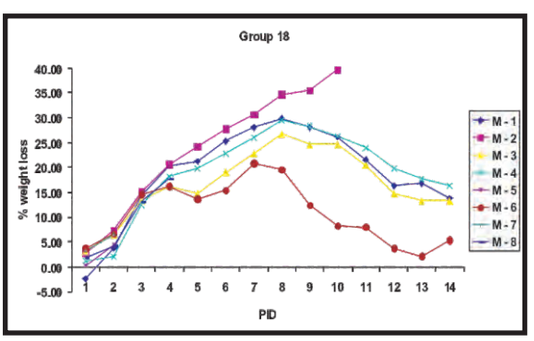

Figure 27a: Weight loss in mice immunized with 5µg HA DNA + Cad B

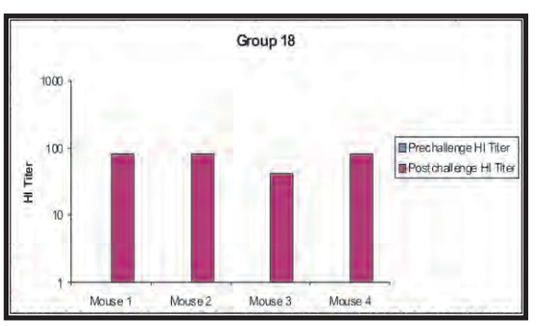

Figure 27b: HI titres in mice immunized with 5µg HA DNA + Cad B

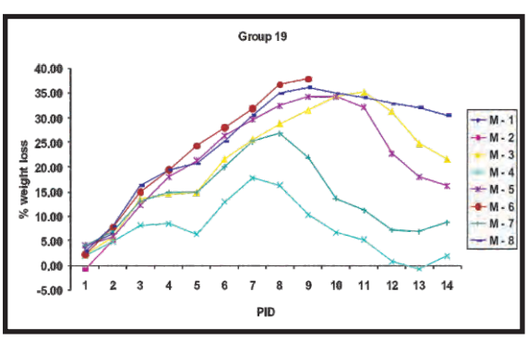

Figure 28a: Weight loss in mice injected with PBS

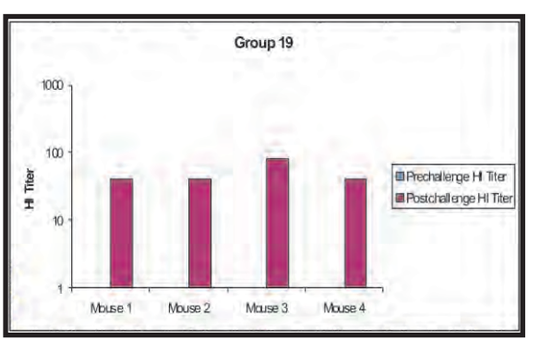

Figure 28b: HI titres in mice injected with PBS

New proposals submitted for approval Extramural:

Submission of R & D project for DBT funding, project title: 'Characterization of Hepatitis E virus putative helicase protein' by K. S. Lole

Technical support / consultancy provided:

Virological / Serological testing

A Total 687 of samples were checked for HCV RNA These included 598 non-clinical blood products from drug controller of India (DCI).and 89 clinical samples from different hospitals. Out of 89 clinical samples tested for HCV RNA, 26 were positive. The positive samples were further processed for genotyping. The genotypic distribution of the positive samples are as follows Eleven belonged to 3a, 4 belonged to 1b, 4 belonged to 1a, 4 belonged to 3i, 2 belonged to 3b and the rest one belonged to 5a

Evaluation of kits with control panel sera for HCV and HBsAg

A total of 32 kits were received by United Nations Office for Project Services (UNOPS), New Delhi and from different private companies for evaluations.

Chronic Hepatitis B and C patients:

A total of 82 and 18 patients were tested for the presence of HCV RNA and HBV DNA respectively in PCR & 90 for HBV viral load.

Sporadic acute viral Hepatitis patients:

A total of 258, 199 & 112 serum samples were tested for the detection of anti-HAV-IgM, anti-HEV-IgM and HBsAg respectively.

Epidemics of viral Hepatitis:

3758 sera representing 5 outbreaks of viral hepatitis were tested for hepatitis A, B & E virus (Gujrat (Modasa), Tasgaon (Dist. Sangali), Khed Shivapur (Dist. Pune), Roha (Dist. Raigad), Adbalgaon (Dist. Ahmednagar)

Providing Sequencing facility (3130XL Genetic Analyzer) for the other epartments:

A total of 8703 samples provided by other departments were sequenced and chromatograms were provided.

Sequencing Run	Sample
JE	1341
Virus Repository	209
Microarray	6
Rota	3033
Influenza	2389
Pune University	8
Measles	15
M.Sc. Lab	24
Dengue	314
Avian Influenza	1092
HCL & VR	270
Chandipura	2
	8703

Equipment procured

Sr. No	Equipment	Quantity/ No.
1	Split AC, 2Tm Cap	4
2	Microcentrifuge	2
3	Flow Cytometer (FACS Aria)	1
4	- 800C Ultra low freezer	1
5	HP Laserjet printer CP 1215	1
7	Hot Air Oven	1
8	Single pan balance (range 1-300 gm)	2
9	ELISA Reader, gift from LabIndia	1
10	-800C Ultra low freezer, gift from LabIndia	1
11	Remi Centrifuge 8RC	1

Resource generation:

By Providing technical services Rs. 25,56,659/-

Japanese Encephalitis Virus Group

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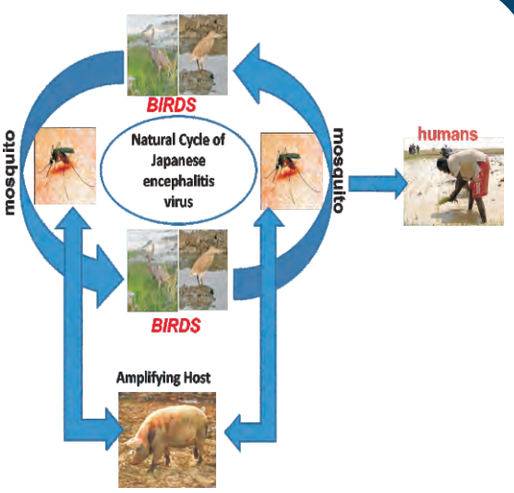
Ms. S. Harini, SRF

Project staff

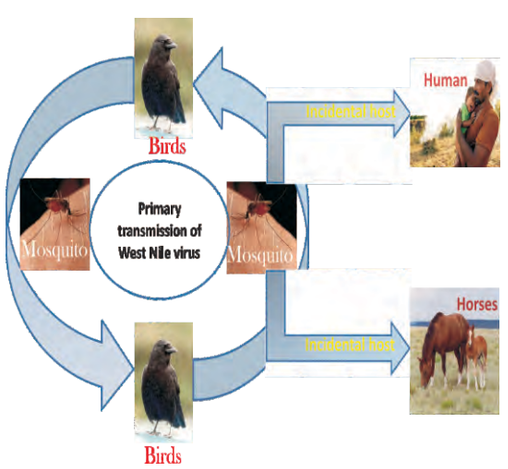
Mr. Pradip Fulmali,
Technical Officer Project

Japanese Encephalitis Virus Group



Projects

- Investigations of the Gorakhpur acute encephalitis syndrome (AES) outbreak, 2009 -10.
- Molecular epidemiology of Japanese encephalitis virus in India.
- Determinants of peripheral pathogenesis of West Nile virus.
- Development of molecular techniques for the rapid diagnosis of agents infecting Central nervous system.
- Cloning and expression of envelope protein of JEV and WNV envelope proteins for use in diagnostic tests.
- Role of innate immune response in Japanese encephalitis virus infection in humans.
- Selective expression of recombinant viral proteins in immunocompetent cells
- Identification of antibody reactive peptide epitopes on Japanese encephalitis virus
- Laboratory diagnosis of suspected JE cases
- Development of conjugate for JE virus specific IgM ELISA for human clinical specimens



Investigations of the Gorakhpur acute encephalitis syndrome (AES) outbreak, 2009 10

V. P. Bondre, G. N. Sapkal, S. Athawale, P. V. Fulmali, S. Mahamuni, S. Walkoli, S. Vidhate, V. Shankararaman and M. M. Gore

Gorakhpur region of UP state has documented a massive encephalitis outbreak during 2009. The acute encephalitis syndrome (AES) patients were hospitalized in BRD Medical College Gorakhpur. NIV Unit Gorakhpur collected 874 Cerebrospinal fluid specimens from the patients during the acute phase of illness (March 2009 to January 2010). These specimens were transferred to NIV, Pune, in cold chain for further investigations.

Objective

- To test the human CSF specimens for Japanese encephalitis and Enteroviral infection.

Work done

CSF specimens were processed for detection of JE and Entero virus which are endemic in the region and are associated with AES cases. Standard one step RT-PCR assays were performed using the JEV and Enterovirus diagnostic primers previously standardized in the Lab. The RT-PCR amplified JEV and EV specific products were confirmed by sequence analysis.

Results

Only 11 out of 874 specimens (1.26%) were positive by the EV specific diagnostic RT-PCR. The assay amplified a 407 nucleotide product from 5' NCR region. Sequence analysis of nine amplicons showed a maximum of 99% nucleotide identity with Coxsackie virus A11 and 2 sequences showed 98.5% sequence identity with HEV-B.

Seventy three (8.35%) of 874 CSF specimens were positive for JEV-C-prM gene (334-bp amplicon) as determined by RT-PCR. BLAST analysis confirmed that 43/73 sequences were closely related to genotype III strains while 30/73 sequences were closely related to genotype-I strains of JEV. In phylogenetic analysis two distinct nucleotide sequence divergence pattern was seen among the 66 JEV C-prM sequences. The dendrogram using the C-prM gene sequences showed that 27 sequences formed cluster with the genotype-I sequences which showed ≥12% nucleotide divergence when compared with other JEV genotypes. The remaining 39 JEV sequences clustered together with genotype III (Figure 1). The comparison of the 39 genotype III JEV sequences showed PNI of 92.80-100 with JEV strains P20778 (AF080251), GP78 (AF075723), and 014178 (EF623987) isolated in India.

Conclusion

This study showed JEV genotype-I sequences for the first time from encephalitis patients in India. The JEV viral RNA detected in CSF specimen from patients hospitalized with acute encephalitis syndrome showed closed identity with JEV genotype-III and genotype-I strains. Genetic analysis of JEV sequences from this study with the JEV sequences from Gen Bank clearly showing the introduction JEV genotype -I strain in Gorakhpur region of India and also suggest co-circulation of three distinct JEV strains belonging to genotype-III. Recently, several studies on JEV detection and phylogenetic analysis in different Southeast Asian countries documented emergence and reemergence of JEV genotype-I strains.

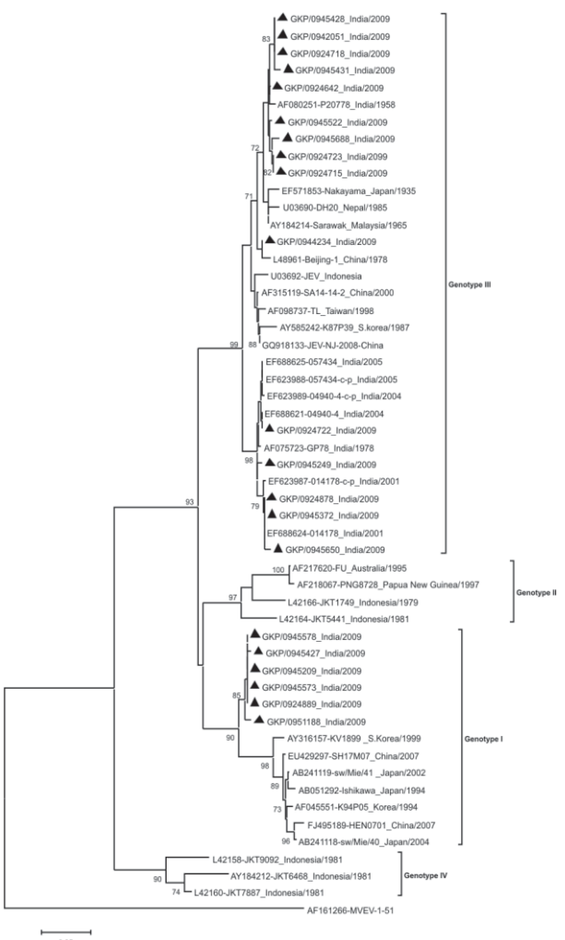


Figure 1: C-prM gene sequence based phylogenetic analysis of recently detected JEV sequence from Gorakhpur, India

Molecular epidemiology of Japanese encephalitis virus in India.

V. P. Bondre, G. N. Sapkal, S. Athawale, P. V. Fulmali, V. M. Ayachit, V. Sonowane and M. M. Gore.

JEV genotype-I sequences have been detected in CSF of encephalitis patients during 2009 epidemic in Gorakhpur. The C-prM gene sequence based analysis suggested distinct genetic nature of sequences from the patients. Hence, the CSF specimens were used for isolation and molecular characterization of the JEV strains.

Objective

- Isolation and molecular characterization of JEV from CSF of encephalitis patients from Gorakhpur, India.

Work done

Attempts were made to isolate JEV from the PCR positive specimens. The specimens were inoculated in BHK-21 cells and were observed for virus induced cylopathological changes. For molecular characterization envelope gene sequence was amplified from all the isolates and full genome sequencing was also done for one JEV genotype-I isolate.

Results

Three of the JEV PCR positive specimens showed CPE like changes after 8 days in BHK-21 cell. This specimen was further passage for 3 times in BHK-21 cell line and consistent CPE like changes were observed. The envelope gene was amplified and sequenced from the three isolates. BLAST analysis of the 1500 nucleotide of amplified E gene sequence confirmed them as JEV genotype-I sequence which showed sequence similarities of 96.7 - 98.5% and 87.1 - 87.8% with genotype-I and genotype-III strains respectively available in GenBank. Phylogenetic analysis using partial E gene sequences showed that Indian sequences form a separate sub-cluster within JEV genotype I sequences and showed maximum PNI of 98.5% with Japanese genotype I swine isolate of 1995 (AY377579/95-167/1995/swine/Japan).

Complete genome sequence 10976 nucleotide was generated for the isolate gkp095054/India. Phylogenetic analysis using the complete genome showed close relationship between gkp095054/India and Sw/Mie/41/2002 isolate from Japan with percent nucleotide identity (PNI) 97.60% (Fig 2).

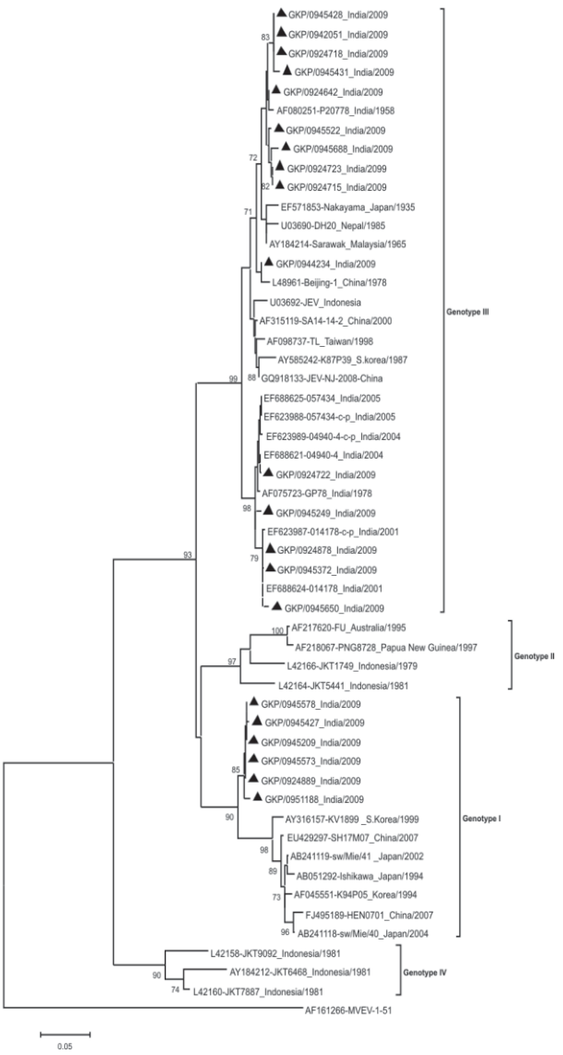


Figure 2: Complete genome based phylogenetic analysis of recently isolated JEV genotype I virus from Gorakhpur India.

Determinants of peripheral pathogenesis of West Nile virus.

G. N. Sapkal, V. P. Bondre, V. M. Ayachit, S. Mahamuni, P. V. Fulmali and M. M. Gore

Genetic variation among West Nile Virus (WNV) strains reflects on their differential pathogenecity. Lineage I strains are peripherally pathogenic when studied in murine model as compared to strains from other genetic lineages. Pathogenecity of the virus depends on

its replication potential and ability to evade host immune system.To understand this phenomenon, neutralizing escape (NE) variants were developed from the peripherally pathogenic lineage-I strains; replication potential and innate cytokines responses were studied in mice model.

Objective

- To study in-vivo replication potential and modulation of innate cytokines of neutralizing escape variants of WNV compared to parent strains.

Work done

Our previous studies demonstrated loss of peripheral pathogenicity of NEIF1A7 1.1 and reduction in TNF α secretion in peritoneal exudate cell (macrophage) of murine origin as compared to parent strain and NE IVC3F10 1.2. To understand the mechanisms observed in vitro, in vivo studies were carried out using the murine model.

Adult Swiss mice were inoculated i.p with 10^5 PFU of parent and both NE variants. Tissues from three mice were dissected and processed for viral determination after every alternate day by qRT-PCR and infectious assay. qRT-PCR demonstrated that on day 4 PI there was no significant differences in viral load in blood of mice infected with parent and both neutralizing escape variants. On contrary, at this point NE IF1F7 1.1 showed very low infectious titer approximately 101 TCID50/ ml as compared to parent and NE IVC3F10 1.2 (Figure 3 A). Virus titres determined by qRT-PCR were seen to be more than 2 orders of magnitude higher than titres determined by CPE assay.

On day 6 PI, mice infected with parent and NE IVC3F101.2 had a significant viral load in blood as compared to NE IF1A7 1.1. Animals infected with parent showed a peak of replication at 6 day PI as determined by both qRT-PCR and CPE assay, where it exhibited the presence of virus in both spleen and brain. In NE IVC3F10 1.2 infected mice the peak of replication was delayed until day 10 where it showed presence of virus in both spleen and brain. The maximum viral load in spleen observed by qRT-PCR was 3.3 x 10^5copies/gm, while it was 10^4-4.5 TCID50/ gm by CPE assay. However, mice inoculated with NE IF1A71.1 virus was detected in spleen only and no virus was detected in brains of these mice at any of the monitored time points in both qRT-PCR and CPE assay.

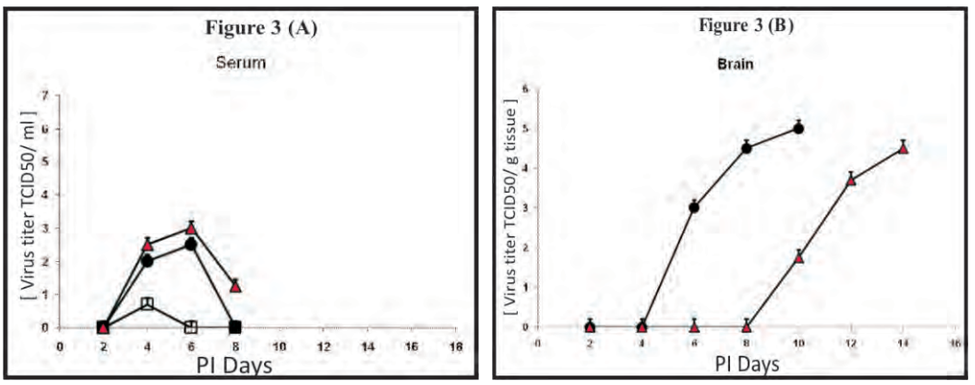


Figure 3: Viral burden in serum (A), spleen (B) and brains (C) of Swiss mice inoculated i.p. with 10^5 PFU of the parent WNV 68856 (), NE IF1A71.1()and NE IV3F101.2(). Tissue and serum specimens were titrated individually on PS cells. The titers are expressed as the average of three mice. Viral titers are expressed as log10 TCID50/g or ml of tissue or blood. Mean (±SD) titres are for triplicate cultures.

The levels of inflammatory cytokines in the sera of mice infected with parent and variant viruses

To further understand the significance of TNF-α and IL1-β *in-vivo* during WNV infection, levels of these cytokines in infected mice sera (parent and variants inoculated) were also monitored. In case of IL1-β, its concentration increased continuously with no significant differences between parent and both neutralizing escape variants was observed up to 4 days pi. However, its concentration increased to statistically significant levels at 6-8 and 10-12 days pi in parent and NE IVC3F10 1.2 respectively (Fig 4 A).

Mice infected with parent and NE IVC3F10 1.2, TNF α level also increased steadily during infection and reached its maximum at 6-8 and 10-12 days pi in parent and NE IVC3F10 1.2 respectively. During this period all the mice demonstrated severe sickness with paralysis and/ or 100% mortality ($p>0.01$). However, in mice infected with peripherally non-pathogenic variant NE IF1A71.1, we observed 3-4-fold lower TNF α levels in sera as compared to parent and NE IVC3F10 1.2 (Figure 4 B).

The levels of other inflammatory cytokines did not significantly differ in sera of mice infected with parent and variant viruses.

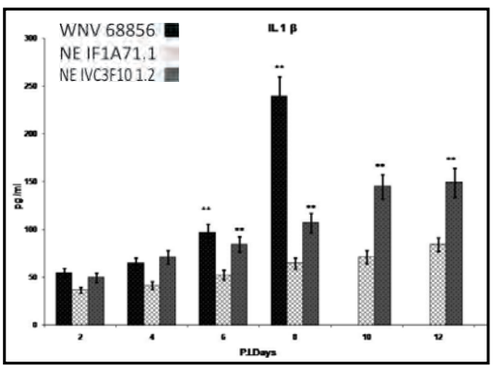


Figure 4 (A)

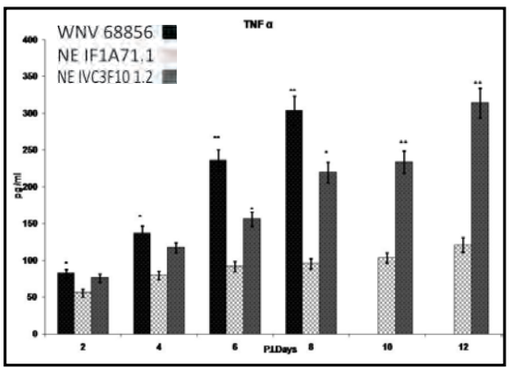


Figure 4 (B)

Figure 4: (A) IL 1 β and (B) TNF-α in the sera of mice infected with WNV 68856, NE IF1A71.1 and NE IVC3F10 1.2. Each dataset represents sera from a pool of mice tested in duplicate per time point. The error bars indicate the standard errors. Significant differences between groups are denoted by an asterisk. Asterisk (*) show the pairs that exhibit significant (P 0.05) and two asterisks (**) highly significant (p 0.005) differences by Student T test in the graph.

Development of molecular techniques for diagnosis of agents infecting Central nervous system.

V. P. Bondre, G. N. Sapkal, P. V. Fulmali, and M. M. Gore

Human central nervous system infections are becoming major health problems worldwide. With the strengthening of diagnostic capabilities, newer viral agents are being identified as cause of human CNS infections. Till date, molecular diagnostic tests for JEV and WNV have been developed and are being evaluated for their specificity and sensitivity.

HSV 1 is one of the main cause of serious neurological complication with a wide spectrum of clinical manifestations. Effective antivirals therapy is available for therapeutic intervention of HSV infection and therefore, rapid laboratory diagnosis becomes essential. Study to develop and standardize a SYBR green based real time PCR assays for detection of HSV was initiated to improve the diagnostics for clinical specimens referred throughout the country.

Objective

- Development and standardization of quantitative SYBR green based real time PCR assays for detection of HSV 1 and 2.

Work done

Primers were designed by aligning glycoprotein gene of different strains which is considered as conserved gene among of the HSV 1 and 2 strains available in Genebank. Four primer pairs were designed to detect all genetic variants of HSV 1 and 2 strains.

The target genomic region was amplified by using external primers and cloned in Zero Blunt PCR cloning vector. The recombinant vector was transformed into MAX efficiency *E. coli* DH5α and the recombinants were selected by antibiotic pressure. The recombinant plasmid was confirmed by sequencing and restriction digestion, amplification with virus specific primers and sequencing.

The SYBR green based real time PCR assay was standardized with template DNA, virus specific primers using Superscript SYBR green QPCR kit. The assay detected 37 copies/ml of HSV with similar efficiency in three replicate experiments (Table 1). Sensitivity was determined by using different dilutions of the recombinant plasmid DNA spiked in blood, serum and CSF. The HSV789F and HSV1070R primer pair specifically detected HSV and non-specific amplification in blood, serum and CSF was not observed. The melting curve analysis indicates amplification of a single product at the end of reaction. The melted temperature of the amplified product was determined to be 91.5-91.8°C (Table 2).

Conclusion

The quantitative SYBR green based real time PCR assays detected up to 37 copies/ml of HSV DNA. However, this assay has to be developed with Taqman chemistry to increase the specificity.

Future plan

Development and standardization of quantitative SYBR green based real time PCR assays for detection of HSV has been completed and work on evaluation of the assay using human clinical specimens and validation by comparing with other standard assay is in progress. Further the assay will be developed using Taqman real time chemistry.

Table 1: Determination of sensitivity to detect the HSV genomic copies

No	Colour	Name	Type	Ct	Given Conc (copies/ml)	Calc Conc (copies/ml)	% Var
1		1E+09	Unknown	11.70		8.47E+09	
2		1E+08	Unknown	15.72		3.57E+08	
3		1E+07	Unknown	20.37		9.11E+06	
4		S1	Standard	23.23	1.00E+06	9.59E+05	4.1%
5		S2	Standard	25.99	1.00E+05	1.09E+05	8.7%
6		S3	Standard	29.07	1.00E+04	9.59E+03	4.1%
7		1E+02	Unknown	33.41		3.15E+02	
8		1E+01	Unknown	36.07		3.87E+01	

Table 2. The melting curve analysis of HSV specific real time PCR

No.	Name	Curve Peak 1
1	1E+09	91.5
2	1E+08	91.5
3	1E+07	91.5
4	Standard 1	91.5
5	Standard 2	91.3
6	Standard 3	91.2
7	HSV DNA 1E+02	91.5
8	HSV DNA 1E+01	91.8
9	NTC	00.0

Cloning and expression of envelope protein of JEV and WNV for use in diagnostics.

V. P. Bondre, G. N. Sapkal, V. Shankararaman, S. Mahamuni, S. Walkoli and V. M. Ayachit

JEV envelope glycoprotein (E) and non-structural protein 1 (NS1) are being explored for early diagnosis of JEV infection, To improve the sensitivity and specificity of existing serological assays, cloning and expression of E and NS1 in mammalian cell culture was attempted. The expressed proteins will be explored for its possible applications in serological diagnosis.

Objective

- Cloning and expression of the E and NS1 genes in mammalian cells
- Development of stably transfected cell line with recombinant E and NS1 genes

Work done

JEV strain 014178 was used for amplification of the E and NS1 coding protein fragments. These amplicons were made with signal sequences for transport and cloned into mammalian expression vector pcDNA4/TO. These constructs were transfected in BHK 21 cells and the transformants were selected for five passages on Zeocin (750 µg/ml) and Restriction mapping of these sequences was done. The transformants were screened for expression of NS1 protein by immune-fluorescence assay.

Results

JEV NS1 protein was expressed in BHK-21 cells and detected by IFA (Figure. 5).

Future plan

The JEV E and NS1 protein expression in BHK21 cells will be further confirmed by ELISA and Western Blotting. The time kinetics of expression will be studied to prepare higher amount of protein. The purified protein will be explored to determine its utility as purified virus antigen in IgM ELISA.

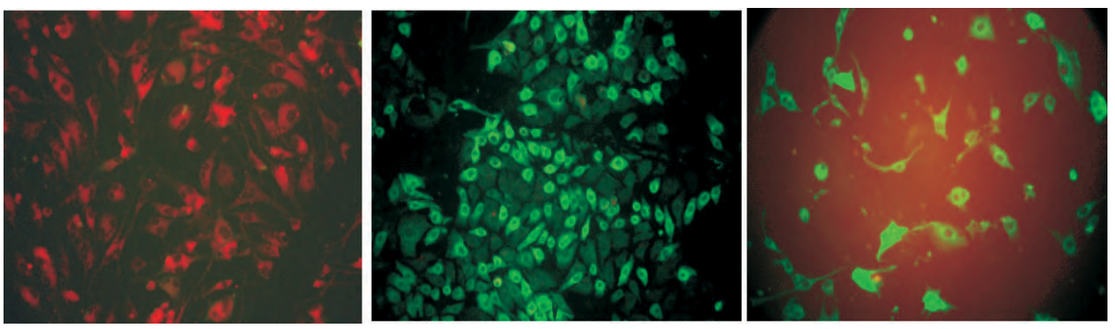


Figure 5. Expression of JEV 014178 NS1 protein in BHK21 cells detected by IFA using JEV specific polyclonal antibodies. A. Uninfected cells (negative control); B. JEV infected cells (positive control) and C. Stably transfected cells after selected on Zeocin for five passages expressing NS1 protein

Role of innate immune response in Japanese encephalitis virus infection in humans.

M. M. Gore and S. Harini

Only a small proportion of non-immune infected individuals develop clinical features during JEV infection. As fate of the host is determined before/during the initial stages of the adaptive immunity setting-in, it is expected that the innate immunity would play a crucial role in combating the invading pathogen. Studying the interaction of JEV with human macrophages (Mϕ) and DC in terms of their susceptibility to viral infection and subsequent response of co-stimulatory molecules on APC and cytokine and chemokine milieu created by these cells could possibly help us in understanding difference of clinical and subclinical infection.

Objective

- To standardize human DC culture.

Work done

Standardization of Dendritic cell culture

The monocytes from PBMC were isolated using CD14+ beads, and checked for CD14+ purity. The purity levels were almost ~90%. These pure monocytes were then cultured in presence or absence of GM-CSF (50ng) and IL4 (50ng) per 10_6 monocytes. After 5-7days of

incubation, purity level of immature Dendritic cell (DC) was checked using DC marker CD1a. The cells (with / without cytokine treatment) were stained with a cocktail of antibody CD1aFITC and CD80PE, and the corresponding isotype control were also maintained (Fig. 6).

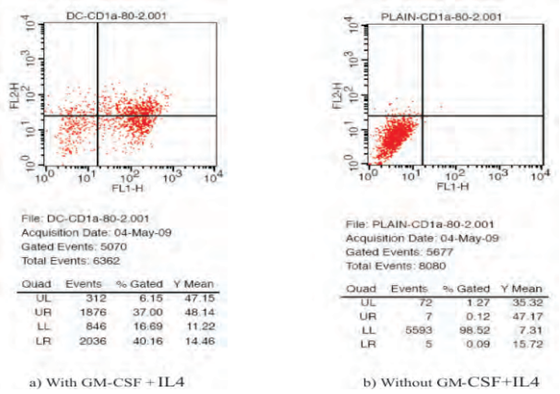


Figure 6: CD1aFITC + CD80PE

Functional status of Macrophages and DCs after JEV infection

From our preliminary experiment, we observed that JEV infection in whole PBMC was able to stimulate CD86 in monocytic population, and a difference in the ability of two different JEV strain in inducing CD86 levels were also noted. Hence, to further estimate the ability of JEV to induce CD86 levels in other antigen presenting cells (APCs) like macrophages (Mϕs) and DC was also performed. Mϕ s and DCs were infected with 1MOI of different JEV strain, i.e wild-type JE057434, and vaccine SA-14-14-2; and 48hrs post infection the levels of co-stimulatory marker CD86 were determined using FACS caliber (Fig. 7).

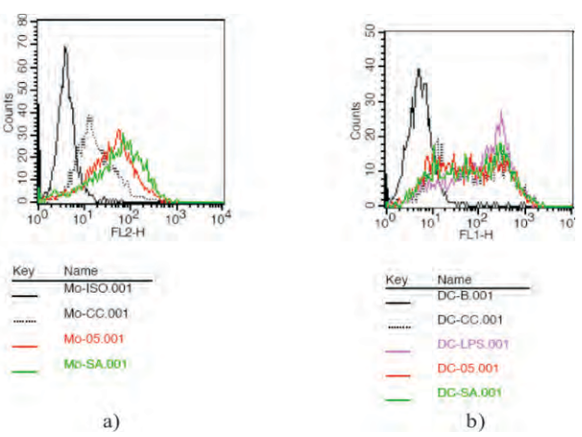


Figure 7: a) Macrophage, b) DC

The differential ability of vaccine JEV strain to induce higher CD86 levels than wild-type strain is more clearly visible in Mϕ than DC, as observed previously in monocytic population of JEV infected whole PBMCs.

Selective expression of recombinant viral proteins in immunocompetent cells.

M. M. Gore and M. F. Ahsan

In conventional DNA vaccines the expression is controlled by a nonspecific (CMV) promoter making it possible to express the protein in all the cells, which get transfected including that of nonprofessional APCs. This might affect the overall intensity and longevity of immune response in-vivo. Hence, the approach is to express the antigen only in professional APCs, this could be achieved by using cell specific promoters.

Objective

- To study the cloning and expression of JE virus structural gene under the control of immune cell specific promoters and study immune response and protection in-vivo when those recombinants are used as a plasmid vaccine.

Work done

Expression studies of various promoter constructs

Protocol was standardized with Lipofectamine-2000 for the transfection of plasmid (pAcGFP1-N1) with CMV as a promoter in APC and Non APC cells. Opti-MEM was used as a medium. After 24 hours of post transfection of various promoter constructs, cells were observed for fluorescence under fluorescent microscope (Fig 8). As a negative control, promoterless vector was used.

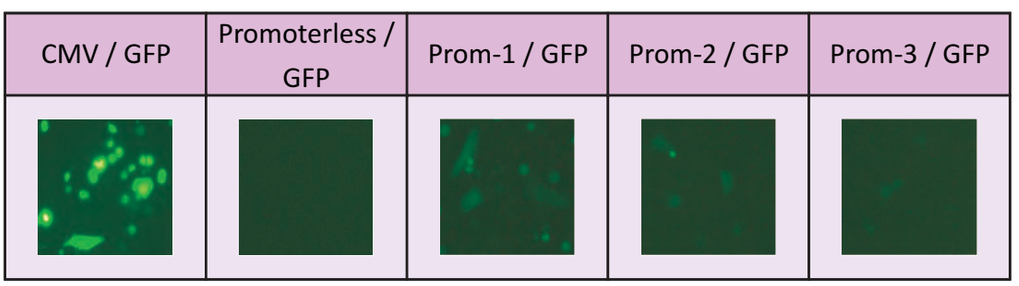


Figure 8: APC cells

Standardization of Real time PCR for GFP using TaqMan probe-based chemistry.

- The amplicon size of the primers is 100bp with probe in between the FAM as fluorophore and BHQ-1 as a quencher.

- For in-vitro transcription, linearization of the cloned vector is required from the 3' end of the insert. SpeI restriction enzyme was used from the MCS of the pGEMT vector.
- In-vitro transcription was done using MEGAscript kit (Ambion)
- Sensitivity is <= 100 copies of RNA with 10 fold diluted standards

Cloning of APC specific promoter with E gene of JEV

Based on the expression studies of various promoters with GFP, Promoter-1 was selected and cloned with E gene of JEV & confirmed through sequencing for in-vivo studies.

Results

- Expression studies of various promoter constructs using GFP as a reporter in APC and Non APC cell lines
- Standardization of Real time PCR for GFP
- Cloning of APC specific promoter with E gene of JEV

Future plan

Experiments on Real time PCR and Flow cytometry along with in-vivo studies using mouse model is ongoing.

Identification of antibody reactive peptide epitopes on Japanese encephalitis virus.

Roopesh Singh Gangwar, M. M. Gore

Japanese encephalitis (JE) virus infections are endemic in Northeast region of India. Diagnosis of JE infection mainly depends on IgM antibody detection as an indication of primary immune response. The primary IgM antibody response to flaviviruses is anti Egp and is also type specific, the secondary IgG response tends to be cross-reactive and HI positive.

Antibody reactivity on complete protein would always be beset with cross-reactivity. Hence we have identified small stretches of virus specific peptide epitopes (B-cell epitope) on different virus encoded protein. The serological response to these epitopes has been studied by carrying out ELISA.

Objective

- To analyse anti-JE virus antibody response in clinical and sub clinical JE infections at peptide level.

Work done

Earlier we have reported B-cell epitopes (JE specific and WN specific) and ELISA were standardized on synthetic peptides using known sera. The two peptides were selected for further studies. These peptides have been used to screen the patient samples from the Dibrugarh region of Assam. The results were compared with NIV MAC ELISA kit, ELISA on whole virus particle and neutralization test.

The peptide ELISA results were found similar with ELISA on whole virus Ag and it showed promising results. Out of 116 paired samples tested, 20 samples (acute) have been specifically differentiated between JE (14) and WN (6) positive. This should be noted here that these samples could not be differentiated by ELISA on whole virus particle. The mice were immunized with chimeric peptides of these sequences. The experiment is in progress.

Findings

The peptides JE40 and WE147 can be used as a diagnostic tool for differential diagnosis of JE and WN infections.

Laboratory diagnosis of suspected JE cases.

G. N. Sapkal, S. A. Mahamuni, S. Walkoli, V. M. Ayachit, and V. P. Bondre

NIV is recognized as WHO reference center for diagnosis of encephalitic viruses. Human clinical specimens referred by different hospitals in the country are diagnosed for presence IgM antibodies specific JEV infection. The findings are communicated to the respective hospitals.

Work done

A total of 598 human clinical specimens that include 346 sera and 252 cerebrospinal fluids (CSF) from hospitalized encephalitic patients from all over India were tested by JEV specific IgM ELISA. 12/ 252 (4.76%) CSF and 13/346 (3.75%) showed presence of JEV specific IgM antibodies.

Development of conjugate for JE virus specific IgM ELISA for human clinical specimens.

G. N. Sapkal, S. A. Mahamuni, S. Walkoli, and S. Vidhate

JE virus specific monoclonal antibody (HX-2) developed by JE group is purified and conjugated with biotin, purified, titrated and supplied throughout the year for preparation of JEV diagnostic kit developed by Arbovirus Diagnosis division.

Technical support

Ventri Biologicals, Vaccine division, Pune

Technical support on MDV hybridoma revival and cryopreservation is provided to Ventri biologicals, Pune.

Bharat Biotech International Ltd., Hyderabad

As per request by BBIL, JE group provided data on antigenic analysis of master virus bank, working virus bank and concentrated JE virus bulk

Monoclonal antibodies against E protein of JEV were provided to BBIL for QC Analysis

Dengue Group

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Dengue Group

List of Projects

- Investigations of Dengue outbreaks in Pune
- Evolution of dengue viruses in the country
- The role of host/viral factors in dengue immunopathogenesis
- Immunogenetic profiling of dengue infected patients from Pune, Maharashtra

Investigations of Dengue outbreaks in Pune

Paresh Shah, Anand Singh, Rupali Bachal, Asha Bhagat, Cecilia D.

The current year is the last year of the five-year study we had undertaken to determine the pattern of dengue in Pune. The cases that occurred in two major hospitals KEMH, Pune, YCMH, Pimpri and five clinics spread out in the city were actively investigated. The objectives set for the current year and work carried out is presented below.

Objectives

- Determine the demographics, the disease severity and the circulating serotype/s.
- Investigate periurban outbreaks.
- Determine the duration of IgM positivity in dengue.

Work done

The 2009 outbreak of dengue in Pune was larger than the previous five years. A total of 2047 suspected cases were tested for the presence of DENV-specific IgM and 1401 (68.4%) were found positive. The age/sex distribution of cases is shown in figure 1. The number of cases was highest in the age group of 21-30 yrs (n=355, 25%). The male to female ratio was highest in the 21-30 yr age group (4:1) followed by the 11-20 yr age group (3.4:1) (Figure 1).

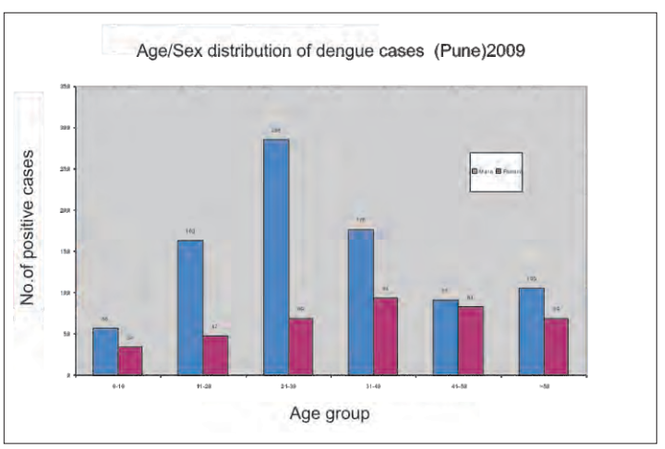

Figure 1: Age and sex distribution of dengue cases

There were a few dengue cases, starting from January (39 cases), which dropped to single digit numbers from February to April. From the month of May onwards, the number of cases started rising and peaked in October. The percentage of positive cases remained consistent, 70-75% from June to September. In November-December the percentage dropped down to 55-60% (Figure 2).

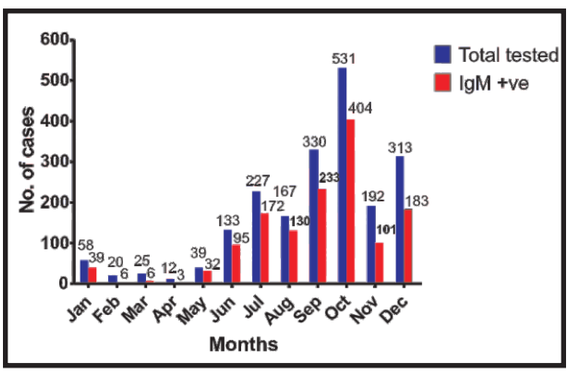

Figure 2: Monthly distribution of dengue cases in Pune 2009

Disease severity was lower this year than the previous years with only 7% showing DHF as categorized by WHO in 2007 (Figure 3). The numbers of fever cases seeking medical assistance were higher perhaps because of the H1NI scare, resulting in a greater representation of dengue fever than the last four years.

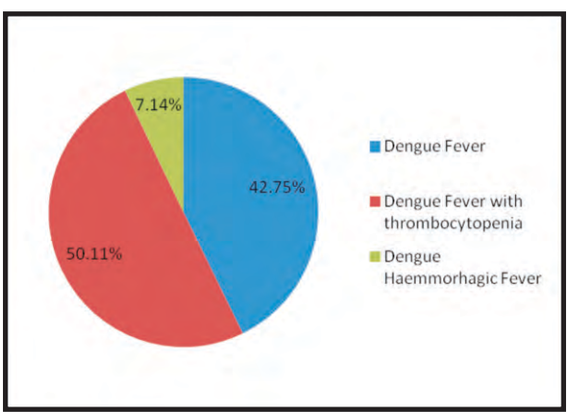

Figure 3: Distribution of dengue cases based on disease severity

The number of individuals in whom the serotype could be identified was 35, with all four serotypes being represented. There were 7 cases of DENV-1, 13 cases of DENV-2 and 14 cases of DENV-3 and one case of DENV-4. The distribution of serotypes depicted in figure 4 which has been generated on a google map of Pune, shows that DENV-1 was possibly introduced into the heart of the Pune city from the North while DENV-3 was introduced from southwest. DENV-2 on the other hand seems resident. DENV-4 was a late entrant and was perhaps imported from outside the city (Figure 4). DENV-4 has not been detected in the city since the last 6 years and has not been reported since 1970s. The travel history of the patient did not offer any clues.

