

**11.1 Kyasanur Forest Disease (KFD)**

**11.1.1.Genotyping of KFD virus isolates in India:**

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**Introduction**

The Kyasanur forest disease (KFD) was first recognized in 1955 in the forested areas of Shimoga district, Karnataka State, India, which is the only geographical area in this country, which is endemic for this disease. NIV has isolated several strains of KFD viruses. Study was undertaken to sequence these strains using NS5 gene.

**Objectives**

- Genotyping of KFD virus strains isolated during last 3-4 decades.
- Establishing diagnostic-PCR.

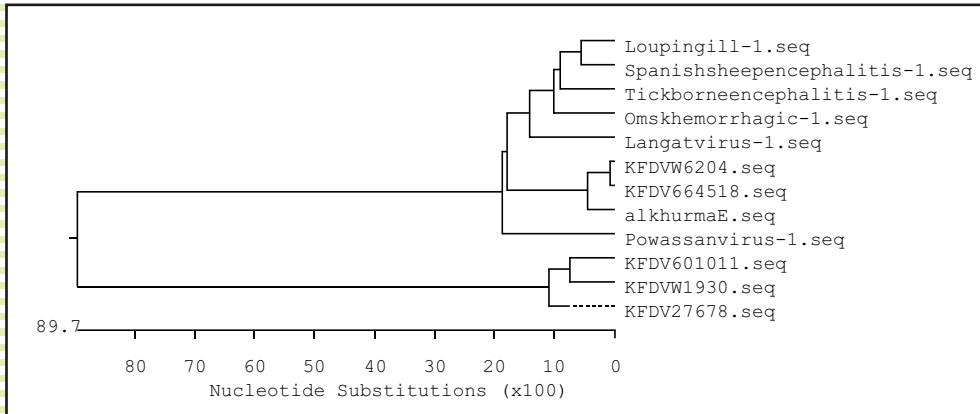
**Work done**

**11.1.2. Sequencing of complete “E” gene, NS-3 and NS-5 gene of KFD virus**

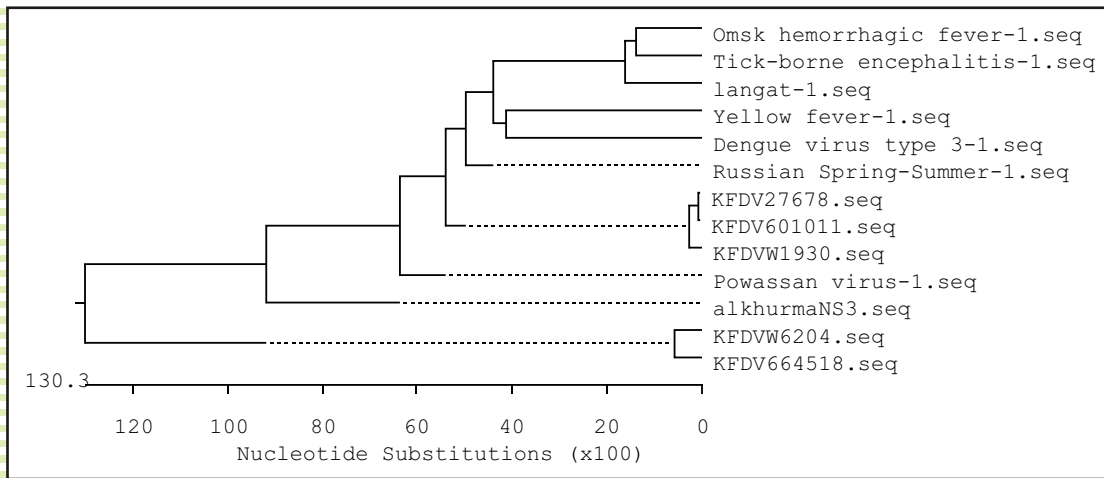
Five KFD virus strains (W6204, W1930, G-27678, 601011 & 664518) isolated in the past from Karnataka (Table-1) were used for this study. These were first propagated in Swiss albino infant mice, on 3<sup>rd</sup> PID, brain suspension of infected mice were passage in Vero cell line, which showed CPE at 4th PID and the propagated TCF material used to extract RNAs. cDNA was prepared using random hexamers, using standard AMV reverse transcriptase reaction (Promega). E gene, NS-3 and NS-5 regions were amplified using various internal primers. Primers were designed for Envelop gene from genomic location 751-1566 to 1465-2382 and two products were amplified i.e. S1 of 815 and S2 917bp. NS-3 gene primers made for three small fragments of 693 bp, 743 bp and 881bp were amplified from 6385-4409 which cover total NS-3 gene. For NS-5 gene again four sets of primers made which cover 7475 - 10439 genomic location. After getting proper size of products, DNAs were purified using Promega Wizard DNA purification kit. Purified products were used for Sequencing. Phylogenetic analysis performed using mega 3.0 software for Envelop gene, NS3 & NS5 genes. (Fig. 1 to 3).

