Understanding *Acanthamoeba* Keratitis in India: A Microbiological, Cellular and Molecular Approach

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PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled " **Understanding *Acanthamoeba* Keratitis in India: A Microbiological, Cellular and Molecular Approach**" and submitted by **Pasricha Gunisha** ID NO **2001PHXF030** for award of Ph. D. Degree of the Institute, embodies original work done by her under my supervision.

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PUBLICATIONS

LIST OF PRESENATATIONS AND AWARDS

ABBREVIATIONS

µg	: Microgram
µl	: Microliters
µM	: Micromolar
AA	: Anti-*Acanthamoeba*
AB	: Anti-bacterial
AF	: Anti-fungal
AIDS	: Acquired immunodeficiency syndrome
AK	: *Acanthamoeba* keratitis
ASA.S1	: *Acanthamoeba* specific amplimer
ATCC	: American type culture collection
AV	: Anti-viral
BCL	: Bandage contact lens
bp	: Base pair
C12MDP-L1P	: Liposomes containing dichloromethylene diphosphonate
CCMB	: Center for Cellular and Molecular Biology
CD	: Cluster designation
CFW	: Calcofluor white
CHx	: Chlorhexidine
CI	: Confidence interval
CLW	: Contact lens wear
CNS	: Central nervous system
CPE	: Cytopathic effect
CS	: Corneal scrapings
DAB	: 3 3' Diaminobenzidine tetrahydrochloride
DF	: Diagnostic fragment
dNTPs	: deoxy Nucleotide Triphosphate
EMBL	: European Molecular Biology Laboratory
ER	: Endoplasmic reticulum
FF	: Fungal filaments
FISH	: Fluorescent in situ hybridization

FN : False negative
FP : False positive
GAE : Granulomatous amoebic encephalitis
GNB : Gram negative bacilli
GPC : Gram positive cocci
GSP : Group specific probe
HBsAg : Hepatitis B surface antigen
HIV : Human immunodeficiency virus
HLA-DR : Human leukocyte antigen – DR
HSV : Herpes simplex virus
IFA : Immunofluorescent assay
KOH : Potassium hydroxide
KTZ : Ketoconazole
LSU : large subunit
LTFU : Lost to follow up
LVPEI : L V Prasad Eye Institute
mAbs : Monoclonal antibodies
MCC : Minimum cysticidal concentration
$MgCl_2$ Magnesium chloride
MIP-2 : Macrophage inflammatory protein 2
n : Number
NADPH :Nicotinamide adenine dinucleotide phosphate
ND : Not done
NJ : Neighbor joining
nm : nanometer
NNA : Non-nutrient agar
NPV : Negative predictive value
PBS : Phosphate buffer saline
PCR : Polymerase chain reaction
PDA : Potato dextrose agar
PHMB : Polyhexamethylene biguanide
PK : Penetrating keratoplasty
pM : picomole

PPV	: Positive predictive value
PVI	: Povidone Iodine
PYG	: Proteose peptone yeast glucose broth
rDNA	: ribosomal DNA
RFLP	: Restriction fragment length polymorphism
rns	: Ribosomal mitochondrial sequences
Rns	: Ribosomal nuclear sequences
SDA	: Sabouraud dextrose agar
srRNA	: small ribosomal subunit RNA
SSU	: small subunit
St	: Steroid
ST4P	: Sequence type T4-specific probe
TA	: Tissue adhesive
TAE	: Tris Acetic acid EDTA
TNF α	: Tumor necrosis factor α
TUNEL	: Terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling
TSC	: Tandem scanning confocal
UHF	: Unidentified hyaline fungus
UK	: United Kingdom
UPGMA	: Unweighted pair group with arithmetic mean sequential clustering program
US	: United States
UV	: Ultra violet rays

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Diseases affecting the cornea are a major cause of blindness worldwide, next to cataract in overall importance (Thylefors *et al* 1995). The prevalence of corneal diseases varies from country to country and from one population to another. The epidemiology of corneal blindness is complicated and encompasses a wide variety of infectious and inflammatory eye diseases that cause corneal scarring, which ultimately leads to functional blindness. Of all the indications of corneal transplantations in India, infectious keratitis and its complications rank the highest (26.8%), which is probably underreporting owing to the diagnostic difficulties involved (Dandona *et al* 1997). Comprehensive studies addressing various aspects of infectious keratitis such as epidemiology, etiology and pathogenesis are lacking from India. Though reports of specific etiologic agents like fungus and bacteria in infectious keratitis are available for years in India, the fact that *Acanthamoeba*, a protozoan, is responsible for causation of infectious keratitis is a relatively recent development.

The aim of this thesis was to document epidemiological features and risk factors of *Acanthamoeba* keratitis (AK). It also aimed at evaluating clinical features and conventional microbiological techniques for diagnosis of AK and also developing an assay that is sensitive and aids rapid and easy detection of *Acanthamoeba* in clinical specimens. We also, attempted to gain insight into pathogenesis of AK and taxonomic position of *Acanthamoeba* isolates from keratitis patients in India. A

review of literature of the type and nature is given to provide rationale for the aims of this thesis.

1.1 *ACANTHAMOEBA*: THE ORGANISM

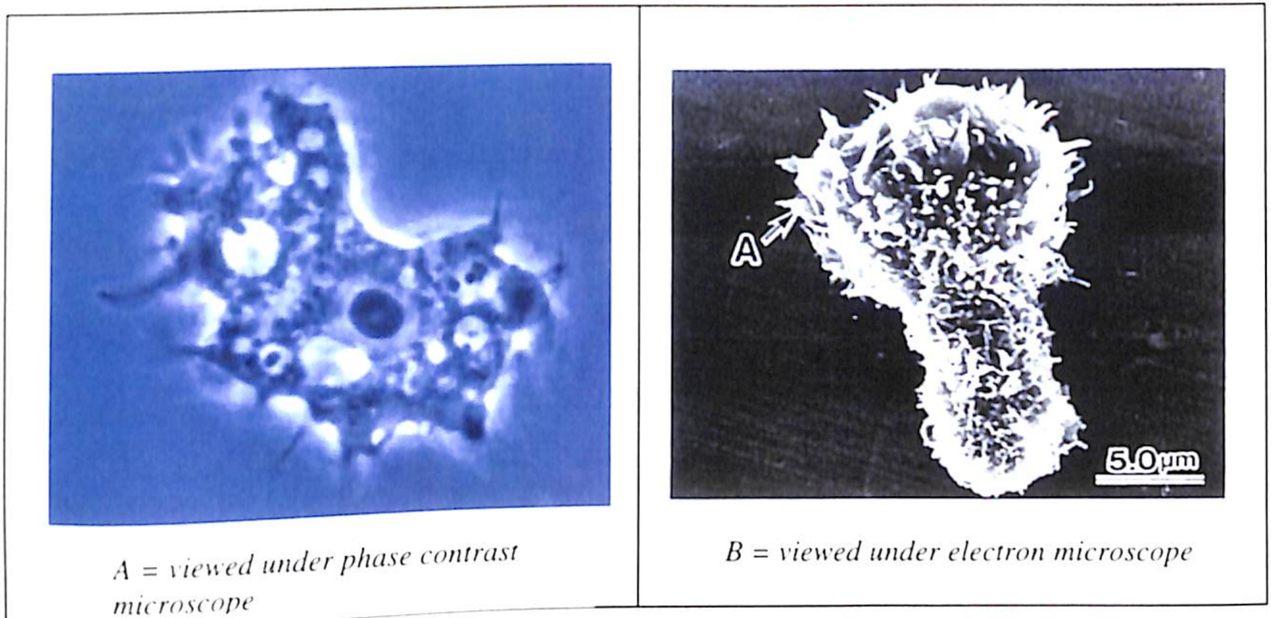
Rosel Von Rosenhof first described free-living amoebae in 1755 (Rosel von Rosenhof 1755) and later Dujardin (1841) found numerous limax amoebae (the term "limax" was used for small amoebae with sluggish movement) from water samples collected from the river Seine in France. In the early 1900s, accurate descriptions of so called limax or sluglike, amoebae were made by Vahlkampf (1905), Naegler (1909), Hartmann (1910) and other researchers. *Acanthamoeba* was first isolated and described in 1913 and it was not considered to have pathogenic potential (Puschkarew 1913). In 1930, Castellanii discovered that *Acanthamoeba* could grow in bacterial and fungal cultures and this observation led to methods for culturing free-living amoebae (Castellanii 1930). Douglas placed this amoeba in the genus *Hartmanella* and named it *Hartmannella castellanii* in 1930 (Douglas 1930) and later Volkonsky created the genus *Acanthamoeba* in 1931 (Volkonsky 1931). In 1961, Culbertson demonstrated that *Acanthamoeba* were pathogenic since they produced cytopathic effect (CPE) in cultures of monkey kidney cells (Culbertson *et al* 1958; 1959).

Acanthamoeba has been classified as (Corliss 1998):

Kingdom	: Protista
Subkingdom	: Protozoa
Phylum	: Sacromastigophora
Subphylum	: Sarcodina
Superclass	: Rhizopoda
Class	: Lobosea
Subclass	: Gymnamoebia
Order	: Amoebida
Suborder	: Acanthopodina
Family	: Acanthoamoebidae
Genus	: *Acanthamoeba*

1.1.1 Morphology and life cycle

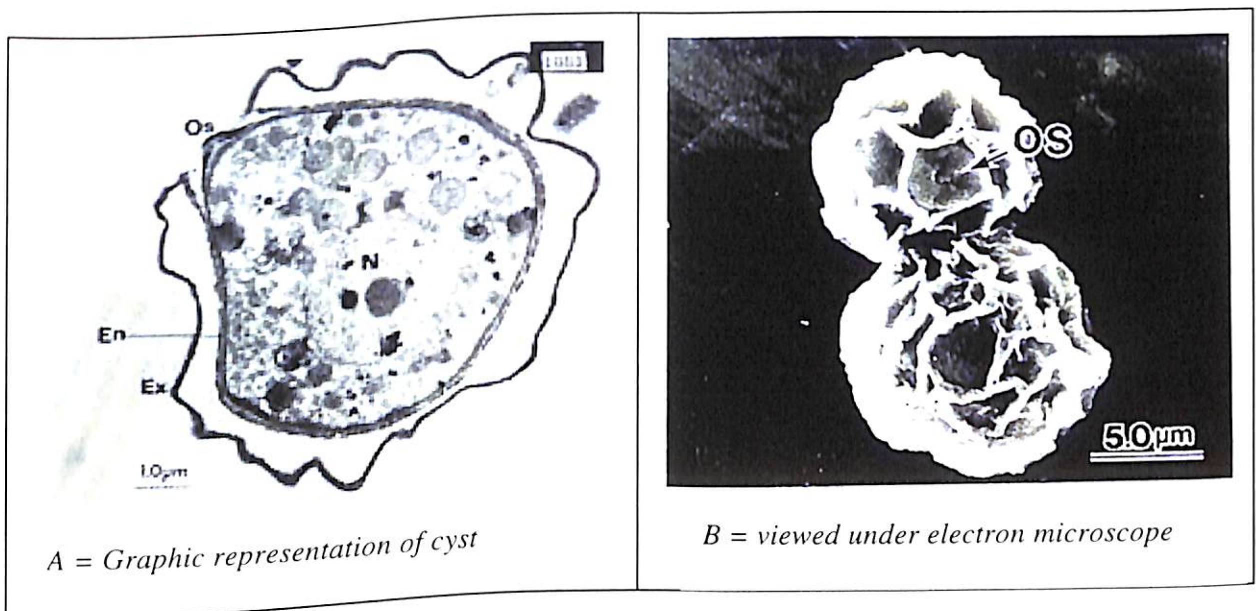
The life cycle of *Acanthamoeba* comprises of two distinct stages, an actively feeding, dividing trophozoite (Figure 1.1) and a dormant cyst (Figure 1.2). The trophozoites vary in size depending on the species between 25-40μm in length (Armstrong 2000). They are irregular ir shape, uninucleate (Jones 1986), flat, aerobic and sluggishly motile (Armstrong 2000). Motility is polydirectional (Visvesvara 1991) and during the movement of trophozoites, a hyaline pseudopodium slowly extends from the amoeba and when moving on a surface, small processes called filopodia extend between the amoeba and the surface that play a role in mobility (Illingworth *et al* 1998). Also, many thin processes called acanthopodia (Figure 1.1B) project from the body and their purpose is not clearly understood (Illingworth *et al* 1998). It has a central cytoplasmic contractile vacuole, the function of which is to expel water (Illingworth *et al* 1998). The trophozoites phagocytose food and any other small particles into cytoplasmic vacuoles, which was demonstrated experimentally using latex beads (Armstrong 2000). Cell division is by mitosis, during which the nuclear membrane, nucleolus and nucleus disappear. The nucleus is characterized by a large central nucleolus and nuclear membrane without chromatin granules. These features enable differentiation of members of the genus *Acanthamoeba* from those of the genus *Entamoeba* such as *E.histolytica*, on histological examination. Other intracellular features of the genus *Acanthamoeba* include mitochondria, cytoplasmic fat globules, centriole like bodies and distinctive water regulatory, contractile vacuoles (Armstrong 2000).



A = viewed under phase contrast microscope

B = viewed under electron microscope

Figure 1.1: Acanthamoeba trophozoites
Courtesy Dr M Nagata (http://protist.i.hosei.ac.jp)



A = Graphic representation of cyst

B = viewed under electron microscope

Figure 1.2: Acanthamoeba cyst
Courtesy Dr M Nagata (http://protist.i.hosei.ac.jp)

Depletion of the food source and other adverse conditions such as desiccation, extreme heat, extreme cold and assault by antimicrobials and chemicals, result in encystment (Armstrong 2000). Cyst is double walled, 10-25μm in size (Figure 1.2A), with a wrinkled outer wall (exocyst) and a stellate polygonal inner wall (endocyst). The cyst wall consists of polysaccharide, one third of which is cellulose (Armstrong 2000). The two walls meet at several places giving it a polygonal appearance and pores or ostioles (Figure 1.2A and 1.2B) are evident at the junction of the walls. Each pore is closed by a plug called as the operculum (Visvesvara 1991), which is made up of mucopolysaccharide and is a target for acanthamoebicides. The purpose of the ostiole is to maintain communication with the outside environment (Armstrong 2000). The cyst is resistant to freezing, desiccation, standard chlorination of water supplies and a variety of antimicrobial agents (Jones 1986). It can also survive exposure to temperatures between -20°C and + 42°C, sodium chloride concentration of 0.85% (Auran *et al* 1987) and a pH range of 3.9-9.75 (Armstrong 2000). Cysts may remain viable for many years until it is exposed to a food source, when it again assumes the trophozoite form. It is not known or understood how the cyst recognizes a renewed food source, although it readily excysts in the presence of both liquids nutrients and bacteria.

Cells typically are uninucleate, but nuclear division is relatively easily uncoupled from the cytoplasmic division e.g., by growth in suspension cultures, in which multinucleated cells are often found. Nuclei also can undergo amitotic divisions that result in nonviable progeny. Most typical duration of the total cell cycle ranges from ~ 6-12 hours (Byers *et al* 1991). *Acanthamoeba* can be transmitted by insect vectors

including cockroaches and flies (Auran *et al* 1987). *Acanthamoeba* are carriers of intracellular bacteria, especially *Legionella* species, which have the ability to reproduce within the trophozoites. It has been proposed that this may be of importance in the persistence and spread of these organisms in the environment (Illingworth *et al* 1998).

1.1.2 Ecology and distribution

Acanthamoeba (Acanth = spine or thorn) are free-living amoebae. They are ubiquitous throughout the world and are among the most prevalent protozoa found in the environment. (Mergeryan 1991; Rodriguez-Zaragoza 1994). They have been isolated from soil, dust, air, natural and treated water, seawater, swimming pools, sewage, air-conditioning units, domestic tap water, drinking water treatment plants, bottled water, eyewash stations, contact lenses and their cases and as contaminants in bacterial, yeast and mammalian cell cultures (Castellani 1930; De Jonckheere 1991; Jahnes *et al* 1957; Kingston and Warhurst 1969; Rivera *et al* 1987;Mergeryan 1991; Michel *et al* 2001; Paszko-Kolva *et al* 1991). *Acanthamoeba* spp. have been isolated from vegetation, animals including fish, amphibia, reptiles and mammals (Dykova *et al* 1999; Sesma 1988; Sesma *et al*, 1989; Walochnik *et al* 1999), from the nasal mucosa and throats of apparently healthy humans (Cerva *et al* 1973; Newsome *et al* 1992), from infected brain and lung tissue, from skin lesions of immunosuppressed patients and from corneal tissue of patients with AK (Lalitha *et al* 1985; De Jonckheere *et al* 1991; Martinez and Visvesvara 1997). Thus, *Acanthamoeba* is inhabitant of two different niches; as phagotrophs in aquatic habitats where they feed on bacteria and as opportunists where they infect the eye and the central nervous system (Armstrong 2000). *Acanthamoeba* is also described as amphizoic (Gr. Amphi

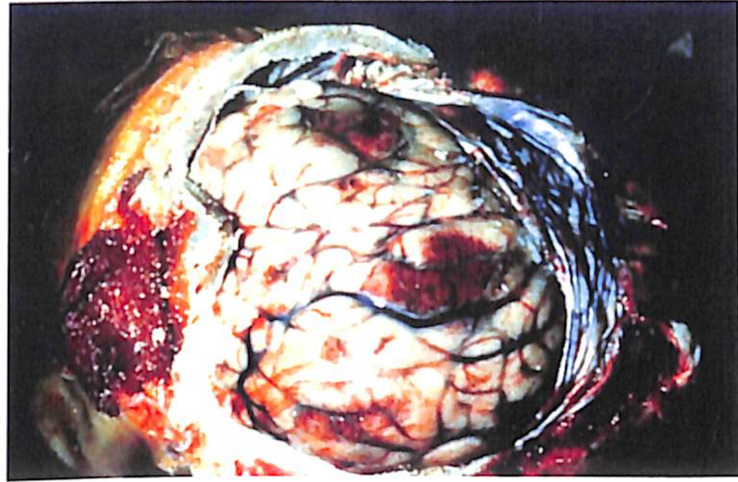
on both sides) i.e., the ability of amoeba to exist both as free-living organisms and endoparasites (Armstrong 2000).

1.1.3 Infections caused by *Acanthamoeba*

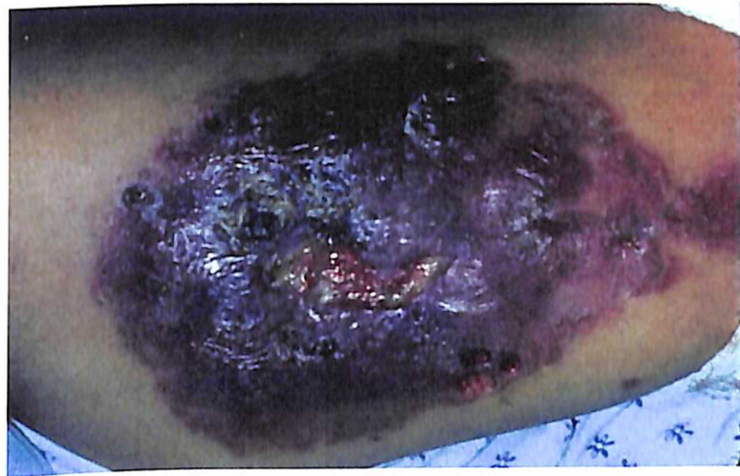
Acanthamoeba infections in humans are infrequent despite their ubiquitous presence in the environment (Auran *et al* 1987). The two main populations at risk from *Acanthamoeba* infections are at the two extremes of health. At one end of the spectrum there is normal healthy contact lens wearer or an individual with mild trauma to the cornea and at the other end is malnourished or immunosuppressed patient (Armstrong 2000). *Acanthamoeba* keratitis (AK) is result of an accidental trauma to the cornea; however, most cases are associated with contact lens wear. Granulomatous amoebic encephalitis (GAE) is a rare but fatal infection usually seen in AIDS patients. It is expected that with the rise in contact lens wear together with the escalating spread of AIDS, the incidence of *Acanthamoeba* infections will continue to increase (Armstrong 2000).

1.1.3.1 Non-ocular infections

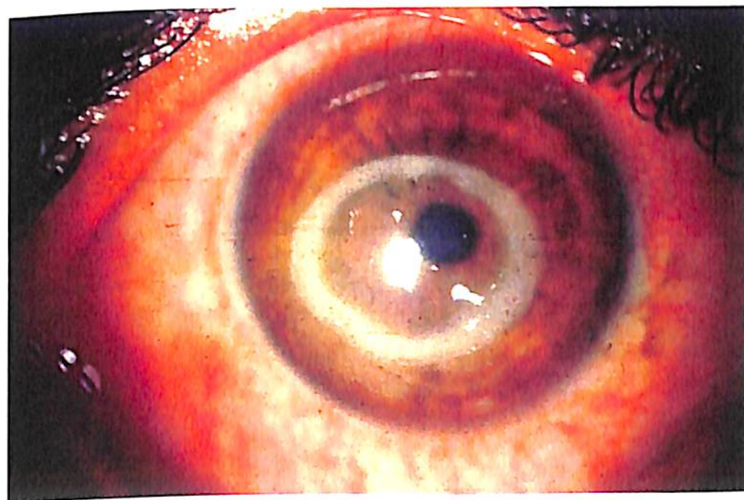
GAE is a sub-acute infection, which generally, but not always, occurs in debilitated or immuno-compromised individuals (Auran *et al* 1987). GAE is characterized by a chronic protracted slowly progressive CNS (Central nervous system) infection (Duma *et al* 1978). *Acanthamoeba* is found in the CNS in both trophozoite and cyst form and characteristically produces nectorizing granulomata with multinucleated giant cells, accompanied by a panarteritis with amoebic invasion of the vessel wall (Auran *et al* 1987). Focal neurological defects progress over days or weeks resulting in diffuse meningoencephalitis (Figure 1.3A) and death (Armstrong 2000).



A: Brain tissue (autopsy) in GAE



B: Skin ulcer (gross)



C: Ring shaped corneal infiltrate in AK

Figure 1.3: Infections caused by Acanthamoeba

A and B: Courtesy Dr W Keith Hadley (http://labmed.ucsp.edu)

First case of *Acanthamoeba* infection in AIDS patients was reported in 1986 (Gonzalez *et al* 1986), since then increasing number of cases of disseminated *Acanthamoeba* infection have been reported in individuals with AIDS (Marciano-Cabral and Cabral 2003). Most of these infections are diagnosed postmortem. It has been postulated that impairment of the host defense mechanisms in immuno-compromised individuals results or contributes to the infection which can spread from the primary site of infection to other organs and tissues (Marciano-Cabral and Cabral 2003). Infections in AIDS patients include GAE (Gardner *et al* 1991), chronic sinusitis (Kim *et al* 2000a) and cutaneous lesion with *Acanthamoeba* present in sinus lesions and skin ulcers (Bonilla *et al* 1999). These skin lesions (Figure 1.3B) are most often the presenting manifestation of *Acanthamoeba* infection in AIDS patients and nasal passage is thought to be the portal of entry (Marciano-Cabral and Cabral 2003).

1.1.3.2 Ocular infections

Keratitis is the most common ocular infection caused by *Acanthamoeba* (Auran *et al* 1987; Figure 1.3C)). It is a sight threatening disease and is characterized by prolonged morbidity and significant loss of visual acuity for up to 15% of patients (Duguid *et al* 1997; Radford *et al* 1998). Infection of the eye without corneal involvement is extremely rare, although optic neuritis and macular disease (Schlaegel and Culbertson, 1972), uveitis (Jones *et al* 1975), chorioretinitis (Johns *et al* 1988) and endophthalmitis (Heffler *et al* 1996) have been reported in the literature.

1.2 *ACANTHAMOEBA* KERATITIS

1.2.1 Corneal inflammation

Cornea is the transparent anterior portion of the fibrous coat of the eye (Figure 1.4) consisting of five layers: stratified squamous epithelium, Bowman's membrane, stroma, descemet's membrane, and mesenchymal endothelium (Figure 1.5). It serves as the first refracting medium of the eye. It is structurally continuous with the sclera, is avascular, receiving its nourishment by permeation through spaces between the lamellae. It is innervated by the ophthalmic division of the trigeminal nerve via the ciliary nerves and those of the surrounding conjunctiva, which together form plexuses (Gipson 1994). Inflammation of the cornea is called keratitis, which is a significant cause of ocular morbidity around the world. Keratitis may be of ulcerative (breach in corneal epithelium with underlying infiltration of inflammatory cell) or non-ulcerative type, which in turn might be infectious or non-infectious. AK is an infectious ulcerative type of corneal inflammation (Sharma 2001).

1.2.2 Epidemiology

Ocular infection due to *Acanthamoeba* was first reported in 1973 in a healthy, 7-year old boy, who developed fever, sore throat, nausea, vomiting, headache and iridocyclitis of the left eye. He died 29 days later following rapidly progressive meningoencephalitis. Amoebic trophozoites were identified in the ciliary body of the left eye and in many brain sections. It did not involve the cornea (Jones *et al* 1975). *Acanthamoeba* keratitis was first reported in June 1973 by *Jones et al (*1975) where cysts and the trophozoites were found on smears and on a blood agar plate that contained corneal scrapings from a Texas rancher who had splashed himself in the eye with tap water from contaminated river sources (Jones *et al* 1975). The first

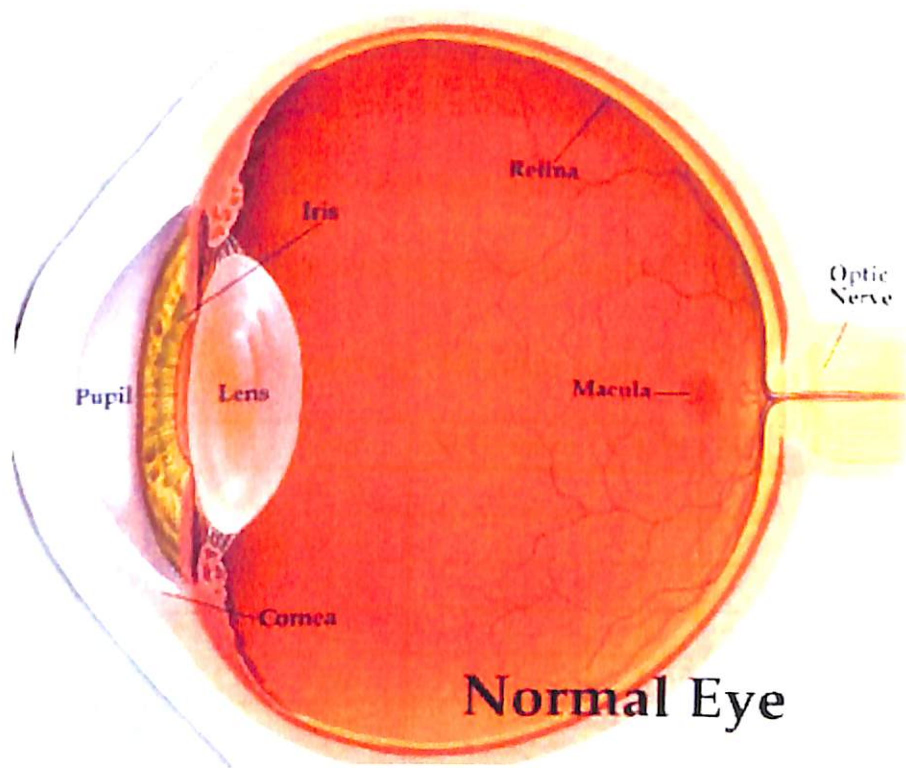


Figure 1.4 Diagrammatic representation of the anatomy of the eye

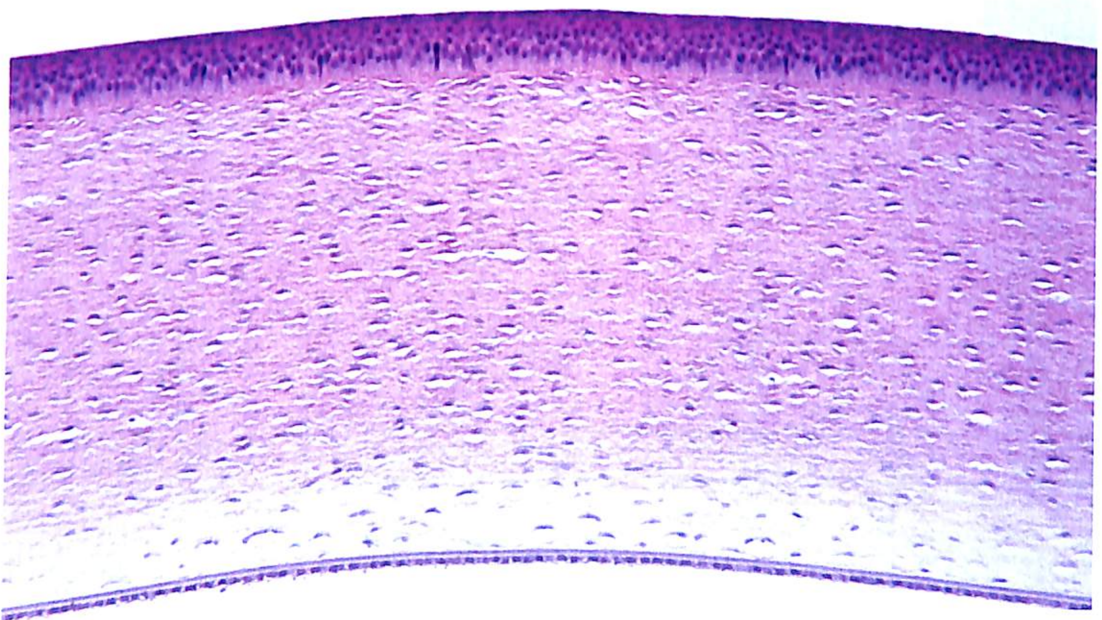


Figure 1.5 Histopathological section of normal human cornea (Haemotoxylin and eosin stain x10)

published report of confirmed *Acanthamoeba* infection of the eye appeared in Lancet in 1974. This report described two cases: the first occurred in a female school teacher, who had progressive infectious keratitis that was recalcitrant to treatment. The second described a male farmer who had ocular injury from a tree branch a year earlier. After this injury the eye healed quickly and had not bothered the patient for a year, when the first symptoms of *Acanthamoeba* appeared (Naginton *et al* 1974). In the next 10

Table 1.1: Some important reports of AK cases from 1973

No	Reference	Year	No.of cases	CLW	NCLW	Country
1	Jones *et al* 1973	1973	1	0	1	UK
2	Naginton *et al* 1974	1974	2	0	2	US
3	Key *et al* 1980	1978	1	0	1	US
4	Ma *et al* 1981	1981	11	0	11	US
5	Samples *et al* 1984	1984	1	1	0	US
6	Hirst *et al* 1984	1984	1	1	0	US
7	Blackmann *et al* 1984	1984	1	1	0	US
8	Scully *et al* 1985	1985	1	1	9	US
9	Moore *et al* 1985	1985	3	3	0	US
10	Theodore *et al* 1985	1985	3	0	3	US
11	Cohen *et al* 1985	1985	4	2	2	US
12	US Department 1987	1985-1986	24	20	4	US
13	Auran *et al* 1987	1973-1987	35	17	18	US, UK, Netherlands, Germany, Belgium
14	Yeoh *et al* 1987	1985-1987	4	0	4	UK
15	Moore *et al* 1987	1987	11	11	0	US
16	Tseng et al 1989	1989	1	0	1	Taiwan
17	Rabinovitch *et al* 1990	1990	1	1	0	Canada
18	Stehr-Green *et al* 1989	1973-1988	189	160	29	US
19	Bacon *et al* 1993	1984-1992	72	64	8	UK
20	Radford *et al* 1998	1992-1996	243	225	18	UK
21	Radford *et al* 2002	1997-1999	106	93	13	UK

CLW: Contact lens wearer   NCLW: Non contact lens wearer

years only 11 cases (in non-contact lens wearers) of AK were reported among which 5 were reported from US and 6 from three countries of Europe (Jones 1986). In 1960,

the first hydrophilic polymer to be used as a contact lens material was invented in Czechoslovakia (Wichterle and Lim 1960) and was introduced in the US in 1970 where these soft contact lenses became extremely popular (Schaumberg *et al* 1998). In 1984, the first case of *Acanthamoeba* keratitis in soft contact lens wearer was reported. This patient had been wearing soft contact lenses while using an outdoor hot tub from which *Acanthamoeba* was later isolated (Samples *et al* 1984). Most of the cases were subsequently reported from the US and UK (Table 1.1) though they have also been reported from other parts of world like Australia (Gebauer *et al* 1996), Brazil (Alves *et al* 2000), Ghana (Leck *et al* 2002), India (Sharma *et al* 2000), Japan (Tachikawa *et al* 1995) and other countries. The incidence of AK has been reported to

Table 1.2: AK cases reported from India from 1984

No	Reference	Year	No.of cases	CLW	NCLW	State
1	Kulkarni 1984	1984	1	0	1	Tamilnadu
2	Sharma *et al* 1988	1987	1	0	1	Tamilnadu
3	Sharma *et al* 1990	1990	4	0	4	Tamilnadu
4	Thomas *et al* 1990	1990	3	0	3	Tamilnadu
5	Sharma *et al* 1990	1990	9	0	9	Tamilnadu
6	Srinivasan *et al* 1993	1993	1	1	0	Tamilnadu
7	Singh *et al* 1994	1994	1	1	0	Delhi
8	Srinivasan *et al* 1997	1997	3	0	3	Tamilnadu
9	Davamani *et al* 1998	1997	44	0	44	Tamilnadu
10	Narang *et al* 1999	1999	3	0	4	Maharashtra
11	Sharma *et al* 2000	2000	39	0	39	Andhra Pradesh
12	Parija *et al* 2001	2001	11	0	11	Pondicherry
13	Narsimhan *et al* 2002	2001	19	0	19	Tamilnadu
14	Srinivasan *et al* 2003	2003	103	0	103	Tamilnadu
15	Sharma *et al* 2003	2003	1	1	0	Andhra Pradesh

be 1/10,000 in CLW annually in UK (Seal *et al* 1996) while a study from US estimated it to be 1.65-2.01/million CLWs (Schaumberg *et al* 1998). Another study from UK estimated the incidence to be 0.14/100,000 (Radford *et al* 1998) and the

same group later in 2002 reported it to be as high as 21.14 million CLW; they attributed the increase in incidence to the geographical location of the population (Radford *et al* 2002).

First case of AK from India was a patient from Sankara Nethralaya, Chennai, who was later diagnosed in Boston, US in 1984. The patient was a high myope wearing soft contact lenses. She developed bacterial keratitis and was referred to Boston for keratoplasty (Kulkarni 1984). The corneal button on histopathology examination revealed *Acanthamoeba*. In 1987, the first case of AK was diagnosed in India at Aravind Eye hospital, Madurai in a patient who did not wear contact lenses (Sharma *et al* 1988). Since then only few reports of AK in India have been published in the literature and most of these are from south India and in non-contact lens wearers (Table 1.2). Prevalence of AK has been reported to vary between 1-3% in keratitis patients (Sharma *et al*,2000; Davamani *et al* 1998).

1.2.3 Predisposing factors

Historically, AK has been associated with corneal trauma and exposure to contaminated water. However, since 1986, an association of AK with contact lens wear became apparent (Stehr-Green *et al* 1989) and AK was/is a potential threat to all contact lens wearers who use unsterile solutions to rinse, store or lubricate their contact lenses (Moore *et al* 1987). Use of soft contact lenses has been considered as the major risk factors for AK (Chynn *et al* 1995). In a case control study of soft contact lens wearers, factors like; using homemade saline, disinfecting lenses less than recommended by the manufacturer and wearing lenses while swimming were associated with AK (Stehr-Green *et al* 1987). AK has also been documented in

patients who use contact lenses other than soft contact lenses also. These include hard (Moore *et al* 1987), rigid gas permeable (Koeing *et al* 1987) and even disposable contact lenses that theoretically should not carry the risk of contaminated lens solution and containers (Ficker *et al* 1989). *Acanthamoeba* has been isolated from the solutions and cases of contact lens-wearers with AK. It is likely that all of these solutions and lens cases are concurrently contaminated with bacteria or fungi that provide the food source for the amoeba (Donzis *et al* 1989). Once in the solution, the organism probably gains access to the eye (Figure 1.6) through either application of contaminated solutions directly to the eye or by means of contact lenses (Brady and Cohen 1990). Exposure to vegetative or organic material: dust or water contaminated with *Acanthamoeba* and preexisting corneal infection are the major predisposing factors for AK in non-contact lens wearers (Srinivasan *et al* 2003; Auran *et al* 1987).

1.3 CLINICAL FEATURES OF *ACANTHAMOEBA* KERATITIS

1.3.1 Clinical Symptoms

Subclinical infection with *Acanthamoeba* is more common than the actual infection itself, this is because of the fact that *Acanthamoeba* is ubiquitous and infection with non-pathogenic strains of *Acanthamoeba* generates or induces both cellular and humoral immunity which in turn protects individuals against infection by pathogenic strains of *Acanthamoeba* (Auran *et al* 1987). But when the infection occurs, the symptoms mainly include severe pain, tearing, photophobia, blepharospasm, blurred vision and foreign body sensation (Auran *et al* 1987). The ocular pain may be severe, often disparate to the degree of corneal and intraocular inflammation (Jones 1986), and has been reported in most of the patients with AK (Dougherty *et al* 1994). Severe ocular pain disproportionate to the degree of keratitis was the presenting symptom in

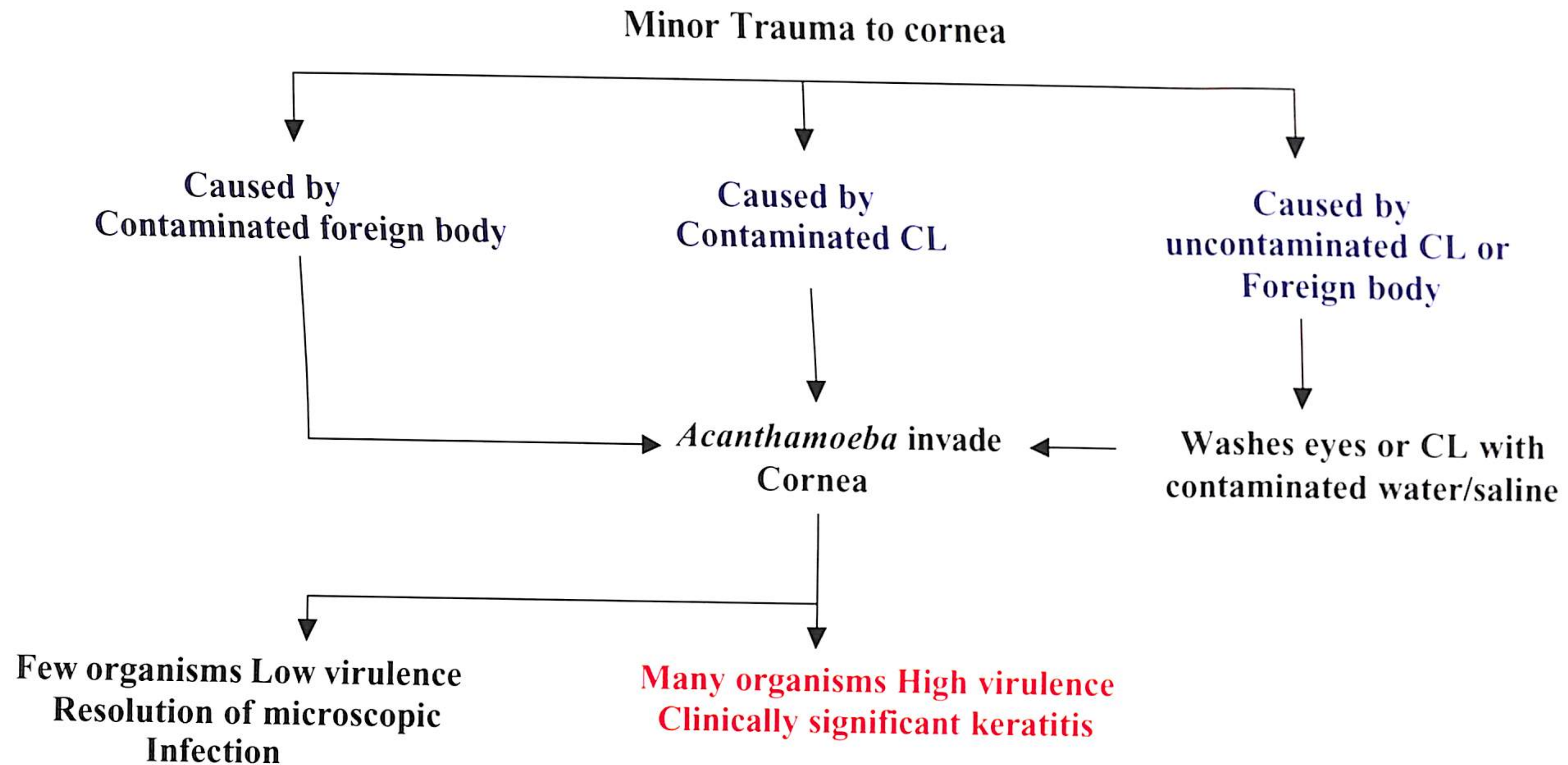


Figure 1.6 Risk factors of Acanthamoeba keratitis (Moore et al 1987)

91% of the patients in a paper published by Chynn *et al* (1995). However, Mathers *et al* (1996) found that only 51% of the patients complained of pain while 48.8% of the patients described only a mild irritation or foreign body sensation in the eye and 4.65% of the patients did not experience any pain at all. Sharma *et al* (2000) also did not record severe ocular pain in any of their 39 AK patients, which was unlike the earlier reports in which severe ocular pain was well documented and regarded as most common initial clinical symptom.

1.3.2 Clinical Signs

Clinical signs of AK have been divided into early and late stages on the basis of median interval between the onset of symptoms and diagnosis of AK. If it is less than 15 (Srinivasan *et al* 2003) or 30 days (Chynn *et al* 1995; Sharma *et al* 2000) it is categorized as early and if it is later than that it has been considered as late stage of the disease. Srinivasan *et al* (2003) did not find any significant difference in the clinical features between the two stages. They reported a large case series of 103 cases of AK and found that 74% of the patients presented with epithelial defect while, corneal edema was present in 66%, diffuse infiltration in 62%, ring infiltrate in 51%, and hypopyon in 42% of the patients. They observed satellite lesions in 18.4%, endothelial plaques were seen in 5.8% of the patients while only 2 cases (1.9%) showed radial keratoneuritis (Srinivasan *et al* 2003) which is unlike the other reports which have suggested that radial keratoneuritis occurs frequently in cases with AK and can be used as one of the criterion to diagnose AK (Chynn *et al* 1995; Bacon *et al* 1993). Similarly, Sharma *et al* (2000) in their case series of 39 patients of AK reported only 1 (2.5%) patient to have radial keratoneuritis. Radial keratoneuritis is the presence of infiltrate along the corneal nerves, and is usually found in the

midstroma, beginning paracentrally and extending to the limbus in a radial pattern (Moore *et al* 1986). Unlike the above reports from India, Bacon *et al* (1993) reported 15 cases of AK in which an early diagnosis was made i.e., within 30 days of the onset of symptoms and they found that 11 of 15 cases (73.3%) of AK showed perineural infiltrate. Similarly, Chynn *et al* (1995) reported that 45% of their patients developed radial keratoneuritis. Apart from these features Auran *et al* (1987) observed adenopathy in 2 of 35 cases described by them. Chemosis, conjunctival injection and tarsal or limbal follicles were also present in few cases. Initial corneal changes, which were described by them include, opaque streaks, fine epithelial and sub-epithelial stippling and microcystic edema.

Mannis *et al* (1986) and Srinivasan *et al* (2003) have reported that the clinical picture of AK is similar to any suppurative keratitis and thus does not offer any clue for the diagnosis of AK in most of the cases. In a case series of 26 AK cases 57% of the patients developed a distinct ring infiltrate, 93% developed a persistent corneal erosion, iritis was present in all cases, only 39% of the cases were severe enough to produce hypopyon and scleritis was reported in 14% of the patients (Mannis *et al* 1986). The range of intraocular inflammation in AK is variable and can range from trace cell and flare to marked uveitis with keratic precipitates and hypopyon (Brady and Cohen 1990). Sharma *et al* reported hypopyon in 44.4% and 53.8% of their patients in two separate reports on AK (Sharma *et al* 1990;2000). Chynn *et al* (1995) compared the clinical features observed in both contact lens associated and non-contact lens associated AK cases and they found that there was no significant difference in the clinical presentation between the two groups but the patients with

non-contact lens related AK had worse outcome, only for the fact that the diagnosis in these cases was comparatively delayed.

The hallmark of AK is presence of ring infiltrate in the stroma (Figure 1.3C). Ring infiltrate rarely may occur in other conditions such as bacterial corneal ulcers, but in the appropriate clinical setting it is pathognomonic for AK (Theodore *et al* 1985). Ring develops usually two to three months into the course of the disease and begins as non-confluent stromal infiltrate in the paracentral cornea and then with time the infiltrates coalesce into a partial, complete 360°, double or concentric ring (Moore *et al* 1987). Auran *et al* (1987) reported that this ring or annular infiltrate is progressive and often causes stromal thinning or furrowing, along with variable overlying epithelial defect. Ring infiltrate presumably develops by the interaction of polymorphonuclear leucocytes with intact organisms, antigens or by products of the infection. The central stroma within the ring appears coarsely granular (Jones *et al* 1986). Jones *et al* (1986) suggested that ring infiltrate usually occurred in the late stage of the disease, but was not thought to be true by Srinivasan *et al* (2003) and Sharma *et al* (2000) where they reported the presence of ring infiltrate in both early and late stages of the disease, with no significant difference. In some cases, usually in the later stages of the disease, the stromal infiltrate may be homogenous without ring like character (Auran *et al* 1987). The stromal inflammation may also be well circumscribed with homogenous edema with minimum cellular infiltrate and intact epithelium thereby simulating herpes simplex disciform stromal keratitis (Jones *et al* 1986). Holland *et al* (1991) encountered six cases of AK with infiltrates that were unusual because they were sub-epithelial and occurred late in the course of the disease. These authors attributed this infiltrate to either inflammatory response

against active *Acanthamoeba* infection or adverse reaction to topical medication or possibly by an immune reaction directed against *Acanthamoeba* antigen.