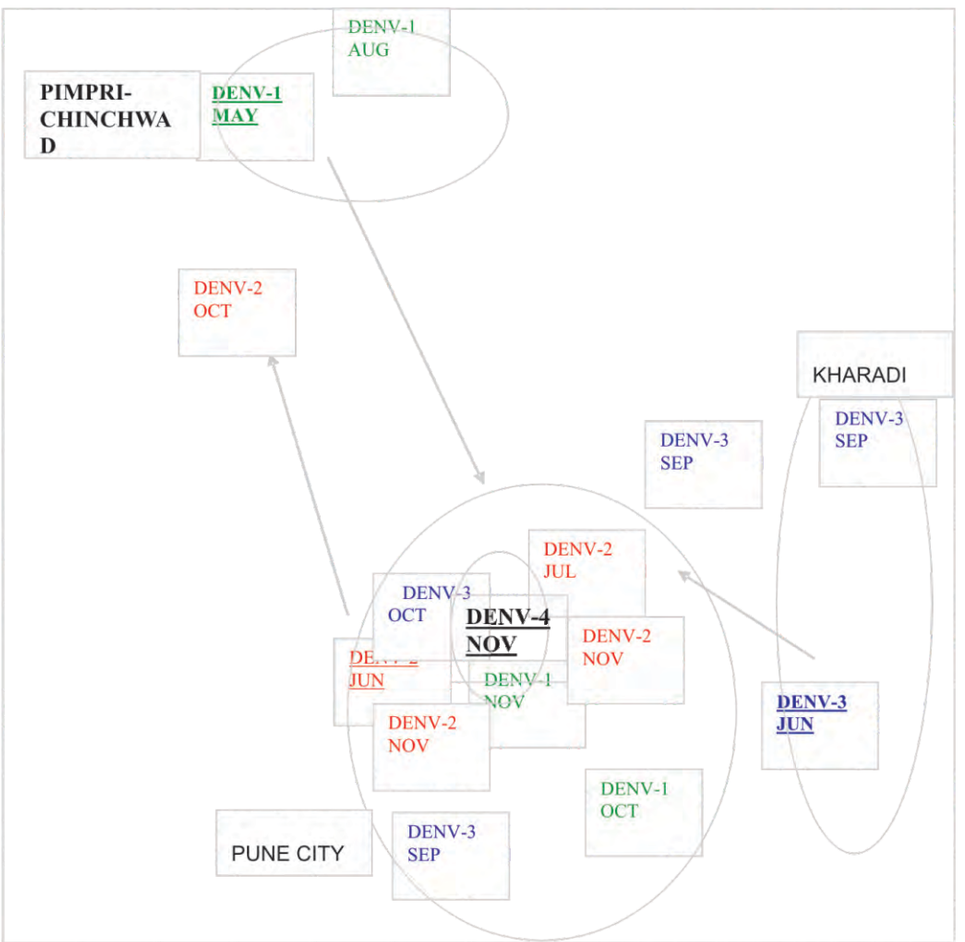

Figure 4: Distribution and spread of dengue serotypes in Pune city

We conducted a pilot contact survey in four localities of Pune city on the basis of the samples we received from these areas. Taking the index cases (IgM positive) as referral points for sampling, samples were collected from contacts. For the survey we took help of the PHC workers of that locality. The survey was conducted within 15 to 20 days of the first cases being reported from the locality, the areas covered in each locality had a radius of < 0.5 km. Considering IgM data, (fig.7) the ELISA reading of the samples indicated that the cases were detected within a month of the infection. Therefore within a month, the exposure rate was highest in Talegaon followed by Bhosari. The index case of the locality in Talegaon was a fatal case. The adult population was more affected in all the localities (Table 1).

Table1: Exposure rate of dengue among contacts and neighbors of dengue cases

Area of sample collection	Total no. Tested	Mean Age of Total no. tested	Pos (IgM)%	Mean Age of IgM positives
Hanumannagar 04.08.09	126	28.57	23(18.25)	31
Bhosari 30.06.09	69	31.65	30(43.47)	30
Wadgoansheri 09.07.09	91	28.73	12(13.18)	31
Talegaon Dabhade 2.12.09	21	30.47	13(61.9)	32

The ORG group had collected samples from three different areas for determining exposure to H1N1. Whether dengue virus was circulating in the same populations during the outbreak was investigated. The presence of dengue infections (presence of dengue specific IgM) was determined. There was one dengue IgM positive case in a boarding school of Panchangani (July 2009). In the slum area of Kashewadi (August 2009), only 13 serum samples were IgM positive of 227 tested. In the Bhimnagar (October 2009) slum area, 11 samples were IgM positive out of 85 tested. Considering the duration of IgM response we could surmise that the percentage dengue cases occurring in Kashewadi during July-August 2009 was 5.7% while in September-October 2009 it was 13% in Bhimnagar.

Future plans: Data analysis and reporting

Evolution of dengue viruses in the country

K R Gurukumar, J A Patil, Paresh Shah, Cecilia D, George Jacob, Sam Peter

Our earlier studies on genomic analysis of dengue viruses had identified the genotypes of the four serotypes circulating in the country. The only genoytpe switch was reported for dengue 2 serotype.

Objective:

- To detect inter and intra outbreak variations in the E gene of dengue viruses
- To characterize the viruses circulating in Kerala at molecular level

Work done:

Inter and Intra outbreak variations

A partial region of the E gene, domain 3 (ED3) from 23 DEN-Positive serum samples, collected from 2005 to 2008, including 9 DENV-1 (nt-727-1407) 6 DENV-2 (nt-865-1480) and 8 DENV-3 (nt-805-1479) was amplified and sequenced. The nucleotide and amino acid divergence for the three serotypes of DENV was compared, between and within the three years of study.

Inter outbreak variation analysis

The nucleotide and amino acid divergence of DENV-1 between the three years was 4% and 2.6% respectively. DENV-2 sequences of 2007 and 2008 were highly similar (99.7% nt/100% aa) and differed from 2005 sequences by 2% at nucleotide and 0.8% at the amino acid level. DENV-3 viruses sequenced over the two years, 2005 and 2007 were about 1.4% divergent from each other at both the nucleotide and amino acid levels.

Analysis of inter outbreak variations indicated that DENV-2 and DENV-3 were more conserved and evolved at a lower rate when compared to DENV-1, which showed a greater divergence at both the nucleotide and amino acid level.

Intra outbreak variation analysis

p-distance analysis of the sequences obtained during the three years of study revealed that DENV-1 was less divergent in 2005 (1.6%/1.5% divergence of NT/AA) than in 2007 (2.7%/2.3% divergence of NT/AA). There was only one sample that could be sequenced in 2008.

DENV-2 on the other hand showed more divergence in 2005 (3.4%/1.3% divergence of NT/AA) than 2008 (0.6%/0 divergence of NT/AA). 2007 had only one sample that could be sequenced. Similar to DENV-2, DENV-3 was also more divergent in 2005 (1.5%/1.6% divergence of NT/AA) compared to 2007 (1%/0.4% divergence of NT/AA). Therefore while DENV-1 seems to be diversifying, DENV2 and DENV-3 seem to be stabilizing.

Characterization of dengue viruses circulating in Kerala at molecular level

Last year, there was a large outbreak of fever cases in Kanjirapally. One hundred samples which were collected within 7 days post onset of illness were brought to NIV to determine the serotype/genotype of dengue virus circulating in the area. The samples collected at early POD which were negative for DEN & CHIK specific IgM antibodies were tested by DENV-specific multiplex RT-PCR. 28 were found to be positive for DENV RNA. The multiplex RT-PCR revealed that two serotypes were circulating in the area. Nine cases were identified as DENV-2 and 17 were DENV-3. Based on the partial core sequence, the DENV-2 from Kanjirapally was closest to Srilanka 2004 and Gwalior 2006/2008 isolate. The DENV-3 was closest to Gwalior 2004 sequence.

Future plans

The samples from Kerala that have been found positive by RT-PCR will be used for virus isolation. These will be characterized to determine their phylogenetic relationship with the viruses circulating in the rest of the country.

The role of host/viral factors in dengue immunopathogenesis

Gurukumar KR, Priyadarshini D, Rupali Bachal, Anand Singh, Naik SF, Paresh Shah, Alagarasu K, Cecilia D

The significance of viral load in disease severity of dengue has been shown in the previous years using the group specific real time RT-PCR established in our laboratory (Annual Report 2006-2007). In the present year, we have studied the effect of serotypes on the viral load and disease severity. We have also been dissecting the antibody response in dengue patients.

Objectives

- To determine the effect of serotype on the association of viral load with disease severity.
- To determine the duration of IgM response in dengue patients

Work done

Effect of serotypes on the association of viral load with disease severity

To determine the effect of serotype on viral load, a subset of the samples were tested in the serotype specific real time RT-PCR, using primers and probes described earlier. The following correlations were drawn.

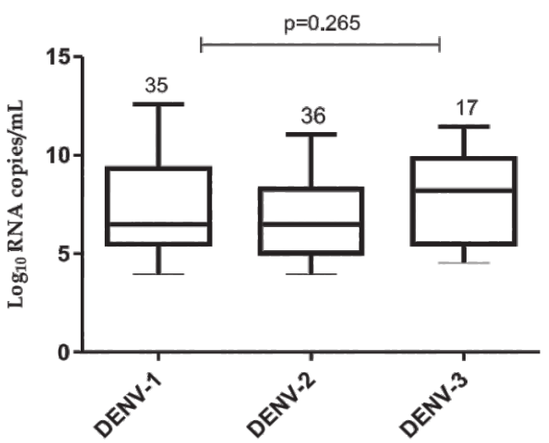

Figure 5: Viral load in different dengue serotypes

When the viral load was compared in dengue infections of the three serotypes in circulation, no difference was observed between DENV-1, 2 and 3 as shown in the figure 5.

When the viral load with the different serotypes was compared in DF and DHF cases, it was observed that the DHF cases had significantly higher viral load than DF cases in DENV-1 and DENV-2 cases (Figure 6). However in DENV-3 cases there was no difference. This perhaps indicates that the mechanism of disease pathogenesis may not be the same for all the serotypes.

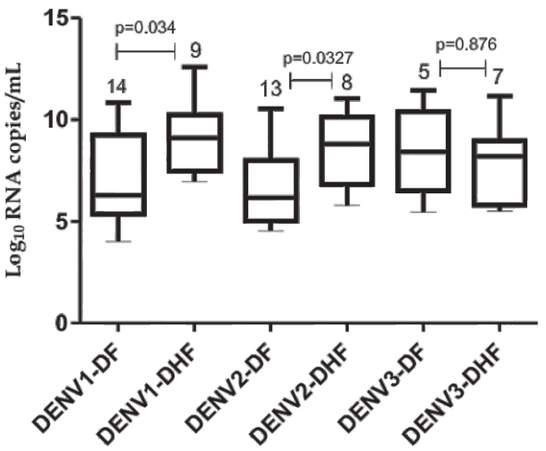

Figure 6: Viral load in DF and DHF cases of different dengue serotypes

Duration of IgM response in dengue cases

We further attempted to determine the duration of IgM response in dengue cases (Figure 5). Paired samples were obtained for 28 individuals. The IgM response was surprisingly short lived. The OD values dropped to below cut-off levels by 40 to 49 days in all cases, indicating that the IgM response lasted just a month and a half compared to the assumption that IgM response can be detected for 3-6 months. Whether the cases were primary or secondary has not been determined, but if one assumes that the IgM response is high in primary infections and low in secondary, the range of optical density values observed ranged from 0.3 to 2.0 and should include primary and secondary infections.

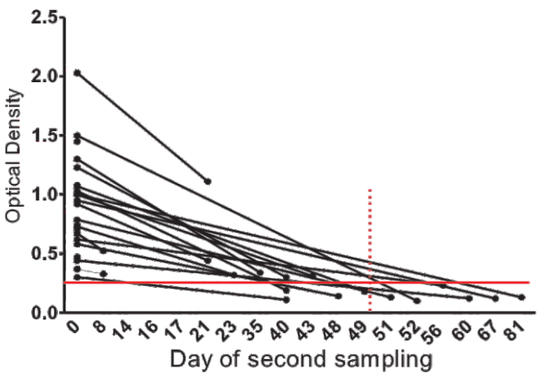

Figure 7: Duration of IgM response in dengue cases

Future plans:

The viral load and antibody response will be studied with a larger number of samples representing all four serotypes.

Immunogenetic profiling of dengue infected patients from Pune, Maharashtra

Alagarasu K, Shah PS, Cecilia D

Introduction

The outcome of DENV infection is determined by various factors including viral and host genetic factors. Among the different host factors, Human Leukocyte Antigens (HLA) plays a major role in regulating the immune responses and the pathogenesis of DENV infection.

Objectives

To find out whether dengue fever or dengue hemorrhagic fever are influenced by

- Human leukocyte antigen class I (HLA- A, -B and C) and
- HLA class II alleles (HLA-DRB1 and HLA-DQB1).

Work Plan

The work plan for the first six months includes

1. Standardization of HLA typing for class I and class II alleles
2. Sampling and HLA class II typing in 50 apparently healthy subjects (HS) who have no known history of hospitalization for fever with dengue like illness.

Work Done

Polymerase chain reaction based techniques using sequence specific primers for typing HLA alleles were standardized. Blood samples were collected from 46 apparently HS and six

patients who were hospitalized for dengue fever and confirmed by presence of dengue specific IgM antibodies. DNA was isolated from the blood samples and typing for HLA-DRB1 alleles was performed. HLA-DRB1*15 was the most frequent allele observed among studied NHS followed by HLA-DRB1*04, *07, *03, *10 and *14.

Future Plans

More samples from dengue patients and HS will be collected and studied for their HLA profile to find out the association of HLA alleles with DF or DHF.

Services

Altogether 3950 samples were tested for dengue IgM using the kits received from Arbovirus diagnostic unit and the reports were given to the concerned hospitals, the Joint Director State Health Services and the NVBDCP.

Eighteen lots of the kit prepared by Arbovirus diagnostic units were validated using panel of positive and negative sera created by our group.

Extra work load due to Swine Flu

The supply of reagents for swine flu testing to different centers all over the country was co-ordinated by our group including one Scientist E, one Scientist B and one RA. Kits were supplied to 18 centers and reports of reagent status were sent regularly to the ICMR and Ministry. One Scientist C, One Scientist B, One SRF and two technicians were involved in influenza (H1N1) diagnosis.

Training offered

1. Dr. PS Shah was invited by WHO to facilitate training at National Public Health Laboratory ,Katmandu, Nepal for Pandemic influenza diagnostic techniques.(August 2009).
2. Five students completed their M.Sc., dissertation work during the year 2009-2010.

Chikungunya Group

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Chikungunya Group

Projects

- Investigation of Chikungunya-like illness in Tirunelveli district, Tamil Nadu
- Studies on differential infectivity of two strains of Chikungunya virus in Vero E6 and C6/36 cell lines
- Studies on differential infectivity of two strains of Chikungunya virus in different mosquitoes using real time RT-PCR
- Evaluation of RNAi (RNA interference) technology in inhibiting Chikungunya Virus replication *in-vitro* and *in-vivo*
- *Chikungunya pathogenesis study in laboratory animals*
- *CHIKV routine diagnostic Activity*

Investigation of Chikungunya-like illness in Tirunelveli district, Tamil Nadu

Sudeep A. B., Balasubramanian R., Jacob G. P., Arankalle V. A. and Mishra A. C.

Introduction

Cases of fever with acute arthralgia/arthritis in a massive scale were reported from several districts of Tamil Nadu during November-December 2009. NIV has investigated the outbreak in January 2010 to determine the etiological agent.

Objectives

To determine the IgM antibody status among the people.

To isolate the etiological agent/s responsible for the outbreak and characterize at serological and molecular levels.

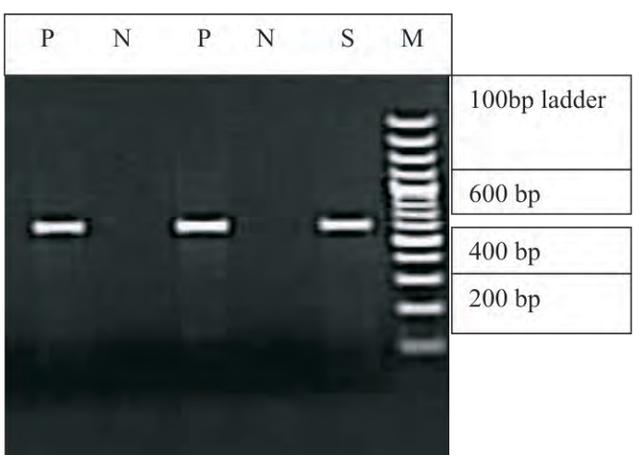
To determine the role of vector mosquitoes in the outbreak.

Work done

Blood (n=217) and mosquito samples were collected by NIV team from two badly affected areas in Tirunelveli district *i.e.* Melapalayam and Kadainallur. The blood samples were tested by MAC-ELISA test and found 107 and 22 samples with detectable levels of Chikungunya virus (CHIKV) and dengue virus specific IgM antibodies respectively.

Attempts to isolate the etiological agent from 12 acute samples resulted in the isolation of one strain of CHIKV. The isolate was amplified in Vero E6 cell line and confirmed by RT-PCR targeting partial sequences of E1 and NS4 genes (Fig 1). The strain belonged to African genotype (ESCA) without any mutation (E1: 226A). None of the mosquitoes pools (n=4) yielded virus isolation.

Figure 1: Gel electrophoresis RT-PCR amplicon of CHIKV.M=Molecular weight marker; N=Negative control; S= Tirunelveli isolate (CHIKV)



Studies on differential infectivity of two strains of Chikungunya virus in Vero E6 and C6/36 cell lines

Sudeep A. B., Parasher D., Arankalle V. A. and Mishra A. C.

Introduction

Since not much information is available on the Chikungunya virus infection and morphogenesis at cellular level, an attempt was made to study the replication kinetics of two strains of CHIKV in a vertebrate and an invertebrate cell line using different parameters.

Objectives

To study the growth kinetics of two strains of CHIKV in Vero E6 and C6/36 cell lines.

To study the commencement of cytopathic effects and plaque morphology in Vero E6 cell line with the above strains of CHIKV.

Work done

Growth kinetics of two strains of Chikungunya virus (061573, Andhra Pradesh 2006; 074831, Kerala 2007) was carried out in Vero E6 and C6/36 cell lines. Virus infected cells and supernatants were harvested at different post infection hours (4hr interval) and assayed for virus infectivity by $TCID_{50}$ method.

Though both strains showed virus replication in the cell line from 4hr PI, virus yield in supernatant was comparatively more with 061573 strain initially. At 8^{th} hr PI, the virus yield was 3.63 and 5 $\log_{10}$ $TCID_{50}$/ml for 074831 and 061573 strains respectively. A rapid increase in titer was observed with the latter strain reaching a titer of $\log_{10}10^{7.63}$ $TCID_{50/}$ml while the respective titer with the former remained at $\log_{10}$ $10^{5}TCID_{50/}$ml. However, after 16hr PI virus yield was almost similar with both the strains. In cells, though the initial adsorption of virus was more in case of 061573 strain than 074831 strain, virus yield at 8 hr PI was slightly more with 074831 strain ($\log_{10}10^{7.63}$ $TCID_{50}$/ml) than 061573 strain ($\log_{10}$ $10^{7}TCID_{50}$/ml).

An almost identical pattern of virus growth was observed in C6/36 cells as compared to Vero E6 with the two strains. The virus adsorption and subsequent growth was also similar to that observed in Vero E6 (cells). With 061573 strain, virus yield in the cells and supernatant at 48hr PI was 10^{8} $TCID_{50}$ per ml which was maintained till 72^{nd} hr PI. However, with 074831 strain, the same titer was obtained at 40 hr PI and maintained up to 72 hr PI. Further studies are in progress.

Studies on differential infectivity of two strains of Chikungunya virus in different mosquitoes using real time RT-PCR

Parashar D., Gokhale M. D., Sudeep A. B., Mishra A. C. and Arankalle V. A.

Work done

Ae. aegypti and *Ae. albopictus* mosquitoes were membrane fed with AP (E1-226A) strain, harvested at a daily interval from 0 to 7 days and processed by real time PCR. Analysis of post feeding mosquitoes at 0 hr showed an average titer of $10^{5.58}$ ($\pm 10^{0.52}$) and $10^{5.62}$ ($\pm 10^{0.51}$) RNA copies in *Ae. aegypti* and *Ae. albopictus* respectively. Analysis on the 3rd day PI demonstrated 3 log increase in RNA copies. Further studies are in progress.

Evaluation of RNAi (RNA interference) technology in inhibiting Chikungunya Virus replication *in-vitro* and *in-vivo*

Parashar D., S. Kumar, D. Patil, A. B. Sudeep, A. C. Mishra and V. A. Arankalle

Introduction

RNA Interference, a phenomenon of sequence-specific degradation of RNAs mediated by double-stranded RNA holds promise as a potential therapy for CHIKV infection. In continuation of the last years study *in vitro*, systems an attempt was made to evaluate two siRNAs (Chik 1 and Chik2) against CHIKV infection at *in vivo.*

Objective

To evaluate the potential of siRNAs in inhibiting CHIKV replication in laboratory mice.

Work done

Two siRNAs, *i.e*. Chik - 1, Chik 5 were mixed with CHIKV in three combinations (Chik-1 alone, Chik 5 alone and a mixture of Chik 1 and Chik 5 in combination with Hiperfect reagent) and inoculated subcutaneously in 4-6 days old Swiss albino mice with appropriate positive and negative controls. The study did not yield any conclusive results and further studies are in progress.

Chikungunya pathogenesis study in laboratory animals

D. R. Patil and V. A. Arankalle

Introduction

The post 2005-06 CHIKV outbreak in India and Indian Ocean Islands has seen reports describing unique clinical manifestations in humans. It is speculated that CHIK disease could be an immunopathology, though very little information is available. To study immunopathological aspects of CHIKV induced disease, we developed a mouse model, which showed hind limb stiffness and severe myopathology. We used the mouse model to study

CHIKV induced local and systemic immune response, viral RNA kinetics and pathology produced in different organs.

Objective

To study serum cytokine levels and local immune response in CHIKV infected mice.

Work done

Serum Cytokine profile in infected mice: Serum samples from infected animals and mock inoculated controls collected at different days post infection (PI) were assayed for the concentration of different cytokines using a multiplex biometric immunoassay (Bio-plex pro Mouse cytokine 23 plex Assay, Bio-Rad), containing fluorescent dyed beads, conjugated with a monoclonal antibody specific for a target protein, according to manufacturer's instructions. Our results indicated IL-13 has no significant role in CHIKV infection. However, the Chemokines *viz*. KC, MCP-1, and RANTES levels were significantly elevated at early time points *i.e*. on 3rd day PI and declined sharply subsequently. Similar elevation was observed for IL-6 and IL-10 levels, but their decline after 3rd day PI was gradual. On the contrary, IL-2 and IL-17 concentrations peaked at 6th and 8 day PI respectively. IL-3 and IL-5 levels have shown uniform increase upto 8th day PI.

Gene expression profile (Immune panel) in infected thigh of the mouse: RNA was extracted from thigh muscles of infected (3, 8 and 17 day PI) and mock infected mice (one sample) using Ribopure RNA isolation Kit (Ambion, USA) according to manufacturer's instructions. cDNA was prepared from 500 ng of total RNA per sample and run in duplicate on 7900-HT fast real time PCR machine (ABI, Foster City, CA). Data analysis was done using RQ software, with respect to control (uninfected) mice values. 18S was used as endogenous control. Statistical analysis for TLDA was performed using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA).

Out of 96 genes, expression of 79 genes were found to be up-regulated by at least 2.5 folds in infected mouse hind limbs as compared to the hind limbs of mock-infected mice at different time points. On the contrary, down regulation was noted for only two genes *ie*. CD19 and CSF3 by 4 fold and 66 folds on 8th PID and 17th PID respectively.

Expression of CXCL10 and CXCL11 was elevated by more than 1000 folds on day 3 PI, which declined sharply on 8 day PI. But the level remained higher than normal. Expression of the receptor to these chemokines, CXCR3 was found elevated on day 3 PI, which peaked on day 8 PI. Expression of CCR-2 increased concomitant to the expression of its ligand CCL-2 whereas CCL19 marginally increased on 3rd day PI and declined thereafter, becoming comparable to controls. Notably the expression of CCR-4 and CCR-7 remained comparable with controls throughout the period of study.

Expression of CD3E on day 3 PI increased, peaking at day 8 PI. Notably, the expression of CD8A was also elevated on day 3 PI, which peaked on 8th day PI and remained at higher levels upto day17 PI whereas, CD4 level was moderate on 8 and 17 day PI. H2Ea and H2Eb expression was normal on day 3 PI but increased sharply on day 8 PI. The expression of H2Eb has shown increase upto 17th day PI while no further increase in the level of H2Ea was observed. CD68 expression increased on 3rd day PI, which peaked at 8th day PI. On the contrary CD19 expression was down regulated on day 3 and 8 PI and became normal on 17 day PI. Expression of CD28 was weaker as compared to CTLA-4 throughout the study.

Expression of IFN , a hallmark of Th1 activity, was very high on day 3 to day 8 PI, but showed decline on day 17 PI though remaining on the higher side. IL-12 expression was also elevated; in particular, IL-12b expression was more compared to IL-12a. IL-18 expression peaked on day 3 PI but declined on day 8 PI. Similarly, IL-6 expression peaked on day 3 PI, which dropped sharply on day 8 PI. Notably, expression of IL-17 was very high on day 3 PI, augmented further on day 8 PI and maintained higher levels upto day 17 PI.

Expression of skeletal endothelium activation markers (E-selectin, P- selectin, Endothelin 1, VCAM-1, and ECE-1) was significantly elevated on day 3 PI. Notably the expression for all these molecules subsided significantly on day 8 PI and was at par with control mice.

We noted significantly elevated expression of granzyme and perforin at all time points studied, although expression was maximum on day 3 PI. Fas expression was elevated on day 3 and day 8 PI but declined to the level of controls on day 17 PI. Fas L expression increased on day 3 PI and peaked at day 8 PI and remained elevated upto 17th day PI. Expression of another pro apoptotic molecule, Bax was found to be increased at day 3 PI, which became normal at later time points. Notably, we found normal expression of anti apoptotic molecules Bcl2 and Bcl2 L during the study.

CHIKV routine diagnostic Activity

V. A. Arankalle, Supriya Hundekar and Mishra A. C.

Objective

To provide laboratory diagnosis for samples of suspected viral etiology.

Work done

Two thousand three hundred and fourteen samples were tested for CHIKV IgM using indigenously developed MAC-ELISA test (NIV Kit), which resulted in 1207 positives. The maximum number of cases was reported from Maharashtra with a positivity of 44% while Tamil Nadu reported a high positivity (78%). Month-wise and state-wise incidence of CHIKV positive cases and the number of samples tested at NIV during 2009-10 is given in Table 1 and Table 2.

Table 1: Month-wise distribution of CHIKV positive cases

Month	Total Samples tested	CHIKV IgM positive cases
April 09	57	15
May 09	89	61
June 09	150	70
July 09	136	67
August 09	142	70
September 09	161	63
October 09	284	110
November 09	289	141
December 09	292	167
January 10	325	220
February 10	259	149
March 10	130	74
	Total 2314	1207

Table 2: State-wise distribution of CHIKV cases

SR. NO.	State	Samples tested	CHIKV IgM POSITIVE
1	Maharashtra	1711	750
2	Tamil Nadu	558	438
3	Kerala	23	6
4	Gujarat	17	11
5	Andhra Pradesh	03	0
6	Orissa	02	2
Total		2314	1207

Evaluation of MAC-ELISA kits

Eleven MAC-ELISA kits for detection of anti-CHIKV IgM were validated during the year 2009-10.

Chandipura virus group

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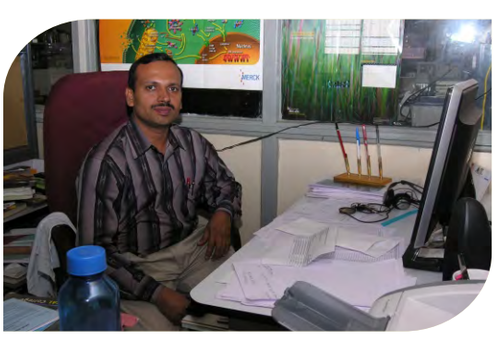

Chandipura virus group

Projects

- Host immune response to age susceptibility of Chandipura virus in murine model- DBT.
- Oncolytic effect of Chandipura virus nucleo (N) protein on experimentally induced tumor in murine model-DST.
- Laboratory diagnosis and Development of Diagnostics for Chandipura Virus- Service project

Host Immune response to age susceptibility of Chandipura virus in Murine Model

Funded by DBT

B. Anukumar and A. C. Mishra

Introduction

The immense clonal expansion of T lymphocytes that often follows infection probably represents an effort by the host to completely overwhelm an invading pathogen, thus minimizing the amount of tissue pathology that develops. However, other factors, such as the anatomical positioning of a pathogen can shift the balance in favour of dire immunological consequences. This activated T lymphocytes are continuously generated during immune response against infection and it should be removed from the immune system to prevent the bystander tissue injury as well as maintain the cellular homeostasis. There are several mechanisms in the immune system to control the proliferation and activation of lymphocytes to maintain cellular homeostasis. This study mainly focus on role of B cells, brain infiltrating lymphocytes (Bil) and T regulatory (Treg) cells during Chandipura virus infection.

Objective

- To study the various immune cells in pathogenesis and regulation in Chandipura virus infection.

Work done

Interaction of Chandipura virus with B lymphocytes through toll like receptor 4 (TLR4)

Chandipura virus induces IgM response within 48h post infection in young susceptible mice. Normal course of immune response to antigen, the antigen presentation, T cell activation and B cell activation collectively takes 4 to 5 days to secrete antigen specific IgM. To find out the mechanism of early IgM response, the B cells were purified from Swiss albino mice and infected with virus. Antigen specific proliferation, activation, IgM secretion and virus replication were the parameters tested in the infected B cells. The infected B cells were proliferated and secreted significant level of antigen specific IgM at 96h PI($p<0.05$). These cells were not permissive for virus replication. The infected B cells also up regulated the activation markers CD25 and CD69. To confirm whether Chandipura virus interact with B cells through TLR4, the TLR4 was blocked with anti TLR4 antibody. The blocked B cells did not proliferate and did not secrete undetectable amount of IgM after infection. These results were further confirmed by infection of B cells from TLR4 mutant C3H/J mice and same results were observed. The present study concluded that Chandipura virus directly interacts with B cells through TLR4 and the interaction might leads to secretion of antigen specific IgM antibody.

Infiltration of lymphocytes into the brain during Chandipura virus infection

Chandipura virus infection causes encephalitis in children as well as experimental mice. Encephalitis may be due to damage of brain cells by replication of the virus or immune response to infected cells. Under normal circumstances, CNS is not surveyed by circulating lymphocytes as in peripheral system. This is due to the physical nature of the blood-brain barrier (BBB) that typically excludes circulating cells, paucity of MHC antigen expression and other immune regulatory molecules on neural elements and lack of a formal lymphatic drainage system within the brain. But in case of injury or invasion by infectious agents, the state of immunological silence must be overcome very rapidly in order to efficiently defend the integrity of the organ. The immune mediated cell damages in brain are mainly due to the effect of infiltrating lymphocytes from peripheral immune system. This study aims at elucidating the role of brain infiltrating lymphocytes in pathogenesis using mice as the model system.

Mice were infected intracerebrally with Chandipura virus. Brain infiltrating lymphocytes were isolated from the mice at 48h post infection by Percoll density gradient method. Isolated lymphocytes were morphologically characterized by Leishman staining. It showed presence of atypical lymphocytes. These cells were also phenotypically characterized by staining with different phenotypic markers viz. TCR-β, CD45, CD14, CD4, CD8 and CD19. Presence of CD45+ and TCR-β+ cells was noticed (Fig.1). Percentage of CD4+, CD8+ and CD19+ cells were comparatively more in infected mice as compared to control. This suggests that there is infiltration of lymphocytes in infected mice. These lymphocytes may contribute to pathogenesis.

T regulatory cells in Chandipura virus infection

Chandipura virus induces reduction in CD4 and CD8 positive cells in susceptible mice. The reduction might be due to either direct virus infection of lymphocytes or regulatory mechanism of immune system like Fas/Fas L mediated apoptosis, suppression of proliferation by T regulatory (CD4+CD25+Foxp3+) cells etc to eliminate the activated lymphocytes to prevent the tissue injury. This study analyses the Fas/Fas L expression and T regulatory cells during infection. The direct virus infection mediated reduction of CD4+ and CD8+ cells were ruled out by direct infection of splenocytes. Initially the activation of CD4+ cells from infected mice was checked by expression of CD69 and secretion of IFN-γ. The CD4+ cells were found to be expressed in greater level of CD69 and secreted IFN-γ than uninfected mice. The activated CD4+ cells expressed significant amount of Fas/Fas L at 48h PI. The CD4+ cells were also positive for CD25 and expressed FoxP3, IL-10 and TGF-β transcripts. These results suggested that the immune system might control the activated lymphocytes by expression of Fas/Fas L and T regulatory cells.

Oncolytic potential of Chandipura virus nucleo (N) protein in experimentally induced tumour in Murine model

B. Anukumar, Prajakta Sahir and Poonam shawale

Introduction

The concept of using viruses in the treatment of cancer dates back to the beginning of this century when it was noted that patients with various malignancies experienced spontaneous tumour regression after rabies vaccination or about with a viral illness. Animal experiments in the 1920s confirmed that viruses were capable of infecting and lysis of experimental murine tumours. During 1950s it was demonstrating that Newcastle disease virus (NDV) and influenza virus were potent oncolytic agent of murine tumours. VSV is a well studied prototype for the large group of negative- strand RNA viruses. It is one of the most rapidly cytocidal viruses due to the potent induction of apoptosis in infected cells. The oncolytic potential of VSV against a variety of cancers has been established. Chandipura virus belongs to same genus of VSV and induces cytopathic effect in several cell lines. The current study was carried out to test the oncolytic potential of Chandipura virus.

Objective

- To study the oncolytic potential of Chandipura virus in experimentally induced tumour in murine.

Work done

Apoptosis induction of Chandipura virus in 3T3 cells

Induction of apoptosis in virus infected 3T3 cell line was tested by four different techniques viz production of apoptotic bodies, expression of caspase-3, changes in mitochondria membrane potential and TUNEL positivity of cellular nuclei. The cells were infected with Chandipura virus and at different time points, fixed and then used for further study. Apoptotic bodies were noticed at 4h hour PI cells in propidium iodide staining (Fig.2). Similarly mitochondrial membrane leakage was noticed from 120 min PI onwards (Fig.3). Caspase-3 expression was noticed from first hour PI onwards and at the same time the cells were positive for Chandipura viral antigen also (Fig.4). In TUNAL assay, TUNEL positive nucleus was noticed in virus infected cells (Fig.5). Collectively these results indicated that Chandipura virus induced apoptosis in 3T3 cells.

Tumour regression in murine tumour model

Tumour was induced in NIH nude mice by inoculation of BHK21 cells through subcutaneous route. After the appearance of visible tumour, the volume was measured and inoculated with live virus directly onto the tumour. The tumour volume was measured at

seven days interval upto 21 days PI. It was observed that after infection the tumour was regressed and then reappeared in some mice (Fig.6). The median survival time for infected mice was 22 days compared to the control (10 days). This result concluded that Chandipura has oncolytic potential.

Service projects

B. Anukumar, Rashmi. G and Poonam Shewale

Laboratory diagnosis of Chandipura virus

Total 385 samples received from various parts of India throughout the year were processed for Chandipura virus IgM. Fifty samples were positive for Chandipura virus IgM. Out of 385 samples 162 samples were processed for diagnostic RT-PCR and 68 samples were positive for CHP RNA.

Disease investigation

The Additional Director (Health), Gujarat invited NIV to investigate the encephalitis cases in Panchamahal District. NIV investigated possible involvement of Chandipura virus in encephalitis. Five hundred and eighty seven sera from fever cases and 1000 sand flies were collected from affected area. The sera were tested for Chandipura IgM and IgG, Dengue IgM and Chikungunya IgM. CHP IgM positive samples were 10.56% and 35.77% positive for CHP IgG. Three samples were positive for dengue IgM and 12 samples for Chikungunya IgM. None of the sand flies were positive for Chandipura RNA. None of the samples showed JE virus specific IgM antibodies.

Preparation of diagnostics (Monoclonal antibody)

Splenocytes from Chandipura immunized mice was fused with SP2/O cells. The fused cells were screened for anti Chandipura virus antibody and one clone was found positive for Chandipura virus. Characterization of the clone is in progress.

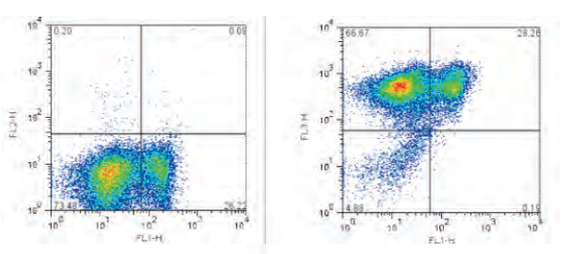

Figure 1: Cells from brain are positive for CD45 (common leukocyte antigen), TCR-β (T cell receptor-β) and negative for CD14 (marker for macrophages)

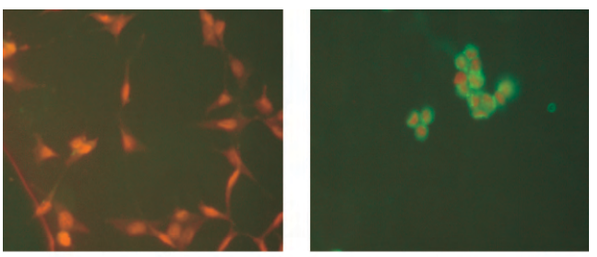

Figure 2: Chandipura virus a) uninfected as well as b) infected 3T3 cells stained with anti Chandipura antibody and counter stained with propidium iodide. The infected cells showed apoptotic bodies.

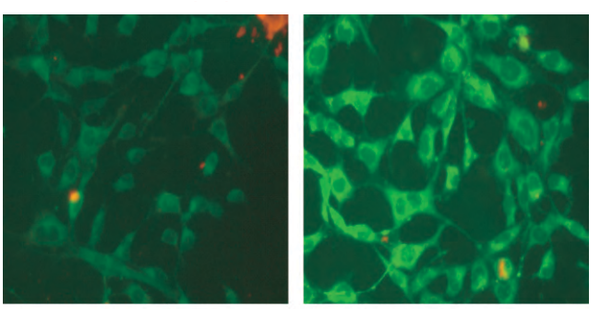

Figure 3: Chandipura virus a) uninfected as well as b) infected 3T3 cells stained with mitocapture reagent to test the mitochondrial membrane potential. The virus infected cells accumulated green fluorescence indicated the apoptosis.

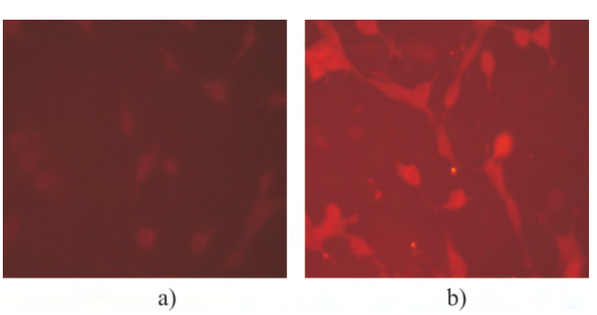

Figure 4: Chandipura virus a) uninfected as well as b) uninfected 3T3 cells stained with anti Caspase3 antibody. The red fluorescence in infected cells indicated caspase3 positivity.

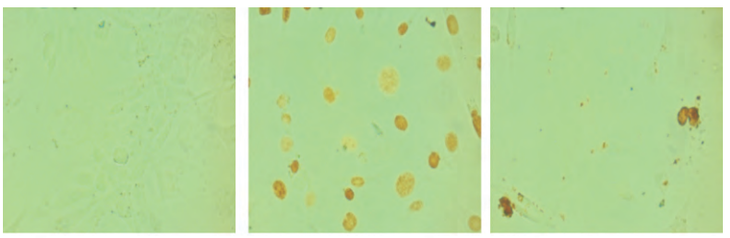

Figure 5: Brown colour TUNAL positive nucleus in the virus infected cells indicated the Chandipura virus infection mediated apoptosis.

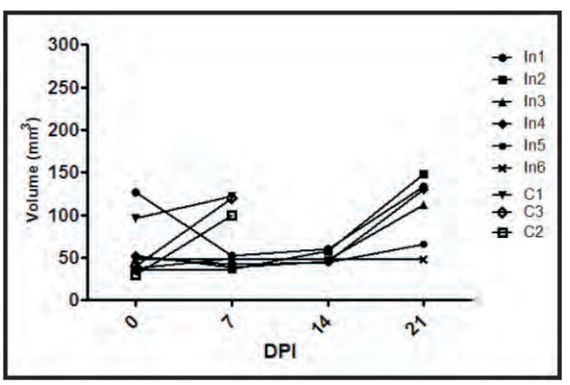

Figure 6: The graph represented the tumour volume of individual mouse both control (C) and infected (In).

Human Influenza Group

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Human Influenza Group

Projects

- Investigations of Pandemic Influenza A (H1N1) 2009.
- Multsite epidemiological and virological monitoring of human influenza surveillance network in India, phase II.
- Enhanced surveillance for pandemic H1N1 (pH1N1).
- Study of incidence, morbidity, mortality, immunology & risk factors for complications of Pandemic H1N1 influenza like illnesses (ILI) in HIV infected individuals, at Pune.
- Development of multiplex PCR for detection of respiratory disease viruses.
- Influenza Disease Burden in rural communities India
- Participation in External Quality Assurance

Investigation of Pandemic Influenza A (H1N1) 2009

M.S. Chadha and Influenza group

Introduction

Beginning May 2009, India experienced a pandemic caused by influenza A (H1N1) 2009 virus. Our network with its pandemic preparedness took a frontline role in providing diagnosis, as well as carrying out genetic analysis. Pandemic investigation and mitigation activities were undertaken together with the local and central health authorities. With information of novel influenza in Mexico and the US, Influenza department stepped up surveillance activities in Pune. We provided diagnosis for patients from various parts of the country. Clinical samples were received from Andaman & Nicobar Islands, Tamil Nadu, Delhi, Karnataka, West Bengal, Maharashtra, Goa and Kerala. NIV got 1st sample from a traveler returned from USA on the 2nd of May 09. The 1st positive was detected from Hyderabad on 14th May 09. Till 15th June 09 NIV and National Centre for Disease Control, New Delhi tested 283 samples in parallel. After 16th June, 2009, NIV continued to test samples from various states. 1st positive from Pune was reported on 9th June 09 and 1st death from Pune on 3rd August 09.

Objective

Diagnosis by Real time RT-PCR

Influenza virus isolation and antigenic characterization.

Genetic analyses of pandemic influenza.

To determine susceptibility of pH1N1virus to antiviral drugs.

Strengthening of regional laboratories for pandemic H1N1 detection.

Work done

1.1 Diagnosis:

During May 2009 to March 2010 out of, 25488 clinical samples were referred to NIV by various state health authorities for etiological diagnosis. They were tested by real time PCR for detection of influenza A and sub typed for pandemic H1N1. 5345 (21%) samples were found positive for pandemic H1N1 and 2077 (8.1%) for Seasonal Influenza A (Figure 1, 2)

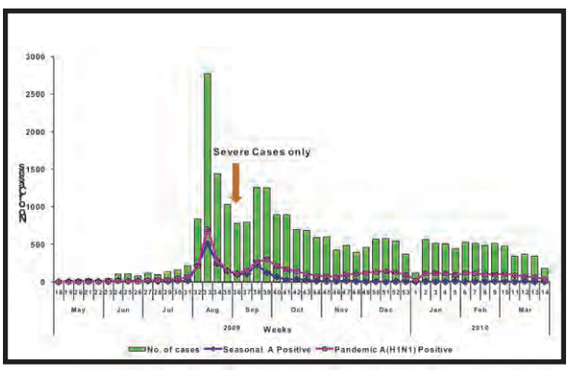


Figure 1: Clinical samples received per week for confirmation of pandemic influenza (H1N1)

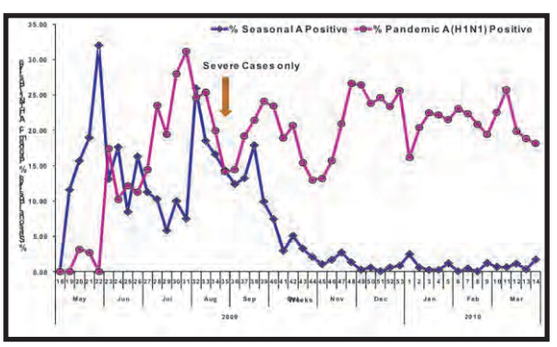


Figure 2: Percent positivity of Seasonal Influenza A & Pandemic A (H1N1)

Of the above referred clinical samples (1stAugust to 31st October 2009), 800 from Pune were randomly selected for detection of seasonal influenza H1N1, H3N2 and Type B. These samples when tested by real time RT-PCR showed 7.12% seasonal influenza positivity: 2%(16), A (H1N1),4%(32)A (H3N2),1.1%(9) Type B; whereas 5.5%(44) influenza A positives were un-typable. Of this subset 16 %(129) samples were p(H1N1) positive.