**Table 1: Details of KFD isolates used for Sequencing**

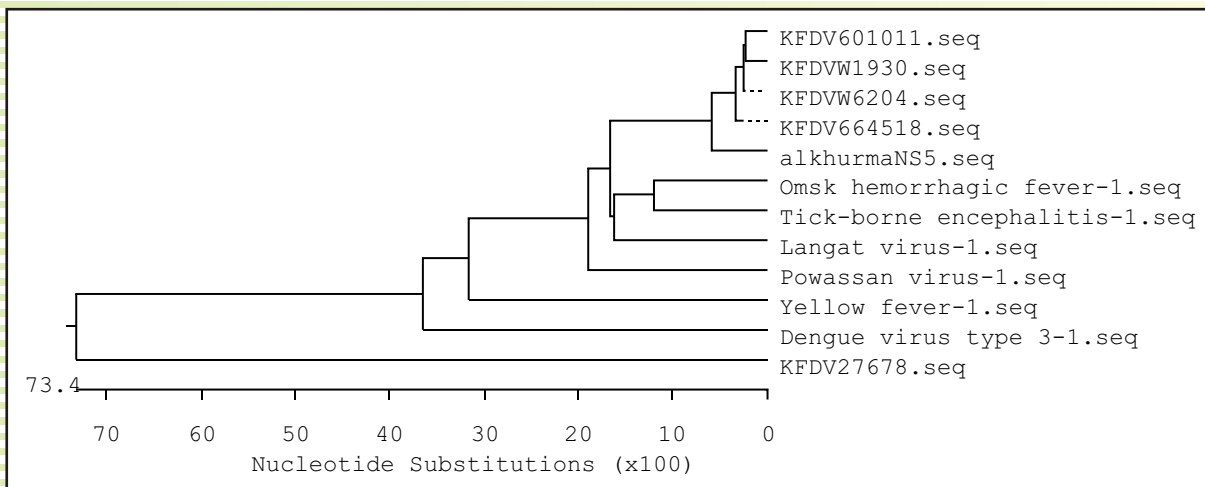
Strain No.	Year of isolation	Place of isolation	Original source
W 6204	1959	Kannahalbi, Karnataka	<i>Semnopithecus entellus</i>
W 1930	1958	Kopalagadde, Karnataka	<i>Semnopithecus entellus</i>
G-27678	1959	Chimnoor, Karnataka	<i>Heamophysallis spinigera</i>
601011	1960	Chikkasakuna, Karnataka	Human serum
664518	1966	Hennagene, Karnataka	<i>Heamophysallis turturis</i>



**Figure 1 : Phylogeny of Envelop gene of KFDV and other flaviviruses.**



**Figure 2 : Phylogeny of NS3 gene of KFDV and other flaviviruses.**



**Figure 3: Phylogeny of NS5 gene of KFDV and other flaviviruses.**

**11.1.3. Attempts to raise hyper-immune serum against KFD virus**

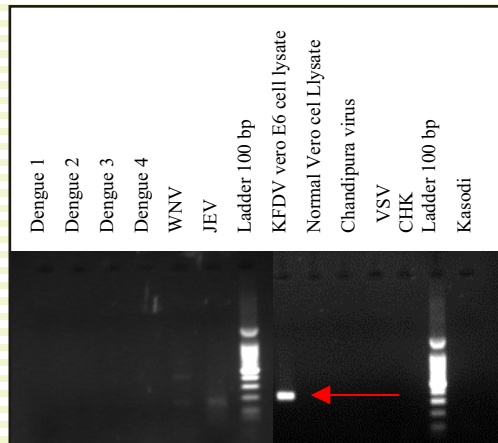
Swiss albino mice and rabbits were immunized with KFD virus strain w 1930. First two dosages were formalin-inactivated antigens and these were followed by three live virus dosages. Titration of immune sera was done using IFA of cell line on 4<sup>th</sup> PID of infection with KFDV. The titer was 1:150.

**11.1.4. Susceptibility studies of *Ae. aegypti* mosquitoes to KFD virus after intrathoracic inoculation**

Susceptibility of *Ae. aegypti* mosquitoes was determined after intrathoracic inoculation with KFD virus, with a view to develop quick assay system for qualitative detection of antibodies in sera. After 8th PID the head squashes of mosquitoes showed positivity. Further studies are being performed to compare the rate of multiplication of KFD virus with other mosquito-borne Flaviviruses.