1.3.3 Complications

Clinical signs of severe disease include, scleritis, glaucoma and cataract (which presumably is secondary to the prolonged and at times severe iritis), which are usually signs in late presenting disease (Bacon *et al* 1993). Involvement of the posterior segment is rare and was described in two cases by Auran *et al* (1987). In 1988, Johns *et al* (1988) reported a case of chorioretinitis in the right eye of the patient already having contact lens associated AK in the left eye. Authors hypothesized that chorioretinitis may have developed from the hematogenous dissemination from the corneal infection. Burke *et al* (1992) reported panophthalmitis and associated tractional retinal detachment in a patient diagnosed to have AK. Two months post AK diagnosis, the patient developed anterior nodular scleritis, followed by iritis, band keratopathy, ocular hypotension and significant lens opacities. Ultrasonography showed choroidal and retinal detachment, finally after three months requiring enucleation. *Acanthamoeba* endophthalmitis has also been reported in a patient with AIDS. The patient had disseminated *Acanthamoeba* infection of the skin and lung along with granulomatous uveitis, wherein aqueous and vitreous specimens showed the presence of the parasite (Heffler *et al* 1996). As in bacterial and fungal keratitis, advanced infection in AK also produces necrotizing stromal suppuration and corneal perforation (Jones 1986). Recurrence of *Acanthamoeba* infection in the cornea was noted in 7 of 72 cases of AK reported by Bacon *et al* (1993).

Acanthamoeba sclerokeratitis is an uncommon complication of AK and in its most severe form it is associated with significant morbidity and is seen as a difficult therapeutic challenge (Lindquist *et al* 1990). *Acanthamoeba* sclereokeratitis is marked by recent or concurrent ipsilateral corneal infection with *Acanthamoeba* in association with severe anterior scleritis, manifesting as deep scleral pain with globe tenderness, engorgement of deep episcleral blood vessels and/or the presence of scleral thickening on ultrasonography (Lee *et al* 2002). In a case series from Moorfields eye hospital, London, UK, out of 200 cases of AK, 19 (9.5%) developed *Acanthamoeba* sclerokeratitis (Lee *et al* 2002). The apparent preference of *Acanthamoeba* for cooler environment such as the anterior cornea (35°C) may also be a factor in the decreased tendency for invasion of higher core temperature tissues such as sclera. Scleritis is usually anterior and diffuse but also might be nodular in distribution (Lee *et al* 2002). There is only one published report describing diffuse posterior scleritis and optic neuritis associated with AK (Mannis *et al* 1986).

1.4 ANTI-*ACANTHAMOEBA* DRUGS AND SURGICAL MANAGEMENT

Until recently, there has been a limited medical therapeutic armamentarium for treating AK. The literature attests to a variety of drugs providing variable efficacy against different *Acanthamoeba* species or strains both *in vivo* and *in vitro*. The first medical cure for AK was reported in 1985 with a combination of propamidine and neomycin (Wright *et al* 1985). A very important determinant of successful outcome or treatment is the ability of a compound to penetrate in deeper part of stroma of the cornea. The compound may have apparent sensitivity *in vitro* but might not be effective *in vivo* if it cannot penetrate. The ability of medical therapy alone to eradicate *Acanthamoeba* from the cornea is variable. Success of therapy depends on

many confounding factors like stage of disease, depth of involvement, individual host response, virulence and size of inoculum of the infecting strain and variable sensitivity of *Acanthamoeba* spp. to anti-microbial agents (Illingworth and Cook 1998). Several compounds have been used with varying effect in clinical setting for the therapy of AK, some of which are described below:

1.4.1 Diamidine derivatives

Diamidines act as inhibitors of S-adenosylmethionine decarboxylase (Hugo and Byers 1993) or interact directly with the amoeba's nucleic acids (Greenidge *et al* 1993) or inhibit cytoplasmic enzymes (Arnott *et al* 1994). Diamidines are well tolerated by ocular tissue when applied topically (Lindquist 1998). Some of the diamidine derivatives, which have been used against AK, include:

- **Propamidine isethionate (Brolene 0.1%)**

  It is an aromatic diamidine and the medical cure with propamidine therapy is only achieved when it is commenced early in the course of the disease (Bacon *et al* 1993; Moore and McCulley 1989). Prolonged treatment with propamidine leads to toxic keratopathy, which clears gradually after discontinuation of the drug (Johns *et al* 1988; Alizadeh *et al* 1997).

- **Hexamidine:** It was found to have greater cysticidal activity than propamidine (Brasseur *et al* 1994; Gray *et al* 1996).

1.4.2 Cationic Antiseptics

Chlorhexidine is a biguanide, while polyhexamethylene biguanide (PHMB) is a polymeric biguanide. Both act by compromising the integrity of the mucopolysaccharide plug that seals the ostiole of the *Acanthamoeba* cyst. Irreversible loss of essential cellular components through the damaged plasmalemma results in cell death (Armstrong, 2000). While cytoplasmic precipitation is a secondary event (Seal *et al* 1996). Corneal epithelial toxicity (clinically) is minimal for chlorhexidine and PHMB at a concentration of 0.02% (Lindquist 1998). Both chlorhexidine and PHMB have both amoebicidal and cysticidal activity (Elder *et al* 1994; Hay *et al* 1994).

- **Chlorhexidine**

  It is the most prevalent biocide used in antiseptic products. It is commonly used in disinfectants, preservation and in hand-washing and oral products (McDonnell and Denver 1999). It has a persistent effect on tissues for up to 24 hours after application in a concentration of 0.02% (Seal *et al* 1996). Kosrirukvongs *et al* (1999) achieved successful treatment of AK with 0.006% chlorhexidine, but recommend that early diagnosis is very crucial for the successful outcome. At concentrations of ≥ 0.2%, chlorhexidine has toxicity to skin, conjunctiva and corneal epithelial cells and fibroblasts, but at concentrations of 0.02% there is no apparent ocular toxicity (Seal *et al* 1996a; Green *et al* 1980).

◆ **Polyhexamethylene biguanide (PHMB)**

PHMB is manufactured principally as an industrial grade sterilant. It is used in cosmetics and soaps as preservatives, as an algastatic compound in swimming pools and a constituent of contact lens disinfecting fluids (Seal *et al* 1996). In early 1990, cationic disinfectant PHMB was found to be highly effective in killing both cysts and trophozoites in *in vitro* studies (Illingworth and Cook 1998). Larkin *et al* (1992) reported its successful clinical use at a concentration of 0.02%. In their study, all 6 patients had failed to respond to conventional anti-*Acanthamoeba* therapy, which included propamidine and neomycin. PHMB is non-toxic to mammalian epithelia at concentration of ≤ 20% (Berry and Easty 1993). Its also lacks corneal toxicity clinically (Larkin *et al* 1992; Bacon *et al* 1993). However, single first line use of PHMB in therapy of AK was questioned by Tseng *et al* (1998) who detected and cultivated *Acanthamoeba* from a cornea biopsy of an apparently successfully treated (PHMB prescribed for 4 months) case of contact lens associated AK. Also Lam *et al* (2000) have reported that topical PHMB monotherapy leads to persistence of infection and hence suggested use of combination therapy in treatment of AK. PHMB has advantages over propamidine in having high consistent cysticidal activity (Elder *et al* 1994) and no toxicity (Berry and Easty 1993).

1.4.3 Antibacterial agents

Initially, in absence of anti-amoebic agents, antibacterials were used with almost no success for the treatment of AK (Illingworth and Cook 1998). Aminoglycosides such

as paromomycin and neomycin (Lindquist 1998) were used effectively in conjunction with other topical drugs for the treatment. They are largely ineffective against cysts. Topical use of neomycin for long period of time is toxic to the cornea and can induce hypersensitivity reaction (Wilson 1991). Also, Imidazoles such as miconazole, clotrimazole, ketoconazole and itraconazole have been used for the treatment (Lindquist 1998), however these antibiotics are only amoebastatic. Among these imidazoles, ketoconazole has been reported to be most effective against *Acanthamoeba* (Lindquist 1998). In recent times, antibacterials have been recommended only for prophylaxis of the infection as in presence of persistent epithelial defect, there is always a great risk of secondary bacterial infection.

1.4.4 Other drugs

- **Povidone-Iodine (PVI):** It has been used at a concentration of 0.5 to 2.5% for the treatment of AK and the authors found that it had better anti-amoebic activity than chlorhexidine both against trophozoites and cysts of *Acanthamoeba* (Gatti *et al* 1998).

- ***Alkylphosphocholines (APCs)***

  APCs are a group of compounds, which consist of phosphocholines esterified to various long chains aliphatic alcohols (Eibl and Unger 1990). They are active against *Leishmania donovani* (Croft *et al* 1987), *Trypanosoma cruzi* (Santa-Rita *et al* 2000) and *Entamoeba histolytica* (Seifert *et al* 2001). Walochnik *et al* (2002) tested 8 of these compounds against *Acanthamoeba* isolates and found that seven of the eight APCs had amoebastatic activity,

while only hexadecylphosphocholine exhibited highest degree of cytotoxicity against trophozoites resulting in complete cell death at a concentration as low as 40μM and also displayed significant cysticidal activity. Thus the authors suggested that hexadecylphosphocholine is a promising new candidate for the topical treatment of AK (Walochnik *et al* 2002).

- **Corticosteroids:** Topical corticosteriods have been used to treat AK, but their use is controversial, as some authorities consider them to be contraindicated because of their role in suppression of host immune response (D'Aversa *et al* 1995; Stern and Buttross 1991). Steroids have been reported to inhibit both excystment and encystment (Osato *et al* 1986). This, in theory, has the advantage of rendering the amoebae more susceptible to destruction by the immune defenses, but it also has a disadvantage in that the cysts are more resistant to antiamoebic agents. However, in contrast to the above observation, McClellan *et al* (2001) indicated that exposure of *Acanthamoeba* trophozoites and cysts to dexamethasone increased the pathogenicity of the organism. In their *in vitro* studies, exposure to dexamethasone increased the number of trophozoites through excystment and growth. Their results emphasized the importance of maintaining adequate amoebicidal therapy if a topical steroid is used in the management of AK (McClellan *et al* 2001). Illingworth *et al* (1995) used steroids in 18 out of 23 eyes infected with *Acanthamoeba* and found no apparent adverse effects, while Rabinovitch *et al* (1991) suggested that use of corticosteriods was single most important factor predicting failure of medical therapy. Hence the question of when to use topical steroids is not

satisfactorily resolved as yet and if at all they are used they should be used with caution, particularly at the commencement of antiamoebic therapy (Illingworth and Cook 1998).

1.4.5 Surgical management

Debridment : Corneal debridement in combination with medical therapy was used successfully in treatment of AK. Brooks *et al* (1994) treated 2 cases of AK only with epithelial debridement, without any concomitant treatment with anti-amoebic agents. Although Illingworth *et al* (1995) suggested that experience with debridement is too limited for it to be recommended without medical therapy.

Penetrating keratoplasty (PK): Since the introduction of antiamoebic agents and their successful use in treatment of AK, PK is now usually unnecessary in the acute phase of the disease. Although, if PK is performed in inflamed eyes, there is a possibility of graft failure due to recurrent infection. In a study by Cohen *et al* (1987), two of five eyes and in another study six of seven grafts failed in such circumstances (Ficker *et al* 1993). However, Illingworth *et al* (1995) had encouraging results in all the nine grafts, when PK was done on inflamed eyes.

Cryotherapy: Reports where cryotherapy has been recommended for treatment of AK were described before the availability of cationic antiseptic agents, thereby relegating cryotherapy to a limited role (Illingworth and Cook 1998). *In vitro* studies have shown that cryotherapy only kills the trophozoites

and not cysts (Meisler *et al* 1986). However, success with cryotherapy coupled with other medical treatment has been reported in five cases by Binder (1989) and one additional case by Lindquist *et al* (1998).

Deep lamellar keratectomy and conjunctival flap: Cremono *et al* (2002) found that deep lamellar keratectomy with a conjunctival flap is a suitable approach to help control the infection and to help relieve pain in patients with advanced AK. Investigators proposed that, if the eye is inflamed and painful, a conjunctival flap could provide symptomatic relief and help quieten the eye in preparation for a future PK. The aim of deep lamellar keratectomy with conjunctival flap is to excise and remove necrotic tissue. In addition conjunctival flap brings conjunctival vessels and lymphatic channels that may enhance the immune response against the amoebae (Cremono *et al* 2002).

Amniotic membrane: Kim *et al* (2001) have reported good results with the use of amniotic membrane transplantation in three patients with AK. Amniotic membrane contains several inhibitors (Kim *et al* 2000) and anti-inflammatory proteins (Hao *et al* 2000). Cremeno *et al* (2002) suggested that these properties of amniotic membrane might facilitate the healing process and prevent necrosis in infective keratitis patients. They also believed that amniotic membrane in combination with lamellar keratectomy may shorten the surgical time and may also facilitate faster recovery of corneal transparency compared with conjunctival flap.

Thus, AK is a difficult disease to treat and requires prolonged therapy, especially to eradicate cysts, which are the most difficult obstacles. Many agents have been studied to assess their antiamoebic effect against AK. Clinical reports suggest that selection of drugs has been haphazard and relationship between drug susceptibility and clinical efficacy remains unproven. Although antiamoebic therapy with combination of cationic antiseptics, diamidines and antibiotics has been successful, a continued testing of isolates with new drugs is important.

1.5 IMMUNOLOGY AND PATHOGENESIS

The pathogenesis of AK and the immune response against *Acanthamoeba* are of interest since the organism is ubiquitous in water and soil but only small fraction of people acquire the disease. The low incidence of AK may be due to atleast two mutually compatible explanations: firstly, *Acanthamoeba* is a weak pathogen and secondly, there is high degree of innate host resistance against it (Garner 1993).

1.5.1 Immune response

The immune defense mechanisms that operate against *Acanthamoeba* have not been well characterized (Marciano-Cabral and Cabral 2003) moreover studies on it are very limited as only corneal transplantation specimens are available for study and in most cases such patients have, been intensively treated with anti-inflammatory agents prior to surgery. Thus, most reports describe the late stages of the disease probably modified by drug therapy (Garner 1993; Auran *et al* 1987; Mathers *et al* 1987). High titer of IgM antibody against *Acanthamoeba* has been detected in the serum of healthy asymptomatic individuals, suggesting that exposure to *Acanthamoeba* is common but not the fatal infection (Cursons *et al* 1980). However, protection from lethal or fatal

infection involves both innate and acquired immunity (Cursons *et al* 1980). There have been few studies to assess the interaction of *Acanthamoeba* with specified cells of the immune system. It has been reported that the earliest response of the host to amoebae consists of influx of neutrophils to the site of infection (Ferrante and Abell 1986) and later it was proved that macrophages play a more important role than neutrophils in killing *Acanthamoeba* (Marciano-Cabral and Toney 1998). The fact that these cells play an important role in the immune system has been established by *in vivo* (animals model) and *in vitro* (in cell lines) experiments. Apart from the above cells, complement activation and IgM antibody production add to defense against invading *Acanthamoeba* infection (Niederkorn *et al* 1999).

Complement

It is an innate resistance factor that is activated by *Acanthamoeba* and provides the first line of defense against invading organisms (Ferrante and Rowan-Kelley 1983). The complement is activated by the alternative pathway. The C5, C6, C7, C8 and C9 fraction of complement system, when activated, act together to cause membrane damage of amoebae (Ferrante and Rowan-Kelley 1983). Although, the main function of complement activation is to generate opsonic factors such as C3b for recognition of the amoebae by phagocytic cells, it also leads to generation of mediators of inflammation such as anaphylotoxins like C3a and C5a which contribute to the pathogenesis of the AK (Ferrante 1991).

Antibodies

Titers of antibodies to *Acanthamoeba* in human serum ranges between 1:20 and 1:80. Antibodies are mainly of IgM and IgG isotypes. Antibodies are also present in cord

blood suggesting that the antibodies to *Acanthamoeba* are transferred placentally. Antibodies function in various ways to limit invasiveness of *Acanthamoeba*. Addition of antibodies to free living amoebae *in vitro* prevents their adhesion and spreading (Ferrante 1991). Antibodies also inhibit phagocytic property of amoebae and promote neutrophil mediated killing of amoeba (Ferrante 1991).

Macrophages

Macrophages are extremely important effector cells in ocular inflammation and are often detectable in acute ocular infections. They serve three primary functions: 1) as antigen presenting cells for T-lymphocytes. 2) as inflammatory effector cells and 3) as regulators in other processes, such as fibrosis (Cousins and Rouse 1996). Stewart *et al* (1992) in their study showed that macrophages demonstrate a strong chemotactic response to *Acanthamoeba* and can directly kill trophozoites *in vitro*. van Klink *et al* (1996) in their study on Chinese hamster selectively depleted out macrophages with liposomes containing dichloromethylene diphosphonate (C12MDP-L1P) and found that macrophage depletion affected the incidence, severity and chronicity of keratitis. The incidence of infection in normal animals was approximately 60% but rose to 100%. The clinical appearance was much more severe and there was a major change in chronicity of keratitis with earlier onset and a prolonged and chronic course in the C12MDP-L1P treated hamsters. C12MDP-L1P treatment prevented the antigen presentation to T cells by conjunctival macrophages, thus preventing the generation of an *Acanthamoeba* specific immune response. The profound exacerbation of AK in hamsters treated with C12MDP-L1P strongly suggested that macrophages played an important role in corneal infection. Macrophages prevented the disease from becoming chronic because of their extensive attack early in the infection, which

possibly prohibited the spread and invasion of the *Acanthamoeba*. The authors explained that macrophages are usually present in the acute phase of the disease hence their absence has been documented in corneal biopsy specimens and corneal button from penetrating keratoplasty patients. They believed that macrophages serve as an important barrier to corneal infection and exert their effect by preventing the initiation of infection and appearance of clinical signs. Thus they suggested that macrophages act as a first line of defense and eliminate significant numbers of *Acanthamoeba* trophozoites (van Klink *et al* 1996).

Polymorphonuclear cells (neutrophils)

Neutrophils are the most abundant grauolocytes in the blood. They are efficient phagocytes and readily invade tissues and degrade ingested material. They act as important effector cells through the release of granule products and cytokines (Cousins and Rouse 1996). In order to determine importance of neutrophils in the pathogenesis of AK, Hurt *et al* (2001) inhibited the conjunctival neutrophils migration by sub-conjunctival injection with an antibody against macrophage inflammatory protein 2 (MIP-2), a powerful chemotactic factor for neutrophils, secreted by the cornea and by using intraperitoneal injection of anti Chinese hamster neutrophil antibody. The inhibition of neutrophils to the cornea resulted in an earlier onset and more severe infection. Authors also intrastromally injected recombinant MIP-2 into the cornea and found an initial intense inflammation that resulted in rapid resolution of the corneal infection. Thus the profound exacerbation of AK was seen when neutrophil migration was inhibited and rapid clearing of the disease in the presence of increased neutrophils. Both these observations strongly suggested that neutrophils play an important role in combating *Acanthamoeba* infection in the cornea.

Neutrophils kill amoeba only when they are activated by lymphokines. In addition, these altered neutrophils cannot act in the absence of complement or antibody (Ferrante 1991). Therefore, combined action of lymphokines, complement and antibody is needed for killing mechanism of neutrophils. Neutrophils killing mechanism involves both the oxidative respiratory system and enzyme which are found in its azurophillic granules e.g., myeloperoxidase. T cells and macrophage cytokines augment both oxidative respiratory system i.e., respiratory burst and release of lysosomal enzymes from neutrophils. TNFα is responsible for significant stimulation of the respiratory burst (NADPH oxidase activation) which results in the production of oxygen derived reactive species and the release of azurophillic granules (Ferrante 1991).

Lymphocytes

The lack of vigorous lymphocytes infiltration is either because of lack of recruitment by macrophages or secondary to a lack of vascularization. If vascularization is present, lymphocytic and plasmolytic infiltration are usually observed mainly in the immediate vicinity of blood vessels in the corneal stroma or in the vascular pannus (Mathers *et al* 1987).

1.5.2 *In vivo* and *in vitro* models

The mechanisms involved in corneal tissue damage and invasions by the amoeba are poorly understood, especially those related with early events of amoebae-cornea interaction. Several animal models (van Klink *et al* 1993; He *et al* 1993; Cote *et al* 1991; John *et al* 1991; Badenoch *et al* 1991) have been used to study AK. In efforts

to develop an animal model of AK. Niederkorn *et al* (1992) in a series of *in vitro* studies examined the ability of *A.castellanii* to adhere, invade and damage normal intact corneas of 11 mammalian and one avian species. They revealed that parasites not only failed to produce cytopathic effect (CPE) but also did not even bind to corneal epithelium of mice, rats, cotton rats, horses, guinea pigs, cows, chickens, dogs and rabbits. However parasites adhered, invaded and produced severe damage to human, pig and Chinese hamster cornea. Their results indicated that *A.castellanii* exercises rigid host specificity at the host cell surface and the parasite recognized species-specific surface molecules on the corneal epithelium. However, disadvantages of these methods include lack of reproducibility between experiments and the need to sacrifice animals on a regular basis (Halenda *et al* 1998).

Non-corneal cell lines have also been used for *in vitro* studies of the pathogenicity of *Acanthamoeba* (Curson and Brown 1978), but these studies yield data that is not specific to cornea-pathogen interaction (Halenda *et al* 1998).). Recently primary corneal cultures (Kahn *et al* 1993) and immortalized human corneal epithelial cell lines (Araki *et al* 1995) have been developed which represent greater characteristics of the *in vivo* situation.

1.5.3 Pathogenesis

Acanthamoeba is a ubiquitous parasite; hence its inoculation in the cornea can be via contaminated water, soil or any foreign body in the eye. Some researchers believe that initial insult to the cornea in form of trauma, chemicals, organic matter, insect or microtrauma because of contact lens wear is required for the infection to occur (Roussel *et al* 1985; Theodore *et al* 1983). On the other hand Omana- Molina *et al*

(2001) in their study on Chinese hamsters, have described that *Acanthamoeba* spp. are capable of producing damage to intact hamster cornea, without producing a previous artificial lesion. Once the amoeba is present on the cornea, an important first step in the infectious cascade of AK is its binding to the corneal epithelium. Thus AK occurs in a sequential manner and is initiated by the adhesion of the pathogens to the host cells, followed by invasion of the corneal stroma (Leher *et al* 1988). Adherence and penetration may be the two-step process necessary for the *Acanthamoeba* to establish corneal infection (Moore *et al* 1991).

- **Adherence**

  In the initial stages of adhesion, cytoplasmic projections or acanthopodia of the trophozoites come in contact with the superficial cells of the cornea. Soon after trophozoites adhere completely and separate the cell junction of the corneal epithelial cells and eventually desquamate them (Omana-Molina *et al* 2001). Trophozoites can adhere more intensely with the epithelial surface, thus trophozoites are probably more important than the cysts in initiating human corneal disease (Ubelekar *et al* 1991).

Stopak *et al* (1991) suggested that studying the human corneal constituent that act as substrates or an attachment site for *Acanthamoeba*, will definitely lead to a better understanding of the pathogenesis of AK. Yang *et al* (1997) have demonstrated that corneal epithelium expresses *Acanthamoeba* reactive mannose glycoprotein receptor and the parasites express a mannose-binding protein. Therefore, the authors proposed that one mechanism of *Acanthamoeba* adhesion to the corneal surface involves interaction between

the mannose binding protein of the amoebae and mannose glycoprotein receptor of the corneal epithelium (Yang *et al* 1997). Leher *et al* (1988) were in agreement with the above investigators and also found that engagement of the mannose receptors induces the release of serine protease, which mediates contact independent cytolysis of corneal epithelial cells. Their study implied that the adherence of trophozoites to corneal epithelial cells is essential for initiating the cytolytic machinery of *Acanthamoeba* but is unnecessary once the mannose receptor is engaged. Authors proposed that *Acanthamoeba* trophozoites are capable of mediating both contact dependent and contact independent cytopathic effect. The mannose receptor is crucial for both these processes (Leher *et al* 1988). Studies on SIRC (rabbit corneal epithelial) cell lines suggested that adherence of *Acanthamoeba* to the monolayer of cells is a time and temperature dependent process. They also observed an interstrain difference in adherence of *Acanthamoeba* to the cell line suggesting that adherence of *Acanthamoeba* may correlate with observed variation in the rate of progression and virulence *in vivo* (Morton *et al* 1991).

After adhering to corneal epithelial cells, *Acanthamoeba* requires cellular elements for its sustenance. The cell surface of the *A.castellanii* is a highly specialized region that is not active in the active transport of solutes, but is involved directly in the uptake of nutrients by endocytosis, membrane fusion events and cell motility (Ubelaker *et al* 1991). It feeds on complex macromolecules found most commonly in living cells for its nutrition (Stopak *et al* 1991). *Acanthamoeba* feeds directly on the dense cellular epithelial cells causing disruption of the layer and eventually allowing access to the corneal

stroma, which provides further nutritional support through its keratocytes. This plentiful food supply allows the organism to subsist in the stroma for long period of time (Stopak *et al* 1991).

- **Penetration**

Moore *et al* (1991) suggested that the trophozoites of *A.castellanii* use two methods to penetrate human corneas *in vitro*. The first method involves the secretion of material, which mainly includes enzymes, that interferes with the junction of the surface squamous epithelium. *Acanthamoeba* are known to have several enzymes that include ribonucleases, phosphatase, proteinase, α-glucosidase, β-N-acetylglucosaminidase and β-glucuronidase (Moore *et al* 1991). Plasma membrane of *Acanthamoeba* has enzymes like phospholipase A, lyophospholipase, acetyl Co-A hydrolase, palmitoyl Co-A synthetase alkaline phosphatase and 5`-nucleotide activities and Mg^{++} adenosine (Victoria *et al* 1975). Acyl Co-A:lysolecithin acyltransferase, CDP choline: 1,2-diacylglycerolcholine phosphotransferase are present in the microsomal fraction (Victoria *et al* 1975). Thompson and Shultz (1971) reported that substantial levels of two phospholipases, glucose-6-phosphatase and 5`-nucloetidase in both rough and smooth endoplasmic reticulum (ER) but found that NADPH cytochrome C reductase and rotenone insensitive NADPH cytochrome C reductase were present only in smooth surface membrane. Moore *et al* (1991) suggested that rough ER plays an important role in elaborating substances that break the desmosomes of the squamous epithelium. Because of the enzymatic action, trophozoites separate adjacent

surface cell, extend pseudopodia into the separated area and move under the surface of the epithelium without causing damage to overlying cells. This finding may explain the clinical signs of stromal infiltrates without associated epithelial signs (Moore *et al* 1991). Apart from many enzymes, collagenase was also attributed to pathogenicity of *Acanthamoeba*, since collagenase from the axenic cultures of *A.castellanii* digested collagen shields and type I collagen *in vitro*. This finding further suggested that the stromal degradation in AK might be caused in large part by parasite-derived collagenase (Alizadeh *et al* 1996).

The second feature of penetration is phagocytosis of epithelium (Moore *et al* 1991). Ferrante and Bates (1988) have shown that *Acanthamoeba* contains the proteolytic enzymes elastase and Cursons *et al* (1978) found phopholipase A production by *Acanthamoeba*. There have been many reports determining the basis of pathogenesis in *Acanthamoeba* and in one of the studies authors found that pathogenic isolates of *Acanthamoeba* displayed plasminogen activator activity. Based on this, the investigators hypothesized that pathogenesis of AK involves the activation of plasminogen to active plasmin, which in turn promotes the parasite penetration into the corneal epithelium (Alizadeh *et al* 1996).

1.5.4 Histopathology

The histopathologic features of AK vary. Initially, amoebae are restricted to the corneal epithelium but as the disease progresses they invade the underlying stroma and cause extensive damage and provoke mild to severe inflammation (Larkin and

Easty 1990; Larkine and Easty 1991; Marciano-Cabral and Cabral 2003). Epithelial ulceration, destruction of the Bowman's layer, inflammation in the stroma, necrosis and the presence of trophozoites and/or cysts of *Acanthamoeba* in the stroma are the common histopathologic features seen in AK (Vemuganti *et al* 2000).

1.5.4.1 Inflammatory responses

The inflammatory response is usually acute with varying degrees of fibroblastic response and necrosis (Auran *et al* 1987). Polymorphonuclear cells with few macrophages are the predominant cells noted in the anterior two third of the stroma (Auran *et al* 1987; Kinota *et al* 1993; Marciano-Cabral and Cabral 2003). Conspicuous absence of lymphocytes is noted by many, and is attributed to the absence of vascularization in the cornea (Mathers *et al* 1987; Garner 1993; Kremer *et al* 1994; Vemuganti *et al* 2000). Lymphocytic and plasmocytic infiltration in the cornea is closely associated with vascularization, wherein the lymphocytes and plasma cells are observed mainly in the vicinity of blood vessels (Mathers *et al* 1987). Mathers *et al* (1987) hypothesized that there is ineffective lymphocytic recruitment either because the organism has the ability to mask its antigen from the cellular immune system or the organism has the capability to suppress the function of infiltrating macrophages. Lack of lymphoid cells does not suggest that there is no immune reaction, since Garner *et al* (1993) observed lymphoid cells in both limbal conjunctiva and the anterior uvea in 5 of 30 cases which underwent evisceration. Similarly, Vemuganti *et al* (2000) have reported the presence of lymphocytes and plasma cells at the limbus in two of the cases of AK where evisceration was done.

The infiltrate in the stroma mainly consists of intact neutrophils, nuclear dust and large fragments of karyorrhectic nuclear form and degenerate neutrophils. Garner (1993) reported the presence of neutrophil infiltration in the anterior stroma in 86.7% (26 of 30) of the cases while 33.3% (10 of 30) of the cases showed infiltration in posterior stroma. Kremer *et al* (1994) observed that degree of inflammation roughly correlated with the number of organisms. Mathers *et al* (1987) performed immunophenotyping of the various cells in the corneal buttons sections of two patients diagnosed to have AK and found that 98% of the cells in the corneal stroma were positive for HLA-DR antigen. Anti-HLA-DR antibody stains all the cells that express Major histocompatability complex class II antigen that includes macrophages and keratocytes. Authors thus found intense infiltration of macrophages, although they were ineffective in recruiting lymphocytes into the cornea. They also observed neutrophils predominantly at the site of active ulceration in both cases, while sparse lymphocytes were seen only in one case (Mathers *et al* 1987).

1.5.4.2 *Acanthamoeba* in corneal stroma

Both cysts and trophozoites have been observed in the histopathologic section of the corneal buttons of AK cases. While cysts stain easily with haematoxylin and eosin and other stains, trophozoites can be missed as keratocytes. The trophozoites exhibit prominent nuclei and basophilic intracellular organelles (Kinota *et al* 1993). Garner (1993) observed the presence of trophozoites and cysts both in anterior and posterior stroma in 93.3% of their cases. Against this, inflammatory cells, though observed in anterior stroma, were present only in 33.3% of cases in the posterior stroma, thus suggesting that cysts were present in the posterior stroma in the absence of inflammatory cells in their vicinity (Garner 1993). Blackman *et al* (1984) also made

similar observations as above and suggested that this phenomenon might be due to the capacity of the *Acanthamoeba* to camouflage their antigenicity. This protective mechanism has been observed in other parasites and might be due to a mucinous material surrounding the live parasite which fades away when the parasite dies and then is exposed to varied defense system of the host (Blackman *et al* 1984). Vemuganti *et al* (2000) reported intact and degenerated trophozoites with few cysts in the anterior and mid stroma and cysts predominantly in the deeper quiet stroma. They suggested that when the parasite invades the deeper stroma it undergoes morphogenesis into cystic form as a defense mechanism and hence the absence of inflammatory cells around it. Kremer *et al* (1994) also reported presence of viable cysts in the posterior stroma, close to Descemet's membrane. Neutrophil and macrophage response in AK thus, is a result of the secondary inflammatory response against the necrotic amoebic organism and not because of the intact organism (Mathers *et al* 1987). .

1.5.4.3 Stromal changes

Like the inflammatory reaction, necrosis and stromal loss are also limited to the superficial stroma (Garner 1993; Vemuganti *et al* 2000). Vemuganti *et al* (2000) reported inflammatory reaction in posterior stroma in two of five cases, while one case showed vessels in the stroma without any associated lymphocytic infiltration. Apart from neutrophils and macrophages, they also reported the presence of eosinophils in the stroma. Another interesting feature, which was reported by them, was the loss of keratocytes in all layers of the stroma. Kremer *et al* (1994) also found depletion of keratocytes in both anterior and posterior stroma and keratocyte loss in posterior stroma was independent of the inflammatory reaction. Usually loss of

keratocytes is attributed to phagocytic activity of the parasite, but Vemuganti *et al* (2000) observed keratocyte loss independent of the parasite load in the vicinity, thus suggesting additional modes of cell loss. Using TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling) staining on corneal button sections from patients undergoing PK for advanced *Acanthamoeba* keratitis, they attributed the keratocyte loss to apoptosis in association with phagocytic activity of the parasite and necrosis.

1.5.4.4 Atypical features

Granulomatous inflammation in AK cases is thought to be a rare finding and Mietz and Font (1997) were the first ones to document a case of granulomatous inflammation in AK. They observed a granulomatous necrotizing stromal keratitis associated with a florid granulomatous reaction in the anterior chamber along the plane of Descemets membrane. Granulomatous inflammation with multinucleated giant cells and epitheloid histiocytes were noted in deep corneal stroma. They were unable to explain why granulomatous reaction was confined only to posterior corneal stroma along the plane of Descemet's membrane and in the anterior chamber. Granulomatous reaction has also been documented in the scleral nodules developed in a patient with *Acanthamoeba* sclerokeratitis (Key *et al* 1980). The scleral inflammation in *Acanthamoeba* sclerokeratitis has been hypothesized to result either from secondary immunologic phenomenon related to primary corneal infection or from direct scleral infection by organism from the cornea (Auran *et al* 1987; Garner 1993). Key *et al* (1980) were the first to demonstrate the presence of *Acanthamoeba* cysts in the scleral biopsy.

Garner (1993), based on results of a histopathological study of 30 cases of AK envisaged four stage pathogenetic sequence of events after initial breaching of the epithelium by *Acanthamoeba*. They are:

Stage I: Initial infection: This involves breaching of the surface epithelium. At this stage, there is no inflammatory response, because according to many reports intact amoebae do not induce inflammatory response. Hence, at this stage opsonization of the parasite by antibody and complement must be occurring.

Stage II: Keratocyte depletion: In the second stage of the infection, keratocyte depletion is seen in anterior part of stroma.

Stage III: Inflammatory response: The composition of the infiltrate is predominantly neutrophils with some macrophages.

Stage IV: Stromal necrosis: Reduced thickness of stromal collagen accompanied by acute inflammatory cell infiltration is observed. This is attributed to lysis of stromal collagen by enzymes released by neutrophil and other collagenolytic enzymes. There is minimal or no neutrophil infiltration in this stage.

1.6 DIAGNOSIS

1.6.1 *In vivo* methods

1.6.1.1 Clinical features

Clinical symptoms and signs have been described in detail in section 1.3. Earliest signs of AK are non-specific and not relied upon completely to make final diagnosis of AK, though clinical features do give a clue and are used in conjunction with

laboratory findings. Severe ocular pain is well documented in AK and regarded as most common initial clinical symptom by some authors (Chynn *et al* 1995). Presence of ring infiltrate in the stroma has been described as a hallmark of AK. Ring infiltrate rarely may occur in other conditions such as bacterial corneal ulcers, but in the appropriate clinical setting it is said to be pathognomonic for AK and thus offers diagnostic clue (Theodore *et al* 1985). Chynn *et al* (1995) and Bacon *et al* (1993) suggested that radial keratoneuritis occurs frequently in cases with AK and can be used as one of the criteria to diagnose AK. However, other authors have not found this sign to be frequently present (Sharma *et al* 2000; Srinivasan *et al* 2003). Clinical suspicion of AK in contact lens wearer is much higher than in patients who do not wear contact lens. Thus, increased level of clinical suspicion is very crucial for the diagnosis of AK, in non-contact lens wearers. Clinicians should avoid waiting for the "classic" clinical signs to appear and should initiate confirmatory diagnostic investigations (Schaumberg *et al* 1998).

Auran *et al* (1987) in their review article found that in many of the AK cases, the initial diagnosis made was bacterial, fungal or of viral etiology, hence the diagnosis was delayed in the range of 11 days to 15 months. Also the initial treatment with antibacterial, antifungal or antiviral agents in such cases showed initial improvement but clouded the diagnosis further. Similarly, HSV keratitis has been reported to be initial diagnosis in many cases (Mannis *et al* 1986). The other entity, most often mistaken for amoebic infection, is fungal disease. Both fungal and amoebic keratitis cannot be distinguished from each other because of the similar stromal keratitis, history of trauma with organic matter and the chronicity of the disease. Sharma *et al* (2000) found that the most common clinical suspicion in 45.4% AK cases was of

fungal keratitis. The only bacterial pathogen producing a clinical picture that might be confused with *Acanthamoeba* is the lesion produced by *Mycobacterium* spp. Lesions produced by *Mycobacterium*, like *Acanthamoeba*, are associated with soil trauma to the eye (Mannis *et al* 1986). Thus, clinically, AK has the potential to mimic other forms of keratitis.

1.6.1.2 Confocal Microscopy

Confocal microscope has a simultaneous focus of both the illuminating source and the objective lens on a single point of tissue. Both the illumination source and the objective have the same focal point and therefore it is called confocal microscope. Pinpoint illumination and focus creates high resolution images and produces minute field of view which is obviated by building a large image from the rapid summation of many pinpoint fields of view acquired in an ordered gridlike manner (Winchester *et al* 1995). Tandem scanning confocal (TSC) microscope was first developed in 1964 by Mojmir Petran and Milan Hadravasky. As an ophthalmic imaging device the TSC microscope is effective in imaging the cornea. Cornea is a semi-transparent, low contrast structure. The TSC microscope has the ability to create high resolution, high contrast optical section of the cornea *in vitro*. Confocal microscope also provides optical section that can be stacked to build a three dimensional view (Winchester *et al* 1995). Confocal microscope offers superior resolution and contrast compared to slit lamp. It offers magnification of x200 to x500, optical sectioning of the cornea, increased contrast and ability to see through optical opacities (Chew *et al* 1992).

Acanthamoeba cysts were visualized as highly refractile round bodies of 10-15µm in diameter (Chew *et al* 1992), while Auran *et al* (1994) observed a 26µm diameter object resembling the *Acanthamoeba* cysts using confocal microscope. Winchester *et al* (1995) reported the use of confocal microscope on eight AK patients, wherein the cysts appeared spherical and the size ranged from 10-25µm in size, they were larger than leukocytes and smaller than epithelial cells. Pfister *et al* (1996) demonstrated, apart from double walled cysts, trophozoites with their extending pseudopodia. Confocal microscope was tolerated by the patients except that the patients experienced decreased vision in the examined eye for 10 minutes due to the intensity of the microscope light source. There was no evidence of corneal trauma, such as epithelial abrasions in any of the patients. The disadvantages mainly include small eye movement's interference with confocal microscopy imaging. A moderate degree of patient co-operation is required to keep the eye still for sometime, therefore, the procedure cannot be used on children, debilitated patients and patients with nystagmus (Winchester *et al* 1995).

However, it has advantages in situations where obtaining adequate samples from corneal scrapings or the more hazardous biopsy is difficult. Confocal microscopy helps in rapid diagnosis therefore leads to early treatment and subsequent avoidance of penetrating keratoplasty. It is a non-invasive technique, hence can be performed even when there is fairly low index of suspicion for this disorder. It also helps in monitoring the response to treatment and can keep check on recurrences (Winchester *et al* 1995).

1.6.2 *In vitro* methods

1.6.2.1 Smears and cultures

For the diagnosis of AK, only two specimen types are suitable a corneal scrape and a corneal biopsy. Scraped material is inoculated over the agar plates for culture and smears are made on glass slides for staining immediately (Armstrong 2000). Identification of *Acanthamoeba* organisms can be accomplished using several stains like Gram, Giemsa (Culbertson 1961), calcofluor white stain (Wilhelmus *et al* 1986), Fungiflora Y stain (Inoue *et al* 1999), lactophenol blue (Thomas *et al* 1990) and acridine orange (Hahn *et al* 1998) to stain *Acanthamoeba* in the clinical specimens.

Although the *Acanthamoeba* can grow (not as colonies) on chocolate and blood agar it is preferable to grow it on non-nutrient agar (NNA) which is also called Page's agar, with overlay of bacterial cells, usually *E.coli* (Page 1967). NNA is used to give support and minimize growth of toxic, competitive, or inedible bacteria on which the trophozoites feed (Das 1974). Once inoculated with scraped material, the plates are incubated at 30°C for up to seven days and the plates are examined daily under low power of the microscope. Trophozoites may be seen moving across the field of vision leaving tracks behind on lawn of *E.coli* on NNA plate (Armstrong 2000). Penland *et al* (1998) compared the use of buffer charcoal-yeast extract agar (BCYE), NNA (with dead *E.coli*) and Tryptic soy agar (with horse or sheep blood) for the growth of *Acanthamoeba* and found that the recovery rates were 73%, 71% and 70% respectively. However, NNA with a bacterial lawn was recommended as the method of choice for the isolation of *Acanthamoeba* (Armstrong 2000).

Acanthamoeba culture has also been reported in Sorbarod filters, which consist of compacted concertina of cellulose fibers encased in a cylindrical paper sleeve. Sorbarod filter enhances the growth of *Acanthamoeba* by providing provision for a wide surface area for the attachment of the amoeba and the inter fiber spaces act as an area for scavenging and multiplication of *Acanthamoeba*. The cellulose fiber is not a source of nutrition, but its flaky and pitted nature provides large surface area and stimulates the growth and multiplication of *Acanthamoeba*. This method of biofilm culture can be used for studies on anti-*Acanthamoeba* drug efficacy and analysis of protein profiles of *Acanthamoeba* species (Armstrong 2000a).

1.6.2.2 Histopathologic examination

Histopathologic sections have been stained using hematoxylin and eosin, periodic acid-Schiff, Wright`s, trichrome, Fields`s stain, Heidenhain`s iron hematoxylin-eosin, Gomori-methenamine-silver, Wilder`s reticulum, Hemacolor and Bauer chromic acid (cited in Auran *et al* 1987). Also fluorescein-conjugated lectins, concanavalin A and wheat germ agglutinin have been used to view *Acanthamoeba* cysts and trophozoites in corneal button histopathologic sections (Robin *et al* 1989).

Hiwatashi *et al* (1997) developed 14 monoclonal antibodies (mAbs) against a strain of *Acanthamoeba castellanii* isolated from human cornea. Nine mAbs were specific for *A castellanii* strain while other five mAbs reacted with *Acanthamoeba* spp. belonging to morphological Group II. They utilized these antibodies to detect *Acanthamoeba* using IFA, Western immunoblot analysis and slot blot analysis and highlighted the probable value of this techniques and antibodies in detection of *Acanthamoeba* in clinical specimens.

1.6.2.4 Molecular Methods

Both hybridization and amplification techniques have been used for the detection of *Acanthamoeba* in clinical samples. Gast *et al* (1995) described the use of specific probes based on the sequences of small ribosomal subunit RNA (srRNA) for the identification of *Acanthamoeba*. They suggested that these probes are promising new diagnostic agents which can be used to detect *Acanthamoeba* in clinical samples and from the environment. FISH (Fluorescent in situ hybridization) has been used for rapid and unequivocal identification of *Acanthamoeba* in clinical samples (Stothard *et al* 1999). FISH with help of GSP (Group specific probe) and ST4P (Sequence type T4-specific probe) probes which complement 18S rDNA have been used by Stothard *et al* (1999). GSP identifies all members of the genus *Acanthamoeba*, while ST4P is specific for the subgenus group that is most commonly identified in *Acanthamoeba* keratitis infections. This technique could detect *Acanthamoeba* in 12 (80%) of the 15 samples, which were culture positive with overall sensitivity of 88%.

Gast (2001) developed a novel reverse dot blot method for the detection of *Acanthamoeba* wherein oligonucleotide specific for the ribotypes described by

Stothard *et al* (1998) were developed. They applied these probes in a reverse dot blot method format i.e. oligonucleotides bound to the membrane. Approximately 750bp of 18S rRNA gene was amplified from *Acanthamoeba* isolates and hybridized with oligonucleotides bound to the membrane. Considerably good results were obtained with this hybridization assay however the limitations of it being expensive and cumbersome existed, since this assay had be to be performed both under high and low stringency condition which made it time consuming and required fluorescent microscope which is expensive.

Polymerase Chain Reaction (PCR) is a molecular biological technique developed in 1985 by a team of scientists at Cetus Corporation, led by Kary Mullis (Mullis 1986). PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in target DNA. PCR technique involves series of cycles; template denaturation, primer annealing and the extension of the annealed primers with the help of DNA polymerase enzyme. This results in exponential accumulation of the specific fragment (Erlich 1992).

Vodkin *et al* (1992) were the first to devise a PCR based assay for the detection of *Acanthamoeba*. Two primer sets specific to 18S rDNA were selected which included one short target of 272bp which was a genus specific marker and a long target that offered the promise for distinguishing pathogen from non-pathogen. Apart from 18S rDNA, the 26S rRNA gene has also been explored to develop a diagnostic marker. Lai *et al* (1994) developed a non-radioactive DNA probe (ArDNA-a) complementary to 26S rDNA. This probe was 126bp *BamH1-Sst1* restriction fragment of 925bp 26S

rDNA unit, which they used for detection of *Acanthamoeba* from the crude lysate of its culture and proposed that it can be used for detecting *Acanthamoeba* in clinical specimens.

Lehmann *et al* (1998) were the first ones to use PCR for the detection of *Acanthamoeba* in clinical samples, which included corneal scrapings, and tear samples. PCR was carried out with two markers belonging to 18S rDNA, both the primers sets were genus-specific. Mathers *et al* (2000) used three primer sets, which were based on 18S rDNA for the detection of *Acanthamoeba* in the clinical samples and also for the confirmation of their results obtained from confocal microscopy. Using PCR they confirmed the presence of *Acanthamoeba* in cases, which were positive by confocal microscopy and epithelial biopsy. Schroeder *et al* (2001) also developed primer pair called JDP1 and JDP2 for the detection of *Acanthamoeba* from corneal scrapings. This primer pair amplified a subset of 18S rRNA gene and they called it as ASA.S1 (*Acanthamoeba* specific amplimer) amplimer. ASA.S1 amplimer obtained from JDP1 and JDP2 primers is highly genus specific and could be amplified from all the genotypes (Schroeder *et al* 2001).