1.2 Virus isolation:

P. Kulkarni, B. More, M. Rangole, A, Kadam, A. Kiravale, M. Chadha

Representative real time positive pandemic and seasonal samples were attempted for influenza virus isolation.

Pandemic H1N1 2009: Four hundred and ninety eight samples were inoculated in MDCK for virus isolation of which 361 samples were simultaneously inoculated in embryonated chicken eggs. 200 pandemic H1N1 isolates were obtained; 118 in MDCK, 47 in eggs alone and 35 in both MDCK and eggs.

Seasonal Influenza: One hundred and sixty three seasonal influenza positives were inoculated in MDCK cell line and yielded 8 seasonal influenza virus isolates [6 seasonal A (H1N1), 2 A (H3)]

1.3 Genetic Analysis:

In collaboration with Bioinformatics group

V. Potdar, M. Dakhave, S. Jadhav, S. Cherian, M. Chadha

Full genome analysis:

We sequenced and studied whole genome of six Indian isolates from 3 fatal and 3 recovered cases. These positive cases of pH1N1 virus were detected from Pune (3), Bangalore (2) and Hyderabad (1) between May and September 2009.

Sequence analysis of the whole genome of six Indian isolates revealed >99% nucleotide identity with the A/California/04/2009 (H1N1) prototype strain in all the gene segments. Similarly, 99.06% amino acid identity was noted in the HA of the Indian isolates with respect to A/California/04/2009. The percent amino acid divergence (PAD) within each gene segment of the six Indian isolates ranged from 0% (in PB2 and M) to 0.66% (in HA).

Phylogenetic analysis of the six concatenated whole genome sequences was performed along with the whole genomes of global isolates (684 global isolates) available in the Gen Bank. In an earlier concatenated whole genome analysis of 240 pH1N1 virus isolates upto July 2009; seven discrete clades of the pH1N1 viruses circulating globally were observed. Six Indian concatenated whole genome analyses revealed that the Indian isolates belonged to three different clades. The earliest Indian isolate (A/India-Hyd/NIV51/2009) of May 2009 clustered with clade 5 isolates, one Indian isolate (A/India-Pune/NIV6196/2009) of mid August belonged to clade 6 while four Indian isolates (A/India-Blore/NIV236/2009, A/India-Blore/NIV310/2009, A/India-Pune/NIV6447/2009, A/India-Pune/8489/2009) of the period from June end to August 2009 clustered into clade 7.(Figure 3)

The clade specific signature mutations were noted in the Indian isolates. With respect to the prototype isolate A/California/04/2009 the specifc mutations were seen in Indian isolates that were listed in Table1. The amino-acid changes in the NP (V100I) 128 and NA (V106I and N248D) as noted in clade 5, clade 6 and clade 7 isolates were also observed in the single clade 5 Indian isolate, A/India-Hyd/NIV51/2009. In addition to the above 3 mutations, the clade 6 Indian isolates possessed the 2 mutations, K2E and Q310H in the HA that are unique to clade 6, while the clade 7 Indian isolates possessed S220T specific to the clade 7. The mutation I123V in NS1 that was found to be specific for clade 7 was noted in 3 out of the 4 Indian isolates wherein A/India-Pune/NIV8489/2009 retained I123.Mutations P100S and I338V in HA along with P224S in PA present in the Indian isolates were observed in all the non-clade1 isolates (Table 1).

Table 1: Mutations in the gene segments of the six whole genomes of pH1N1 Indian isolates with respect to A/California/04/2009

Gene segment	Residue Number	A/California /04/2009	A/India-Hyd/NIV51/2009	A/India-Blore/NIV236/2009	A/India-Blore/NIV310/2009	A/India-Pune/NIV6196/2009	A/India-Pune/NIV6447/2009	A/India-Pune/NIV8489/2009
PB1	61	T						I
	254	F		L		L		
PA	28	P	Q					
	224	P	S	S	S	S	S	S
	271	P			R			
	581	M					L	
NP	100	V	I	I	I	I	I	I
	232	T	P	P			P	
NA	30	I	V	V		V	V	
	106	V	I	I	I	I	I	I
	189	N				S		
	248	N	D	D	D	D	D	D
	256	F		V				
NS1	28	G				S		
	123	I		V	V		V	
	154	G						R
NS2	115	A	T					

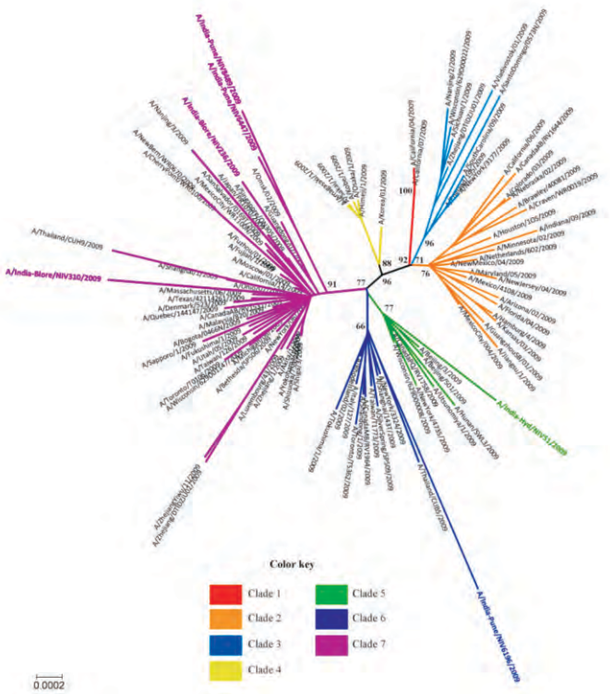


Figure 3: Phylogeny of six concatenated genomes of P (H1N1)

HA gene analysis:

To monitor the antigenic drift and to study specio-temporal pattern, 13 representative P(H11N1) isolates from May to September 2009, including 6 full genomes mentioned above were sequenced for complete HA gene. Sequence analysis showed the percent amino acid divergence (PAD) within 13 HA genes were 0.58 ± 0.17%. HA-based Bayesian phylogeny analysis of 13 Indian isolates when compared to the concatenated whole genome-based phylogenetic tree, clustering was maintained for clades 6 and 7 (Figure 4)

HA of the Indian isolates as in the other pH1N1 viruses possess D190 in the receptor binding site that is known to confer binding of H1 viruses to human receptors probably supporting the efficient transmissibility of these viruses in human. Majority of Indian isolates 11 of 13 as well as pH1N1 viruses possess D225 in the receptor binding domain (RBD) though two of the Indian isolates (A/Pune/NIV9355/2009, A/India-Pune/NIV10278/2009) had D225G (D222G as per pH1N1 numbering). Notably, this mutation was observed in the isolates from two fatal cases reported in August and September. Mutation in HA gene of 13 p (H1N1) Indian isolates with respect to A/California/04/2009 were listed in Table 2

PB2 gene analysis; A partial sequencing of 60 isolates was done to check K340N mutation which is marker for virulence along with D225G HA mutation. Two out of 60 isolates showed the K340N mutation; out of which one from fatal case. Notably these two isolates did not have D225G mutation in HA gene. Hence correlation of both the mutation with virulence was not seen.

Table 2: Mutations in the HA gene of 13 pH1N1 Indian isolates with respect to A/California/04/2009

Residue position (pH1N1 numbering)	Residue position in HA (without signal peptide)	A/California/04/2009	A/India-Hyd/NIV51/2009	A/India-Blore/NIV236/2009	A/India-Blore/NIV310/2009	A/India-Pune/NIV6196/2009	A/India-Pune/NIV6447/2009	A/India-Pune/NIV8489/2009	A/India-Delhi/NIV3610/2009	A/India-Mum/NIV5442/2009	A/India-Pune/NIV9355/2009	A/India-Mum/NIV9945/2009	A/India-Pune/NIV10278/2009	A/India-Delhi/NIV3704/2009	A/India-Pune/NIV10604/2009
2	-	K	·	·	·	E	·	·	·	·	·	·	·	·	E
4	-	I	·	·	·	·	T	T	·	·	T	·	·	·	·
15	-	A	T	·	·	·	·	·	·	·	·	·	·	·	·
100	83	P	S	S	S	S	S	S	S	S	S	S	S	S	S
171	154	K	·	·	·	·	·	·	E	·	·	·	·	·	·
214	197	T	A	A	A	A	A	A	A	A	A	A	A	A	A
220	203	S	·	T	T	·	T	T	T	T	T	T	T	T	·
239	222	D	·	·	·	·	·	·	·	·	G	·	G	·	·
240	223	Q	·	R	·	·	·	·	·	·	·	·	·	·	·
251	234	V	·	·	·	·	·	I	·	·	·	·	·	·	·
310	293	Q	·	·	·	H	·	·	·	·	·	·	·	·	H
338	321	I	V	V	V	V	V	V	V	V	V	V	V	V	V
356	339	G	·	·	·	R	·	·	·	·	·	·	·	·	·
387	370	N	·	·	·	·	·	·	·	·	K	·	·	·	·
468	451	S	·	N	·	·	·	·	·	·	N	·	N	·	·
564	547	I	·	T	·	·	·	·	·	·	T	·	T	·	·

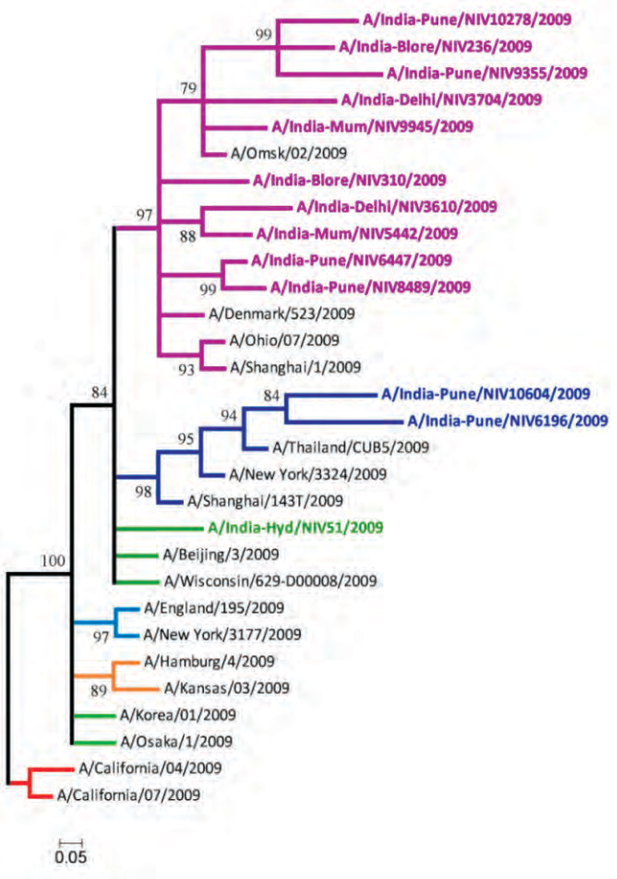


Figure 4: HA-based Bayesian phylogeny of p (H1N1)

Drug Susceptibility Testing

Since the beginning of pandemic influenza A (H1N1) virus, pH1N1 virus was susceptible to oseltamivir and zanamivir (neuraminidase inhibitors), and resistant to amantadine and rimantadine (M-2 channel blockers, or adamantanes). Monitoring of p(H1N1) for Neuraminidase Inhibitor (NAI) drug resistance was carried out. Partial Na gene sequencing for detection of H275Y mutation, a known marker for Oseltamivir resistance was carried out for two hundred p (H1N1) isolates. All the 200 isolates were sensitive to Oseltamivir drug.

Developments of Rapid molecular test to detect H275Y mutation in clinical samples

Real time RT-PCR was standardized for detection of H274Y mutation in clinical samples. 87 isolates which had already been assayed by conventional RT-PCR foll0wed by sequencing were

tested by Real time RT-PCR method standardized by us. All the 87 isolates were sensitive to oseltamivir drug and the results were concordant. Corresponding clinical samples were also tested by Real time RT-PCR and all were found to be sensitive. Further 302 clinical samples were tested by this method and result showed that the clinical samples were sensitive to oseltamivir drug.

Training & Technical Support to Other Laboratories

India experienced first case of pandemic H1N1 in May 2009. This emphasizes a need for timely diagnosis of this novel influenza virus and strengthening of all regional and designation of new laboratories for pandemic H1N1 detection. The staff of Referral center actively participated in an international workshop as trainers for other south East Asian countries and organized 3 National workshops for regional Laboratories. All International & National laboratories were trained for diagnosis of pandemic H1N1 2009 by real time PCR and conventional RT-PCR. Technical support, Standered operating procedure's (SOP), diagnostic reagents & kits (for 70,000 tests) were provided to international and national Laboratories.

Future Plans:

Surveillance for pH1N1 virus and drug resistance will be continued. Evolution of the virus also will be tracked by genetic analysis.

Multisite epidemiological and virological monitoring of human influenza, surveillance network in India, phase II

Objectives

- To continue the established epidemiological and virological influenza surveillance network in different geographical areas of India.
- Human resource development by way of training and strengthening of infrastructure.
- Timely dissemination of information generated and improve awareness.

Contribute the influenza strains and information generated to the global influenza surveillance network.

Pandemic preparedness

NIV acts as a regional and referral centre for influenza activity in India. Regional center carries out surveillance in and around Pune and Referral center has the responsibility for reconfirm and drug susceptibility testing of isolates received from regional laboratories. Further, contribution of strains and data was made to global network. Quality control and strengthening of regional laboratories was done through training and technical support.

Work done

Regional Activity: Surveillance in Pune

ILI surveillance was carried out among patients attending the various Pune Municipal Corporation (PMC) general outpatient (OPD) clinics and one pediatric OPD and IPD in KEM Hospital. OPDs were visited 3 times a week for sample collection.

34 samples collected during March and April 2009, were processed for virus isolation in MDCK cell line. None yielded an isolate.

From June 2009 onwards, testing strategy was changed for influenza surveillance; Real Time RT PCR was used as a frontline test and PCR positives wee further processed for virus isolation. Virological surveillance data (Samples tested by real time PCR) in Pune during June 09-March 2010 is summarized in Table 3. All sub types of influenza were circulating in Pune during this period and Pandemic H1N1 2009 was predominant amongst all.

Table 3: Real time PCR Results for OPD cases

No of samples Tested	No of samples Positives (%)	Pandemic H1N1 2009	H1	H3	H1+H3	B
531	139 (26.2%)	65	16	42	1	15

Influenza Virus isolation:

One hundred thirteen samples positive by real time PCR were processed for influenza virus isolation in MDCK cell line and 17 samples yielded influenza virus isolates: 10 Pandemic (H1N1) 2009 , 5 influenza type B and 1 H1 and H3 each.

Activity of Referral Centre:

NIV as referral center reconfirms the isolates and performs genetic analysis for isolates received from other regional laboratories. Referral center also contributes strains to WHO collaborating center and surveillance data to global influenza surveillance network (GISN).

Reconfirmation:

NIV received isolates/ clinical samples from all regional labs. These isolates were re-inoculated in MDCK and reconfirmed. Clinical samples of 21 patients who were influenza real-time RT- PCR positives from Dibrugarh were received for reconfirmation. Results are shown in Table 4.

Table 4: Influenza Isolates/ clinical samples received and reconfirmed by referral centre

Regional Center	No of Isolates received	No of isolates reconfirmed
National Institute of Virology, Pune	17	17
All India Institute of Medical Sciences, New Delhi	85	81
Christian Medical College, Vellore	23	22
National Institute of Cholera and Enteric Diseases, Kolkata.	54	51
Vallabbhai Patel Chest Institute (New Delhi)	25	25
Regional Medical Research Center Dibrugarh.Nagpur	21(real time PCR positive clinical samples)	4
Indira Gandhi Medical College, Nagpur	2	2
Total	206 isolates and 21 clinical samples	202

2.3 Genetic Analysis of influenza isolates:

V. Potdar, S. Tikhe, M. Dakhave, A. Salunkhe, M. Chadha

Reconfirmed isolates were subsequently characterized to determine phylogenetic relation with the recommended vaccine strains for the year 2009- 2010. For phylogenetic analysis HA1 gene was sequenced for A (H1N1), A(H3N2) and type B isolates. Drug susceptibility was also studied. To check the known markers for amantadine and neuraminidase drug, M2 gene and NA gene were sequenced.

A (H1N1): Thirty nine 2008-2009 H1 isolates were analyzed by sequencing for HA1 gene. City and year wise details of isolates were as follows; 10 isolates of 2008 from Chennai and 29 isolates of 2009 from Pune (10), Delhi (8), Kolkata (7), Chennai (4).Phylogenetic analysis clearly indicated that 38 strains of H1N1 were found to be close to A/Brisbane/59/2007, which is the recommended vaccine strain from 2008 to 2009 & 2009-10 for northern hemispheres. One strain (2009) from Delhi clustered with A/New Caledonia/20/99 which was 2001-2007 vaccine components. (Figure 5)

A (H3N2): One hundred and eight isolates of 2008- 2009 from Kolkata-17(09), Vellore- 2(08) &12(09), Delhi 63(09), Chennai -5(08) & 4(09), Pune2 (09), Dibrugarh 3(09) were analyzed for HA1 gene by sequencing and phylogenetic analysis.The results showed that 13 (2008) isolates and two (2009) isolates (Delhi &Kolkata) were clustered with A/Brisbane/10/2007 which was 2008-2010 vaccine components while all other 2009 isolates were clustered with A/Perth/16/2009, 2009-2010 vaccine component of southern hemisphere.(Figure 6)

Type B: Forty eight Type B isolates of 2008-2009 from Delhi 39(08) & 2(09), Vellore 2(08) & 1(09), Kolkata 3(09), Pune 1(08) were analyzed for HA gene. Phylogenetic analysis showed that 28 (2008) Delhi isolates and 4 (2009) isolates from Kolkata and Vellore were clustered with B/Brisbane/60/2008 which was 2009-2010 vaccine component. At the same time 2008(14) and 2009(2) Delhi isolates were clustered with B/Florida/4/2006, which was 2008-2009 vaccine component. Co-circulation of both the lineages was seen in Delhi.(Figure 7)

Drug susceptibility testing:

Adamantine Drug Susceptibility: 39 A(H1N1) and 108(H3N2) isolates of 2008-2009 were subjected to M2 gene sequencing for observing the presence of known molecular markers (amino acid positions 26,27,28,30 and 31). All H3 isolates were resistant to adamantine by S31N mutation. Majority A (H1N1) isolates were sensitive to adamantine drug except two 2008 isolates from Vellore, which were resistant to amantadine by S31N mutation. One Delhi isolate of 2009 had L26F known mutation leading to amantadine resistance this mutation is first time report from India. 38 A (H1N1) isolates were sensitive to adamantine.

Neuraminidase Drug Susceptibility: 39 A(H1N1) and 108(H3N2) isolates of 2008-2009 were tested for Oseltamivir resistance by NA gene sequencing to check the amino acid sites E119V, R292K, N294S(N2) , H275Y (N1) that responsible for drug resistance. All 108 H3 isolates were susceptible to Neuraminidase inhibitor drugs. Out of 39 A (H1N1) isolates, 31 isolates were resistant to Oseltamivir by H275Y mutation where as eight isolate from Pune (4) and Chennai (4) were sensitive to oseltamivir. Results indicts that co-circulation of neuraminidase resistant and sensitive viruses were observed in India.

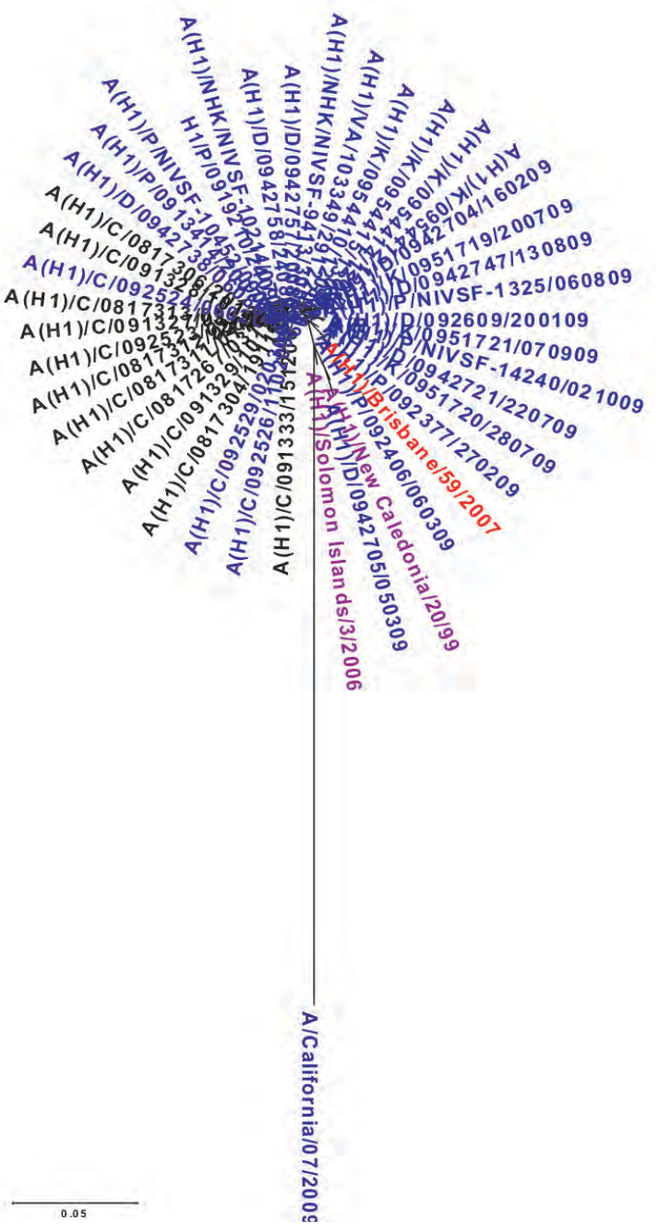


Figure 5: Phylogenetic Analysis of HA gene of A (H1N1) isolates

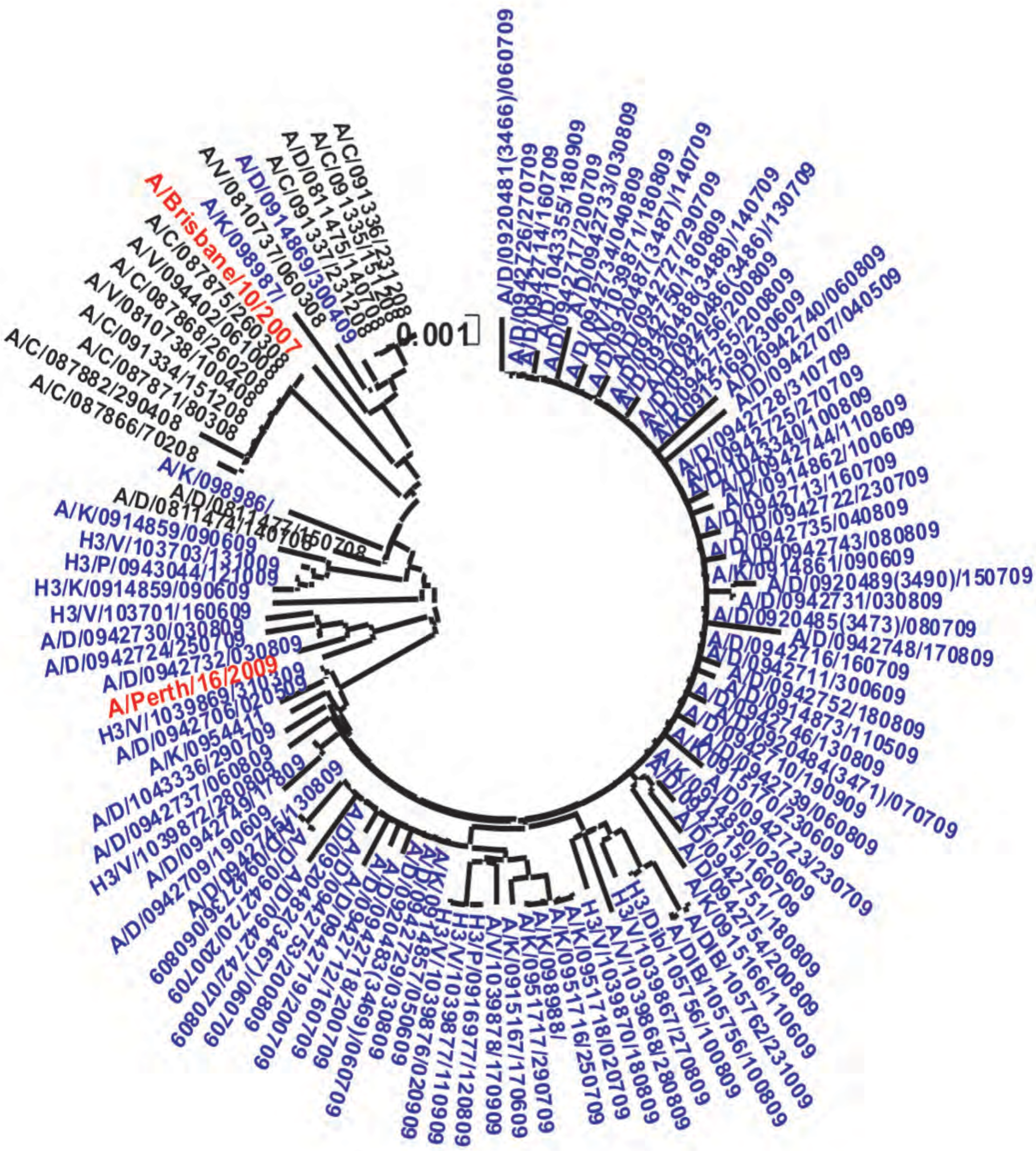


Figure 6: Phylogenetic Analysis of HA gene of A (H3N2) isolates

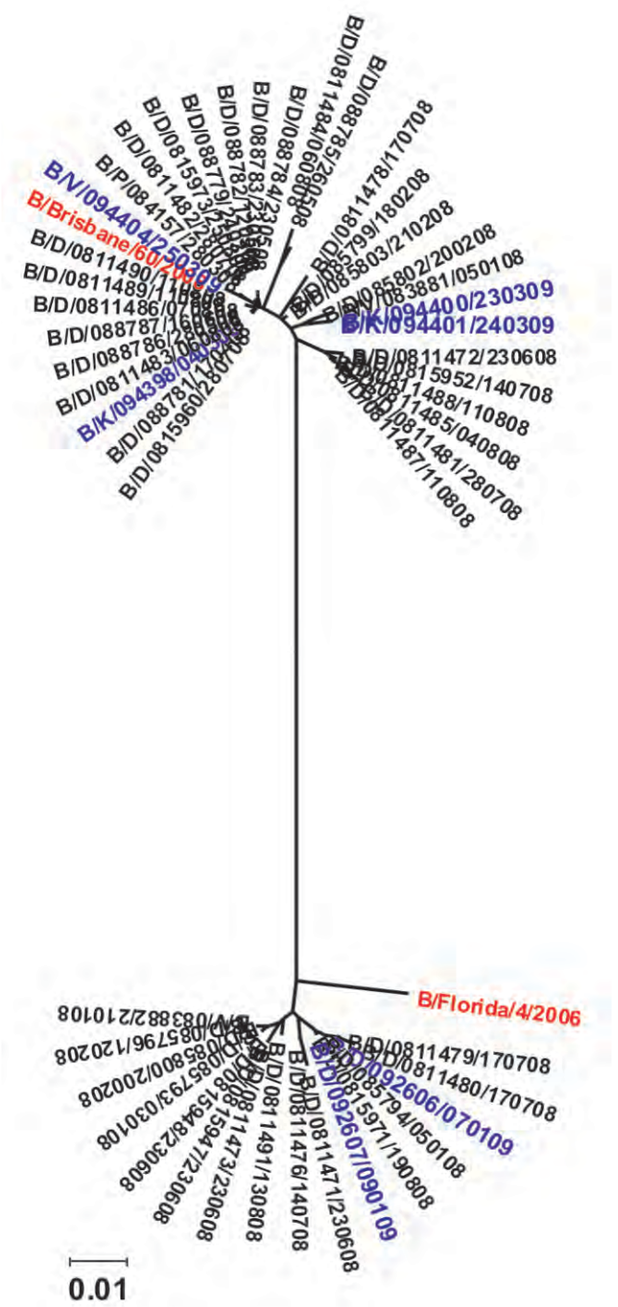


Figure 7: Phylogenetic Analysis of HA gene of Type B isolates

2.4 Quality Control of Regional Laboratories:

For capacity building of regional centers, for diagnosis of pandemic A (H1N1) panel of 5 coded samples (including seasonal strains as well as pandemic H1N1 strains) was sent to regional laboratories (NIV Pune, AIIMS Delhi, KIPM Chennai, CMC Vellore, NICED Kolkata, RMRC Dibrugarh and VP Chest, Delhi). Results were 100% matched for all the laboratories except VPCI New Delhi who reported mismatched for two samples.

2.5 Shipment to CDC:

Representative 10 strains were sent twice to WHO collaborating center CDC in December and January respectively.CDC re-confirmed the isolates. CDC Results were concordant for 19 strains except for one isolate which could not grow.

2.6 Contribution to Global Network: Data Contribution to WHO Fluent:

Flu Net WHO is an electronic platform, of the Influenza Surveillance data on global levels. NIV continues to enter Virological and epidemiological data, collected from 9 regional centers in India.

Enhanced surveillance for pandemic H1N1 (pH1N1)

Objective

- The aim of this study is to determine the incidence of novel H1N1 influenza virus infection in hospitalized acute lower respiratory tract infections and pneumonia in children & adults, (and exacerbation of chronic lung disease for adults in India study spectrum and seasonality of clinical illness caused by novel H1N1 influenza virus.

The study has been undertaken at four major sites, with existing Influenza surveillance capacity. These include National Institute of Virology Pune (West), All India Institute of Medical Sciences New Delhi (North), National Institute of Cholera and Enteric Diseases, Kolkatta (East) and King Institute of Preventive Medicine Chennai (South).

Work done

This study is in collaboration with Sassoon general hospital. SARI cases from adults and pediatric groups were screened for influenza A & B infection. Clinical samples received from Sassoon hospital were subjected for real time PCR. Among adults 2/39 patients had p H1N1 influenza whereas in children, 8/128 patients, had p H1N1 influenza and 6 had influenza type B.

Future plans

This surveillance will be carried out for one calendar year.

Study of incidence, morbidity, mortality, immunology & risk factors for complications of Pandemic H1N1 influenza like illnesses (ILI) in HIV infected individuals, at Pune.

Objective

Surveillance for pH1N1 in HIV infected individuals. In collaboration with

- National AIDS Research Institute, Pune (NARI),
- Pune Municipal Corporation (PMC),
- Pimpri Chinchwad Municipal corporation (PCMC)

Work done

Surveillance for pH1N1 is necessary in high risk groups HIV individual is one of the high risk group.

6 HIV patients having Influenza like Illness (ILI) were tested for Influenza and none were positive.

Development of multiplex PCR for the detection of respiratory disease viruses

Infections with respiratory disease viruses have a significant social and economic impact. For quick response to a potential outbreak, it is desirable to have a fast, accurate, and comprehensive diagnostic method capable of simultaneously typing and sub-typing respiratory viruses. However, virus-specific RT-PCR assay which require separate amplification of each virus, are resource intensive.

Objective

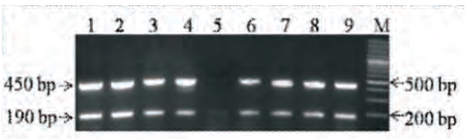
To develop a one-step multiplex RT-PCR for respiratory disease viruses (Influenza A&B, Parainfluenza 1-4 (PIV), Human Respiratory Syncytial Viruses (RSV A&B), Human Metapneumovirus (hMPV), Human Corona Viruses (CoV), Human Rhinoviruses (HRV), Adenoviruses (AdV), and human Enteroviruses) on viral isolates and clinical samples.

Work done

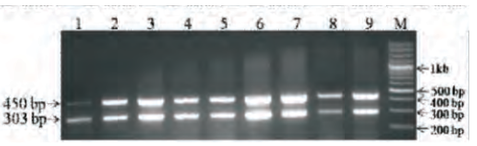
Primers were designed for Influenza A, Seasonal H1, Seasonal H3, Pandemic H1, Influenza B, Respiratory Syncytial Viruses A and B, Human Metapneumovirus, Parainfluenza virus 1-4, Human corona virus OC43, Corona virus 229, Corona virus NL63. One step multiplex PCR with Influenza A, Influenza B, Seasonal H1, Seasonal H3 and Pandemic H1 was standardized with virus isolate as well as on clinical specimens. One step multiplex PCR results were matched with real time RT-PCR upto 37 Ct value. Sizes of the different genes were: Pandemic H1 599 bp; Influenza B 515 bp; Influenza A 450 bp; Seasonal H3 303 bp and Seasonal

Multiplex PCR with clinical samples

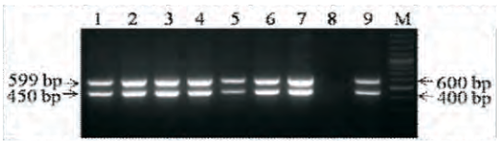
(A)Seasonal H1 (all samples are positive)



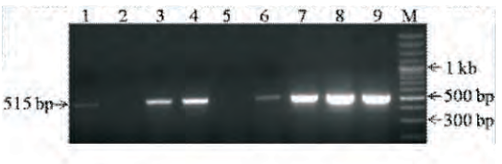
(B) Seasonal H3 (all samples are positive)



(C) Pandemic H1 (all samples are positive except sample 8 which was negative by real time RT PCR also)



(D) Influenza B (all samples are positive except sample 2&5 which were negative by real time RT PCR also)



H1 190 bp. This test showed 100% specificity. Influenza A, Influenza B, Seasonal H1, Seasonal H3 and Pandemic H1 genes were cloned in pGEMT vector for positive control and to check sensitivity of one step multiplex PCR. Other sets of multiplex PCR for respiratory viruses are under progress.

Influenza Disease Burden in rural communities India

Objectives

This study is undertaken to determine the burden of influenza in two rural areas of India. The study will also determine risk factors for severe illness. The study sites are Vadu,(40 kilometers southwest of Pune,) and Ballabhgarh, located (40 kilometers south of Delhi). These locations have ongoing demographic surveillance studies.

Specific objectives:

- Estimate the incidence of laboratory confirmed influenza among persons hospitalized for acute respiratory illnesses and acute exacerbations of chronic medical conditions.
- Estimate the incidence of laboratory confirmed influenza among medically attended outpatients seeking care for acute respiratory illnesses and acute exacerbations of chronic medical conditions

- Characterize the clinical spectrum of inpatient and outpatient disease related to influenza, determine risk factors for severe disease and describe the seasonality of influenza.

Work done

In this study patients with Severe Acute Respiratory Infection (SARI) cases and OPD cases were enrolled and respiratory specimens were collected at the Vadu and sent to NIV for identification of influenza. These samples were tested by real time PCR.

Table 5: Results of tests carried out in IPD and OPD patients

Cases	No of samples Tested	No of samples Positives by real time PCR (%)	Pandemic H1N1 2009	H1	H3	B
IPD	1394	321*(23 %)	151	20	100	50
OPD	605	105** (17.3%)	53	2	2	48

*PCR positives were processed for virus isolation in MDCK cell line. Of the IPD cases, 60 real time positives were processed for virus isolation which yielded 1 type B isolate.

Among 27 real time positives from OPD cases, virus isolation in MDCK yielded 5 virus isolates (4 pandemic H1N1 2009, 1 Type B).

Participation in External Quality Assurance

V. Potdar, S. Tikhe, S. Salunkhe, M. Dakhave, K. Patil, M. Chadha

External quality control of PCR is necessary for referral center and hence referral center took part in external quality assurance program. The Referral Centre took part in the WHO External Quality Assurance Panel 7(EQAP) testing program. The panel is mainly for molecular testing of influenza A along with Novel A (H1N1) and Type B influenza. Panel contains coded lyophilized RNA with reconstitution buffer. Reconstituted RNA were then subjected to various in-house and commercial real time and conventional RT-PCR which were regularly been used by referral centers.

WHO EQAP Panel 6: Panel 6 comprising of 10 coded RNA was received in June 2009.

WHO EQAP Panel 7: Panel 7 with 10 coded RNA received in January 2010.

Both panels were tested for Type A, H5, H1, H3, N1, Pandemic H1 by real time PCR and one step PCR. Results for both the panel were 100 % concordant with EQAP results.

Avian Influenza Group

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Project staff

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Mr. B. Pal, Research Assistant (till August 2009)
Mr. B. J. Payyapilly, Research Assistant
Mr. V. Ghule, Laboratory Attendant

Avian Influenza Group

Projects

- Avian Influenza Outbreak investigations
- Avian Influenza (AI) surveillance
- Pandemic H1N1 outbreak investigations
- Seroepidemiology of pandemic influenza A (H1N1) 2009 virus infections in Pune, India
- Pre-clinical efficacy study of pandemic influenza (H1N1) 2009 inactivated (IIV) and live attenuated (LAIV) vaccine in mice.
- Protective efficacy of H5N2 inactivated vaccine against highly pathogenic H5N1 AI virus in chickens
- Generation of immune sera using inactivated influenza virus isolates for influenza diagnosis
- Follow-up of the respiratory disease outbreak in Murshidabad district, West Bengal

Avian influenza outbreak investigations

J. Mullick., S. D. Pawar., S. S. Koratkar, S. Cherian, S. Jadhav

Introduction

Highly pathogenic avian influenza (HPAI) outbreaks are caused by Influenza A viruses belonging to the family *Orthomyxoviridae.* Globally, the HPAI are known to belong to H5 subtypes causing up to 100% mortality in poultry and among these the H5N1 viruses continue to pose a serious threat to global public health during HPAI outbreaks. There has been a wide spread infection of H5N1 in backyard as well as commercial poultry in West Bengal, Tripura and Assam in 2008 and 2009. These outbreaks are now continuously monitored which help to contain the spread of H5N1 viruses. During this period, the Avian influenza department has provided diagnosis of the infected poultry received from various regions of the country and isolated the viruses from them. Full genome sequencing and characterization of the viruses has portrayed a candid picture about the introduction of the virus into the country and its spread.

Objective

- Diagnosis of H5N1 in the country
- Isolation and characterization of the various strains of H5N1

Work done

Diagnosis of H5N1: Dead bird samples received from Hemtabad block of Kantor district from Uttar Dinajpur, West Bengal suspected for H5N1 were processed by RT-PCR , Real Time PCR and virus isolation. One step RT-PCR using WHO sets of diagnostics primers specific for influenza A, HA (H5) and NA (N1) and Real Time PCR using ABI influenza A/H5 and N1 kits were performed. The results of the positive samples were submitted to the Government of India. Simultaneously, the virus was isolated using 10 day-old embryonated chicken eggs and MDCK cell line. Identification of the virus was performed by sequencing HA, H5 and N1 gene fragments and by hemagglutination inhibition (HAI) assay. The HAI assay including a panel of antisera that included antibodies against influenza A(H5N1)-Navapur A/ Ck/ India/ 33487/ 2006-H5N1, A(H5N2), A(H7N3), A(H9N2), NDV and normal ferret and sheep sera concluded that the positive samples had the H5 antigen. Sequencing of the HA gene showed presence of the characteristic polybasic endo-proteolytic cleavage site GERRRKKR which is a known molecular marker for HPAI virus. Molecular characterization for markers such as E627K substitution at the PB2 gene (pathogenic marker), substitution of H274Y in the NA gene (marker for resistance to Oseltamivir and Zanamivir drugs), and sensitivity to Amantidine on the basis of markers in the M gene was performed which confirmed the highly virulent virus.

Isolation and Characterization of the H5N1 strains: Characterization of the H5N1 strains received during the outbreak investigations shown in Figure 1 were performed to understand the genetic diversification of the Indian isolates. The virus was isolated from the representative set of samples that were positive for H5N1 from 2008 outbreaks in Assam and West-Bengal and 2009 outbreaks in West-Bengal. A total of 8 full genome sequences were performed for all the eight segments. Molecular and phylogenetic analyses for all the segments were further carried on. In the HA gene phylogenetic analysis, all the 2008-09 Indian isolates belonged to the EMA sublineage of clade 2.2 (Figure. 2). Based on the molecular and phylogenetic characterization of the Indian H5N1 viruses, this study suggested introduction of the H5N1 virus for the third time into the country, suggestive of a possible endemicity in the eastern and northeastern regions of the country.

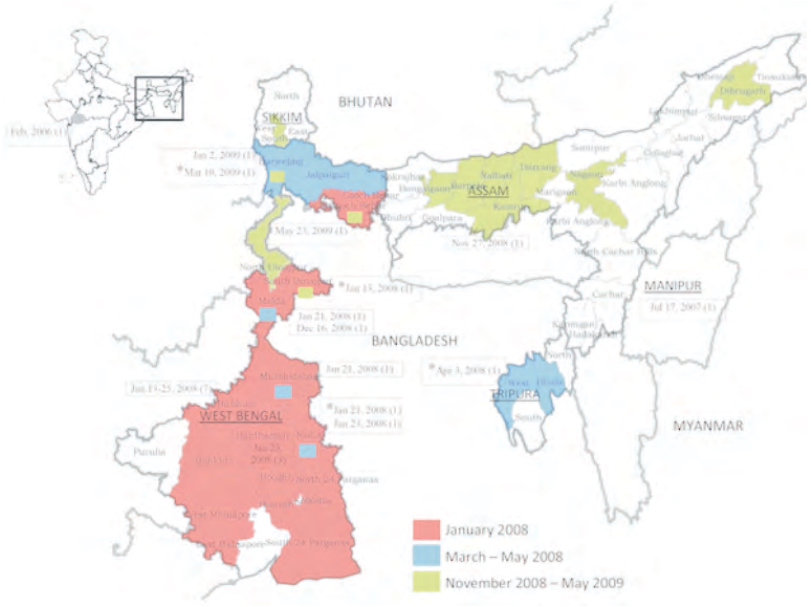
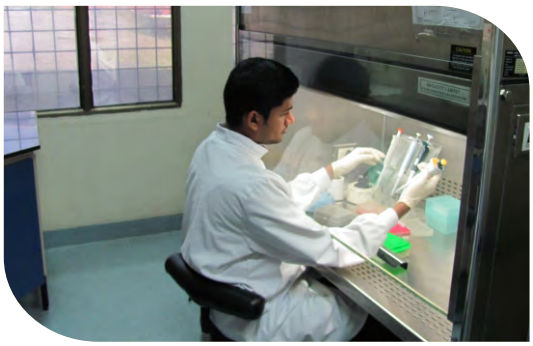


Figure 1: Location of the H5N1 outbreaks during 2008-2009 in India



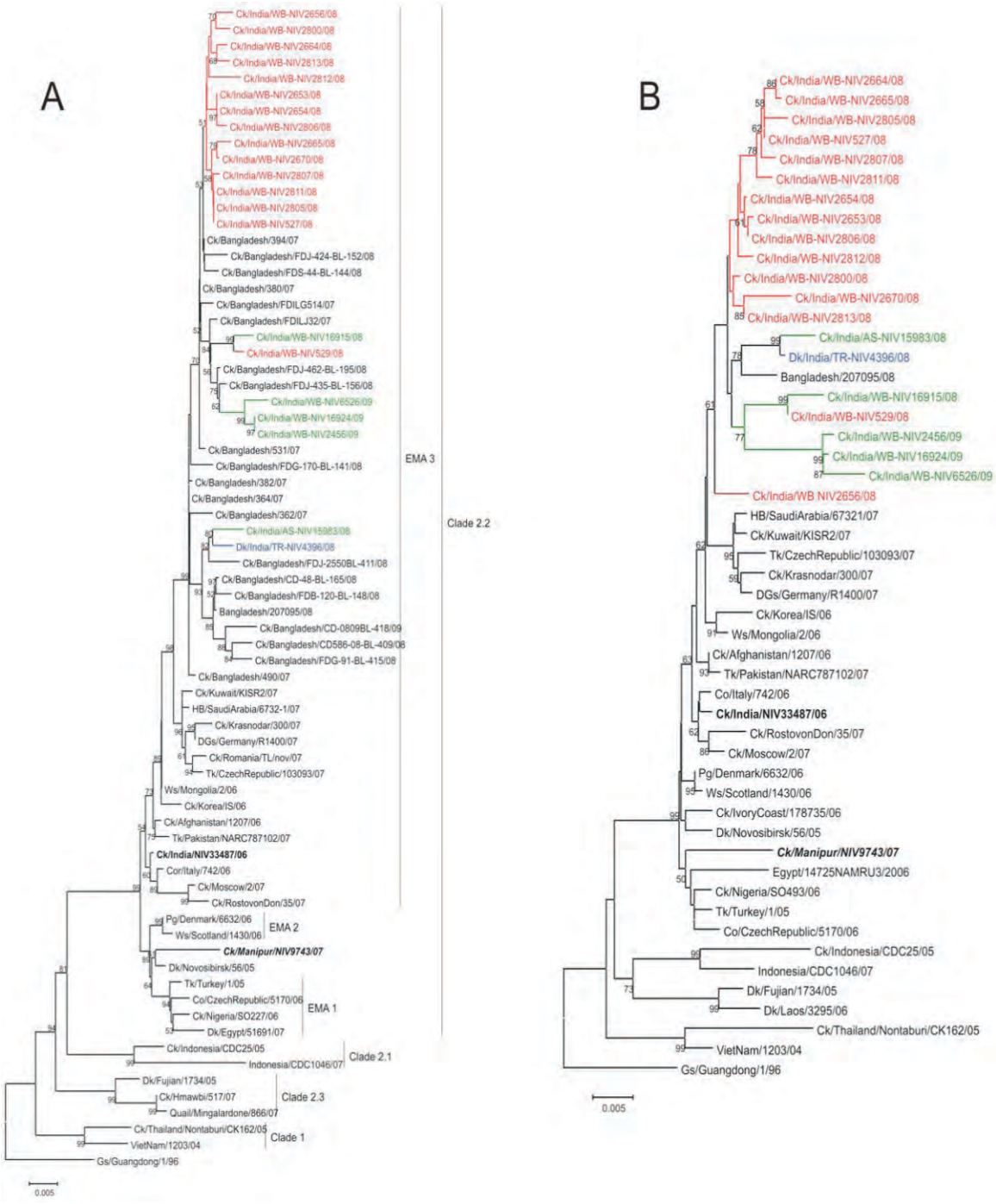


Figure 2: Phylogenetic analysis of the HA (A) and the NA (B) genes of the H5N1 Indian isolates using the neighbour joining method

Avian influenza (AI) surveillance

S. D. Pawar, C. G. Raut, S. S. Koratkar, J. Mullick, A. C. Mishra

Wild birds and avian species associated with aquatic habitats such as ducks, geese, swans, gulls, shorebirds, etc. are considered as primordial reservoirs of the AI viruses. Much less is known about the influenza viruses of these aquatic birds in the Indian habitat. Since India has envisaged H5N1 outbreaks at different phases, the role of migratory birds, poultry and ducks cannot be ruled out in the possible transmission of H5N1. The AI department had earlier initiated AI surveillance in wild migratory birds and poultry in pune districts jointly with the Ela foundation, Pune. In the light of the multiple H5N1 outbreaks in West-Bengal, a state with a very unique habitat, the AI surveillance studies have been extended to understand epidemiology of AI viruses in these species.

