Materials and methods
MATERIALS & METHODS

In order to characterise Thai sacbrood virus, various technique used in virology were standardised to study the nature of virus, the particle infectivity and other such properties of the virus. Isolation of the virus is of essence to study these properties. Successful purification of Thai sacbrood virus depends on many factors. All the factors governing the purification of the virus were met and the virus was isolated. The size of the virus particles, the molecular weight of the protein, the localisation of the virus in tissues, the serodiagnostic aspects along with the pathological changes in the host tissue, the colony performance, the symptomology and the intensity of the Thai sacbrood disease were recorded in the present study.

2.1 Study area

Thai sacbrood disease is the first virus disease that played a major havoc to the beekeeping industry in Karnataka. Major beekeeping areas of Karnataka were selected to assess the damage and destruction to the bee colonies by the disease.

The study area comprised of part or whole of Hoskote, Magadi and Dodballapur of Bangalore rural district; Yalahanka, Kumbalgud and Shivanahally of Bangalore urban district; Mudagere and Sakrepatna of Chikamangalur district; Puttur and Sullia of Dakshin Kannad district; Hassan and Sakleshpur of Hassan district; Madikeri, Somvarpet, Virajpet and Bhagmandala of Kodagu district; Kolar and Chikballapur of Kolar district; Kollegal
and B.R.Hills of Mysore district; Shimoga and Bhadravathi of Shimoga district, Tumkur and Chelur of Tumkur district and Sirsi and Kumta of Uttara Kannada district.

A proforma sheet was prepared to record the loss of the colonies during the study period. The data was collected from beekeepers, bee scientists, bee societies, apiaries and bee personnel. Data on the incidence of Thai sacbrood disease for a period of three years starting from May 1994 to April 1997 was collected. The outbreak and spread of Thai sacbrood disease during the test period in the selected twenty-six study centres was recorded. Observations were made on the status of the disease. The relative infection of Thai sacbrood disease in black and yellow strains of *Apis cerana* was also recorded during the period from May 1994 to April 1997 (Appendix A).

### 2.2 Collection of diseased samples

Honeycombs infected with Thai sacbrood disease were collected from various study centres, wrapped in paper covers and were brought carefully to the laboratory for further analysis. The combs were transported as quickly as possible to prevent any larval decay.

### 2.3 Preservation of diseased samples

The larvae were removed from the combs using forceps or matchsticks. Infected larvae were pooled in groups of thirty each and placed in plastic vials. The larvae were preserved at 4 °C for further use. However, the larvae were preserved at −20 °C when they were required for a longer duration.
2.4 Disposal of used samples

Proper disposal of the used diseased samples was done by burning. The spread of the virus was thus prevented.

1.5 Symptoms of Thai sacbrood disease

1.5.1 Identification of colony symptoms

Performance of a healthy colony was noticed and the variation in the performance of the Thai sacbrood diseased colonies based on the hive traffic was determined.

2.5.2 Identification of brood symptoms

The differences exhibited by the brood of the Thai sacbrood diseased colonies in terms of the size, shape and colour at different stages of larval and pupal development were studied.

2.5.3 Identification of adult bee symptoms

Behavioural changes exhibited by the nurse bees, guard bees and forager bees in a Thai sacbrood diseased colony were recorded.

2.6 Purification of Thai sacbrood virus

The method of Bailey et al. (1982) was followed for the purification of Thai sacbrood virus. A total of thirty larvae/pupae were ground in 0.5mg Potassium phosphate buffer with pH 8 under cold conditions. 10-20 mM Diethyl dithio carbamate (DIECA), 5-50 mM Sodium ethylene diamine tetra ascetic acid (EDTA) and one part of Carbon tetra
chloride (CCl₄) were added to prevent the action of polyphenol oxidases and enzymatic activity and also to remove membranes and organelles. The extract was clarified by low speed centrifugation at 15,000 g for 15 minutes for the removal of precipitated host contaminants. The supernatant was taken and used for the high-speed centrifugation at 70,000 g for three hours at 4 °C. The pellet so obtained was re-dissolved in 0.01 M Potassium phosphate buffer and left at 4 °C overnight. 4-5 ml of the virus suspension was layered on a continuous density gradient of 5 percent to 40 percent sucrose. Ultracentrifugation was done at 80,000 g for three hours at 4 °C. Virus band was visualised by top light. The virus band was removed, repelleted again by centrifugation at 24,000 g for one hour. The pellet so obtained was re-suspended in a minimal volume of phosphate buffer and stored at -20 °C in small vials.