1.7 TAXONOMY AND CLASSIFICATION

Although the genus *Acanthamoeba* was first established in 1931, considerable confusion about its taxonomic classification existed in the literature until recently. Volkonsky (1931) established the genus *Acanthamoeba* in 1931. He divided the then existing genus *Hartmannella* into three genera i.e., *Hartmannella*, *Glaeseria* and *Acanthamoeba*. He created genus *Acanthamoeba* since amoebae were characterized by the appearance of pointed spindles at mitosis and double walled cysts with ostioles

and an irregular outer layer (Volkonsky 1931). In 1952 Singh and in 1970 Singh and Das stated that classification of amoebae by form, locomotion and appearance of cysts has no phylogenetic value and the shape of mitotic spindle was inadequate as a generic character and thus they discarded the genus *Acanthamoeba*. Meanwhile in 1966, Pussard agreed with Singh (1952) that spindle shape should not be used for inter-generic differentiation but considered the distinctive morphology of cyst to be decisive character at the generic level and recognized genus *Acanthamoeba*. In 1967, Page also concluded that spindle was doubtful criterion for classification, but the presence of acanthopodia and structure of the cyst was sufficiently distinctive and concluded that generic designation of *Hartmannella* and *Acanthamoeba* are justified. In 1975, Visvesvara and Balamuth identified definable and demonstrable differences in the trophozoite and cyst stages of *Acanthamoeba* and *Hartmannella* including differences in their nutritional requirement and serological responses. Singh and Hanumaiah (1979) accepted the genus *Acanthamoeba* but placed in the family *Hartmannellidae*. Sawyer and Griffin (1975) created a new family *Acanthamoebidae* and Page (Bovee1965) designated the sub-order Acanthpodina under the order Amoebida.

On the whole, classification of *Acanthamoeba* at the genus level is relatively clear, but since it is an asexual organism, the concept of the species is unclear. Many approaches have been used for the subgenus classification of *Acanthamoeba*, which mainly include:

1.7.1 Morphological

In 1977, Pussard and Pons proposed the classification based on the morphology of cysts (Figure 1.7). They established 18 different species in 3 distinct groups (Table 1.3)

Table 1.3 : Morphological classification of Acanthamoeba

Group I	Group II	Group III
A. astronyxis	*A.castellanii*	*A.culbertsoni*
A.comandoni	*A.rhysodes*	*A.royreba*
A.echinulata	*A.mauritaniensis*	*A.palestinensis*
A.tubiashi	*A.divionensis*	*A.lenticulata*
	A.griffini	*A.pustulosa*
	A.polyphaga	
	A.lugdunensis	
	A.quina	
	A.triangularis	

Criteria for morphological classification

Group I: This group members have large trophozoites and cysts and average diameter of the cyst is ≥ 18μm. The ectocyst and endocysts are widely separated and the ectocyst is smooth or gently wrinkled, while the endocyst is more or less stellate and meets the ectocyst at the ends of arms or rays. Operculum is normally at level of the ectocyst.

Group II: The mean diameter of the cyst in this group is 18μm. The ectocyst and endocyst are close together or widely separated. Ectocyst may be thick or thin, and usually is wrinkled or mamillated. Endocyst may be stellate, polygonal, triangular or sometimes round or oval. *Acanthamoeba* belonging to this group do not have well

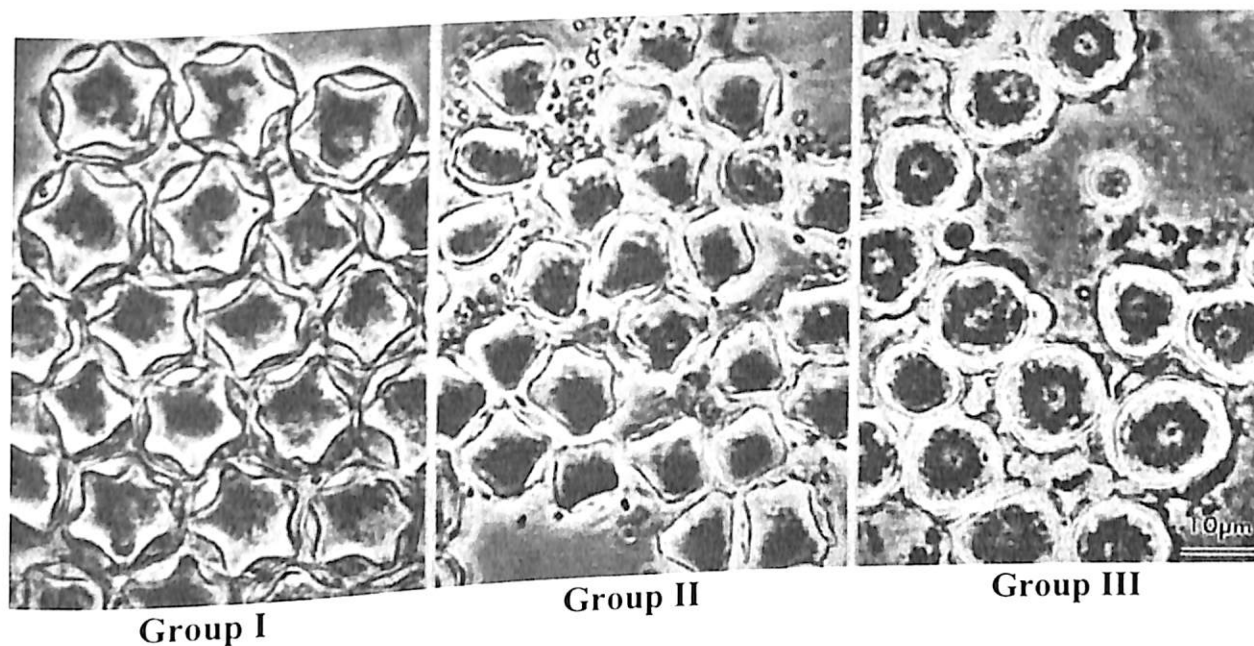


Figure 1.7 Morphological groups of Acanthamoeba

Courtesy Dr Y Tomiyama (http://protist.i.hosei.ac.jp)

developed arms or rays. Operculum is at the junction of endocyst and ectocyst and is in the depression formed by the infolding of the ectocyst. Most widespread and commonly isolated *Acanthamoeba* belong to this group.

Group III: In this group the mean diameter of the cyst is <18μm. Ectocyst is thin and is either gently rippled or unrippled. Endocyst is usually round but has 3-5 gentle corners.

Morphological characteristics have been used earlier for classification but they vary in their evolutionary rates and are subject to parallel and convergent evolution (Web address 1). The systematic classification of *Acanthamoeba* based on cyst morphology has been deemed ambiguous and vague. It can define an isolate up to the genus level but variations occur in cyst forms within the species and a clonal population. This fact makes classification-using morphology a very subjective process. Also, this system does not show genetic relationship between the strains (Pilar *et al* 2001). Sawyer (1971) observed that the ionic strength of the growth medium could alter the shape of the cyst walls, thus substantially reducing the reliability of cyst morphology as a taxonomic characteristic.

1.7.2 Biochemical and immunological

In last decade, several groups have used analysis of isoenzyme electrophoretic patterns to address intrageneric relationship and to test morphological classification. Moura *et al* (1992) found good agreement between isoenzyme pattern and morphological groups, but their study was limited and included only one Group 2

isolate i.e., *A castellanii*. Using esterase and acid phosphatase isoenzymes pattern on starch gel electrophoresis, Costas and Griffiths (1986) divided 32 *Acanthamoeba* strains into 12 groups. These groups did not always correspond with the species designation. De Jonckheere (1983) carried out isoelectric focussing for 30 strains of *Acanthamoeba* belonging to different species. He compared zymograms of acid phosphatase, leucine aminopeptidase, malate dehydrogenase, propionyl esterase, glucose phosphate isomerase, phosphoglucomutase and alcohol dehydrogenase. He also analyzed protein patterns separated by agarose isoelectric focussing in a pH gradient of 5-8. The result suggested changes in the taxonomy within the morphology Group 2 of Pussard and Pons (1977). The drawback of this method of classification was that, different zymoderms existed within a species, so he suggested that neither isoenzyme pattern nor morphological analysis should be used alone for subgenus classification (De Jonckheere 1983).

Daggett *et al* (1985) carried out extensive phylogenetic analysis of *Acanthamoeba* based on isoenzyme electrophoretic patterns of 71 isolates, which included 15 different species. They identified 15 different lineages, but found inconsistencies since eight out of 15 lineages had more than one species in them and many of the species occurred in multiple lineages. The authors assumed this to be due to inconsistency in morphological classification (Dagget *et al* 1985). Jacobson and band (1987) reported that the environmental isolates changed pattern when they are grown axencially under the laboratory condition. This type of change can be due to changes in gene expression or posttranslational process of the enzymes. This could be problematic for isoenzyme-based classification. Thus, each study classified isolates of *Acanthamoeba* into several different groups that often were inconsistent with

species and or morphological group designations. Each study suggested a need for revision of classification. Apart from the isoenzyme pattern, antigenic differences between *Acanthamoeba* isolates have also been used for typing (Visvesvara and Balamuth 1975; Stevens *et al* 1977).

1.7.3 Molecular

In the 1960 many breakthrough ideas of modern molecular biology were published which included, the detailed composition or sequences of biomolecules like protein and DNA. This new knowledge was applied to determine the generic relationship and evolutionary lineages between various animal kingdoms and microorganisms. Homologous molecules were discovered in different organisms, and the comparison revealed that the basic biomolecular framework of all living things is the same; an observation consistent with the very Darwinian notion that all life is, ultimately, monophyletic. Typically, DNA sequences are used to determine phylogenetic relationship, for relatively recent events, for example, the human and chimpanzee split, where protein sequences are too conserved to be useful. Protein sequences are desirable for more ancient events, for example, human divergence from insects, when DNA sequences are usually too divergent to make accurate estimates on the basis of patterns of nucleotide substitutions (Web address 1).

The most useful single molecule, which has been used to classify and determine genetic relationship, is ribosomal RNA (rRNA). Evolutionary studies of the rRNA caused a revolution among the phylogenists (Web address 2). Ribosomes are composed of two subunits, one large and one small. Each of the subunits is a complex of RNA and protein. The specific type of RNA in ribosome is called **ribosomal RNA**,

or **rRNA**. There are four specific types of rRNA in eukaryotes, and these are designated by their size: 28S or 26S, 18S, 5.8S, and 5S. Of the ribosomal RNAs, the 28S or 26S, 5.8S and 5S are found in the large subunit and the 18S is found in the small subunit. Subunit sizes and general makeup of the eukaryotic ribosomal subunits are shown in Figure 1.8 (web address 3).

The rRNA genes are ubiquitous and abundant in all active cells (Olsen and Woese 1993). The genes for the rRNA precursor appear in multiple copies and are organized in tandem arrays separated by non-transcribed spacers along the eukaryotic nuclear genome. Each repeating unit consists of three rRNA genes (18S, 5.8S and 28S or 26S) and two internal and one external transcribed spacers. Each of the 18S and 28S or 26S rRNA genes are organized into several highly conserved cores interrupted by variable divergent domains also called expansion segments. The fundamental importance of rRNA in protein synthesis imposes evolutionary constraints to its overall structure (Melen *et al* 1999). Hence rRNA genes are conserved in structure and function through billions of years hence called an ancient molecule. It changes slowly enough to provide information over the entire evolutionary spectrum. It has also resisted "lateral transfer" of the genes between different species (web address 2) Thus, rRNA is conserved enough to identify and yet it contains enough variability to determine evolutionary relationships (web address 3) The conservation in secondary structure of rRNA can help in providing a basis of accurate alignment. Furthermore, the large size of molecule minimizes statistical fluctuations. Finally because it is relatively easy to sequence rRNA genes, a large and continuously growing database of published sequences is available (web address 2). Using rRNA sequences it is possible to estimate the historical branching order of the species, the total amount of

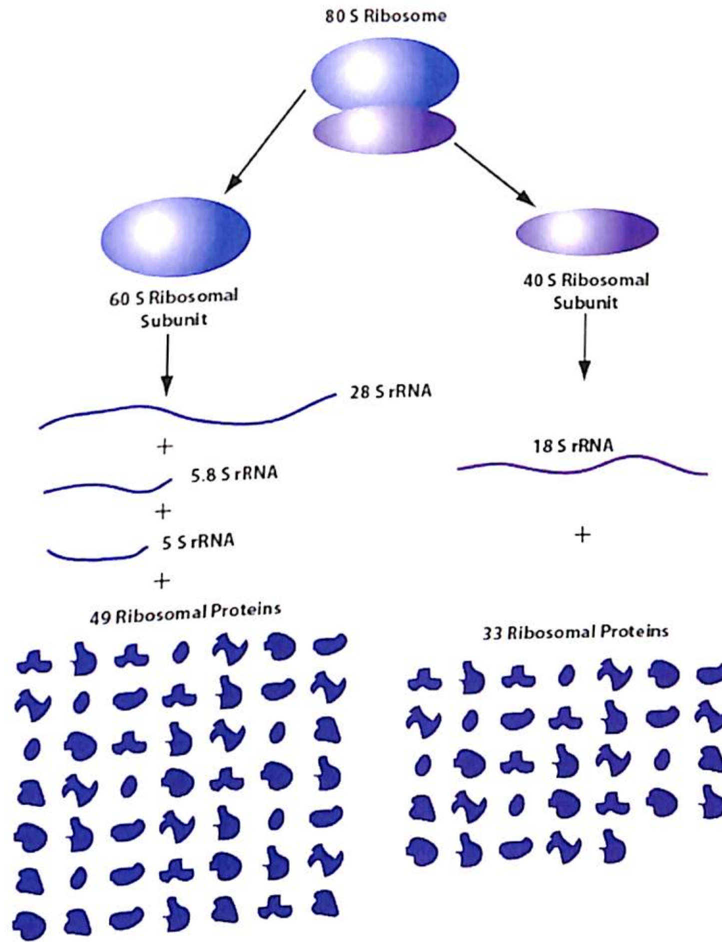


Figure 1.8 Eukaryotic ribosomal subunits and their make up

sequence change and the "genetic line" of descent and hence it is called the molecular clock of evolution. The molecular clock hypothesis posits that a given biological molecule exhibits a relatively constant rate of change over time, irrespective of the taxonomic lineage within which it evolves (Zuckerkandl and Pauling 1965).

The total cellular DNA content of *Acanthamoeba* Neff strain ranges between 1 to 2 pg for uninucleate amoebas although the ploidy level is uncertain. The size of haploid genome of *Acanthamoeba* has been estimated to be ~ 4-5 x 10^7 bp (Byers 1986; Byers *et al* 1990). In *Acanthamoeba*, the transcription unit of ribosomal RNA has been identified to be 12kbp in length, of which 9.7 kbp includes the external transcribed spacer, internal transcribed spacers, 18S rDNA, 5.8S rDNA and 26S rDNA (D'Alessio *et al* 198'). The remaining 2.3 kbp is the intergenic spacer (Yang *et al* 1994).) Approximately 600 copies of the repeat unit are present in each *Acanthamoeba* cell (D'Alessio *et al* 1981).

The *Acanthamoeba* 18S rRNA gene has been sequenced in its entirety (Gunderson and Sogin 1986) and its coding sequence is 2303bp long (Byers *et al* 1990). *Acanthamoeba* has the second largest 18S rRNA gene sequenced to date (Byers *et al* 1990). 18S rRNA gene sequences are highly conserved and have strong functional constraints and hence change slowly and reveal phylogenetic relationship between organisms (Sogin *et al* 1989). Therefore they are being used to determine the taxonomic status of *Acanthamoeba* and till date sequences of all known *Acanthamoeba* species are available in the public databases (Gast *et al* 1996; Stothard *et al* 1998). At present sequencing of complete 18S rRNA gene is considered as most

reliable method of genotyping (Seal 2003). 18S rRNA gene has the right combination of conserved and variable domains, which makes it an appropriate phylogenetic marker. If there is too much change within the gene, then the sequences become randomized, and if there is too little change (if the gene is too conserved), then there is little or no change between the evolutionary branching of interest, and it will not be possible to infer close genus or at species level.

Like all eukaryotes, *Acanthamoeba* have both cytoplasmic and mitochondrial ribosomes. Both types of ribosomes consist of large subunit (LSU) and small subunit(SSU) ribonucleoproteins. Rns are the nuclear genes coding for 18S rRNA found in the cytoplasmic SSU, while rns are mitochondrial genes coding for 16S rRNA found in mitochondrial SSU. The total length of Rns sequence for *Acanthamoeba* is 2250-2650bp while that of rns sequence is 1578-1514bp (Byers *et al* 2001). Recently the above have been explored to perform genotyping of *Acanthamoeba* isolates. The techniques mainly include Restriction fragment length polymorphism (RFLP) of the complete cellular DNA and mitochondrial DNA, Riboprinting or PCR-RFLP of ribosomal nuclear or mitochondrial genes and complete or partial sequencing of the ribosomal nuclear or mitochondrial genes. All these recent molecular techniques, which have been used for genotyping or subgenus classification of *Acanthamoeba*, are described below:

1.7.3.1 RFLP of the ribosomal mitochondrial DNA

Byers *et al* (1983) carried out RFLP on 13 strains of *Acanthamoeba* which included 10 strains of *A.castellanii*, two of *A.polyphaga* and one of *A.astronyxis*. They found relatively high degree of molecular diversity among strains, which have been

classified as a single species. Yagita and Endo (1990) on the other hand analyzed 8 *Acanthamoeba* isolates and similarly revealed a large degree of interstrain genetic diversity. Similar results were reported by Bogler *et al* (1983) who also examined mitochondrial DNA fragment patterns obtained from 15 *Acanthamoeba* strains. Gautom *et al* (1994) typed 15 *Acanthamoeba* clinical isolates and compared it to 25 environmental isolates and 10 *Acanthamoeba* ATCC strains. Seven different fingerprint groups emerged from their analysis of clinical isolates with six selected restriction enzymes. They concluded this technique could be used to determine taxonomic relationships between *Acanthamoeba* isolates.

1.7.3.2 RFLP of the whole cell DNA

McLaughlin *et al* (1988) characterized four *Acanthamoeba* species by performing RFLP on their total cellular DNA. Total DNA was digested with three enzymes and later hybridized with 5.2 kilobase long *A.castellanii* mitochondrial DNA probe. The RFLP results revealed that this method of classification was in agreement with the previous classification methods like the ones based on isoenzyme pattern and morphology of *Acanthamoeba*. Kilvington *et al* (1991) typed 33 *Acanthamoeba* isolates belonging to Group II morphological group. Using this technique they differentiated these isolates into seven multiple strains and three single-strain groups and found that keratitis causing isolates grouped together in a single group.

1.7.3.3 PCR-RFLP of the ribosomal nuclear genes

Chung *et al* (1998) amplified approximately 2.3 kilobases of 18S rDNA of 23 *Acanthamoeba* isolates and subsequently digested them using 10 restriction enzymes. The restriction pattern were analyzed and the dendrograms obtained were in

agreement with the morphological grouping of Pussard and Pons (1977) and with the dendrogram constructed by Stothard *et al* (1998), which is based on complete 18S rDNA sequences. Kong and Chung (2002) used the similar strategy as above and typed 24 *Acanthamoeba* strains, however the numbers of restriction endonucleases used by them were four instead of ten. They provided a scheme to classify *Acanthamoeba* based on the restriction pattern in combination with morphological features of cysts of *Acanthamoeba*. They believed this key or combination could be used for subgenus classification of *Acanthamoeba* especially when large numbers of clinical and environmental samples have to be typed. Khan and Paget (2002) performed riboprinting for 8 known *Acanthamoeba* isolates (known species) and 16 unknown isolates from environment and clinical specimens. They partially amplified the 18S rRNA gene using the primers described by Gunderson and Sogin (1986) and then digested the amplified products with 8 restriction enzymes. The analysis of the restriction pattern highlighted ambiguities in the morphology-based classification. Comparison of the restriction pattern of known and unknown *Acanthamoeba* isolates revealed that all unknown clinical isolates either belonged to the species *A.castellanii* or *A. polyphaga*.

1.7.3.4 PCR-RFLP of the mitochondrial genes

Yu *et al* (1999) amplified 1550 bp of the mitochondrial ribosomal gene from 25 *Acanthamoeba* strains and digested them with 8 restriction enzymes. They preferred mitochondrial gene over nuclear because of the fact that mitochondrial genes do not have introns. The results obtained were in concurrence with classification based on morphology and the one based on riboprinting of 18S rRNA gene (Chung *et al* 1998).

1.7.3.5 Nucleotide sequence based methods

Complete sequencing of the genes

- Johnson *et al* (1990) were the first to use ribosomal nucleic acid sequences to study the phylogeny of the genus *Acanthamoeba*. The results obtained by them were concordant with the morphological classification of Pussard and Pons (1977) but their study included only a limited number of strains.

- Gast *et al* (1996) proposed four distinct sequence types based on analysis of complete sequences of 18S rRNA gene from 18 *Acanthamoeba* strains. They were designated as sequence types T1-T4. T1 included *A.castellanii* V006. T 2 included *A. palestinensis* Reich, T3 included *A.griffini* S7, while T4, the fourth sequence type included 15 isolates classified as *A.castellanii, A.polyphaga, A.rhysodes* and 10 other isolates of *Acanthamoeba* obtained from keratitis patients. Interstrain differences between T4 was 0%-4.3% whereas differences among sequence types were 6%-12%. They found that T4 has a worldwide distribution, since isolates from Asia, Europe and North America belonged to this group. Data also indicated that T4 includes representatives of three different species *A.castellanii, A.polyphaga* and *A.rhysodes*. These findings confirmed the inconsistencies of the morphological classification.

- Stothard *et al* (1998), in extension to 4 sequence types proposed by Gast *et al* (1996), further sequenced 35 strains of *Acanthamoeba* making a total of 53 strains representing 16 species. They identified eight additional lineage

sequence types T5-T12. Eight of 12 sequence types represented only a single species, while other 4 included more than one nominal species suggesting that sequence types could be equated with species in some cases and with complexes of closely related species in others. The largest complex sequence type T4 contained 6 closely related species and 24/25 isolates from keratitis patients. One keratitis causing isolate belonged to genotype T3. Six species, which were included in the T4 sequence type, included *A.castellanii, A.polyphaga, A.rhysodes, A.hatchetti, A.culbertsoni* and *A.lugdunensis*. Even this classification was insufficient for full phylogenetic resolution of branching orders within the T4 sequence type but the mixing of species observed at terminal nodes confirmed that traditional classification of isolates is inconsistent. Walochnik *et al* (2000) genotyped three *Acanthamoeba* strains isolated from keratitis patients from Austria and found that two isolates belonged to the T4 genotype while, one belonged to T6 genotype.

- Horn *et al* (1999) proposed another two genotypes T13 and T14 based on complete sequences of 18S rRNA gene. Each genotype consisted of one *Acanthamoeba* strain each and both isolates exhibited <92% sequence similarity to each other and to other *Acanthamoeba* isolates. Two years later Gast (2001) also designated two *Acanthamoeba* strains isolated from stool specimens as T14 genotype based on the complete sequence of 18S rRNA gene. The two genotypes showed 11% dissimilarity from the existing *Acanthamoeba* isolates belonging to other genotypes.

- A recent publication has examined the mitochondrial rns sequences (Ledee *et al* 2003). They analyzed complete rns sequences (16S rDNA) of 68 strains of *Acanthamoeba*. These included 35 unique sequences and represented 11 of 12 Rns genotype. rns sequences also showed that mT4 is large clad, which includes 52 different strains with 22 different, but closely related rns sequences. Clade was supported by bootstrap values of 99%. Sequence dissimilarity within mT4 genotype was 0.7%. It included 18 strains currently classified into 6 different species and 35 unclassified strains. They sub-divided the T4 genotype into 8 subtypes and designated them from T4a – T4h, and this was done based on the high bootstrap values between the subtypes. The phylogeny based on mitochondrial rns sequences was mostly consistent with that observed with nuclear Rns DNA. The exceptions being that, several mT4 strains with identical mitochondiral 16S rDNA sequences, had different nuclear sequences. The reason for this could be either of the below; faster evolution of nuclear genes over mitochondrial genes, which is not quite possible but cannot be ruled out; true sexual process which occurs, followed by a mitotic sorting out of mitochondria; or because of parasexual nuclear process or cytoplasmic exchange in the absence of nuclear exchange. The advantages which rns sequencing has over Rns sequencing is that the mitochondrial gene is much shorter and more consistent in length, have a higher percentage of alignable sequences and have none of the complications caused by multiple alleles or introns which are occasionally found in Rns.

Partial sequencing of the 18S rRNA gene

- DF3 fragment is part of ASA.S1 amplimer of 18S rRNA gene and has been used previously by few researchers to genotype *Acanthamoeba* isolates (Schroeder *et al* 2001; Booton *et al* 2002; De Jonckheere *et al* 2003). Schroeder *et al* (2001) used DF3 to genotype 12 South African isolates and 6 corneal scrape specimens from Scotland. A total of 71 *Acanthamoeba* strains which included 53 *Acanthamoeba* strains belonging to the 12 genotypes described by Stothard *et al* (1998) and the ones mentioned above, were used to construct the tree. They found that using these sequences genotype T4 could not be distinguished from the closely related T3 and T11 genotype. They also found that the trees obtained were not as robust as those obtained with complete sequence of 18S rDNA. Booton *et al* (2002) sequenced the same fragment of the 18S rRNA gene from 13 corneal scrapes obtained from keratitis patients from Hong Kong. They were successful in genotyping the isolates using this marker and highlighted the use of this diagnostic fragment where axenisation of the culture is not prerequisite. De Jonckheere *et al* (2003) also performed genotyping of the *Acanthamoeba* isolates obtained from infected cornea of the keratitis patients, from their contact lens, contact lens boxes and saline solutions using DF3 fragment and found that all keratitis causing isolates belonged to genotype T4.

- Khan *et al* (2002) amplified partial Rns (910-930 bp) and genotyped 14 isolates of *Acanthamoeba*. They grouped them as pathogenic and nonpathogenic based on osmotic tolerance, extracellular proteases and cytopathic effect on rabbit corneal epithelial cell line. They found that ten of

the fourteen *Acanthamoeba* isolates were pathogenic and belonged to genotype T3, T4 and T11. It was for the first time that a keratitis causing *Acanthamoeba* isolate was designated to genotype T11.

- Hewett *et al* (2003) genotyped 6 environmental *Acanthamoeba* isolates based on 1475 bp of 18S rRNA gene and allocated them to a new genotype named T15.

1.7.3.6 Random amplified polymorphic DNA (RAPD)

RAPD was used for genotyping 19 *Acanthamoeba* strains isolated from Brazil. RAPD profiles showed highly polymorphic pattern among most of the isolates, although few closely related isolates formed clades. They suggested that RAPD is fast and informative technique but can only be used for gross characterization of new isolates and for assessment of genetic relatedness between the isolates. When it comes to determining more precise relationships, other techniques like sequencing of ribosomal DNA subunits should be performed (Alves *et al* 2000).

CHAPTER 2: AIMS

2.1 Introduction

As seen from the literature review, to date AK has been described primarily from developed countries with several studies suggesting soft contact lens wear as the greatest risk factor. In contrast, the reports from India and other developing countries are few and have mainly been in non-contact lens wearers. This low prevalence of *Acanthamoeba* keratitis in India has apparently been due to the belief that the disease is related mainly to contact lens wear - a factor usually absent in most cases of keratitis from this part of the world, and also because of the unavailability of simple and sensitive diagnostic tools for its clinical detection. A consequence of this low reporting is the skeptical acceptance of *Acanthamoeba* as a pathogenic organism by the medical fraternity as well as the health authorities in India. Hence due to the above facts there are very few centers in India who actually diagnose AK and even fewer who take up research projects in it.

2.2 AIMS

2.2.1 Primary aims

- To determine the epidemiological features and predisposing factors for *Acanthamoeba* keratitis patients in India.
- To document the clinical features in non–contact lens associated keratitis and determine its utility in the diagnosis of AK.
- To evaluate the conventional microbiological techniques for diagnosis of AK.
- To develop molecular diagnostic markers to aid rapid and accurate diagnosis of AK.

- To determine taxonomic status of *Acanthamoeba* and perform sub-genus classification using conventional morphological method and recent nucleotide sequence based methods.

2.2.2 Secondary aims

- To determine the minimum cysticidal concentrations for drugs commonly used in the treatment of AK and compare their efficacy.
- To determine the histopathologic and immunohistochemical features of AK.

CHAPTER 3: EPIDEMIOLOGY, PREDISPOSING FACTORS, CLINCIAL FEATURES AND TREATMENT OUTCOME IN *ACANTHAMOEBA* KERATITIS PATIENTS

3.1 INTRODUCTION

Acanthamoeba keratitis (AK) has now been reported from Europe (Bacon *et al* 1993), Asia (Houang *et al* 2001), Australia (Gebauer *et al* 1996), America (Stehr-Green *et al* 1989) and parts of Africa (Leck *et al* 2002). Studies have suggested that contact lens wear is the leading risk factor for AK (Auran *et al* 1987; Stehr-Green *et al* 1989; Bacon *et al* 1993; Radford *et al* 1998). In contrast, the reports from India (Davamani *et al* 1998; Sharma *et al* 2000; Srinivasann *et al* 2003) and other developing countries (Alves *et al* 2000; Xuguang *et al* 2003) are few and have mainly been in non-contact lens wearers. The prevalence of contact lens associated AK seems to be declining. Radford *et al* (2002) have reported that there is reduction in the cases of AK from 1995 in UK mainly because of improvement in contact lens hygiene and widespread dissemination of scientific knowledge about AK in the public. There is no study describing the prevalence of AK in India, although earlier report from this institute estimated it to be 1-3% in keratitis patients (Sharma *et al* 2000). Literature focusing on the clinical features of AK has been mainly from contact lens wearing keratitis patients (Moore *et al* 1985; Stehr-Green 1989) with few reports describing the clinical picture of AK in Indian patients (Sharma *et al* 2000; Srinivasan *et al* 2003). Hence there is dearth of knowledge about the epidemiology and clinical features of AK patients from India.

This report highlights the epidemiological features, predisposing factors, clinical features, and treatment outcome of AK patients diagnosed at the L V Prasad Eye Institute (LVPEI), a tertiary eye care center, located at Hyderabad, India. To the best of our knowledge this is the largest series of human *Acanthamoeba* keratitis patients ever reported in literature.

3.2 MATERIALS AND METHODS

3.2.1 Patients

Patients who presented to the Cornea Services of the LVPEI between January 1995 and December 2003, and who were subsequently diagnosed as having AK by culture and/or smear were included in this study. A total of 191 individuals were diagnosed as AK among the 8537-keratitis patients seen during the period 1995-2003. However medical records of 172 patients were accessible to us and thus were reviewed for demographic and clinical data.

3.2.2 Investigative procedures

The investigative procedures followed at LVPEI are:
At presentation, a detailed history of the patient was recorded on a predesigned proforma (Appendix 1). This included socio-demographic information; duration of symptoms, predisposing factors, and prior therapy received, if any, and associated ocular and systemic diseases.

The patients were then subjected to a thorough ocular examination for size, location and clinical features of the ulcer using a slit-lamp biomicroscope. In suspected cases of infectious keratitis, corneal scrapings were collected by the ophthalmologist under

local anesthesia and processed for microbiological investigations by smear and culture. Complete clinical and microbiological data was captured in the proforma for subsequent entry in the corneal ulcer database and the data was subsequently analyzed using this database. Further details on AK patients were also manually collected from medical records of the patients.

3.3 RESULTS

3.3.1 Epidemiological Features

The number of AK patients increased through the years i.e. from 1995 till 2003, however the increase was parallel to number of keratitis patients seen during the same period (Figure 3.1 and 3.2). The demographic features of patients with *Acanthamoeba* keratitis are shown in Tables 3.1 and 3.2.

Table 3.1 :. Gender distribution of patients with AK (n=172)

Male			Female		
Number	%	95% CI	Number	%	95% CI
115	66.9	59.5-73.4	57	33.1	26.5-40.4

Table 3.2 : Age distribution of patients with AK (n= 172)

Range (Years)	Number	%	95% CI	Mean Age
0-20	22	12.8	8-18	-
21-40	99	57.56	50-64.7	-
41-60	43	25	19-31	-
> 61	8	4.65	2.2-9.0	-
Total	172			35.1yrs

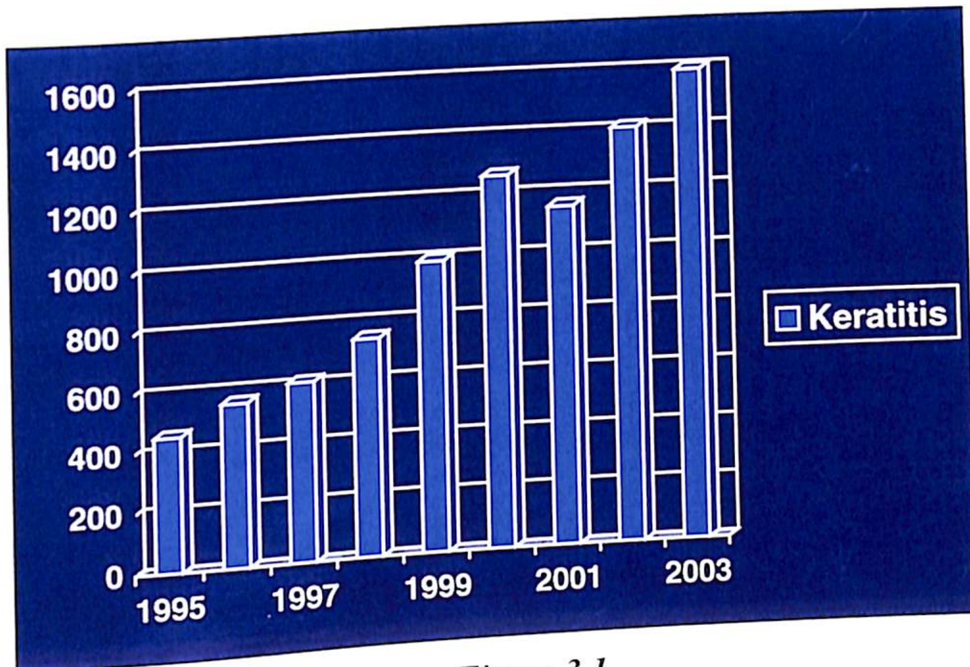


Figure 3.1
No. of Keratitis patients seen at LVPEI 1995-2003 (n=8537)

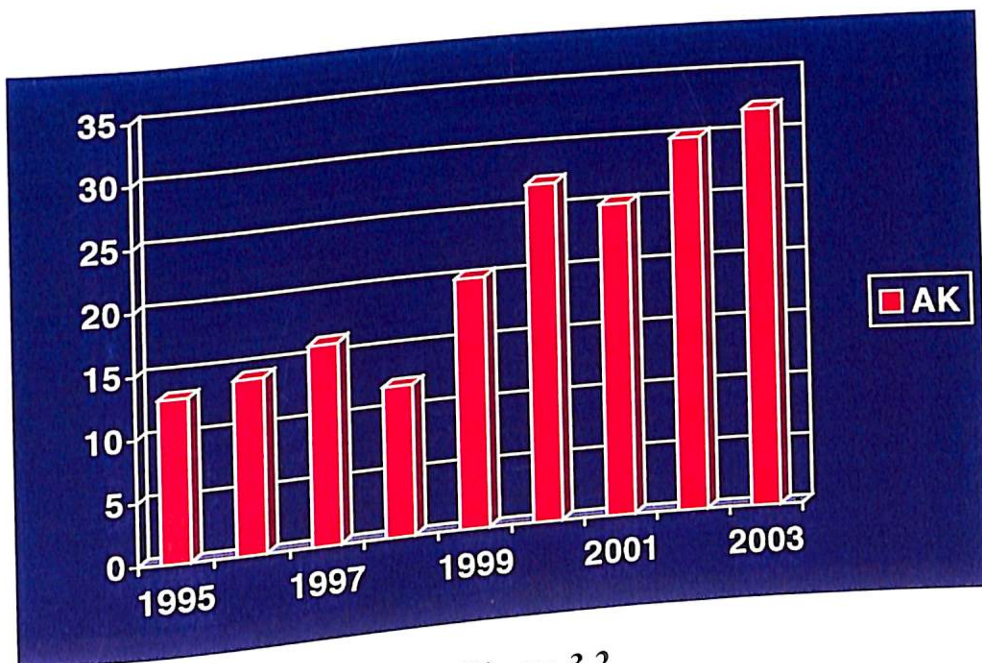


Figure 3.2
No. of AK patients seen at LVPEI 1995-2003 (n=191)

Among the 8537 keratitis patients diagnosed at the LVPEI between January 1995 and December 2003, 172 (2%) were diagnosed as AK, either proven by culture or smear examination of the corneal scrapings. Among these patients 115 (66.9%) were males and 57 (33.1%) were females (ratio 2:1). The average age at presentation was 35.1 years and it ranged from 2–70 years. A majority of the affected individuals belonged to the age group that ranged between 21 and 40 years.

The affected individuals were engaged in various occupations, which are listed in Table 3.3.

Table 3.3 : Occupational status of patients with AK (n=172)

Occupation	Number	%	95% CI
Agriculture	59	34.3	27.6-41.6
Manual labour -agriculture	39	22.7	17-29.5
Household work	25	14.6	9.9-20.6
Office	9	5.2	2.6-9.7
Unemployed	31	18	12.9-24.4
Student	9	5.2	2.6-9.7

Ninety-eight patients (57%) were engaged in agricultural – related work or manual labor. Table 3.4 shows the duration of symptoms in patients prior to their presentation to the Cornea services of LVPEI. Thirty-eight patients (22%) were examined within one to two weeks of onset of symptoms; the duration varied between two to four weeks in 88 patients (51.2%) and > 1-2 months in 25 patients (14.3%).

Table 3.4: Duration of symptoms prior to presentation in AK patients at LVPEI (n=172)

Duration	Number	%	95% CI
Less than 1 week	9	5.2	2.6-9.7
1 to 2 weeks	38	22	16.5-28.8
2 to 4 weeks	88	51.2	43.7-58.5
> 1 to 2 months	25	14.3	9.9-20.6
> 2 to 3 months	9	5.2	2.6-9.7
> 3 months	3	1.7	0.3-5.2

An analysis of the incidence of AK over an eight year period (between 1996 and 2003) revealed that AK was prevalent in the summer (February to May) and monsoon (June to September), while very few cases were reported in the winter (October to January) as depicted in Figure 3.3.

An analysis of the socio-economic status of 172 patient revealed that 122 (71%) of the patients were non-paying patients (salary less than Rs.1000/month) and the rest 50 (29%) were paying (Table 3.5).

Table 3.5 : Socio-economic status of patients with AK (n=172)

Paying			Non-paying		
Number	%	95% CI	Number	%	95% CI
50	29	22.7-36.2	122	71	63.7-77.2

3.3.2 Predisposing Factors

A history of recent injury to the affected eye was reported in 66 (38.3%) of 172 patients. The object causing the trauma was not identified in 7 (10.6%) patients. Among the objects identified (Table 3.6), injury with plant or agriculture material led to AK in 23 (35%) individuals. Seventeen patients (25.6%) complained of dust falling

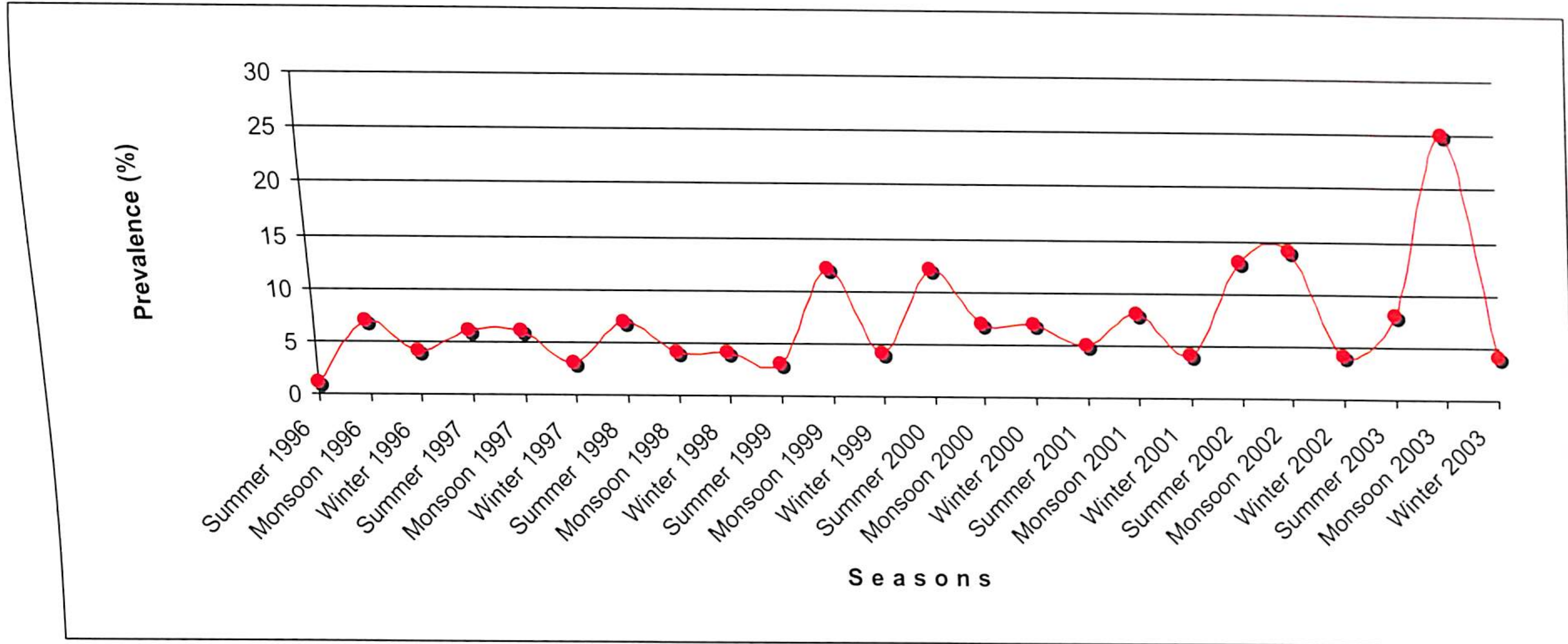


Figure 3.3
Seasonal variation in the prevalence of Acanthamoeba keratitis
The peaks indicate a higher prevalence in summer and monsoon season

into the eye. Other objects responsible for injury were stones, cow horn/tail, chemicals and fingernail. Apart from trauma nine patients (5.2%) gave history of washing their face or eyes prior to the onset of symptoms with previously stored water. Among the 172 individuals with AK, only one patient was a contact lens wearer (0.6%) while other 171 had never worn contact lenses.

One patient had undergone cataract surgery one month before onset of symptoms in the affected eye; while another patient developed AK in the eye in which penetrating keratoplasty was performed for corneal dystrophy. One patient was immunocompromised since the patient was positive for both HIV and HBsAg, while another patient was only HBsAg positive. Another patient had a history of ocular albinism from childhood.

Table 3.6 : Risk factors identified in patients with AK (n= 76)

Risk factor	Number	%	95% CI
(i) Trauma	66	86.8	77.2-92.8
Plant / agriculture	23	34.8	24.4-46.9
Dust	17	25.7	16.6-37.5
Stone	2	3	0.2-11
Animal	7	10.6	4.9-20.6
Chemical	5	7.5	2.9-16.9
Human (nail)	5	7.5	2.9-16.9
Unknown	7	10.6	4.9-20.6
(ii) Washing eyes with stored water	9	11.8	6.1-21.2
(iii) Contact lens wea.	1	1.3	0-7.7
Total	**76**	**44.1**	**36.9-51.6**

Thus, based on the medical records of the patients with AK, risk factors could be associated only in 76 individuals (44.1%). The majority of the patients 96 (55.8%) were not aware of the exact mode of infection.

The median interval between onset of symptoms and diagnosis was 28.3 days (range 4 to 150 Days). One hundred and twenty two (71%) of the patients had already been treated for keratitis with antibacterial, antifungal, or antiviral drugs either alone or in combination, prior to visiting our center (Table 3.7).

Table 3.7 : Treatment received by AK patients prior to presentation at LVPEI (n=122)

Drugs (Alone)	No.	Drugs (in combination)				
		+AB No.	+AF No.	+AV No.	+St.No.	AB+AF+AV
AB	46	-	-	-	-	6
AF	17	22	-	-	-	-
AV	10	12	1	-	-	-
St	1	1	2	1	-	-
AA	3	-	-	-	-	-

AB: Antibacterial; AF:Antifungal; AV:Antiviral; St: Steroid; AA: Anti-*Acanthamoeba*

3.3.3 Clinical Features

All patients presented with redness, watering and decrease in vision. Severe pain was noted in 17 (9.9%) of the patients. Various clinical signs noted in our patients are shown in Table 3.8.