Avian influenza surveillance in poultry, domestic ducks and in wild migratory birds in districts of West Bengal (WB)

Introduction

A collaborative project with the State Key Laboratory of Emerging Infectious Diseases, (SKLEID), University of Hong Kong, Hong Kong (WHO reference laboratory) has been initiated. During this period the teams from NIV and SKLEID, Hong Kong visited the local poultry markets and collected cloacal swabs from the various poultry centres to initiate the local poultry market surveillance. The teams also visited epicenters of H5N1 poultry outbreaks in WB

Objective

- Set up epidemiological surveillance of AI in poultry, domestic ducks and in wild migratory birds in WB
- Study the seroprevalence of AI in ducks and poultry in WB.

Work done

A total of 186 samples from ducks and poultry were collected and 54 samples were also collected from poultry (chickens, quails) Pune, Maharashtra (Fig.3). All the samples were inoculated in 10-day-old embryonated chicken eggs for virus. One sample from poultry market was positive for New Castle Disease (NDV) and none of the other samples were positive for AI viruses.

During the second visit to WB, a total of 606 serum, cloacal and tracheal samples were collected from ducks and chickens from various blocks of Murshidabad and Nadia districts of West Bengal. The details of serum samples are shown in Table-1.

Table 1: Details of samples

Sr. No	Type of Samples	Species		Total
		Duck	Fowl	
1	Blood (Serum)	200	20	220
2	Tracheal swabs	203	19	222
3	Cloacal Swabs	149	15	164
	Total	552	54	606

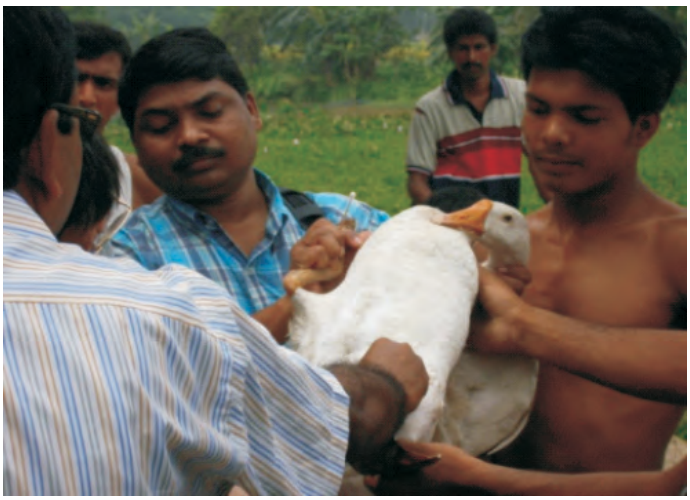


Figure 3: Collection of cloacal swab from ducks in WB

All the collected 220 sera were tested by hemagglutination inhibition (HI) assay for detection of antibodies against AI viruses and 386 tracheal and cloacal swab specimens were processed for virus isolation. The results of serology and virus isolation are as follows;

Serology results: Sera from ducks and chickens were tested by hemagglutination inhibition (HI) assays using horse and turkey red blood cells with influenza H5N1, H7N1, H9N2 and NDV viruses.

Ducks showed antibodies against H5N1 and H7N1 viruses while chickens did not show the presence of antibodies against H5N1 and H7N1 viruses indicating past exposure of ducks to H5N1 and H7N1 viruses. Ducks showed 20.7% antibody positivity against H9N2 virus suggesting active circulation of H9 viruses in ducks. Ducks and chickens also showed positivity against New Castle Disease (NDV) virus (Table 2).

Table 2: HI antibody percent positivity in duck and chicken sera (HI titer Cut Off: >/=10)

	Virus Antigen used			
Sera from	H5N1	H7N1	H9N2	NDV
Ducks	18/198 (9.0%)	8/198 (4.0%)	41/198 (20.7%)	6/198 (3.0%)
Chickens	0/18	0/18	2/18 (11.1%)	5/18 (27.7%)
Total sera	214	216	216	216

Virus Isolation results:

Cloacal and tracheal swab specimens from ducks and chickens were processed for virus isolation using 10-day-old embryonated chicken eggs. A total of 386 samples were processed for virus isolation. Allantoic fluids from inoculated eggs were tested by hemagglutination (HA) test. Allantoic fluids which were positive by HA tests were further tested in HI assay for virus identification. A total of 16 allantoic fluids were positive by HA test and all were identified as influenza A(H4) viruses.

AI surveillance in domestic poultry markets in Pune during May-July, 2009

SD Pawar, SS Keng, MR Khude, SK Waghmare.

Introduction

AI surveillance in domestic poultry markets in Pune was carried out during May-July 2009. The samples included cloacal and tracheal swabs, cage swabs, drinking water samples and tissue samples. Birds that were screened consisted of broiler, backyard chickens and quails (total birds sampled were 609). A total of 1189 samples were collected from various wet poultry markets in and around Pune City.

Work done

A total of 693 samples were processed for virus isolation. Allantoic fluids positive by HA tests were then identified by HI assay using H5, H7, H9 and NDV antisera. A total of 43 influenza viruses were isolated from these samples and were identified as influenza H9 viruses. Partial sequencing of HA and NA genes were done and further characterization of these isolates are in progress.

AI surveillance in wild and migratory birds in districts of WB

S. D. Pawar, S. Pande, A. S. Rawankar, S. D. Kale, M. R. Khude, Ela foundation, Pune and RDDL, WB

Introduction

In the light of the highly pathogenic avian influenza (HPAI) H5N1 outbreaks in WB, AI surveillance was undertaken in wild and migratory birds in districts of WB to study the role of these birds in influenza virus transmission. This work was undertaken in collaboration with Ela foundation, Pune and RDDL, WB.

Work done

A total of 3522 fecal and environmental samples were collected from ten districts of WB, during 13th February to 13th March 2010. 512 representative samples were processed for virus isolation. No virus was isolated from these samples. Further processing of the remaining samples for virus isolation is in progress.

Pandemic H1N1 outbreak investigations

V. A. Potdar, M. S. Chadha, S. M. Jadhav, J. Mullick, S. S. Cherian & A. C. Mishra

Introduction

The first influenza pandemic of the 21st century was declared with the emergence of a novel Influenza A (H1N1) strain in April 2009 in Mexico and USA that further spread globally infecting people worldwide and causing deaths. The virus was first detected in India in May 2009 following which there were many outbreaks of the virus in other parts of India.

Objectives

- Diagnosis of samples by rRT-PCR.
- Characterization of the pH1N1 strains of India.

Work done

Diagnosis and detection of swine flu was done by Real time RT-PCR as per CDC protocols during the surge of samples at NIV. Clinical samples were received from the Human Influenza department and report was subsequently sent to the department and the respective centre.

Members of the detection and diagnosis team: J Mullick, MR Khude, SS Kode, SS Koratkar, SS Keng, BJ Payyapilly, SK Waghmare, V Ghule, JPN Babu (AI Group); P Yadav, P Barde, DR Patil, DK Singh, S Chauhan, CP Upadhyaya, BSL 3 Lab support staff, (VR-HCL Group); Vasudha P, P Phulmali. S Rautela, R Singh, Feraz (JE Group); Hirawati (Microarray); MD Gokhale, George, O Mandke,(Entomology Group).

Characterization of pH1N1 virus: In an attempt to understand the molecular nature of the virus full genome sequencing of pH1N1 from different Indian isolates were performed. During this period all the eight segments of 6 Indian isolates including both recovered and death cases from various places in India was completed. One of the isolate was the first Indian pH1N1 case from Hyderabad detected for pH1N1 infection. Analysis of the full genomes from the six Indian isolates showed the circulation of virus belonging to clade 5, 6 and 7. Sequencing the HA gene of 28 additional isolates representing various time-points and regions and its analysis determined introduction of any mutations during the course of time of infection. Analysis of the HA gene of these 34 isolates showed that two isolates out of 34 had a D222G mutation. Further studies are underway to understand the correlations of the specific mutations to viral fitness and adaptability in the country.

(J Mullick, SS Keng, BJ Payyapill (AI Group); VA Potdar, Chadha M (Influenza); AC Mishra)

Seroepidemiology of pandemic influenza A (H1N1) 2009 virus infections in Pune, India

B. V. Tandale, S. D. Pawar, Y. K. Gurav, M. S. Chadha, S. S. Koratkar, V. N. Shelke, A. C. Mishra

Introduction

India reported the first confirmed pandemic influenza (H1N1) 2009 case from Hyderabad on 16th May 2009 and Pune reported its first case on 23rd June 2009. Seroepidemiological study of the pandemic influenza (pH1N1)2009 infections was undertaken by HI assay.

Work done

HI assay using turkey Red Blood Cells (RBCs) was standardized and used for serodiagnosis of pandemic influenza (H1N1) 2009. Pandemic influenza (H1N1) 2009 virus isolate was grown in 10-day-old embryonated chicken eggs, was inactivated using beta-propiolactone and was used in the assay. This was undertaken in collaboration with Outbreak Response Group of NIV.

Initially, pre-pandemic archived sera (103 no.), collected during the year 2008 were assayed for the determination of baseline seropositivity. These sera were obtained from Dengue Group. All of the 103 sera collected during the year 2008 had a titre of <1:10 against the pandemic influenza A (H1N1) 2009 virus. A titre of ≥1:10 was considered positive. This work was done at Biosafety Level -2 with BSL-3 practices. All the collected sera were also tested with the following seasonal influenza viruses;

- Influenza A(H1N1)
- Influenza A(H3N2)
- Influenza B Victoria
- Influenza B Yamagata

Sero-surveys were conducted among the risk groups and general population to determine the extent of pandemic influenza A (H1N1) 2009 virus infections. Serum samples were from the risk groups such as hospital staff, general practitioners (GPs), school children

and staff and general population. HI assays were performed using turkey RBCs employing standard protocols.

Detailed results reported by the ORG Group.

Conclusion

Pandemic (H1N1) 2009 infection was widespread in all sections of community. However, infection was significantly higher in school children and general practitioners. Hospital staff had the lowest positivity suggesting the efficacy of infection-control measures.

An Outbreak of pandemic influenza (H1N1) 2009 in a residential school in Panchgani, Maharashtra, India.

Y. K. Gurav, S. D. Pawar, M. S. Chadha, V. A. Potdar, A. S. Deshpande, A. H. Hosmani & A. C. Mishra

Introduction

An outbreak of influenza in a residential school during June and July 2009 was investigated with an objective to determine the etiology, clinico-epidemiological features and dynamics of the infection.

Work done

HI assay was performed on entire study population (352 students and 63 staff members) for detection of antibodies for pandemic (H1N1) 2009, seasonal H1N1, H3N2 and Influenza B/Yamagata (BY) and B/Victoria (BV) lineages.

Results

Serosurvey carried out showed HI antibodies to pandemic (H1N1) 2009 virus in 216 (52 %) subjects in the school and 22 (9%) in the community.

Detailed results reported by the ORG Group.

Conclusions

The first outbreak of pandemic (H1N1) 2009 due to local transmission was confirmed among students in a residential school at Panchgan, Maharashtra, India. This is the first report of outbreak of pandemic (H1N1) 2009 in a residential school in India.

Serosurveys for Pandemic Influenza A(H1N1) 2009 virus infections among hospitals and schools as high-risk settings/groups in Mumbai (Feb. 2010)

S. D. Pawar, B. V. Tandale, Y. K. Gurav, S. S. Koratkar, M. S. Chadha and A. C. Mishra

Work done

In continuation of the sero-surveillance work, a total of 458 sera from school children and hospital staff were collected from Mumbai by the ORG Group. These samples were tested for pandemic influenza (H1N1) 2009 antibody. 56.7% school children and 25.6% hospital staff

showed HAI antibodies to pH1N1 virus. Detailed results are discussed in the quarterly report of the ORG Group.

Serology Team: MR Khude, SS Kode, Mr. Walekar, Mr. Sonawane, Mr. Anand Singh, Rupesh Singh, Miss. Harini Suryanarayan, Mr. JPN Babu, Mr. V. Ghule and Members of ORG Group.

Serodiagnosis of swine origin influenza H1N1 infections by Microneutralization (MN) assay

S. S. Kode, S. D. Pawar, A. C. Mishra

Work done

Standardized of MN assay for serodiagnosis of pandemic influenza (H1N1) 2009. Human sera from Pune and Panchagani were tested in MN test. Positivity to both pandemic and seasonal H1N1 viruses was noted in MN test. Further analysis and work to study cross-reaction of pandemic (H1N1) antibodies with influenza (H1N1) viruses is in progress.

Pre-clinical efficacy study of pandemic influenza (H1N1) 2009 inactivated (IIV) and live attenuated (LAIV) vaccine in mice.

S. D. Pawar, L. R. Yeolekar, S. S. Koratkar, M. Ganguly, R. M. Dhere, A. C. Mishra

Introduction

After rapid global spread of a pandemic influenza (H1N1) 2009, there is need for development of effective vaccines for mass vaccination.

Objectives

- Investigation of the immunogenicity and protective efficacy of inactivated influenza vaccine (IIV) and live attenuated influenza vaccine (LAIV) of pandemic influenza (H1N1) 2009 in BALB/c mice as a pre-clinical animal model.

Conclusion

Both one and two doses of IIV rendered protection from the disease after challenge with the wild-type virus. Two doses of LAIV were required for protection in BALB/c mice.

Phase II & Phase III clinical trials of pandemic influenza (H1N1) 2009 live attenuated (LAIV) vaccine (SIIL-NIV).

S. D. Pawar, L. R. Yeolekar, S. S. Kode, S. Parkhi, T. Barde, R. M. Dhere, A. C. Mishra

Work done

Validation of Hemagglutination (HA), HI and Microneutralization (MN) assays was undertaken. Analysis of results is in progress. During the phase II and III clinical trials, sera from vaccinated individuals from Pune, Ahmedabad, Bangalore and Indore will be tested by HI and MN assays.

Detection of swine influenza targets in Phase-I clinical trials of pandemic influenza (H1N1) 2009 live attenuated (LAIV) vaccine (SIIL-NIV)

J. Mullick, L. R. Yeolekar, S. S. Keng, B. J. Payyapilly, R. M. Dhere, A. C. Mishra

Work done

Prior to the detection of the samples from the clinical trials four different lots of the vaccine were tested for the detection of flu A, swine A, swine H1 and an internal control gene RNase P. Following this, nasal swabs from159 trials were tested for the flu targets. RNA was isolated from the swabs received from SIIL and subjected to Real time RT-PCR. The tests and the analysis are in progress.

Protective efficacy of H5N2 inactivated vaccine against highly pathogenic H5N1 AI virus in chickens

S. D. Pawar, S. S. Koratkar, J. Mullick, A. C. Mishra

Introduction

With the view of the vaccination of domestic poultry as a tool to combat AI H5N1, we received two bottles of the H5N2 vaccine (M/s Intervet India Pvt. Ltd., Batch No. M10622708/01-2010 on 6/3/2009) from the Animal Quarantine and Certification Services (NR), Government of India, New Delhi for evaluation of vaccine efficacy.

Objective

- Evaluation of efficacy of H5N2 vaccine in chickens.

Work done

The efficacy study of the same vaccine lot was undertaken in broiler chickens as per the manufacturers' protocol. Briefly, a total of ten 3-week-old broiler chickens were vaccinated with 0.5ml vaccine per chicken by intramuscular route. The birds were bled after post-vaccination day 10 and 21 and sera were tested in HI assay to check the antibody titers against H5N1 and H5N2 viruses.

Results

On post-vaccination day 10, all the vaccinated birds were negative for the antibodies against H5N1 and H5N2 viruses. At post-vaccination day 21, only two birds showed seroconversion against homologous antigen (with a titer of 20 in HI test). As per reports, vaccine-induced HI titers of ≥ 40 are considered a measure of efficacy. Further vaccine lots were requested from the Animal Quarantine and Certification Services (NR), New Delhi for repeating the experiment.

Generation of immune sera using inactivated influenza virus isolates for influenza diagnosis

S. D. Pawar, S. S. Koratkar, J. Mullick

Introduction

Antibodies against newly isolated influenza A(H11N1) virus are required for the diagnosis of influenza.

Objective

- Generation of immune sera for influenza diagnosis.

Work done

H11N1 was inactivated using Beta propiolactone. Chickens were immunized to raise immune sera.

Results

Sera from immunized birds were tested in HI test and were positive for influenza antibodies. These antibodies will be used as reagents for identification of newly isolated influenza viruses.

Follow-up of the respiratory disease outbreak in Murshidabad district, West Bengal

S. D. Pawar, M. R. Khude, M. S. Chadha

Introduction

An outbreak of respiratory disease was reported during September-December 2008 in the Murshidabad district, West Bengal. NIV investigated the outbreak and respiratory syncytial virus (RSV) was found to be the etiological agent. This year, follow-up of the outbreak was carried out to monitor the situation in the district.

Work done

A total of 114 throat swabs were collected from outpatient department and in-patient wards of Berhampur District Hospital, Berhampur, Murshidabad. Data from hospital records was also collected for analysis. These samples were tested in RT-PCR for virus detection.

Results

One sample was positive for pandemic influenza (H1N1) 2009 virus and one was positive for RSV. Report was sent to the hospital and Principal Secretary, Health Department of West Bengal.

Detailed results reported by the Human Influenza Group.

Enteric Viruses Group

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Enteric Viruses Group

Projects

1. Epidemiological studies on rotaviruses
2. Characterization of rotaviruses
3. Preparation of egg yolk antibodies against rotaviruses for oral immunization in human
4. Hospital based surveillance and characterization of non-rota enteric viruses in acute gastroenteritis cases
5. Additional studies
   - Investigation of an outbreak of acute gastroenteritis for rapid detection of rotavirus
   - Investigation of viral etiological agents associated with Hand, Foot and Mouth Disease in India, 2009-10
   - Detection and molecular characterization of enteroviruses in cases with acute encephalitis syndrome identified in Gorakhpur District, Uttar Pradesh, North India
   - Studies on hepatitis A

Epidemiological studies on rotaviruses

Hospital based surveillance of rotavirus disease and strains in children

S.D. Chitambar

Rotavirus infections are the major cause of severe dehydrating diarrhea in children. Among the different group A rotavirus genotypes, G1P[8], G2P[4], G3P[8], G9P[8] genotypes circulate most commonly all over the world. In addition, uncommon and untypeable rotavirus strains co-circulate and mixed infections with different rotavirus genotypes occur in developing countries. Currently two live oral vaccines from Merck (Rota Teq) and Glaxo Smith Kline (Rotarix) have been licensed in more than 100 countries and are being introduced into routine immunization programs in the US and some other countries. Epidemiological and molecular studies would help to define the need for and benefits of rotavirus vaccines in India.

Objectives

- To estimate the proportion of rotavirus diarrhea
- To find out prevalent rotavirus types among hospitalized children

Work done

Fecal specimens collected from 194 children hospitalized for diarrhea showed 33.5% rotavirus positivity by ELISA. All ELISA positive specimens were subjected to multiplex PCR for rotavirus VP7 (G) and VP4(P) typing. Nearly 95.3% and 89.2% of the strains were typed for G and P genotypes respectively while 80% were typed for both. Sequence based typing was attempted for rotavirus strains untypeable for G or P or both genotypes by multiplex PCR. The study showed circulation of G1P[8] (17.3%), G9P[8] (17.3%), G2P[8] (13.5%), G2P[4] (9.6%), G2P[6] (3.8%), G1P[4] (1.9%), G4P[4] (1.9%), G12P[8] (7.7%), G12P[6] (3.8%) and G9P[4] (3.8%) rotavirus strains (Fig 1). Mixed infections {G9,G12P[6] (7.7%), G9G12P[8] (1.9%), G2G12P[8] (1.9%), G2G9P[6] (1.9%), G1G9G8P[8] (1.9%) G2P[6]P[8] (1.9%) and G9P[8]P[4] (1.9%)}with rotavirus strains were detected in 19.1% of the specimens (Fig 1). Only 4.6% strains remained nontypeable for both genes.

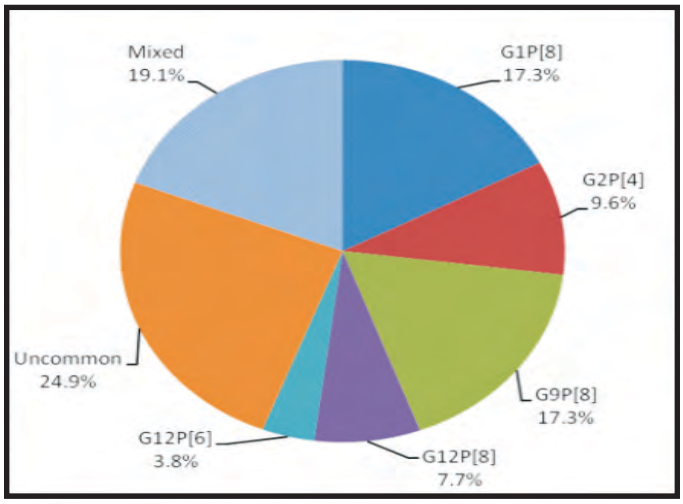

Fig 1: Distribution of G (VP7 gene) and P (VP4 gene) types among typeable rotavirus strains isolated from children.

Fecal specimens were collected during November 2008-December 2009 from 143 children aged ≤10 years hospitalized at Jawaharlal Nehru Medical College (JNMC), Belgaum for diarrhea. Rotavirus positivity was 15.4% by ELISA. All ELISA positive specimens were subjected to multiplex PCR for G and P typing. Seventy three percent and 46% of the strains were typed for G and P genotypes, respectively and 41% were typed for both. Five (22.7%) strains remained nontypeable for both genes. The study showed circulation of G9P[8] (33.3%), G2P[4] (22.2%) and G9P[4] (22.2%) Strains. Mixed infections of G9, G10P[8] (11.1%) and G3,G9,G10,P[4] (11.1%) were detected.

Future Plan

Rotavirus surveillance will be continued to monitor the common, uncommon and emerging rotavirus genotypes causing acute gastroenteritis (AGE) among children.

Surveillance of rotaviruses in adolescent and adult cases of acute gastroenteritis

V.S. Tatte, A. Lahon and S.D. Chitambar

Group A rotaviruses are the major cause of AGE in children. These viruses are also known to infect adolescents and adults. Recent studies carried out among adults in the developing as well as developed countries have shown that the group A rotavirus infections are on the rise.

Group B rotaviruses (GBR) are also known to cause diarrhea. These viruses have been isolated only from China, India, Bangladesh and Myanmar. However, limited data is available.

Objective

- To determine the prevalence and genotypes of rotaviruses in adolescent and adult cases of AGE

Work done

Fecal specimens were collected from 62 adolescent and adult cases of AGE visiting local hospitals of Pune city during the year 2009-2010. Thirteen of 62 (21%) were detected positive for rotavirus by ELISA. Among these specimens, 7/13 (53.8%) were typed for VP7 (G) and 8/13 (61.5%) were typed for VP4(P) genes. Both types were established in 6/13 (46.2%) of the specimens by multiplex PCR, while 4/13 (30.8%) remained untypeable for both genes. Predominance of G9P[4] (50.0%) followed by G1P[8] (16.7%) and G2P[4] (16.7%) was noted. Mixed infections with G1,G9 P[4] (16.7%) were detected (Fig 2).

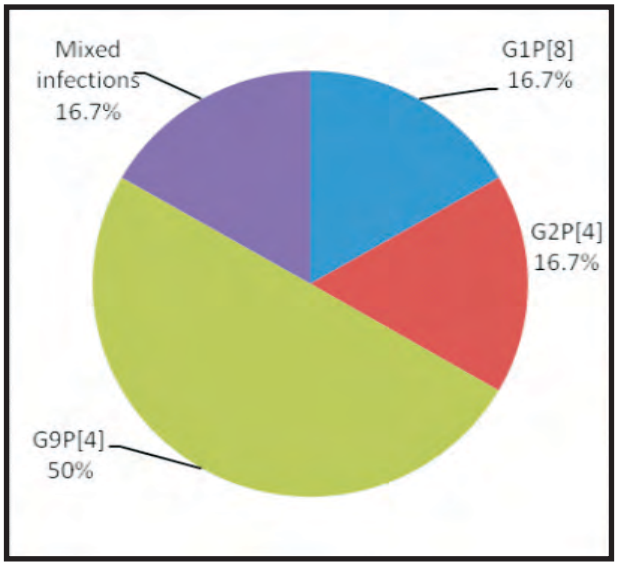

Fig 2. Distribution of G (VP7 gene) and P (VP4 gene) types among typeable rotavirus strains isolated from adolescents and adults

Fecal specimens were collected from 24 adolescents and 29 adults hospitalized for diarrhea at JNMC, Belgaum. Rotavirus positivity was detected to be 25% and 20.7% respectively by ELISA. All ELISA positive specimens were subjected to multiplex PCR for typing of VP7 (G) and VP4 (P) genes. In adolescents and adults 67% and 50% of the strains were typed for both genes, respectively. Circulation of G9P[8]/G1P[8]/G9P[6]/G1, G3,P[8] each at 25% and G9P[8]/G9P[4], G3,G9, P[8] each at 33.3% was noted.

Three hundred and forty five stool specimens were collected during 2004-2009 from adolescent (n=50) and adult patients (n=296) visiting OPDs for acute diarrhea. All specimens were subjected to RT-PCR using primers specific for GBR NSP2 gene followed by sequencing and phylogenetic analysis. Thirty four (9.8%) of 345 specimens showed positivity to HuGBRs. The GBR positivity was distributed in all age groups ranging from 11-19 to >50 years and throughout the year. Phylogenetically all strains (n=34) of the present study showed 97.3-100% nucleotide identity with the strains from Indian-Bangladeshi lineage. Eighteen percent of the GBR positive specimens showed positivity to group A rotavirus. The study indicates that GBR is also commonly circulating viral agent of AGE.

Future Plan

Group A and B rotavirus strains will be characterized to study temporal variations at genomic level.

Detection of GBR infection in Alappuzha, Kerala

An outbreak of water borne disease was reported in June, 2009 in certain parts of Alappuzha district. Stool specimens collected from 22 patients (1.5-83 years) with diarrhea were received at NIV, Pune. Group A rotavirus was detected in 3/22 samples by ELISA. One of 22 samples showed presence of GBR by RT-PCR.

Surveillance of rotaviruses was continued in the same district. Of 110 specimens collected, 8 (7.3%) showed positivity to GBRs. Of the 8 positive specimens, 3 (37.5%) belonged to children, 1(12.5%) belonged to adolescent and 4 (50%) belonged to adults. Mixed infection with group A rotavirus was identified in only 1 (12.5%) specimen. Phylogenetically, all strains (n=8) of the present study clustered with Indian-Bangladeshi lineage (Fig 3). The data shows circulation of GBR in southern region of India.

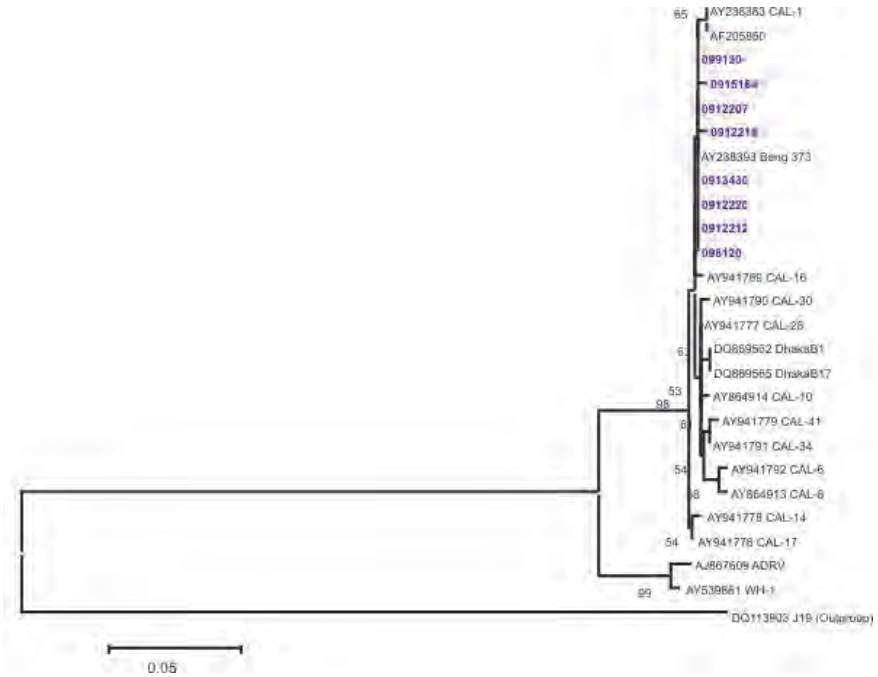

Fig 3 : Phylogenetic analysis of GBR strains from Alappuzha, Kerala based on Partial NSP2(87-369 bp) gene sequence

Characterization of Rotaviruses

Genetic analysis of rotavirus strains from pediatric, adolescent and adult cases of acute gastroenteritis

V.S. Tatte, S.S. Ranshing, K.N. Rawal and S.D. Chitambar

Studies to understand rotavirus evolution have important implications for development and use of rotavirus vaccines. Such studies have documented that point mutations, rearrangements, reassortment, recombination and interspecies transmission are the potential factors that contribute to the diversity of rotaviruses. In this context, several studies have reported identification of different lineages within the VP7 (G1, G2, G3 and G9), VP4 (P[4], P[8],P[6]), VP6 and NSP4 genes of rotavirus.

Objectives

- To analyze the rotavirus NSP4 and VP6 gene sequences and their linkage
- To analyze rotavirus VP4 gene sequences

Work done

Sequence analysis of NSP4 genes of human rotavirus strains circulating in children, Pune, western India.

In continuation with earlier studies (AR 2008-09) conducted on characterization of rotavirus VP6 genes nearly full-length NSP4 genes from 23 group A rotavirus strains recovered from children hospitalized for diarrhea in Pune during 2004-2007 were amplified by RT-PCR and sequenced. A phylogenetic dendrogram was constructed with nucleic acid sequences by neighbor joining method to identify NSP4 genotype.

NSP4 genotypes were identified in 22/23 (95.7%) rotavirus strains with one remaining unidentified. The distribution was found to be 68.2% in genotype A and 31.8% in genotype B. This data was compared with the previously reported analyses of VP6 genogroups (AR 2008-09) of the same rotavirus strains isolated from children. Fourteen strains showed combination of VP6 and NSP4 genes as SGI/ Genotype A whereas seven strains showed SGII/ Genotype B. Only one of 22 (4.54%) strains displayed unusual combination of VP6 and NSP4 genes i.e. SGII/ Genotype A in children.

Analysis of linkage between the NSP4 and VP6 genes of rotavirus strains from adolescents and adults at two time points

Work done

NSP4 and VP6 genes of 118 rotavirus strains, detected in adolescent and adult cases of AGE in 1993-1996 and 2004-2007 were characterized to determine their diversity and genetic linkage. Eighty-two percent and 89% of the strains showed amplification of NSP4 and VP6 genes, respectively in RT-PCR. These included 71/75 (94.66%) and 26/43(60.46%) rotavirus strains from the years 1993-1996 and 2004-2007 respectively for the NSP4 gene and 71/75 (94.66%) and 34/43 (79.1%) strains from 1993-1996 & 2004-2007 respectively for VP6 gene. Sequencing and phylogenetic analysis of the VP6 genes showed distribution of genogroups in the lineages I-1 (1.4%), I-2 (50.7%) and II-4 (47.9%) in the 1990s and I-2 (73.5%) and II-4 (26.5%) in 2000s, indicating diversity in both genogroups. Amino acid divergence within the genogroup II strains from 1990s and genogroup I strains from the 2000s was noteworthy (4.7-6.7%). Sequencing and phylogenetic analysis of the NSP4 genes showed almost equal distribution (45.0-55.0%) of genotypes A and B. However, there was a higher amino acid divergence within the genotype B strains (up to 9.3%) than in genotype A strains (up to 2.9%) at the two-time points. Nearly 70% of the strains showed NSP4-A - VP6 I or NSP4-B - VP6 II genetic linkage. The discordance in the linkage noted in 29.7% of the strains was predominated by NSP4-B and VP6-I combination and appeared strikingly high in the infections caused by unusual and mixed rotavirus strains. This is the first report describing the phylogenetic analysis of rotavirus NSP4 and VP6 genes and their discordance in adolescent and adult cases with AGE from India. The extensive diversity within the rotavirus genes and their relationship revealed by this study emphasizes the need for evaluation of the rotavirus vaccines being used currently.

Sequence analysis of rotavirus VP4 genes from adolescents and adults

VP4 genes of 131 rotavirus strains recovered from adolescents and adults hospitalized for AGE in Pune, western India at two time points (1993-1996 and 2004-2007) were characterized by multiplex PCR and sequencing to determine the circulating VP4 genotypes and their lineages. A total of 73 P[4] and 69 P[8] VP4 genotypes were detected. The distribution of these genotypes into lineages and their nucleotide sequence identities are described in Table 1.

Table 1. Analysis of rotavirus VP4 genes

Gene (Vp4)	Total positivity	Lineages	No positive (%)	% NT identity with reference strains	% NT identity within strains
P[8]	69/131 (52.67%)	P[8]-2	9 (13.4%)	95.7-96.8%	96.4-100%
		P[8]-3	59 (85.5%)	97.1-100%	96-100%
		P[8]-4	1 (1.4%)	97.9%	-
P[4]	73/131 (55.72%)	P[4]-1	3 (4.1%).	96.6-100%	96.6-98.2%
		P[4]-5	70 (95.9%)	95.3-99.5%	95.9-100%

Molecular characterization of rotavirus strains adapted to cell culture

R. Arora and S.D. Chitambar

Group A rotaviruses (RVs), members of the family *Reoviridae,* are important enteropathogens of human and a large variety of mammals and birds worldwide. The RV genome consists of 11 segments of double stranded RNA (dsRNA) ranging from 0.6 to 3.3 kb that encode six structural proteins (VP1-4, VP6, VP7) and six nonstructural proteins (NSP1-6). Characterization of rotavirus genes, especially of VP7 and VP4 from wild type rotavirus strains recovered from fecal specimens, is widely carried out. However, limited studies have reported genomic changes that occur in human rotaviruses during cell culture adaptation.

Objectives

- To isolate rotavirus strains from acute diarrhea patients
- To study the genomic changes in rotavirus strains during adaptation to cell culture

Work Done

Studies conducted earlier on isolation of rotavirus strains from acute diarrhea patients were continued further. Presence of rotavirus strain in infected MA-104 cells was confirmed by antigen capture ELISA. RT-PCR and sequencing were carried out for all structural (VP1-VP4,

VP6 & VP7) and non structural (NSP1-NSP5) genes of wild type and culture adapted (at passage 5 and passage 10) strains.

Sequences were compared with prototype strain KU from Japan. Nucleotide substitutions were noted for all structural (VP1-VP4, VP6 and VP7) and nonstructural (NSP1 and NSP3) genes. The nucleotide and amino acid substitution detected in the genes is summarized in Table 2

Table 2: Comparative nucleotide and amino acid substitutions in culture adapted G1P[8] rotavirus strains.

Gene	Number of nucleotide / amino acid changes as compared to their wild type counter part		
	K05	K44	Sh36
VP1	7/1	NC*	23/7
VP2	23/4	NC*	34/4
VP3	NC*	NC*	18/3
VP4	3/1	1/1	64/12
VP6	NC*	NC*	17/1
VP7	NC*	NC*	7/1
NSP1	NC*	NC*	23/11
NSP2	NC*	NC*	NC*
NSP3	4/1	NC*	NC*
NSP4	NC*	NC*	NC*
NSP5	NC*	NC*	NC*

*NC : No Change

The results indicate that the adaptation of rotavirus to cell culture involves VP4 gene and that the involvement of other genes is strain specific. According to the new classification system based on 11 gene segments of rotavirus strains, the study strains (wild type and culture adapted K05, K44 and Sh36) were classified as G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 representing the gene segments VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4 and NSP5 respectively.

Detection and molecular characterization of rotaviruses in animal species from Nagpur and Gorakpur regions

S.D. Chitambar, V.S. Tatte and C.G. Raut

(In collaboration with Drs. V.V. Ingle and M. Jadhav, Department of Veterinary Microbiology

and Animal Biotechnology, Nagpur Veterinary College, Nagpur)

Rotaviruses are enteric pathogens in young ones of a number of animal species including human, cattle, and pigs. These viruses cause a significant morbidity and mortality in animals leading to a considerable economic loss.

To date 6 P-types (P6 [1], P7 [5], P8 [11], P [14], P [17] and P [21]) and 9 G-types (G1, G2, G3, G6, G7, G8, G10, G11 and G15) types have been reported among group A bovine rotaviruses (BoRV-A). Epidemiological studies of porcine rotaviruses (PoRV) in several countries have identified at least four main G types - G3, G4, G5, and G11, as the common types. Other porcine rotaviruses such as G1, G2, G6, G8, G9 and G10 have also been reported occasionally. However, in India limited data is available on the rotavirus types causing asymptomatic infections in animals.

Objective

- To characterize rotavirus infections in animals

Work Done

Three hundred fecal specimens from different species, comprising cattle (n=100), buffalo (n=100) and porcine (n=100) from Nagpur region showed 11% rotavirus positivity in group A rotavirus antigen capture ELISA. Reverse transcription PCR of all these specimens followed by multiplex genotyping PCR showed G10P[NT] and G4P[6] genotypes predominantly in cow / buffalo calves and piglets respectively. In piglets 73.9% of the strains were G-typed (G2 (17.6%), G3(5.8%), G4(52.9%), G8(5.8%), G10(5.8%)) and 47.8% of the strains were P-typed (P[6] (72.7%) and P[8] (18.1%)). Among both G and P typed strains (47.3%) in piglets, unusual strains with G2P[8], G3P[6] P[8] and G1G3P[6] were also detected in 11.1% of the samples. Sequence analysis of all these strains confirmed their human origin.

Phylogenetic analysis of the VP6 gene of all the strains from bovine and porcine species showed identity with human rotavirus VP6 genogroups I and II strains respectively.

One hundred and thirty six rectal swabs were collected from asymptomatic pigs, buffalos and cows from Gorakhpur, North India. Thirteen of 136 (9.6%) specimens showed positivity to rotavirus by each of ELISA and RT-PCR of VP6 gene. VP7 (G) and VP4(P) genes were characterized by multiplex PCR. All positive specimens belonged to porcine species. Multiplex PCR detected G9P[19], G12P[4], G4P[NT] and G[NT]P[4] specificities in rotavirus strains. Phylogenetic analysis of partial sequences of VP6 and VP7 genes showed maximum identity with human strains while VP4 genes were closer to human P[4] and porcine P[19] strains.

Future Plan

Surveillance, characterization and isolation of animal rotavirus strains will be continued.

Preparation of egg yolk antibodies against rotaviruses for oral immunization in human

M. Burgohain, G.S. Dhale, C. G. Raut and S.D. Chitambar

Objective

- To quantitate the viral load in a mouse model developed for rotavirus diarrhea

Work done

Real Time PCR of stool and tissue samples

Genomic RNA was extracted from stool and intestinal tissue samples using Trizol-LS and Trizol-R respectively. cDNAs (380 bp length) of VP6 gene were synthesized in the presence of MMLV reverse transcriptase enzyme.

The resulting cDNA and Quantitect SYBR Green PCR mastermix kit (Qiagen, USA) were used in real time PCR. Eight serial dilutions of the plasmid containing the rotavirus VP6 fragment (10^{-1} to 10^{-8} copies per sample) included in each reaction served as positive controls to derive the standard curve as well as rotavirus quantitation in the experimental specimens.

Rotaviral RNA was detected in diarrheic stools of infected animals with the shedding of virus highest at 24 hour post inoculation (hpi) and decreased subsequently. In the initial stage of infection (24 hpi) rotaviral RNA was detected in all three parts (duodenum, jejunum and ileum) of the small intestine, while it was detected continuously only in the ileum during the period of study.

Future Plan

The data obtained in various assays will be analyzed to determine the correlation of viral load in feces and tissue samples with clinical/pathological features of rotavirus infection in mice.

Hospital based surveillance and characterization of non-rota enteric viruses in acute gastroenteritis cases

S.D. Chitambar and V. Gopalkrishna

Acute gastroenteritis is one of the most common diseases in humans and continues to be a significant cause of morbidity and mortality worldwide. Among enteric viruses, rotavirus is the leading viral agent associated with severe diarrhea especially in infants and young children. However, some patients develop diarrhea with non-rotavirus infections indicating involvement of other enteric viral or bacterial pathogens. Recently, association of other enteric viruses such as Calici, Astro, Adeno, Entero viruses have been reported in sporadic cases and outbreaks of diarrhea in Asian, European countries and United States of America. In India, limited studies are reported on other enteric viral pathogens. The causative agents in such cases are rendered unidentified in the absence of concerted efforts in most of the

episodes of gastroenteritis. It is essential to study the spectrum of unknown viruses in sporadic cases and outbreaks of gastroenteritis.

Identification and molecular characterization of Norovirus strains in patients with acute gastroenteritis from western India

P. Chhabra and S.D. Chitambar

Full-length human NoV genome sequencing has been done for more than 100 strains from around the world. However, the data on NoV genomes, their diversity and evolution from Indian subcontinent are not available to date.

In a study conducted on NoV surveillance in AGE cases from western India during 2005-2007, GII.4 and recombinant GII.b/GII.3 strains predominated in the years 2005-06 and 2006-07, respectively (Chhabra et al. 2009). Also, the study indicated occurrence of "novel GII.4 variants" in the year 2007 on the basis of 300 bp region of 5'end of ORF2 (Chhabra et al. 2009). One strain each from GII.4 (PC15) and GII.b/GII.3 (PC52) specificities and "new GII.4 variants" (PC51) was selected for complete-genome sequencing and analysis.