2.7 Substrate preparation of grids for electron microscopy

Copper grids of 400 mesh were used for holding the virus particles. The grids were made sticky by means of glue. Formvar coated plastic was used as a substrate for the copper grids. Glass slides dipped in Formvar were dried and the sticky grids were placed on the slide.

2.8 Negative staining

A drop of the virus suspension was placed on the grid and was allowed to absorb for 15 seconds. The grid was rinsed with ammonium acetate buffer and further stained with uranyl acetate. The excess stain was removed by a filter paper and the grid was air-dried. This grid was used for observing the particles under Transmission Electron Microscope (Hitchborn and Hills, 1965).
2.9 **Sample tissue preparation for electron microscopy**

Tissue preparation was done by fixing the Thai sacbrood diseased larvae/pupae, the brain and hypopharyngeal glands of adult worker bees in glutaraldehyde.

Post fixation was carried out for three hours at room temperature in 1 percent osmium tetroxide in phosphate buffer. The post fixed tissue was dehydrated by acetone in graded series of 32 to 100 percent.

Propylene oxide was used for treating the tissue and to help in infiltration. Embedding media of 1:1 ratio of propylene oxide : araldite followed by further embedding in fresh araldite was done and poured in a mould.

2.10 **Ultramicrotomy of the fixed sample**

Sections were cut on an ultramicrotome and put on water surface. Grey coloured and silver coloured sections of about 13 nm thickness were taken, placed on the grids stained with 2 percent uranyl acetate, rinsed with distilled water and post stained with 0.2 percent lead citrate solution. The sections were again washed with distilled water and air-dried. Grids prepared as above were observed under the Transmission Electron Microscope to record the presence of Thai sacbrood virus particles (Gupta *et al.*, 1989).

2.11 **Samples for serodiagnosis**

A total of 250 samples were collected randomly from the study centres. Visual based detection and Enzyme Linked Immunosorbent Assay (ELISA) based detection were employed for the detection of Thai sacbrood virus.
2.12 ELISA for detection of Thai sacbrood virus

Direct antibody coating ELISA (DAC-ELISA) (Hobbs et al., 1987) was used. Preparation of viral antigen was done by taking about 1 gm of larvac, weighing and titrating in presence of carbonate buffer pH 7.4 containing 0.01 M sodium diethyl dithio carbonate as a 10 percent solution (w/v) under sterile conditions. Larvac and pupae of worker bees (1 gm) and drone bees (1 gm) were titurated and the material was centrifuged at 2000g for 10 minutes. The supernatant extract was collected and concentrated by dialysing against 1 percent PEG. The final amount of suspension was diluted in 1 ml of PBS-TPO buffer. Dilution was done to the level that permits reliable detection.

Reference antiserum against Thai sacbrood virus was obtained through the courtesy of Dr. Brenda V. Ball, of Rothamsted Experimental Station, U.K. and was used throughout the study.

Different dilutions of the antibodies (1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400, 1:450 and 1:500) were studied. Background healthy reaction in ELISA test was prevented by absorbing the antibodies with healthy larval protein prepared by grinding the larvae with 0.2 percent DIECA in 0.85 percent saline and centrifuging the suspension at 5000 rpm/8000 g for 10 minutes. Glycerol was added to one volume of the supernatant.