Epithelial defect was recorded in 153 (89%) of the patients. Radial keratoneuritis, which has been described as specific sign for the diagnosis of AK was seen in only in 27 (15.7%) of the patients. Anterior stromal infiltrate was observed in all patients

Table 3.8 : Clinical signs in patients with Acanthamoeba keratitis (n= 172)

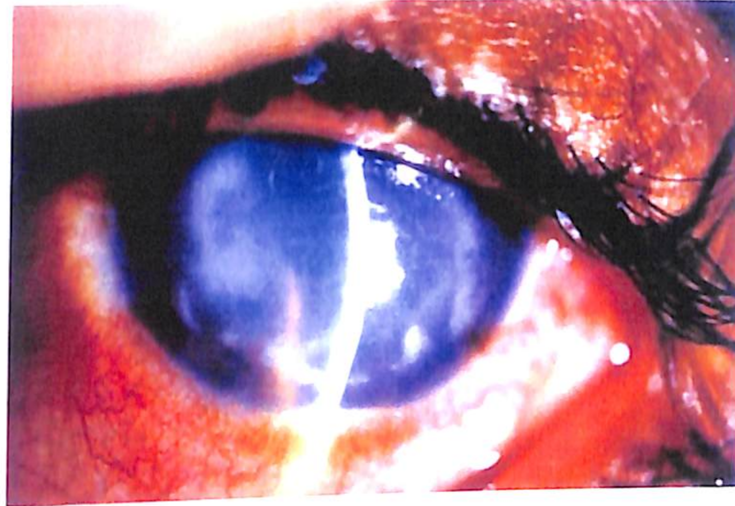
Clinical signs	Number	%	95% CI
Epithelial defect	153	89	83.3-92.8
Radial keratoneuritis	27	15.7	10.9-21.9
Endothelial plaque	3	1.75	0.3-5.2
Hypopyon	90	52.3	44.8-59.6
Deep vascularization	84	48.8	41.4-56.2
Limbitis	40	23.2	17.5-30.1
Scleritis	18	10.5	6.5-16
Corneal sensation decrease	24	14	9.5-19.9
Dendrite	3	1.7	0.3-5.2
Severe pain	17	9.9	6.1-15.3

Table 3.9: Type of infiltrate seen in AK patients (n= 172)

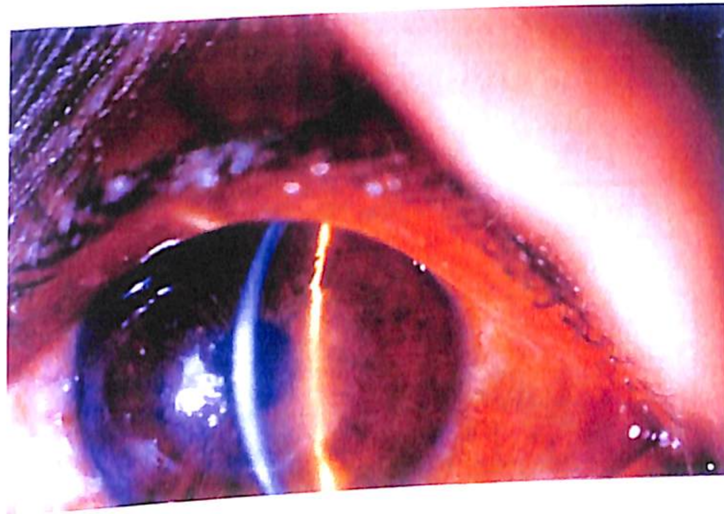
Type of infiltrate	Number	%	95% CI
Ring infiltrate	75	43.6	36.4-51
Satellite infiltrate	24	14	9.5-19.9
Diffuse infiltrate	73	42.6	35.3-49.9

except one who presented to us with a corneal scar. Ring infiltrate was the presenting sign in 75 (43.6%). satellite infiltrate was seen in 24 (14%) and diffuse infiltrate was recorded in 73 (42.6%) of the patients (Table 3.9 and Figure 3.4).

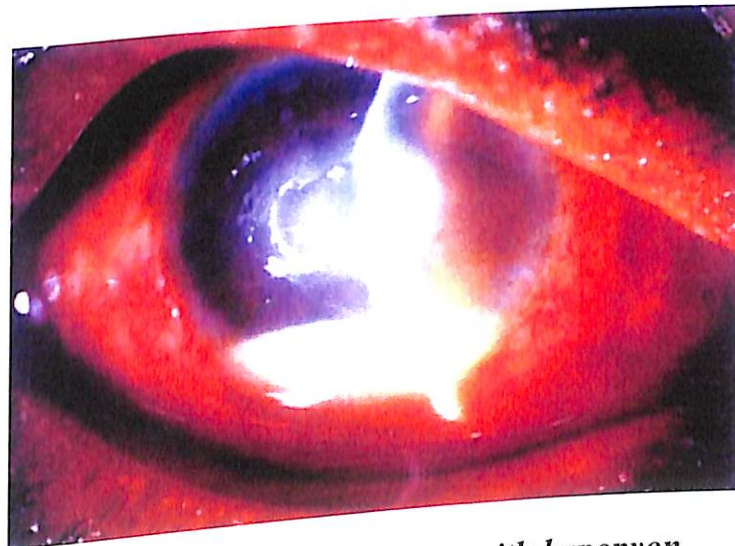
More than half of the patients i.e., 90 (52.3%) had anterior chamber reaction with hypopyon from trace to 6.5 mm. Three patients (1.74%) presented with dendritiform epithelial lesion mimicking viral keratitis, while in another 3 (1.74%) patients endothelial plaques were reported. Decreased corneal sensation was recorded in 24 (14%) of the patients. Later in the course of *Acanthamoeba* infection, some of the



A = Satellite lesions



B = Diffuse infiltrate



C = Ring shaped infiltrate with hypopyon

Figure 3.4: Slit lamp photographs of the cornea under optical section

patients developed complication wherein limbus and sclera got involved. Scleral nodule developed in 18 (10.5%) of the patients, while limbal involvement was recorded in 40 (23.2%) of the patients. In course of the treatment, deep vascularization was recorded in almost half of the patients 84 (48.8%).

3.3.4 Treatment

3.3.4.1 Medical treatment

Once the diagnosis was established by microbiological investigations the treatment was started with a combination of polyhexamethylene biguanide 0.02% (PHMB,

Table 3.10: Treatment receeived by AK patients at LVPEI (n=172)

Drug	Number	%	95% CI
PHMB + CHx	88	51.2	44.9-57.3
PHMB + CHx + KTZ	22	12.8	8.5-18.6
PHMB + CHx + AB	10	5.8	3-10.5
PHMB + CHx + Steroid	10	5.8	3-10.5
PHMB + CHx + AF	2	1.2	0.05-4.4
PHMB + CHx + Brolene	3	1.7	0.3-5.2
PHMB + CHx + Neosporin/ PI	5	2.9	1-6.8
PHMB + Brolene + Neosporin	24	14	9.5-19.9
CHx + Brolene + Neosporin	2	1.2	0.05-4.4
CHx + Brolene + KTZ/Itraconazole	4	2.3	0.7-6
Ciplox	1	0.6	0-3.5
Neosporin	1	0.6	0-3.5

PHMB: Polyhexamethylene biguanide; CHx: Chlorhexidine; KTZ: Ketoconazole
AB: Antibacterial; AF: Antifungals; PI: Povidone Iodine

Bacquasil, ICI, USA) and chlorhexidine digluconate 0.02% (Chx, Sigma, C-9394) in 140 patients (81.4%). Among these 140 patients, 88 (51.2%) were treated only with the combination of PHMB and CHx, while other 52 were treated with a combination

of PHMB + CHx, along with either anti-bacterials (32) like ciprofloxacin, ofloxacin, Ketoconazole (KTZ) etc; or anti-fungals (2) like natamycin; or anti-*Acanthamoeba* drugs (8) like brolene, neosporin, povidone-iodine; steroids in 10 cases (Table 3.10). The other 30 patients were treated with a combination of drugs like PHMB, Brolene, neosporin, CHx, KTZ and itraconazole. While one patient was prescribed only ciprofloxacin and another 1 patient was treated with neosporin alone.

3.3.4.2 Surgical treatment

Surgical procedures were performed in 53 (30.8%) patients. The surgical procedures performed on the patients included: therapeutic penetrating keratoplasty (Th PK) in 20 patients (11.6%), evisceration in 12 (7%), cataract surgery in 8 (4.6%), tarsorrhaphy in 2 (1.2%), superficial keratectomy in 1 (0.6%), tissue adhesive (n-butyl cynoacrylate tissue adhesive (TA) application with bandage contact lens (BCL) in 17 (9.9%) and optical keratoplasty was performed in 6 (3.5%) of the patients (Table 3.11).

3.3.5 Treatment outcome

Of the 172 AK patients, corneal infiltrate resolved following medical treatment in 117 (68%) patients. Nine patients among these underwent optical PK for improvement in vision. Twenty patients were lost to follow up and on the last day of follow up, still had active keratitis. Three patients worsened with treatment and were advised to undergo therapeutic penetrating keratoplasty but they refused and later were lost to follow up (Table 3.12).

Table 3.11 : Surgical intervention in AK patients in the study (n=172)

Surgery	Number	%	95% CI
Therapeutic PK	14	8.14	1-6.8
Optical PK	5	2.9	4.8-13.3
Evisceration	9	5.2	2.6-9.7
TA + BCL	10	5.8	3-10.5
Tarsography	1	0.6	0-3.5
Optical PK + Cataract	4	2.3	0.7-6
Therapeutic PK + Cataract	2	1.2	0.05-4.4
TA + BCL + Cataract	1	0.6	0-3.5
TA + BCL + Therapeutic PK	2	1.2	0.05-4.4
Therapeutic PK + Evisceration	1	0.6	0-3.5
TA + BCL + Evisceration	2	1.2	0.05-4.4
Superficial keratectomy + TA + BCL	1	0.6	0-3.5
TA + BCL + Tarsorrhaphy + Cataract + Therapeutic PK	1	0.6	0-3.5
Total	**53**	**30.8**	**24.3-38**

PK: Penetrating keratoplasty;TA: Tissue adhesive; BCL: Bandage contact lens

Among the 20 patients who underwent therapeutic keratoplasty, only 4 (20%) patients had a clear graft and their vision improved, while in other 12 patients (60%) the graft failed and in another four the graft was either edematous or ectactic (Table 3.13).

Table 3.12 : Treatment outcome in AK patients (n=172)

Outcome	Number	%	95% CI
Resolved keratitis	117	68.02	60.7-74.5
LTFU	23	13.4	9-19.3
Therapeutic PK	20	11.6	7.5-17.3
Evisceration	12	7	3.9-11.9

LTFU: Lost to follow up; PK: Penetrating keratoplasty

Table 3.13 : Outcome of Therapeutic PK in AK patients (n=20)

Outcome	Number	%	95% CI
Clear graft	4	20	7.4-42.1
Failed graft	12	60	38.6-78.1
Edematous graft	3	15	4.3-36.8
Ectactic graft	1	5	0-25.4

3.4 DISCUSSION

Corneal ulceration is a worldwide cause of monocular blindness and surprisingly there are only few studies evaluating the etiological factors predisposing a population to the corneal infection (Di Bisceglie *et al* 1987; Upadhyay *et al* 1991). Gonzales *et al* (1996) reported the incidence of corneal infection in Madurai, south India to be 11.3 per 10 000 population which is at least 10 times higher than the incidence reported from the US (Erie *et al* 1993). The epidemiological pattern of corneal ulceration varies significantly from country to country and even from region to region. Thus, in order to develop a comprehensive strategy for the diagnosis, treatment and ultimately for the prevention of the corneal infections, the pathogenic organisms causing the ulceration should be known (Srinivasan *et al* 1997).

Acanthamoeba keratitis has been described primarily from developed countries with several studies suggesting soft contact lens wear as the greatest risk factor (Stehr-Green *et al* 1987; Radford *et al* 2002). In contrast, the reports from India and other developing countries are few and have mainly been in non-contact lens wearers wherein the prevalence of AK was estimated to be 1-3% in keratitis patients (Davamani *et al* 1996; Sharma *et al* 2000;). This low incidence of *Acanthamoeba* keratitis has apparently been due to the belief that the disease is related mainly to

patients were treated with anti-bacterial, antifungal, antiviral drugs and in some cases steroids alone or in combination with antimicrobial agents was prescribed.

The majority of the patients (55.8%) were not aware of the exact mode of infection. Risk factors could be established in only 44.1% of the patients, which included trauma to the cornea in 38.3%, washing eyes with stored water in 5.2% and contact lens wear in 1 patient (0.6%). To the best of our knowledge, on literature survey we found that only 15 research articles describing 243 AK patients have been published from India (refer Table 1.2). Among these only three AK patients were contact lens wearer (Srinivasan 1993; Singh *et al* 1994; Sharma *et al* 2003). Thus, neither the mode of injury nor the patient's occupation, nor the clinical history of the cases gave any suggestion that any of this information could be used as an index of suspicion for AK.

The clinical picture of AK patients in this study did not offer any particular clues for diagnosis. Radial keratoneuritis, considered specific for AK (Bacon *et al* 1993) was present only in 27 cases (15.7%), limiting the potential to use this clinical feature for the diagnosis of AK. Seventeen patients (9.9%) of the patients in our series complained of severe pain that was disproportionate to the size of ulcer. Severe pain is regarded as the most common initial clinical symptom in AK cases, but this was not found by us and others (Srinivasan *et al* 2002; Sharma *et al* 2000). The hallmark of AK is presence of ring infiltrate and its presence is considered pathognomonic for AK (Theodore *et al* 1985). We found that ring infiltrate was present in 43.6% of the patients. Perry *et al* (1995) reported that significantly decreased corneal sensation is a frequent finding in early AK and therefore physicians should consider AK as an

Thirty one (18%) of the patients, categorized as un-employed, were not doing regular jobs and belonged to lower socio-economic strata of the society. Similar results have also been reported by Srinivasan *et al* (1997) from Madurai, south India and Upadhyay *et al* (1991) from Nepal in microbial keratitis patients. We found that AK was more prevalent in warmer weather i.e. summer and monsoon seasons rather than winter season. Similar association of AK with warmer weather was evidenced by Kyle and Noblet (1986). Shortage of water in summer may lead to usage of contaminated water and rainy season may also cause contamination of unprotected water.

Of importance is the fact that 88 (51.2%) of our patients presented to our institute after 14-28 days after the start of the ocular symptoms, while only 5% presented to us within the first week of their illness. This might be due to the fact that this Institute is a tertiary eye care center; where in many of the patients are referred from rural eye care primary centers and other ophthalmologists running private clinics in rural centers. Also, of note is the fact, that 71% of the patients before presenting to us consulted a healthcare provider of some kind and were prescribed medication. Among 122 patients who received treatment prior to presentation at LVPEI only three (2.5%) were previously treated for AK and specific anti-*Acanthamoeba* drugs were started for them. This fact is of concern and highlights the ignorance of eye care practitioners in rural areas about *Acanthamoeba* as an etiology for keratitis. However, this could also be due to overlap of clinical features between keratitis of varied etiologies like bacterial, fungal and viral and also because of relatively high prevalence (50%) of fungal keratitis in India (Srinivasan *et al* 1997). Remaining 119

patients were treated with anti-bacterial, antifungal, antiviral drugs and in some cases steroids alone or in combination with antimicrobial agents was prescribed.

The majority of the patients (55.8%) were not aware of the exact mode of infection. Risk factors could be established in only 44.1% of the patients, which included trauma to the cornea in 38.3%, washing eyes with stored water in 5.2% and contact lens wear in 1 patient (0.6%). To the best of our knowledge, on literature survey we found that only 15 research articles describing 243 AK patients have been published from India (refer Table 1.2). Among these only three AK patients were contact lens wearer (Srinivasan 1993; Singh *et al* 1994; Sharma *et al* 2003). Thus, neither the mode of injury nor the patient's occupation, nor the clinical history of the cases gave any suggestion that any of this information could be used as an index of suspicion for AK.

The clinical picture of AK patients in this study did not offer any particular clues for diagnosis. Radial keratoneuritis, considered specific for AK (Bacon *et al* 1993) was present only in 27 cases (15.7%), limiting the potential to use this clinical feature for the diagnosis of AK. Seventeen patients (9.9%) of the patients in our series complained of severe pain that was disproportionate to the size of ulcer. Severe pain is regarded as the most common initial clinical symptom in AK cases, but this was not found by us and others (Srinivasan *et al* 2002; Sharma *et al* 2000). The hallmark of AK is presence of ring infiltrate and its presence is considered pathognomonic for AK (Theodore *et al* 1985). We found that ring infiltrate was present in 43.6% of the patients. Perry *et al* (1995) reported that significantly decreased corneal sensation is a frequent finding in early AK and therefore physicians should consider AK as an

alternative diagnosis in patients with presumed HSV keratitis with decreased corneal sensation. In contrast to the above, we found that there was decrease in corneal sensation in very few patients and this cannot be used as a diagnostic criteria. Of note is presence of deep vascularization in almost 50% of the patients though this was not present at the time of presentation.

The final visual outcome, as measured by resolution of the infiltrate and healing of the ulcer, was achieved in 118 (68.6%) of the patients. We used combination of PHMB and CHx in 140 patients (81%). Use of combination of two cationic antiseptics i.e., PHMB and CHx is not common since both of them belong to same group of drugs. They are usually used in combination with either diamidine derivatives or anti-bacterial drugs. Combination of PHMB and CHx was first used by Tirado-Angel *et al* (1996) and they found them to have synergistic activity. Therapeutic penetrating keratoplasty was performed in 20 patients, with little success since graft survival was achieved in only 4 (20%) of the patients. Similar results were obtained by Cohen *et al* (1987) and Ficker *et al* (1993), however Illingworth *et al* (1995) had got encouraging results with PK.

Brady and Cohen (1990) suggested that AK could be strongly suspected based on the presence of characteristics symptoms and signs particularly in the presence of appropriate risk factors. This holds true for the patients who are contact lens wearers, where in there is high level of suspicion of AK. In contrast, in non-contact lens wearers, where the risk factors are not established in most of the patients, clinical features are often not suggestive of AK.

To summarize, among the AK patients, males were more predominant. The most common profession of the patients was related to agriculture and they belonged to lower socio-economic strata. Of interest was the fact that incidence of AK in winter was minimal. Except one, all patient were non-contact lens wearers and risk factors could be established in less than half of the patients. Clinical features observed in patients were not pathognomonic for AK and could not offer much to clinch the diagnosis.

CHAPTER 4: DIAGNOSIS OF *ACANTHAMOEBA* KERATITIS USING CONVENTIONAL MICROBIOLOGICAL TECHNIQUES AND ANTI-AMOEBIC DRUG SUSCEPTIBILITY TEST

4.1 INTRODUCTION

AK is a potentially blinding corneal infection that is often misdiagnosed (Chynn *et al* 1995). Early detection and diagnosis is critical to the outcome of the clinical course of AK (Marciano-Cabral and Cabral 2003). The diagnosis of AK is high on the differential diagnosis in contact lens wearers and therefore patients are diagnosed early (Bacon *et al* 1993). However, in non-contact lens wearers the diagnosis of AK is not easy, and often delayed (Chynn *et al* 1995). This is mainly because AK may mimic other forms of keratitis: a previous study from India reported that 45.4% of AK had been misdiagnosed as fungal keratitis (Sharma *et al* 2000), and also the fact that risk factors for non contact lens associated AK are variable. A definitive diagnosis of AK cannot be made based only on clinical features but have to be done in conjunction with laboratory tests. Chynn *et al* (1995) attributed worse outcome in non contact lens associated AK patients mainly due to the diagnostic delay. Confirmed diagnosis of AK is extremely important since the treatment and therapy of AK is specific, prolonged and might be toxic to the cornea.

Once the diagnosis is achieved, appropriate anti-*Acanthamoeba* drug therapy is required. Novel approaches to chemotherapy of AK continue to be forthcoming from time to time. Over the years, a multitude of antiseptic, antibacterial and anti-fungal drugs have been tried for their anti-cysticidal and trophozoite amoebicidal efficacy (refer section 1.4) with variable results both *in vivo* and *in vitro*. The use of the biguanides has revolutionized the treatment of early cases of AK and has been very

successful for patients presenting within eight weeks of start of initial symptoms (Seal 2003). Biguanides like CHx and PHMB have been used in combination with diamidine group of drugs like propamidine and hexamidine with considerable success (Larkin *et al* 1992; Duguid *et al* 1997; Seal 2003).

The objectives of this study were:

1. To evaluate the conventional microbiological techniques for the diagnosis of AK.
2. To compare the sensitivity of smear examination with culture technique for the detection of *Acanthamoeba* in corneal scrapings.
3. To perform *in vitro* drug susceptibility test and compare minimum cysticial concentrations (MCCs) of PHMB, CHx and Propamidine on *Acanthamoeba* strains isolated from corneal scrapings.

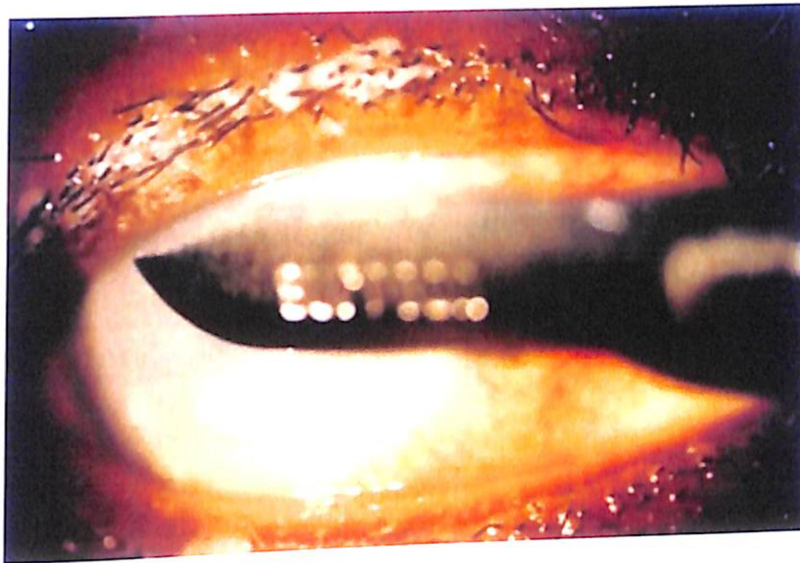
4.2 MATERIALS AND METHODS

4.2.1 Patients

Microbiology records were reviewed for a total of 172 patients who were diagnosed as AK based on smear and/or culture results.

4.2.2 Collection of corneal scrapings

Using standard techniques (Jones *et al* 1981), corneal scrapings were obtained in the clinic using sterile blade no 15 on Bard Parker handle by ophthalmologists with the aid of slit-lamp biomicroscope (Figure 4.1a). In all cases the cornea was topically anaesthetized prior to scraping with topical anesthetic (4% Lignocaine hydrochloride). Scrapings were taken from the base and edge of the ulcer.



A = Slit lamp photograph of collection of corneal scrapings using blade no.15 on Bard parker handle

B = Laboratory tray containing media, slides, blades, pencil, KOH+CFW reagent etc

Figure 4.1: Collection of corneal scrapings

4.2.3 Conventional microbiological techniques

Multiple scrapings from the base and edge of the ulcer were taken and placed on presterilized glass slides for staining and inoculated on an array of media for culture (Table 4.1, Figure 4.1b).

Table 4.1: Staining techniques and culture media used for the diagnosis of non-viral keratitis including AK

Smear and culture	Detection/growth of microorganisms
Staining techniques	
KOH/CFW	Fungus; *Acanthamoeba*
Gram stain	Bacteria; Fungus; *Acanthamoeba*
Giemsa	Cellular profile;Bacteria;Fungus;*Acanthamoeba*
Culture Media	
Blood agar - aerobic	Bacteria; Fungus; *Acanthamoeba*
Blood agar - anaerobic	Anaerobic bacteria
Chocolate agar	Bacteria; Fungus; *Acanthamoeba*
Brain heart infusion broth	Bacteria; Fungus
Thioglycate broth	Anaerobic bacteria
Non-nutrient agar	*Acanthamoeba*
Sabourauds' dextrose agar	Fungus
Potato dextrose agar	Fungus

As part of standard protocol for microscopic evaluation of corneal scrapings, Gram and Giemsa stainings were performed and observed under bright field microscope, while for KOH/CFW staining, one drop of 10% KOH and one drop of 0.1% calcofluor white-sigma, USA (CFW) with 0.1% Evans blue solution was added onto the corneal scrape and coverslip was placed on it and observed under fluorescence microscope (for preparation of CFW see Appendix 2).

at 37°C. Blood agar plates were incubated under aerobic and anaerobic conditions and chocolate agar was incubated with 5% carbon dioxide.

While inoculating NNA with a blade, only a single streak was made at the center of the plate without piercing too much into the agar. Subsequently using sterile pipette, 2-3 drops of live *Escherichia coli* suspension was added on the surface of NNA taking care to cover the inoculum. The plate was tilted on all sides to allow the bacterial suspension to spread over the plate surface. The plate was kept in upright position till the suspension dried and then it was sealed with parafilm and incubated. NNA plates were observed daily under the x4 objective lens of the microscope (magnification x40) for tracks and trophozoites without opening the lid to avoid contamination (for preparation of NNA and *E.coli* suspension refer Appendix 2).

Certain criteria were followed to determine the significance of growth in culture, especially for bacteria and fungus, since some of them are normal flora of the eyelids and conjunctival sac and can appear on culture media as contaminants.

A culture of bacteria or fungus was considered significantly positive when there was

- Growth of the same organism on two or more media, or
- Confluent growth at the site of inoculation on one solid medium, or
- Growth in one medium with consistent direct microscopy findings, or
- Growth of the same organism on repeated corneal scrapings.

The diagnosis of AK was however made if the cysts of *Acanthamoeba* were demonstrated in any of the smears and/or if culture was positive on NNA.

All bacterial and fungal isolates were identified as per the standard procedure (Isenberg 1996). *Acanthamoeba* was identified at the genus level by morphology of cysts in smear and cultures (Visvesvara 1995).

4.2.4 *In vitro* anti-amoebic drug susceptibility test

4.2.4.1 Clinical isolates

Seventy nine *Acanthamoeba* strains isolated from corneal scrapings of 79 patients were included in the study. These 79 were among the 172 patients described before in the previous chapter. All the isolates were grown on NNA with *E.coli*. NNA plates with trophozoites were incubated at 30^{0}C for 7 days to obtain mature *Acanthamoeba* cysts. Medical records of these 79 patients were retrospectively reviewed.

4.2.4.2 Standardization of inoculum

The cysts were recovered from NNA-*E.coli* culture by flooding the plate with 10mL of one-quarter strength Ringer solution and then the amoebae were gently dislodged from the plate using a swab or bent glass rod. The cyst suspension was transferred to a 15mL centrifuge tube and spun at 500g for 5 minutes. The pellet containing *Acanthamoeba* cysts was washed twice with the same buffer solution. Subsequently the concentration of the cysts was adjusted to 1 X 10^4 cysts/ mL with one-quarter ringer lactate solution.

4.2.4.3 Anti-amoeba drugs and the dilutions

Anti-amoebic susceptibility test was performed with three anti-*Acanthamoeba* drugs: PHMB (Bacquasil, ICI, USA), CHx digluconate (Sigma-Aldrich C-9394, St Louis; MU USA.) and Propamidine isethionate (Brolene; Rhone-Poulenc Rorer Australia

PTY LTD, NSW, Australia). A working stock solution of 1mg/mL was made for all the drugs. The test was performed in flat-bottomed 96 well microtitre plate and all procedures were performed in laminar flow hood (Figure 4.2A). For the purpose of dilution of the drugs, 100µL ringer lactate solution was added to all the 12 wells of the microtitre plate. In the first and the second well 100µL of 1mg/mL of the stock drug was added, wherein the first well serves as drug control or negative control. From the second well onwards two-fold dilutions were performed till the eleventh well, from where 100µL of the drug was discarded. The 12th well was the amoebae control or the positive control where no drug was added. Thus the concentration of the drugs in the wells ranged from 500 to 0.49µg/mL (Table 4.2).

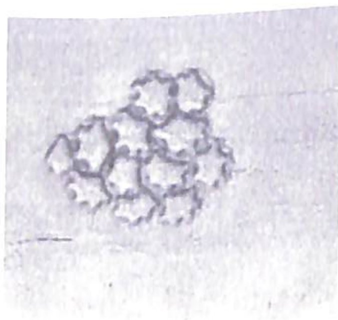
4.2.4.4 Test procedure

After the drug dilutions, 50µL of the standardized *Acanthamoeba* suspension was added to all the wells except the negative control (Table 4.2). Subsequently, the plate was sealed with adhesive film and incubated at 30°C for 48 hours. After incubation, using a multichannel pipette, the solution was gently removed from all wells and refilled with 200µL of quarter strength ringer solution, taking care to add the buffer

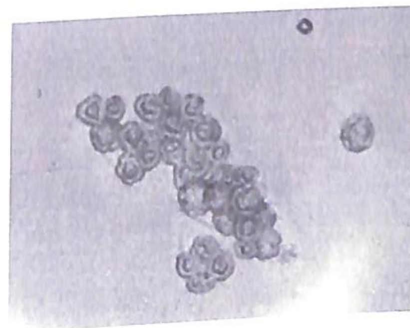
Table 4.2: Outline of the procedure for anti-amoebic drug susceptibiltiy test

	1	2	3	4	5	6	7	8	9	10	11	12
Ringer solution (microliters)	100	100	100	100	100	100	100	100	100	100	100	100
Drug (microliters)	100	100	100	100	100	100	100	100	100	100	100	-
Cyst suspension (microliters)	-	50	50	50	50	50	50	50	50	50	50	50
Drug concentration (microgram/ml)	500	500	250	125	62.5	31.25	15.6	7.8	3.9	1.9	0.97	0.49

A = Done in laminar flow hood with help of multichannnel pipette and microtitre plate



B = Healthy cysts



C = Dead cysts

Figure 4.2: In vitro drug susceptibility test

solution on the sidewalls of the wells. The plate was left undisturbed for 5-10 minutes and then the wells were washed with ringer solution and this step was repeated twice. After the final wash, 100μL of heat killed *E.coli* suspension (OD at 540nm – 0.1-0.2) was added in all wells and the plate was resealed and incubated at 30°C for 7 days.

4.2.4.5 Recording the results

The minimum cysticidal concentration (MCC) is the lowest concentration of test solution that results in no trophozoite excystment after seven days of incubation. Using the 10x objective of an inverted microscope (magnification x100), the wells were examined daily for up to 7 days for the presence of excysted trophozoites. This test was performed in duplicates for all the isolates and if either the positive or negative control did not show the desirable results the test was repeated.

4.3 RESULTS

4.3.1 Microbiological findings

Among the 172 AK patients analyzed, complete microbiological data was available for 166 patients. For the six patients, the microbiological data was not complete wherein, either, one of the staining techniques was not performed or culture was not done. Among the 166 smear and/or culture proven cases of AK, *Acanthamoeba* alone was the etiological agent in 132 patients (79.5%). Bacteria and fungus co-existed with *Acanthamoeba* in 32 (19.2%) and two (1.2%) patients respectively (Table 4.3). By direct microscopic examination of the smears, KOH/CFW was positive in 147 patients (88.5%), Gram was positive in 135 (81%) and Giemsa was positive in 122

Table 4.3: Microbiological findings in Acanthamoeba keratitis patients (n = 166)

Tests	Number	%	95% CI
Cysts in smears			
Gram	135	81	74.6-86.5
Giemsa	122	73.5	66.2-79.6
KOH + CFW	147	88.5	82.7-92.6
Culture			
Acanthamoeba	135	81	74.6-86.5
Bacteria	32	19.2	13.9-25.9
Fungus	2	1.2	0.05-4.5

patients (73.5%) (Figure 4.3), while culture results showed positivity in 135 patients (81.3%) (Figure 4.4). The number of days taken to show culture positive for *Acanthamoeba* ranged from 1-10 days with an average of 3.5 days.

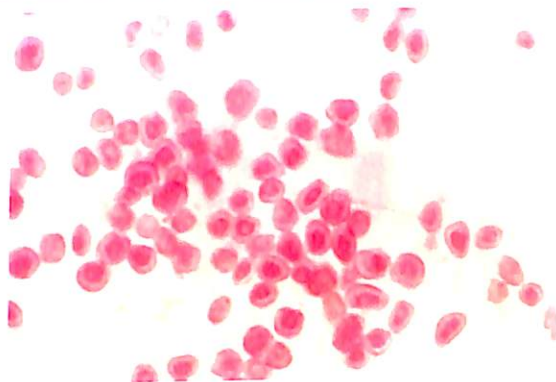
When the comparison of each of the staining technique was made with the gold standard (Table 4.4), i.e., culture, KOH +CFW had the highest sensitivity of 87% (95% CI: 82.7-92.6), followed by Gram with 81% (95% CI: 74.6-86.5) and least sensitive among the three was of Giemsa staining with 70% (95% CI: 66.2-79.6).

Table 4.4: Comparison of the staining techniques with culture in detecting *Acanthamoeba* in corneal scrapings (n = 166)

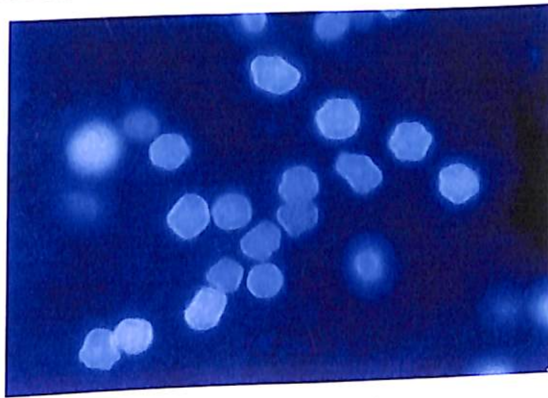
	KOH/CFW		Gram		Giemsa		Total
	+	-	+	-	+	-	
Culture +	118	17	109	26	95	40	135 (81%)
Culture -	29	2	26	5	27	4	31
Total	147 (87%)	19	135 (81%)	31	122 (70%)	44	**166**

4.3.2 Anti-amoebic drug susceptibility test

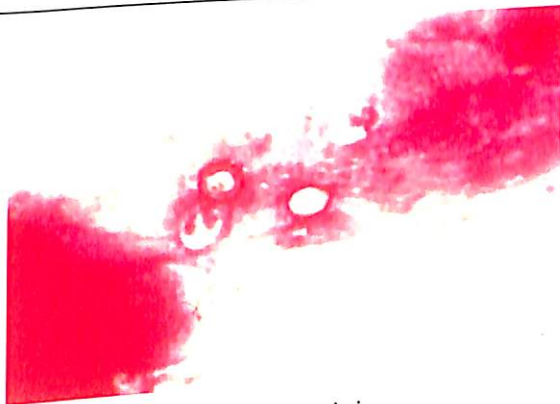
Table 4.5 summarizes MCCs of PHMB, CHx and Propamidine isethionate (Brolene) for 79 strains of *Acanthamoeba* isolated from keratitis patients. Figure 4.2C shows



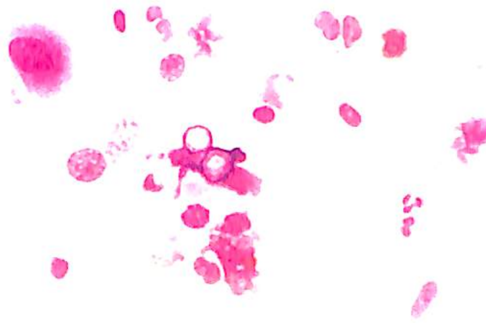
A = KOH with Evans blue
(Bright field microscope x250)



B = KOH +CFW staining
(Fluorescence microscope x500)_



C = Gram staining
(Bright field microscope x500)



D = Giemsa staining
(Bright field Microscope x500)

4.3: Corneal scraping smear exam. for detection of Acanthamoeba cysts

A = Cluster of cysts
(Bright field microscope x100)

B = Tracks of the trophozoites
(Bright field microscope x40)

Figure 4.4 : Acanthamoeba grown on NNA with E.coli

Table 4.5: Results of the MCC of the drugs against Acanthmoeba isolates from keratitis patients (n = 79)

Drug microgram/mL)	Range	Mean	Median	SD
PHMB	0.49 - 500	66.9	7.8	137.6
CHx	0.49 - 500	57.7	3.9	133.4
Brolene	1.9 - 500	334.6	500	184.4

the cyst morphology under the effect of anti-*Acanthamoeba* drug. The geometric mean of MCC for PHMB, CHx and Brolene was 66.9±137.6µg/mL, 57.7±133.4µg/ml and 334.6±184.4µg/ml respectively. Comparison between each of the drugs (using student's 't' test) revealed that PHMB and CHx were better drugs than Brolene ($p<0.0001$), however, when PHMB and CHx were compared, CHx was better drug than PHMB since its mean MCC level was lower than PHMB. This was, however, not statistically significant ($p = 0.56$).

Among 79 isolates tested for MCC, medical records were available for 77 patients. Three out of 77 patients were not treated with either of the drugs (PHMB, CHx and Brolene). Seventy four patients were treated with either of the combinations i.e.,

Table 4.6 Treatment outcome in AK patients treated with PHMB+CHx (n=69)

Outcome	Number	%	95% CI
Therapeutic PK	4	5.7	1.85-14.4
Evisceration	6	8.7	3.7-18
Resolved	52	75.3	63.9-84
LTFU	7	10.1	4.7-19.7

LTFU: Lost to follow up

PHMB + CHx (69 patients), PHMB + Brolene (3 patients) and CHx + Brolene (2 patients). Among 69 patients treated with combination of PHMB and CHx, corneal infiltrate had resolved in 75% of the patients, while drug toxicity was seen in none (Table 4.6).

4.4 DISCUSSION

Acanthamoeba keratitis is a chronic progressive infection that has been increasingly identified worldwide. Initial clinical features of AK are varied and it can be misdiagnosed as fungal, bacterial or viral keratitis wherein the treatment is delayed and outcome is poor. Thus definitive diagnosis of AK is extremely important. For some time now, clinical features and a combination of conventional microbiological techniques like smear examination and culture are being used but with variable results. According to a protocol at our institute a combination of three staining techniques i.e., Gram, Giemsa and KOH + CFW along with culture are used for diagnosis of non-viral keratitis. We evaluated these staining techniques and determined their sensitivity against culture, which is the gold standard for the detection of *Acanthamoeba*. KOH/CFW had the maximum sensitivity (87%) to detect cysts in the clinical samples, followed by Gram (81%) and culture (81%) while Giemsa (70%) had least sensitivity among the four.

Among the 166 patients 31 (18.7%) cases were culture negative and smear positive (either of the stain). All these 31 patients were treated based on the smear report, this is in contrast to the criteria, which is followed for bacteria and fungus (mentioned earlier in the chapter) where the patients are not treated only based on the smear report. Morphology of cyst is characteristic and sufficient to identify the genus

Acanthamoeba in the clinical specimens. For 17 patients (10.2%) smear was negative (none of the three staining technique was positive) and culture was positive, hence in these 10.2% of the patients the definitive diagnosis was delayed by an average of 3.5 days. Successful use of CFW to detect *Acanthamoeba* cysts in clinical samples was first demonstrated by Wilhelmus *et al* (1986). Calcofluor white is a chemofluorescent dye, which has an affinity to bind to the polysaccharide polymers of amoebic cysts. This staining technique requires only five minutes after the fixation of the slides in methyl alcohol (Auran *et al* 1987). Thomas and Kuriakose (1990) however mentioned that staining with CFW might be simple and rapid but needs expensive, elaborate use of fluorescent microscope, which is not available to many laboratories. They recommended the use of lacto phenol cotton blue for staining *Acanthamoeba* cysts in clinical samples.

According to a protocol at our Institute, the first scrape from the keratitis patient is always used for KOH +CFW staining. In our study, we found that the sensitivity of Gram and Giemsa was higher that what is reported in literature; we believe that this higher sensitivity might be due to the fact that these two smears are always seen after the KOH/CFW smear. Therefore, if a smear stained with KOH +CFW is positive, in view of CFW result the probability of finding *Acanthamoeba* cysts in the smear stained with Gram and Giemsa will be much higher. Therefore, it is possible that sensitivity of Gram and Giemsa is high only because they are seen in conjunction with KOH+ CFW, however this hypothesis needs to be proved. Though these three staining techniques have high sensitivity to detect cysts of *Acanthamoeba*, they fail to detect trophozoites, which mainly predominate in the initial stages of infection (Stothard *et al* 1999). Being more susceptible to treatment than cysts if trophozoites

are detected in early stages of the disease, it will lead to better prognosis since trophozoites are the ones, which are present in early stage of the disease (Stothard *et al* 1999).

Culture offers definitive diagnosis but Bacon *et al* (1993) reported only 44-74% success in growing *Acanthamoeba* from clinical samples, while others have reported much less satisfactory results (Auran *et al* 1987; Mathers *et al* 1996). The inability to culture *Acanthamoeba* from some corneal epithelial specimens may be the result of either inadequate sample, presence of non-viable or small number of organisms in the clinical specimen early in the course of the infection or can also be due to lack of expertise in collecting the epithelial scraping/biopsy or in processing it (Bacon *et al* 1993). However, culture remains the gold standard. The time taken for culture to show growth varied from 1-10 days with an average of 3.5 days.

The co-isolation of bacteria from corneal scrapes has been recognized in up to 58% of AK patients (Bacon *et al* 1993). However, in our series of 166 AK patients, we found that 32 (19.22%) of the patients had bacteria as their co-isolate, which mainly included Gram positive bacteria like *Staphylococcus epidermidis* and various *Corynebacteria* spp. In two patients *Fusarium* spp. and unidentified hyaline fungus were the co-isolates. The presence of bacterial keratitis in combined infection, both clinically and on culture, may delay the diagnosis of AK and therefore appropriate treatment (Bacon *et al* 1993).

The first effective treatment of AK was developed 20 years ago with propamidine (Brolene) and neomycin (Wright *et al* 1985) but only half the patients responded.

After considerable research, this regime was replaced seven years ago with CHx and propamidine (Hay *et al* 1994;Seal *et al* 1996) or the polymeric equivalent PHMB. These three drugs are among the most popular drugs, which have been used in recent times for treatment of AK. We wanted to determine the efficacy of these drugs *in vitro*, hence MCC for each of the drug was determined using method described by Hay *et al* (1994). We found that Brolene had very high MCC value ranging from 1.9 - 500 with a mean of 334.6μg/mL indicating its inefficacy in killing cysts and hence we recommend that this drug should not be used alone as drug of choice in the treatment of AK. However, many have used this diamidine in combination with cationic antiseptics like CHx and PHMB (Hay *et al* 1994). When we compared the efficacy of PHMB and CHx as cysticidal agents we found that CHx had lower MCC value than PHMB, but it was statistically not significant. Therefore, we believe that both the drugs are equally effective against cysts of *Acanthamoeba*. Efficacy of these two drugs were also tested by Narasimhan *et al* (2002), however, the *in vitro* method used to determine the MCCs was different from the one used by us. They carried out the test in Durham tubes instead of microtiter plates and determined MCC by plating cyst (treated with anti-*Acanthamoeba* drugs) on NNA plates seeded with *E.coli*. They tested 19 *Acanthamoeba* strains, isolated from keratitis patients and found that MCC values of CHx were lower than PHMB (a finding similar to ours) and this was statistically significant, thus concluding that CHx had a better *in vitro* cysticidal activity than PHMB.

Seal (2003) recommended the use of cationic antiseptic (either PHMB or CHx) with diamidine derivative (Propamidine or hexamidine) for treatment for AK. He

suggested that there is no advantage by treating with a combination of PHMB and CHx and stated that the combination would be more toxic to the cornea. Synergistic action of PHMB and CHx *in vitro* has been reported by Tirado-Angel *et al* (1996), but this combination had not been evaluated in patients. This study for the first time reports the efficacy of these two drugs. Sixty-nine patients were treated with a combination of PHMB and CHx in this study and we found that in 75.3% of the patients, the corneal infiltrate had resolved with no drug toxicity recorded for any of the patients.

Both PHMB and CHx belong to same group of drugs i.e., cationic antiseptics and act by binding their highly charged positive molecules to the mucopolysaccharide plug of the ostiole of the cyst, resulting in penetration into the amoeba, where they bind to phospholipid bilayer of the cell membrane. This results in membrane damage with irreversible loss of calcium followed by cell electrolytes from the cytoplasm to cause cell lysis and death (Seal 2003). CHx is a smaller molecule than PHMB and thus may penetrate better in corneal tissue (Seal *et al* 1996).

In summary, conventional microbiological techniques aided diagnosis of AK in all 166 patients. KOH + CFW is a simple, sensitive and rapid staining technique for the detection of *Acanthamoeba*. Grams and Giemsa are also sensitive methods for the detection of *Acanthamoeba* in our series. Success rate of culture was much better than what is reported in literature. Thus, our results suggest that the conventional microbiological techniques in combination with clinical features can be used for rapid and definitive diagnosis of AK.

To conclude, efficacies of Brolene as cysticidal agent is minimal *in vitro*, while both PHMB and CHx have low MCC. Combination of CHX and PHMB seems to have effective amoebicidal action within the cornea. If this combination of drugs is used, it could shorten the time and frequency of anti-acanthameobic drugs treatment, which in turn may reduce the toxic reaction of the drugs and obviate effects of inherent or acquired resistance to the drugs.

CHAPTER 5: HISTOPATHOLOGIC AND IMMUNOHISTOCHEMICAL STUDIES ON *ACANTHAMOEBA* KERATITIS PATIENTS

5.1 INTRODUCTION

In recent years, with the use of newer anti-amoebic agents, the medical treatment of *Acanthamoeba* keratitis has improved significantly. Despite medical treatment, some patients may develop complications and require corneal transplantation or evisceration. In addition, patients with healed corneal ulcers on medical therapy may require optical keratoplasty for visual rehabilitation. The histologic changes in the corneal button or eviscerated materials obtained from AK patients include, epithelial ulceration, stromal inflammation and necrosis, presence of cysts and trophozoites of *Acanthamoeba* (Garner 1993; Kremer *et al* 1994; Yang *et al* 2001), apoptosis of keratocytes (Vemuganti *et al* 2000) and rarely granulomatous inflammation of the corneal stroma (Mietz and Font 1997). We studied clinical, histologic and immunohistochemical features in five cases of *Acanthamoeba* keratitis who underwent keratoplasty or evisceration for rapidly progressive, non responding disease of the cornea.

5.2 MATERIALS AND METHODS

5.2.1 Patients

At LVPEI, all patients diagnosed as AK are treated using a standard protocol. Decision for surgical intervention is taken if there is: 1) large infiltrate at presentation, 2) progression of the disease despite the initiation of anti-*Acanthamoeba* treatment for 2 weeks 3) If there is involvement of limbal region, or 4) impending perforation. Over a period of 8 years i.e., between 1995-2002, the ophthalmic pathology services of our Institute received 18 corneal buttons/eviscerated materials from AK patients.

All these 18 specimens were subjected to histopathological examination. Among these histopathological features of five tissues (3-corneal buttons, 2-eviscerated contents) were reviewed and were subjected to immunophenotyping. Medical records of these five cases were also reviewed and the clinical pictures at the time of presentation, medical treatment and its duration and indication for surgical intervention were noted.