Objective

- To amplify full-length genomes of 3 NoV strains (PC15, PC51 and PC52) to determine the extent of conserved and variable features of the Indian NoV strains

Work done

Amplifications of full-length genomes of 3 NoV strains (PC15, PC51 and PC52) were carried out using nine overlapping sets of forward and reverse primers. Full-length genomes of all of the 3 strains were characterized by phylogenetic, SimPlot, selection pressure and hydrophilicity analyses.

Phylogenetic analysis

The strain, PC15 was placed in a GII.4 Hunter subcluster. An intragenotype recombination event between ORFs 2 (new GII.4 variant) and 3 (DenHaag subcluster) of the strain, PC51 was detected for the first time in this study. The strain, PC52 showed the presence of commonly detected intergenotype recombination, GII.b/GII.3.

A sixteen amino-acid signature code (TDVVYYAGASQPRDDI) was also identified in ORF2 of single and recombinant GII.3 specificity strains, which may serve as a differentiating genetic marker for these strains.

Simplot analysis

To authenticate the recombination event, a continuous stretch of 2,429 bp covering complete VP1 (ORF2-1, 622 bp) and VP2 (ORF3, 807 bp) genes was subjected to SimPlot analysis. The estimated recombination breakpoint was at position 6,706 (^{2}sum = 27, $p < 0.01$) i.e. at ORF2/3 overlap according to PC51 genome (Fig. 4a). The strain, PC52 carried a

recombination of known type-GII.b/GII.3 that showed 97.9% nucleotide identity with the strain, Sydney C14 of the same specificity in a continuous stretch of 3,232 bp of RdRp and capsid genes (Fig. 4b).

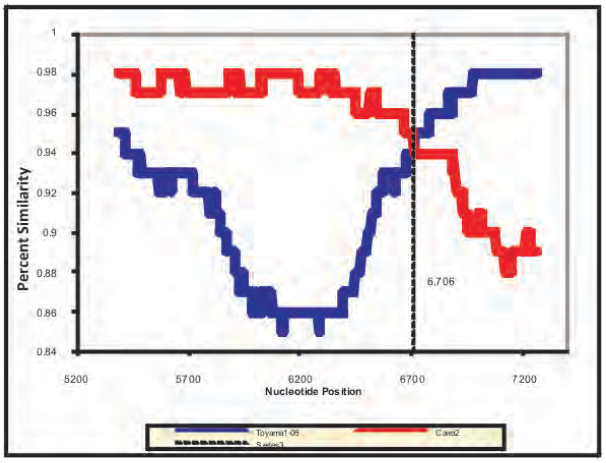

Fig. 4a

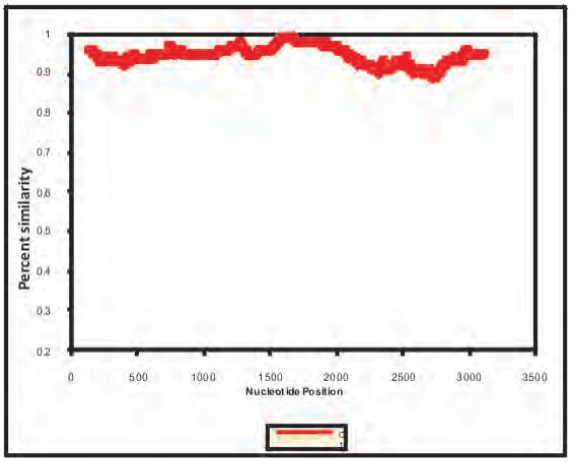

Fig. 4b

Fig 4: SimPlot analysis of nucleotide sequences of a) ORF2 and ORF3 sequences of the strain PC51 and b) ORF1 and ORF2 sequences of the strain PC52 identified in western India. Window size: 600bp; Step size: 3bp. Vertical line indicates the putative recombination breakpoint.

Selection pressure analysis

The comparative analysis of deduced amino acid sequences of complete ORF2 region of selected strains (inclusive of PC15 of the present study) from all of the known six subclusters of GII.4 and seven strains (inclusive of PC51 of the present study along with OC07138, Cairo-2, Cairo-4, Cairo-8, SSCS and RIS) of new GII.4 variant type (7^{th} subcluster) indicated 13 {174, 196, 297, 333, 340, 352, 357, 368, 372, 382, 393, 394 (except Camberwell and Grimsby clusters) and 407}sites under selection pressure. A pairwise comparative analysis of these sites identified a constant amino acid change in every new epidemic variant, The study indicates adaptation of NoVs in the environment to escape the host immune response and persist in the population.

Hydrophilicity Index

As compared to the Toyama1 strain of 2006b variant type, the PC51 (new GII.4 variant) showed amino acid substitutions at 306 (L-Q), 352 (Y-L), 357 (P-D), 259 (T-A) and 364 (S-R) positions in hypervariable region (ORF2-P2 subdomain) at pH 7.0 indicating increase in the hydrophilicity of the protein. Similarly, there was an increase in the hydrophilicity of GII.3

capsid protein of the strain PC52 (GII.b/GII.3) as compared to the strain TV24 of single GII.3 specificity due to amino acid substitutions at 289 (T-V), 333 (A-G), 389 (P-Q), 391 (Q-K) and 394 (K-R) positions in hypervariable region.

Future plan

NoV surveillance will be continued to identify the NoV genotypes, variants and recombinant strains.

4.2. Detection and characterization of Astro and Aichi viruses in acute gastroenteritis patients

H. Verma, S.D. Chitambar and V. Gopalkrishna

AGE is considered as a cause of morbidity and mortality worldwide. Group A rotaviruses, calci, astro, enteric adenoviruses (serotype 40-41), aichi, picobirna and toro viruses have been recognized as etiological agents. Although reports on non-rota enteric viruses such as adeno, astro and aichiviruses in sporadic and outbreak cases of AGE are available throughout the world, no such studies are available in Indian population especially from western India, Maharashtra state where outbreaks of gastroenteritis are often reported.

Objectives

- To estimate the proportion of AGE caused by Astro and Aichi viruses in sporadic cases from western India
- To detect and characterize the most prevalent circulating strains of Astro and Aichi viruses in western India

Work done

Molecular typing of Human astroviruses (HAstVs)

A five-year (2004-2008) study was conducted on patients with AGE from different cities of Maharashtra, western India to detect and characterize astrovirus infections. One thousand two hundred and forty fecal specimens were collected from sporadic cases from Pune, Aurangabad and Nagpur cities. All specimens were subjected to astrovirus specific RT-PCR followed by sequencing and phylogenetic analysis. The overall positivity to astrovirus was found to be 3.1% with highest number of infections in winter months. A high prevalence of astrovirus was observed in children $\leq$ 1 year of age. Phylogenetic analysis of the partial ORF1a (serine protease) and ORF2 (capsid gene) regions showed circulation of three probable recombinant types with different ORF1a/ORF2 specificities (HAstV-8/HAstV-1, HAstV-7/HAstV-2, HAstV-4/HAstV-5) along with HAstV-8 of a single specificity in the study population. HAstV-8/HAstV-1, specificity predominated (67.7%) in the region followed by HAstV-7/HAstV-2 (9.7%), HAstV-4/HAstV-5 (6.5%) and HAstV-8 (16%) types.

Detection and characterization of Aichivirus strains

One thousand two hundred and forty specimens were collected from hospitalized children with acute gastroenteritis from Pune, Aurangabad and Nagpur cities Maharashtra state, western India. Among these 912 samples from Pune (January 2004 - December 2008), 100 from Aurangabad (May 2005 - February 2006) and 228 from Nagpur cities (June 2006 - August 2007). Aichi virus RNA was detected in 1.3% (12/912), 1% (1/100) and 0.4% (1/228) of fecal specimens from AGE patients from Pune, Aurangabad and Nagpur cities respectively by RT-PCR using 3C and 3D junction primers. An overall prevalence of 1.1% was detected.

Phylogenetic analysis of the Aichi virus strains showed 94.2-96.8% nucleotide identity with genotype B strain of Bangladesh (EF079157, B-171/05). Among positive samples, six of 14 (42.9%) were typed on the basis of VP1 gene. All six strains showed 92.6% - 98.2% nucleotide sequence homology with genotype B strain of Bangladesh (EU143276/B-302) (Fig.5).

Fig 5. Phylogenetic tree based on partial nucleotide sequences (200 bp) of 3CD region of Indian Aichivirus strains.

Future Plan

Full length genome sequence data of HAstV and Aichi virus strains circulating in western India will be obtained.

Detection and molecular characterization of enteroviruses from patients with acute gastroenteritis

P.R Patil, S.D. Chitambar and V. Gopalkrishna

The genus Enterovirus (EV) of the family *Picornaviridae* includes nonenveloped viruses comprising a 7,500 nucleotide single- stranded positive RNA genome. The genome encodes seven nonstructural (2A-3D) and four structural (VP1-VP4) proteins. The human Enteroviruses (HEV) are now classified into four species on the basis of genome organization and sequence similarity as well as biological properties: HEV-A (CA-2,3,5to8,10,12,14,16,EV-71,76,89,90,91), HEV-B (CB-1to6,CA-9, all Echos, EV-69,73 to 75 and 78 to 88,EV-97,100 and 101), HEV-C (CA-1,11,13,17,19 to 22,24 and PV 1 to 3, EV-96), HEV-D(EV-68 and 70)

Objective

- To detect and type enteroviruses in patients with AGE

Work Done

Seven hundred and sixty seven fecal samples from sporadic (Pune, Nagpur and Aurangabad, Maharashtra) and 170 from outbreak (Mumbai, Maharashtra) cases of AGE were collected during 2005-2008. All the samples were tested for the presence of EV-RNA by nRT-PCR using 5'NCR. Prevalence of EV was found to be 15.4 %(118/767) and 20.6 %(35/170) in sporadic and outbreak cases respectively. A prevalence of EV RNA at 4.0% (6/150) level was found in non-diarrheal cases. Twenty three of 28 (82.1%) and 12 of 35(34.2%) EV strains, respectively from sporadic and outbreak cases (2006) and 5 of 6 EV strains detected in non-diarrheal cases (2008) were serotyped by using VP1 gene.

In order to evaluate VP2 gene specific primers for serotyping, 7 EV strains with known and 16 with unknown types (VP1) were subjected to RT-PCR and sequencing using test (VP2) primers. Six of 7 strains with known types showed amplification of VP2 gene indicating same types and thus indicated 86% correlation between VP1 and VP2 gene based typing. Eight of 16 nontypeable strains typed by VP2 gene specific primers (Fig. 6) suggested utility of VP2 gene as an alternative method for EV typing.

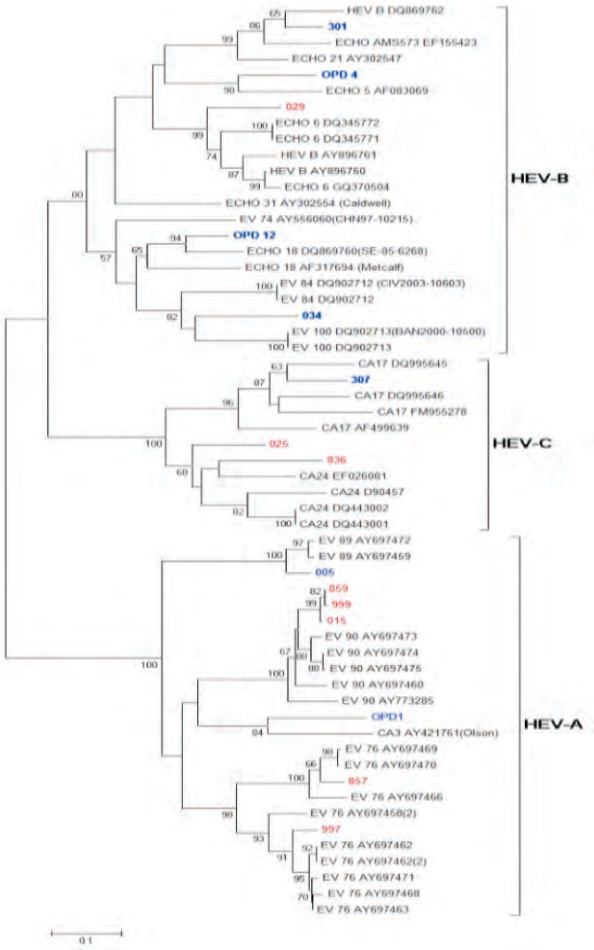

Fig 6. Phylogenetic analysis of EV on the basis of VP2 gene

Future plan

The work will be continued to type nontypeable strains and obtain the data on full length genomes of commonly circulating and novel enterovirus strains.

Additional studies

(I) Investigation of an outbreak of acute gastroenteritis occurred in Sangli district, Maharashtra state

S.D. Chitambar, V. Gopalkrishna, A. Lahon, V.S. Tatte, P. Chhabra, G.P. Patil and H. Verma

Objective

- To detect and characterize enteric viruses in the clinical specimens collected during an outbreak of AGE

Work done

Twenty nine stool/rectal swab specimens were collected from adolescent (11-19 years) and adult (>19 years) patients with acute diarrhea in Kalambi, Bhose and Khanderajuri villages of Sangli district. All specimens were subjected to RT-PCR using primers specific for NSP2 gene followed by sequencing and phylogenetic analysis. Of 29 specimens, 5(17.2%) showed positivity to HuGBRs. GBR positivity was higher (30.8%) in adults aged 20-35 years than in adolescents (25%). Group A rotavirus positivity was found to be 3(10.3%). However, other enteric viruses like enteric adenoviruses, astroviruses, enteroviruses and noroviruses were not detected in any of the samples.

(ii) Investigation of viral etiological agents associated with Hand, Foot and Mouth Disease (HFMD) in India, 2009-10

V. Gopalkrishna, P.R. Patil, G.P. Patil and S.D. Chitambar

Hand, foot and mouth disease (HFMD) is a childhood viral infection. The illness is manifested by fever followed by pharyngitis, mouth ulcers and rashes on the hands and feet. The disease is usually self limiting with occasional complications leading to pneumonia, meningitis and encephalitis. The disease is reported to be caused by Coxsackie-A16 (CA-16) virus and Enterovirus 71 (EV-71). Other EV types such as CA-4 to CA-7, CA-9, CA-10, CB-1 to CB-3, CB-5, Echo-4 and Echo-19 have also been found associated with both sporadic infections and outbreaks of HFMD. HFMD cases were reported from Kerala, West Bengal, Orissa during June-Oct 2009 and Tamil Nadu, January-February 2010.

Objective

- To investigate the HFMD cases for the association of enteroviruses.

Work done

Seventy eight samples (23 vesicular fluids, 36 sera, 13 stools and 6 throat swabs) were collected from 54 HFMD cases identified in Alappuzha and Pathanamthitta districts, central Kerala (n=30); Kolkata, West Bengal (n=8); Bhubaneswar, Orissa (n=7) and Ooty, Tamil Nadu (n=9). Thirty nine of 78 (50%) specimens which included 17 vesicular swabs, 8 sera, 2 throat swabs and 12 stool samples from 32 of 54 (59.2%) patients showed presence of EV-RNA by RT-PCR targeted against 5'NCR. Overall, EV-RNA positivity detected in HFMD patients from Kerala, West Bengal, Tamil Nadu and Orissa states was 53.3% (16/30), 62.5% (5/8), 66.6% (6/9) and 71.4% (5/7) respectively. BLAST analysis of the sequences indicated homology of the Kerala strains to CA-6(n=16), EV-71(n=1), ECHO-9(n=1) and that of the West Bengal (n=9), Orissa (n=5) and Tamil Nadu (n=7) strains to CA-16(n=21) in 5'NCR.

Thirty three of 39(84.6%) strains amplified in 5'NCR also showed amplification of VP1/2A junction region or VP1 region. Sequence analysis of the PCR products revealed the presence of CA-16 in West Bengal (n=9), Orissa (n=5) and Tamil Nadu (n=7) and that of CA-6 (n=10), EV-71(n=1) and Echo-9 (n=1) in Kerala.

(iii) Detection and molecular characterization of enteroviruses in cases with acute ephalitis syndrome (AES) identified in Gorakhpur District, Uttar Pradesh, North India.

V. Gopalkrishna, G.P. Patil, P.R. Patil, M.S. Joshi, K.N. Rawal B.V. Tandale, Y.B. Gurav, M.M. Gore and S.D. Chitambar

Acute viral encephalitis has been associated with wide range of viruses and can occur either in sporadic or as an outbreak episode. Viral etiological agents such as Herpes virus, Alpha virus, Influenza A virus, Rabies virus, Flavi virus, Chandipura virus have been found associated with the syndrome of encephalitis. Recently, certain serotypes of enteroviruses have also been found to be associated in encephalitis, meningitis and other neurological disorders from various part of Indian subcontinent.

An acute encephalitis syndrome (AES) was reported in and around Gorakhpur district of UP, India during Oct 2008- Oct 2009. Investigation was carried out to determine the association of enteroviruses.

Objective

- To detect and characterize enteroviruses in AES cases from Gorakhpur

Work done

AES cases admitted to BRD Medical college, Gorakhpur during the study period were investigated for enterovirus infections.

RT-PCR (5'NCR) based detection of enteroviruses:

In continuation to the previous carried out in study 2008, AES cases were continued to be investigated in 2009. One thousand eight hundred and eighty one (n=1881) rectal swabs were screened for the presence of enteroviruses by RT-PCR using 5'NCR gene specific primers followed by sequencing of amplicons. Enterovirus RNA positivity was detected in 30.1% (566/1881)of the specimens. Distribution of EV genogroups is shown in Table 3.

Table 3. Genogroup wise distribution of enteroviruses in AES patients in Gorakhpur districts (UP), 2008-09

Genogroups 2008	Serotypes 2008	Genogroups 2009	Serotypes 2009
HEV-A	EV-76,EV-89,EV-90,EV-91,CA-7,HEV-A	HEV-A	CA-4,5, 6, EV-76, 89, 90,91,
HEV-B	Echo1,11,14,19,21,26,33, EV73,79,81,86,100,101,102, CB4,HEV-B	HEV-B	EV-99,85,84,74, E-30,13,11,19,6,4,HEV-B ,CB-3
HEV-C	CA1,13,19,20,22,24,EV-99 PV-2	HEV-C	CA-1, 11, 13, 17, 19, 20, 22, 24, PV-1, PV-3
HEV-D	NIL	HEV-D	NIL

(Iv) Studies on Hepatitis A

Guillain Barre' Syndrome (GBS) associated hepatitis A virus strain

M.S. Joshi, S.S. Cherian, S. Bhalla and S.D. Chitambar

Hepatitis A usually occurs as a self-limiting infection of the liver, though a variable spectrum of the disease ranging from subclinical to severe forms has also been reported. In addition, neurological syndromes such as myeletis, peripheral neuropathy, Guillian Barre' Syndrome (GBS) have been described in association with hepatitis A. GBS is viewed as a reactive, self-limited, autoimmune disease that causes rapidly progressing muscle weakness that in severe cases can result in paralysis. The infections caused by Cytomegalovirus (CMV), herpes, Epstein-Barr virus (EBV) and hepatitis viruses are the most common viral infections that precede the GBS. It is not clear why some patients develop GBS while others do not. A GBS patient investigated in earlier study (AR 2006-07) was classified as a case of subtype acute motor axonal neuropathy (AMAN) on the basis of physical, central nervous system and electrodiagnostic examinations. Although the patient showed strikingly high anti HAV IgG levels, presence of HAV RNA was detected up to 126 days by RT-PCR. Molecular characteristics of hepatitis A virus (HAV) have been studied widely. However, there is a paucity of data on their correlation with virological and serological findings.

Objectives

- To map the unique and heterologous amino acid substitutions observed in GBS-IND strain on to experimentally known B cell epitopes

- To predict the three dimensional structure of capsid proteins, determine sequential and conformational epitopes, estimate the surface charge distribution and map the observed mutations

Work done

As reported previously (AR 2006-07) percent nucleotide divergence displayed by the Indian strains (CP-IND, PN-IND and GBS-IND) varied from 3 to 6 while percent amino acid divergence varied from 0.1 to 0.7 as compared to the other HAV IIIA strains (n=5) available in the GenBank. The GBS-IND strain showed a higher rate of nonsynonymous substitutions as well as a higher number of unique and heterologous amino acid substitutions compared to the HAV IIIA GenBank strains. These amino acid substitutions in the GBS-IND strain were detected in a nonstructural protein (2C-251F) and B cell epitope regions of structural proteins (VP1-29E, VP1-91S, VP3-50Y and VP4-5S). In a comparative analysis of HAV strains, homology based model of the HAV capsid proteins indicated a localized alteration in the surface charge distribution on the VP1 protein of GBS-IND strain and involvement of its unique amino acid substitutions in the predicted antigenic determinants. Overall, the study suggests that the unique amino acid substitutions in the GBS-IND strain may have contributed to neutralization escape of the virus leading to a longer duration of viremia.

Hepatitis A in Acute Encephalitis Syndrome cases from Gorakhpur, North India

S.D. Chitambar, M.S. Joshi, S. Bhalla, G.N. Sapkal, BV Tandale, KP Kushwaha and M.M. Gore

AES was reported in several cases admitted to BRD medical college hospital, Gorakhpur during 2006- 2009. In 2008, 73% and in 2009 (with clinical/biochemical data available only for 55% cases), 46% cases showed abnormal liver functions. These cases mainly involved pediatric population and hence investigations were carried out for viral hepatitis A.

Objective

- To investigate the AES cases from Gorakhpur, north India for recent infection of hepatitis A

Work done

Among the AES cases admitted to the BRD Medical college hospital, Gorakhpur, 7.5% (144/1912), 2% (44/2098), 35% (564/2200) and 100% (2182/2182) cases respectively from the years 2006, 2007, 2008 and 2009 along with control sera (n=174) collected in June, 2009 were subjected to anti-hepatitis A virus IgM capture ELISA developed indigenously at NIV, Pune.

Hepatitis A infections were detected in 10-*33%* of AES cases. These infections were high up to 6 years of age and occurred throughout the year. Fecal excretion of hepatitis A virus in 12-13% and viremia in 31% cases of hepatitis A indicated recent infection of HAV.

Presence of hepatitis A virus RNA in saliva and urine specimens of hepatitis A patients

S. Bhalla, M.S. Joshi and S.D. Chitambar

Hepatitis A virus (HAV) infection is highly endemic in developing countries. It is the most common cause of infectious hepatitis especially where inadequate sanitary conditions prevail. The virus perpetuates from one susceptible individual to other by fecal - oral route and majority of the population acquires this infection in their childhood. Although stool and serum samples are widely used for detection of HAV/HAV RNA other specimens have been rarely reported for this purpose.

Objective

- To determine the presence of markers of HAV infection in saliva and urine specimens of hepatitis A patients

Work done

Serum, urine, saliva and stool specimens were collected from seventy pediatric cases (1.6 to 15yrs) of hepatitis during Oct 2007 - Jan 2009. All specimens were subjected to anti-HAV IgM capture ELISA and RT-PCR targeting partial RNA polymerase region (3D) on the day of collection. PCR products of the specimens indicating amplification of HAV RNA were sequenced. Of the 70 patients, 65 were tested positive for serum anti-HAV IgM and confirmed to have recent infection of hepatitis A. Seroreactivity was used as gold standard for the assay. Nearly 98.2% and 86.1% of the patients were positive for salivary and urinary anti-HAV IgM antibodies respectively. HAV RNA was detected in 67.6% of the serum samples, 52.2% of the stool samples, 12.3% of the urine samples and 8.7% of the saliva samples. Phylogenetic analysis of partial RNA polymerase region showed close homology with subgenotype IIIA strains. The study indicated possible risk of transmission of hepatitis A infection through urine and saliva.

Technical Support/Consultancy provided (2009-2010)

1. Testing was provided for 368 stool samples for rotavirus detection.
2. Testing was provided for 50 clinical specimens for enterovirus detection Any other information for inclusion of Annual Report 2009-2010

Training/Workshops/Seminars Organized

- Training provided to Dr. Shashank Purwar, Assistant Professor, Dept of Microbiology, J.N. Medical College, Belgaum for the diagnosis of Rotavirus infection from 14th Feb to 26th Feb. 2010.

Training provided for the completion of M.Sc./M.Tech. Dissertaion on the following topics:

M.Sc. (Biotechnology)

- Molecular Detection and Characterization of group B rotavirus strains
- Detection and molecular characterization of group A rotavirus strains from asymptomatic animals
- Molecular characterization of CA-6 detected in HFMD, India

M.Sc. (Virology)

- Molecular characterization of genotype G1 rotaviruses from Pune, western India.
- Isolation and molecular characterization of enteroviruses associated with Hand, Foot and Mouth disease (HFMD)

M.Tech. (Biotechnology)

- Detection and molecular characterization of human group B rotavirus strains in patients with acute gastroenteritis from western India.

Measles Group

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Measles Group

Projects

- Measles Aerosol Vaccine: Phase II/III Clinical Trial
- Case Based Study (Case Based Study for Measles virus at Measles Aerosol Vaccine: Phase II/III Clinical Trial area)
- Molecular Epidemiology of Measles in North East region of India

Measles Aerosol Vaccine: Phase II/III Clinical Trial

Herve, Ramesh. S. Jadi

World Health Organization (WHO) reported 30 million cases of Measles per year causing 777,000 deaths. The disease is vaccine preventable and vaccination is highly effective. Vaccination of susceptible population is not possible because of logistical constrains in administering a vaccine by subcutaneous route. Vaccination program has reduced the incidence by about 72% and mortality by 82%. Failure to deliver at least one dose of vaccine to all infants is the leading cause for high Measles morbidity and mortality that exists even today.

In nature Measles virus is transmitted by aerosol route. Immunization of susceptible population by aerosol vaccine would be important and promising route.

Objective

- Immunogenicity of Measles vaccine by aerosol and subcutaneous route.
- Whether vaccine delivered by an aerosol route is safe and immunogenic.

Work done

Installation of Automated ELISA workstation (Behring ELISA processor III) and other instruments was installed and hands on training were provided to the staff. Project related SOP's (Standard operating procedures) were prepared as per the WHO guidelines and implemented. The cases from pre and post immunized serum samples were collected and sent to NIV Pune by collaborative centre (Vadu centre). Two thousand serum samples were registered and aliquoted for ELISA, PRNT, repeat test, and for storing (archiving). Measles IgG ELISA test was standardized. Samples received for IQA and EQA were processed for ELISA (both automated and manual method) as well as PRNT and Shwert & Westgaurd plot was generated. Measles group passed this proficiency panel test sent from HPA (U.K) with 100% match in the result.

Summary and future plan

The 2000 samples that are collected from Measles aerosol vaccine phase II/III clinical trial will be processed for quantization of IgG antibody to understand the Immunogenicity of Measles vaccine immunized through aerosol route.

Case Based Study

Case Based Study for Measles virus at Measles Aerosol Vaccine: Phase II/III Clinical Trial area

Ramesh. S. Jadi

Introduction

The Measles aerosol vaccine group has selected five PHC areas (approximate

population 300,000) in three Blocks (approximate population 1.6 million) in Pune district (Maharasthra state, India) for conducting pivotal trials of measles aerosol vaccine in India. Case Based Study has been initiated to obtain incidence rate in the population in and around the Measles Aerosol Project study site areas. The laboratory confirmation of the reported clinical cases of measles will be done through serological testing for measles IgM antibodies.

Objective

- To obtain a measles incidence rate in the population in and around the Measles Aerosol Project study site.

Work done

56 suspected Measles samples were collected (January to March 2010) from Measles aerosol vaccine phase II/III clinical trial area (Shirur - 09 PHCs, Khed- 12 PHCs and Haveli- 12 PHCs). ELISA test was performed on these samples for Measles IgM antibody and Measles negative samples (19 samples) were tested for Rubella IgM. Details are as shown in the table.

Table 1: Measles and Rubella IgM results

		Measles IgM Results				Rubella IgM Results		
Number of Serum Samples Received	Number Tested for Measles	Positive	Negative	Equivocal	Number Tested for Rubella	Positive	Negative	Equivocal
56	56	37	19	0	19	05	14	0

Genotyping study from Measles Aerosol Vaccine surveillance area: Clinical samples (Blood, Throat swab, Oral swab & Urine) were collected from two measles suspected cases in December 2009 from Measles aerosol vaccine clinical trial area (Sanaswadi, Shirur). Serum samples of these cases were found positive for measles IgM antibodies by ELISA. Throat swab and oral swab samples were subjected to RT-PCR and sequencing for N gene. Both the cases were RT-PCR positive. Sequence analysis showed that measles virus strains belonged to genotype D8.

Future Plan

Genotyping studies along with serosurveillance will be conducted for suspected measles outbreaks from MAV clinical trial areas.

Molecular Epidemiology of Measles in North East region of India

Ramesh. S. Jadi, J. Mahanta (RMRC, Dibrugarh)

Lack of baseline data on measles molecular epidemiology in northeastern part of India spurred ICMR- NIV to create the regional network of premier medical institutions and pediatricians in the region. The NIV-ICMR initiated network is investigating measles outbreaks, performing laboratory studies like serology, virus isolation, RT-PCR and sequencing to know the indigenous genotypes circulation in NE region. Systematic investigations of the outbreak would generate baseline data to understand epidemiology of measles, and would lead to formulation of measles control strategies in the NE region.

Objective

- Establishment of sentinel Surveillance network of Pediatrician and medical establishments in northeast India.
- Investigating outbreaks and sporadic cases of Measles in the region.
- Serology confirmation, Virus isolation studies and PCR- sequencing on the specimens collected through the network.
- Establishing the sequence database of measles sequences from NE region and tracking the transmission pathways of the virus strains circulating in the region.

Work done

RMRC, Dibrugarh referred 10 serum samples for measles serology and genotyping studies in December 2009. Results showed that all 10-serum samples were negative for measles as well as rubella IgM ELISA. Genotyping studies were carried out on 4-throat swab samples collected from Nagaland, and 1 throat swab sample each from Meghalaya (Shillong) and Manipur (Imphal). Genotyping study revealed that 3/4 and 1/1 throat swabs from Nagaland and Meghalaya respectively were positive where as the throat swab sample obtained from Meghalaya was negative for measles N gene RT-PCR. Sequence analysis showed that measles virus strain belonged to genotype D8.

Six suspected cases from Anjaw district, Arunachal Pradesh were referred (February 2010) to NIV, Pune by RMRC, Dibrugarh, for measles genotyping study. 4/6 of these cases were confirmed as measles by IgM ELISA. RNA extracted from four serum and two throat swab samples were referred to NIV by RMRC Dibrugarh for genotyping studies. Sequence analysis revealed that 2/6 (RNA extracted from throat swab samples) were positive for measles N gene RT-PCR. The measles virus strain belonged to genotype D4 (Figure-1)

Future Plan

To strengthen NIV-ICMR measles surveillance network to cover all the seven states and establish the sequence database of measles sequences from NE region.

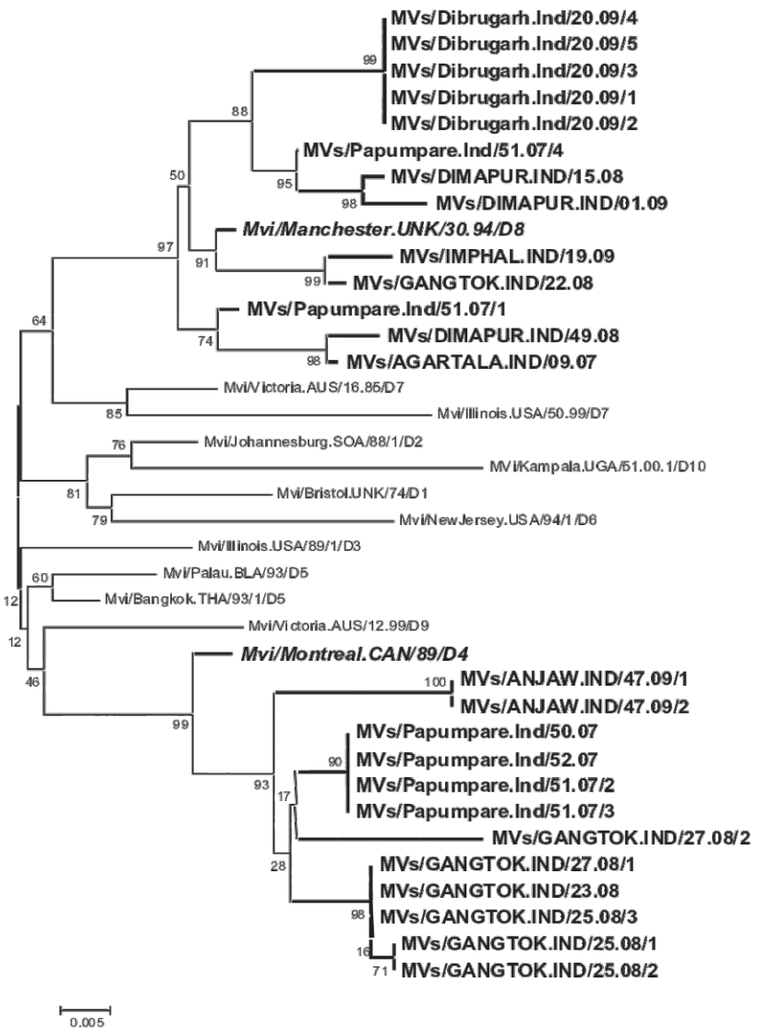

Figure 1: Phylogenetic analysis of N gene sequences obtained from NE region.

Validation of Measles laboratory

- Measles laboratory was renovated as per WHO standards from March-July 2009. Lab equipments were reinstalled; authorized engineer performed validation and calibration.

Other Activities:

- Measles group contributed in Swine flu studies; like sample aliquoting, molecular biology, VTM preparation and sero surveillance work.
- Referred serum samples were tested for measles and rubella IgM by ELISA. (Table 2)

Table 2: Measles and Rubella IgM results

		Measles IgM Results				Rubella IgM Results		
Number of Serum Samples Received	Number Tested for Measles	Positive	Negative	Equivocal	Number Tested for Rubella	Positive	Negative	Equivocal
76	76	32	43	01	43	02	39	02

Measles group also passed Proficiency Test for Measles and Rubella IgM ELISA conducted by WHO.

Audits

International Audits

- Measles laboratory, NIV, passed audit for annual WHO accreditation of Regional Reference Measles/Rubella laboratories (30 Nov- 02 Dec 2009). Audit was done by Dr. David Featherstone (WHO, Geneva) and Dr. Nalini Ramamurty (WHO-SEARO New Delhi).
- Measles lab was also audited by international scientific lead auditor (Rita Marta) and passed the audit without major suggestions.

National Audit

- Diagnosearch an Indian Clinical Research Organization audited the process of aliquoting, storage and retrieving of clinical trial samples.
- Documents related to Quality Control process and SOP's were verified. Audit was cleared with minor suggestions.

Medical Entomology & Zoology Group

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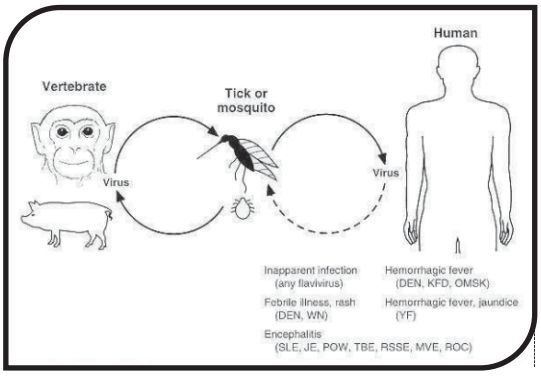
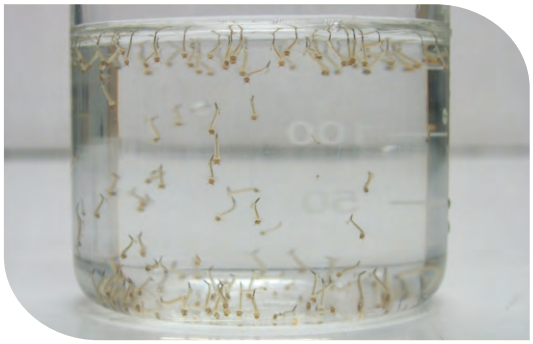
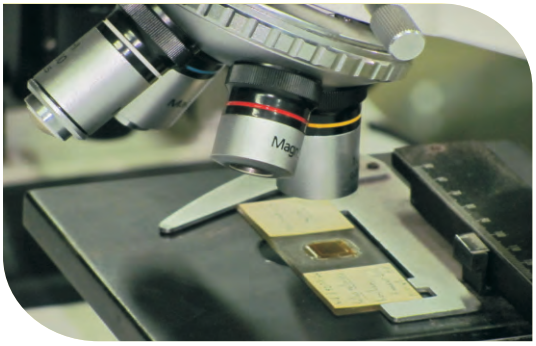




Medical Entomology & Zoology Group



Projects

- Studies on the risk factors for JE/AES in Gorakhpur District (UP) using GIS based approach
- Ecological studies during the outbreaks of suspected viral etiology
- Virus-Vector Interaction studies

Studies on the risk factors for JE/AES in Gorakhpur District (UP) using GIS based approach

Gokhale M. D., Gore M. M. and Jamgaonkar A. V.

Studies were continued during the period to generate an in-depth database of JE/AES cases in and around Gorakhpur. Both the rural as well as urban areas of the district have reported cases. The data is generated with reference to socio- economic and hygienic status of the patient along with the geographic location. The present and the previous year information are being analyzed using the computational program software. This would lead to better understanding of correlating factors responsible for JE/AES cases in Gorakhpur.

Objective

- To understand the risk factors leading to JE/AES cases in Gorakhpur district.
- To analyze distribution of the cases.
- Develop a computational program to understand disease association with ecological factors

Work done

Geographic Information System (GIS) studies were conducted in three PHC blocks viz; Jungle Kauria, Piprauli and Derwa. Three hundred sixty four GPS spots were mapped and information was gathered with reference to ecological parameters. A software development platform is generated with the help of the GS Lab. This map locator platform enables features such as Google earth view, exploration of the map layers, visualization of AES cases on the Gorakhpur map, the customized legend feature support, time line filter layers with reference to day/month/quarter/year etc (Figure 1 & 2). The case data can be correlated with ecological features such as precipitation, temperature etc. Distribution of cases is displayed in color grid format for better visualization. Correlation between cases and different parameters are compared using statistical tools.

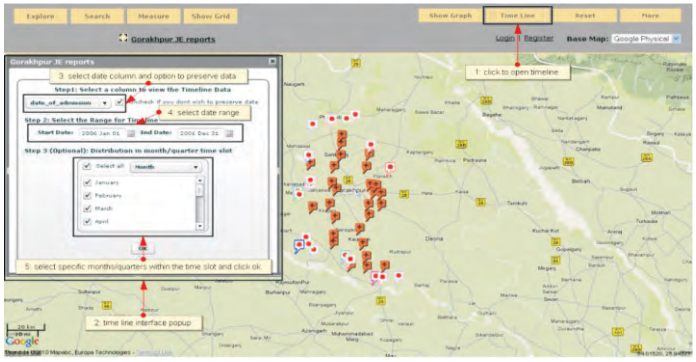


Figure 1: Time Line Feature

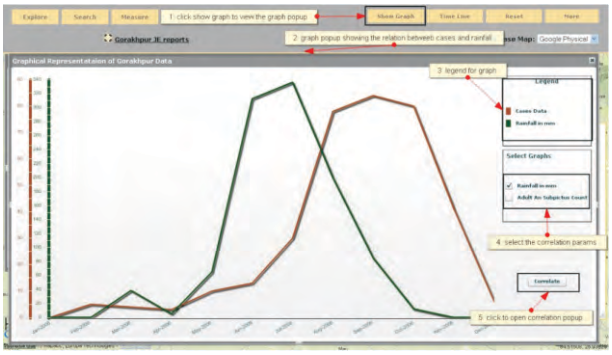


Figure 2: Graphs: Comparing two parameters

Future plan

Information gathered through GIS Portal would be studied in the context of disease distribution, spread and prediction.

Ecological studies during the outbreaks of suspected viral etiology

Gokhale M. D. and Jamgaonkar A. V.

Studies on chikungunya virus

Entomological studies were conducted during the chikungunya outbreaks in the following areas viz ; Kozhikode and Allapuzha district in Kerala, Pune and Thane district in Maharashtra respectively.

In Pune, four rural localities in Bhor PHC and in Thane, two localities in Kasara PHC and two localities in Kalyan Urban Agglomeration were visited. The presence of *Aedes aegypti* mosquito was confirmed by larvae and adult collections. The Breteau index and the adult house index ranged between 10-20 and 5-10%.

Studies on chandipura virus

Wild pools of sand flies were received from two chandipura virus affected districts of Gujarat viz; Panchmahal and Kheda. Ecological studies were conducted in a follow-up visit. A total of eleven villages in three districts were visited and sand fly collections were made. 1009 sand fly individuals were collected. These belong to Sergentomyia (874 individuals) and Phlebotomus (135) individuals. The collected individuals were distributed in 37 pools and processed for chandipura detection using real time PCR technique.

Studies on the bird fauna in Gorakhpur

Faunistic study on the avian fauna in Gorakhpur has been initiated. 23 water bodies were visited in Gorakhpur and Sant Kabirnagar districts. In all 43 bird species were sighted in the study area. There were 7 species of water birds, 17 species of water frequenting birds and

19 species of terrestrial birds. Presence of large number of Egrets and Herons is noteworthy as these are considered to play a role in the natural cycle of JE Virus.

Future plan

Ecological and entomological studies would be continued in response to outbreak investigation studies.

Virus-Vector Interaction studies

M. D. Gokhale and Mrs. M. S. Mavale

Persistence of chikungunya virus in dried Aedes aegypti mosquito

Studies were conducted on the persistence of chikungunya virus in dried Aedes aegypti as a potential surveillance tool. Mosquitoes were infected with chikungunya virus by inoculation. After confirmation of infection they were killed by brief freezing and applied to the sticky adhesive tape. They were desiccated at 28, 37 and 45 degree centigrade to mimic the moderate, hot and dry environment. At weekly intervals a sample collection of dried mosquitoes were tested by real time PCR. It was observed that virus could be detected up to 4 month period.

Persistence of chandipura virus in dried Aedes aegypti mosquito

Studies on the chandipura virus in sand fly have been initiated. As a precursor to this exercise Aedes aegypti mosquito is selected as an experimental host for chandipura virus. Adult mosquito was infected by inoculation and after confirmation of infection was killed by brief freezing. These were applied to the sticky adhesive tape. Gradual desiccation was ensured at 28 degree centigrade. At weekly intervals a sample of dried mosquito were stored at -70 degree centigrade freezer. The first week collection has been tested by real time PCR and found positive. While second week onwards mosquito were negative.

Susceptibility of Aedes aegypti to dengue 2 (TR1751) virus

Comparative studies were done on the susceptibility of Aedes aegypti to dengue 2 virus by inoculation and oral feeding route. The infected mosquitoes were checked for the detection of virus by Immuno fluorescence test at periodic intervals. It was observed in inoculated mosquito batch that head squash positivity appeared by 6th day post infection and gradually increased .While very low positivity was observed in the orally fed batch even up to 21st day post infection incubation.

Studies on the growth of chikungunya virus (Kerala Strain) in Aedes aegypti and Aedes albopictus mosquito

Growth kinetics studies on chikungunya virus infection in Aedes aegypti (Figure 3) and Aedes albopictus were carried out.

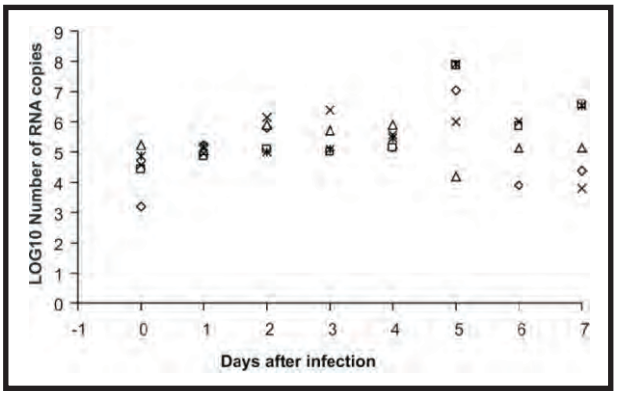


Figure 3: Growth Kinetics of CHIKV African strain (Kerala) in *Aedes aegypti*

When both the species were infected by oral route there was a gradual increase in the quantum of the virus by third day post infection. Real time PCR results indicate a rise of 1000 fold increase in the virus copy number. In inoculate mosquito batch the virus multiplication peak was observed at 24 Hours and gradually stabilized by 80 hours of post infection.