The purified antigen was used as a coating solution. 96 well, polystyrene microtitration plates were used. Coating solution (200 μl) was distributed per well and after an overnight incubation at 37 °C, the plates were washed with deionised water and thoroughly dried.
Controls were also loaded and incubated. The excess of antigen was removed and blocking solution (milk) was filled in the wells. The plates were rinsed and washed 2-3 times leaving the buffer each time for 1-2 minutes in the plate. 200 µl of the diluted antiserum was dispensed in each well and incubated for two hours. Rinsing of the plates was again repeated. Diluted secondary antibody-Goat Antimouse Alkaline Phosphatase (1:1000) (conjugate antibody) was prepared and 200 µl was dispensed in each well. The solution was again incubated for two hours. Rinsing and washing of the plates with PBS-tween was done. 0.2 ml/well of the substrate-chromogen which was prepared freshly in diethanolamine substrate buffer was added and was incubated for half an hour until yellow colour developed. The reaction was terminated by adding 50 µl/well of 3M NaOH. Results were recorded by visual observation and measuring the absorbance in a ELISA plate reader at 405 nm. Twice the absorbance value of the control antigen was the limiting value for the DAC ELISA assay. Statistical analysis to test the significance of ELISA method was done using the hypothesis testing method Z.

2.13 Immunodiffusion for detection of Thai sacbrood virus

The immunodiffusion test or the Ouchterlony test was performed as per the method of Mansi (1958). The test was performed in glass petriplates using purified agarose. 0.8 gms of agarose was melted in 100 ml PBS containing 0.5 percent SDS in boiling water bath. The agarose medium was cooled to about 60°C and was poured in the petriplate. Six wells were made using the templates. Antiserum dilutions were taken with PBS as dilutant. Antigen was prepared by grinding the tissue and clarifying by centrifugation (2000 g / 10 min). 3 percent SDS was added to 1 ml of antigen. The petriplate was taken and after removing the agarose plugs, few microlitres of molten agarose was added to seal the
bottom of the wells. Normal serum and healthy larval extract were used as controls. A programme sheet was prepared to record the values at different dilutions of the antigen and the antibody. The petriplates were incubated at 37 °C in a humid box for 24 to 48 hrs. The precipitated lines were observed with different dilutions of the antigen and antibodies in each well and a record was made.

2.14 Determination of absorbance of Thai sacbrood virus

For measuring the absorbance, one cuvette (a rectangular cell) filled with solution containing virus and another cuvette filled with buffer solution without the virus were inserted in the spectrophotometer. The wavelength dial was set at 260 nm. The blank was positioned in the light beam and the scale was set to read "zero" absorbancy. The absorbance of the cuvette with the virus solution was read. 15 λ fractions of virus suspension was taken and made upto 1 ml with the buffer. Absorbance of each of the solution was read at 260 nm and 280 nm. The absorbance (Λ) indicated log I₀/I where I₀ = log of the ratio of the incident radiation with the blank interposed in the beam and I = the transmitted radiation of the cuvette with the virus solution. The UV absorption of proteins was measured by A₂₆₀/₂₈₀ = 1.4

2.15 Determination of molecular weight of Thai sacbrood virus protein

The procedure of Maniatis et al., (1982) was used for the molecular weight determination of proteins. The separating gel was prepared by the vertical glass slab gel units which were assembled in the casting mode using 1 mm spacers. The separating gel solutions were mixed. APS and TEMED were added just before use by swirling. The solution was poured into the gap between the two glass plates and sufficient space was left
for the stacking gel. Water was poured over the gel to prevent the oxygen diffusing into the gel. After polymerisation, the water was poured out and washed with deionized water. The stacking gel was prepared and APS and TEMED were added just before pouring. Teflon comb was inserted taking care not to trap any air bubbles. The gel was allowed to set for 30 minutes. When the stacking gel was polymerising, the samples were prepared by mixing 15 λ fractions of 1x SDS gel loading buffer. A drop of bromophenol blue was added, heated on a water bath for 5 minutes before loading. A marker protein sample of known molecular weight and the control protein taken from healthy larvae were also prepared the same way for loading.

