MATERIALS AND METHODS
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SELECTION OF PATIENTS

From May 1996 to Aug 1998 a total of 3252 patients with corneal ulcers were subjected to detailed investigation at the Regional Institute of Ophthalmology and Govt. Ophthalmic Hospital Chennai (Madras). The criteria for suspecting *Acanthamoeba* keratitis are as follows: Ring infiltrates of the cornea associated with intense pain disproportionate to clinical signs, moderate lid oedema with little chemosis, absence of response to antimicrobials and ring ulcers with a crescentric notch.

Under a collaborative project with Aravind Eye Hospital and Post Graduate Institute of Ophthalmology, from 1994 –1998. *Acanthamoeba* cultures and serum samples were obtained from *Acanthamoeba* keratitis cases. Detailed histories of all keratitis cases were collected.

Sample collection

Corneal scrapings were collected aseptically from patients with keratitis. One portion was examined under KOH for the presence of cysts other portion was used for culture. Blood samples were collected aseptically and allowed to clot. Serum was separated and stored at -20°C until use.
Blood agar, Nutrient agar and Brain heart infusion broth were used for bacterial isolation. Sabouraud's dextrose agar was used for fungi and Non nutrient agar for *Acanthamoeba*.

**Culture of *Acanthamoeba* spp.**

**Plate Culture**

1.5% agar plate was prepared and overlaid with *E.coli* (ATCC 25922), to culture *Acanthamoeba* spp. The samples were directly inoculated on to the surface of the plates. The growth of the amoebae was observed from 48 hours onwards.

**Slide culture**

On to a 25mm x 75mm microscopic slide, 2-3ml of the of 2% non-nutrient agar was poured aseptically and allowed to solidify. Then a thick suspension of *E.coli* was smeared on to the surface. These slides were placed in a sterile 75ml screw capped polypropylene container to prevent drying [Plate 1]. The corneal scrapings were directly inoculated on to the surface of the agar and incubated at 37°C, and after 24- 48 hours, the slides were directly examined in a microscope under aseptic conditions for the presence of trophozoites / cysts of *Acanthamoeba* species. The incubation was continued for 21 days, if no growth was observed, it was considered as negative (Plate 1).

**A study on the Antibody response in patients with corneal ulcers**

At random 200 serum samples from patients with corneal ulcer were collected along with the history of the patients. These included cases of Bacterial,
PLATE I

SLIDE CULTURE FOR ACANTHAMOEBA

Slide with 2% Agar
E. coli suspension
Plastic container
fungal, viral and *Acanthamoeba* keratitis. Serum samples were collected from culture proven cases of *Acanthamoeba* keratitis from Chennai and Madurai. All the samples were tested by indirect haemagglutination test to find the antibody response for *Acanthamoeba* spp.

### Isolation of *Acanthamoeba* for experimental study

A clone of the *Acanthamoeba* isolate derived from a single cell was used for the experimental study. *Acanthamoeba* suspension was diluted in such a way that 5-8 cells were seen on an average of 10 fields. Two hundred microliter of this diluted suspension was poured over a non-nutrient agar plate and incubated at 37°C for one hour. An agar block that contained single amoeba was cut under a microscope a transferred to a fresh plate and the resultant clone was used for further work.

### Preparation of *Acanthamoeba* suspension

1.5% non-nutrient agar (NNA) plates overlaid with *E.coli* served as medium for the growth of *Acanthamoeba* spp. To get the maximum yield of *Acanthamoeba* in plate culture the plates were inoculated and kept inside a chamber for 14 days at 37°C. The 14 day old cultures were flooded with sterile distilled water and *Acanthamoebae* scrapped off from the surface of the agar. The harvested suspension was washed thrice in sterile distilled water by centrifuging at 1500 rpm for 10 minutes.
**Purification of *Acanthamoeba* Cysts**

To get pure viable cyst the *Acanthamoeba* suspension was treated with 1N HCl for 30 minutes. After the treatment the suspension was washed 4 to 5 times in sterile distilled water. Then the pH was adjusted to 7.0 – 7.2, and the suspension was washed twice in distilled water. By sub-culturing the purified cysts on NNA the viability of the cysts was tested. A drop of the suspension was inoculated on Nutrient agar and Blood agar to check the presence of any viable bacterial cells. When the cysts grew in NNA plates and no bacterial growth was observed on nutrient agar and blood agar the *Acanthamoeba* suspension was used for experimental study.