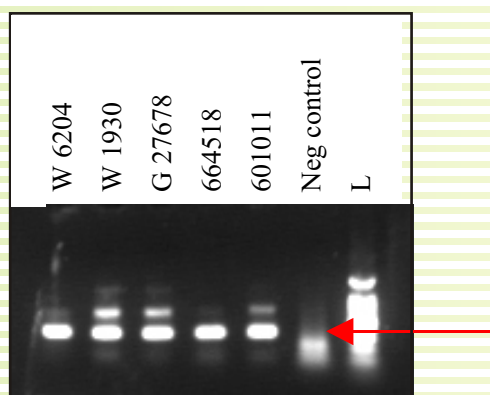
**11.1.5. Establishment of diagnostic RT-PCR for the KFD**

Diagnostic RT-PCR is a useful tool to confirm KFD from other haemorrhagic viruses in India. Study was undertaken to standardize this PCR. Primers were designed from NS5 gene region. Primers from highly conserved region of KFD & differing in other flaviviruses were established from multiple sequence alignment. The primers chosen gave 355 bp product. PCRs were optimized using temperature gradients. Results showed that test was specific and could distinguish KFD virus from all the other flavivirus. The NCBI non-redundant database for cross reactivity showed that possibly these primers would also discriminate KFDV from many other hemorrhagic causing viruses present worldwide which was later confirmed in wet lab experiment (Fig 4).



**Fig. 4 : Specificity of primer sets against different flaviviruses.**

Different isolates of KFDV was taken & standardized protocol was employed to check the reproducibility of reaction (Fig 5). Sensitivity of these primers is being worked out. Similarly serologically KFD confirmed field collected samples will be tested to understand the utility of this test.



**Fig. 5 : Reproducibility of PCR reaction confirmed using different isolates of KFDV.**

#### 11.1.6. IgM ELISA for KFD virus

standardization was started using 10 acute serum samples of KFD patients (confirmed by virus isolation) & ten acute serum samples (with no virus isolation) using Alkhumrah virus ELISA reagents from CDC. In addition, 5 sera samples of the lab workers were taken after 30 days of post vaccination with the single dose of formalin inactivated 1.0 ml subcutaneous KFDV (Kysanur Forest Disease Virus) vaccine. Sera from above mentioned 10 patients were supposed to contain anti-KFDV IgM antibodies. No differences in positive and negative samples could be established. In another trial three negative samples were included from normal person unexposed to KFD, which again gave the same results.

#### 11.2 Development of diagnostic facility for Hantavirus DR Patil

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Hantavirus genus in bunyaviridae family consist of some of the zoonotically important viruses with potential to cause severe disease in human. HTN, SEO, DOB are important strains associated with old world rodents where as SNV, NY and Andes are associated with new world rodents.

Attempts are being made to standardise IgM ELISA for Hanta viruses with the help of CDC. This test will be useful as diagnostic test during suspected outbreaks.

#### 11.3 Molecular characterization of viruses (family Bunyaviridae) isolated from India

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##### Introduction

The Bunyaviruses are spherical particles of 80-120 nm in diameter and shares a common genetic organization of three predominantly negative stranded RNA segments (S, M and L). They belong to Bunyaviridae family. Based on antigenic, genetic and ecological relatedness these viruses are subdivided into five genera. Most viruses in this family are transmitted by arthropods, primarily mosquitoes, ticks, sand flies, or thrips. Hantaviruses are exceptions; that are rodent borne and transmitted in aerosolized rodent excreta. Viruses in this group were earlier classified on the basis of antigenic relationship.

**Objectives**

- Characterization at serological and molecular level to assist in right taxonomic placement.

**Work done**

**1. Studies on Ingwavuma virus (ING) virus isolated in India**

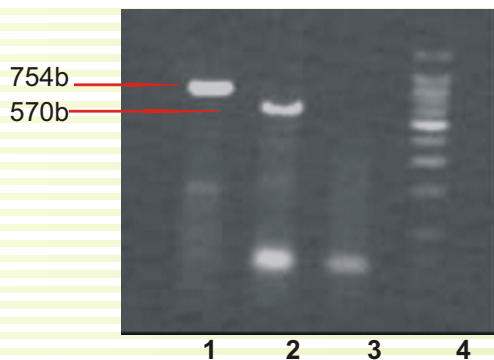
The Ingwavuma virus belongs to Bunyaviridae family, genera Orthobunyavirus. This genus includes about 60 viruses, which cause disease in humans. In India, it was isolated in 1976 from a human case and a pig, since then no studies were made to understand its prevalence and extent of disease caused by this virus in our country.