Future plan

Virus- vector interaction studies would be continued at the molecular level

Service Project

- Detection of virus from arthropod samples: During the period *Aedes aegypti* mosquito received from Surat, Bhosari and Parbhani. 54 male and 209 female mosquitoes were processed by mosquito inoculation; all were negative by IFA.
- Supply of mosquito species: Larval and adult stages of *Aedes aegypti* and *Culex quenquefasciatus* were supplied to Ross life Science company for pesticide evaluation. Total revenue generation towards this service is Rs.10,000.00/- only.

Most Important Findings

The comparative studies on virus vector interaction using real time PCR technique and IFA test are being standardized. This would give a clear perspective on quantitative and qualitative aspect of the interaction respectively.s

Bioinformatics & Data Management Group

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Bioinformatics & Data Management Group

Projects

- Docking studies of Dansylcadeverine in the Vesicular stomatitis virus glycoprotein
- Transmission Dynamics of 2009 H1N1 pandemic Influenza in a residential school in Panchgani, Maharashtra: Mathematical **modeling and simulation studies**
- Whole genome analysis of Chandipura virus isolates and comparative analysis with other rhabdoviruses to understand the molecular markers of pathogenicity
- Core facility services

Docking studies of Dansylcadeverine in the Vesicular stomatitis virus glycoprotein

Sarah Cherian, Arpita Banerjee

Introduction

Rhabdoviruses include Vesicular stomatitis virus (VSV) as well as notable human pathogens such as the Rabies virus and the Chandipura virus for which there are limited therapeutic agents. Modeling of target receptors and binding site identification is an important step in developing new therapeutic agents. Ligands that bind selectively to proteins of the membrane fusion pathway can retard or block viral entry and thus serve as antivirals. We selected the surface fusion glycoprotein G of the VSV, as a potential antiviral target. Dansylcadaverine, Rimantadine and Amantadine are known drugs which have been shown to prevent VSV internalization, though the molecular mechanism of their inhibition is not well understood. We therefore tried to explore the potential of these ligands as fusion inhibitors by undertaking in-silico binding site identification and docking studies.

Objectives

- Scan the prefusion structure of VSV glycoprotein for probable ligand binding pockets using computational softwares used for in-silico drug discovery.
- Target the predicted site with the best score to carry out the docking studies of the three known inhibitors, Dansylcadaverine, Rimantadine and Amantadine.

Results

A ligand binding pocket was predicted in the the G trimer interface. Docking of Dansylcadaverine into this pocket resulted in the ligand making contacts with the residues 269-274 in one chain and 27-32 in another chain of the trimer. These residues coincide with the residue stretches required for the formation of the central helix during the fusogenic transition. Ser28 and Glu276 from each of the chains which are very critical for the structural transition of the glycoprotein were H-bond donors to the three molecules of the ligand. Also, Glu276, which is one of the acidic amino acids that are solvent exposed in the prefusion state, gets buried at the trimer interface in the postfusion conformation. Dansylcadaverine thus made energetically favourable contacts with residue stretches required for the structural transition of the protein to the fusion active form. Rimantadine and Amantadine failed to dock in the binding pocket. The results suggest that Dansylcadaverine could prevent viral entry by stabilizing the G protein in the prefusion conformation. The findings of this study can be extended to design antivirals against other viruses of the same family.

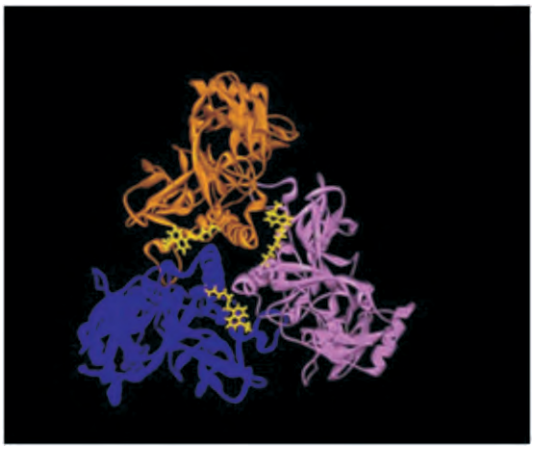

Figure 1: Docking of Dansylcadeverine molecule in the G protein

Transmission Dynamics of 2009 H1N1 pandemic Influenza in a residential school in Panchgani, Maharashtra: Mathematical modeling and simulation studies

P. Shil, Y. K. Gurav, M. S. Chadha, A. C. Mishra

Introduction

Mathematical modeling of epidemics has great potential for better understanding of the transmission pattern of diseases and predictions of outcome of different control strategies. In the present work, the transmission dynamics of an outbreak of the pandemic influenza A H1N1 (2009) during June July 2009 in a residential school in Maharashtra state of India has been studied.

Objectives

- To determine the epidemiological features of the outbreak in an Indian residential school setting (clustering of population).
- Qualitative assessment of the effectiveness of the control measures.

Results and discussion

A mathematical model of the type Susceptible-Exposed-Infectious-Asymptomatic-Recovered (SEIAR) has been adopted for the purpose and simulations performed using MATLAB® package. Analyses of epidemiological data revealed that close clustering within population resulted in high transmissibility with basic reproduction number R0 = 2.61 and transmission rate (β) being 1.566×10^{-3} per day. The doubling time of the epidemic was estimated to be 2.14 days. The average serial interval was estimated as 3.5 days (1.5 days, incubation time + 0.5 × 4 days, infectious period). The model had predicted that without interventions, the outbreak could have continued for 60 days generating a total of 281 symptomatic cases. Timely interventions like imposition of control measures and administration of Oseltamivir (both prophylactic and treatment) lead to control of the

outbreak which ended by 38th day after generating 176 symptomatic cases. The study also helped us in determining the parameters for asymptomatic cases (duration: 4 days; infectiousness ~60% to that of Symptomatic).

Conclusion

The model has successfully described the dynamics of transmission in the residential school setting and helped to ascertaining the epidemiological parameters for asymptomatic cases and in evaluating the effectiveness of the control measures. The work presents a framework for studying similar outbreaks of influenza involving clustered population.

Whole genome analysis of Chandipura virus isolates and comparative analysis with other rhabdoviruses to understand the molecular markers of pathogenicity

Sarah Cherian, V.A. Arankalle

Introduction

The Chandipura virus (CHPV) belonging to the Rhabdoviridae family, has recently been associated with a number of encephalitis epidemics, with high mortality in children, in different parts of India. No full length genome sequences of CHPV isolates are currently available in GenBank and little is known about the molecular markers for pathogenesis. The complete genomic sequences of four isolates from epidemics during 2003-2007 along with the deduced sequence of the prototype isolate of 1965 were analysed using phylogeny, motif search, homology modeling and epitope prediction methods. Comparison with other Rhaboviruses was also done for functional extrapolations.

Objectives

- Sequence/ structure-based characterization of the deduced protein sequences, genomic termini and the gene junctions of the isolates from the 2003-07 CHPV epidemics vis-à-vis the prototype isolate.
- Comparisons with other members of the Rhabdoviridae family such as VSV, Isfahan virus (ISFV) (genus:Vesiculovirus), rabies virus (RABV) (genus: Lyssavirus) and Bovine Ephemeral fever virus (BEFV) (genus: Ephemerovirus) for functional motifs.

Results

The CHPV isolates possessed phosphorylation motifs in the nucleoprotein N and phosphoprotein P and both phosphorlation and glycosylation motifs in the large protein L. Probable links to axonal transport involving the glycoprotein G and P were noted. The isolates also maintained other functional motifs observed in Rhabdoviruses suggesting the pathogenic potential of CHPV. With respect to the prototype isolate, significant additional mutations were acquired in the 2003-2007 isolates. Several mutations in G mapped onto probable antigenic sites. Mutations in N mapped onto regions crucial for N-N interaction and a putative T-cell epitope. A mutation in a potential Caesin kinase II phosphorylation site in P may attribute

to increased rates of phosphorylation. Gene junction comparison suggested altered transcription rate of the matrix protein M and that of P in the 2007 isolates. Overall, important insights into the possible virulence determinants of the CHPV have been determined.

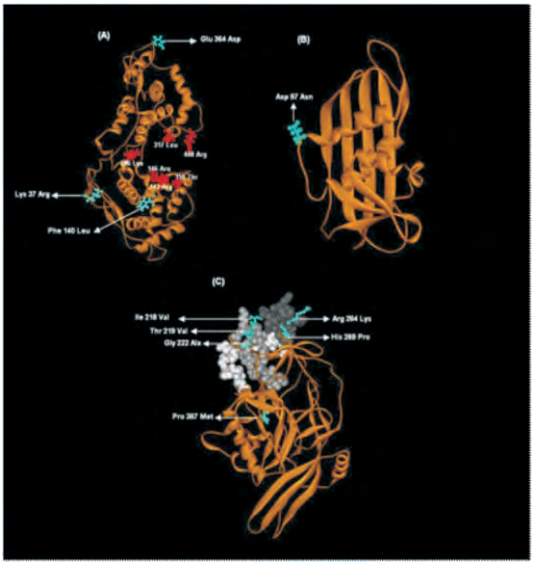

Figure 2: Homology models of CHPV proteins and mapping of significant functional residues. All observed mutations in cyan. (A) Nucleoprotein: RNA binding residues shown in orange, residues part of RNA binding groove in blue and loop Ser340-Val375 in yellow. (B) Matrix (C) Glycoprotein: Antigenic site G3 shown in shades of gray

Core Facility Services

Statistical data analysis

Regular services to the various experimental groups at NIV and MCC, Pashan, in the areas of statistical data analysis of Epidemiological and serological data, bioinformatics sequence analysis, phylogeny, evolutionary dynamics, structural modeling etc.

Computer related services

Management of the LAN and internet services at NIV, Pune and MCC, Pashan.

Awards Received

- Dr Sarah Cherian, “Pioneers in Genomics Education” Class of 2010, Ocimum Biosolutions and Gene Logic, Hyderabad, for outstanding contribution to the field of Genomics Education, 2010.
- Dr Pratip Shil, 2000 Intellectuals of the 21st Century Awards 2009-2010, International Biographical Centre, Cambridge, UK, for valued contribution in experimental and computational biophysics.

High Containment Lab & Virus Repository

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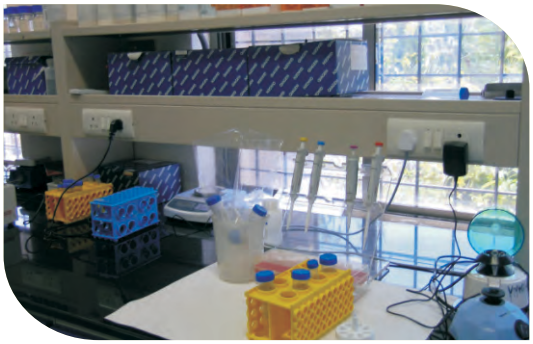
High Containment Lab & Virus Repository



Projects

- Development of serological and molecular diagnostic facility for Kyasanur Forest Disease virus
- Establishment of Maximum Containment Facility at MCC, Pashan, Pune
- Molecular characterization of viruses (Family: *Bunyaviridae*) isolated from India
- Studies on the Densoviruses of *Aedes* mosquitoes and their interaction with susceptibility of mosquitoes to Chikungunya virus
- Surveillance of viruses of potential zoonoses from bats of India
- Laboratory diagnosis of referred samples of unusual cases

Development of serological and molecular diagnostic facility for Kyasanur Forest Disease virus

D. T. Mourya, P. D. Yadav, P. V. Barde

Objective

- Screening of clinical samples for KFDV by indigenously developed ELISA assays.

Work Done

Total 130 clinical samples received from Shimoga state laboratory, Karnataka were tested using standardized IgM capture ELISA assay. Additionally 30 convalescent samples were received and tested by the standardized IgG capture ELISA test.

Some experiments were designed and conducted to establish Dot-blot ELISA using known KFD positives and negatives sera samples. Known amount of KFDV mice brain inactivated antigen was blotted onto nitrocellulose membrane, after blocking with 4% BSA the blots were incubated with known positive and negative immune serum in various dilutions (1:10, 1:50, 1:100). The blots were then probed with anti human IgM antibody tagged with AP.

Total 41(31.53%) and 29 (22.30%) samples were found positive for IgM and RT-PCR respectively. Total 20 convalescent sera were found positive for IgG. Encouraging initial results were observed for Dot-blot ELISA for KFD, further work to fine-tune the technique is in progress.

Establishment of Maximum Containment Facility at MCC, Pashan, Pune

D. T. Mourya and A. C. Mishra

Objective

- To create a specialized maximum containment facility to handle the pathogens of BSL-4.

Work Done

- Prepared the on-site and stand alone equipments list for up-coming BSL-4 project.
- Preparation of document for scientific staff's training requirement for operation of BSL-4 facility.

Considerable progress is going on about development of internal different laboratories', procurement of related equipments and supporting gadgets.

Molecular characterization of viruses (Family: Bunyaviridae) isolated from India

P. D. Yadav and D. T. Mourya

Objective

- Characterization of unidentified bunyaviruses at molecular level to assist in right taxonomic placement.

Work Done

The present study involves phylogenetic comparison of selected Ganjam virus isolates from India with other nairoviruses based on complete N gene of the virus. Eight strains of GANV isolated at NIV during 1954-2002 from different parts of India were used in this study. GANV were propagated in *Vero E6* cell line and the harvested virus suspensions were used as the source of RNA to be used in further experiments. The N gene of GANV was amplified either as a complete gene in one reaction or in fragments whenever necessary. For the sequencing of complete N gene of GANV (1590bp) 15 different primers were used. The sequences obtained were analyzed; annotated to get a consensus sequence, aligned against the sequence of prototype strain of GANV as reference and then aligned with all the other representative nairoviruses. Conversion from nucleotides into amino acids was done and analysis was done at both nucleotide and amino acid levels. A phylogenetic tree was constructed to compare the GANV isolates with other nairoviruses (CCHF, DUGV, HAZV, KUPV and NSDV) whose complete S segment sequences were available on the web in GenBank database (NCBI).

Further to know about this virus, Complete L (11.8 kb) and Complete M (4.9 kb) gene was amplified for strain No. 779 and sequencing was done. Few more GANV strains are in process of amplification and sequencing.

The phylogenetic data at both the nucleotide and amino acid levels showed that all the strains of GANV along with the NSDV strain form one monophyletic lineage. This confirmed that these two are minor variants of the same virus. CCHFV and HAZV together formed another clad whereas DUGV and KUPV made a separate branch in the tree. Distance analysis showed that the prototype GANV G619 and the GANV strain 779 have 100% homology at the nucleotide as well as amino acid level indicating that these two are the same strains of GANV. The different strains of GANV showed 9-10% difference with NSDV at the nucleotide level and 3-4% difference at the amino acid level, with maximum difference of 4% in case of strain 616287. HAZV showed 37-38% and 37% difference with GANV as well as NSDV at nucleotide and amino acid levels respectively. However, the present data obtained, suggests that GANV and NSDV are minor variants of the same virus.

Once the sequencing of L and M gene is complete, phylogenetic comparison will made to see the changes at nucleotide and amino acid with other Nairoviruses and NSDV which will answer why NSDV and Ganjam virus are having so much difference in the pathogenicity.

Studies on the Densoviruses of *Aedes* mosquitoes and their interaction with susceptibility of mosquitoes to Chikungunya virus

D. T. Mourya, M. D. Gokhale, Aruna Shivram and P. D. Yadav

Introduction

The Densonucleosis viruses (DNV) are the etiological agents of insect disease known as Densonucleosis. This virus was first isolated from *Galleria mellonella* while the first mosquito DNV was isolated from *Aedes aegypti*. These belong to family Parvoviridae, subfamily Densovirinae, and genus Brevidensovirus. They are small icosahedral, non enveloped particles of 18-26 nm diameter with a ss linear DNA genome, encapsidated equimolecularly as + & - strand. It has a genome size of 3.9 5.9 kb. In mosquitoes, anal papillae are the primary site of infection, from which it spreads to fat bodies & other tissues including muscle fibers & nerves. The significance of DNV arises from the report that it can be a candidate for biological control activity of mosquitoes, killing up to 100% of early stage larvae. It can also be used as a system for delivering foreign genes in mosquitoes.

Objective

- To determine the effect of co-infection of DNV and CHIKV in *Ae. aegypti* mosquitoes.
- To determine the effect of DNV on the life span of *Ae. aegypti* mosquitoes.
- To determine the effect of temperature on the replication of DNV in *Ae. aegypti* mosquitoes.

Work Done

Study 1: 3000 first instar larvae of *Ae. aegypti* were infected with 10-1 of DNV which corresponds to DNA copy number of 6.07x10-8. The females that emerged were infected by oral feeding with CHIKV whose genomic copy number corresponds to 5.53E+06 RNA molecules. Fully fed adults were segregated and counted. Controls used included larvae infected either with DNV or with CHIK and uninfected *Ae. aegypti* larvae. Adults were stored every alternate day post CHIKV infection starting from 0day to 8th day. 12 individual mosquitoes were stored daily in 2 batches with 6 individuals in each batch. One batch was subjected to DNA extraction and the other to RNA extraction. PCR and RT-PCR was done to confirm the presence of DNV and CHIKV respectively. The quantitation of viruses was done by real time PCR. The result indicated no change in the copy number of CHIKV after co infecting with DNV.

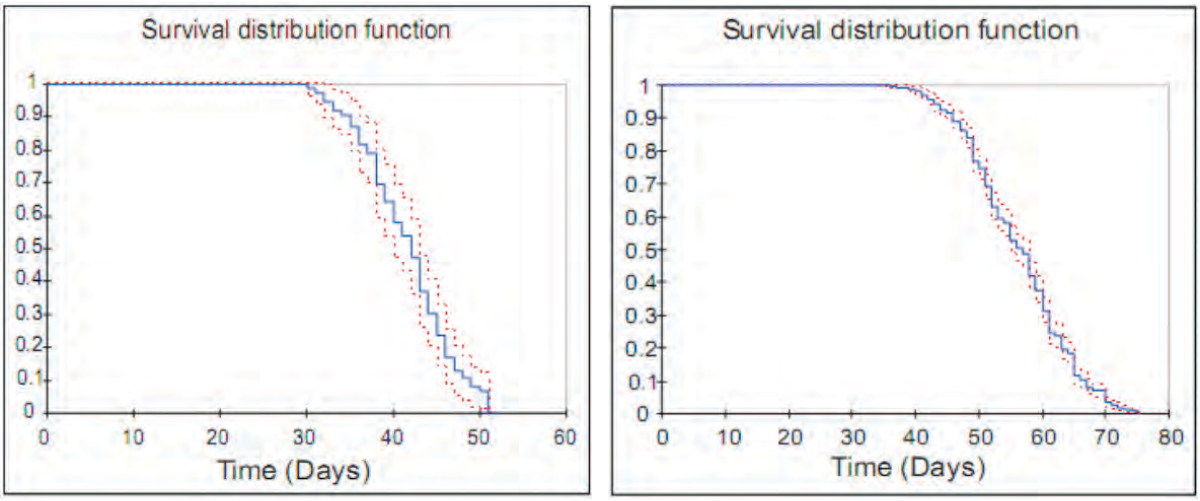
Table 1: Effect of co-infection of DNV and CHIKV in *Ae. aegypti* mosquitoes. The values are approximated to three decimal points.

Days post infection	LOG of RNA copy number of CHIKV (±SD)		LOG of DNA copy number of DNV (±SD)	
	In presence of DNV	In absence of DNV	In presence of DNV	In absence of DNV
0	3.345(±0.130)	3.173(±0.081)	1.906(±0.185)	2.389(±0.308)
2	3.220(±0.501)	3.573(±0.554)	1.709(±0.205)	2.642(±0.029)
4	3.613(±0.482)	3.807(±0.057)	1.040(±0.338)	1.574(±0.308)
6	3.893(±0.542)	3.897(±0.601)	1.409(±0.307)	1.043(±0.105)
8	5.354(±0.110)	5.269(±0.237)	0.765(±0.050)	1.410(±0.364)

Study 2: 200 first instar larvae were infected with 10-1 (DNA copy number 6.07E+08) dilution of DNV. 200 uninfected first instar larvae were taken as control. Larvae were maintained under standard conditions and counts were taken daily until the death of the last adult. Statistical analysis of the effect of DNV on the mortality of *Ae. aegypti* maintained at different temperature was performed using Kaplan Meier method and its statistical significance was derived using log rank test as implemented in the software XL STAT. Real time PCR was performed to determine the DNA copy number of DNV at every metamorphic stage of *Ae. aegypti*. In infected larvae, the duration of the larval stage decreased by 15 days. The total mortality of the infected larvae were found to be significantly more than that of uninfected mosquitoes by t-test ($p=4.12E-05$). A total mortality of 92% was seen of which 88% mortality occurred at the larval stage and 4% at the pupal stage. The survival period of adult mosquito was also affected significantly with infection. The median life span of the infected group was dropped by around 19 days. The median life span among the control group was found to be 58.39 days (±0.660) while that of infected group was found to be 39.25 days (±0.586). The adult males among the control group had a mean survival for 57.0 days (±0.697) and females had a mean survival of 60.8 days (±1.40) while for the infected group it was 39.0 days (±0.577) and 40 days (±1.581) respectively. Log rank analysis of the adult survival showed a $\chi 2$ value of 442 on 1 degree of freedom and a $p<0.001$. Parameters affected by infection of DNV in *Ae. aegypti* mosquitoes is represented in table 1. Fig 1 and 2 represent the Kaplan Meier analysis and log rank analysis of adult survival respectively.

Table 2: Parameters affected by infection of DNV in *Ae. aegypti* mosquitoes

Parameters	DNV infected	Control
Larval duration	24.26 ± 4.57	28.38 ± 2.10
% larval mortality	85	8.5
% pupal mortality	10	2.18
% total mortality	95	10.68
Average survival of males	39.0 ± 0.577	57.0 ± 0.697
Average survival of females	40.0 ± 1.581	60.8 ± 1.40
Average survival of adults	39.25 ± 0.586	58.39 ± 0.660



a) Infected group

b) Control group

Figure 1: Kaplan Meir analysis of adult mosquitoes of the infected group and control group

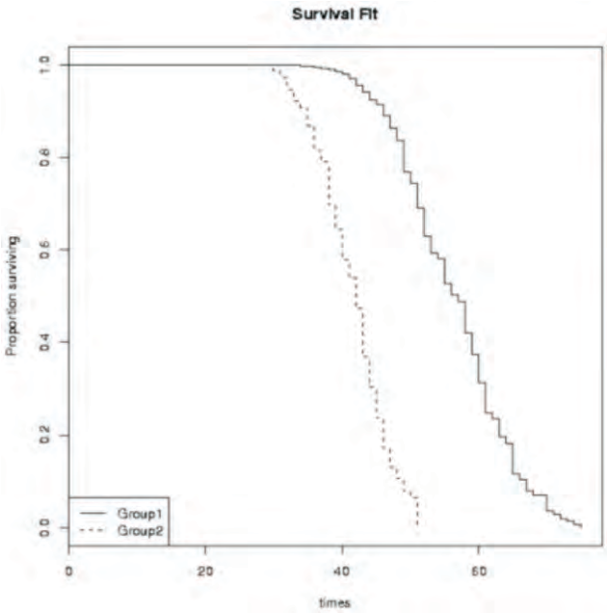


Figure 2: Log rank analysis of adult survival

Maximum multiplication of the virus was found during the 3rd and 4th instar of the larvae. The log of DNA copy number of DNV during the 3rd instar of larvae was found to be 9.87±1.51 while that during the 4th instar of larvae was found to be 9.63±1.43. Minimum multiplication was found in the adult stage with log of DNA copy number 3.60±0.42. Table 3 represents multiplication of DNV at different metamorphic stages in terms of log of genomic copy number.

Table 3: Multiplication of DNV at different metamorphic stages of *Ae. aegypti* mosquito

Stage	Log of copy number
First instar	6.33 ± 0.29
Second instar	6.66 ± 1.46
Third instar	9.87 ± 1.51
Fourth instar	9.63 ± 1.43
Pupal stage	4.07 ± 0.37
Adult stage	3.60 ± 0.42

Study 3: 50 first instar larvae were taken in 3 different beakers and infected with 10-1 dilution of DNV. Larvae were maintained at 28°C, 30°C and 37°C. 50 uninfected larvae were used as controls in each temperature. Counts were taken daily until the death of the last adult in each case. Real time PCR was performed for the 4th instar larvae (5 individuals) to determine the amount of viral nucleic acid present in each temperature. Though the mortality was significant between the infected and control group maintained at each temperature, analysis of statistical significance of day wise mortality by t-test suggested that the variation in mortality with temperature was insignificant ($p>0.05$). The copy number of DNV did not vary significantly with temperature ($p>0.05$). Parameters affected by infection of DNV at different temperatures are represented in table 4. Fig 3 represents the multiplication of DNV at different temperatures in terms of log values of genomic copy number.

Table 4: Parameters affected by infection of DNV at different temperatures

Parameters	37°C		30°C		Room temperature	
	Infected	Control	Infected	Control	Infected	Control
Larval duration	17.83 ± 5.56	34.02 ± 5.19	18.73 ± 2.79	34.7 ± 5.23	21.22 ± 7.89	35 ± 5.34
% larval mortality	76.0	10.0	78	8	82	10
% pupal mortality	8.33	2.22	9.10	0	11.11	0
% total mortality	84.33	12.22	87.10	8	93.11	10
Average survival of males	43.5 ± 1.80	61.00 ± 0.56	36.00 ± 1.87	59.5 ± 0.84	36.62 ± 0.50	56.17 ± 0.94
Average survival of females	45.25 ± 1.03	58.75 ± 2.22	36.83 ± 0.60	62.1 ± 0.81	41.17 ± 0.92	57.02 ± 0.27
Average survival of adults	45.0± 0.91	60.19 ± 0.74	36.63 ± 0.65	61.29 ± 0.85	41.6 ± 0.87	56.22 ± 1.33

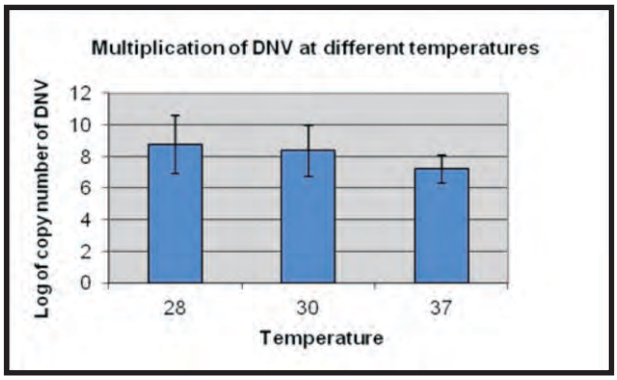


Figure 3: Multiplication of DNV at different temperatures. Each bar represents the average of log of copy number of DNV DNA at different temperatures

Surveillance of viruses of potential zoonoses from bats of India

D. T. Mourya and C. G. Raut

Introduction

Bats are the reservoir of several viruses. Other countries have isolated and identified viruses of BSL 1- 4. They are ecologically playing important role in harboring and shedding the viruses. In India, scanty reports are available on the mentioned aspect. To understand recent status of the bats in India, we have collected some samples and processed for *in vitro* virus isolation and serology of different influenza viruses as the priority.

Work done

Bats [*Rousettus leschenaulti*] have been trapped from Robbers cave, Mahabaleshwar, Maharashtra, India by using mist net. Bats were anesthetized and collected blood by heart puncture, further tissue samples were collected by euthanizing the bats. Tissue samples were processed for virus isolation in Vero E6 cell line, and as a priority serology was performed for different influenza viruses.

No virus isolation indication was observed from 30 samples (kidney, liver and urine) in a week's period of time. Furthermore serologically all the thirty blood samples were also found negative for influenza viruses (H5, H1, H3, and NDV).

Laboratory diagnosis of referred samples of unusual cases

D. T. Mourya

Objective

- To provide the diagnosis of unusual human cases by performing different tests

Work done

A total 75 clinical samples were received from NIV field unit Kerala on 27-1-2010. Of these [40 samples which were negative for Dengue and Chikungunya] were processed for determining Ross River Virus. Of these 40 samples only 9 were sufficient enough for RNA extraction hence these were tested for Ross River Virus, Murray Valley Encephalitis and Sindbis Virus, which were found negative. However, these 40 samples were also found negative for Hantaan IgM by CDC kit. Similarly, these 40 samples did not yield any virus isolation.

Five human serum samples were received from Rajkot (2), Pune (2) and Mumbai (1) tested for Hantaan IgM by CDC kit and found negative. Samples from Rajkot, Gujarat were also checked for the presence of CCHF and RVF by RT-PCR, which were found to be negative for both the viruses.

Dengue and Chikungunya negative samples from Kerala were further processed for RRV, MVE and Sindbis virus were also found negative by RT- PCR. Also sample received from different part of India suspected for Hantan Virus were found negative by Hantaan IgM CDC ELISA kit.

Services provided

- Chikungunya virus investigation: A field visit to Kannur district of Kerala state, which was badly hit by Chikungunya was made and collected 20 blood samples from acute cases. Entomological team collected Ae. albopictus mosquito samples from the affected areas. The samples were brought to laboratory in wet ice and are being processed for virus isolation and characterizing.
- Screening of clinical samples for Hantaan virus: Laboratory diagnosis was provided for Hanta virus. Six samples received from District panchayat Kutch, Gujarat and two samples from Ahmadabad, Gujarat were found negative for the virus using three Nested RT-PCR for all group of Hantavirus. Twenty eight serum samples from Mysore were tested by ELISA & PCR for Hantavirus and found negative.
- Infrastructure development at NIV Kerala Unit: Dr AB Sudeep was deputed to NIV Kerala Unit Alappuzha from 6th August 2009 to 2nd September 2009 to initiate the construction of main laboratory for Virology at Alappuzha. Meetings were held with CPWD and KSEB engineers regarding the construction and electrical requirement. Requirements for the proposed laboratory and administrative block along with design were given to CPWD architect to finalize design and costing.
- Bioassays were conducted on Biolarvicides (Bti) for efficacy testing using *Aedes aegypti* larvae. (Two samples from Govt. of Maharashtra).

Bangalore Unit

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Bangalore Unit

Projects

- Aetiological and epidemiological studies on flavivirus infections in Karnataka and neighbouring areas with particular reference to Japanese Encephalitis (JE) and dengue (DEN) viruses.
- Studies on Chikungunya virus: Aetiological epidemiological studies on Chikungunya (CHIK) virus infections in Karnataka state and neighbouring states.
- Studies on Poliovirus: Surveillance of Acute Flaccid Paralysis (AFP) cases from Karnataka State as a part of WHO-SEAR Polio Lab Network in the WHO's Global Eradication of Poliomyelitis Programme.
- Studies on Poliovirus: Intratypic differentiation of poliovirus isolates from AFP cases received from National Polio Laboratory as a part of WHO-SEAR Polio Lab Network in the WHO's Global Eradication of Poliomyelitis Programme.
- Studies on Measles: Surveillance of Measles cases from Karnataka State and Kerala State, as a part of WHO-SEAR Measles Laboratory. Network in the WHO's Global Measles Elimination Programme

Aetiological and epidemiological studies on flavivirus infections in Karnataka and neighbouring areas with particular reference to Japanese Encephalitis (JE) and dengue (DEN) viruses

P. N. Yergolkar and Hanumaiah

Introduction

JE and DEN viral infections in Karnataka and neighbouring areas have remained to be important Public Health Problems. There is continuous need to study the problem for an early diagnosis for patient management and undertaking prevention and control measures.

Objectives

- To diagnose and establish JE and DEN viral infections in suspected patients, to study epidemiological features, establish serum/virus bank for these diseases.
- To provide laboratory diagnosis for patient management and for prevention and control measures by the State Health authorities.

Work done

Studies on JE in Karnataka State and neighbouring districts

Specimens received from a total of 6 suspected cases of encephalitis from 3 districts of Karnataka (4 cases) and 1 district of Andhra Pradesh (2 cases) were screened for JE IgM antibodies. Only the two cases from Nellore district of Andhra Pradesh were positive for JE IgM antibodies. JE positive cases were male child aged 4 years and 7 years and were reported in the months of April 2009 and February 2010 respectively.

Studies on dengue (DEN) in Karnataka State and neighboring districts

Serum specimens were received from a total of 4356 suspected cases of CHIK/DEN. Of these 4240 cases were from Karnataka, 69 from Andhra Pradesh, 28 from Tamil Nadu and 6 from Kerala states and remaining 13 were from other states.

Out of 4356 cases, samples from 3322 cases were tested for dengue IgM antibodies by MAC-ELISA. Specimens with < 3 Post Onset Days, CHIK IgM positives were not included for dengue IgM testing. Of the 3322 cases tested, 928 (27.9%) cases were positive for dengue IgM antibodies. DEN virus isolations if any from 343 acute sera processed in C6/36 cell line are to be confirmed. Details of district wise cases are presented in Table 1. Dengue activity was observed throughout the year with peak in August and involved all age groups in both sexes.

Future plan

There is continued need for surveillance, diagnosis and virus isolation for molecular epidemiology of JE and dengue infectious for undertaking preventive and control measures by the Health authorities and better management of cases by the clinicians.

Table 1: District wise Diagnosis of DENGUE cases from Karnataka and bordering States April-2009 to March-2010

District	DEN IgM		
	No. Tested	No. Positive	No. Negative
Bangalore City	1940	629	1311
Chikkaballapur	246	21	225
Ramanagara	180	46	134
Davanagere	127	18	109
Kodagu	109	11	98
Bangalore Rural	101	40	61
Bidar	91	6	85
Tumkur	77	26	51
Gulbarga	65	3	62
Hassan	50	11	39
Kolar	47	13	34
Bangalore Urban	44	16	28
Mandya	33	8	25
Chitradurga	14	2	12
Mysore	10	6	4
Bellary	9	7	2
Raichur	5	2	3
Chikmagalur	5	2	3
Shimoga	4	0	4
Belgaum	1	1	0
Haveri	1	1	0
Udupi	1	1	0
Chamarajanagar	1	0	1
Dharwad	1	0	1
Uttara Kannada	1	0	1
Address Not Known	48	14	34
Karnataka Total	3211	884	2327
Andhra Pradesh	66	22	44
Tamil Nadu	26	7	19
Kerala	6	4	2
Other states	13	11	2
Grand Total	3322	928	2394

Studies on Chikungunya virus: Aetiological epidemiological studies on Chikungunya (CHIK) virus infections in Karnataka state and neighboring states

P. N. Yergolkar, P. S. Sathe, A. C. Mishra

Introduction

Chikungunya cases are still being reported from Karnataka State since 2005 There is need to study and monitor CHIK infections.

Objectives

- To diagnose CHIK infections and outbreaks for prevention and control.
- To study epidemiological features and molecular characterization of virus isolations from humans.

Work done

Serum specimens were received from a total of 4356 suspected cases of CHIK/DEN. Of these 4240 cases were from Karnataka, 69 from Andhra Pradesh, 28 from Tamil Nadu and 6 from Kerala states and remaining 13 were from other states.

A total of 3810 cases were tested for Chikungunya IgM antibodies by MAC-ELISA. Specimens with < 3 days of post fever onset, DEN IgM confirmed cases and cases from already CHIK outbreak confirmed areas were not included for CHIK IgM testing. Of these 1434 (37.6%) were positive for CHIK antibodies. During the period a total of 343 samples were processed for virus isolation in C6/36 cell line and 85 (24.8%) CHIK virus isolations were made and confirmed by IFA from 8 districts of Karnataka. In total 1519 (39.9%) cases were diagnosed as CHIK infection by serology and virus isolation during the period. District wise details of CHIK cases diagnosed are presented in Table 2. Chikungunya cases were reported throughout the year by virus isolation or serology with peak activity in the month of July. Chikungunya activity involved all age groups in both sexes.

Future plan

There is continued need for surveillance, diagnosis and virus isolation for molecular epidemiology of CHIK infectious for undertaking preventive and control measures by the Health authorities and better management of cases by the clinicians.

Table 2: District wise Diagnosis of CHIKUNGUNYA cases from Karnataka and bordering States April-2009 to March, 2010

District	DEN IgM			CHIK Virus Isolation (VI)		Total Pos. For CHIK (VI + IgM)
	No. Tested	No. Positive	No. Negative	No. Processed	No. Pos.	
Bangalore City	2276	908	1368	227	57	965
Chikkaballapur	273	70	203	19	2	72
Ramanagara	216	104	112	32	14	118
Davanagere	143	41	102	1	0	41
Kodagu	136	54	82	4	1	55
Bangalore Rural	104	26	78	11	1	27
Bidar	92	40	52	6	4	44
Tumkur	91	29	62	4	0	29
Gulbarga	68	33	35	4	0	33
Hassan	58	21	37	7	2	23
Bangalore Urban	51	17	34	3	0	17
Kolar	48	16	32	6	3	19
Mandya	39	14	25	3	0	14
Chitradurga	14	6	8	-	-	6
Mysore	10	2	8	1	0	2
Bellary	7	0	7	-	-	0
Raichur	6	2	4	-	-	2
Chikmagalur	5	0	5	-	-	0
Shimoga	4	1	3	-	-	1
Chamarajanagar	1	1	0	-	-	1
Dharwad	1	1	0	-	-	1
Belgaum	1	0	1	-	-	0
Haveri	1	0	1	-	-	0
Udupi	1	0	1	-	-	0
Uttara Kannada	1	0	1	1	0	0
Address Not Known	49	15	34	3	0	15
Karnataka Total	3696	1401	2295	332	84	1485
Andhra Pradesh	68	17	51	5	0	17
Tamil Nadu	27	15	12	1	0	15
Kerala	6	1	5	1	0	1
Other states	13	0	13	4	1	1
Grand Total	3810	1434	2376	343	85	1519

Studies on Poliovirus: Surveillance of Acute Flaccid Paralysis (AFP) cases from Karnataka State as a part of WHO-SEAR Polio Lab Network in the WHO's Global Eradication of Poliomyelitis Programme

P. N. Yergolkar and Hanumaiah

Introduction

Acute Flaccid Paralysis Surveillance for Global Eradication of Poliomyelitis Programme was initiated in India by the Govt. of India in collaboration with the WHO/WHO-SEARO/WHO-NPSP since June 1997. NIV Bangalore Unit has the responsibility of processing all stool specimens from Karnataka and Kerala state and Bareilly division of Uttar Pradesh specimens since July 2007. In the Global Polio Eradication Initiative (PEI), acute flaccid paralysis is defined as "Any case of AFP in a child aged <15 years, or any case of paralytic illness in a person of any age when polio is suspected". Any case meeting this definition undergoes a thorough investigation to determine if the paralysis is caused by polio.

Objectives

- To isolate and identify wild poliovirus from AFP cases until a global decision on discontinuation of AFP Surveillance.
- To participate in WHO-SEAR Polio Laboratory Network as per conditions and guidelines of WHO's Global Polio Eradication Programme and to coordinate with State, National and WHO Programme Managers.

Work done

A total of 7183 stool specimens from 3592 AFP cases were processed for virus isolation. AFP cases investigated in Karnataka were- 756, Kerala-316 and Uttar Pradesh- 2468. Other state cases investigated were Bihar-15, Andhra Pradesh-14, Tamil Nadu-11, Maharashtra-7, West Bengal, Delhi, Uttaranchal and Jharkand-1 each. One case was also tested from Maldives which was investigated in Kerala state.

Results for 3592 AFP cases were, Wild P1-4, Wild P3-50, P2-VDPV-5, Polio vaccine viruses-340, NPEV-836, NEV-1, Negative-2242 and results are pending for 14 cases. Wild polioviruses were isolated from Uttar Pradesh only. Results of AFP cases tested are presented in Table 3.

Wild poliovirus was isolated in Uttar Pradesh in the months of April-2, May-9, June-16, July-8, October-4, November and December-7 each and January-1. Majority (55.6%) of the wild cases was in children in 1-2 years age group and 96.3% of the cases were in children up to 10 years. Isolations were from both male (40) and female (14) children. P1 wild cases were one each from Bareilly, Moradabad, Rampur and Shahajahanpur districts. P3 wild cases were from the districts of Rampur-19, Badaun-15, Bareilly-9, Shahajahanpur-5 and Pilibhit-2.

During the period specimens from 135 contacts of 27 index AFP cases, 21 from Uttar Pradesh, 5 from Karnataka and 2 from Kerala were tested for virus isolation. Wild poliovirus-3 was isolated from 3 contacts from Uttar Pradesh from Badaun district. Other results were 19-polio vaccine viruses, 64-NPEV only and 49- negative.

Table 3: Virological results of AFP cases investigated in NIV Bangalore Unit

State	AFP Cases tested	No. Of samples tested	Wild P1 virus	Wild P3 virus	VDPV P2 virus	Polio Vaccine viruses	NPEV Only	NEV	Neg
Uttar Pradesh	2468*	4932	4	50	5	313	717	1	2242
Karnataka	756*	1514	0	0	0	23	83	0	549
Kerala	316	640	0	0	0	4	29	0	283
Bihar	15	29	0	0	0	0	1	0	14
Andhra Pradesh	14	27	0	0	0	0	4	0	10
Tamil Nadu	11	20	0	0	0	0	1	0	10
Maharashtra	7	13	0	0	0	0	1	0	6
Delhi	1	2	0	0	0	0	0	0	1
Uttaranchal	1	1	0	0	0	0	0	0	1
Jharkhand	1	2	0	0	0	0	0	0	1
West Bengal	1	2	0	0	0	0	0	0	1
Maldives (Country)	1	1	0	0	0	0	0	0	1
Grand Total	3592*	7183	4	27	5	340	836	1	2242

* Results are pending 14 days of receipt for 13 UP cases and 1 Karnataka case.

Polio Laboratory at NIV Bangalore has been fully accredited by the WHO for the period 2009-10 during on-site review visit by the WHO's expert in December 2009. Accreditation conditions were changed with the implementation of New (Alternate) Algorithm since January 2008 and up-gradation of NIV Bangalore to Polio ITD laboratory since May 2009. It is a combined Accreditation procedure for NPL and ITD Laboratories.

Future plan

Need to continue AFP Surveillance and laboratory diagnosis as per programme requirements. Functioning of National Polio Laboratory and Polio Intratypic Differentiation (ITD) Laboratory at NIV Bangalore are very essential as both are inter linked in the WHO accreditation process.

Studies on Poliovirus: Intratypic differentiation of poliovirus isolates from AFP cases received from National Polio Laboratory as a part of WHO-SEAR Polio Lab Network in the WHO's Global Eradication of Poliomyelitis Programme

P. N. Yergolkar and Hanumaiah

Introduction

Programme adapted New Algorithm in April 2007 for testing / identification of polioviruses by micro-neutralization test. CPE positive isolates were referred to the Regional Reference Laboratory (RRL) for identification and Intratypic Differentiation (ITD). Reporting time was reduced from 28 to 14 days for primary isolation and from 14 to 7 days for ITD reporting. It was decided to have >60 % of samples to be tested in the same laboratory for ITD in order to achieve the expected reporting days for AFP cases globally. National Polio Laboratory (NPL) at NIV Bangalore was upgraded to ITD Laboratory from May 2009 considering the capabilities and 11 years performance in the Network after fulfilling all ITD Laboratory Accreditation conditions.

ITD of polioviruses involves testing of poliovirus isolates received from the NPL, NIV, Bangalore by diagnostic PCR and ELISA. All isolates are referred to the Global Specialized Laboratory at ERC, Mumbai for sequencing and Banking.

Objectives

- To identify poliovirus serotypes (P1, P2 and P3), mixtures of polioviruses and carryout intratypic differentiation as wild (Non Sabin Like) or vaccine (Sabin Like) polioviruses from isolates of AFP cases received from the NPL.
- To participate in WHO-SEAR Polio Lab Network as Polio ITD Laboratory as per conditions and guidelines of WHO's Global Polio Eradication Programme and to coordinate with State, National and WHO Programme Managers.

Work done

Polio Intratypic Differentiation (ITD) Testing Laboratory in the WHO-SEAR Polio Laboratory Network at NIV, Bangalore was fully functional from May 2009 onwards after completion of WHO poliovirus PCR Proficiency test panels in April 2009 with 100% results. On-site review visit of ITD Laboratory by WHO was completed in December 2009 and Laboratory was fully accredited by the WHO for the years 2009 and 2010. WHO Proficiency test panels of 2009 for poliovirus diagnostic PCR and ELISA was passed with 100% score in the months of August and December 2009 respectively.