5.2.2 Microbiological investigations

Retrospectively the microbiological data was reviewed wherein the corneal scrapings from all patients were subjected to smear examination by three methods, viz., potassium hydroxide with calcofluor white, Gram and Giemsa stains and culture on media for bacteria, fungus, and Acanthamoeba, as described earlier in Chapter 4.2.3. Corneal buttons, whenever available for microbiologic studies, were processed similarly for bacteria, fungus and *Acanthamoeba*.

DNA was extracted from paraffin sections of corneal buttons and eviscerated materials with the commercially available DNAzol solution (Helena BioSciences, UK) using a procedure described by Chomczynsky *et al* (1997). DNA extracted from these sections were tested for presence of herpes simplex virus DNA by PCR, using primers specific for glycoprotein D gene of herpes simplex virus (Aurelius *et al* 1991).

5.2.3 Histopathology

Keratectomy eviscerated material was fixed in 10% buffered formalin. Paraffin sections were stained with haematoxylin – eosin and periodic acid stains.

5.2.4 Immunophenotyping

Immunohistochemistry was performed using monoclonal mouse anti-human antibodies (Dako, Denmark) against T cell CD 3, Macrophage CD 68 and B cell CD 20 antigens. After deparaffinizing, immunohistochemistry was performed on the tissue sections as per the procedure described by Sharma *et al* (2001). After deparaffinizing the sections, the endogenous peroxidase acitivity was neutralized using 100% methanol and 0.4% H_2O_2. Incubating the sections with prewarmed citrate buffer for 15 minutes in hot air oven maintained at 100°C retrieved the antigenic epitopes of the corneal section. Non-immunologic binding of antibodies was blocked by incubation with bovine serum albumin. Incubation with all the primary antibodies was carried out in a moist chamber at 4°C overnight. On the following day, after thorough washing with phosphate buffer saline, secondary biotinylated goat anti-mouse antibody (Dako, Denmark) was added and incubated at room temperature in moist chamber for 30 minutes. This was followed by incubating the sections with avidin-biotin complex wherein the biotin was conjugated with horseradish peroxidase enzyme (Dako, Denmark) for 45 minutes. The peroxidase activity was visualized by incubation with freshly prepared 3 3' Diaminobenzidine tetrahydrochloride (DAB) containing 0.0015% H_2O_2. The slides were counterstained with hematoxylin, dehydrated and cleaned in xylene and mounted. The slides were viewed under x500 magnification of light microscope. Corneal button section, without the incubation

with primary antibody, was used as negative control, while tonsil section served as positive control. The phenotype of the inflammatory cells was assessed in the region of the granulomatous inflammation, surrounding stroma and limbus.

5.3 RESULTS

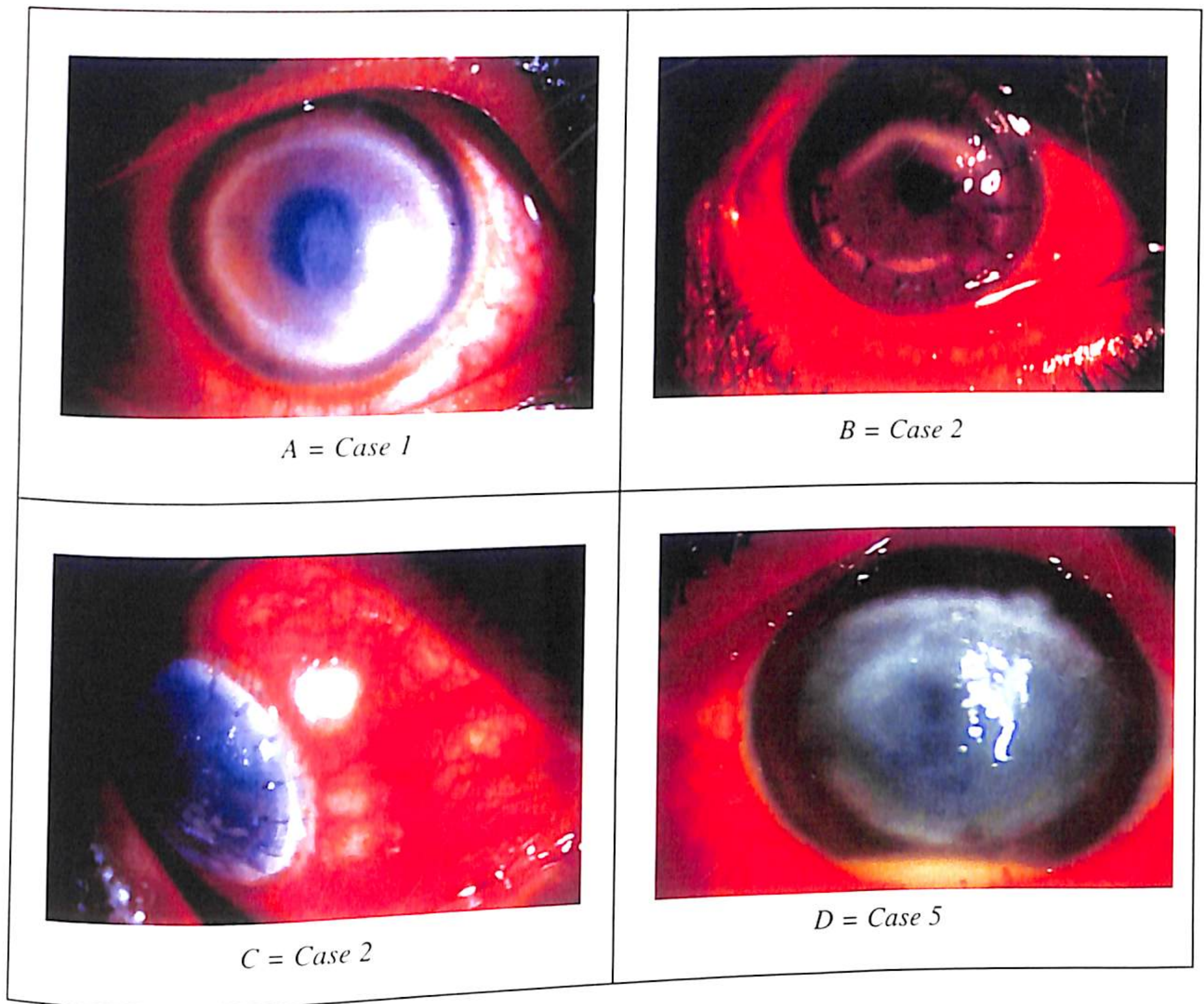
5.3.1 Demography

Among the 18 corneal button/eviscerated specimens five (27.7%) displayed granulomatous cell reaction. Five patients included 3 males and 2 females and their age at the time of surgery ranged from 20 - 65 years (median 45 years). Two patients underwent evisceration while the other three had therapeutic penetrating keratoplasty done.

5.3.2 Case histories

Case 1

A 30-year-old male patient presented with complaints of pain, redness, watering and decreased vision of two months duration following an injury to the eye. He was diagnosed as a case of corneal ulcer and was treated with antibiotic eye drops for two months before being referred to our institute. At presentation, he had a vision of hand movements at 2 meters with inaccurate projection of light. The conjunctiva was congested. Cornea showed a ring infiltrate (Figure 5.1A) involving the limbus. Fundus could not be visualized. B scan revealed choroidal thickening with no gross vitreous opacities. Corneal scrapings revealed 0-4 cysts of *Acanthamoeba* per high power field. Patient was diagnosed as *Acanthamoeba* keratitis and treated with 0.02% CHx



A = Case 1

B = Case 2

C = Case 2

D = Case 5

Figure 5.1: Slit lamp photograph in diffuse illumination of cornea (x16)

A: Large ring shaped infiltrate with surrounding edema
B: Ring shaped granular infiltrate involving the graft host junction
C: Clear graft and congested sclera showing four abscesses of varying size, sparing the graft host junction
D: Ring shaped infiltrate with surrounding edema. Note the presence of hypopyon

and 0.02% PHMB for a period of one month. There was progression of the ulcer, marked thinning and ectasia of cornea with impending perforation. The patient underwent evisceration and the excised contents were subjected to histologic examination.

Case 2

A 65-year-old lady, who underwent uncomplicated penetrating keratoplasty with extracapsular cataract extraction and posterior chamber lens implantation in her right eye for granular dystrophy, presented to her ophthalmologist three months post-operatively complaining of pain, photophobia, and decreased vision. The ophthalmologist diagnosed her condition as graft rejection. She was treated with hourly topical prednisolone acetate. After three days of therapy the epithelial line increased in size and developed areas of epithelial defect. The surgeon suspected recurrent HSV keratitis, therefore reduced the frequency of prednisolone acetate and started topical acyclovir (5%) 5 times a day. Seeing no improvement, he referred the case to us. At initial examination at our institute her visual acuity was hand movements in the right eye and 20/125 in the left eye. The conjunctiva was injected. Cornea showed two circumferential epithelial defects, about 2 mm inside the graft host junction, associated with granular infiltrate (Figure 5.1B). Rest of the graft showed diffuse stromal haze associated with multiple keratic precipitates. The host cornea demonstrated minimal superficial vascularization and all interrupted sutures were intact. Anterior chamber was deep and the intraocular pressure appeared digitally normal. Microscopic examination and culture of the

corneal scrapings were positive for *Acanthamoeba*. A review of the patient's medical and social history revealed that she was using pond water for washing face and taking bath. The patient was treated with half hourly topical PHMB 0.02% and chlorhexidine 0.02% and oral itraconazole 100mg twice daily. Owing to no improvement, a therapeutic penetrating keratoplasty was done after 2 weeks and the excised corneal button was sent for histopathology. On the first postoperative day, there were exudates in the anterior chamber. There was evidence of suture abscess, epithelial defect and endothelial pigments on the 7th post-operative day, which progressed to scleral abscess at the end of 5 weeks (Figure 5.1C). The final visual acuity at the end of 6 weeks was perception of light.

Case 3

A 45- year- old man presented with severe pain, redness, watering, pricking sensation and reduced vision of 10 days duration. He gave a history of sand particles falling into his left eye. He consulted a local ophthalmologist who diagnosed the case as hypopyon corneal ulcer and referred to our institute. On examination, he had edema of lids with pseudoptosis. Conjunctiva was congested and chemosed. Cornea showed an epithelial defect 2 x 2.5 mm associated with underlying stromal infiltrates of 8mm. Anterior chamber was deep with 1mm hypopyon. Initial corneal scrapings did not reveal any organisms on microscopic examination of smear while a repeat scraping revealed cysts of *Acanthamoeba*. The patient was treated for five weeks with 0.02% PHMB and 0.02% CHx eye drops along with oral itraconazole. There

was no response to the above medication. The ulcer progressed to involve the limbus and the sclera with increase in intraocular pressure, thereby, necessitating evisceration.

Case 4

A 20-year-old woman presented to us with the complaints of pain, redness, watering, photophobia and diminished vision in her left eye of two and half months duration. She gave history of unknown particles falling into her eye. At the time of presentation the visual acuity in her affected eye was light perception and accurate projection of rays in all quadrants. On examination, the left eye showed a ring shaped corneal infiltrate 1mm inside the limbus. The corneal stroma within the ring showed a granular infiltrate. Surrounding cornea was edematous with deep vascularization in two quadrants. Anterior chamber was deep. Posterior segment appeared normal on B-scan ultrasonography.

Corneal scrapings revealed *Acanthamoeba* cysts on microscopic examination. The patient was treated with topical 0.02% PHMB and 0.02% CHx and oral ketoconazole. Over the next 10 days the infiltrate increased in density and showed progressive vascularization. We added prednisolone acetate 1% every three hourly. With this therapy the central infiltrated area showed progressive thinning. Therefore, we advised penetrating keratoplasty. The corneal tissue was processed for histopathology. At the end of 6 weeks, the graft was clear with a visual acuity of 20/50 in the left eye.

Table 5.1. : Results of Microbiological investigations on Corneal Scrapings, Corneal Buttons, and Evisceration material from five cases included in the study

Patient No	Sample	Smear Results			Culture	PCR for HSV-1 DNA
		KOH + CFW	Gram	Giemsa		
1	Corneal scraping	+	+	+	*Acanthamoeba spp.* *Staphylococcus epidermidis*	
	Evisceration material	ND	ND	ND	ND	-
2	Corneal scraping	+	+	+	*Acanthamoeba spp.* *Neisseria spp.*	
	Corneal button	ND	ND	ND	-	-
3	Corneal scraping	+	-	-	-	
	Evisceration material	ND	ND	ND	ND	-
4	Corneal scraping	+	+	+	*Acanthamoeba spp.*	
	Corneal button	ND	ND	ND	*Acanthamoeba spp.*	-
5	Corneal scraping	+	+	+	-	
	Corneal button	ND	ND	ND	*Acanthamoeba spp.*	-

ND: Not done   + : Positive   - : Negative

either of three staining methods. Eviscerated material from the two patients were not subjected to microbial investigations while corneal button from the three patients were subjected to only culture examination. Two of these corneal buttons showed positivity in culture. PCR for HSV1 DNA was negative for all five cases.

5.3.4 Histopathology

The histopathologic features of the corneal buttons from the five cases are given in Table 5.2. There was epithelial ulceration with destruction of Bowman's layer in all the cases. The stroma showed inflammatory infiltrates consisting of neutrophils in the anterior two-thirds of stroma (Figure 5.2). Vascularization of stroma was noted in mid and deep peripheral stroma in two cases (Figure 5.3). Viable and degenerated cysts of *Acanthamoeba* were seen in the stroma (Figure 5.4). In addition, the deeper stroma and the region around Descemet's membrane showed a few aggregates of epitheloid cells, lymphocytes and multinucleated giant cells (Figure 5.3). Some of the giant cells and occasional keratocytes showed cysts of *Acanthamoeba* in the cytoplasm, suggesting the phagocytosed parasites (Figure 5.5). Limbal tissue, when identified in the sections, showed dense lymphoplasmacytic infiltrates admixed with few eosinophils.

5.3.5 Immunophenotyping

The inflammatory cells in the corneal stroma were found to be of T cell population. In the granulomatous regions, the cells were positive for T cells (Figure 5.6A), CD 68 (Figure 5.6B) and negative for B-cell marker, suggesting a predominance of T lymphocytes with macrophages. The detailed results are depicted in Table 5.2.

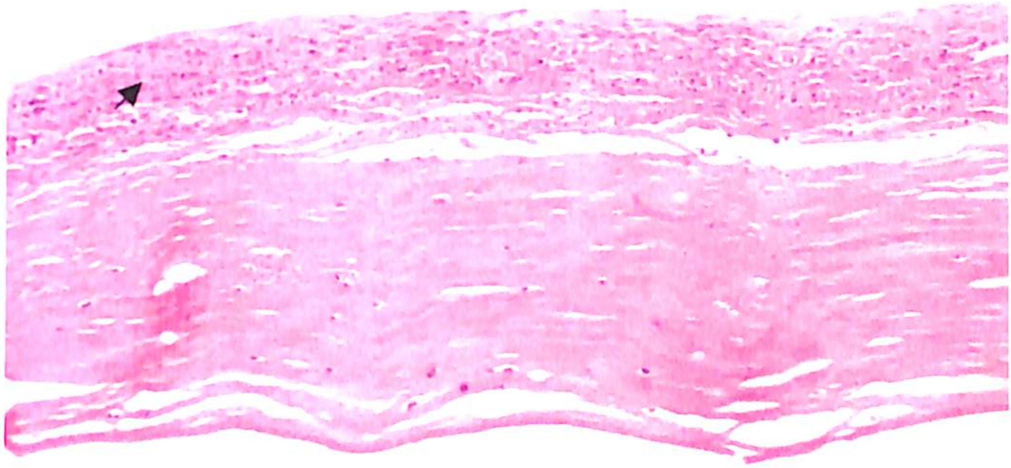


Figure 5.2: Corneal button section showing epithelial ulceration and inflammatory infiltrates in the anterior one-third of corneal stroma
(haematoxylin and eosin, x40)

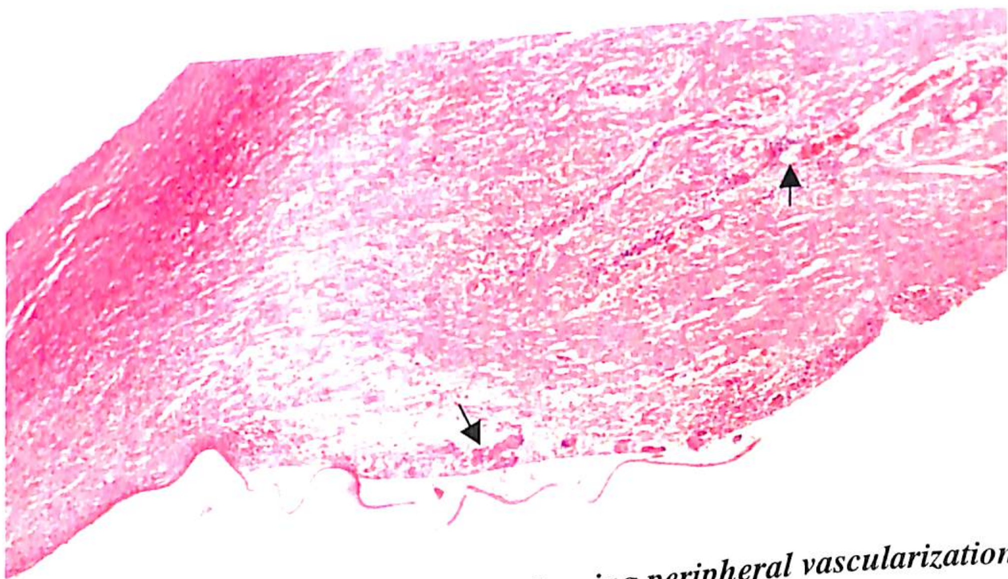


Figure 5.3: Corneal button section showing peripheral vascularization and corneal inflammation involving full thickness of stroma.
Note the presence of granulomatous inflammation and multinucleated giant cells in the deep stroma with an artifactually detached Descemet's membrane (haematoxylin and eosin, x20)

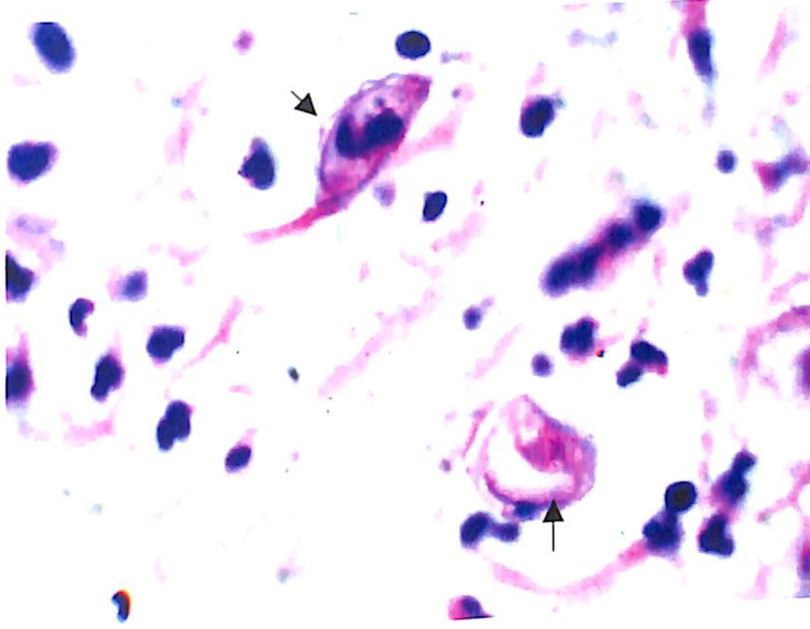


Figure 5.4: The corneal stroma shows two cysts of Acanthamoeba with a double-layered wall (Periodic acid and Schiffs stain, x1000)

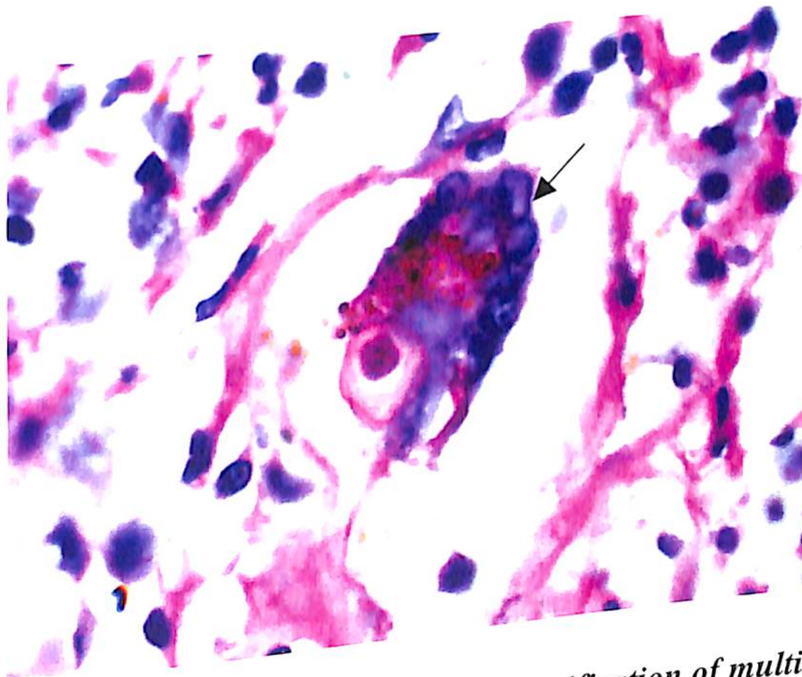
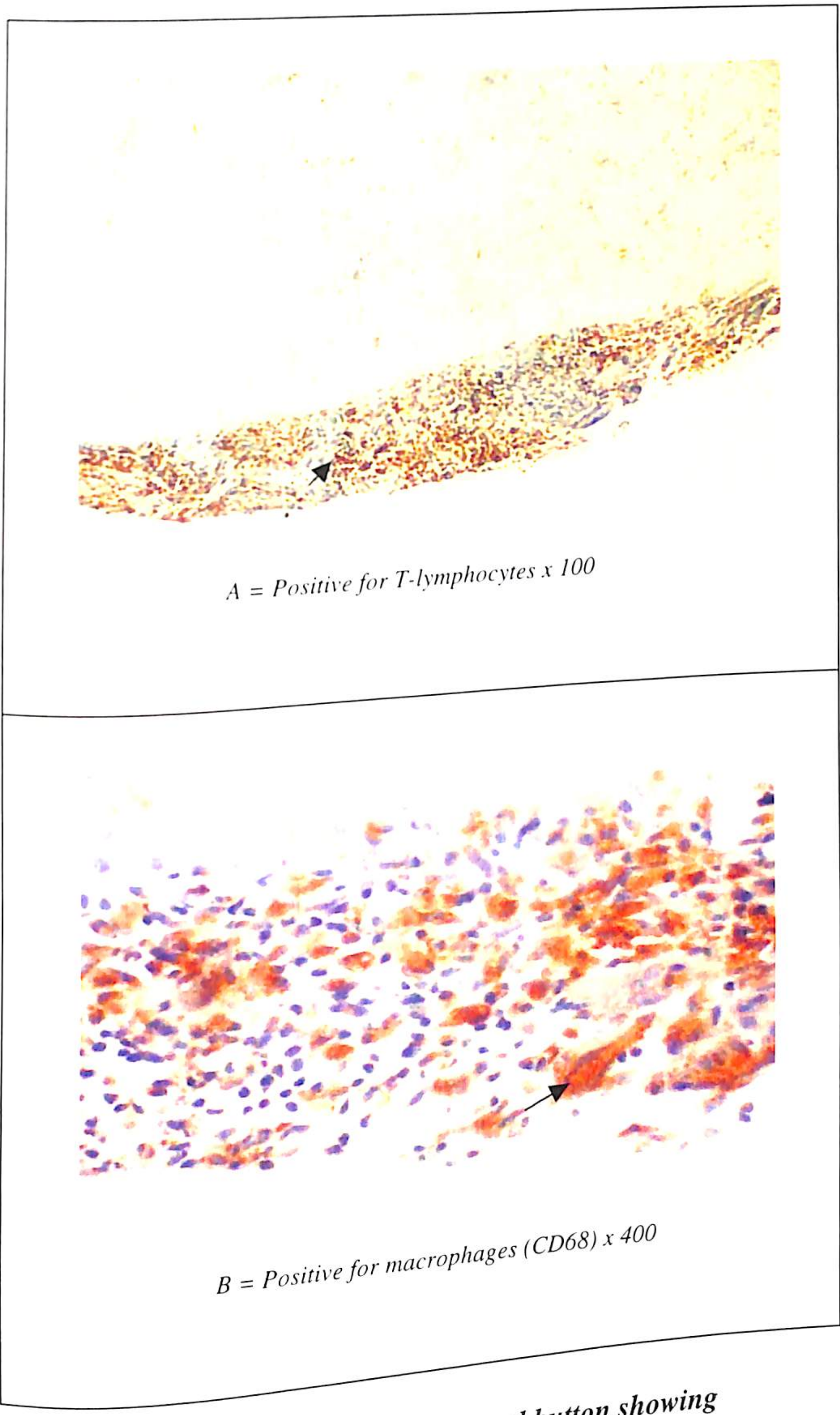


Figure 5.5: Section shows a higher magnification of multinucleated giant cell with a engulfed cyst of Acanthamoeba within the cytoplasm (periodic acid schiffs stain, x1000)



A = Positive for T-lymphocytes x 100

B = Positive for macrophages (CD68) x 400

Figure 5.6: Section of corneal button showing immunoperoxidase positive staining. (DAB chromogen and counterstained with hematoxylin)

Table 5.2: Histologic features of 5 cases of Acanthamoeba keratitis included in the study

Case No	Epithelium	Bowman's layer	Stromal inflammation	Vessels	Necrosis	Granuloma	Phenotype	*Acanthamoeba* load
1	Ulcerated	Absent	Severe (M, P)	-	+	+	T, CD : + B: -	Cysts 3 +
2	Ulcerated	Absent	Diffuse	+	+	+	T, CD: + B: -	Cysts 3 +
3	Ulcerated	Absent	Severe (M, P)	-	-	+	T, CD : + B:-	Cysts 3 +
4	Ulcerated	Absent	Severe (A, M)	-	+	+	T, CD : + B:-	Cysts 3+; trophozoites 2+
5	Ulcerated	Absent	Severe (M, P)	+	+	+	T, CD : + B:-	Cysts 2 +

M : Mid stroma A : Anterior stroma P : Posterior stroma - : Negative + : Positive

5.4 DISCUSSION

Histologically, the corneal tissues in *Acanthamoeba* keratitis show evidence of epithelial ulceration, polymorphonuclear infiltrates, stromal necrosis along with the presence of trophozoites and/or cysts in the corneal stroma (Garner 1993). Despite the prolonged clinical course of the disease, a few unique observations have been made in AK which include: a) absence of vascularization (Kremer *et al* 1994), b) scarcity of lymphocytes (Garner 1993), c) keratocyte loss through apoptosis,d) the presence of cysts in the deep stroma, unaccompanied by inflammatory cells (Vemuganti *et al* 2000). Though acanthamoebic infections of brain usually evoke granulomatous inflammation (Dougherty *et al* 1994), this is rarely reported in AK. In this report we describe five cases of AK presenting with granulomatous inflammation in the posterior corneal stroma, four of which presented with rapidly progressive AK involving limbus and sclera. To understand the significance of these findings we performed the immunophenotyping of the inflammatory cells and attempted a clinicopathological correlation.

Clinically, AK is characterized by severe pain with an early superficial keratitis; followed by radial perineural infiltration, ring infiltration and rarely limbitis and scleritis (Bacon *et al* 1993). Sclerokeratitis in AK is often associated with severe inflammation and is a therapeutic challenge to the ophthalmologist (Lee *et al* 2002). The limbal and scleral inflammation has been reported to increase on initial intensive topical anti-amoebic therapy and this has been related to immune mediated response to dead or dying amoebae within the cornea (Lee *et al* 2002). Fortunately limbal and

scleral extension of AK remains a rare complication. None of the patients in our earlier reported series of 39 patients had developed this complication (Sharma *et al* 2000). Three of five cases reported in this series had a severe clinical course that progressed despite adequate doses of supervised medical treatment, necessitating surgical intervention. One case presented with total corneal ulcer, while the other presented as a graft infiltrate, clinically mimicking a rejection phenomenon. The median duration of medical treatment was 2 weeks (1- 8 weeks). History of trauma was elicited in 4 cases. Though prolonged medical treatment is usually advised for *Acanthamoeba* keratitis, penetrating keratoplasty has been advocated in cases, which threaten the integrity of the eye (Cohen *et al* 1987).

Histologically, the tissues showed epithelial ulceration and destruction of Bowman's layer. The stroma showed dense inflammatory infiltrates predominantly consisting of polymorphonuclear infiltrates in all cases, as has been reported in most studies (Garner 1993; Kremer *et al* 1994; Yang *et al* 2001). The deeper stroma showed lymphocytes, macrophages, epitheloid granulomas and giant cells. Though polymorphonuclear cells are believed to be the first line of defense in all infections, including AK, recent evidence points towards the role of macrophages (van Klink *et al* 1996). They performed conjunctival macrophage depletion in Chinese hamsters to determine the importance of macrophages in AK. They selectively eliminated macrophages using macrophagicidal drug C12MDP-L1P (Liposomes containing dichloromethylene diphosphonate). They found profound exacerbation of AK in hamster treated with this drug, strongly suggesting that macrophages play an important role in the corneal infection with *Acanthamoeba*, probably by acting as a

first line of defense and eliminating significant numbers of *Acanthamoeba* trophozoites.

Two of the five cases showed evidence of vascularization in our series. This is different from the observations made by Kremer *et al* (1994) who noted the conspicuous absence of vascularization in 10 cases reported by them. In general, it is believed that lymphocytic infiltration in the cornea is closely associated with vascularization (Mathers *et al* 1987). When vascularization is present, lymphocytic and plasmacytic infiltrates are usually observed predominantly in the immediate vicinity of blood vessels in the corneal stroma or in the vascular pannus. An immune response to chronic inflammation can be expected to further involve macrophages, lymphocytes and macrophage derived epitheloid cells (Mathers *et al* 1987).

Garner (1993) interpreted that absence of lymphoid cell may be due to absence of stromal vascularization and consequent barrier to invasion by relatively immotile cells. In all our cases, lymphomononuclear cells were noted in the deep stroma accompanied by macrophages, epitheloid cells and multinucleated giant cells. Though Auran *et al* (1987) reported the presence of granulomatous inflammation in 5 cases; best illustration of this finding was reported by Mietz and Font (1997). Granulomatous inflammation extending to sclera has been reported by Doughtery *et al* (1994). In the two eviscerated tissues, there was no evidence of granulomatous inflammation in the sclera or other layers.

The frequency of granulomatous inflammation in corneal tissues varies from 2% to 25% depending on the type of tissues included in the study (Weiner *et al* 1985; Green *et al* 1967; Holbach *et al* 1990). Granulomatous inflammation in 13.8% (23 of 167) of fungal keratitis have been reported by Vemuganti *et al* (2002) and now we report 27.7% (5 of 18) in *Acanthamoeba* keratitis. Though it can be seen in various other infective and non-infective corneal diseases, there is enough evidence that it is most commonly associated with disciform herpes simplex keratitis (Holbach *et al* 1990). In this study, the DNA isolated from the paraffin sections of the corneal tissue and eviscerated material were negative for herpes simplex virus DNA, thereby ruling out any associated or pre-existing herpes virus keratitis. Though definite pathogenesis of the granulomatous reaction in general remains unknown, the process may have a non-immune or immune aetiology (Weiner *et al* 1985). The non-immune response is the well-known foreign body granuloma. There is an influx of macrophages due to chemotaxis and these cells persist in the area if the foreign material is poorly soluble (Williams and William 1983). The immune pathway is the result of sensitized T cells releasing lymphokines and causing the accumulation of macrophages (Taussing 1984). The presence of T lymphocytes, as found in this study, suggests that granulomatous inflammation in cornea appears to be an immune-mediated process. These T cells could either be sensitized to microbial antigens, altered cellular and/or basement membrane structures from the host, or both. Holbach *et al* (1990) support the role of viral antigens while Weiner *et al* (1985) suggest a non-viral antigen in the aetiopathogenesis of this type of inflammation. Though the clinical implications of this type of inflammation is not clearly documented, it has been suggested that granulomatous inflammation around Descemet's membrane can be identified

clinically and should be considered as an indication for penetrating keratoplasty in herpes stromal keratitis. We speculate that it may be the same for AK. What is important to note is that all four of five cases had a rapid clinical worsening with extension of the inflammation to the limbus, with involvement of sclera in four cases, necessitating an early surgical intervention, suggesting the possibility that it could be a poor prognostic maker. However, whether the granulomatous inflammation is the cause or the effect of the advanced disease cannot be commented upon by these five cases but it is likely that the two are related.

To summarize, granulomatous inflammation, is not an uncommon finding and could be seen in rapidly progressive form of *Acanthamoeba* keratitis, not responding to medical treatment. However further studies are warranted to understand the varied presentations of this disease and their clinical implications.

CHAPTER 6: SUBGENUS CLASSIFICATION OF *ACANTHAMOEBA* STRAINS ISOLATED FROM KERATITIS PATIENTS IN INDIA

6.1 INTRODUCTION

Taxonomy of *Acanthamoeba* at species level has had a confusing evolution wherein the earliest classification was based on the morphology of the *Acanthamoeba* cysts/trophozoites (Pussard and Pons 1977). Several groups have used isoenzyme electrophoresis (De Jonckheere 1983) and antigenic differences (Stevens *et al* 1997) between the isolates for typing of *Acanthamoeba*. More recently molecular techniques like restriction fragment length polymorphism (RFLP) of the mitochondrial (Byers *et al* 1983) and whole cell DNA (McLaughlin *et al* 1988); PCR-RFLP of the nuclear (Chung *et al* 1998) and mitochondrial ribosomal DNA (Yu *et al* 1999) have been used for subgenus classification of *Acanthamoeba* but with variable success. The small subunit ribosomal RNAs (ssu rRNAs) have been recognized as a well-suited marker for estimating phylogenetic relationship (Woese 1987) and this approach has been employed to determine the taxonomic status of *Acanthamoeba* (Gast *et al* 1996;Stothard *et al* 1998). Stothard *et al* classified 53 strains of *Acanthamoeba* into 12 sequence types based on the complete sequences of 18S rRNA gene (Stothard *et al* 1998). This classification system is presently considered robust and consistent. It seems to be based on more readily interpretable characters and has revealed inconsistencies in the earlier classification systems (Gast *et al* 1996; Stothard *et al* 1998). This phylogenetic clustering of *Acanthamoeba* isolates has been further confirmed using complete mitochondrial ribosomal sequences or 16S rRNA gene (Ledee *et al* 2003). So far, the 18S rDNA typing has mainly been done with *Acanthamoeba* isolates from contact lens wearers and the genotype assigned to most of them using complete sequencing is, T4 (Gast *et al* 1996; Stothard *et al* 1999).

Although sequencing of 18S rDNA and 16S rDNA yields highly reliable data complete sequencing of genes is generally expensive and time consuming (Alves *et al* 2000). If found a equally reliable, a partial sequencing may serve the same purpose within limited time and resources

In this study, genotype and taxonomic status of *Acanthamoeba* spp. isolated from corneal scrapings of keratitis patients with no history of contact lens wear was determined. Phylogenetic inferences were obtained from complete sequencing vis-à-vis partial sequencing of seven amplimers of 18S rRNA gene of *Acanthamoeba*.

6.2 MATERIALS AND METHODS

6.2.1 *Amoebae* strains

A total of 23 amoebae isolates were included in this study, consisting of 15 isolates from keratitis patients (13 from this institute and 2 from Sankara Nethralaya, Chennai), 1 environmental isolate, 1 each from Pakistan and Argentina and 5 standard strains of *Acanthamoeba* (Table 6.1).

6.2.2 Amoebae isolation

6.2.2.1 Corneal scrapings

Corneal scrapings taken by the ophthalmologists from suspected cases of AK were inoculated in an array of media for the isolation of bacteria, fungi and *Acanthamoeba*. Non-nutrient agar (NNA) with overlay of live culture of *E.coli* was used for the isolation of *Acanthamoeba*.

Table 6.1: Acanthamoeba strains used in the study and their morphological classification

S.No	Amoebae	Source	Geographical location	Morphological group*	Sequencing
1	*A.castellanii* ATCC 50374†	Yeast culture	UK	-	Partial
2	*A.castellanii* ATCC 50370†	Keratitis	USA	-	Partial
3	*A.castellanii* Neff ATCC 50373†	Soil	USA	-	Partial
4	*A.polyphaga* ATCC 50372†	Soil	Japan	-	Partial
5	*A.culbertsoni* Diamond CDC†	Keratitis	USA	-	Partial
6	*A. spps*	-	Argentina	II	Partial
7	*A.spps*	-	Pakistan	II	Partial
8	*A.spps* L 402/97	Keratitis	Hyderabad, India	I	Partial
9	*A.spps* L 565/97	Keratitis	Hyderabad, India	II	Complete
10	*A.spps* L 773/96	Keratitis	Hyderabad, India	I	Complete
11	*A.spps* L 1060/96	Keratitis	Hyderabad, India	II	Complete
12	*A.spps* L 407/95	Keratitis	Hyderabad, India	II	Partial
13	*A.spps* L 473/00	Keratitis	Hyderabad, India	II	Partial
14	*A.spps* L 749/98	Keratitis	Hyderabad, India	I	Complete
15	*A.spps* L 1019/99	Keratitis	Hyderabad, India	I	Partial
16	*A.spps* L 1002/99	Keratitis	Hyderabad, India	I	Complete
17	*A.spps* L 1035/99	Keratitis	Hyderabad, India	III	Partial
18	*A.spps* L 1629/99	Keratitis	Hyderabad, India	III	Complete
19	*A.spps* L 98/00	Keratitis	Hyderabad, India	I	Complete
20	*A.spps* L 478/00	Keratitis	Hyderabad, India	I	Partial
21	*A.spps* SN-2	Keratitis	Chennai, India	III	Partial
22	*A.spps* SN-3	Keratitis	Chennai, India	III	Partial
23	*A.spps*	Water	Hyderabad, India	-	Partial

*- Based on present study; † - Standard strains

6.2.2.2 Environment

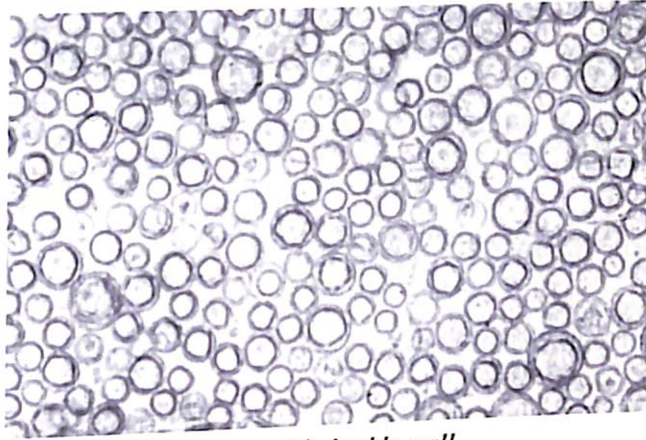
In order determine if contaminated water was the source of the infection, eight attempts were made to isolate *Acanthamoeba* from water that was collected and brought by AK patients. The patients were explained that water should be collected from the water source which he/she uses for the daily chores likes bathing etc and that the water should be collected in a clean container. Patients were asked to collect approximately 2 liters of well water. Amphotericin (fungicidal) at a concentration of 2.5mg ml was added to the water and then centrifuged at 4500 rpm for 15 minutes. The pellet obtained was inoculated on NNA plates, which were pre-seeded with *E.coli* suspension. The NNA plates were observed daily for the growth of *Acanthamoeba*. Among the many attempts only one strain of *Acanthamoeba* could be isolated.

6.2.2.3 Axenic culture of *Acanthamoeba*

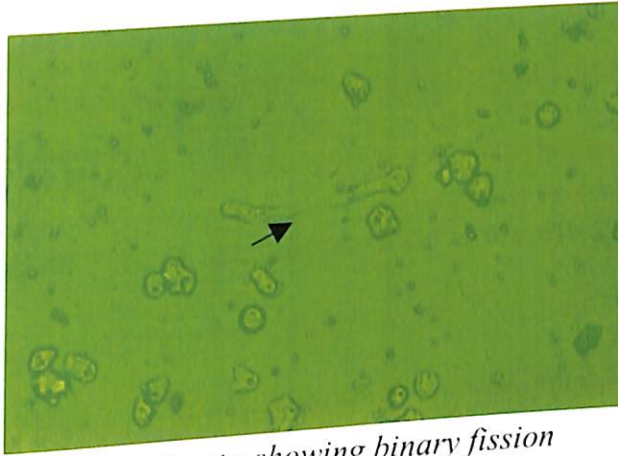
In order to genotype and perform morphology based classification, cultures grown on NNA were axenized in Proteose Peptone Yeast Glucose broth (PYG; for preparation see Appendix 2). A small piece of the NNA with growth of *Acanthamoeba* was cut and placed in 25cm^2 sterile tissue culture flask to which 5 mL of PYG medium containing ciprofloxacin (60µg/mL) was added. The flasks were incubated at 30°C and observed for growth daily (Figure 6.1).

6.2.3 Morphological classification

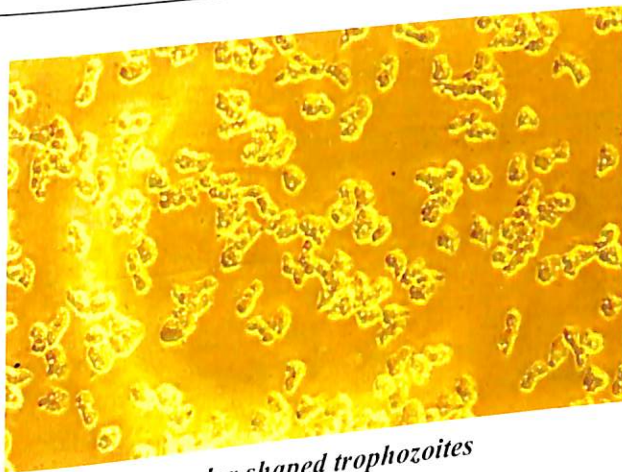
Classification based on morphology was done for all isolates except the 5 standard strains. These 17 isolates were axenized and allowed to encyst in PYG medium and



A = cysts with ***double wall***



B = Trophozoite showing binary fission



C = irregular shaped trophozoites

Figure 6.1: Acanthamoeba cysts and trophozoites in axenic culture under phase contrast microscope, x400)

later classified based on morphological features of both cyst and trophozoite, as described by Page (Page 1967) and Visvesvara (Visvesvara 1991).

6.2.4 DNA extraction

Total DNA was extracted from the 23 amoebic cultures using the UNSET (Urea, NaCl, Sodium dodecyl sulphate, EDTA, Tris: for preparation see Appendix 2) lysis buffer method (Hugo *et al* 1992). To isolate DNA from cultures, amoebae were harvested from 4-5 days old 5 ml confluent cultures (~1 x 10^6 amoebae, containing ~ 9:1 ratio of trophozoites and cysts) by centrifugation at ~1000 g for 5 minutes. The harvested cells were washed twice using 5 ml of PBS (Phosphate buffer saline) and resuspended in 0.5 ml of UNSET lysis buffer for DNA isolation. The aqueous lysate was extracted twice with 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was finally precipitated using 0.1 volume of 3M NaCl and 2 volume of ethanol and dissolved in 200 μl of 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

6.2.5 Amplification of 18S rRNA gene domains

18S rRNA gene is the most commonly used rRNA subunit to estimate phylogenetic relatedness. Ribosomal RNA genes are conserved through billions of years and resist lateral transfer of genes between the species, hence evolve slowly which is a prerequisite to estimate phylogenetic relatedness. 18S rRNA gene has been sequenced for all the species of *Acanthamoeba* and the data is available in the public database. Various primer pairs specific to 18S rRNA gene of *Acanthamoeba* have been described in literature and among which three were chosen by us to evaluate their ability to be used as a phylogenetic marker. Three sets of primers i.e. ASA.S1

(*Acanthamoeba* specific amplimer: Schroeder *et al* 2001), ACARNA (*Acanthamoeba* –18S rRNA gene based primer: Vodkin *et al* 1992), GP- P1 (Genus specific primer: Lehmann *et al* 1998) spanning within 932 - 2076 bp (*A. castellanii* Neff strain: GenBank Accession No.U07416: Figure 6.2) of the 18S rRNA gene, were used for amplification (Table 6.2).

The primers were synthesized at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. All PCR reactions were carried out in a laminar flow hood after 30 minutes of UV irradiation to decontaminate surfaces and all supplies within the hood. Pre-sterilized PCR tubes, double autoclaved Milli Q water and positive displacement tips and pipettes were used to reduce the possibility of contamination. The primer pairs were tested initially for amplification of *A.castellanii*, clinical isolates of *Acanthamoeba*, *Pseudomonas aeruginosa*, *Aspergillus* species, Herpes simplex virus, and human leukocytes DNA. The amplification profile for each of the primers is given in Table 6.2. Each 20 µl PCR reaction comprised: 1 µl of DNA (~10 ng genomic DNA), 200 µM dNTP, 2 pM of each primer, 1x standard PCR buffer (containing 1.5 mM $MgCl_2$) and 1 unit Taq DNA polymerase (Gene Taq, MBI Fermentas, Lithuania). Amplifications were performed in MJ Research PTC 200 thermocycler. The PCR products were run in a 1.5% agarose / TAE gel containing 0.5 µg/ml ethidium bromide and the results were visualized and recorded on a UV gel documentation system (UVItec Ltd, Cambridge, UK).