The combs were removed from the gel and the gel was mounted on the electrophoretic apparatus. Tris-glycine electrophoretic buffer was added to the top and bottom reservoir. 15 μl of the samples was loaded in each well. The unit was connected to the power supply and was turned off when the dye reached bottom. Staining was done for 1 to 2 hours in Coomassie Brilliant Blue and destaining with acetic acid, methanol and water. Gel was transferred to the gel storage solution and photographed.

2.16 Infectivity assay of Thai sacbrood virus

Diseased honeybee larvae were taken from the combs infected with Thai sacbrood disease. Extract of these diseased larvae was prepared by macerating a group of these larvae in water, filtering the suspension through filter paper and finally through a Millipore filter (0.45 μ) to remove all the bacteria. (Hitchcock, 1966). Heat sterilised filtrate and pure water were used for the controls. Infectivity tests were done by feeding the colonies with the virus extract and sugar syrup (1:1) in the initial case and the colonies with sugar syrup
n the later case. Ten colonies were tested against the suspension by making serial dilutions of the viral suspensions and feeding it. The percent Thai sacbrood infection was recorded.

Administration of the filtrate to the individual cells was done by marking a single frame. A small droplet (few μl) of the partially purified suspension was added to the food surrounding each larva by means of a micro syringe. The position of the larvae was identified with a calibrated frame. Alternate cells of the comb were fed with the extract. The control cells were fed with water in one series of experiments and with head-fed extracts in another series of experiments. Larvae in their first, second, third and fourth stages (identified by the size of the larvae) were fed with the extract. A total of 150 larvae were tested under each experiment. This experimental comb was kept back in the hive box and examination of the appearance of disease symptoms was carried out once every two days. The experiment was repeated for a total of twenty colonies.

2.17 Determination of brood rearing activity

The amount of brood including the area of the eggs, larvae and pupae was measured by means of a grid and is expressed in cm². The brood area of normal and diseased colonies was determined fortnightly over a period of three years. The larval area and the pupal area was measured. Based on the values obtained, the percent reduction in brood rearing activity was calculated by using the following formula:

\[(\text{Normal brood area} - \text{Diseased brood area}) \times 100 \div \text{Normal brood area}\]
Determination of the size of foraging population

Foraging activity of *Apis cerana* both in the diseased and normal colonies was compared. A total of ten colonies were selected for the purpose of comparative study of foraging activity. The foraging activity during the peak foraging time between 8.00 hrs and 11.00 hrs and again between 15.00 hrs and 18.00 hrs was studied. The hive conditions of the test colonies were adjusted so that the size of the adult population and the areas of the brood and food source were more or less same for each group of normal and infected colonies.

During the sampling, the hive was temporarily closed for ten minutes and the incoming bees were collected by using a sweep net (25 to 30 bees). The bees with pollen in their corbiculae were classified as pollen foragers and bees with no pollen in their corbiculae were classified as nectar foragers. The honey stomach of each collected specimen was drawn out by pulling the abdomen over No.1 filter paper and the contents were blotted on the paper (Park, 1926). Based on the appearance of the stain, the bees were classified as nectar carriers or water carriers (Erickson *et al.* 1973). Two way analysis of variance (Two way ANOVA) was conducted and the significance of pollen foragers, nectar foragers and pollen and nectar foragers at different stages of diseased condition was observed. The area of brood, pollen and honey in the control and early, later and advanced stages of infection were measured twice a month during the study period using a grid of 1 cm² (Reddy, 1980). The size of the bee population was estimated by the number of frames covered with bees on both the sides.
2.19 Sectioning of Thai sacbrood diseased larvae and pupae

Second and third stage larvae and pupae infected with sacbrood disease were collected and fixed in Bouin's fluid. The larvae were processed in different grades of ascending alcohol for dehydration and were embedded in paraffin wax (58° – 60° melting point) for two hours in two changes. Paraffin blocks were prepared and serial sections were cut at a thickness of 5 – 6 μ using Heitz rotary microtome. Transverse sections were taken from the anterior regions, middle regions and posterior regions. The paraffin sections were deparaffinised in two changes of xylene and descending grades of alcohol. The deparaffinised sections were stained in haemotoxylin eosin stain and subjected to histopathological observations (Weesner, 1968).