**Exflagellation Experiment**

All *Acanthamoeba* species do not possess a flagellated trophozoite stage in their life cycle whereas the *Nagleria* species have this stage. In order to differentiate *Acanthamoeba* from *Nagleria*, Exflagellation experiment was carried out on all *Acanthamoeba* species. *Acanthamoebae* were grown on non-nutrient agar for 2-3 days and harvested. A suspension was made with distilled water in a sterile glass tube and mixed well. A drop from the above suspension was placed on a clean sterile glass slide and covered with a cover glass. The edges were sealed with nail polish and the set up was placed inside a moist chamber at 37°C for 2-3 hours. The tube with the suspension was also incubated in the moist chamber. Periodically the slide and a drop of the suspension were observed for the presence of free swimming flagellates.
Experiments to study the growth Pattern of *Acanthamoeba* on NNA

A standard inoculum of 50 microliter of *Acanthamoeba* with $10^7$ cysts was inoculated to NNA plates seeded with *E.coli*. The cysts were inoculated on day 0 and evenly spread over the plate. Observations were made from day 1 to day 15 to document the process of excystation, precysts and cyst formation over a period of time. A sterile 2mm punch of the agar surface was cut off from the plate for observation and the results were recorded. The experiment was performed at three different occasions with representative samples from GroupI, GroupII and GroupIII of *Acanthamoeba* spp. and the mean values were recorded.

**Temperature tolerance test**

It is reported by Ma et al.\(^1\) that the pathogenic strains of Acanthamoeba can grow at elevated temperature (42°C). In order to find out the growth pattern of the clinical isolates, the temperature tolerance test was done. 120 samples of *Acanthamoeba* spp. isolated from corneal ulcers were subjected to growth at different temperatures. A standard inoculum of 20μl of the *Acanthamoeba* containing 10,000 amoebae was placed on the center of the plate. The plates were incubated at 4°C, 25°C, 37°C and 42°C. Observations for the growth of *Acanthamoeba* was made 25mm away from the inoculation site and the plates were observed for a maximum period of 14 days. The test was done in triplicates and growth on two of the plates was taken as positive.
Morphometry of *Acanthamoeba*

The morphometry was done to classify the *Acanthamoebae* based on the description of Singh et al. and Visveswara. The characteristic features of 35-40 cysts were observed and measured on three different occasions and the mean values were recorded. The following features were observed: The size of the cysts, the characteristics of ectocyst whether they are smooth/wrinkled/mammillated or rippled, and the characteristic feature of endocyst such as round/oval/stellate/triangular/polygonal or irregular were observed. The distance between the endocyst and ectocyst was measured and the presence/absence of Operculum recorded.

Animal experiments

All the animals used in our study were obtained from the Department of Laboratory Animal Medicine, Tamil Nadu Agriculture and Veterinary Science University, Madhavaram, Chennai.

The investigations with all the animals were performed in accordance with the ARVO Resolutions on the use of animals in Research and compiled with Declaration for a project under the Council of Scientific and Industrial Research (CSIR), New Delhi.

The animals were fed with commercially available pellets supplemented with fresh vegetables, soaked cereals and clean water. The animals were maintained in the departmental animal house throughout the period of study.
Antiserum for *Acanthamoeba* in Rabbit

Healthy Outbred Newzealand white Rabbits weighing 1.5 - 2.0 Kg were selected. A suspension of $10^7$ cysts/ml was used to prepare the whole cell antigens. The first dose of antigen in complete Freund’s adjuvant was injected intramuscularly at different region of the thigh. Booster dose of the antigen was given with incomplete adjuvant. The rabbits were bled at regular intervals. Antibodies were raised against three different groups of *Acanthamoeba* spp.

The mouse pathogenicity test

Outbred, 10 to 14 days old Swiss Webster mice of either sex were anesthetized with ether and 20μl of *Acanthamoeba* suspension containing 10,000 amoebae was instilled into the nostril of each mouse using a 27G hypodermic syringe or using micropipette. The mice were fed with commercial feed and soaked cereal and were inspected every day for the signs of the disease, until they died or were sacrificed. Representative sections of brain, lung, eye and liver were subjected to impression smears on non-nutrient agar plates and fixed in 10% formalin for histopathology.

Experimental *Acanthamoeba* keratitis in a Rabbit model

Healthy out bread 6-8 weeks old Newzealand white Rabbits were used for this experiment. Eyes were examined before the experiments to exclude animals with preexisting lesions and anomalies.
The experimental animals were anaesthetized with pentobarbitone sodium (40mg/kg). 10% xylocaine was used for local anesthesia for the experimental eye. On the 0 day 0.01 ml of *Acanthamoeba* suspension, containing $10^7$ amoeba/ml was injected into the cornea using a 27G hypodermic needle. The inoculation was made slightly away from the center of the cornea, and the other eye of the animal served as a control. 30 strains of *Acanthamoeba* were selected at random and 3 animals per strain were used for this study.

On day 3,7,14,21 and 28 the animals were anaesthetized, the corneal scraping was taken for culture and the lesion produced was measured in a microscope fitted with ocular micrometer and photographed in frontal views. An infection index was calculated using the following formulae.

\[
\text{Percentage Infection Index} = 100 \times \frac{\text{Diameter of the Lesion}}{\text{Diameter of the Cornea}}
\]

Blood samples were collected on day 7, 14 and 21 for immunological study. Rabbits were then sacrificed with intravenous injection of pentobarbitone sodium and corneal tissues were subjected to culture and histopathological examination. Procedures for these experiments were based in part on earlier experiments by Font et al.\textsuperscript{134,135} Schlaegel and Culbertson\textsuperscript{136}.