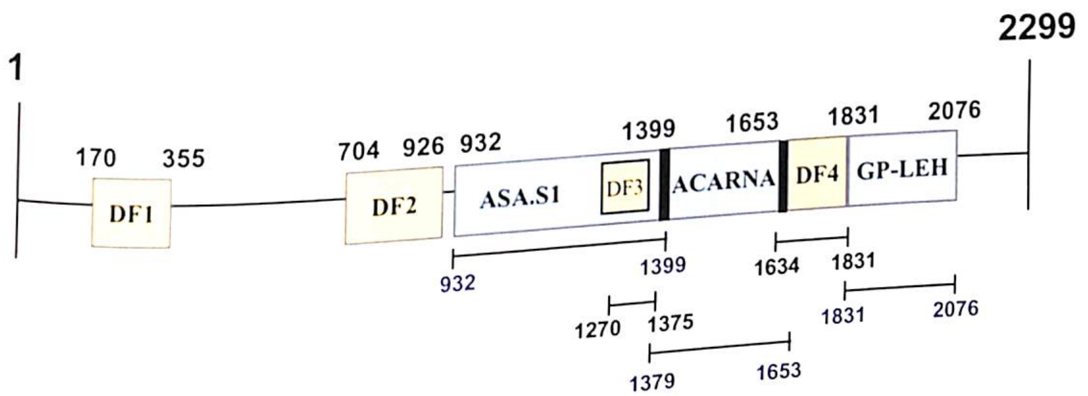


Figure 6.2 *Diagrammatic representation of 18S rRNA gene of Acanthamoeba showing the seven domains. The yellow colored boxes represent the diagnostic fragments for which the analysis was done in silico while the boxes colored blue are the domains which were amplified by PCR and sequenced.*

(Nucleotide positions for all the domains are in reference to Acanthamoeba castellanii Neff strain (Accession No. U07416)

Table 6.2: Details of primers and PCR conditions used in the study

S.NO	Gene	Primer name	Specificity	Primer sequence	Nucleotide position*	PCR conditions	Size
1	18S rDNA	ASA.S1	Genus	F - 5'-GGCCCAGATCGTTTACCGTGAA-3' R - 5'-TGACTCCCCTAGCAGCTTGTGAGA-3'	932-954 1375 -1399	94°C - 1 min 94°C - 30 secs } 35 cycles 65°C - 30 secs } 35 cycles 72°C - 30 secs } 35 cycles 72°C - 5min	467bp
2	18S rDNA	ACARNA	Genus	F - 5'-TCCCCTAGCAGCTTGTG-3' R - 5'-TAACGAACGAGACCTTAAC -3'	1379-1396 1634 -1653	94°C - 1.5min } 35 cycles 50°C - 1.5min } 35 cycles 72°C - 1.5min } 35 cycles 72°C - 5min	272bp
3	18S rDNA	GP-P1	Genus	F - 5' GTTTGAGGCAATAACAGGT-3' R - 5' CCTAGTAAGCGCGAGTC -3'	1831- 1850 2059-2076	94°C - 30secs 94°C - 15secs } 35 cycles 56°C - 20secs } 35 cycles 72°C - 10secs } 35 cycles	253bp

* - Nucleotide position based on *Acanthamoeba castellanii* Neff strain Accession No. U07416

PCR amplification of the complete 18S rRNA gene was done at Ohio State University, Columbus, USA, using SSU1 and SSU2 oligonucleotide primers described earlier (Weekers *et al* 1994).

6.2.6 DNA sequencing

6.2.6.1 Three fragments of 18S rRNA gene

Sequencing was performed for all the three PCR amplimers for 23 amoebae using the same sets of primers at concentration of 1pM i.e., half of the concentration used for PCR. Sequencing was done for both the strands with the 'BigDye Terminator sequencing kit' (Applied Biosystems, Foster city, CA, USA) as per the manufacturer's protocol. The sequencing amplification conditions were: 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min for 30 cycles. The amplicons were precipitated using 1 µl of 3M sodium acetate (pH 4.6) and 50 µl of ethanol and incubated on ice for 10 min. The pellet was recovered by centrifugation (15,000 rpm/20 min/4°C), washed with 70% ethanol, dried and dissolved in 10 µl of diluted HiDi formamide (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Partial sequencing of these three amplimers was performed at CCMB, India, on an automated DNA sequencer (ABI PRISM 3700) using dideoxy terminator sequencing chemistry.

6.2.6.2 Complete 18S rRNA gene

Complete sequencing for the 18S rRNA gene was done only for 7 amoebae isolates. Direct double stranded Dideoxy sequencing (dsCycle Sequencing kit, GIBCO/BRL, Gaithersburg, MD) of the complete 18S rRNA gene was done at Ohio State University using protocol as described by the manufacturer. The primers used for the sequencing of the entire gene have been described earlier (Weekers *et al* 1994).

6.2.7 Alignment of the sequences

6.2.7.1 Three fragments of 18S rRNA gene

The partial sequences of 23 isolates obtained from all the three sets of primers were edited using sequence editor program of ABI PRISM and the forward and reverse strands of each sample were assembled using AUTOASSEMBLER program of ABI PRISM. Sequences at both 5' and 3' end of the gene determined by the primers were excluded from further analysis. Sequences obtained from the PCR products were blasted against sequences in EMBL database and 7 reference sequences of *Acanthamoeba* belonging to T4 genotype (Table 6.3) showing 97-100% homology to the blasted sequence were selected. Apart from the above, 11 reference *Acanthamoeba* strains were also selected, which belonged to genotype T1 to T3 and T5-T12. All the sequences were aligned with their corresponding reference sequences from the EMBL database using CLUSTAL X program.

6.2.7.2 Complete 18S rRNA gene

Complete 18S rDNA sequences of the 7 *Acanthamoeba* isolates were aligned using program ESEE (Eye ball sequence editor; Cabot and Beckenbach 1989). This was done after identification of homologous position on the basis of secondary structure of the 18S rRNA gene. All the sequences were aligned with the same set of 18 *Acanthamoeba* reference strains (Table 6.3) including 7 belonging to T4 genotype and a master alignment was made. Schroeder *et al* (2001) have earlier described three diagnostic fragments (DF1-DF3; Figure 6.2) within the GTSA.B1 (Genus specific amplimer) domain of 18S rRNA gene wherein DF1 is 185 bp in size (170-355 bp) with two variable regions, DF2 is 222 bp amplimer (704-926 bp) having 4 variable regions and DF3 which is part of ASA.S1 amplimer and is 105 bp long (1270-

Table 6.3: Details of Acanthamoeba reference strains used in the study

S.No	Reference *Acanthamoeba* species	rDNA genotype clade	Source	Geographical location	GenBank Accession Number
1	*A. castellanii* CDC:0981:V006	T1	GAE	USA	U07400
2	*A. polyphaga* OX-1	T2	old distilled water	USA	AF019051
3	*A. griffini* H37	T3	keratitis	UK	S81337
4	**A. hatchetti 3ST**	**T4**	**keratitis**	**Austria**	**AF260723**
5	**A. rhysodes**	**T4**	**Tap water**	**Austria**	**AF260720**
6	**A. royreba Oak Ridge ATCC 30884**	**T4**	**Soil**	**UK**	**U07417**
7	**A. polyphaga Naginton**	**T4**	**keratitis**	**UK**	**AF019062**
8	**A. castellanii CDC:0184:V014**	**T4**	**keratitis**	**India**	**U07401**
9	**A. sp. Liu-E1**	**T4**	**keratitis**	**China**	**AF019055**
10	**A. polyphaga HC-2**	**T4**	-	**Mexico**	**AF019056**
11	*A. lenticulata* strain 118	T5	Nasal Mucosa	Germany	U94736
12	*A. palestinensis* 2802	T6	Soil	France	AF019063
13	*A. astronyxis* Ray & Hayes	T7*	Lab water	USA	AF479546
14	*A. tubiashi* Lewis & Sawyer	T8*	Freshwater	USA	AF479545
15	*A.comandoni* Pussard	T9*	Soil	France	AF479544
16	*Acanthamoeba culbertsoni* Lilly A-1	T10	Human cell culture	USA	AF019067
17	*Acanthamoeba stevensoni* RB:F:1	T11	Marine sediment	USA	AF019069
18	*Acanthamoeba healyi*	T12	GAE	BWI	AF019070

Acanthamoeba strains in bold face – T4 genotype; * - Not used in the analysis since generated large gaps in the alignment

1375bp) consisting of single variable region (Schroeder *et al* 2001). In order to evaluate the ability of these three amplimers in giving phylogenetically informative data, master alignment was made with same set of reference sequences.

In addition, another 197 bp amplimer (1634-1831 bp) of 18S rRNA gene, which we designated as DF4, was also evaluated (Figure 6.2). All the nucleotide positions for all the amplimers were in reference to *Acanthamoeba castellanii* Neff strain, GenBank Accession No. U07416. Therefore, altogether seven regions (ASA.S1, GP-1, ACARNA, DF1-DF4) of 18S rDNA were analyzed and compared to the complete sequencing of the same gene (Figure 6.2).

6.2.8 Phylogenetic analysis

Confidence in genetic affiliation inferred by different sequence data sets was tested by bootstrap analysis. For this purpose the sequence data was resampled 100 times using SEQBOOT, and each of the resampled data set was then used to calculate genetic distance estimates using DNADIST. The multiple distance matrices were then used to reconstruct the distances tree/topologies showing genetic relationship between the isolates and reference microorganisms using UPGMA (Unweighted pair group with arithmetic mean sequential clustering program). Majority-rule (50%) consensus trees were constructed for the topologies using CONSENSE. All these analyses were done using the PHYLIP package version 3.6 (Felsenstein 1985).

6.2.9 Calculation of the percent dissimilarity values

Apart from constructing trees for each of the regions, dissimilarity values were calculated as a percentage of mismatched bases in pairwise comparison of sequences

without the removal of unique gaps or ambiguous positions. Distances were calculated by DNADIST program of the PHYLIP package version 3.6 using Kimura 2 parameter model. Dissimilarity values were calculated with sequences obtained from seven 18S rDNA domains and complete 18S rRNA gene. These values were calculated to determine the taxonomic status of Indian isolates and to compare the diagnostic values of each of these amplimers of 18S rDNA gene with complete sequence of the gene. Such a comparison was possible only for seven Indian isolates for which sequence data for complete gene and seven domains were available. Therefore the master alignment for this analysis included seven Indian isolates (Table 6.1) and a set of 18 *Acanthamoeba* reference strains (Table 6.3)

6.3 RESULTS

6.3.1 Morphological classification

Seven among the 17 amoebae isolates tested belonged to group I while 6 of them belonged to Group II and 4 isolates belonged to group III (Table 6.1).

6.3.2 Genotyping using 18S rRNA gene sequences

6.3.2.1 Complete 18S rDNA

Complete 18S rRNA gene sequences were obtained for seven *Acanthamoeba* isolates. The size of the gene ranged from 2193 - 2250 bp. The sequences of the seven Indian isolates when aligned with 18 reference sequences yielded 3199 bp long aligned sequence set and this was without the removal of ambiguous bases, gaps and intronic region. Manual viewing of the sequence data set revealed large gaps in the alignment, which were mainly attributed to the genotype T7-T9. Hence sequences specific to

these genotype were removed and the alignment now yielded 2961 bases. In continuation, removal of 662 bp intronic region specific to T3 and T5 reference genotype reduced length of the aligned sequences to 2299 bases.

The phenogram revealed that all Indian isolates grouped together with reference sequences belonging toT4 genotype (Figure 6.3). None of the Indian isolates grouped with T3 and T11 genotype although they have been documented in literature to be closest genotypes to T4. The genetic identities were supported by high bootstrap values.

Dissimilarity values were calculated using master alignment of 2299 bp. Minimum average sequence dissimilarity between the genotypes was 3.73%, which was between genotype T3 and T11 (Table 6.4). Hence, any *Acanthamoeba* isolate with Sequence dissimilarity value more than 3.73% was grouped in another genotype.

Table 6.4: Average dissimilarity values in pairwise comparison of complete 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4-ref	Cs-Ind
T1										
T2	9.54									
T3	7.42	8.03								
T5	37.12	**37.87**	36.44							
T6	10.42	4.24	8.93	38.40						
T10	10.56	11.51	10.91	38.26	11.62					
T11	7.60	8.18	**3.73**	36.13	9.10	10.84				
T12	11.79	12.55	12.77	39.64	12.32	7.47	12.05			
T4-ref	6.60	8.5[illegible]	5.27	0.36	9.72	10.89	4.85	12.96	2.14	
Cs-Ind	6.68	8.56	5.31	36.47	9.62	10.84	4.89	12.98	2.12	2.08

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

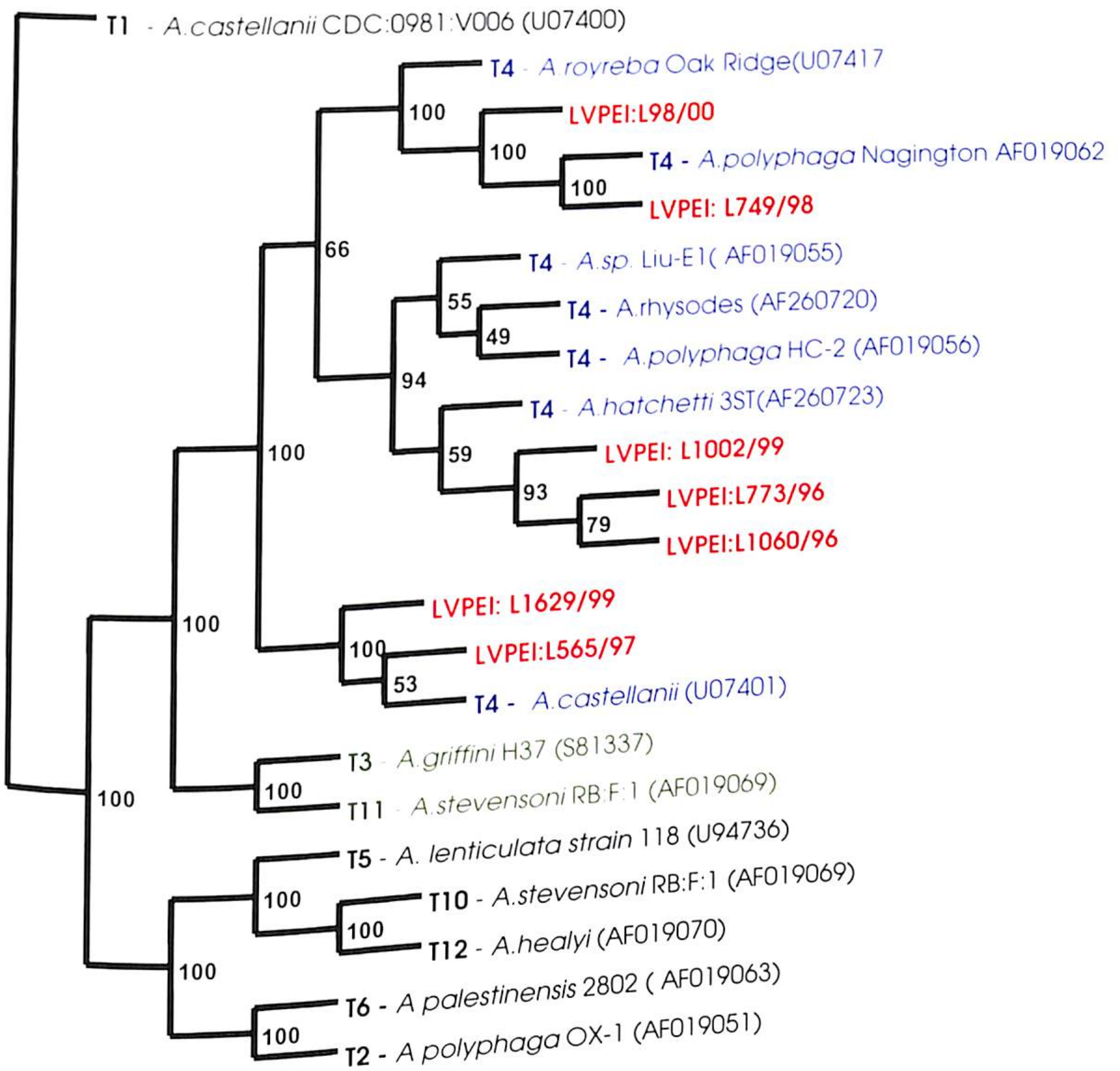


Figure 6.3: UPGMA tree based on complete sequences of 18S rRNA gene for 7 Acanthamoeba strains isolated from non-contact lens associated keratitis

Note that all the isolates from India (entries labels with prefix LVPEI) are included in the T4 genotype. The 7 Acanthamoeba isolates included in the study are represented in red color. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis.

dissimilarity between Indian isolates and reference sequences belonging to T4 genotype ranged from 0.01 – 2.9% with an average of 2.12%. This value was less than 3.73% hence all Indian isolates belonged to the T4 genotype. Average sequence dissimilarity between T3 and T4 was 5.27%, while between T4 and T11 was 4.85% (Table 6.4).

6.3.2.2 ASA.S1 amplimer

This domain of 18S rRNA gene was amplified for all 23 amoebae isolates mentioned in Table 6.1. Approximately 460 bp were amplified from each of the isolates (Figure 6.4). Primer sequences were omitted for analysis. GenBank Accession numbers of 22 of the 23 *Acanthamoeba* isolates analyzed in the study are AF534135-AF534156. Number of alignable bases for ASA.S1 amplimer was 431bp. The UPGMA tree constructed based on these sequences revealed that 13 Indian isolates along with those from Pakistan, Argentina, 1 environmental isolate and the standard *Acanthamoeba* stains grouped together with T4 genotype reference sequences (Figure 6.5). Here also the grouping was supported by high bootstrap values.

The minimum dissimilarity value between the genotypes was 4.55%, which was between genotype T10 and T12. Percent dissimilarity ranged from 0-3.24% with an average of 2.07%, between Indian isolates and T4 genotype. The average sequence dissimilarity between T3 and T4 was 5.91% and between T4 and T11 was 5.50% (Table 6.5).

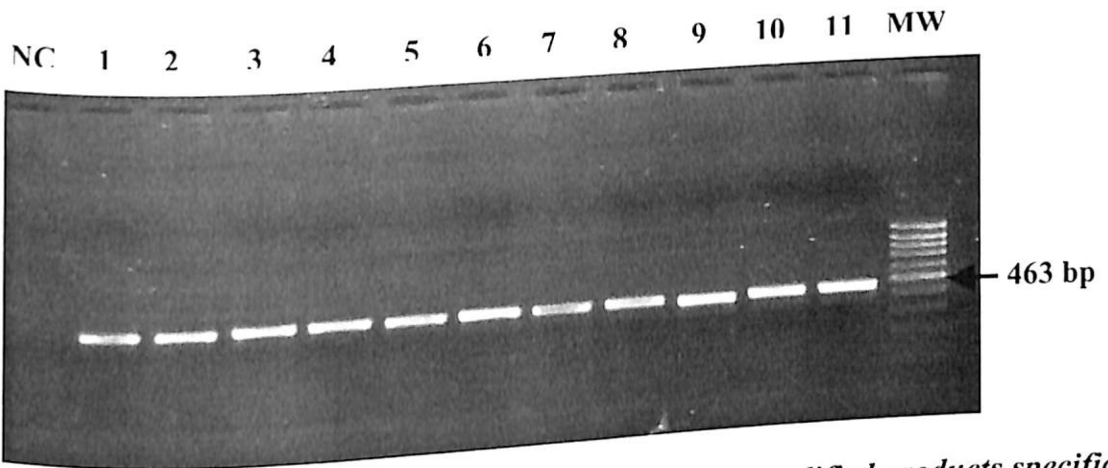


Figure 6.4: Representative 1.5% agarose gel showing the amplified products specific to ASA.S1 domain of 18S rRNA gene obtained from Acanthamoeba strains isolated from non-contact lens associated keratitis patients.

Lanes: NC- Negative control; 1-11-DNA from Indian *Acanthamoeba* isolates; MW – Molecular weight marker (100 bp ladder)

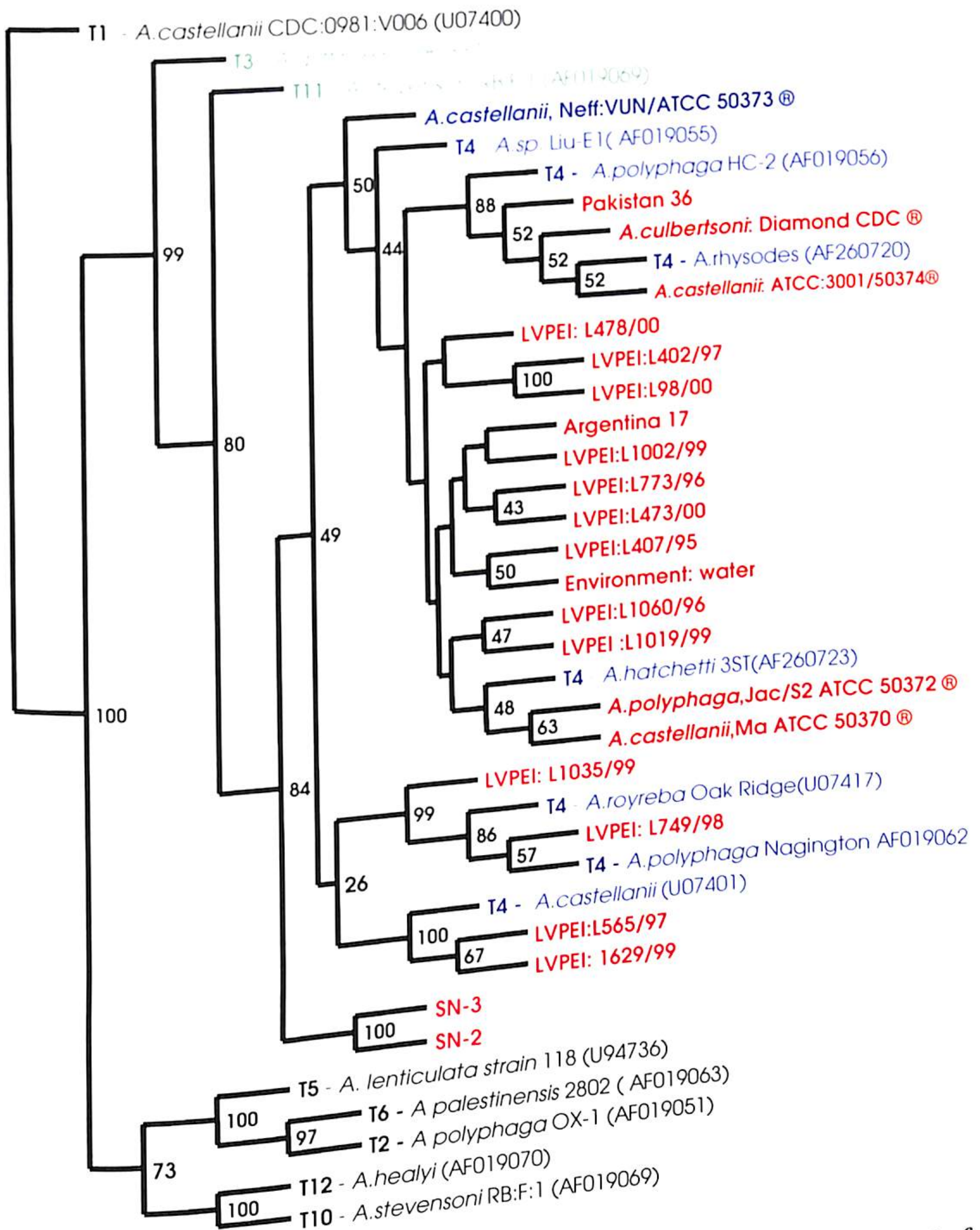


Figure 6.5: UPGMA tree based on partial sequences of ASA.S1 fragment of 18S rRNA gene for 23 Acanthamoeba strains isolated from non-contact lens associated keratitis

Note that all the isolates from India (entries labels with prefix LVPEI and SN) are included in the T4 genotype. The 23 Acanthamoeba isolates included in the study are represented in red color. The entries marked with ® are the reference Acanthamoeba spp. sequenced in the present study. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis

Table 6.5: Average dissimilarity values in pairwise comparison of partial sequences of ASA.S1 amplimer pecific to 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4	Cs-Ind
T1										
T2	17.02									
T3	9.96	16.76								
T5	13.22	8.44	12.19							
T6	16.45	4.74	15.94	7.87						
T10	10.99	15.51	12.56	11.82	14.73					
T11	9.34	**17.63**	7.99	12.20	16.75	11.63				
T12	10.79	16.94	11.24	12.31	16.37	**4.55**	11.78			
T4 ref	9.36	17.04	5.91	13.16	16.26	12.00	5.50	11.95	2.48	
Cs-Ind	9.53	17.33	6.32	13.27	16.28	12.18	5.16	11.87	2.07	2.18

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

6.3.2.3 GP-P1 amplimer

Approximately 253 bp were amplified from each of the 23 *Acanthamoeba* isolates (Figure 6.6). After omitting the primer sequences number of alignable bases for this amplimer was 242. The tree topology obtained from this sequence alignment was identical to the one obtained by ASA.S1 amplimer, although it was not supported by very high bootstrap values (Figure 6.7). Minimum sequence dissimilarity between genotypes was 1.32% that was between T3 and T11 genotype, hence the cut off value, to group any isolate in a genotype was 1.32%. Percent dissimilarity between Indian isolates and reference T4 genotype strains ranged from 0-6.04% with an average of 3.73%. Dissimilarity value between T3 and T4 was 5%, while between T11 and T4 was 5.05% (Table 6.6)

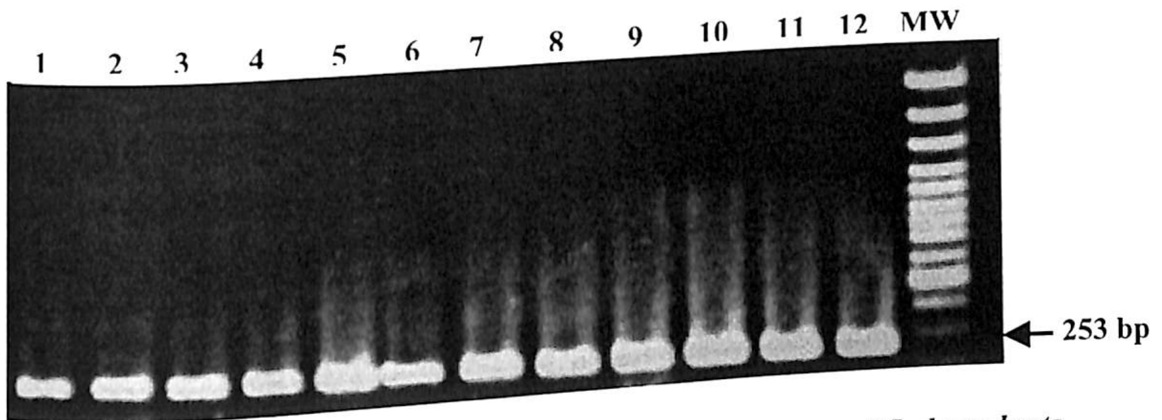


Figure 6.6: Representative 1.5% agarose gel showing the amplified products specific to GP-P1 domain of 18S rRNA gene obtained from Acanthamoeba strains isolated from non-contact lens associated keratitis patients.

Lanes:; 1-12-DNA from Indian *Acanthamoeba* isolates;
MW – Molecular weight marker (100 bp ladder)

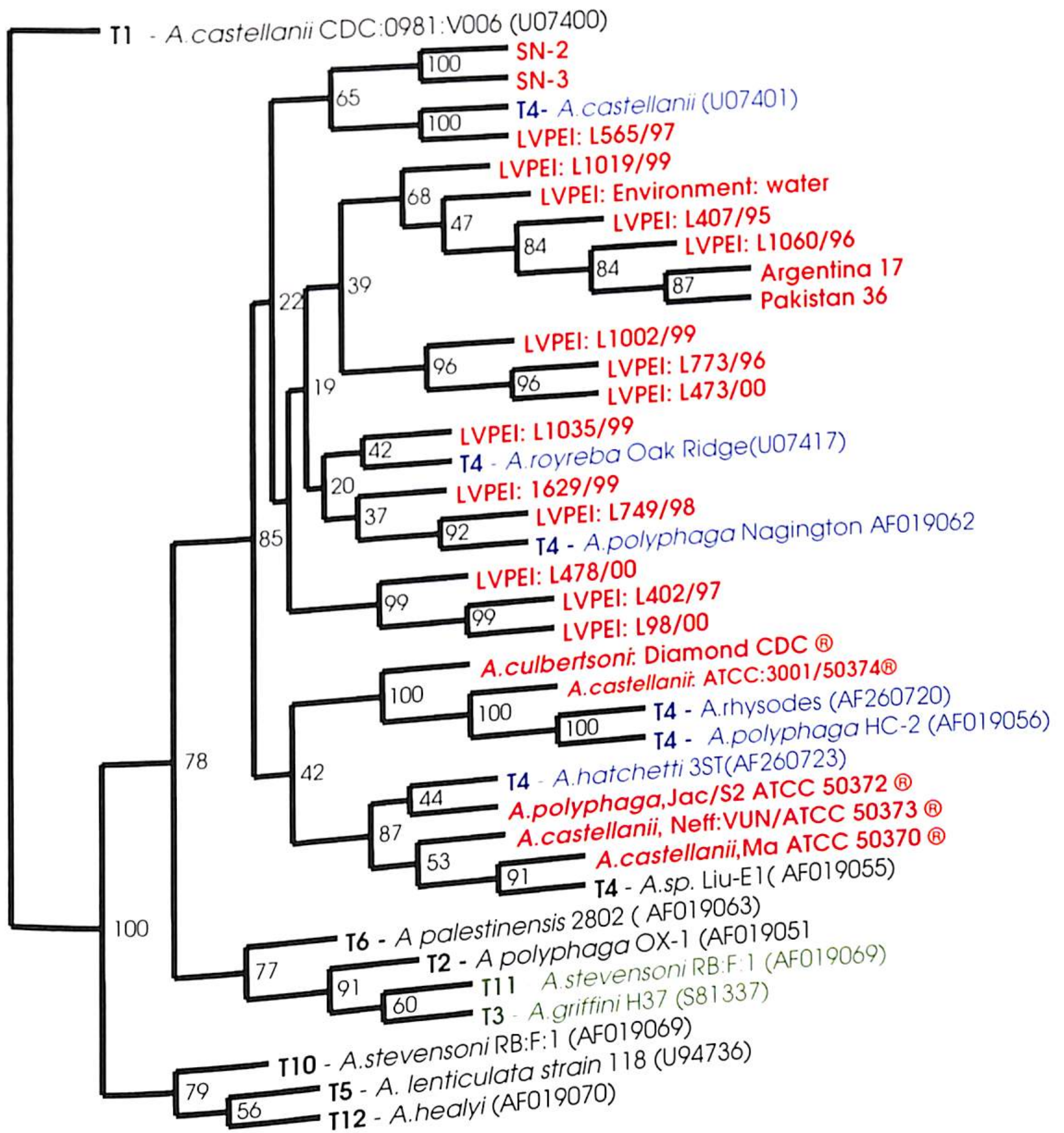


Figure 6.7: UPGMA phylogenetic tree based on partial sequences of GP-P1 fragment of 18S rRNA gene for 23 Acanthamoeba strains isolated from non-contact lens associated keratitis.

Note that all the isolates from India (entries labels with prefix LVPEI and SN) are included in the T4 genotype. The 23 Acanthamoeba isolates included in the study are represented in red color. The entries marked with ® are the reference Acanthamoeba spp. sequenced in the present study. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis

Table 6.6: Average dissimilarity values in pairwise comparison of partial sequences of GP –P1 specific to 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4-ref	CS-IN
T1										
T2	[illegible]									
T3	[illegible]	[illegible]								
T5	[illegible]	[illegible]	9.61							
T6	[illegible]	[illegible]	6.02	12.39						
T10	[illegible]	9.03	8.96	**17.16**	11.09					
T11	[illegible]	[illegible]	**1.32**	9.99	6.46	8.96				
T12	[illegible]	9.94	10.30	16.77	12.59	15.34	10.76			
T4-ref	8.58	7.36	5.00	11.52	8.21	10.86	5.65	15.45	3.40	
CS-IN	8.70	7.47	5.05	11.73	8.48	12.10	6.29	16.01	3.72	3.83

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

6.3.2.4 ACARNA amplimer

Approximately 272 bp were amplified from each of the 23 *Acanthamoeba* isolates (Figure 6.8). Numbers of alignable sequences were 237 bases after removal of primer sequences. Master alignment of the sequences revealed no sequence variation between the genotypes and Indian isolates except for *A.castellanii* (GenBank Accession No. U07401; T4 genotype), which showed variation from Thymine to Adenine at 86th position of the alignment. In addition to this we also included *Balamuthia mandrillaris* (Accession No AF477022), *Hartmannella vermiformis* (Accession No. AF 426157) and *Comanodonia operculata* (Accessions No AY033896) in the master alignment and found that only *Hartmannella* showed variation in 12 bases (95% homology with *Acanthamoeba* standard strains), while *Balamuthia* and *Comanodonia* showed 100% homology with *Acanthamoeba* isolates. Dissimilarity values were not determined for this amplimer, since it was derived from conserved domain of 18S rRNA gene.

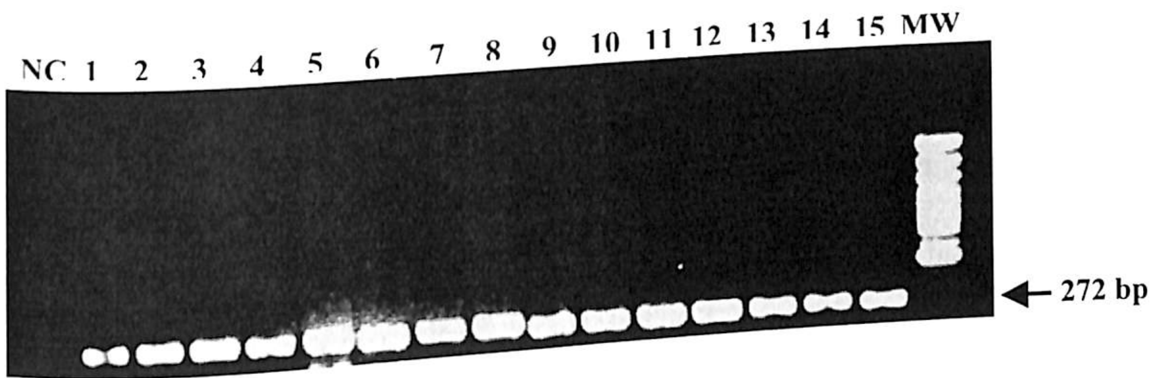


Figure 6.8: Representative 1.5% agarose gel showing the amplified products specific to ACARNA domain of 18S rRNA gene obtained from Acanthamoeba strains isolated from non-contact lens associated keratitis patients.

Lanes:; NC; Negative control 1-15-DNA from Indian *Acanthamoeba* isolates; MW – Molecular weight marker (100 bp ladder)

6.3.2.5 Diagnostic fragments: DF1, DF2, DF3 and DF4

Sequences for these amplimers were extracted from complete 18S rDNA sequences of the 7 Indian pathogenic isolates. Number of alignable nucleotides for DF1, DF2, DF3 and DF4 were 213bp, 288bp, 115bp and 219bp respectively. Tree constructed from the DF1 fragment revealed that Indian isolates grouped together with T4 genotype reference strains but it was not supported by high bootstrap values (Figure 6.9), while phenogram from DF2 sequences showed that Indian isolates grouped together with T4, T3 and T11 genotype reference sequences (Figure 6.10). Trees obtained from DF3 and DF4 sequences clearly indicated that all the Indian isolates formed a clade with T4 reference sequences, supported by high bootstrap values (Figure 6.11 and 6.12) and that T3, T4 and T11 genotype are closely related genotypes, similar to the observation obtained from the complete sequence of 18S rRNA gene and ASA.S1 amplimer, but Two way table made to determine the dissimilarity value between the

Table 6.7: Average dissimilarity values in pairwise comparison of partial sequences of DF1 fragment specific to 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4-ref	CS-IN
T1										
T2	24.87									
T3	12.94	20.34								
T5	30.57	25.06	25.41							
T6	23.17	7.65	20.64	27.69						
T10	16.85	27.38	21.89	**36.86**	27.99					
T11	16.05	17.86	**7.16**	26.54	19.69	25.48				
T12	14.31	34.91	22.98	41.81	31.18	19.77	23.20			
T4-ref	19.25	13.95	16.12	25.51	16.67	26.87	12.84	29.86	7.21	
CS-IN	18.96	14.04	15.20	26.29	15.68	26.52	13.09	29.07	6.36	6.13

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

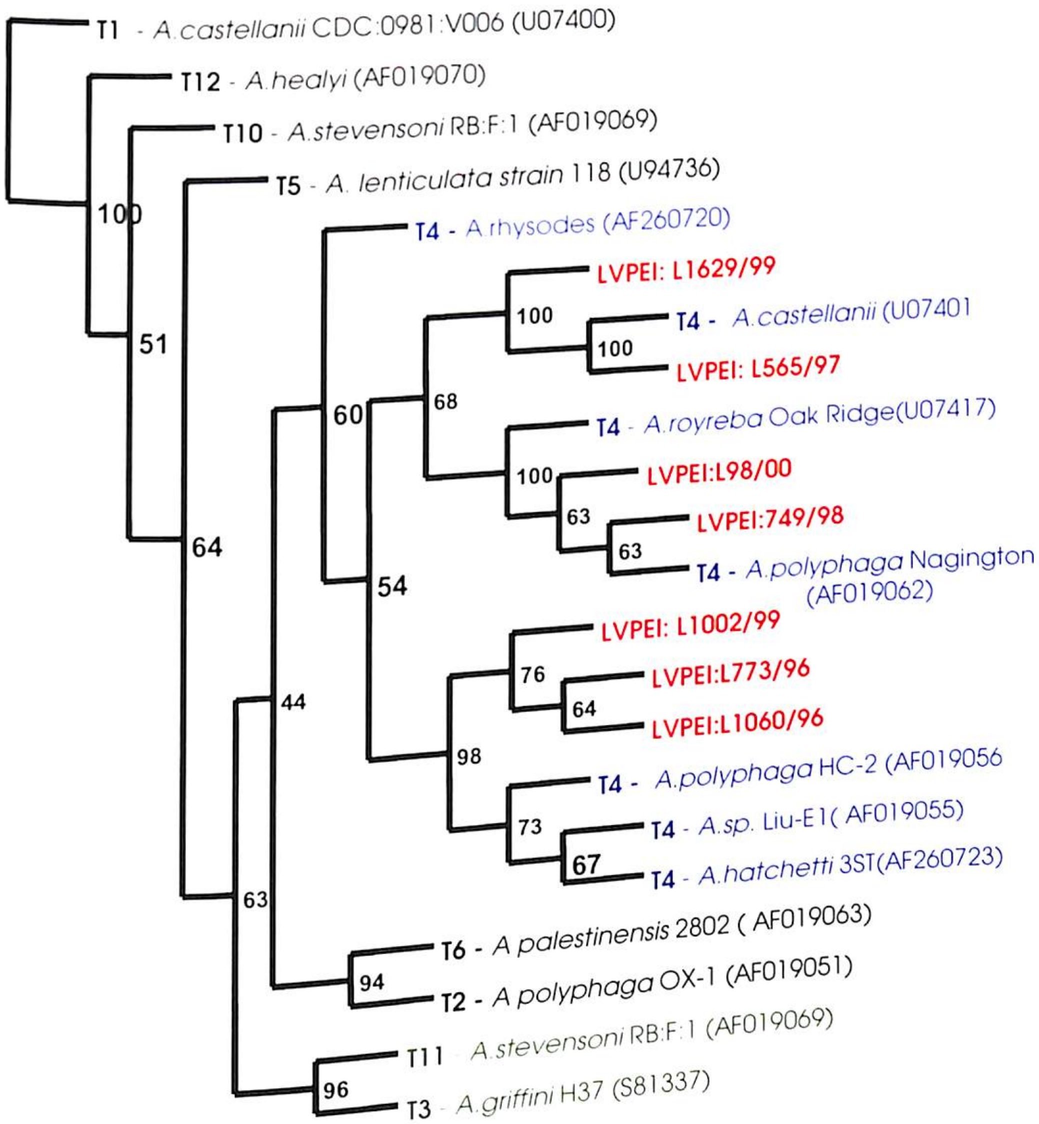


Figure 6.9: UPGMA tree based on partial sequences of the DF1 fragment of 18S rRNA gene for 7 Acanthamoeba strains isolated from non-contact lens associated keratitis

Note that all the isolates from India (entries labels with prefix LVPEI) are included in the T4 genotype. The 7 Acanthamoeba isolates included in the study are represented in red color. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis

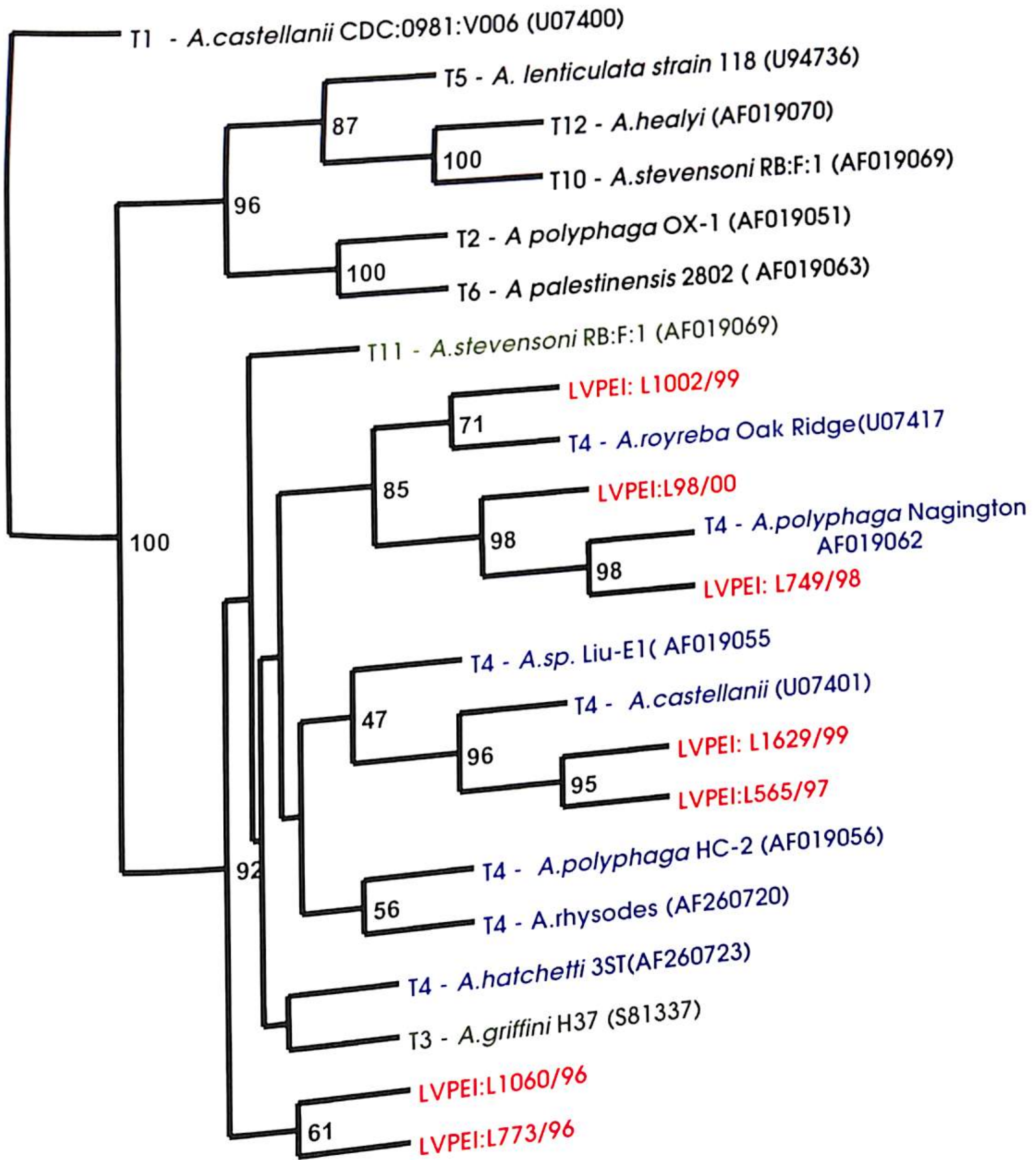


Figure 6.10: UPGMA tree based on partial sequences of the DF2 fragment of 18S rRNA gene for 7 Acanthamoeba strains isolated from non-contact lens associated keratitis

Note that all the isolates from India (entries labels with prefix LVPEI) are included in the T4 genotype. The 7 Acanthamoeba isolates included in the study are represented in red color. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis

T1 - *A.castellanii* CDC:0981:V006 (U07400)
T3 - *A.griffini* H37 (S81337)
T11 - *A.stevensoni* RB:F:1 (AF019069)
T4 - *A.royreba* Oak Ridge(U07417
LVPEI: L749/98
T4 - *A.polyphaga* Nagington AF019062
LVPEI:L98/00
LVPEI:L1060/96
LVPEI:L773/96
LVPEI: L1002/99
T4 - *A.hatchetti* 3ST(AF260723)
T4 - *A.sp.* Liu-E1(AF019055)
T4 - *A.polyphaga* HC-2 (AF019056)
T4 - A.rhysodes (AF260720)
T4 - *A.castellanii* (U07401)
LVPEI: L1629/99
LVPEI:L565/97
T5 - *A. lenticulata strain* 118 (U94736)
T6 - *A palestinensis* 2802 (AF019063)
T2 - *A polyphaga* OX-1 (AF019051)
T10 - *A.stevensoni* RB:F:1 (AF019069)
T12 - *A.healyi* (AF019070)
88
77
100
60
84
82
47
46
100
70
46
73
100
63
100
76
92
99

Figure 6.11: UPGMA tree based on partial sequences of the DF3 fragment of 18S rRNA gene for 7 Acanthamoeba strains isolated from non-contact lens associated keratitis

Note that all the isolates from India (entries labels with prefix LVPEI) are included in the T4 genotype. The 7 Acanthamoeba isolates included in the study are represented in red color. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis

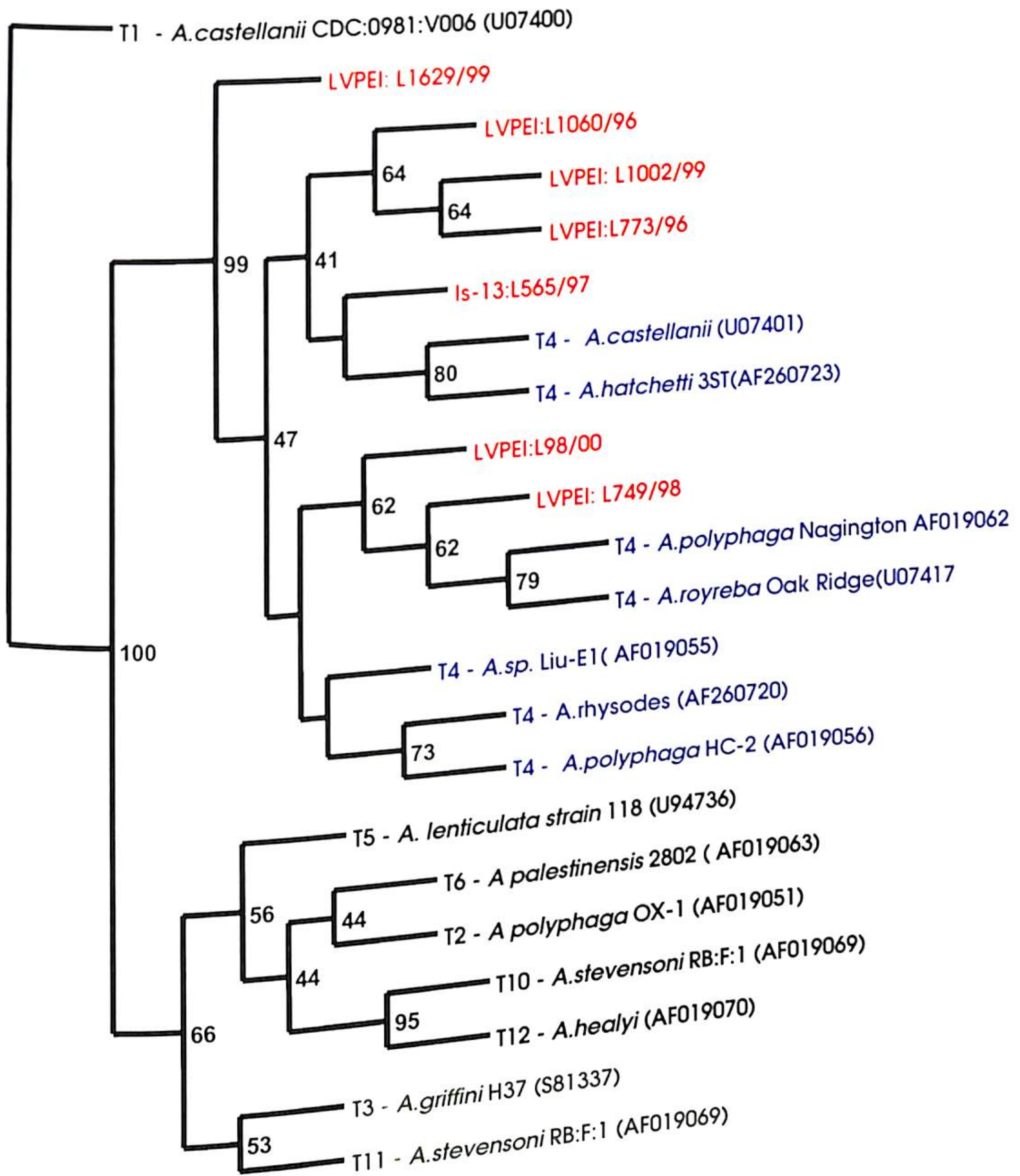


Figure 6.12: UPGMA tree based on partial sequences of the DF4 fragment of 18S rRNA gene for 7 Acanthamoeba strains isolated from non-contact lens associated keratitis

Note that all the isolates from India (entries labels with prefix LVPEI) are included in the T4 genotype. The 7 Acanthamoeba isolates included in the study are represented in red color. The numbers at the node are the bootstap values. The accession numbers of the reference sequences obtained from EMBL data base are given in the parenthesis

Table 6.8: Average dissimilarity values in pairwise comparison of partial sequences of DF2 fragment specific to 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4-ref	CS-IN
T1										
T2	[illegible]									
T3	[illegible]	[illegible]								
T5	[illegible]	34.92	28.79							
T6	[illegible]	**5.20**	21.96	37.29						
T10	[illegible]	35.86	35.78	**53.41**	34.97					
T11	16.38	21.30	9.03	24.02	22.16	34.97				
T12	47.14	40.93	46.85	47.04	42.11	27.36	42.78			
T4-ref	[illegible]	22.29	8.08	26.64	23.73	40.47	8.63	50.67	6.02	
CS-IN	15.48	22.35	9.52	27.31	23.80	39.96	9.22	50.30	6.39	7.01

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

Table 6.9: Average dissimilarity values in pairwise comparison of partial sequences of DF3 fragment specific to 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4-ref	CS-IN
T1										
T2	**133.37**									
T3	46.94	113.87								
T5	89.26	20.07	71.54							
T6	116.14	**12.92**	91.77	16.77						
T10	77.24	100.44	54.42	64.24	89.76					
T11	48.96	112.97	41.86	69.56	103.04	53.98				
T12	68.67	111.39	46.08	60.21	101.37	19.59	45.44			
T4-ref	35.72	110..24	26.00	73.88	91.58	51.68	22.51	55.27	9.88	
CS-IN	39.41	102.67	28.30	72.65	89.25	53.26	22.51	56.53	9.02	8.98

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

Table 6.10: Average dissimilarity values in pairwise comparison of partial sequences of DF4 fragment specific to 18S rDNA sequences from 7 Acanthamoeba isolates

	T1	T2	T3	T5	T6	T10	T11	T12	T4-ref	CS-IN
T1										
T2	[illegible]									
T3	[illegible]	[illegible]								
T5	[illegible]	[illegible]	[illegible]							
T6	[illegible]	12.86	18.29	28.90						
T10	[illegible]	26.74	19.85	25.31	26.51					
T11	[illegible]	[illegible]	**6.41**	14.58	20.27	22.71				
T12	[illegible]	[illegible]	[illegible]	**29.08**	23.82	13.96	20.17			
T4-ref	[illegible]	[illegible]	[illegible]	14.70	23.45	20.38	7.26	24.60	0.71	
CS-IN	[illegible]	[illegible]	11.42	15.25	22.94	20.14	6.81	24.36	1.03	1.08

The values in bold face represent the maximum and minimum average dissimilarity values between the genotypes

genotype revealed that minimum sequence dissimilarity between the genotypes was 7.16% (between T3 and T11) for DF1, 5.20% (between T2 and T6) for DF2, 12.92% (between T2 and T6) for DF3 and 6.4% (between T3 and T11) for DF4. Average dissimilarity value between Indian isolates and T4 genotype reference sequence was 6.36% (range 0-15.3%) for DF1, 6.39% (range 0-13.6%) for DF2, 9.02% (range 0-16.6%) for DF3 and 1.03% (range 0-2.1%) for DF4 amplimer (Table 6.7 – 6.10).