**A: In vivo & in vitro propagation**

There were two strains of this virus isolated from Pig (strain no. 86208-3) and human (strain no. 86627-2) were available in the repository. These were propagated in infant Swiss albino mice, which showed sickness at 72 hour, and in Vero cell line also it showed CPE at 48 hour. Data was raised on its susceptibility to various cell lines, which showed its profuse multiplication in PS, Vero, RD, and C6/36 cell lines and further confirmation done by IFA in all these cell lines at 2nd and 3rd PID.

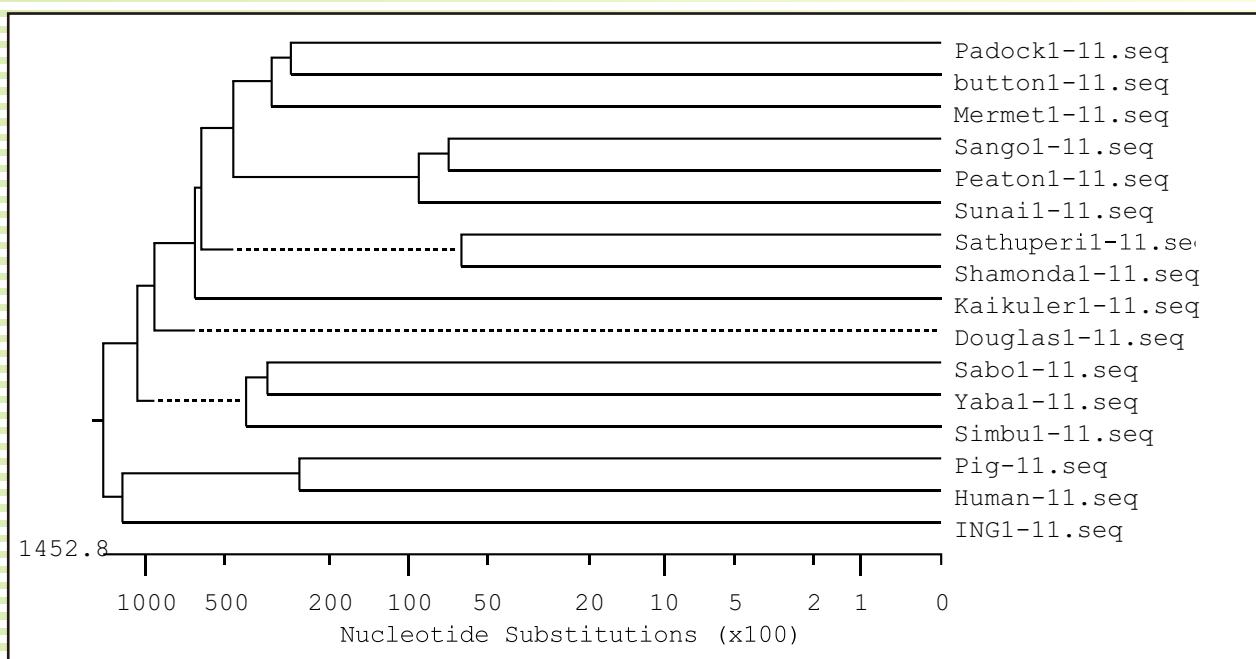
**B: Mosquitoes susceptibility studies**

The virus isolated from human was inoculated in *Ae. aegypti* mosquitoes which showed that this mosquito species was susceptible after ITI. However, when mosquitoes were fed on the high titred blood virus mixture they did not become infected and transmission attempts on infant mice were also unsuccessful. Head squash and the salivary glands of all the orally fed mosquitoes (55 females) were also found negative to ING virus antigen by IFA test.



**Fig 6: Gel photo of Ingwavuma virus for Nucleocapsid and Glycoprotein gene**  
 Lane 1: Vero cell lysate of Ingwavuma virus for N gene  
 Lane 2: Vero cell lysate of Ingwavuma virus for M gene  
 Lane 3: Normal Vero cell lysate  
 Lane 4: 100 Bp DNA Ladder (Promega)

Both the isolates of Ingwavuma virus were propagated in Vero cell line. Tissue culture fluids were used for RT - PCR using Glycoprotein and Nucleocapsid (N & NSs) gene specific primers (Fig-6). The amplified products of 754bp (N gene) and 570bp (G1 gene) were extracted from the gels and sequenced. Phylogenetic data suggest that both the Indian isolates (human & pig) of Ingwavuma virus make a monophyletic tree (Fig-7) and showing highest homology with AF362395, Ingwavuma virus strain An4165. Sathuperi and other Simbu viruses falling in different phylogenetic lineage.