During the period a total of 1135 virus isolates from 396 AFP cases and 40 isolates from 23 Contacts were received. Of these as per programme requirement 649 isolates from AFP

cases and 23 isolates from contacts were tested initially by diagnostic PCR assay and of these 535 poliovirus vaccine isolates from AFP cases and 20 from Contacts were tested by poliovirus ELISA. Isolates with discordant PCR and ELISA results are referred to the Poliovirus Global Specialized Laboratory (GSL) at ERC, Mumbai for sequencing results to confirm Vaccine Derived Polioviruses (VDPVs) which are programmatically considered as wild polioviruses. Final combined results of PCR, ELISA and sequencing for AFP cases are, P1 wild virus-8, P3 wild virus-92, P3 wild virus and P1 vaccine virus mixture-3, P2 VDPV-11, P1 vaccine virus-177, P2 vaccine virus-153, P3 vaccine virus- 132, poliovirus vaccine mixtures-59, NPEV only-13 and Non Enterovirus (NEV)-1. Final results for Contacts are P3 wild virus-3, P1 vaccine virus-6, P2 vaccine virus-8, P3 vaccine virus-4 and poliovirus vaccine mixtures-2.

Future plan

To carry out full fledged ITD testing and reporting of poliovirus isolates as per Programme conditions. To establish Real Time RT-PCR assay for ITD of polioviruses and report as per rRT-PCR algorithm of the Programme. Equipment supply and training have been completed in the month of March 2010.

Studies on Measles: Surveillance of Measles cases from Karnataka State and Kerala State, as a part of WHO-SEAR Measles Laboratory Network in the WHO's Global Measles Elimination Programme

P. N. Yergolkar and Hanumaiah

Introduction

Surveillance of Measles outbreaks was initiated by the Govt. of Karnataka in collaboration with Government of India and WHO/WHO-SEARO/WHO-NPSP since June 2006 with NIV Bangalore as Measles/Rubella diagnosis and virus isolation Laboratory in the WHO-SEAR Network. Samples from Kerala State were received from November 2007 onwards.

Objectives

- To diagnose Measles and Rubella cases by detection of IgM antibodies by ELISA.
- To isolate and identify Measles and Rubella virus and refer isolates for Genotyping to RRL at NIV, Pune.
- To participate as National Measles Laboratory in WHO-SEAR Measles Laboratory Network as per conditions and guidelines of WHO's Global Measles Elimination Programme and to coordinate with State, National and WHO Programme Managers.

Work done

Laboratory diagnosis of Measles and Rubella cases by IgM ELISA: During the period a total of 174 case serum specimens from 35 outbreaks were received from 12 districts of Karnataka and

10 districts of Kerala. Of these 108 (62.1%) were positive for Measles and 39 (22.4%) positive for Rubella and 27 (15.5%) were negative. Of the 35 outbreaks investigated 25 (71.4%) were Measles, 7 (20.0%) were Rubella, 2 (5.7%) were both Measles and Rubella mixed and 1 (2.9%) were negative for both. District wise serological results from Karnataka and Kerala States are presented in Table 4.

Measles and Rubella virus isolation: During the period 18 urine samples were processed for virus isolation in Vero/SLAM cell line. One Measles virus was isolated from Kasargod district in Kerala in March 2010. Measles virus was confirmed by Chemicon IFA kit and isolate has been referred to the Regional Reference Laboratory at NIV, Pune. This is the first Measles virus isolation from Kerala state at NIV Bangalore Unit.

Measles laboratory was fully accredited by WHO after onsite review visit by the WHO's expert in May, 2007 for further period. WHO Measles and Rubella proficiency test panel was passed with 100% score in September, 2009. All laboratory accreditation conditions are fulfilled.

Future plan

Surveillance work to be continued as per Global Programme for Elimination of Measles. To establish diagnostic PCR facility for identification of Measles and Rubella viruses as per Programme requirement.

Table 4: District wise diagnosis of Measles and Rubella cases (IgM positive) from Karnataka and Kerala States

District	No. Tested	Measles Positive	Rubella Positive	Negative
Bellary	10	8	1	1
Bijapur	11	11	0	0
Chitradurga	5	5	0	0
Dakshina Kannada	5	0	4	1
Dharwad	5	2	1	2
Haveri	4	1	0	3
Gadag	6	6	0	0
Kodagu	5	0	0	5
Kolar	5	3	0	2
Koppal	15	10	1	4
Hassan	5	0	1	4
Udupi	10	0	10	0
Total, Karnataka	86	46	18	22
Idukki	5	5	0	0
Kannur	14	13	0	1
Kasaragodu	15	12	3	0
Kollam	5	0	5	0
Kozhikode	5	5	0	0
Malappuram	10	7	0	3
Palakkad	9	8	0	1
Thrissur	5	0	5	0
Trivandrum	10	7	3	0
Wayanad	10	5	5	0
Total, Kerala	88	62	21	5
Grand Total	174	108	39	27

Gorakhpur Unit

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Gorakhpur Unit

Projects

- Investigations of Outbreak of Acute Encephalitis Syndrome at Gorakhpur, 2009
- Post-licensure efficacy of a single dose of live attenuated SA 14-14-2 vaccine against Japanese encephalitis in India
- Studies on the rist factors for JE/ AES in Gorakhpur district (UP) using GIS based approach

Establishment of NIV Unit at Gorakhpur UP

The NIV Unit at Gorakhpur has already been set up as a functional laboratory. Electrical & civil work, rewiring of the unit, commissioning of generator sets has been completed. Construction of compound wall and water tank has also been completed. Flooring and laboratory furniture has been installed. Project and contract staff has been appointed. The Unit has fully functional ELISA laboratory and laboratory diagnosis of all samples collected by Gorakhpur unit are being processed at GKP laboratory.

Investigation of Outbreak of Acute Encephalitis Syndrome at Gorakhpur, 2009

M. M. Gore, K. P. Kushwaha, A. K. Thacker, D. K. Srivastava, S. D. Chitambar, V. P. Bondre, V. Gopalkrihna, G. N. Sapkal, V. K. Sirvastava, A. K. Pandey

A total of 2663 cases of acute encephalitis syndrome (AES) were admitted in the pediatric and Medicine wards of BRD Medical College. Majority of the cases were reported during July-October (Figure 1).

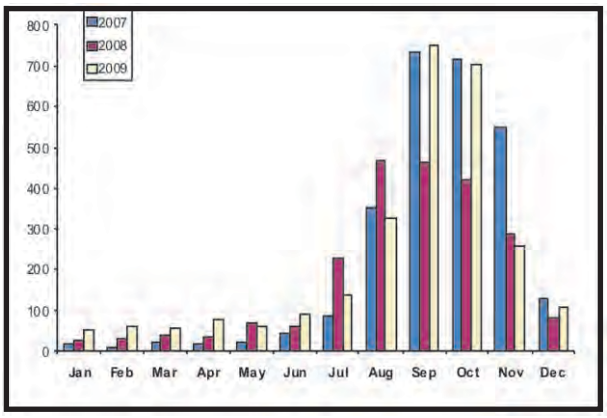

Figure 1: Monthly distribution of AES cases during 2007 -09

Majority of the cases (73.07%) were from Gorakhpur, Kushinagar, Mahrajganj and Deoria districts of eastern UP. There were 1940 AES cases (15.12 cases/100,000 population) for the total population of 12.83 million (2001 census). Distribution of cases has been plotted as high (>mean+1SD), average (mean +/-1SD) or low incidence (< mean -1SD). All these cases were adjacent to major water bodies or stagnant water bodies.

BRD Medical College staff collected CSF samples, while blood and rectal swabs were collected by NIV staff. A total of 6585 samples consisting of 1704 CSF and 2494 acute serum samples & 2387 rectal swabs were collected. Detailed case history was recorded in computerized format for more than 2000 cases. The cases are being analyzed as per the etiology.

A total of 375 cases had detectable levels of IgM antibodies against JE virus. In addition, CSF samples were tested for presence of JE genome and 75 samples were identified to be JE genome positive. Highlight of the study was detection of Genotype I JE virus in the CSF samples. This has been found for the first time in India, in addition to currently prevalent Genotype 3 strain.

Enterovirus diagnosis was done by PCR. CSF of 13 out of 693 CSF tested were positive for enterovirus. Majority of the samples showed Coxsackie A 11 and A 22. In addition, out of 2117 rectal swab samples tested 597 (28.2%) showed presence of enterovirus genome. This indicated extensive enterovirus mediated encephalitis cases in eastern UP. In addition, 2038 serum samples were tested for presence of IgM antibodies against Hepatitis A virus. 214 (10.5%) sera showed presence of anti HAV IgM further supporting the enterically transmitted etiology of AES in this region.

Clinical and epidemiological analysis of AES cases in 2009.

Based on the diagnosis obtained AES cases were grouped into two categories, Confirmed JE and Probable Enterovirus encephalitis. Out of 2663 AES cases clinical details were available for 2038 cases. JE diagnosis was obtained in 438 cases however, 103 of these cases showed presence of enterovirus in their gut, as evidenced by EV positivity of rectal swabs. Thus, clinical analysis has been done for only 332 cases. 494 cases have been classified into “Probable EV”. Further, these cases were classified as age <15 and >15 based on age of vaccination for JE (Figure 3).

Age wise classification of cases revealed that EV cases were predominantly in the age group of 1-10 whereas JE cases were in >15 yr of age.

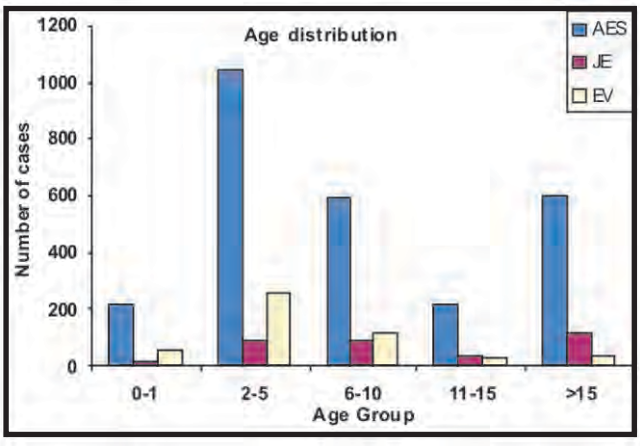

Figure 3: Age wise classification of AES, JE and EV cases

A differential clinical diagnosis of AES cases was carried out by the clinicians. A few cases were diagnosed with bacterial or tubercular meningitis and treated accordingly (Table 1). In addition, associated syndromes like myocarditis and protein malnutrition were also recorded. Table gives the details of the cases according to the clinical diagnosis in different age groups. As such the difference in each of the group was not significant indicating the overlap of associated symptoms and the infections.

Table 1: Clinical diagnosis and associated major syndromes with cases

	AES		JE only		EV	
	<15	>15	<15	>15	<15	>15
Total	2064	599	218	114	458	36
Bacterial meningitis	64	1	4	0	16	0
Tubercular Meningitis	29	14	4	1	8	0
Myocarditis	220	1	27	1	50	0
Protein malnutrition	76	2	8	0	15	0

Although the peak number of cases were observed both for JE and EV in the months of July to October, EV cases occurred throughout the year (Figure 4). This was evident from the monthwise distribution of positive cases in each group.

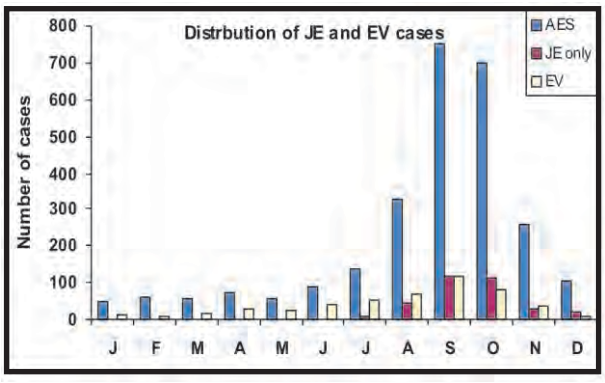

Figure 4: The month wise distribution of JE and EV cases

Mortality in AES

Overall mortality in the admitted cases was 19.4% excluding cases that were discharged against the medical advice. Further analysis showed that 60% of the deaths occurred within the first three days of admission indicating that all the intervention and critical care needs to be concentrated on the initial period after the onset of symptoms (Table 2).

Table 2: Mortality rates in AES, JE and EV cases

	AES		JE		EV	
	<15	>15	<15	>15	<15	>15
Days after admission						
0	0	25	5	2	1	5
1	83	7	7	1	19	0
2	110	16	10	2	25	3
3	58	27	6	7	9	1
4	30	13	9	3	8	0
5	23	7	3	0	8	0
6	14	7	4	2	1	0
7	11	3	2	0	3	0
8-15	40	16	3	0	14	0
>15	17	5	0	2	8	0
Total deaths	411	106	46	18	100	4
Total Cases	2064	599	218	114	458	36
% Mortality	19.9	17.7	21.1	15.8	21.8	11.1

Clinical symptoms in AES JE and EV cases

Clinical histories were available in >2000 cases. Analysis of associated symptoms at the time of onset of disease indicated that in both JE and EV encephalitis cases fever was always associated. Most cases showed high grade fever at the time of onset of symptoms. Duration of fever varied. It is supposed that as the encephalitis is a seves condition most cases reached hospital within two days of onset of neurological symptoms. However, duration of sample collection and the onset of fever indicated that only about 25% of the cases got admitted within 3 days of onset of fever (Table 3). The low positivity of CSF for enterovirus might be related to this phenomenon. Associated symptoms included vomiting, abnormal behavior, loose motions. frothing form mouth and difficulty in breathing were also noted in many cases. Most cases did not have rashes. In Associated symptoms, headache in adult cases was more prominent as compared to children. Also loose motions before the onset of disease was seen at a higher rate in EV encephalitis than in JE.

Table 3: Clinical symptoms in AES JE and EV cases

		AES		JE		EV	
		<15	>15	<15	>15	<15	>15
Duration of fever to Sample collection (Days)	0-3	421	111	47	23	117	6
	4-7	768	240	93	52	166	15
	8-15	661	157	64	32	133	10
	>15	125	57	12	3	25	4
Fever	High Grade	995	265	121	62	207	12
	Mild	317	80	34	15	60	3
	Moderate	210	57	23	13	51	4
	Total	1522	402	178	90	318	19
Associated Symptoms	Vomiting	930	340	101	56	199	24
	Headache	240	378	36	76	34	24
	Loose motions	207	23	8	5	54	1
	Cough	144	23	11	3	36	2
	Swelling	146	7	14	0	25	0
	Abnormal behavior	19	3	0	1	6	0
	Difficulty in breathing	63	2	4	0	16	0
	Frothing from mouth	233	14	25	2	50	4
	Rash	30	2	0	0	6	0
	Total	1221	512	154	97	328	34

Values are number of cases in each category cases have been classified as15 and >15 yrs of age.

Neurological symptoms associated with JE and EV cases

Various neurological and other clinical symptoms were noted at the time of admission in majority of the cases. Majority of the cases showed altered sensorium. A substantial number ~50% of the cases in both JE and EV encephalitis were unconscious at the time of admission (Table 4). Large number of cases (>60%) also had spasms and these were mainly of tonic clonic type. Neck rigidity and Kernig's signs were seen in about 35% of cases. Deep tendon reflexes were absent in majority of the cases. Similarly, majority of the cases also showed bilateral plantar extensor reflexes. In few cases plantar reflexes were mute or unilateral. Decreased tone was recorded in higher number of cases in EV encephalitis than in JE however, unlike

earlier years the difference was not significant. Pallor and edema was seen in minority of the cases. Hepatomegaly turned out to be significantly associated with EV encephalitis than in JE. In few cases splenomegaly was also seen along with hepatomegaly.

Table 4: Neurological and other clinical symptoms associated with AES, JE and EV

		AES		JE		EV	
		<15	>15	<15	>15	<15	>15
Mental status	Unconscious	926	62	106	11	244	6
	Altered behavior	40	17	2	4	9	
	Altered sensorium	718	430	94	87	117	26
	Total histories	1497	171	178	39	322	11
Spasms	Tonic	218	10	8	1	74	3
	Tonic clonic	1330	163	173	38	266	8
Seizures		18	25	1	1	9	1
Convulsions		70	4	5	0	25	0
Uprolling of eyes		1654	180	192	37	370	8
Neck Rigidity		297	287	39	54	56	20
Kernig's signs		16	226	26	45	31	13
DTR	Pos	1833	233	201	39	23	15
	Neg	77	197	4	43	409	13
Plantar	BL E	1564	257	182	42	338	16
	BL F	342	225	29	48	83	15
	Mute	24	34	3	6	11	3
	Uni E	35	13	1	3	12	2
SAR	Pos	538	28	54	5	124	3
	Ab	1189	159	133	34	263	5
Tone	Inc	586	39	65	8	150	0
	Nor	1126	163	115	28	235	6
	Dec	153	2	12	2	40	3
Pallor		25	82	1	17	6	4
Edema		46	11	3	1	16	2
Hepatomegaly		994	15	95	3	215	0
splenomegaly		466	13	49	2	88	0
Hepato spleeno-megaly		448	11	49	2	84	0

Laboratory investigations in AES, JE and EV cases BL-Biateral UM-Unilateral

Lab investigations were carried out in order to understand various liver and renal functions along with the routine examinations (Table 5). Mean Hemoglobin values were within the normal range in all patients. There was slight increase in the mean total leukocyte count. The differential count also did indicate any major abnormality in most cases. Mean SGOT and SGPT values were raised, while mean blood urea and creatinin levels were within normal range. Number of cases showing higher than normal values for SGOT, SGPT, Urea and creatinin were higher in EV encephalitis than in case of JE encephalitis indicating multiorgan involvement in EV encephalitis. Serum anti Hepatitis A virus IgM antibodies was determined in 2038 samples of AES cases. Percent positivity to HAV IgM was very high in EV encephalitis cases as compared to JE encephalitis cases. This was true even for adult cases.

Table 5: Laboratory investigations in AES, JE and EV cases

	AES		JE		EV	
Mean values	<15	>15	<15	>15	<15	>15
HB (gm/dL)	10.8	10.7	11.0	10.9	10.8	11.3
TLC (No./mm3)	12.5	10.5	13.1	10.3	13.0	10.5
DLC-Poly (%)	61.1	72.1	64.9	70.5	61.5	75.2
DLC-Lympho (%)	36.4	24.1	32.9	24.2	36.1	23.0
Random blood sugar(mg/dL)	114.0	112.7	125.7	107.4	124.6	124.8
SG OT (unit)	73.9	78.7	56.5	62.5	67.4	50.9
SG PT (unit)	59.4	72.7	43.1	91.5	48.4	68.5
Urea (mg/dL)	37.1	65.8	33.1	92.8	39.4	35.7
Creatinin (mg/dL)	0.9	1.4	0.7	1.5	0.8	1.1
No of cases	1497*	171*	178*	39*	322*	11*
CSF Pro >40 (mg/dL)	181	163	16	31	45	16
CSF pro <40 (mg/dL)	215	84	18	11	55	7
SGOT>40 (unit)	384	23	25	4	106	3
SGPT>35 (unit)	364	74	22	9	88	6
Urea >45 (mg/dL)	129	20	8	4	37	1
Creatinin >1.8 (mg/dL)	23	42	0	7	7	0
% HAV IgM +ve	2.9	12.9	9.1	1.0	13.5	12.5

The data is definitely indicative of massive water borne infections probably by enterovirus leading to encephalitis. *Indicate total no. Of cases in each grop

Post-licensure efficacy of a single dose of live attenuated SA 14-14-2 vaccine against Japanese encephalitis in India

M. M. Gore, B. V. Tandale, S. A. Khan, V. Tsu, A. Khalakdina, S. Khare

There have been no studies of SA 14-14-2 vaccine in Indian populations. The National Technical Advisory Group on Immunization (NTAGI) recommended that ICMR undertakes a case-control study to understand the effectiveness of the SA 14-14-2 vaccine in field conditions in India.

A community-based matched case-control study was conducted in Uttar Pradesh (UP) and Assam. One to four (cases to controls) matched design was used with pair-wise matching of age, sex and village of residence during the risk period, with a target sample size of 201 cases and 804 controls. Vaccination history for JE and other childhood vaccines was elicited from the child's guardian orally, vaccination cards were examined when available, and health center records were also searched for confirmation of vaccine history.

A total of 202 cases and 796 controls met the initial criteria for analysis. The analysis was done on four groups: total sample (n=998: 202 cases & 796 controls), reconfirmed as per-protocol (n=438: 88 cases & 350 controls), PCR+ (n=145: 29 cases & 116 controls), and sample with all cases except PCR+ (n=853: 173 cases & 680 controls) (Table 6). Field efficacy of the vaccine in the four analysis groups varied widely. The overall protective effect was 63.6% (95% CI: 46.8, 75.1), while in the reconfirmed per-protocol sample it was 80.6% (95% CI: 63.3, 89.8). In the PCR+ subgroup, the protective effect was only 11.7% (95% CI: -127.3, 65.7), and in the larger sample with all cases except PCR+ it was 69.0% (95% CI: 53.0, 79.6). Analysis of the antibody levels among controls revealed little difference 1-3 years after the campaigns between those who were vaccinated and those who were not. The report is being prepared and sent to ICMR for approval.

Table 6: JE Vaccine Field Efficacy

	Odds Ratio (OR)	Protective effect 1-0R (%)	95% confidence Interval	
Unadjusted-Total Sample				
Assam (n=250)				
Written confirmation	0.27	73.2%	37.7%	88.5%
Any report on JE Vaccination	0.25	75.2%	47.5%	88.3%
Uttar Pradesh (n=748)				
Written confirmation	1.13	-12.9%	-103.3%	37.3%
Any report on JE Vaccination	0.42	58.1%	34.9%	73.0%
Total (n=998)				
Written confirmation	0.66	34.2%	-6.5%	59.2%
Any report on JE Vaccination	0.36	63.6%	46.8%	75.1%
Unadjusted-Per Protocol				
Assam (n=220)				
Written confirmation	0.32	68.2%	25.1%	86.5%
Any report on JE Vaccination	0.20	79.5%	54.1%	90.9%
Uttar Pradesh (n=218)				
Written confirmation	0.56	43.9%	-88.0%	83.3%
Any report on JE Vaccination	0.18	82.3%	49.5%	93.8%
Total (n=438)				
Written confirmation	0.38	62.0%	23.8%	81.0%
Any report on JE Vaccination	0.19	80.6%	63.3%	89.8%
PCR + (n=145)				
Written confirmation	1.78	-77.6%	-453.4%	43.0%
Any report on JE Vaccination	0.88	11.7%	-127.3%	65.7%
All but PCR+ (n=853)				
Written confirmation	0.54	46.0%	8.0%	68.3%
Any report on JE Vaccination	0.31	69.0%	53.0%	79.6%

Analysis of drinking water from different locations and sources for virological assessment

In order to carry out longitudinal studies on the environmental water sampling, AES prevalence data in the Gorakhpur district was analyzed. It was observed that Khorabar and Chargawan blocks are consistently reporting a very large number of cases within Gorakhpur region. Six villages from these blocks have been chosen for the longitudinal water sampling. Water samples are being collected concentrated and stored for virological analysis.

Studies on the risk factors for JE/AES in Gorakhpur district (UP) using GIS based approach

Gokhale M. D., Gore M. M., Jamgaonkar A. V.

In-depth database of JE/AES cases was generated in and around Gorakhpur. Both the rural as well as urban areas of the district have reported cases. The data is generated with reference to socio- economic and hygienic status of the patient along with the geographic location. The present and the previous year information are being analyzed using the computational software program. This would lead to better understanding towards correlating factors responsible for JE/AES cases in Gorakhpur.

Objective:

- To understand the risk factors leading to JE/AES cases in Gorakhpur district
- To analyze the distribution of the disease
- Develop a computational program to understand disease association with ecological factors

Work done

Geographic Information System (GIS) studies were conducted in three PHC blocks viz; Jungle Kauria, Piprauli and Derwa.A total of 364 GPS spots were mapped and information was gathered with reference to ecological parameters. A software development platform is generated with the help of the GS Lab. using map Locator platform. This program enables features such as Google earth view, exploration of the map layers, visualization of AES cases on the Gorakhpur map, the customized legend feature support, time line filter layers with reference to Day/Month/Quarter/Year etc. The case data can be correlated with ecological features such as precipitation, temperature etc. Distribution of cases is displayed in color grid format for better visualization. Correlation between cases and different parameters are compared using statistical tools.

Some of the achievements of the project were plotting the cases on the map and making the grid in order to carry out analysis of spatial and timescale. A snap shot of location map is given below.

Summary

At present the software platform is developed as NIV GIS Portal. The different aspects of JE/AES data are depicted on the map in timeline visualization, color grid format, correlation with different parameters using statistical tools (Figure 6).

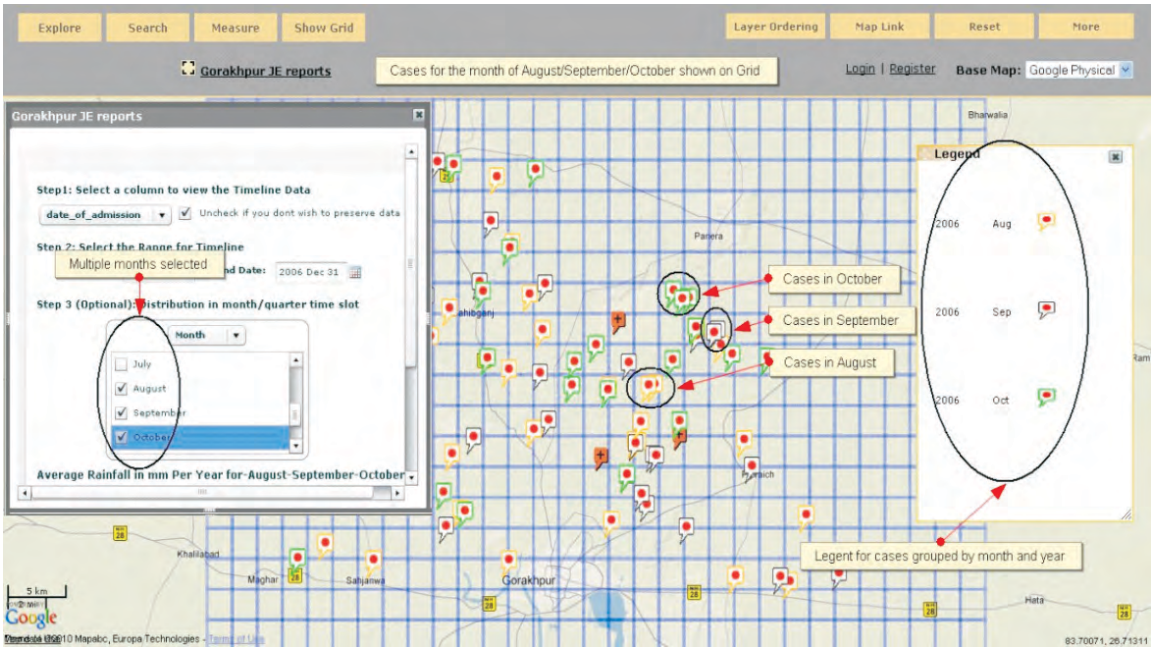

Figure 6: Different aspects of JE/AES GIS data depicted on the map

Entomological studies

A facility to collect and identify mosquitoes at NIV Gorakhpur Unit has been created. In addition to collection of larvae, establishment of colonies of vector species at NIV Pune for various studies. Mosquito collection and insecticide resistance of vector species is being carried out in the specified areas of Gorakhpur District. A project on molecular marker for insecticide resistance has been approved by ICMR.

The seasonal prevalence of mosquitoes collected from Bhathat, Chargawan and Khorabar blocks of Gorakhpur district showed that *Culex quenquefasciatus* was maximum during winter. Occurrence of *Cx. tritaeniorhynchus* and *Anopheles peditaeniatus* observed in transition season i.e. spring season while occurrence of *Cx. tritaeniorhynchus* in month of January was nil due to very low temp. (6 to 9°C). *Mansonia uniformis* showed its presence in March (Table 7).

Table 7: Dusk collection of mosquitoes in Gorakhpur Area

Dusk collection of mosquitoes in Gorakhpur Area Jan-Mar 2010							
Species	Male	Female	Total	Total Pools	Total man-hours	PMHD	Month
An. annularis	2	48	50	10		1.19	Jan
An. subpictus	0	9	9	1		0.21	Jan
An. Peditaeniatus	0	6	6	2		0.14	Jan
Cx. bitaeniorhynchus	11	0	11	2		0.26	Jan
Cx. pseudovishnui	0	6	6	1		0.14	Jan
Cx. univitatus	18	2	20	2		0.48	Jan
Cx. quinquefasciatus	376	763	1139	40	42	27.12	Jan
An. annularis	2	15	17	4	32	0.53	Feb
An. Peditaeniatus	0	247	247	5		7.72	Feb
Cx. tritaeniorhynchus	0	214	214	6		6.69	Feb
Cx. quinquefasciatus	246	826	1072	38		33.50	Feb
An. annularis	0	6	6	2	20	0.30	Mar
An. Peditaeniatus	0	201	201	7		10.05	Mar
Cx. tritaeniorhynchus	0	176	176	5		8.80	Mar
Cx. univitatus	0	6	6	1		0.30	Mar
Cx. quinquefasciatus	28	742	770	25		38.50	Mar
Ma. uniformis	0	60	60	4		3.00	Mar

Major Achievements

- Establishment of NIV Unit at Gorakhpur
- Etiological analysis of more than 2600 cases for JE and enteroviruses conducted
- Isolation of Genotype I JE virus from UP.
- Vaccine efficiacy study condicted for JE attenauted vaccine.

Kerala Unit, Alappuzha

Scientific staff

Dr. George P. Jacob, Scientist D

Dr. R. Balasubramanian, Scintist B

Technical staff

Mr. D. K. Butte

Kerala Unit, Alappuzha

Projects

- Hospital based surveillance of Chikungunya, dengue and Japanese encephalitis viruses
- Outbreak investigation of viral diseases
- Studies on the mosquito fauna of Alappuzha district, Kerala State in relation to Japanese encephalitis virus (JEV)

Hospital based surveillance of Chikungunya, dengue and Japanese encephalitis viruses

George P Jacob, Sudeep AB, Shah PS and Balasubramanian R.

Objective

- NIV Kerala unit at Alappuzha was established to initiate research on viruses prevalent in Kerala as well as to provide diagnostic support to the state government to initiate control measures.

Work done

A total of 1758 Serum/CSF referred samples were screened for IgM antibodies against CHIKV, DENV and JEV by MAC-ELISA using NIV kits. The monthly incidence of CHIKV and DENV cases are given in Table 1. CHIKV cases were reported from many districts of Kerala during the year(fig.1). A total of 463 samples were found positive for CHIKV IgM antibody out of the 1028 samples tested. Of these positive samples, 92% were reported during June-August, 2009. Dengue cases were reported from a few districts and 37 samples tested positive of the 572 samples tested. The summary of the results is presented in Table1.

Table 1: Monthly Incidence of CHIKV/DENV/JEV Cases, Kerala state 09-10.

Month	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Total
CHIK	3/19	9/77	45/ 219	122/ 236	261/ 407	14/ 32	2/9	0/5	2/2	3/9	1/7	1/6	463/ 1028
DEN	1/30	5/88	17/ 192	2/58	2/68	4/58	2/25	2/14	1/10	0/9	0/10	1/10	37/ 572
JE	0/5	0/5	0/4	0/20	0/49	0/28	0/7	0/6	0/11	0/6	0/12	0/5	0/158

*No. of positives/Total No. of Samples.

Outbreak investigation of viral diseases

Sudeep A. B., George P. Jacob, Balasubramanian R. and Tandale B. V.

Introduction

Several disease outbreaks suspected to be of virus origin by clinical pictures were reported from different parts of Kerala and Tamil Nadu. NIV Kerala unit has investigated these outbreaks as part of their objective. Details of the investigations are given below.

(A) Outbreak in Kanjirapally Taluk, Kottayam, Kerala

NIV Kerala unit has investigated a dengue-like outbreak in Kanjirapally Taluk of Kottayam district during April-June 2009 and collected 210 blood samples from four hospitals and

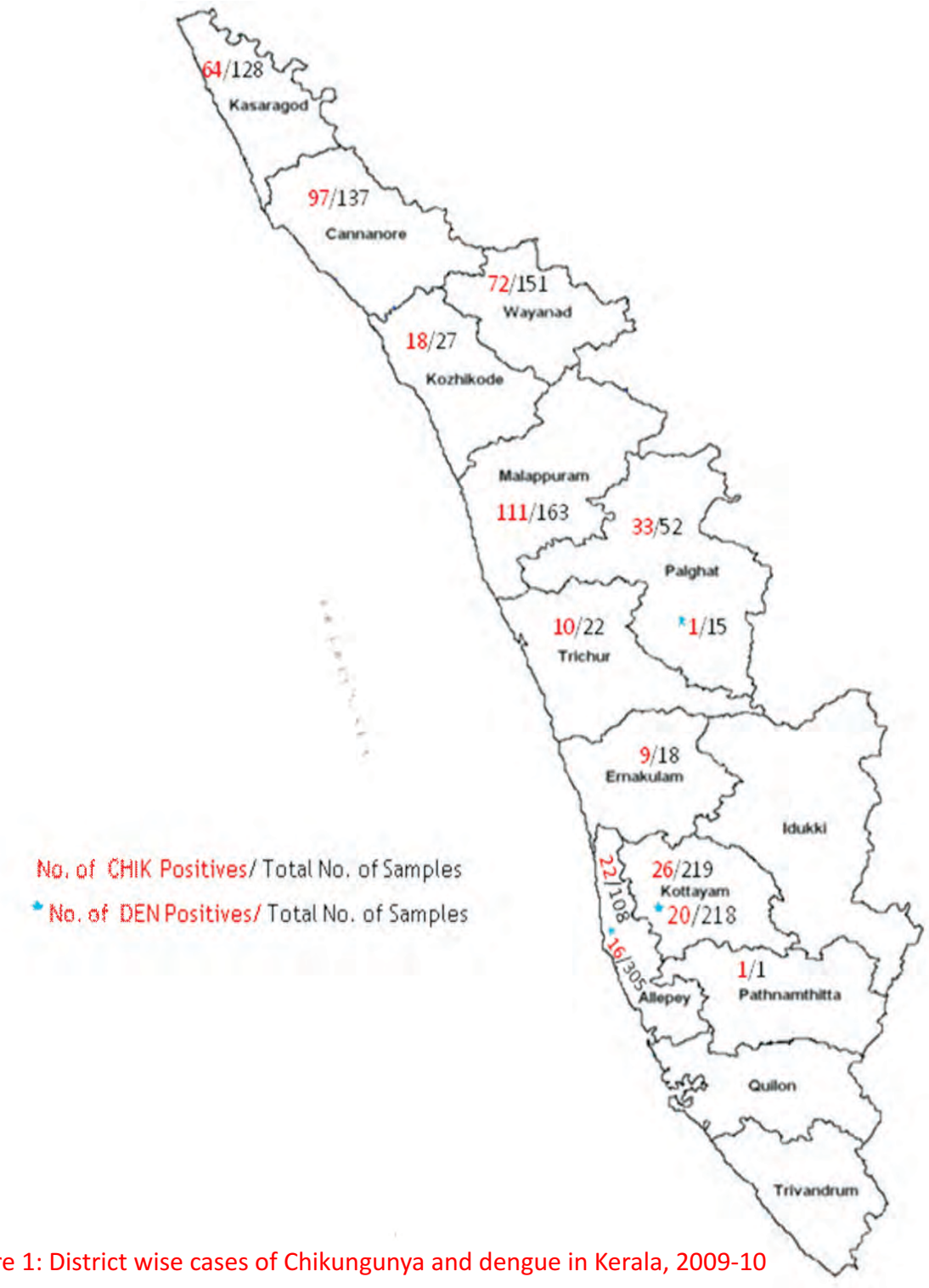

Figure 1: District wise cases of Chikungunya and dengue in Kerala, 2009-10

screened for CHIKV and DENV IgM antibodies. Twenty-three samples tested positive for IgM antibodies against CHIKV and 12 for DENV. Three samples tested positive for both the viruses. Attempts were made to isolate dengue virus yielded two isolates which are being typed to determine the serotypes responsible for the outbreak (Dengue group, NIV, Pune).

(B) Outbreak in Kozhikode district, Kerala

P. George Jacob along with NIV KLU team investigated an outbreak of suspected viral fever in Kozhikode district, Kerala state during 2-6 August 2009 along with the central team (Scientists from NIV, Pune and RMRI, Prot Blair) and submitted report to ICMR. The team attributed the epidemic to Chikungunya based on the results of clinical & epidemiological investigations, laboratory findings (38.5% CHIK IgM antibody positivity) along with the presence of vector mosquitoes viz. *Ae. albopictus* and *Ae. aegypti* in and around households. They observed notable increase in the number of confirmed CHIKV cases in comparison to earlier years. They also observed a high incidence of viral hepatitis, dengue and leptospirosis in the district.

Entomological investigation:

Entomological surveys demonstrated high Breatau Index (Bl >50) and house index (HI) for *Aedes* mosquitoes in 4 out of 9 surveyed areas (Table 2). *Aedes albopictus* was the predominant mosquito observed in rural areas while *Ae. aegypti* dominated in the urban areas.

Serological investigation: Among the 106 human sera tested for anti-CHIKV IgM antibodies, 38 (35.8%) were found positive for CHIKV IgM.

Table 2: A summary of field investigations carried out in Kozhikode district, Kerala State, during a viral fever outbreak, 2-6 August, 2009

Sr. No.	Locality	houses surveyed	persons investigated	No. With fever since June-July 09	No. With joint manifestations (CHIKV)	HI	CI	BI
1	Balussery	20	84	67	66	38	6	38
2	Atholi	15	56	44	33	33	3	33
3	Koilandy	16	74	57	43	12	1	12
4	Panangad	20	92	76	68	56	12	87
5	Kuttiady	20	84	63	45	45	6	65
6	Nadapuram	20	98	51	40	35	4	35
7	Ulliyeri	20	113	65	48	60	16	130
8	Narikunni	20	85	68	60	25	7	45
9	Kodenchery	20	86	21	18	40	10	55
10	Karaparambu	20	89	16	11	75	14	150
11	Vellayil	20	151	22	15	55	19	130

*HI= House Index, BI= Breatau Index, CI= container index

(A) Investigation in Kannur district, Kerala

Team from NIV KLU, collected 11 blood samples from patients with acute sickness from district Govt. hospital, Payyannur, in Kannur district. Seven samples were found positive for CHIK IgM antibodies by MAC ELISA. Entomological investigations showed the presence of *Ae. albopictus* mosquitoes in the affected areas despite source reduction activities. Attempts to isolate virus from mosquito samples were not successful.

(B) Outbreak of mysterious fever in Tirunelveli district, Tamil Nadu

Chikungunya-like outbreak in Thirunelveli district of Tamil Nadu was investigated and blood and mosquito samples were collected to determine the etiological agent. A total of 250 blood samples and four mosquito pools comprising *Aedes aegypti*, *Culex quinquefasciatus* and *Armigeres subalbates* were collected from two badly affected areas of the district viz. Melappalayam and Kadainallur and brought to the laboratory for further processing. IgM antibody assay for CHIKV and DENV using McELISA demonstrated 107 and 22 samples positive respectively (Table 3). Eight samples tested positive for both among the positive samples. None of the 46 samples screened tested positive for JEV IgM antibody. Attempts to isolate the virus from a few acute serum samples yielded one CHIKV isolate. The isolate is being characterized at the molecular level. The mosquito pools did not yield virus isolation.

A few selected samples were also screened for Ross River, Murray Valley encephalitis and Sindbis viruses by Reverse Transcriptase polymerase chain reaction (RT-PCR) at NIV, Pune. IgM antibody test against hanta virus was carried out for 36 samples. All the samples tested negative for the above viruses (Table 3).

Table 3: Details of tests carried out on samples from Tirunelveli district, Tamil Nadu

Sr No.	Test done	Male	Female	Total
1	CHIKV IgM ELISA	*71/140	36/77	107/217
2	DENV IgM ELISA	17/140	5/77	22/217
3	JEV IgM ELISA	0/31	0/15	0/46
4	Ross River virus (RT-PCR)	0/2	0/7	0/9
5	Murray Valley encephalitis virus (RT-PCR)	0/2	0/7	0/9
6	Sindbis virus (RT-PCR)	0/2	0/7	0/9
7	Hantaan virus IgM ELISA	0/22	0/14	0/36
	Virus isolation (in Vero E6 cells)	1/22	0/14	1/36
* No. positive/No. Tested Note: Eight samples tested positive for both CHIKV and DENV				

Studies on the mosquito fauna of Alappuzha district, Kerala State in relation to Japanese encephalitis virus (JEV)

George P. Jacob and R. Balasubramanian

Introduction

This study has been initiated as encephalitis cases (158 cases) were reported from Alappuzha district. The samples tested negative for IgM antibodies against JEV, DENV and CHIKV.

Objective

To study the JEV mosquito fauna and determine the role of these mosquitoes in the transmission of the disease by isolating the etiological agent.

Work done

The monitoring of mosquito population to assess the seasonal prevalence and species composition has been commenced since July 2009 and continued throughout the year, initially in one locality, i.e. Kainakary in Kuttanad Taluk, the rice bowl of Kerala. Subsequently one more locality, i.e. Thakazhy in Alappuzha district was added from March 2010 onwards.

A total of 10,849 mosquitoes comprising 21 species were collected in 28 man hours from the study area. Monthly incidence of mosquitoes collected at the two localities is presented in Table 4. Among all the mosquito species, *Culex tritaeniorhynchus* was the predominant species recorded in the study and accounted for 67% of the total collection. The species mainly breeds in paddy fields and had two peaks; one in August and the other in February-March. Another JEV vector, *Cx. gelidus* was also prevalent in the same localities in high numbers and accounted for 17% of the total catch with a peak in December. The monthly per man hour density of the two species is presented in Fig 2. The other 19 mosquito species collected during the study, represented only in small numbers.

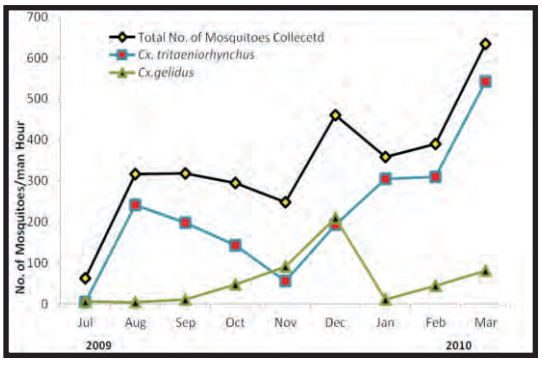

Figure 2: Per man hour density of *Cx.tritaeniorhynchus* and *Cx. gelidus* mosquitoes in Alappuzha

Table 4: Monthly incidence of mosquitoes (dusk collection), 2009-10 in Alappuzha

Sl.No	Species	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total
1	*An.barbirostris*	1	1	0	0	0	0	0	0	0	2
2	*An.peditaeniatus*	0	0	0	115	49	0	11	11	2	195
3	*An.nigerrimus*	0	0	0	0	0	0	0	0	4	4
4	*An.pallidus*	0	0	0	0	0	0	0	0	11	11
5	*An.subpictus*	0	0	1	0	0	0	1	0	6	8
6	*An.vagus*	2	0	0	0	0	0	2	0	0	4
7	*Ae.vexans*	0	0	0	0	0	2	2	0	12	16
8	*Ar.subalbatus*	0	60	30	75	52	36	58	0	9	320
9	*Cx.bitaeniorhynchus*	0	0	8	14	0	0	0	0	0	22
10	*Cx.fuscanus*	0	0	0	0	0	0	0	1	0	1
11	*Cx.fuscocephala*	0	0	0	0	0	1	0	0	0	1
12	*Cx.gelidus*	12	10	33	190	182	837	22	88	488	1862
13	*Cx.infula*	0	0	9	4	0	0	0	0	1	14
14	*Cx.pseudovishnui*	0	0	0	0	0	0	9	0	0	9
15	*Cx.quinquefasciatus*	1	5	2	0	0	0	0	0	0	8
16	*Cx.sitiens*	0	0	0	0	0	0	0	0	2	2
17	*Cx.tritaeniorhynchus*	7	725	596	573	112	770	610	620	3253	7266
18	*Cx.vishnui*	1	5	80	133	106	52	2	46	9	434
19	*Mn.annulifera*	13	1	9	2	0	4	0	0	0	29
20	*Mn.indiana*	9	35	48	134	44	127	0	11	9	417
21	*Mn.uniformis*	79	107	23	6	0	0	0	0	9	224
	Total	125	950	953	1180	496	1840	717	779	3809	10849
	No. of Man Hour	2	3	3	4	2	4	2	2	6	28

Arbovirus Diagnostic Group

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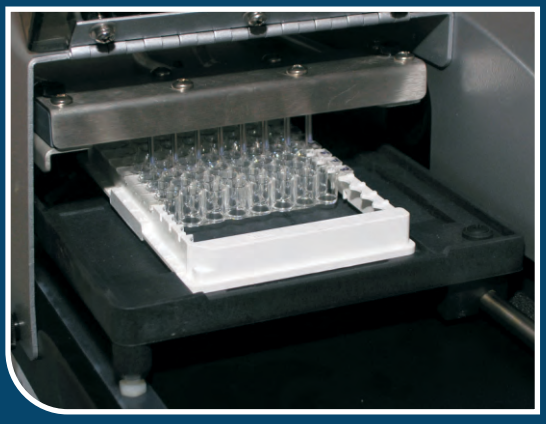

National Institute of Virology,
Annual Report 2009-2010

Preparation of JE, Dengue and Chikungunya MAC ELISA kits on large scale and supply to Sentinel Surveillance Hospitals (SSHs) through National Programme

MAC ELISA kits were supplied to 137 Sentinel Surveillance Hospitals and 15 APEX laboratories in the country for laboratory diagnosis of JE, Dengue and Chikungunya. Month-wise details of kits supplied are given below;

Month-wise supply of MAC ELISA Kits

Development of Monoclonal antibodies against Chikungunya

Month	JE	DENGUE	CHIK	TOTAL
April, 2009	-	62	45	107
May	9	67	53	129
June	20	130	85	235
July	8	159	63	230
August	15	94	55	164
September	14	174	46	234
October	19	143	45	207
November	9	63	8	80
December	9	57	29	95
January, 2010	-	24	27	51
February, 2010	-	20	10	34
March, 2010	-	64	34	98
Total	103	1057	500	1660

Monoclonal antibodies were developed against the Asian strain of Chikungunya virus. The fusion resulted into three hybrids (VF 4, IV B2 and IV G6). The antibodies secreted by hybrid VF 4 were against “C” protein of CHIK virus and were of IgG type and the other two hybrids

secreted antibodies of IgM type. The three hybrids were re-cloned by limiting dilution method to get 2/23 clones.