6.4 DISCUSSION

Over a period of nine years we saw 191 cases of AK and among which only one patient was a contact lens wearer. The fact that 99.4% of our patients were non-contact lens wearers induced us to think if the genetic lineage of these *Acanthamoeba* isolate was different from the one reported from Western countries, which were mainly isolated from patients who wore contact lenses. In an attempt to do so, we in

this study first demonstrated using molecular approach that amoebae, which were isolating were indeed *Acanthamoeba* and subsequently determined the genetic structure and taxonomic affiliations of the *Acanthamoeba* isolates isolated from India using both conventional morphological features of cysts and more recent molecular typing methods. We also evaluated and compared the various domains of 18S rRNA gene for their ability to genotype *Acanthamoeba* isolates rapidly and in an inexpensive way.

Morphological classification

Classification based on morphology of the cyst and trophozoite was not easy, since the morphological characteristics of the amoebae varied in the same clonal population. We found that size of the cyst and trophozoite varied considerably. The reason for this could have been the fact that classification was performed with cysts, which were grown axenically in PYG medium wherein the encystation is not complete (Dr G S Visvesvara: personal communication). Sawyer (1971) observed that the ionic strength of the growth medium could alter the shape of the cyst walls, thus substantially reducing the reliability of cysts morphology as a taxonomic characteristic. Morphometric analysis revealed that all the three morphological groups described in the literature were represented in our isolates. Keratitis causing *Acanthamoeba* have been reported to belong to Group II and III morphological groups (Gast *et al* 1996;Stothard *et al* 1999). Unlike the above, 41.7% of our isolates belonged to Group I while rest 58.3% belonged to Group II and III. While 18S rDNA based genotyping of these 17 isolates revealed that all the isolates belonged to the T4 genotype. Thus our study supports the viewpoint that classification based on the morphology of cysts and trophozoites is not reliable. This morphology based

classification method being ambiguous and subjective over the years have largely been replaced by much more reliable and consistent molecular typing methods, especially those relying on the DNA sequencing of rRNA gene (Gast *et al* 1996;Stothard *et al* 1999;Pilar *et al* 2001). Although morphology based method lacks the ability to resolve taxonomic relationship between *Acanthamoeba* isolates, it's utility to initially identify the organism in the clinical samples cannot be denied. Results of morphological classification stimulated us to pursue, typing our isolates using molecular methods.

Genotyping using 18S rDNA sequences

Parallel advances in the DNA genomics and molecular biology technology tools have made DNA sequencing as the method of choice around the world for genotyping and identification of microbial isolates (Seal 2003). The sequence of rRNA gene is conserved in nature and has number of variable sites within it, this makes it a very useful molecular data for phylogeny and taxonomy (Woese 1987; Gast *et al* 1996; Stothard *et al* 1999). These highly conserved sequences, which have strong functional constraints change slowly during evolution and thus their comparison reveal phylogenetic relationship between organisms (Sogin *et al* 1989). At present sequencing of complete 18S rRNA gene is considered as most reliable method of genotyping (Seal 2003). Therefore we chose 18S rDNA to infer genetic affiliations of the Indian isolates and also to explore the utility of a shorter rDNA amplimer or fragments to genotype *Acanthamoeba* directly from clinical samples rather than using the complete gene.

Complete sequencing of 18S rRNA gene

Complete sequencing of 18S rDNA revealed genetic identity of 7 amoebae isolates. All the isolates were designated as *Acanthamoeba* since they showed ~ 95-99% homology to the reference *Acanthamoeba* strains obtained from the EMBL database. Further phylogenetic analysis of the isolates revealed that the isolates belonged to the T4 genotype, the most common genotype that is associated with keratitis (Gast *et al* 1996; Stothard *et al* 1999). Thus the query about the genetic affiliations of *Acanthamoeba* isolates from non-contact lens keratitis patients was put to rest, since isolates both from contact lens and non-contact lens associated keratitis patients grouped together in the same T4 genotype. Dissimilarity values were calculated to emphasize the amount of sequence variability between the isolates. Analysis of complete sequences showed that minimum sequence dissimilarity between the genotypes was 3.73% (between T3 and T11). We took this value as a cut off value to assign genotype to Indian isolates. Comparison of percent dissimilarity value between Indian isolates and 12 genotype of *Acanthamoeba* revealed that, the Indian isolates showed minimum sequence dissimilarity with the reference sequence belonging to the T4 genotype. The average percent sequence dissimilarity between Indian isolates and T4 genotype sequences was 2.12% (range 0.01 – 2.9%), which was below the cut off value of 3.73%. None of our isolates belonged to the genotype T3 (Stothard *et al* 1999), T6 (Walochnik *et al* 2000) and T11 (Khan *et al* 2002), which have been reported in literature to cause keratitis.

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Our study revealed that there exists a high degree of genetic diversity among the *Acanthamoeba* isolates (Figure 6.3) and all these carry the 'T4 signatures'. Within

themselves, the Indian isolates showed considerable variation suggesting multiple *Acanthamoeba* species. This was emphasized by the fact that representatives of various species of *Acanthamoeba* (T4 reference strains) clustered in T4 genotype. All the three groups (based on morphology) representing various species grouped together in T4 genotype, suggesting that T4 genotype consists of various species and also that the morphological classification cannot be regarded as a tool for speciating *Acanthamoeba*. These results are in conformity with the original work of Gast *et al* (1996), which describes the rDNA sequence types and shows that the 'T4 type sequence' characterizes a heterogeneous group of pathogenic isolates of *Acanthamoeba* comprising of many different species.

Genotyping based on three different sub-domains of 18S rRNA gene

At the moment, sequencing of nearly complete 18S rRNA gene is considered as an ideal method to determine the genetic relatedness between *Acanthamoeba* isolates (Seal 2003). Hence there is still a need to develop a molecular method that is as reliable and sensitive as the previous method but relatively simple and economical. In search of the above we selected out three primer sets belonging to 18S rRNA gene i.e. ASA.S1 (Schroeder *et al* 2001), GP-P1 (Lehman *et al* 1998) and ACARNA (Vodkin *et al* 1992) and using these primer pairs, amplified the DNA from the Indian isolates and subsequently sequenced them. The phylogenetic tree obtained from GP-P1 and ASA.S1 were topographically similar, wherein it showed that Indian isolates belonged to T4 genotype.

The master alignment prepared from ACARNA sequences revealed that there were no variable regions within this amplimer and hence was not fit to be used for

phylogenetic analysis. Since this region was conserved within the genus *Acanthamoeba*, we wanted to investigate if this marker was genus specific for *Acanthamoeba* as claimed by the authors (Vodkin *et al* 1992). We found that it was not so, since the sequences obtained from amoebae related to *Acanthamoeba* (*Balamuthia*, *Hartmannella* and *Comanodonia*) showed 95-100% homology with the sequences obtained from *Acanthamoeba* isolates. Weekers *et al* (1994) evaluated the specificity of the above primer and also concluded that this primer was not specific to the genus *Acanthamoeba* but would also amplify *Balamuthia* and *Hartmannella* as well.

Sequence analysis of ASA.S1 amplimer further confirmed that Indian isolates belonged to the T4 genotype. This amplimer has been previously evaluated by Schroeder *et al* (2001) who did not find this amplimer sufficient enough to differentiate between the closely related T3-T4-T11 clade. *Acanthamoeba* isolates belonging to each of the three genotypes are known to cause keratitis (Stothard *et al* 1999; Walochnik *et al* 2000; Khan *et al* 2002). In contrast to Schreoder *et al* (2001), we found that this amplimer could distinguish between all the genotypes and most importantly could distinguish between the T3, T4 and T11 genotype. Thus being in an Eye Institute, it was important for us to use a marker, which could distinguish between the genotypes that are known to cause keratitis, and ASA.S1 had the ability.

Sequence analysis of the GP-amplimer revealed that it did not have the ability to genotype the Indian isolates although the phylogenetic tree revealed that the Indian isolates grouped together with T4 genotype reference sequences. The percent

sequence dissimilarity values for the Indian isolates and the closely related T3 and T4
was above the cut off value of 1.32%.

Diagnostic fragments belonging to 18S rRNA gene

Phylogenetic trees obtained from the three diagnostic fragments DF1; DF3; DF4
revealed that all the Indian isolates belonged to the T4 genotype. While the tree
generated from DF2 fragment could not distinguish between the T3-T4-T11 clade and
also revealed that the Indian isolates grouped together with all these three genotypes.
When the sequence dissimilarity for these fragments were calculated and compared
with the T4 genotype reference sequences, it revealed that among the four diagnostic
fragments, DF4 was the best domain to determine the phylogenetic inferences,
followed by DF3, DF1 and DF2 (Table 6.11). Percent dissimilarity values for all the
seven Indian isolates (obtained from DF4) when compared to T4 genotype sequences
were below the cut-off value of 6.41%. While for DF3 and DF1 sequences, the values
for all Indian isolates were above the cut-off value of 12.92% and 7.16% respectively,
however their average percent dissimilarity value was below the cut-off value. For
DF2 domain sequences the average percent dissimilarity for the Indian isolates when
compared to the T4 genotype sequences was 6.39% which was above the cut-value of
5.20%, suggesting that this domain is not fit to genotype *Acanthamoeba* isolates.

Comparison between DF1 and DF3 sequences revealed that, the tree obtained from
DF3 was more robust and was supported by higher bootstrap values than the one
obtained from DF1 sequences. DF3 fragment is part of ASA.S1 amplimer of 18S
rRNA gene and has been used previously by few researchers to genotype
Acanthamoeba isolates (Schroeder *et al* 2001; Booton *et al* 2002; De Jonckheere *et al*

Table 6.11 Percent dissimilarity values between genotypes for six domains and complete sequence of 18S rRNA gene of Acanthamoeba

Percent dissimilarity	DF1 213 bp	DF2 288 bp	DF3 115 bp	DF4 219 bp	GP-frag 242 bp	ASA.S1 431 bp	Complete 2299 bp
Average : Indian isolates	**6.13**	**7.01**	**8.98**	**1.08**	**3.8**	**2.18**	**2.08**
Range	0 - 9.8	0 - 13.6	0 - 16.6	0 - 2.1	0 - 6.4	0 - 3.2	0.01 - 2.9
Average: T4 reference strains	**7.2**	**6.02**	**9.88**	**0.71**	**3.4**	**2.48**	**2.14**
Range	0 -15.2	0 -13.67	0 - 17.94	0 - 2.08	0 -6.04	0 - 3.49	0 - 3.2
Average :T4 and indian isolates	**6.36**	**6.39**	**9.02**	**1.03**	**3.7**	**2.07**	**2.12**
Range	0-15.3	0-13.6	0-17.9	0-2.1	0-6.0	0-3.7	0-3.4
Minimum: sequence types	**7.16**	**5.2**	**12.9**	**6.09**	**1.3**	**4.55**	**3.73**
Range	7.16 - 41. 8	5.2 - 53.4	12.9 - 133.3	6.09 - 29. 8	1.3 - 17.2	4.6 - 17.6	3.73 - 39.6

2003). Schroeder *et al* (2001) used DF3 to genotype 71 *Acanthamoeba* strains which included 53 *Acanthamoeba* strains belonging to the 12 genotypes described by Stothard *et al* (1998), 12 South African isolates and 6 scrape specimens from Scotland. They found that using this domain, T4 genotype could not be distinguished from the closely related T3 and T11 genotypes. Also the phylogentic tree obtained from the sequences was not as robust as that of the complete sequence of 18S rDNA. Booton *et al* (2002) also used this fragment to genotype 13 corneal scrapes obtained from keratitis patients from Hong Kong. They were successful in genotyping the isolates and specified its usefulness particularly when axenisation of the culture was not possible. DF3 amplimer was also successfully used by De Jonckheere *et al* (2003) to genotype the *Acanthamoeba* strains isolated from keratitis patients from Belgium, their contact lens, contact lens boxes and saline

Table 6.12: Percent average dissimilarity between genotypes using partial (ASA.S1) and complete sequences of 18S rRNA gene

	T1	T2	T3	T5	T6	T10	T11	T12	T4	Indian isolates
T2	**17.02*** 9.12#									
T3	**9.96** 6.96	**16.76** 7.54								
T5	**13.22** 36.05	**8.44** 35.66	**12.19** 53.33							
T6	**16.45** 10.17	**4.74** 4.30	**15.94** 8.44	**7.87** 35.74						
T10	**10.99** 11.32	**15.51** 12.24	**12.56** 11.30	**11.82** 38.78	**14.73** 12.27					
T11	**9.34** 7.30	**17.63** 7.85	**7.99** 3.73	**12.20** 34.55	**16.75** 8.65	**11.63** 10.80				
T12	**10.79** 12.15	**16.94** 13.00	**11.24** 12.71	**12.31** 40.46	**16.37** 13.09	**4.55** 7.85	**11.78** 12.23			
T4	**9.36** 6.52	**17.04** 8.00	**5.91** 4.81	**13.16** 35.57	**16.26** 9.31	**12.00** 11.70	**5.50** 4.51	**11.95** 13.45	**2.48** 2.20	
Indian isolates	**9.53** 6.52	**17.33** 8.04	**6.32** 4.73	**13.27** 35.52	**16.28** 9.21	**12.18** 11.71	**5.16** 4.45	**11.87** 13.49	**2.07** 3.59	**2.18** 2.17

* Average percentage dissimilarity between genotypes using ASA.S1 sequences of 18S rRNA gene.

\# Average percentage dissimilarity between genotypes using complete sequences of 18S rRNA gene. Shaded values denote the average sequence dissimilarities

Comparison of partial and complete sequencing

In our study among the seven sub-domains of 18S rRNA, which were evaluated, we found that two sub-domains ASA.S1 and DF4 were the best (Table 6.11). Partial sequencing of ASA.S1 and DF4 provided sufficient inter-strain sequence variation to distinguish between all the genotypes and the closely related T3-T4-T11 clade and also successfully genotyped the Indian isolates to the genotype T4. The trees obtained from these two domains were robust and mirrored the tree obtained from complete sequencing of the 18S rRNA gene. Though we found that DF4 sequences were sufficient to genotype, this analysis was done only *in silico* and hence there is a need to further explore its utility *in vitro*. Therefore we believe for now, ASA.S1 amplimer specific to 18S rRNA gene is "the" sub-domain for rapid and robust genotyping of *Acanthamoeba*. Table 6.12 shows the comparison of average percent dissimilarity obtained from ASA.S1 and complete sequences of 18S rRNA gene.

Thus, genotyping using 18S rRNA gene sequences revealed that all Indian isolates belong to the T4 genotype. The phylogenetic inferences obtained from sequencing of ASA.S1 amplimer and DF4 fragment are comparable to those obtained from of the entire 18S rRNA gene sequences. Thus complete sequencing of the gene, which is expensive, cumbersome and time consuming, can be avoided. We strongly believe that ASA.S1 amplimer of 18S rRNA gene is excellent marker both for detection of *Acanthamoeba* in the clinical samples as described earlier (Pasricha *et al* 2003) and for genotyping *Acanthamoeba* directly from clinical samples.

CHAPTER 7: DEVELOPMENT OF MOLECULAR DIAGNOSTIC MARKERS FOR THE DETECTION OF *ACANTHAMOEBA* IN CLINICAL SPECIMENS

7.1 INTRODUCTION

Treatment and therapy of *Acanthamoeba* keratitis is extensive and it is important to have rapid and definitive diagnosis prior to initiating a specific therapy. Clinical features do not offer much hope especially in non-contact lens wearers, where the risk factors do not offer any clue to the diagnosis. Chynn *et al* (1995) reported that the mean time to diagnose *Acanthamoeba* keratitis on an average is 2-5 weeks longer for non-contact lens wearers than for contact lens wearers and this lag time may hamper disease resolution.

Conventional microbiological investigations have been used for the detection of *Acanthamoeba* with variable results. Culture isolation and use of fluorescence microscopy are among the sensitive methods for detection of *Acanthamoeba* but require considerable experience and sophisticated infrastructure that are not available to most ophthalmologists. In comparison, direct detection methods based on routine light microscopy of smears and histological preparations, though widely available to clinicians, are not only less sensitive but are also unreliable. It has been estimated that 60-70% of *Acanthamoeba* keratitis cases are misdiagnosed using such methods (Stothard *et al* 1999). The situation thus calls for newer cost-effective, sensitive, simple and reliable diagnostic tools that can be easily integrated in a small to medium sized clinical set up, leading to more realistic estimates of the disease incidence as well as helping early diagnosis and treatment, for a better prognosis.

Several investigators have demonstrated the usefulness of molecular methods for detection and identification of *Acanthamoeba* (Schroeder *et al* 2001). These methods could be suitable for both clinical and epidemiological purposes; therefore they need to be reliable and sensitive. Polymerase chain reaction (PCR) on corneal scrapings, corneal epithelial biopsy and tear samples for diagnosis of AK has shown promising results (Lehmann *et al* 1998). The technique of fluorescent in situ hybridization (FISH) has also been successfully employed for the purpose (Stothard *et al* 1999).

The objectives of this study were to:

- To evaluate a uniplex PCR assay (based on 18S rRNA gene described by Schroeder *et al* 2001) in a clinical setting and compare the results with conventional microbiological methods for the diagnosis of *Acanthamoeba* keratitis.
- To develop multiplex PCR using primers specific for domains of 18S rRNA and 26S rRNA genes of *Acanthamoeba*.

7.2 18S rDNA BASED UNIPLEX PCR

7.2.1 Materials and Methods

7.2.1.1 Reference samples:

A..castellanii (ATCC 50370) was obtained from American Type Culture Collection (ATCC), Virginia, USA, and maintained in axenic PYG medium. Closely related amoebae i.e. *Balamuthia mandrillaris* CDC:V039 type strain (ATCC 50209) and *Hartmannella vermiformis* (ATCC 50236) were also procured from ATCC and maintained in VERO cell line and *Hartmannella* specific medium, respectively.

Cultures of bacteria (*Pseudomonas aeruginosa*), fungus (*Aspergillus* spp.), and virus (Herpes simplex virus) were clinical isolates from corneal scrapings processed in our laboratory. Human leucocytes were obtained from blood donated by a volunteer. All these reference isolates were used to check the specificity of the primers. To determine the analytical sensitivity of the assay, DNA extracted from the standard strain of *Acanthamoeba* was quantitated. To mimic the actual assay condition i.e. detecting *Acanthamoeba* in corneal scrapings samples, 10 fold of the quantitated *Acanthamoeba* DNA was done in DNA extracted from corneal scraping, which was smear and culture negative for any of the microbial agents. PCR was performed with a varying quantity of *Acanthamoeba* DNA (5μg to 500 femtogram) diluted in DNA obtained from corneal scrapings.

7.2.1.2 Patients

The study was planned in two phases. In the first phase for a pilot study, based on the smear results (later confirmed by culture), corneal scrapings were collected from 66 patients deemed to have either *Acanthamoeba*, bacterial, viral or fungal keratitis. The corneal scrapings were collected in 1 mL of phosphate buffered saline, pH 7.2 and stored at –20°C until tested by PCR.

In the second phase, corneal scrapings were also collected in a similar manner from 53 consecutive cases of suspected microbial keratitis patients seen between March 2002 and June 2002 and stored at – 20°C until tested by PCR. Patients with small corneal infiltrate or those who were otherwise uncooperative were excluded from the study, as additional corneal scrapings could not be collected.

7.2.1.3 DNA extraction from culture isolates and corneal scrapings:

The genomic DNA of *A. castellanii* (standard strain) was isolated using the UNSET buffer using a procedure described earlier in section 6.2.4. While for DNA extraction from corneal scrapings, the eppendorf containing the same were centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded and to the pellet UNSET lysis buffer was added. Subsequently the procedure for extraction was same as that followed for the *Acanthamoeba* isolates except that the organic phase extraction was done only once and final DNA was dissolved in only 30 µL of double autoclaved milli Q water.

The extraction of DNA from *Pseudomonas aeruginosa*, *Aspergillus* species, Herpes simplex virus and human leucocytes followed the procedures described elsewhere (Behzadbehbahani *et al* 1997; Chomcznski *et al* 1997; Pitcher *et al* 1989).

7.2.1.4 Polymerase Chain Reaction (PCR) analysis:

The sequence of the 18S rDNA primer, used in this study, was obtained from Dr. Thomas J Byers (The Ohio State University, Columbus, Ohio, USA) and consisted of forward primer 5'-GGCCCAGATCGTTTACCGTGAA-3' and reverse primer 5'-TCTCACAAGCTGCTAGGGGAGTCA-3'. These primer sequences correspond to nucleotide positions 928-949 and 1367-1390 bp respectively, of *A. castellanii* ATCC 50374 18S rDNA (EMBL Acc. No. U07413). The primers were synthesized at the Centre for Cellular and Molecular Biology, Hyderabad, India. All PCR reactions were carried out in a laminar flow hood after 30 minutes of UV irradiation to decontaminate surfaces and all supplies within the hood. Presterilized PCR tubes, double autoclaved milli Q water and positive displacement tips and pipettes were used

to reduce the possibility of contamination. The primer pairs were tested initially for amplification of *A.castellanii*, *Pseudomonas aeruginosa*, *Aspergillus* species, Herpes simplex virus and human leukocytes DNA. The PCR conditions were modified in the amplification profile and $MgCl_2$ requirement compared to those described earlier (Schroeder *et al* 2001). The amplification profile was: 94°C for 1 min, 61°C for 1 min and 72°C for 1 min for 40 cycles followed by a final extension step of 72°C for 5 min. Each 20 μL PCR reaction comprised: 3 μL of DNA (in case of corneal scraping DNA extracts) or ~10 ng genomic DNA, 200 μM dNTP, 1 pM of each primer, 1x standard PCR buffer (containing 1.5 mM $MgCl_2$) and 1 unit Taq DNA polymerase (Gene Taq, MBI Fermentas, Lithuania). These conditions differed from the ones described by Schroeder *et al* (2001). Amplifications were performed in MJ Research PTC 200 thermocycler. The DNA extracts of corneal scrapings of culture confirmed *Acanthamoeba* keratitis patients were tested initially in a pilot study. On obtaining satisfactory results, corneal scrapings from consecutive patients with microbial keratitis were tested.

The PCR products were visualized by gel electrophoresis using 1.5% agarose / TAE gel containing 0.5 μg/mL ethidium bromide and the results were recorded on a UV gel documentation system (UVItec Ltd, Cambridge, UK).

7.2.1.5 Statistical analysis:

Diagnostic data from corneal scrapings of 53 consecutive patients with suspected microbial keratitis were used for determination of sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of smear examination

and PCR results using culture results as the gold standard. Following formulae were used for calculations.

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{All culture positives (true positives + false negatives)}} \times 100$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{All culture negatives (true negatives + false positives)}} \times 100$$

$$\text{PPV} = \frac{\text{Number of true positives}}{\text{Number of true positives + false positives}} \times 100$$

$$\text{NPV} = \frac{\text{Number of true negatives}}{\text{Number of true negatives + false negatives}} \times 100$$

$$\text{FP} = \frac{\text{Number of false positives}}{\text{All culture negatives (false positives + true negatives)}} \times 100$$

$$\text{FN} = \frac{\text{Number of false negatives}}{\text{All culture positives (false negatives + true positives)}} \times 100$$

7.2.2 Results

7.2.2.1 Sensitivity and specificity of the uniplex PCR

The primers produced *Acanthamoeba* specific amplicon (463 bp) from *A. castellanii* DNA (ATCC 50370) only and not from *Balamuthia, Hartmannella*, bacterial, fungal, viral and human leukocyte DNA (Figure 7.1). The analytical sensitivity of the assay was 5 picograms (Figure 7.2), which is equivalent to 1-2 trophozoites of *Acanthamoeba* (Byers *et al* 1990).

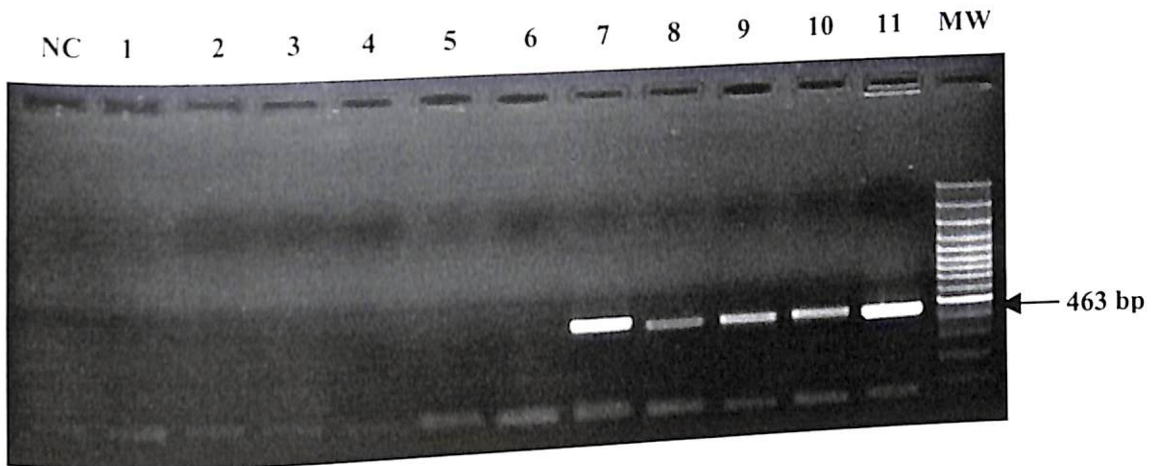

Figure 7.1: Representative gel showing results of PCR using 18S rDNA based primers for direct detection of Acanthamoeba. DNA in Lanes: NC -Negative control; 1- Bacterial (Pseudomonas aeruginosa); 2- Viral (Herpes simplex virus); 3 - Fungal (Aspergillus species); 4- Human leucocytes; 5-11 from corneal scrapings of keratitis patients culture proven to be Bacteria (5), Fungus (6), Acanthamoeba (7-11); 12- positive control A.castellanii (ATCC 50370); and MW- 100 bp ladder (MBI fermentas, Lithuania).

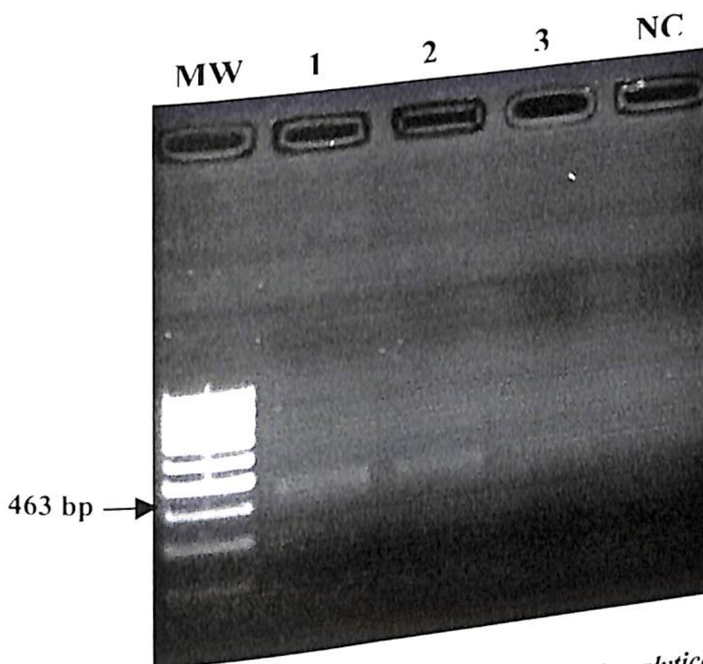

Figure 7.2: Representative gel showing results of analytical sensitivity of the uniplex PCR. DNA in Lanes: MW- 100 bp ladder (MBI fermentas, Lithuania); 1 – 50 pg of A.castellanii (ATCC 50370) DNA; 2 –5pg of A.castellanii (ATCC 50370) DNA; 3- 0.5pg of A.castellanii (ATCC 50370) DNA; NC -Negative

7.2.2.2 Results of phase I (Pilot study)

Corneal scrapings were taken from 66 patients selected on the basis of routine smear and culture results. Twenty-one of 66 patients (31.8%) were diagnosed to have *Acanthamoeba* keratitis based on detection of cysts in smears and /or growth of *Acanthamoeba* in culture. Fifteen of the 66 patients (22.7%) had bacterial keratitis, 20 (30.3%) had fungal and 10 (15.5%) had HSV-1 keratitis. While the corneal scrapings from all 21 cases of *Acanthamoeba* keratitis showed ~463 bp amplicon in PCR, the scrapings from bacterial, fungal and viral keratitis showed no amplification. A brief summary of the clinical findings, diagnosis and treatment outcome of 21 patients with *Acanthamoeba* keratitis included in the pilot study, is given in Table 7.1.

7.2.2.3 Results of phase II:

Fifty-three consecutive patients with suspected microbial keratitis were included for analysis of parameters such as sensitivity, specificity and predictive values. These patients were seen between March and June 2002 and after collection of an average of 7 multiple corneal scrapings for microbiological investigation, an extra scraping was collected for PCR. Therefore, some patients with small infiltrates could not be included in the study as it was not possible to collect an extra scraping. Table 7.2 summarizes the demographic details, predisposing factors, and diagnosis of these patients and Table 7.3 outlines the results of microbiological investigations and their correlation with PCR results on the corneal scrapings of these patients. Considering combined results of culture, smears and PCR, 10 cases of *Acanthamoeba* keratitis out of 53 consecutive patients were identified. Culture was positive in eight of these cases. While six out of eight were positive by both smear and PCR, one was smear positive PCR negative and one was smear negative and PCR positive. The two

culture negative cases were positive in smears while PCR was positive in only one of them. Refer Table 7.4 for the results of comparison of smear and PCR with culture.

Table 7.1: Clinical findings, diagnosis and treatment outcome of 21 patients with AK included in the preliminary evaluation of the PCR (Phase 1)

Characteristics	No. (%) of patients (n=21)
Demographics	
Males	12 (57.1)
Females	09 (42.9)
Mean Age (years)	34.3 ± 14.5
Age range (years)	7 - 63
Predisposing Factors	
Contact lens wear	0
Trauma	06 (28.5)
Foreign body	05 (23.8)
Unknown	10 (47.6)
Detection of *Acanthamoeba* Cysts in corneal scraping smears	
Calcofluor white	20 (95.2)[$]
Gram Stain	18 (85.7)
Giemsa Stain	14 (66.6)
Culture on non-nutrient agar	20 (95.2)[@]
Treatment Outcomes	
Healed with medical therapy	11 (52.4)
Surgical intervention	03(14.3)*
Lost to follow up	07 (33.3)

* Penetrating Keratoplasty - 1, Evisceration – 2
$ One sample was smear positive and culture negative
@ One sample was culture positive and smear negative

Table 7.2: Demographic details, predisposing factors, and microbiological diagnosis in 53 patients with microbial keratitis (Phase 2)

Characteristic	No. (%) of patients (n=53)
Demographics	
Males	38 (71.7)
Females	15 (28.3)
Mean Age (years) ± SD	37.92 ± 19.24
Age range (years)	2 - 86
Predisposing Factors	
Contact lens wear	0
Trauma	29 (54.7)
Diabetes	1 (1.9)
Leprosy	1 (1.9)
Prior surgery	6 (11.3)
Blepharitis	1 (1.9)
Lagophthalmos	2 (3.8)
Spheroidal degeneration	1 (1.9)
Unknown	12 (22.6)
Microbiological diagnosis*	
Bacterial	17 (32.0)
Fungal	14 (26.4)
Acanthamoeba	7 (13.2)
Viral (HSV)	2 (03.8)
Bacterial + *Acanthamoeba*	1 (01.9)
Fungal + Viral	1 (01.9)
Sterile (unknown)	11 (20.7)

*Based on culture of bacteria, fungi or *Acanthamoeba* and antigen/DNA detection of HSV (Herpes Simplex Virus) in corneal scrapings

Table 7.3:Correlation of microbiological findings and PCR results of CS from 53 consecutive patients (Phase 2)

Serial No.	Lab. No.	Direct smear examination			Culture	PCR
		CFW	Gram	Giemsa		
1	400/02	-	-	-	-	-
2	417/02	-	GPC	Cocci	*Streptococcus pneumoniae*	-
3	424/02	-	-	-	*Pseudomonas aeruginosa*	-
4	429/02	FF	FF	FF	*Aspergillus fumigatus*	-
5	432/02	-	GPC	Cocci	*Streptococcus pneumoniae*	-
6	490/02	Microsporidium	Microsporidium	Microsporidium	ND	-
7	503/02	-	-	-	*Staphylococcus epidermidis*	-
8	512/02	-	-	-	*Pseudomonas aeruginosa*	-
9	513/02	-	GPC	Cocci	*Streptococcus pneumoniae*	-
10	569/02	-	-	-	*Staphylococcus epidermidis*	-
11	570/02	-	-	-	-*	-
12	577/02	-	-	bacilli	*Pseudomonas aeruginosa*	-
13	603/02	Actinomycetes	Actinomycetes	Actinomycetes	*Nocardia asteroides*	-
14	604/02	-	-	-	-	-
15	607/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba spp.*	+
16	632/02	FF	FF	FF	UIHF	-
17	652/02	FF	FF	FF	*Aspergillus niger*	-

18	653/02	FF	FF	FF	*Aspergillus niger*	-
19	654/02	FF	FF	FF	UIHF	-
20	661/02	-	*Acanthamoeba* cysts	ND	-	+
21	710/02	-	GPC	Cocci	*Streptococcus pneumoniae*	-
22	753/02	ND	-	ND	-	-
23	788/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba spp.*	+
24	862/02	-	-	-	-	-
25	865/02	-	-	-	*α - haemolytic streptococci*	-
26	877/02	-	-	-	-	-
27	883/02	Actinomycetes	-	-	*Nocardia asteroids*	-
28	886/02	FF	FF	FF	*Bipolaris spp.*	-
29	889/02	-	GPC	Cocci	*Streptococcus pneumoniae*	-
30	918/02	-	GPC	Cocci	*Streptococcus pneumoniae*	-
31	925/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	-	*Acanthamoeba spp.*	+
32	932/02	FF	FF	FF	*Fusarium spp.*	-
33	953/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba spp.*	+
34	956/02	FF	FF	FF	*Aspergillus flavus*	-
35	995/02	FF	FF	FF	UIHF *Streptococcus pneumoniae*	-
36	1001/02	-	GPC, GNB	Cocci, bacilli	*Pasteurella spp.*	-
37	1004/02	-	-	-	-	-
38	1009/02	FF	FF	FF	*Fusarium spp.*	-

39	1014/02	FF	FF	-	UIHF	-
40	1015/02	FF	FF	FF	*Aspergillus flavus**	-
41	1016/02	FF	FF	FF	*Aspergillus flavus*	-
42	1046/02	FF	FF	FF	*Aspergillus flavus*	-
43	1070/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba spp.*	+
44	1132/02	-	GPC	Cocci	α - *haemolytic streptococci*	-
45	1148/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba spp.*	-
46	1177/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	-	-
47	1201/02	-	-	-	-	-
48	1205/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba spp.*	+
49	1210/22	FF	FF	FF	*Aspergillus fumigatus*	-
50	1224/02	-	GPC	Cocci	α - *haemolytic streptococci*	-
51	1226/02	-	-	-	-*	-
52	1243/02	-	-	-	*Acanthamoeba spp.* *Staphylococcus epidermidis*	+
53	1253/02	FF	FF	FF	*Fusarium spp.*	-

FF : Fungal filaments
GPC : Gram positive cocci
GNB : Gram negative bacilli
UIHF : Unidentified hyaline fungus
CFW : Calcofluor white
* : Positive for Herpes simplex virus antigen / DNA
\- : Negative
ND : Not done

Table 7.4: Summary of smear and PCR testing of corneal scrapings for the Acanthamoeba keratitis in comparison to culture based detection (Phase II)

Diagnostic Test	Culture	
	Positive	**Negative**
Smear positive	7 Sens = 87.5% PPV = 77.8%	2 FP = 4.4%
Smear negative	1 FN = 12.5%	43 Spec = 95.6% NPV = 97.7%
PCR positive	7 Sens = 87.5% PPV = 87.5%	1 FP = 2%
PCR negative	1 FN = 12.5%	44 Spec = 97.8% NPV = 97.8%

Sens- Sensitivity
Spec- Specificity
PPV- Positive predictive value
NPV- Negative predictive value
FN - False negative rate
FP - False positive rate

7.2.3 DISCUSSION

It is well known that direct smear examination procedures provide immediate diagnosis while culture may take 1-10 days (average 3.5 days in our series). Therefore, in the face of negative smears, a delay of several days in diagnosis is involved, thus leading to a delay in instituting specific therapy. In order to enhance our diagnostic capability, we decided to use a molecular diagnostic assay that may have an advantage over smear and culture, and may be more sensitive, specific as well as rapid for the detection of *Acanthamoeba* from clinical samples. Although PCR based assays for the detection of *Acanthamoeba* in corneal scrapings have been

described, none have been tested on patients with non-contact lens related keratitis. We decided to evaluate the PCR assay (Schroeder *et al* 2001), which was described to be highly specific and was based on 18S rRNA gene of *Acanthamoeba*.

We evaluated this assay against the appropriate positive and negative controls and found it to be specific for *Acanthamoeba* with modifications in the assay conditions. The specificity of the assay was further confirmed in the phase I of our study where 21 corneal scrapings culture/smear positive for *Acanthamoeba* yielded positive result by PCR and the other 45 corneal scrapings, culture/smear positive for bacterial, fungal and viral keratitis were negative. Convinced of the results of specificity of the assay, we evaluated this test on 53 cases of suspected microbial keratitis. To the best of our knowledge this is the first study to evaluate the sensitivity, specificity and predictive values of a PCR technique vis-à-vis a smear and culture that form the routine diagnostic tests in most ocular microbiology laboratories for the diagnosis of *Acanthamoeba* keratitis.

Our results show that, the sensitivity of PCR was similar (87.5%: 95% CI 50.7-99.8) to that of smear results, which included three methods of smear examination such as calcofluor white, Gram and Giemsa stains (Table 7.3; 7. 4). The long experience of the microbiologists involved in examination of corneal scraping smears in this laboratory may account for the high positivity of smears. Patient samples 20 and 46 (Table 7.3) were culture negative but smear positive, while sample number 20 was found to be culture negative and PCR positive. It is possible that the testing of last scraping by PCR led to negative results (in 2 samples out of 10) and thus the

sensitivity would have been higher if it was tested on initial corneal scrapings of the patients rather than the last.

On the other hand, this study found higher specificity and predictive values of the PCR assay compared to smear methods (Table 7.4), although the difference was statistically not significant. Despite repeated testing, no PCR products were seen in the corneal scrapings of case 45 (smear and culture positive for *Acanthamoeba*) and case 46 (smear positive for *Acanthamoeba* cysts and culture negative), which we attribute to the possible lack of *Acanthamoeba* DNA in the sample. Possibility of PCR inhibitors was ruled out in these two samples by spiking them with *Acanthamoeba* DNA and retesting them. Low DNA yield in clinical samples is known to affect the success of PCR, especially if only mature cysts are present (Schoreder *et al* 2001).