**Figure 7: Phylogeny of N gene from Indian strain of Ingwavuma viruses compared with prototype strain and other Simbu group of viruses.**

#### **D: Retrospective study to detect ING antibodies in human sera collected from Karnataka state**

Both the ING virus isolates were obtained in the past from Karnataka state. 72 sera samples (collected during 2000- 2004) from the patients, which were clinically diagnosed as DHF/DSS but were found negative for dengue by IgM antibodies were tested for the presence of ING antibodies using ELISA (IgG) and head squashes of ING infected mosquitoes as immobilized antigen. Each individual serum was tested on separate slide, which had negative control of head squashes prepared from negative mosquitoes. There were 8 sera samples positive by ELISA (IgG) for ING antibodies. These sera were also found positive by mosquitoes head squash test.

#### **11.4 Studies on Ganjam virus isolated in India**

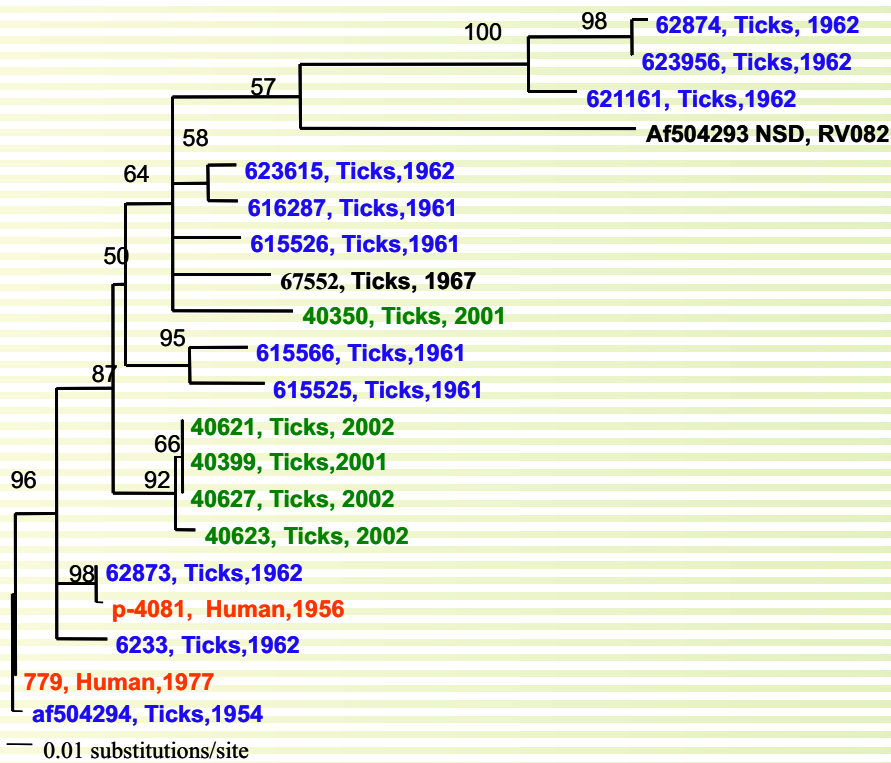
Nairobi sheep disease (NSD) virus, the prototype tick-borne virus of the genus Nairovirus, family Bunyaviridae is associated with acute hemorrhagic gastroenteritis in sheep and goats in East and Central Africa. The closely related Ganjam virus found in India is associated with febrile illness in humans and disease in livestock. Despite the importance of Ganjam virus, a lack of diagnostic tools and genetic characterization have prevented a more precise understanding of the public health and agricultural impact of the virus in India and its relationship with the African NSD virus.

Twenty-three Ganjam virus isolates obtained during 1954 to 2002 from various sources in India were analyzed in this study. The lyophilized virus stocks were revived by intra-cranial inoculation (i.c.) into Swiss Albino mice, and then some stocks were further passaged in Vero or porcine kidney stable (PS) cells. For virus stocks passaged in tissue culture, cytopathic effect (CPE) could be observed on day 4 post infection, and cells were then harvested, centrifuged and supernatant used for viral RNA isolation. Either infected cell supernatant or virus infected mouse brain homogenates were placed in Trizol (Gibco-BRL) and RNA

extracted according to the manufacturer's protocol. Based on the available nairovirus genome sequences, RT-PCR assays have been successfully developed for the detection of Ganjam virus RNA regions encoding the viral nucleocapsid (N) and RNA polymerase (L) gene regions.