The clones were inoculated by intra-peritoneal route in mice and also maintained *in vitro* to get ascetic fluid / culture supernatant containing anti-CHIK antibodies respectively. The antibodies have been biotinylated and are being screened for their utility in the existing IgM Capture ELISA. The monoclonal antibodies did not show any cross reaction with Sindbis virus in Immunofluorescence test.

To Improvise the exiting MAC ELISA Kit

Dengue, JE and Chikungunya Mouse Brain antigens in the MAC ELISA kits have now been replaced with cell culture antigen. The protocol for inactivation has been standardized. The stability of cell culture antigen is underway.

Internal Quality Assurance Audit

Internal Quality Assurance Audit was done for Measles laboratory and Bangalore Field Station for the measles IgM antibody ELISA.

Electron Microscopy & Histopathology Group

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Electron Microscopy & Histopathology Group

Projects

- Core facility research applications
- Studies on the effect of dengue viruses on vascular endothelial cells
- Studies on characterization of cellular lesions in fatal cases of pH1N1, 2009
- Electron tomography studies on dengue virus replication

Core facility research applications

Virus morphodiagnosis referral

A total of 246 specimens in form of tissue culture fluid and other clinical specimen like stool, respiratory secretions, cerebrospinal fluid (CSF) aspirates were referred to the EM core for negative staining examination for viruses. Other specimens referred included cell lines for quality control checks for endogenous viruses and suspected mycoplasma contamination.

Ultrathin sectioning and TEM examination

Experimental tissue specimens from studies on pH1N1 infection in mice were referred for ultratructural studies. Necropsy specimens of lung collected from a total of 35 fatal pH1N1 cases were referred to the EM lab from various hospitals for ultrastructural examination. Majority of these specimens have been analyzed and reported and studies are continuing.

Electron microscopy support to non-ICMR facilities

High resolution TEM support was provided to non-ICR research institutes like NCL, NCCS, Agricultural Universities , ACTREC, ICGEB with appropriate approval.

External Quality Assurance Program (EQA)

As a part of routine quality assurance activity for virus morphodiagnosis, conducted by the Robert Koch the 23rd EQA program was successfully completed with perfect scores.

Upgrading SOP and development of software

The operational standard operating procedures were reviewed and updated as per recent development in the field and active participation in developing the VIRUSLIMS software as applicable to the group was undertaken.

Core pathology

A new program on establishing a core pathology program was undertaken with laboratory setup and initiating support services to programs in Chandipura virus research, experimental enterovirus infection in mice and studies on fatal H1N1cases.

Studies on the effect of dengue viruses on vascular endothelial cells

Background

Endothelial cell infection by dengue viruses occur both *in-vivo* and *in-vitro* (Basu 2009). Although a significant body of research findings strongly implicates altered endothelial functions as a major factor in the etiology of DHF and DSS, the complexity and heterogeneity of endothelial cell physiology-specially cultured primary cells; has been a limitation in feasible interpretation of the data. In the present study we used SK Hep1, a human endothelial cell line of hepatic sinusoidal origin (previously reported as a hepatoma cell) and examined its

susceptibility and physiological responses to infection by all serotypes of DENV *in-vitro*, with an aim to explore the potential of this cell line as a model to investigate DENV-endothelial cell interactions.

SK Hep1 cells were grown in appropriate media, optimized for ideal growth conditions and their susceptibility to the different serotypes of DENV studies in-vitro. The cells were also examined by both Transmission, Scanning and Atomic Force Microscopy to profile the nature of ultrastrucral changes associated with DENV infection and changes in surface topography. Further, viral NS1 protein (purified) was added to the cells and its effect on adhesion and transwell wound healing studied in depth. The E protein of DENV was also cloned into a mammalian expression vector and transient transfection studies were carried out to understand the effect of viral E protein expression.

Results

Results suggested susceptibility of SK Hep1 cells to all serotypes of DENV. Distinct cytopathic effects (CPE) in the form of cell rounding, progressive detachment from the substratum and formation of spindle-shaped elongated adherent cells could be observed for DENV 1, 2 and 3. In our experiments DENV1, 2 and 3 infected C6/36 cells were seen to transmit infection to the SK Hep1 cells as detected by positive IFA for viral antigens in the SK Hep1 cells. Interestingly, in these co-cultures, although CPE was evident within 48 hrs after seeding the co-cultures, degenerative changes were neither acute nor of rapid onset. The infected cells also showed differential capping of ICAM (Figure 1). With infected human PBMCs we used only DV2 TR1751 and it showed a similar transmission like C6/36. In the Matrigel ™ assay for angiogenesis, the control SK Hep1 cells showed extensive “sprout formation” after 18 hrs of incubation with the angiogenic growth factor containing assay media. In comparison, the cells exposed to DV showed significant reduction in individual “cell sprouting” morphology when compared with JEV exposed cells. Interestingly, the Chikungunya virus exposure also showed inhibition of angiogenic growth factor induced “sprout formation”.

The SK Hep1 cells transfected with the expression vector pcDNA 3.1 with the full length E gene showed distinct morphological changes in the form of long cytoplasmic extensions (fig 5, b& c). IFA and flowcytometry analysis of the cells (data not shown) could detect expression of virla E protein 48-72 hrs post-transfection. Importantly, in >30% of the transfected cells, the viral E protein was seen to occur in a clustered form at the tip of the “filopodia-like” extensions with a relatively weaker and diffuse cytoplasmic staining (fig 5d). Complete degenerative changes or necrosis was or necrosis was not evident in the transfected cells observed upto 120 hrs post infection period. Atomic Force and Transmission electron microscopy studies on Dengue virus infected SK Hep 1 cells also showed changes in surface morphology and ultrastructural abnormalities (Figure 2 and 3).

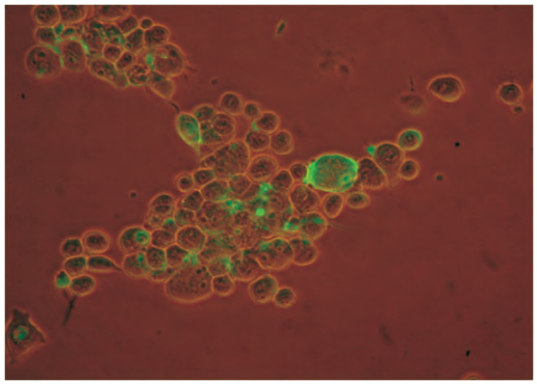

Figure: 1 DIC epifluoresence micrograph of a SK hep1 cell showing the expression of adhesion molecule ICAM.

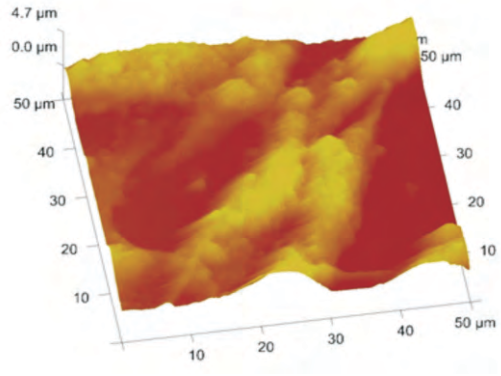

Figure 2: AFM image of contour height profile of SK Hep1 cell showing alteration in surface junctions after DENV infection

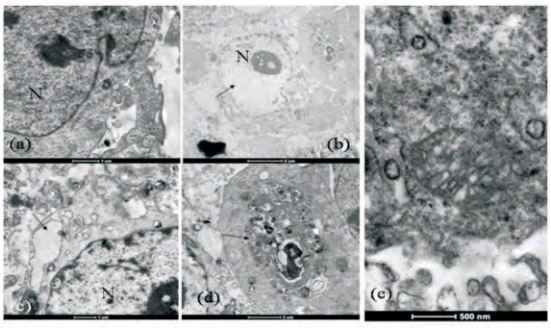

Figure 3: Representative transmission electron micrographs of DV2 infected SK Hep1 cells (a) profile of an uninfected cell (b) advanced nuclear chromatolysis (arrow) (c) dilated endoplasmic reticulum (arrow) (d) cytoplasmic autophagosome and convoluted membranes in infected cell (e) hypertrophied mitochondria with dilated cisternal space. All micrographs have built-in magnification scale bars.

Studies on characterization of cellular lesions in fatal cases of pH1N1 2009

Background

Between 2009-10, Pune had a significant number of pandemic H1N1 infections with fatal cases. Being the National Influenza Reference Laboratory, clinical specimens for diagnosis were reffered to NIV along with necropsy tissues from fatal cases. In a subset of such necropsy tissues with laboratory confirmed H1N1 infections, conventional histopathologic examination and electron microscopy for ultrastructural changes were carried out.

Majority of the lung tissues examined had presence of diffuse alveolar damage and hemorrhagic necrosis typical to viral pneumonia (Figure 4). The novel findings were seen in ultrastructural examination of the lung tissues where distinct presence of Orthomyxovirus particles could be detected in the type II alveolar epithelial cells. Further studies are continuing with immunohistochemistry and possible presence of non-viral pathogen cofactors.

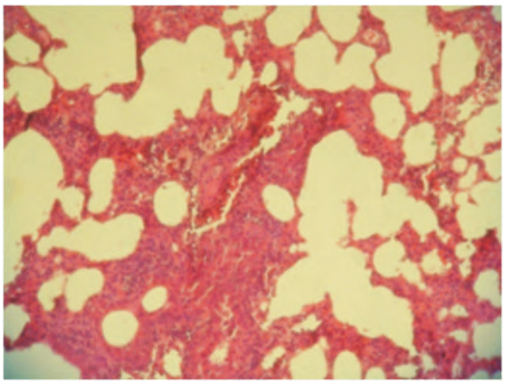

Figure 4: Hematoxylin-eosin stained photomicrograph of diffuse alveolar damage in lung of a fatal pH1N1 case

Electron tomography (ET) of dengue virus replication

Background

Electron tomography has recently emerged to be a very powerful analytical tool that can provide crucial structural information on cellular organelles and virus induced host-cell changes. In the present study ET was used in profiling the endomembrane alterations in dengue virus infected host cells.

Methodology

Virus infected and control cells were epoxy blocked and relatively thick sections > 200 nm were examined under 120KV in a tecnai 12 Biotwin Tem unid equipped with automated tomography platform. The serial tilit ranges of +/- 65 ^{0} were collected and 3D visulaizatons done using an AMIRA imaging suite.

Results

In summary, distinct endomembrane vesicukar changes were seen in the cytoplasm of dengue virus infected cells. 3D reconstruction of these structures showed presence of continuity with ER and other membrane compartments. Further studies areongoing to

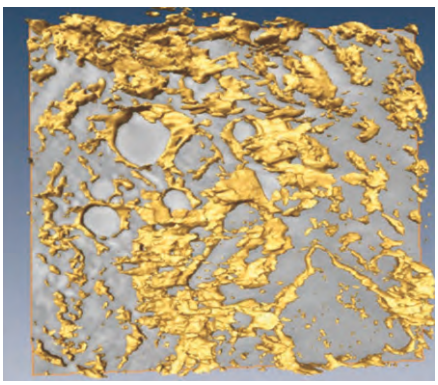

Figure 5: 3D imaging of cellular endomembrane changes associated with dengue virus infection in SK Hep1 cells imaged through ET data series.

Microarry Group

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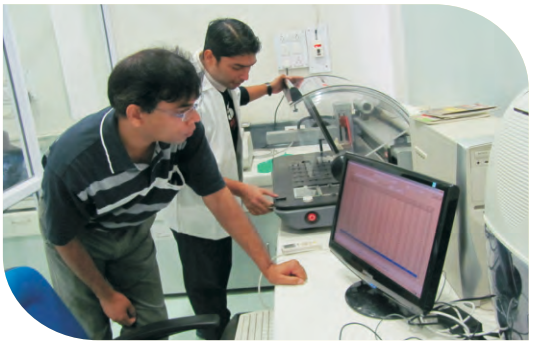


Microarry Group

Projects

The Microarray Group of the National Institute of Virology has well equipped laboratory set up to perform microarray experiments and this group is actively involved in research areas like basic virology as well as diagnostic applications.

Current Projects:

- Host gene expression profiling in highly pathogenic influenza A H5N1 virus infection
- Evaluation of host gene expression profile in case of infection with swine-origin influenza A H1N1 (S-OIV) virus

Host gene expression profiling in highly pathogenic influenza A H5N1 virus infection

Appearance of highly pathogenic avian influenza viruses (HPAI) of the H5N1 subtype in 1997 and cross species transmission to infect human is probably one of the best example of zoonotic transmission of influenza viruses. Frequent outbreaks caused by HPAI in poultry in different countries resulting huge economic loss and depopulation of poultry by distressing infection and transmission of HPAI H5N1 viruses to humans with a case fatality rate of more than 50% is a serious concern over last one decade. HPAI H5N1 influenza viruses have become endemic in poultry populations in Southeast Asia including India, Europe, Africa and Latin America and have spread to more than 60 countries. A fear of pandemic which might arise from this highly devastating subtype of influenza viruses became a continuous threat. Modern Genomics tools such as high-throughput sequencing, mRNA expression profiling has contributed largely towards the understanding of the pathogens. Microarray-based analysis of virus infection is one of the much focal approaches to analyze the molecular basis of the host response and it is possible to focus the study on tens of thousands of genes simultaneously. In addition to the analysis of virus and host factors contributing to the infection caused by HPAI H5N1, host gene response and differential gene expression profiling, we can further explain to the various cellular pathways involved in pathogenicity and virulence. Linking of HPAI and host genomics data with biological outcomes of pathogenicity, high virulence and zoonotic transmission are important strategies to control HPAI.

Objectives:

- Explore differential gene expression pattern of the host cells infected with HPAI
- Mechanisms exercised by HPAI to modulate the cellular host machinery
- Identification of specific gene and their role in virus life cycle
- Involvement of host cellular pathways and identification of new host factor in virus infection

Work Done:

Methods:

Highly pathogenic Avian Influenza A viruses (HPAI), isolated from West Bengal outbreak of 2008, A/chicken/India/WB-NIV2664/2008(H5N1) and reverse genetics modified (RG Modified) low pathogenic H5N1 vaccine reference virus strain, A/India/NIV/2006(H5N1)-PR8-IBCDC-RG7(H5N1) generated from HPAI isolates of NIV (A/Chicken/India/NIV-33498/06) were used to infect A549 cells. Virus infection was carried on in BSL 3 + facility following the safety level to handle HPAI strains. Total RNA was isolated from both the infected and control cells at four different hours post infection (hpi) by trizol method. Analysis and quantification of RNA

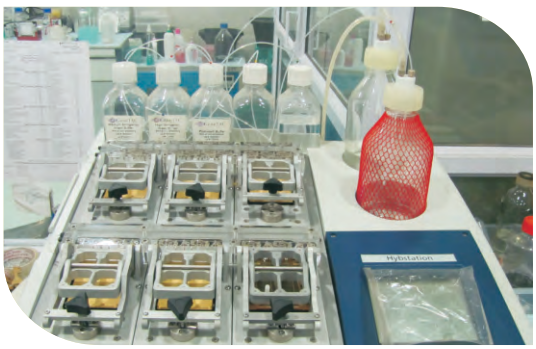
was done on a Bio analyzer. Amplification of RNA and indirect labeling of Cy-dye was done by Amino Allyl MessageAmp II aRNA amplification kit (Ambion) using manufacturer's instruction. One hundred nanograms of RNA from control and infected cells were used for the experiments. The RNA was reverse transcribed and amplified according to the manufacturer's protocol. The purified amino allyl aRNA was labeled with Cy3 and Cy5 for control and experimental samples respectively. Purified samples were lyophilized, resuspended in hybridization buffer (Pronto Universal Hybridization kit, Corning) and hybridized on the Discover human chip (Arrayit corporation, Sunnyvale, CA). Hybridization was carried out in a Hybstation (Genomic Solutions, AnnArbor, MI) and scanning was performed at 5-mm resolutions with the Scan array express (PerkinElmer, Waltham, MI). Grid alignment was done using gene annotation files and raw data were extracted into MS EXCEL.

Data analysis was done using GENOWIZ software (Ocimum Biosolutions, Hyderabad, India). Replicated gene spots were merged and median values of the expression ratios were further considered for the dataset. Dye bias was dealt with by applying loess normalization. Fold change analysis of gene expression was done on log transformed data. Genes with 1.5 folds up/down-regulation were considered as differentially expressed at a p-value<0.05, student's t-test. Functional classification of the genes was performed using Gene Ontology and pathway analysis. Flow chart of the experiments and data analysis is as follows:

Microarray data → Normalization of raw data→ Identification of up regulated and down regulated genes → Determination of differentially expressed genes → Comparative analysis → Gene ontology analysis → Pathway involvement → Linking with host virus interaction biology

Results:

Virus infected cells were collected at four different time post infection (4, 8, 16, 24h). Total cellular RNA isolated from control and infected cells was used to carry out microarray hybridization. Figure 1 shows representative scanned images of the hybridized slides at 24 hpi.



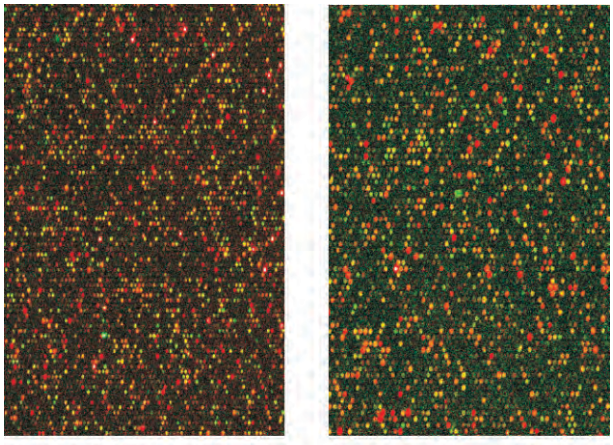


Figure 1. Representative scan images generated after hybridization.

A549 cells were infected with HPAI and RG modified viruses. After 24 hours post infection cells were harvested, RNA isolated, quality and quantity measured and hybridized in Human Discover chips. Scanned image were quantities using Pro-scan array software (Perkins Elmer) and data generated from scanning were analyzed using GENOWIZ software.

The data obtained from hybridization was filtered and normalized using GENOWIZ software. The normalized data was used to calculate differentially expressed genes which were affected by virus infection. The number of differentially expressed genes at different post infection time points with both the viruses (HPAI and RG Modified) has been shown in Table 1.

Table 1. Summary of genes differentially expressed in response to infection with HPAI-H5N1 and RG Modified H5N1 in A549 cell lines.

Time points	Genes qualifying the quality criteria in replicated experiments	Differentially expressed genes (+/-1.5folds, p<0.05)	Up-regulated genes	Down-regulated genes
HPAI- A/chicken/India/WB-NIV2664/2008(H5N1)				
4h	253	40	15	25
8h	208	24	13	11
16h	254	101	53	48
24h	309	111	53	58
RG Modified - A/India/NIV/2006(H5N1)-PR8-IBCDC-RG7(H5N1)				
4h	262	60	36	24
8h	212	128	67	61
16h	237	109	58	51
24h	305	42	22	20

Data generated from all the four end points of infection were analyzed and clustered into 5 different sets. Figure 2 shows Hierarchical clustering of differentially expressed genes observed at different time-points post infection with HPAI-H5N1 (A) and RG Modified H5N1 (B) in A549.

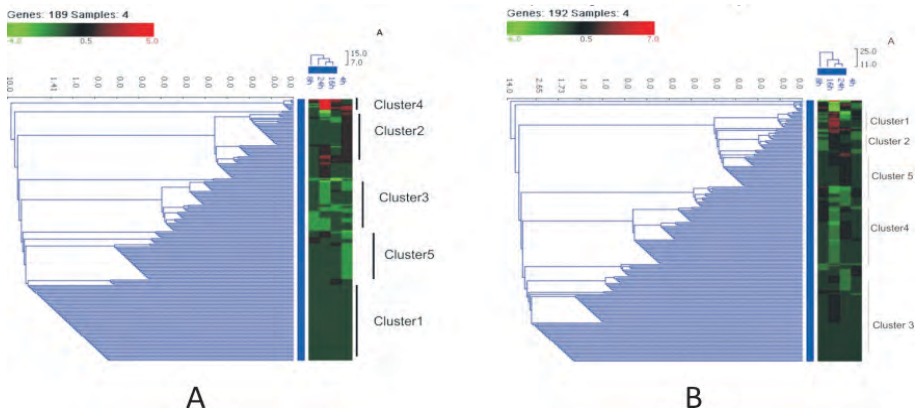


Figure 2. Hierarchical clustering of differentially expressed genes of A) HPAI-H5N1 (A/Chicken/India/WB-NIV2664/2008) and B) RG modified H5N1 (A/India/NIV/2006(H5N1)-PR8-IBCDC-RG7) infected A549 cells at different post-infection time points.

Differentially expressed genes at different time points post infection with HPAI-A/chicken/India/WB-NIV2664/2008(H5N1) and RG Modified H5N1 - A/India/ NIV/ 2006 (H5N1) -PR8-IBCDC-RG7 (H5N1) viruses were determined and list of significantly up and down regulated genes in response to HPAI and RG modified H5N1 virus infection has been shown in Table 2 and Table 3 respectively.

Table 2. List of significantly up-and down-regulated genes in A549 cell lines infected with HPAI- A/chicken/India/WB-NIV2664/2008(H5N1) at different post-infection time points

Gene ID	Description	Fold change
4 hpi		
NM_002963	S100 calcium-binding protein A7 (psoriasin 1)	2.1297483
U69127	far upstream element (FUSE) binding protein 3	-2.124454
8 hpi		
NM_000210	integrin, aRG modifiedha 6	-2.6230335
NM_006206	platelet-derived growth factor receptor, aRG modifiedha	-2.6103954
NM_002268	karyopherin aRG modifiedha 4 (Qip1)	-2.3417711
16 hpi		
NM_004049	BCL2-related protein A1	2.189591
NM_002189	interleukin 15 receptor, aRG modifiedha	2.608685
NM_002198	interferon regulatory factor 1	3.134424
NM_007315	signal transducer and activator of transcription 1, 91k	3.224348
NM_003603	Arg/Abl-interacting protein ArgBP2	3.31811
NM_004048	beta-2-microglobulin	3.339335
NM_001565	small inducible cytokine subfamily B (Cys-X-Cys),	4.358359
NM_002067	guanine nucleotide binding protein (G protein), aRG modifiedha 11 (Gq class)	4.748868
NM_003390	wee1+ (S. pombe) homolog	-14.351
NM_003157	serine/threonine kinase 2	-7.92899
NM_005229	ELK1, member of ETS oncogene family	-4.66394
NM_000221	ketohexokinase (fructokinase)	-4.3185
NM_002117	major histocompatibility complex, class I, C	-3.82267
NM_003467	chemokine (C-X-C motif), receptor 4 (fusin)	-2.5603
NM_004354	cyclin G2	-2.33702
24 hpi		
NM_000584	interleukin 8	2.1922169
NM_001565	small inducible cytokine subfamily B (Cys-X-Cys),	2.3936596
NM_000744	cholinergic receptor, nicotinic, aRG modifiedha polypeptide 4	2.6643968
NM_006180	neurotrophic tyrosine kinase, receptor, type 2	-2.06324

.Table 3. List of significantly up- and down-regulated genes in A549 cell lines infected with RG Modified - A/India/NIV/2006(H5N1)-PR8-IBCDC-RG7(H5N1) at different post-infection time points

Gene ID	Description	Fold change
4 hpi		
NM_001565	small inducible cytokine subfamily B (Cys-X-Cys),	-3.09053
NM_000994	Ribosomal protein L32	-2.09476
NM_005180	murine leukemia viral (bmi-1) oncogene homolog	2.062517
NM_001338	coxsackie virus and adenovirus receptor	2.482498
8hpi		
NM_005859	purine-rich element binding protein A	-5.72255
NM_000629	interferon (alpha, beta and omega) receptor 1	-3.98753
NM_000121	erythropoietin receptor	-3.83773
NM_001338	coxsackie virus and adenovirus receptor	-3.82074
NM_000210	integrin, alpha 6	-2.99664
NM_007294	breastcancer1,earlyonset	-2.97396
NM_002879	RAD52 (S. cerevisiae) homolog	-2.86873
NM_002757	mitogen-activated protein kinase kinase 5	-2.34318
NM_001527	histone deacetylase 2	-2.34189
NM_003286	topoisomerase (DNA) I	-2.15997
NM_005180	murine leukemia viral (bmi-1) oncogene homolog	-2.13859
NM_004504	HIV-1 Rev binding protein	-2.01718
NM_001404	eukaryotic translation elongation factor 1 gamma	2.080469
NM_002155	heat shock 70kD protein 6 (HSP70B`)	2.083674
NM_001032	Ribosomal protein S29	2.203894
NM_006290	Tumor necrosis factor, alpha-induced protein 3	2.693088
NM_002982	small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	2.70706
NM_003516	H2A histone family, member C	2.851324
NM_001565	small inducible cytokine subfamily B (Cys-X-Cys),	7.235646
NM_001924	growth arrest and DNA-damage-inducible, alpha	9.713105
16hpi		
NM_005910	microtubule-associated protein tau	-5.15303
NM_006293	TYRO3 protein tyrosine kinase	-4.34585
NM_001792	cadherin 2, type 1, N-cadherin (neuronal)	-3.43758
NM_001565	small inducible cytokine subfamily B (Cys-X-Cys),	-3.03696
NM_002189	interleukin 15 receptor, alpha	-2.85765
NM_002228	v-jun avian sarcoma virus 17 oncogene homolog	-2.80432
NM_002392	mouse double minute 2, human homolog of; p53-binding	-2.68366
M15329	interleukin 1, alpha	-2.37275
NM_002050	GATA-binding protein 2	-2.36576
NM_021103	thymosin, beta 10	-2.04629
NM_002879	RAD52 (S. cerevisiae) homolog	2.005432
NM_002757	mitogen-activated protein kinase kinase 5	2.170122
X68742	integrin, alpha 1	2.226101
NM_002222	inositol 1,4,5-triphosphate receptor, type 1	2.334975

Gene ontology analysis (Figure 3) explains up regulation of immune related functions up to 16 hours post infection followed by down regulations at 24 hpi, whereas apoptosis related factors show up regulation up to 4 hpi followed by down regulation in the successive end points. These findings can be linked with virus host interaction and further it can be correlated with the gene regulation and its relation to different pathways.

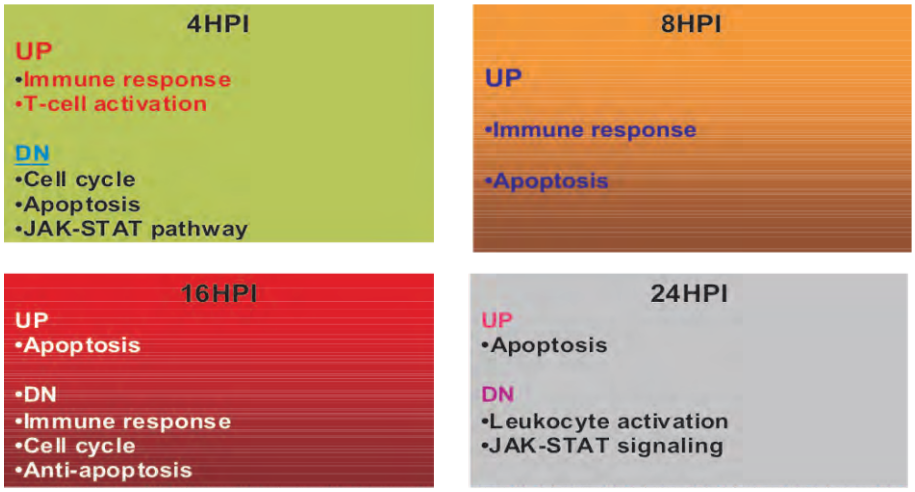


Figure 3. Gene Ontology of differentially expressed genes at 4 different time points in response to HPAI-H5N1

Pathway analysis of differentially expressing genes between the strains:

Based on the data analysis using GENOWIZ software, it has been found genes from the following pathways were induced due to infection with HPAI and RG modified viruses.

- Cytokine-cytokine receptor interaction
- Toll like-receptor mediated signaling-STAT1, JUN
- P53 pathway-Cyclin B1
- MAP-Kinase pathway-Map2k5

Discussion:

Results can be briefly discussed in the following points:

- Association of multiple signaling cascades like Jak-STAT signaling pathway, TGFbeta signaling, MAPK signaling pathway, p53 signaling pathway in host cellular machinery by HPAI.
- Down regulation of apoptosis inducers and upregulation of apoptosis inhibitor at the early stages of infection.

- Much less gene expression can be identified in case of low pathogenic viruses at 24 hpi. This can be explained as HPAI might have robust contribution in modulating host gene expression system to spread lethal infection rapidly
- High pathogenicity of HPAI-H5N1 could be explained by the differential expression of certain immune responsive genes.

Analysis of gene expression, common genes and differentially expressed genes between the HPAI and RG modified virus infected cells at different end points showed general trends that gene expression pattern in both HPAI-H5N1 and RG modified-H5N1 infected cells are qualitatively more or less similar but differs quantitatively. Apoptotic and anti apoptotic gene regulation as well as immune related genes were regulated in such a pattern that virus infected cells can survive till progeny viruses are generated inside the infected cells. However 16 hours post infection time point was most interesting as at this time point maximum number of differentially expressed genes were detected and contrastingly gene response was qualitatively different also. HPAI-H5N1 behaves more virulently leading to devastating disease which is explainable of its ability to induce several chemokines and cytokines. This is the most interesting finding of our study and we are currently analyzing our data in more details, validating and correlating these findings with the relevant biology of virus host interactions

Future Work Plan:

- Validation of data using real time PCR
- Detail study at different time point post infection and comparative analysis of gene regulation and host gene expression in HPAI and RG modifiedAI infection
- Identification of host factors involvement
- Establishment of pathway(s) involvement in flu infection

Laboratory Animals Group

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Mr. R. H. Chavan, Technician
Mr. R. J. Sarpatil, Technician
Mr. S. M. Doke, Technician


C3H/HeJ

Service Project: Care, Breeding & Maintenance of Laboratory Animals

Different species of laboratory animals viz. mouse, guinea pig, rabbit, fowl, goose, turkey and monkey were maintained. Five turkeys were procured from local farm for experimental purpose. A total of 7668 animals (Table - 1) and 1628 ml of Blood samples (Table - 2) were supplied to NIV scientists.

Table 1: Supply of Laboratory Animals to NIV Scientists

Albino Mice	BALB/c Mice	C3H/J Mice	CBA Mice	C57BL/6 Mice	Athymic nude mice	Rabbits	Fowls	Total
3539	3907	88	24	42	63	3	2	7668

Table 2: Supply of Blood Samples to NIV Scientists (quantity in ml)

Guinea pig	Rabbit	Fowl	Geese	Turkey	TOTAL
140	38	439	100	911	1628

Resource generation

A total of 1960 surplus animals as shown in the figure-3 were supplied on request to various research organizations registered with the CPCSEA.

Table 3: Supply of Laboratory Animals to Outside Institutions

Species/ Strain	Albino Mice	NMRI nu/nu Mice	NIH nu/nu Mice	BALB/c Mice	C57BL/6	Total Numbers
Numbers	1080	166	181	521	12	1960

IAEC Activities

- Meeting of the Institutional Animal Ethical Committee (IAEC) meeting was held on 25.05.09. A total of 21 projects were presented by the investigators and discussed.
- Meeting of the Institutional Animal Ethical Committee (IAEC) meeting was held on 18.09.09 for reviewing of health status of rhesus monkeys.
- Meeting of the Institutional Animal Ethical Committee (IAEC) was held on 08.02.2010 to discuss the matters related with maintenance of the animals in the animal house and

for approval of animal experimentation projects Meeting. A total of 12 project proposals were discussed during the meeting.

- Visit of IAEC members arranged at NIV, Pune & MCC, Pashan Animal House facilities on 24th July 2009 for inspection of animals and records.
- Renewal granted by the Director (AW) & Member-Secretary, CPCSEA, Ministry of Environment & Forests, New Delhi for NIV CPCSEA registration for a period of three years i.e. from 11.3.2008 to 10.3.2011.

IBC Activities

- Meeting of IBSC organized on 0.9.2009 to decide on management & disposal of Bio-Medical Wastes generated at NIV, Pune.
- Renewal of Authorization under Bio-Medical Waste (M&H) rules, 1998 BMW Authorization received vide letter MPCB/RO(PAMS)/BMW-PUNE-10/2010 dated 9.3.2010 having validity upto 30.06.2011.

Other activities

- MPCB Annual Report of the bio-medical wastes generated & treated from 1.1.09 to 31.12.10 prepared and submitted to the MPCB, Pune and Mumbai offices.
- Brochure on "Mouse" was prepared in Hindi and released during the Hindi Workshop held at NIV, Pune on 9th December 2009.
- Compilation of training course material containing copies of Environment (Protection) Act, 1986, Ministry of Environment & Forests, New Delhi rules, Hon. High Court, Mumbai directives and Central Pollution Control Board guidelines for Training Workshop on "Bio-medical Waste Management Practices".

Library and Information Services

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Work done

- Compilation of NIV Annual and Quarterly report
- Maintenance and updating web page information like scientist's profile, papers published, major achievements, RTI etc
- Labsys software upgraded to web OPAC version for accessing NIV library services
- ERMED (http://nmlermed.in)online facility continued for online access of >2500 full text journals
- Science direct (www.sciencedirect.com) facility continued for online access of 14 core journals
- ProQuest database (http://proquest.umi.com) facility continued for online access of 1477 journals
- Interlibrary loan facility continued for NIV staff
- Compiled list of papers published by NIV staff, conference / workshops attended / organized (1953-2009).
- Citation Analysis provided for NIV Scientists.
- List of Awards (1953-2009) received by NIV scientist being compiled.
- Services provided for
- Photocopies 34201 pages
- Documents received though inter library loan 730
- Articles / reprints received from authors 1640
- New documents received by IV library
- Books -482
- Bound volumes 617

Achievements

Dr. S. N. Singh

- Received Lifetime achievement award in field of Library and Information Science, Pustakalaya Parishad, Jodhpur 13-2-2010.
- Nominated Member of PhD Committee by Hon'ble Vice Chancellor, University of Pune.
- Editorial Member of Journal/Bulletin: NISARGOPACHAR, National Institute of Naturopathy (Ministry of Health) Pune.
- Ph. D. awarded to one student (Maryam Salami). Use of information communication technologies in pharmacology and allied sciences' library and information centres in Iran.


FIRE

Institutional Bio-Safety Committee

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Activities and work done:

Meeting of IBSC organized on 10.9.2009 to decide on management & disposal of Bio-Medical Wastes generated at NIV, Pune.

Second meeting of the committee was held on November 10, 2009. A format for submitting projects to IBSC with standard operating procedure was prepared. The other activities undertaken are as mentioned below:

Research Project Approval

During the year, the committee screened 50 projects involving animal experimentation for further approval by IAEC.

Other bio-safety issues

- Medical surveillance for workers & staff of BSL-3 [March 2010].
- Reviewed incidence reports obtained during last six months.
- Air pollution survey in the MCC campus was done through MPCB, Pune as per the norms for existence of incinerator in the campus.
- Tender for Effluent Treatment Plant and rainwater harvesting is finalized [March 2010].
- Routine Pest control & Rodent control activity were continued as that of last year [July 2010].
- Incinerator inspection was done by MPCB and incinerator's ash was chemically analyzed. Contract was awarded to a private company approved by MPCB for the disposal of ash.
- Authorization of MPCB was received for the proper functioning of incinerator in the MCC campus.

Scientific Personnel Health Monitoring Program

- As a part of routine health monitoring of staff working in animal house was performed, this included Chest X-ray, AFB in sputum, Haemogram, ECG, Blood Sugar, Lipid Profile. The health parameters of most of the staff members were found within normal limits.
- As a part of routine health monitoring of staff working in BSL-3 laboratory was performed, this included Chest X-ray, Haemogram, ECG, Blood Sugar, and Lipid Profile. The health parameters of most of the staff members were found within normal limits.

Training/workshop/symposium organized on bio-safety and bio-security related issues:

- Organized preliminary training on General Bio-safety in BSL-2 laboratories [27 June 2009].

- In-house Training successfully organized during 11- 19 August 2009 for 72 NIV Group D staff members as per the Sixth Pay Commission recommendations.
- One-day training workshop on "Bio-medical Wastes Management & Handling" organized jointly with the Animal House Group and Institutional Bio-safety Committee on 27th November 2009 to create awareness among the technical and research workers and other staff.
- Organized "Three days training for working in High Containment Laboratory" for NIV staff (15-17 December, 2009).

Table 1: Disposal of Bio-Medical Waste (Animal Carcasses & Allied Wastes) at MCC campus Incinerator [2009-2010]

Sr. No.	Month	Quantity (KG)
1	January 2009	57
2	February 2009	65
3	March 2009	42
4	April 2009	79
5	May 2009	42
6	June 2009	82
7	July 2009	77
8	August 2009	78
9	September 2009	163
10	October 2009	122
11	November 2009	123
12	December 2009	93
	Total	1023

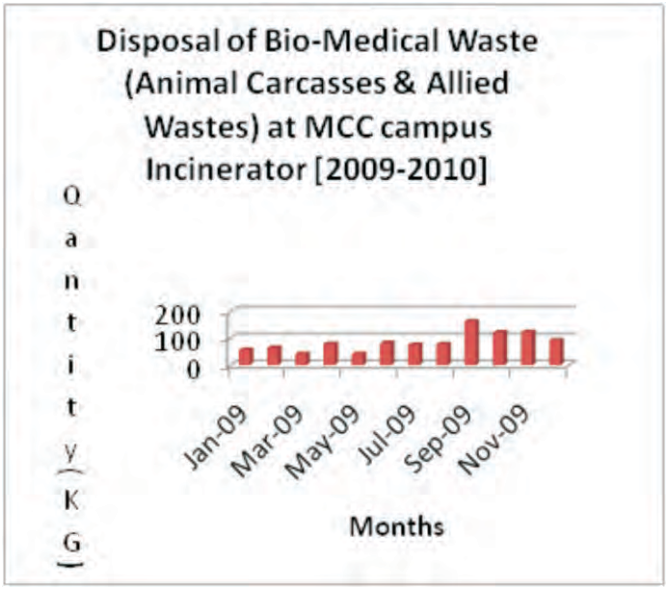

Figure1: Disposal of Bio-Medical Waste (Animal Carcasses & Allied Wastes) at MCC campus

Table 2: Details of shredding material for year 2009-2010

Sr. No.	Month	Quantity (KG)
1	January 2009	192
2	February 2009	310
3	March 2009	275
4	April 2009	296
5	May 2009	277
6	June 2009	232
7	July 2009	282
8	August 2009	412
9	September 2009	422
10	October 2009	502
11	November 2009	483
12	December 2009	425
	Total	4108

Figure 2: Shredding material

Training on working in BSL-3 facility (15-17 December 2009)

M. Sc. Virology

Staff Members

Professor A.N. Latey
Coordinator Academic Programme

Dr.(Ms) Cecilia Dayaraj
Scientist 'E' & Programme Coordinator

Dr.(Mrs) A.S. Tripathy
Scientist 'C' & Programme Coordinator

Mr. S.S. Bedekar, Technical Officer-A

Mr. B.K. Wadke, Personal Assistant

Students

2005-07 batch: 14 students passed out

2006-08 batch: 23 students passed out

2007-09 batch: 19 students passed out

2008-10 batch: 19 students passed out

2009-11 batch: 18 students registered for 2nd year

2010-12 batch: 25 students Registered for 1st year


The Local Cytokine Milieu: A Balancing Act

The M.Sc. Virology course is progressing very well since its launch in 2005. Students of the fourth batch (2008-10) have now completed the course with the following results:

Grade	O	A	B
Number passed	10	5	4

Students of the fifth batch are now in their second year (Semesters III/IV).

Many of our students who have successfully completed this course since 2007 have secured employment in reputed industries and institutes including **Actis Biologics, Bharat Biotech, Hindustan Unilever, National AIDS Research Institute, National Centre for Cell Science, National Institute for Research in Reproductive Health, Serum Institute of India, Tata Institute of Fundamental Research, Venkateshwara Hatcheries and this institute. Several others have secured research positions in** Universities and research institutes in Belgium, Germany, India, Singapore Thailand and the United States of America.

With a view to keep our course upto date and comparable to other similar courses at international level, we have began the exercise of revision of the virology syllabus. It is hoped that this activity will be completed during the next academic year.

Hostel accommodation is available for students. A total of 48 rooms are available in two hostels (Ladies and Gents) at the Pashan campus. The second of these two hostels (meant for lady students) was inaugurated on 10th January 2010 by the Union Minister of Health and Family Welfare, Shri Gulam Nabi Azad. The hostels are provided with all modern amenities. On campus boarding is also provided. Charges for both are very reasonable.

The details of our course are available at

1. http://icmr.nic.in/pinstitute/niv.htm
2. Http://www.unipune.ac.in/pgadmissions/

Administration

Administration is a process of proper planning, organizing, controlling and implementing the policies within a time frame that effective. It is a multi-facet department that and cost effective manner that helper to achieve the organizational goals. Role of NIV administration is Personnel Management, Financial Management, Material management, Contractual Managements and Human Resource Development. It is also an endeavor of the NIV Administration to enrich the knowledge of the employees on the latest developments in the fields of Science, Technology and Administration, so as to enhance the productive role of the individual employee and as a team.

Maintenance

- The services of Maintenance staff and apprentice students of ITI have been efficiently utilized for carrying out periodical / preventive maintenance of equipments /installations along with their regular technical training in the field.
- During this period, the breakdown maintenance has been considerably reduced to about 972 jobs only.
- During this period, major renovation / refurnishing of Animal House passenger lift was done.
- The electrical wiring of guesthouse in NIV campus is very old and load has also increased considerably. Hence, new concealed wiring has been done along with flooring works.
- The compressors of the central AC plant of old building installed during 1950 have lived their life and efficiency has down drastically. Hence, action was being initiated for replacement of the entire chiller units.
- The 400 KVA stand by generator along with AMF panel was very old, about 25 years. It needs major overhauling, which will be un-economical. Also, the power demand has increased. Accordingly, action has been initiated for replacement with suitable capacity DG set, preferably through buy-back system.

Sports & Cultural Activities during NIV Foundation day 2010

Sports & Cultural Activities during NIV Foundation day 2010

SAC Meeting 2009

Recreation & Renovation

NIV staff promoting Garden Activities,
With prizes & medals

List of Publications

1. Agrawal AS, Sarkar M, Chakrabarti S, Rajendran K, Kaur H, **Mishra AC**, Chatterjee MK, Naik TN, Chadha MS, Chawla-Sarkar M. Comparative evaluation of real-time PCR and conventional RT-PCR during two year surveillance for Influenza and RSV among children with acute respiratory infections in Kolkata reveals distinct seasonality of infection. J Med Microbiol. 2009; 58(12):1616-22.
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