**Immunological studies.**

Both Humoral and cell mediated immune responses were studied.
Humoral immune response

Humoral immune response was studied by measuring the antibodies by indirect hemagglutination assay (IHA), using pyruvic aldehyde fixed sheep RBC sensitized with the whole cell antigens of the three different groups of Acanthamoeba. The detailed procedure\textsuperscript{137} is as follows.

Pyruvic aldehyde fixation of SRBC

Sheep blood collected in Alsever’s solution was washed three times in physiological saline and finally made up to 50% packed cell suspension in saline. Four milliliters of pyruvic aldehyde was added to 12ml of 1.7% sodium chloride and the mixture was adjusted to pH 7.0 by the addition of about 35ml of 1% sodium carbonate solution. To this 7ml of Sorenson’s phosphate buffer pH 8.0 was added. 10ml of red cell suspension was then added to the pyruvic aldehyde preparation and the mixture was left at 4°C for 24 hours with occasional agitation. At the end of this period the cells were washed three times in saline and resuspended to approximately 10% packed cell volume in phosphate buffer with pH 7.2 containing 0.1% sodium azide. They were subsequently stored at 4°C.

Preparation of antigen coated SRBC\textsuperscript{137}

Fresh or preserved cells were washed three times with physiological saline or PBS and then packed at 750 x g for 15 minutes. 0.6 ml of the packed cells were pipetted into each of the two bottles and suspended in 10ml of PBS. 10ml of tannic acid (1:20,000) was added to the cell suspension in each bottle and the content of each bottle was mixed well. The bottles were incubated for 15
minutes at 37°C in a water bath. Both the bottles were centrifuged for 5 minutes at 750 x g to bring down the cells and the supernatant was removed and the cells were resuspended and washed once again with about 20 ml of PBS. Finally they were suspended in 10 ml of PBS in each bottle. One bottle was set aside and used for controls and absorption. The other 10ml of RBC’s in PBS were coated with the antigen.

The Acanthamoeba whole cell antigen 2mg/ml was added to 10ml of PBS containing RBC. After gentle mixing, this bottle was incubated at 37°C for 30 minutes in a water bath. The cells in both bottles were spun down and the volume in the bottle of control was made up to 20ml with PBS in order to balance the centrifuge. After the removal of the supernatant, the contents of each bottle were suspended in 20ml of PBS containing 1% heat inactivated and absorbed normal rabbit serum. These antigens coated, sensitized cells were washed three times and resuspended to 50ml in serum saline. For the test, this 1% suspension was used.

**Test sera absorption and the test**

The absorption of serum was done by making an initial 1:10 dropwise dilution of the serum with 1% suspension of uncoated tanned red cells. The mixture was incubated at room temperature for 30 minutes. It was then centrifuged and the supernatant was collected. Then serial dilutions were made from the supernatant. 0.5ml of the sensitized cells were added to each serum dilution. The test was most conveniently carried out in the wells of micro titer agglutination plates. A control consisting of serum at the initial dilution and tanned but uncoated
cells was set up for each titration. Controls with coated and uncoated cells in saline were also prepared for each group of the tests. The plates were incubated at 37°C and the readings were taken after 2-4 hours.

**Cell mediated immune response (CMI)**

The cell-mediated immune response was evaluated by means of leukocyte migration inhibition test (LMI) as described by Soborg\textsuperscript{138} and as modified by Sundararaj\textsuperscript{139,140} and Agarwal\textsuperscript{141,142}, using the antigen extract. These antigens were suitably diluted in complete Earl’s minimum essential medium (MEM) containing penicillin 100 units per ml, streptomycin 100µg per ml and 10% heat inactivated bovine serum so that the concentration was 50µg of protein per ml of the final medium used in the LMI test.

**Leukocyte migration inhibition test**

A leukocyte rich fraction of buffy coat was collected after centrifugation of blood at 2000 x g for 20 minutes. Washing counting and dilution of leukocytes were done following the standard procedure. These cells were drawn into micro capillaries (0.9mm internal diameter and 110mm in length), one end of which was sealed and centrifuged at 1500rpm for 2 minutes. The capillaries were cut at the cell fluid interface and the portion containing cells were fixed with sterile silicon grease in a locally made plastic ring chamber.

The chambers were placed horizontally in moist containers and the cells were allowed to migrate for 24 hours at 37°C. The area of migration was traced on a paper with Camera Lucida and measured with a planimeter. Averages of 6-8
capillaries were taken and the percentage of migration was calculated using the following formula\textsuperscript{143}.

\[
\text{Percentage of migration} = 100 - \frac{100 \times \text{Mean area of migration with antigen}}{\text{Mean area of migration without antigen}}
\]