Sequences were edited and aligned using the PILEUP (Wisconsin Package Version 10.2, Genetic Computers Group, Inc.), CLUSTAL W and BioEdit software version 5.0.6 (North Carolina State University, USA 1997-2001). Phylogenetic analysis was performed using PAUP\*, version 4.0b10 (Sinauer Associates, Inc., Sunderland, MA, USA 2002). The maximum likelihood method was used for analysis of sequence differences among the 318 bp N gene amplicons using a transversion: transition weighting of 6:1. Bootstrap values were calculated by 500 heuristic search replicates. Previously derived NSD RV082 and Ganjam G-619 virus L and N virus sequences were used in the comparison. Sequence divergence was determined using GCG software (Wisconsin Package Version 10.2, Genetic Computer group, Inc), to calculate the average p distance within groups and between groups.

Amplification and nucleotide sequence analysis of the resulting PCR fragments (N - 394bp) from multiple Ganjam virus strains revealed significant diversity among virus isolates obtained from 1954 to 2002 and from various regions of India. Ganjam virus isolates differed by up to 17% at the nucleotide level for the N gene fragments, respectively, with less variation observed at the deduced amino acid level (10.5%). Phylogenetic analysis of virus sequence differences suggests considerable mixing and movement of Ganjam virus strains within India, with no clear relationship between genetic lineages and virus geographic origin or year of isolation. Surprisingly, NSD virus does not represent a distinct lineage, but appears as a minor variant within the Ganjam virus monophyletic group (Fig.8). These findings are consistent with Ganjam viruses being considered as isolates of NSD virus.



**Figure-8: Phylogeny of nucleocapsid gene from Indian strain of Ganjam virus**

## 11.5 Expressed Sequence Tag analysis of mosquito genome

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### Overview of the projec

This project aims at complementing this effort by performing extensive EST (Expressed Sequence Tag) analysis of *Anopheles stephensi*, which is vector of malaria in India. It is proposed make directionally cloned cDNA libraries from infected and uninfected mid gut of this mosquito and sequence these from both ends.

### Work done

#### Maintenance of mosquito colony and infection of mosquitoes

Mosquitoes were fed either on mixture of defibrinated blood and malaria parasite *Plasmodium yoellii* through artificial membrane feeding method or directly on the parasite infected BALB/c mice depending upon the requirement and number of mosquitoes used for experiments. Before blood feeding parasitemia in the circulating blood of infected mice was determined. Experiments were carried out only when the gametocytes percentages were very high in the peripheral blood. Mosquitoes were dissected in batches for obtaining midguts for standardization of cDNA library work.

#### In vivo maintenance of parasites

*Plasmodium* parasites (viz. *Plasmodium yoellii* and *Plasmodium vinckei*) were maintained in adult BALB/c mice (UK/AIIMS strains) by intraperitoneal inoculation (IP) route. Time for effective parasitemia was determined on various post inoculation day (PID). The heparinized blood samples of the parasite infected BALB/c mice were stored in liquid nitrogen, for further studies. Parasites were in-vivo maintained throughout the study.

## 11.6 Molecular markers for identification of some important mosquito vectors in India and their genetic variabilities in different geographical populations

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### Introduction

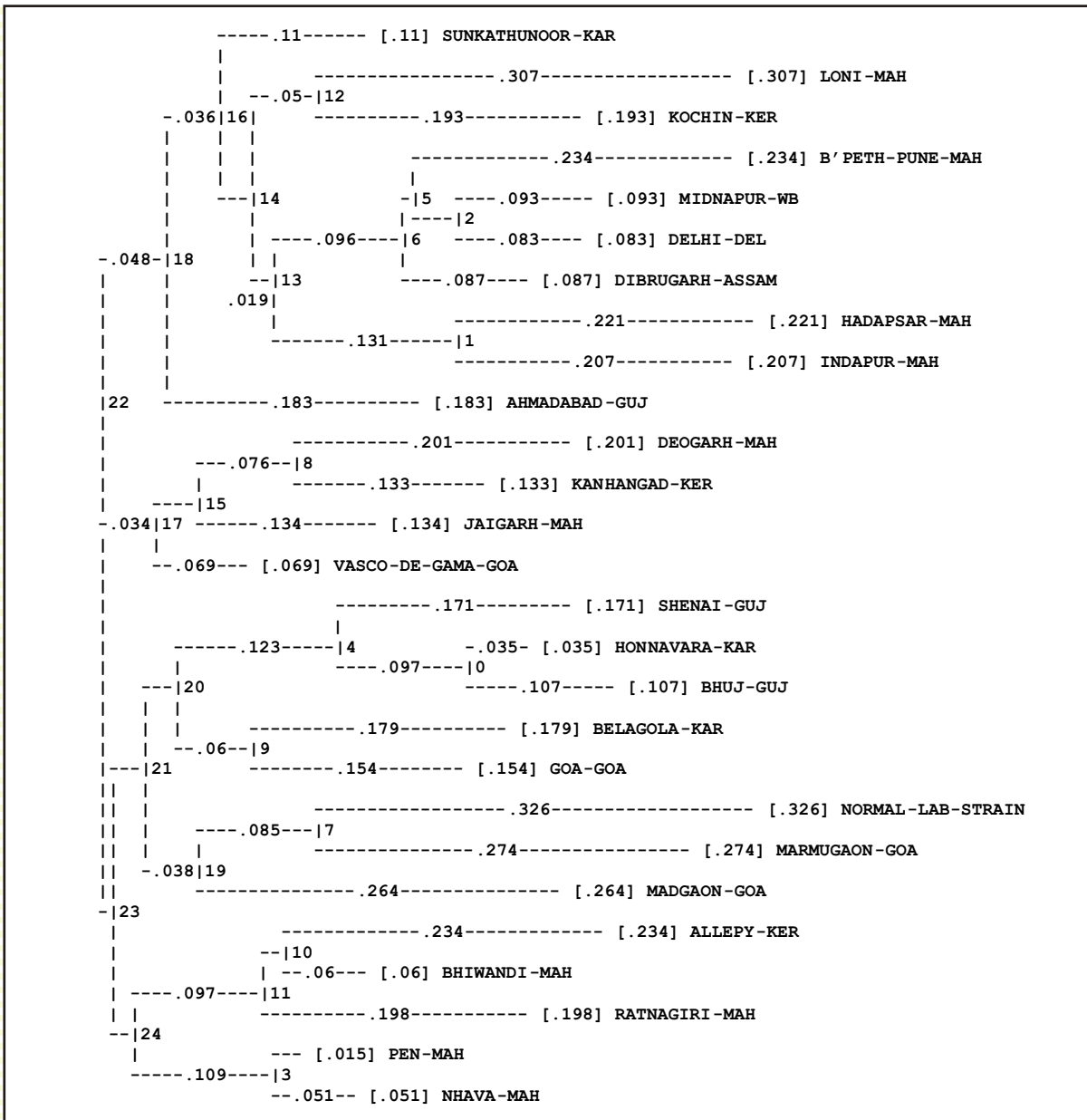
The occurrence of outbreaks of mosquito-borne diseases are mainly linked to the prevalence of the primary mosquito vector and its density. The existing conventional methods do not seem to be adequate, which are being used, for monitoring the vectors. There is a need to develop base line information for molecular and biochemical markers for the important mosquito vectors from various geographical areas.

**Objectives**

- To develop baseline information for molecular and biochemical markers for the mosquito vectors *Aedes aegypti*, from various geographical areas in the country.

**Work done**

Twenty seven strains of *Aedes aegypti* collected from various states were screened-using Random Amplified Polymorphic DNA (RAPD) assay and PCR for gene specific primers from mitochondrial cytochrome-b genes (Fig 9 & 10). Further strains are being analysed.



**Fig 9. Genetic variations in *Aedes aegypti* mosquito populations based on RAPD analysis**

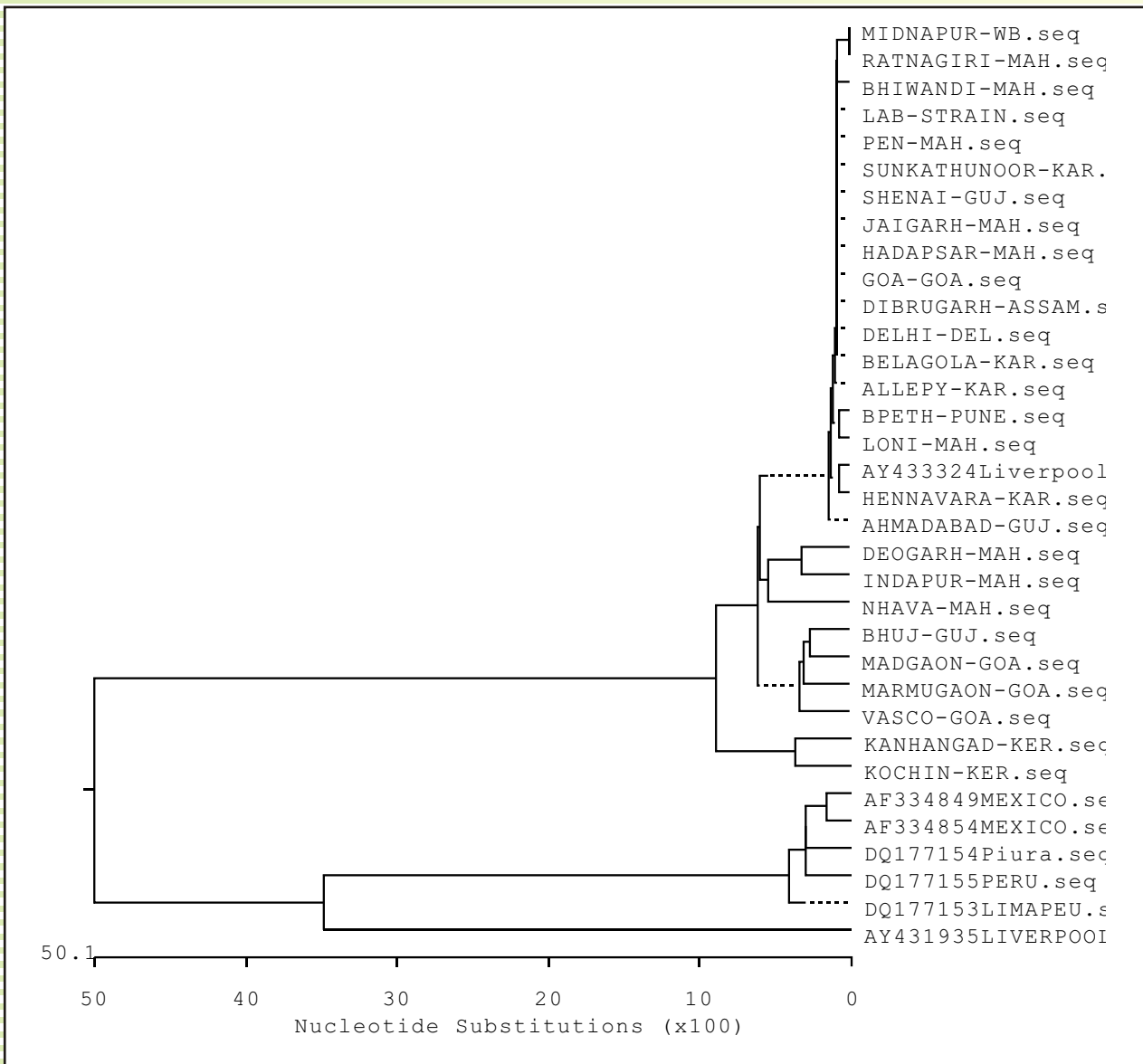


Fig 10. Sequencing of cytochrome-b genes fragment from mitochondria

## 11.7 Preparation of virus pools and immune sera for viral diagnosis

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### A. Preparations of virus pools

#### a) Kaisodi virus

Kaisodi virus, G14132 (KSD) was inoculated in groups of 2-3 day old infant Swiss albino mice for preparation of virus pool and immune serum. Brains of all sick mice were harvested and stored at  $-70^{\circ}\text{C}$  for preparation of virus pool. Kaisodi virus (KSD) G-14132 pool titration was carried out in groups of 2 days old infant Swiss albino mice to determine the pool titre. The pool titre of  $4.8 \text{ LD}_{50}$  was obtained.

#### b) Thotapalayam virus

Thotapalayam virus (66412) was inoculated in groups of 2 days old infant Swiss albino mice for preparation of pool. Brains of all sick mice were harvested and stored at  $70^{\circ}\text{C}$ . Thotapalayam virus (66412) pool was then titrated in groups of 2-day-old Swiss albino mice. Virus dilutions used for the titration were from  $10^{-1.3}$  to  $10^{9.3}$ . The virus titre was  $10^{4.9}$ .

### B. Preparation of immune sera

#### a) Kaisodi virus

Adult Swiss albino mice (3-4 weeks) were immunized with 5 doses of Kaisodi virus (G14132) as 10% infected mouse brain suspension at an interval of one week. All the immunized mice were bled one week after the administration of the last dose. Pooled serum was collected, distributed in small aliquots and stored at  $-200^{\circ}\text{C}$ . The Kaisodi immune serum is being titrated in CF and N tests.

#### b) Thotapalayam virus

Adult Swiss albino mice were immunized at weekly interval with 5 doses of Thotapalayam (66412) virus as 10% infected mouse brain suspension. All immunized mice were sacrificed one week after the administration of the last dose. The immune serum was collected, pooled and aliquoted (1 ml. each) and stored at  $20^{\circ}\text{C}$  for further use. Immune serum against Thotapalayam virus was then titrated against homologous virus in adult Swiss albino mice by i.c route to determine the neutralization index. The immune serum at a dilution of 1:50 neutralized 2.5 logs of virus.

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