Apart from high sensitivity, this study demonstrates high specificity and high positive and negative predictive values of the PCR assay, all of which are hallmarks of a good diagnostic test. Unlike smear and culture techniques that require familiarity with the morphology of cysts and trophozoites of *Acanthamoeba*, apart from the facilities, PCR does not require such expertise. Any laboratory with a molecular biology set up can easily adopt the PCR assay used in this study. Moreover, smear examination techniques, especially calcofluor white, are known to miss trophozoites and a sample with only trophozoites is likely to be labeled as smear negative (Stothard *et al* 1999; Sharma *et al* 2001). In this study, a combination of smear and culture provided diagnosis in similar number of cases as smear and PCR. There are not many ocular microbiology laboratories, which employ multiple smear examination protocol for

corneal scrapings. Moreover, the availability of fluorescence microscope, required for observation of smears stained with calcofluor white is also not very common owing to its high cost factor. Under these constraints, clinical diagnosis based on only smear positivity using only Gram or Giemsa stain is likely to be much lower, as is evident from Table 7.1. Comparatively, the PCR technique can be easily added to the armamentarium of diagnostic methods in a microbiology laboratory. Additionally, the short time taken by the PCR test is a distinct advantage over the culture method. Hence, we strongly believe that the PCR based diagnostic assay, coupled with smear examination, will be very helpful and desirable for rapid diagnosis of *Acanthamoeba* keratitis and be confirmatory in clinically suspected cases with or without culture results.

The high clinical value of PCR in the diagnosis of *Acanthamoeba* keratitis has been earlier shown by Lehmann *et al* (1998) who found a sensitivity of 84%, which is similar to ours (87.5%), although they used clinical diagnosis as the gold standard as opposed to culture in this study. Evaluation of PCR for *Acanthamoeba* in consecutive cases of suspected microbial keratitis, as done in the present study, has further confirmed the applied value of molecular diagnosis in *Acanthamoeba* keratitis. A novel approach by Mathers *et al* (2000) of confirming confocal microscopy diagnosis of *Acanthamoeba* keratitis in contact lens wearers using, PCR highlights the wide scope of utility of PCR assays. Based on their findings, the authors have speculated that *Acanthamoeba* may be responsible for a large percentage of cases that are commonly diagnosed as contact lens over wear. They have alluded to the possible association of *Acanthamoeba* with many forms of corneal epitheliopathy. Similar associations, which may or may not be related to contact lens wear, are expected to

emerge in our set up with improved diagnosis using PCR test for *Acanthamoeba*. Such findings may lead to a revision of the incidence and prevalence data of the ocular or extraocular diseases caused by *Acanthamoeba* species in this part of the world.

Vodkin *et al* (1992) were the first to use PCR for the genus-specific detection of *Acanthamoeba* using primer pair 'ACARNA.for1383' and 'ACARNA.rev1655', which amplifies 272 bp of 18S rDNA. This primer pair was also tested by Lehmann *et al* (1998) in their clinical study, along with a second 18S rDNA based primer pair 'P1GP.for2379' and 'P1GP.Rev2632', which amplifies a 253 bp amplicon. Analyzing complete 18S rDNA sequences of over 80 isolates of *Acanthamoeba*, Schroeder *et al* (2001) have shown that the above two primer pairs could also amplify rDNA of related amoebae i.e., *Balamuthia* and *Hartmannella*. The primers used in our study (JDP1/JDP2) were designed by them from a large database of 18S rDNA sequences and were shown to be genus specific for *Acanthamoeba*. These primers had failed to amplify DNA from closely related amoebae and from several bacterial, fungal and human DNA. Although Schroeder *et al* (2001) used different PCR conditions for achieving high sensitivity and specificity in their study, in this study we have employed only one set of PCR conditions that provide both high sensitivity and specificity. Stothard *et al* (1999) using genus and subgenus specific oligonucleotide probes have shown the specific identification of *Acanthamoeba* in both environmental and clinical samples. Therefore, in our opinion, it would be interesting and gainful to integrate such new PCR techniques in ocular microbiology laboratories dealing with large number of patients with microbial keratitis. This study is the first study to

evaluate a PCR based assay against conventional methods for the diagnosis of AK in a clinical setting.

Inclusion of PCR for *Acanthamoeba* along with conventional methods of diagnosis of non-viral microbial keratitis is expected to improve the diagnosis of *Acanthamoeba* keratitis in ocular microbiology laboratories having molecular biology facilities. While awareness regarding bacterial and fungal keratitis is relatively high in a majority of the eye hospitals in India, as reflected in several publications, information regarding *Acanthamoeba* keratitis is grossly inadequate. The reported incidence of *Acanthamoeba* keratitis in India varies from 1-3% and the cases are predominantly in non-contact lens wearers (Davamani *et al* 1998; Sharma *et al* 2000;Srinivasan 1997). Through this report, we would like to emphasize that with appropriate tests a greater number of cases of *Acanthamoeba* keratitis can be differentiated from bacterial, fungal or viral keratitis and treated appropriately before it is too late.

7.3 18S and 26S rDNA BASED MULTIPLEX PCR

7.3.1 Materials and Methods

7.3.1.1 Reference sample and *Acanthamoeba* isolates

Same set of reference samples (*A..castellanii*-ATCC 50370; *Pseudomonas aeruginosa*; *Aspergillus* spp ; Herpes simplex virus; Human leucocytes) as used in the uniplex PCR assay were used in this study to establish the specificity of the assay. The sensitivity was also determined in the similar manner as described in section 7.2.1.1. In order to standardize the multiplex assay, in the first phase of the study PCR was performed with 23 *Acanthamoeba* isolates which comprised of 16 isolates from India (15 from keratitis patients and one from potable water from one of the

patients' home), one each from Pakistan and Argentina, 4 ATCC strains and one standard strain of *A. culbertsoni*.

7.3.1.2 Patients

In the second phase of the study, for the purpose of testing this PCR assay on the clinical samples, corneal scrapings were collected from 34 patients diagnosed as

Table 7.5 Demographics, clinical findings, microbiological investigation and treatment outcome in AK patients (n=25)

Patient and Sample details	No. (%) of patients
Demographics	
Males	15 (60)
Females	10 (40)
Average age (years)	34.4 +/- 14.7
Age range (years)	7-63
Socio-economic status	
Very Poor	18(72)
Poor-middle class	7(28)
Predisposing factors	
Contact lens wear	0
Injury	6 (24)
Foreign body	6 (24)
Unknown	13(52)
Microbiological Investigations	
Staining techniques	
Calcofluor white	24 (96)
Gram	21 (84)
Giemsa	16 (64)
Culture on NNA	24 (96)
Treatment outcome	
Healed with medical therapy	15 (60)
Surgical intervention	3 (12)
Lost to follow up	7 (28)

either bacterial/fungal/*Acanthamoeba* keratitis during January 2000 to October 2001 in the corneal clinic of our institute. These samples were selected from among the 2213 cases of microbial keratitis with no history of contact lens usage. All the 34 corneal scraping samples were collected in duplicate, of which one set was stored in

phosphate buffered saline (PBS) buffer and the other set was directly investigated for the causative organism by our lab specific protocol. The latter investigation revealed 25 corneal scraping to be culture positive for *Acanthamoeba* and nine for bacteria fungus. The clinical data and results of microbial investigations of all the 25 *Acanthamoeba* positive samples are given in Table 7.5.

In the third phase of the study, in order to evaluate the multiplex assay, the same set of 53 consecutive corneal scrapings samples (as described in section 7.2.1.2) were tested.

7.3.1.3 DNA extraction and Polymersae Chain Reaction analysis

DNA from the isolates and the corneal scrapings was extracted according to procedure described earlier in section 7.2.1.3. For the standardization of the multiplex PCR, two sets of primers were included: one specific to 18S rDNA (Schroeder *et al* 2001) and other specific for 26S rDNA (Lai *et al* 1994) giving 463 bp and 126 bp fragments, respectively. Primer sequences used to amplify the ASA.S1 fragment of 18S rDNA domain have been given before in the Table 6.2, whereas those for 26S rDNA target were: 5`-GGAGCTCCCACGGGAGGCC-3` and 5`-TGGACCGCGTGAGGCTGCGGCT-3` as described by Lai *et al* (1994). For the sake of convenience we will be addressing primer pair described by Lai *et al* (1994) as "Lai" primers.

The fact that ASA.S1 fragment specific primers only amplify DNA from *Acanthamoeba* isolates has been described and established in Chapter 5; wherein DNA from 23 *Acanthamoeba* isolates was amplified and sequenced. In a similar

manner. in order the establish the specificity of "Lai" primers. DNA from the same 23 *Acanthamoeba* isolates was amplified and sequenced using an automated DNA Sequencer ABI-PRISM 3700 available at center for cellular and molecular biology (CCMB). Hyderabad. The sequences were submitted in the GenBank database (Accession nos. AF534157-AF534179) and also blasted using in BLASTn search to determine the homology of these sequences with the ones in the database.

PCR reactions were done in 20 µL reactions using 1 or 3 µL of template DNA (for amoebae and corneal scarping samples respectively). Each PCR reaction contained 1U AmpliTaq Gold (Perkin Elmer. USA). 2 pM of each primer. 200 µM dNTPs and 1.5 mM $MgCl_2$ and was amplified for 35 three-step cycles of 94^0C. 61^0C and 72^0C each for 1 min. followed by final extension of 72^0C/5 min in thermocycler MJR PTC-200. The amplified products were resolved and visualized on ethidium bromide stained 1.5% agarose gel. In all the experiments. negative (water in place of template DNA) and positive (DNA from *A. castellanii*. ATCC-50370) controls were used

In order to standardize the assay. in the first phase of the study PCR was performed with 23 *Acanthamoeba* isolates and in the second phase PCR was performed with DNA extracted from 34 corneal scrapings which were smear and/or culture positive for *Acanthamoeba* (n=25). fungus (6) and bacteria (3). In order to evaluate the PCR assau. in the third phase of the study. PCR was done with corneal scrapings collected from 53 consecutive cases of suspected microbial keratitis patients.

7.3.2 Results

7.3.2.1 Sensitivity and specificity of the assay:

"Lai" primer were found to be specific since it showed 98% homology with *A.castellanii* (EMBL Accession no. X73881). The multiplex PCR assay produced *Acanthamoeba* specific two-band phenotype (463 bp and 126bp) from *A. castellanii* DNA (ATCC 50370) only and not from bacterial, fungal, viral and human leukocyte DNA (Figure 7.3). The analytical sensitivity of the assay was also 5 picogram (Figure 7.4), which is equivalent to 1-2 trophozoites of *Acanthamoeba* (Byers *et al* 1990).

7.3.2.2 Results of Phase I, II and III

All the 23 *Acanthamoeba* isolates were positive for both the primer sets hence giving a two-band phenotype for each isolate. In the second phase of the study, PCR was positive in 24 of the 25 corneal scrapings culture positive for *Acanthamoeba*, while it was negative for the 9 corneal scarping which were culture proven to be either bacterial/fungal.

In the third phase of the study, where PCR was performed on 53 consecutive corneal scrapings specimens, PCR was positive in the same 8 samples, which were positive by uniplex PCR. PCR was negative in the two samples, among which one was both smear and culture positive and the other was smear positive and culture negative.

The demographic, clinical, microbiological and treatment outcome of 25 patients included in the study is given in Table 7.5. Perusal of the patients/clinical data from whom the study samples were drawn did not suggest any bias with respect to the sex and age (Table 7.5) and incidence of *Acanthamoeba* keratitis. Notably, all the patients

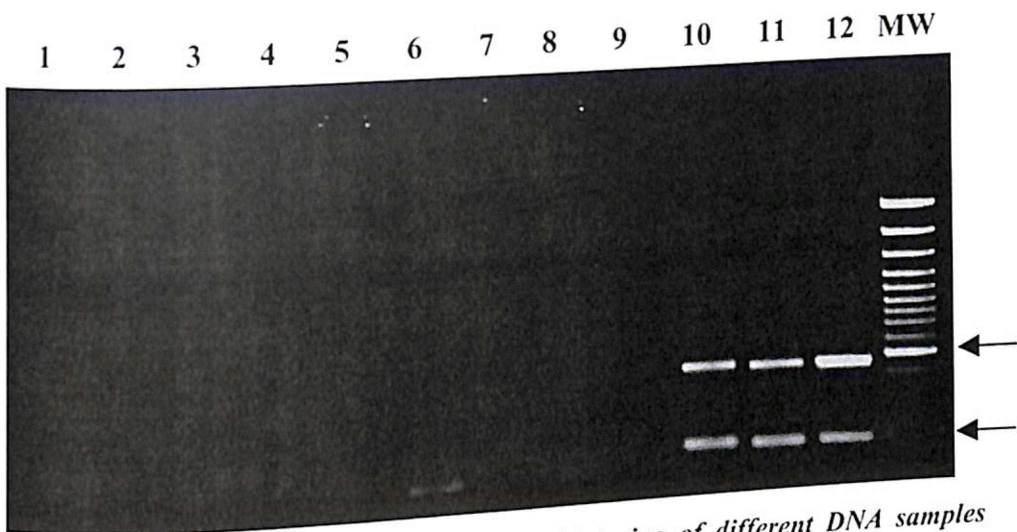

Figure 7.3: Representative gel showing rDNA typing of different DNA samples using multiplex PCR-assay for direct detection of Acanthamoeba. DNA in Lanes: 1- Negative control; 2- Bacterial (Pseudomonas aeruginosa); 3- Viral (Herpes Simplex virus); 4- Fungal (Aspergillus spp.); 5- Human; 6-8- corneal scrapings of keratitis patients culture-proven to be Bacteria (6), Bacteria (7), fungus (8), (9) viral; 10 –11 corneal scrapings from keratitis patients culture proven to be Acanthamoeba and 12- Positive control A. castellanii, MW: 100bp ladder (MBI fermentas, Lithuania). Note amplicons of ~463 and 126 bp only in Acanthamoeba

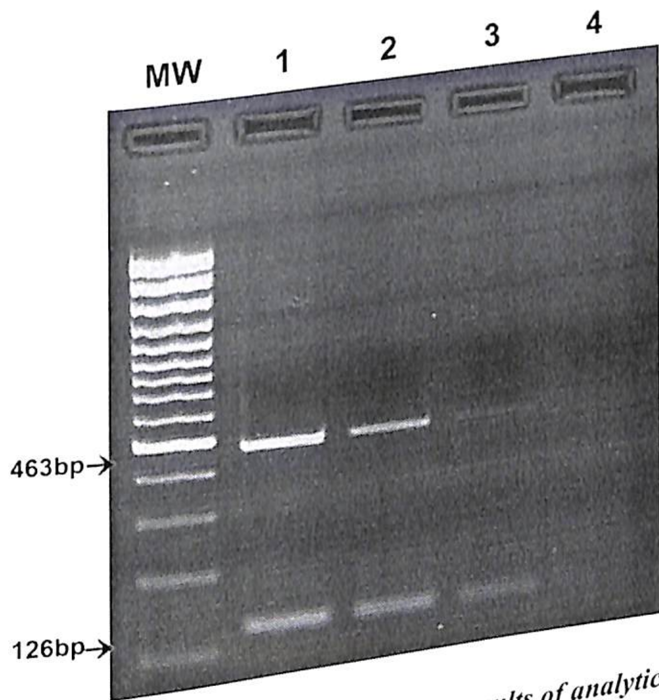

Figure 7.4: Representative gel showing results of analytical sensitivity of the multiples PCR. DNA in Lanes: MW- 100 bp ladder (MBI fermentas, Lithuania); 1 – 500 pg of A.castellanii (ATCC 50370) DNA; 2 –50 pg of A.castellanii (ATCC 50370) DNA; 3- 5pg of A.castellanii (ATCC 50370) DNA; NC -Negative

belonged to relatively poor strata of society, none were contact-lens wearer and for the few cases with known history, mechanical injury/trauma was found to be the main predisposing factor.

7.4 Discussion

In light of the results of the uniplex PCR wherein the PCR did not offer an added advantage over the conventional microbiological techniques, a search for a newer and better diagnostic marker for the detection of *Acanthamoeba* continued and we standardized this multiplex PCR assay. The reasons for selecting markers, specific for 18S rDNA and 26S rDNA for multiplexing were: they both belonged to different genes i.e. one belonged to small subunit and the other belonged to the large subunit of ribosomal RNA, and secondly, the difference in the size of amplified product was approximately 330bp which made it very easy to view the results on routinely used 1.5% agarose gel and the thirdly, the compatibility in annealing temperature between the two primer sets made it very convenient to use these primers in the multiplex assay.

This PCR assay was also specific to *Acanthamoeba* only however; it had an added advantage of utilizing two genetic markers rather than one. A two-point confirmation is always better than one and this assay could achieve that. This assay was sensitive enough to show a positive result even if 1-2 trophozoites/cysts were present in the clinical samples. Thus the sensitivity of the two PCR assay described here were comparable and the only advantage, which the multiplex assay had over the uniplex, was the two- point or two band phenotype confirmation obtained in a single tube, which could not be achieved by uniplex PCR.

CHAPTER 8: SUMMARY AND CONCLUSIONS

Acanthamoeba keratitis (AK) is a sight threatening corneal inflammation, which we believe is underreported in India. It has mainly been reported from south India with very few reports from North India. In our study we determined that agriculture was the occupation for most of the AK patients who had relatively greater risk of damaging their cornea with either vegetative matter or cattle and were also exposed to poor hygienic conditions. In spite of India being a large country with diverse climate and geographical regions, the fact remains that close to 70% of its population is engaged in agriculture related jobs, where they are exposed to similar risks. Though the prevalence of infectious keratitis varies from region to region, we still believe that AK is prevalent but undiagnosed in other parts of the country. Thus, this study calls for epidemiological survey, which should be done across the country to determine the incidence, prevalence and risk factors for AK.

At our center we found that prevalence of AK was 2.3% in keratitis patients and 99.4% of the patients were non-contact lens wearers. ***This report is the largest series of AK patients ever reported in literature from one center.*** Clinical features of AK were not pathognomonic for AK, the initial clinical signs and symptoms of AK resembled any suppurative keratitis of infectious etiology like bacteria and fungus.

Evaluation of conventional microbiological techniques at our institute revealed that that KOH +CFW was the most sensitive technique to detect *Acanthamoeba*, but required the use of expensive fluorescence microscope, which many ophthalmologist cannot afford. Culture was relatively less sensitive, though the positivity rate was

much higher than what is reported in literature. The only disadvantage which culture had was that it took an average of 3.5 days to give positive result, thus delaying the diagnosis, which directly affects the outcome of the disease.

We developed two PCR based diagnostic assays for the detection of *Acanthamoeba* in clinical samples. Both uniplex and multiplex PCR assay were sensitive and specific for the genus *Acanthamoeba*. Comparison of uniplex PCR and smear examination with culture revealed that sensitivity of PCR was same as that of smears, however the specificity and the positive and predictive values of PCR were marginally higher than smear examination although the difference was not significant. Sensitivity of the smears were high in this laboratory because we employ a strategy of examining multiple smears and also have the added advantage of using KOH +CFW staining technique. We concluded that in our setting PCR did not offer much advantage but would definitely be useful in laboratories which have a set up for PCR but are lacking expensive microscopes. ***This study is the first study to evaluate a PCR based assay against conventional methods for the diagnosis of AK in a clinical setting.***

Nucleotide sequence based genotyping of 18S rRNA gene revealed that amoebae isolated from non-contact lens associated keratitis patients were indeed *Acanthamoeba* and belonged to the T4 genotype. The results also revealed that there exists a high degree of genetic diversity among the *Acanthamoeba* isolates. Subgenus classification based on morphology was inconsistent since *Acanthamoeba* isolates belonging to different morphological group, grouped together within the T4 genotype. We also concluded that phylogenetic inferences obtained from ASA.S1 and DF4 domains of 18S rRNA gene are comparable to the ones obtained from the complete

sequence. Hence sequencing the complete gene, which is expensive, cumbersome and labor intensive can be avoided. Thus, this ASA.S1 amplimer can be used as a tool both for sub-genus classification and as marker to detect *Acanthamoeba* in clinical specimens. ***This is first study to establish taxonomic status of Acanthamoeba strains isolated from non-contact lens associated keratitis patients from India.***

In vitro drug susceptibility test revealed that Brolene is not appropriate as, drug of choice for the treatment of *Acanthamoeba*. Both PHMB and CHx had lower MCC but when compared with each other MCC of CHx was lower than PHMB though was not statistically significant. Both PHMB and CHx are excellent drugs for the treatment for AK either alone or in combination.

Review of the histopathologic slides of corneal button/eviscerated material obtained from AK patients revealed that 27% of the samples showed granulomatous inflammation. Immunophenotyping of the cells revealed the presence of T-lymphocytes and macrophages. Thus we conclude that granulomatous inflammation in the posterior corneal stroma is not an uncommon finding in AK and could possibly be immune mediated, though further studies are warranted to prove this.

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Name ____________________
Mr. No. ____________ Episode No. ________

Corneal Ulcer - Patient History Form

LAB No. ____________ Age ______ SEX - M / F VISIT DATE ___ / ___ / ___
FELLOW ____________ PHOTO No. ____________

Affected Eye	OD / OS / OU
Education	Illiterate / Primary / Secondary / University
Occupation	Agriculture Manual labour Office worker / Desk Job Household work
Trauma	No Trauma Stone Plant / Agricultural material Dust / Dirt Animal (e.g. Cowtail) Metal Chemical Human (e.g. Finger poke) Heat / Thermal Injury Not Identified
Chief Complaints	Duration (days) ________ Pain Redness Watering Photophobia Foreign body sensation Defective vision Opacity Discharge
Recurrent attacks	No / Yes
Frequency	[ ]
Prior Consultation	Eye care professional Other physician Traditional healer Self medication Unknown
Prior Investigations	None Scrapings Result Bacterial / Fungi / Acanthamoeba / Viral

PRE-DISPOSING FACTORS

Systemic	Ocular
None	None
Diabetes mellitus	Prior Surgery - PK
Leprosy	Prior Surgery - IOL
Tuberculosis	Prior Surgery - Others
Alcoholism	Prior Ulcer - Same Eye
Rheumatoid arthritis and other C.V.D.*	Prior Ulcer - Other Eye
Stevens Johnson Syndrome	Corneal edema
Vitamin A deficiency	Dry eyes
Extensive burns	Stevens Johnson Syndrome
Measles / Other exanth. Fevers	Entropion
AIDS / Other immune deficiency	Trichiasis
Allergies	Blepharitis
HZO	Xerophthalmia
Not known	Glaucoma
(* C.V.D. - Collegen Vascular Disease)	Dacryocystits
	Proptosis with exposure risk
	Lagophthalmos
	Neurotrophic keratitis
	Conrneal degeneration
	Conrneal scarring
	Not known

MEDICATIONS

Systemic	Ocular
None	None
Antibiotics	Antibiotics
Antivirals	Antivirals
Antifungals	Antifungals
Steroids / Immunosuppressives	Steroids
Others	Native / Home remedy
	Antiglaucoma
	Others

CONTACT LENS WEAR

Yes / No

Type of Lens Hard / Gas permeable / soft / DW / EW / Disposable

Duration of wear each day ______________ (hrs)

Age of CL ______________ (months)

Disinfection method Adequate / Inadequate

L.V. PRASAD EYE INSTITUTE

(Managed by Hyderabad Eye Institute)

Name ______________________________

Mr. No. ______________ Episode No. ________

Corneal Ulcer - Examination Form

1. Nutritional Status		No signs of nutritional deficiency
		Malnurished
2. Visual Acuity		
3. Intraocular Pressure		High
	(Digitally)	Normal
		Low
		Not taken
4. External Examination		
	Ocular Motility	Normal
		Abnormal
	Corneal Sensitivity	Normal
		Decreased
		Absent
		Not assessed
5. Lids		
	Dacryocystitis	Y / N
	Blepharitis	Staph. / Seborheic
		0 1 2 3 4 (0 = none)
	Meibomitis	0 1 2 3 4 (0 = none)
	Lid closure	Normal
		Lagophthalmos
	Edema	0 1 2 3 4
	Lashes	Normal
		Misdirected
6. Tear Film		Normal
		Dry - Mild
		Moderate
		Severe
	Debris	0 1 2 3 4

7. Conjunctiva

Palpebral Congesion	No
	Yes - Papillae 1 2 3 4
	Follicles 1 2 3 4
Bulbar Congestion	Yes / No
Chemosis	No
	Mild
	Moderate
	Severe

8. Location of Ulcer (* within 4 mm of limbus) — Peripheral* / Central / Total

9. Graft Infiltrate — Yes / No

- Involvement — Recipcent / Donor / Both

10. Limbal Involvement — Y / N

11. Scleral Involvement — Y / N

12. Corneal Epithelium

Defect Size Horizontal	0 - 12 ________ mm
Defect Size Vertical	0 - 12 ________ mm
Edema	0 1 2 3 4

13. Bullae — Yes / No

14. Staining Pattern — Rose Bengal / Fluorescein — Dendritic / Geographical / Atypical

15. Corneal Stroma

Infiltrate Single / Multiple

Nature -	Dry / Wet
Edge -	Demarcated
	Hyphate
	Scarred
	Diffuse
	Ring
Size Horizontal	0 - 12 ________ mm
Size Vertical	0 - 12 ________ mm
Depth -	Ant / Mid / Post

	Thinning -	No	
		Yes (%)	
	Perforation -	No	
		Yes - Size ____________	
		Location	Central / Paracentral / Peripheral
	Vascularisation -	Superficial (0 1 2 3 4)	
		Deep (0 1 2 3 4)	
	Edema	0 1 2 3 4	
	Immunering	Yes / No	
	Scar	Yes / No	Sub Epithelial
			Mid Stromal
			Posterior Stromal
	Descements folds	Yes / No	

16. Endothelium

Keratic Precipitates	Y / N
Endothelial Ring	Y / N
Endothelial Exudate	Y / N

17. Anterior Chamber — Not assessible / Assessible

Depth	Deep
	Shallow
	Flat
Cells	0 1 2 3 4
Flare	0 1 2 3 4
Hypopyon	N / Y (mm) ____________

18. Iris — N / Y

	Normal
	Abnormal (Rubeosis)
Synechiae	None / Ant / Post

19. Pupil — N / Y

Size	Normal / Dilated / Mid dilated / Miotic
Reaction	Brisk
	Sluggish
	Fixed
Shape	Regular / Irregular

From N

L.V. PRASAD EYE INSTITUTE
(Managed by Hyderabad Eye Institute)

Name ______
Mr. No. ______ Episode No. ______

Corneal Ulcer - Treatment Form

1. Treatment - Initial — N / Y
   - A) Antibacterials — Ciprofloxacin / F.Genta / F.Cefa / Others
   - B) Antifungals — Natamycin / Miconazole / Clotrimazole / Ketoconazole
     Systemic - Topical / Others
   - C) Anti Acanthamoeba — Neomycin / Dibromopropamidine Isethionate / PHMB / Clotrimazole / Others
   - D) Anti Virals — Acyclovir / Others
2. Treatment Modification — N / Y
   - A) Antibacterials — Ciprofloxacin / F.Genta / F.Cefa / Others
   - B) Antifungals — Natamycin / Miconazole / Clotrimazole / Ketoconazole
     Systemic - Topical / Others
   - C) Anti Acanthamoeba — Neomycin / Dibromopropamidine Isethionate / PHMB / Clotrimazole / Others
   - D) Anti Virals — Acyclovir / Others
   - Reason — No response
     Sensitivity
     Others ______
3. Surgical — N / Y
   - A) BCL + TA — N / Y
     Indication — Thinning / Perforation
   - B) P.K. — N / Y
     Indication — No response to medical treatment
     Failed BCL + TA
     Large Perforation
     Large infiltrate at presentation

Donor size ______ mm
Recipient size ______ mm
No. of Sutures [ ]
Death Utilisation time ______ hrs
Quality of tissue — Excellent / V.Good / Good / Fair / Poor
Additional procedures — N / Y

From No. 12[illegible]

20. Lens	Not Visible Clear Cataract Aphakic Pseudophakic
21. Lacrimal Passage (Clinical / Syringing)	Patent / Blocked
22. Investigations	
Biopsy	Yes / No
Indications	Deep Stromal Infiltrate Negative Scrapings A typical Presentations Others
Date	______ / ______ / ______
23. Initial Diagnosis	Viral Mycotic Keratitis Acanthamoeba Keratitis Bacterial Keratitis Infectious Keratitis Not Specified
24. Final Diagnosis	Viral Mycotic Keratitis Acanthamoeba Keratitis Bacterial Keratitis Infectious Keratitis Not Specified
25. Diagnosis for Viral	HSV-1 Keratitis HSV-2 Keratitis VZV Keratitis Adenoviral Kerato conjuctivitis Chlamydial Kerato conjuctivitis Others
26. Others Specify	

Appendix 2

- **Preparation of Calcofluor White Fluorescent stain**

Stock Solution A

Calcofluor white (Fluorscent brightener Sigma)	1g
Distilled water	100 mL

Stock Solution B

Evans blue (Sigma)	0.05g
Distilled water	100 mL

Working solution

Solution A	1 mL
Solution B	9 mL

- **Preparation of NNA**

15gms of agar was dissolved in 1000ml of Phosphate buffer saline (pH 7.2) and autoclaved and poured in sterile petri plates.

- **Preparation of *E.coli* suspension**

A culture of *E.coli* was maintained on nutrient agar plate. One colony of *E.coli* was suspended in 5ml of PBS (pH 7.2) in a tube (Optical density was adjusted to ~ 0.5 McFarland tube opacity). A fresh lot was made daily.

- **UNSET (Urea, Sodium Chloride, SDS,EDTA and Tris) buffer**

Urea	8M
2% Sodium dodecyl sulphate (SDS)	2%
Sodium Chloride	0.15M
EDTA	0.001M
Tris HCl	0.01M

- **PYG medium for axenic culture of *Acanthamoeba***

Proteose Peptone	20g
Yeast extract	2g
Magnesium sulphate .$7H_2O$	0.980g
Calcium chloride	0.059g
Sodium citrate. $2H_2O$	1g
Ferric ammonium sulphate.6 H_2O	0.02g
Potassium dihydrogen phosphate	0.340g
Disodium hydrogen phosphate.7 H_2O	0.355g
Glucose	18g
Dist. water	1000mL

All the chemicals were added in a sterile conical flask and 900ml of distilled water was added to it and mixed till dissolved. While stirring calcium chloride was added and the pH was adjusted to 6.5 ± 0.2.and then volume of the medium made up to 1000ml and autoclaved.

A mixture of antibiotic and anti-fungal agents were filtered through 0.22µm membrane filter and added to autoclaved medium. One mL of medium was inoculated in brain heart infusion broth for sterility check up.

Mixture of antibiotic and anti-fungal included:

Amphotericin	2.5mg
Pencillin	60mg
Gentamicin solution	50µL
Streptomycin	50mg
Sterile dist water	1mL

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Use of 18S rRNA Gene-Based PCR Assay for Diagnosis of *Acanthamoeba* Keratitis in Non-Contact Lens Wearers in India

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Identification of *Acanthamoeba* cysts and trophozoites in ocular tissues requires considerable expertise and is often time-consuming. An 18S rRNA gene-based PCR test, highly specific for the genus *Acanthamoeba*, has recently been reported in the molecular diagnosis of *Acanthamoeba* keratitis. This PCR assay was compared with conventional microbiological tests for the diagnosis of *Acanthamoeba* keratitis. In a pilot study, the PCR conditions with modifications were first tested on corneal scrapings from patients with culture-proven non-contact lens-related *Acanthamoeba*, bacterial, and fungal keratitis. This was followed by testing of corneal scrapings from 53 consecutive cases of microbial keratitis to determine sensitivity, specificity, and predictive values of the assay. All corneal scrapings from patients with proven *Acanthamoeba* keratitis showed a 463-bp amplicon, while no amplicon was obtained from patients with bacterial or fungal keratitis. Some of these amplified products were sequenced and compared with EMBL database reference sequences to validate these to be of *Acanthamoeba* origin. Out of 53 consecutive cases of microbial keratitis included for evaluating the PCR, 10 (18.9%) cases were diagnosed as *Acanthamoeba* keratitis on the basis of combined results of culture, smear, and PCR of corneal scrapings. Based on culture results as the "gold standard," the sensitivity of PCR was the same as that of the smear (87.5%); however, the specificity and the positive and negative predictive values of PCR were marginally higher than the smear examination (97.8 versus 95.6%, 87.5 versus 77.8%, and 97.8 versus 97.7%) although the difference was not significant. This study confirms the efficacy of the PCR assay and is the first study to evaluate a PCR-based assay against conventional methods of diagnosis in a clinical setting.

Acanthamoeba keratitis has been described primarily from developed countries of the world, with several studies suggesting soft contact lens wear as the greatest risk factor. In contrast, the reports from India and other developing countries are few and have mainly been in non-contact lens wearers (10). This low incidence of *Acanthamoeba* keratitis in developing countries may not be a true picture and calls for detailed epidemiological studies. In all probability, the reported low incidence is due to lack of sensitive diagnostic tools, low awareness, and probably the belief that the disease is related mainly to contact lens wear—a factor usually absent in most cases of keratitis from this part of the world (10). Although we have reported a number of cases, our reports were based on microscopy of the corneal scrapings and culture on nonnutrient agar with *Escherichia coli* overlay (10, 11). Between February 1991 and June 2002, we diagnosed and treated 168 cases of *Acanthamoeba* keratitis. In 25 (15%) of 168 cases, microscopy of the corneal scrapings with calcofluor white, Gram, and Giemsa staining was negative and the diagnosis was based on culture. It is well known that direct smear examination procedures provide immediate diagnosis while culture may take 1 to 10 days (average in our series, 3.5 days). Therefore, in the face of negative smears, a delay of several days in diagnosis is involved, thus leading to a delay in instituting specific therapy.

Several investigators have demonstrated the usefulness of molecular methods for detection and identification of *Acanthamoeba* (9). These methods could be suitable for both clinical and epidemiological purposes; therefore, they need to be reliable and sensitive. PCRs with corneal scrapings, corneal epithelial biopsy specimens, and tear samples for diagnosis of *Acanthamoeba* keratitis have shown promising results (6). The technique of fluorescent in situ hybridization has also been successfully employed for the purpose (13).

Of the several primers used heretofore, Schroeder et al. (9) described a PCR assay using 18S rRNA gene (rDNA)-based primers as being most specific for the genus *Acanthamoeba*. They employed the PCR for the detection of *Acanthamoeba* DNA in corneal scrapings from a limited number of patients. This study aims to evaluate this PCR assay in a clinical setting in an ocular microbiology laboratory with a high volume of microbial keratitis patients and compare the results with those of conventional microbiological methods for the diagnosis of *Acanthamoeba* keratitis.

MATERIALS AND METHODS

Reference samples. *A. castellanii* (ATCC 50370) was obtained from American Type Culture Collection, Manassas, Va., and maintained in axenic PYG (proteose-peptone-yeast-glucose) culture. Cultures of bacteria (*Pseudomonas aeruginosa*), fungus (*Aspergillus* spp.), and virus (herpes simplex virus) were clinical isolates from corneal scrapings processed in our laboratory. Human leukocytes were obtained from blood donated by a volunteer.

Patients. All patients seen at L. V. Prasad Eye Institute with suspected microbial keratitis are routinely required to undergo microbiological investigations before institution of therapy. Patients with suspected nonviral keratitis are investigated for the presence of bacteria, fungi or *Acanthamoeba* by using a common protocol that involves collection of corneal scrapings for smears and cultures. We have described these procedures in detail in an earlier publication (5).

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In general, smears of corneal scrapings are routinely examined after staining with (i) potassium hydroxide plus calcofluor white, (ii) Gram stain, and (iii) Giemsa stain, and the results become available within 15 to 30 min. For a pilot study, based on the smear results (later confirmed by culture), corneal scrapings were collected from 30 patients deemed to have either *Acanthamoeba*, bacterial, or fungal keratitis. The corneal scrapings were collected in 1 ml of phosphate-buffered saline, pH 7.2, and stored at −20°C until tested by PCR.

Corneal scrapings were also collected in a similar manner from 53 consecutive cases of suspected microbial keratitis patients seen between March 2002 and June 2002 and stored at −20°C until tested by PCR. Patients with little corneal infiltrate or those who were otherwise uncooperative were excluded from the study as additional corneal scrapings could not be collected.

DNA extraction from reference samples and corneal scrapings. The genomic DNA of *A. castellanii* and few clinical isolates of *Acanthamoeba* (obtained in our pilot study from suspected cases of keratitis) were isolated using the UNSET procedure (4). Briefly, the harvested cells were washed twice using 5 ml of phosphate-buffered saline and resuspended in 0.5 ml of UNSET lysis buffer for DNA isolation. The aqueous lysate was extracted twice with 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was finally precipitated using 0.1 vol of 3 M NaCl and 2 vol of ethanol and resuspended in 50 µl of double autoclaved milliQ water. The extraction of DNA from *P. aeruginosa*, *Aspergillus* species, herpes simplex virus, and human leukocytes followed the procedures described elsewhere (1, 2, 8).

DNA extraction from corneal scrapings was done by a procedure similar to the one described above except that the organic phase extraction was done only once and final DNA was dissolved in only 30 µl of double-autoclaved milliQ water.

PCR analysis. The sequence of the 18S rDNA primer, used in this study, was obtained from Thomas J. Byers (The Ohio State University, Columbus) and consisted of forward primer 5′-GGCCCAGATCGTTTACCGTGAA-3′ and reverse primer 5′-TCTCACAAGCTGCTAGGGGAGTCA-3′. These primer sequences correspond to bp 928 to 949 and bp 1367 to 1390 bp, respectively, of *A. castellanii* ATCC 50374 18S rDNA (EMBL accession no.U07413). The primers were synthesized at the Centre for Cellular and Molecular Biology, Hyderabad, India. All PCRs were carried out in a laminar-flow hood after 30 min of UV irradiation to decontaminate surfaces and all supplies within the hood. Presterilized PCR tubes, double autoclaved milliQ water, and positive-displacement tips and pipettes were used to reduce the possibility of contamination. The primer pairs were tested initially for amplification of *A. castellanii*; clinical isolates of *Acanthamoeba*, *P. aeruginosa*, *Aspergillus* species, and herpes simplex virus; and human leukocyte DNA. The PCR conditions were modified with regard to amplification profile and $MgCl_2$ requirement compared to those described earlier (9). The amplification profile was 94°C for 1 min, 72°C for 1 min, and 72°C for 1 min for 40 cycles followed by a final extension step of 61°C for 5 min. Each 20-µl PCR mixture comprised 3 µl of DNA (in the case of corneal scraping DNA extracts) or ~10 ng of genomic DNA, 200 µM deoxynucleoside triphosphates, a 1 pM concentration of each primer, 1× standard PCR buffer (containing 1.5 mM $MgCl_2$), and 1 U of *Taq* DNA polymerase (Gene *Taq*; MBI Fermentas, Vilnius, Lithuania). These conditions differed from the ones described by Schroeder et al. (9). Amplifications, which were performed in an MJ Research PTC 150 thermocycler. The DNA extracts of corneal scrapings of culture-confirmed *Acanthamoeba* keratitis patients were tested initially in a pilot study. On obtaining satisfactory results, corneal scrapings from consecutive patients with microbial keratitis were tested.

The PCR products were visualized by gel electrophoresis using 1.5% agarose–TAE (Tris-acetic acid-EDTA) gel containing ethidium bromide (0.5 µg/ml), and the results were recorded on a UV gel documentation system (UVItec Ltd., Cambridge, United Kingdom).

DNA sequencing and comparison. Approximately 450 bp of 18S rDNA-specific PCR products obtained from 12 clinical isolates of *Acanthamoeba* obtained in the pilot study from patients with keratitis were sequenced for both strands using a 2 pM concentration of each of the original primers used for amplification and the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, Calif.) as per the manufacturer's details. The sequencing amplification conditions were as follows: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for 30 cycles. After PCR, the products were precipitated using 1 µl of 3 M sodium acetate (pH 4.6) and 50 µl of ethanol and were incubated on ice for 10 min. The pellet was recovered by centrifugation (18,000 × *g* for 20 min at 4°C), washed with 70% ethanol, dried, and dissolved in 10 µl of diluted Hi-Di formamide (Perkin-Elmer, Applied Biosystems, Foster City, Calif.). Sequencing was performed in an ABI PRISM 3700 DNA analyzer. Raw sequences were edited and assembled using the Auto Assembler program. The sequences obtained in the study were used to identify related reference sequences using a BLASTn search, and these sequences were then retrieved from the EMBL database. All the sequences were finally aligned and used to infer the genetic similarities using CLUSTAL-X (http://www-igbmc.u-strasbg.fr/BioInfo/clustal) software.

Statistical analysis. Diagnostic data from corneal scrapings of 53 consecutive patients with suspected microbial keratitis were used for determination of sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of smear examination and PCR results using culture results as the "gold standard." The following formulae were used for calculations: sensitivity = (number of true positives/total number of culture positives) × 100, specificity = (number of true negatives/total number of culture negatives) × 100, PPV = (number of true positives/number of true positives + number of false positives) × 100, NPV = (number of true negatives/number of true negatives + number of false negatives) × 100, FP = (number of false positives/total number of culture negatives) × 100, and FN = (number of false negatives/total number of culture positives) × 100, where the total number of culture positives is the number of true positives plus the number of false negatives, the total number of culture negatives is the number of true negatives plus the number of false positives, FP is the rate of false positives, and FN is the rate of false negatives.

RESULTS

Specificity of 18S rDNA-based PCR. The primers produced *Acanthamoeba*-specific amplicon (463 bp) from *A. castellanii* DNA (ATCC 50370) and clinical isolates of *Acanthamoeba* but not from bacterial, fungal, viral, and human leukocyte DNA.

Double-strand sequencing of the above 463-bp PCR-amplified products confirmed these to be *Acanthamoeba*-specific amplicons. The sequences obtained in the study (EMBL accession no. AF534143 to AF534154) were found to be most similar (95.6 to 100%) to the reference keratitis-associated pathogenic isolates or species of *Acanthamoeba*. In general these showed an average genetic dissimilarity of 0.023 ± 0.015 for the amplified 18S rDNA from those of the reference sequences (AY148954, U07401, AY026249, AF019062, and U07417). Two of the sequences (AF534149 and AF534151) obtained in the study showed 100% similarity to *A. polyphaga* (AF019062) and *Acanthamoeba* sp. isolate U/E7 (AY026249).

Pilot study on corneal scrapings. Corneal scrapings were taken from 30 patients selected on the basis of routine smear and culture results. Twenty-one of 30 (70%) patients were diagnosed to have *Acanthamoeba* keratitis based on detection of cysts in smears and/or growth of *Acanthamoeba* in culture. Three of the 30 (10%) patients had bacterial keratitis, and 6 of the 30 (20%) had fungal keratitis. While the corneal scrapings from all 21 patients with *Acanthamoeba* keratitis showed a 463-bp amplicon in PCR, the scrapings from patients with bacterial and fungal keratitis showed no amplification. A brief summary of the clinical findings, diagnosis, and treatment outcome of 21 patients with *Acanthamoeba* keratitis included in the pilot study is given in Table 1.

Sensitivity, specificity, and predictive values of the 18S rDNA-based PCR assay. Fifty-three consecutive patients with suspected microbial keratitis were included for analysis of the above parameters. These patients were seen between March and June 2002, and after collection of an average of seven multiple corneal scrapings for microbiological investigation, an extra scraping was collected for PCR. Therefore, some patients with small infiltrates could not be included in the study as it was not possible to collect an extra scraping. Table 2 summarizes the demographic details, predisposing factors, and diagnosis of these patients, and Table 3 outlines the results of microbiological investigations and their correlation with PCR results on the corneal scrapings of these patients. Considering combined results of culture, smears, and PCR, 10 cases of

TABLE 1. Clinical findings, diagnosis, and treatment outcome of 21 patients with *Acanthamoeba* keratitis included in preliminary evaluation of the PCR

Characteristic	No. (%) of patients (n = 21)
Demographics	
Males	12 (57.1)
Females	9 (42.9)
Mean age ± SD (yr)	34.3 ± 14.5
Age range (yr)	7–63
Predisposing factors	
Contact lens wear	0
Trauma	6 (28.5)
Foreign body	5 (23.8)
Unknown	10 (47.6)
Detection of *Acanthamoeba* cysts in corneal scraping smears	
Calcofluor white	20 (95.2)[b]
Gram stain	18 (85.7)
Giemsa stain	14 (66.6)
Culture on nonnutrient agar	20 (95.2)[c]
Treatment outcomes	
Healed with medical therapy	11 (52.4)
Surgical intervention	3 (14.3)[a]
Lost to follow-up	7 (33.3)

[a] Interventions: penetrating keratoplasty (n = 1) and evisceration (n = 2).
[b] One sample was smear positive and culture negative.
[c] One sample was culture positive and smear negative.

Acanthamoeba keratitis out of 53 consecutive patients were identified. Culture was positive in eight of these cases. While six out of eight were positive by both smear and PCR, one was smear positive and PCR negative and one was smear negative and PCR positive (Table 4). The two culture-negative cases were positive in smears while PCR was positive in only one of them.

DISCUSSION

In last 11 years, we have diagnosed and treated 168 patients with *Acanthamoeba* keratitis; among whom only one was contact lens wearer. A total of 197 specimens including corneal scrapings and corneal buttons from these patients had been subjected to smear and culture examination (data not shown). Both smear and cultures were positive in 130 (66%) specimens; the smear was positive and the culture was negative in 27 (14%); the smear was negative and the culture was positive in 33 (17%); and both smear and culture were negative in eight (4%) specimens. In cases where the smear was negative and culture was positive (17%), the diagnosis was delayed for a mean of 3.5 days. In cases where both smear and culture were negative (4%), the initial diagnosis was made by having a high clinical suspicion and the final diagnosis was by detecting *Acanthamoeba* cysts by cultures or smear in repeated corneal scrapings or corneal button obtained during penetrating keratoplasty from the same patient.

In order to enhance our diagnostic capability, we decided to use a molecular diagnostic assay that may have an advantage over smear and culture and may be more sensitive and specific, as well as rapid, for the detection of *Acanthamoeba* from clinical samples. Although PCR-based assays for the detection of *Acanthamoeba* in corneal scrapings have been described, none have been tested on patients with non-contact lens-related keratitis. We decided to evaluate the PCR assay (9), which was described to be highly specific and was based on 18S rRNA gene of *Acanthamoeba*. We evaluated this assay against the appropriate positive and negative controls and found it to be specific for *Acanthamoeba* with modifications in the assay conditions. Using the same primers, we also confirmed that the culture isolates from our patients with no history of contact lens wear were indeed *Acanthamoeba*. The specificity of the assay was further confirmed in our pilot study with corneal scrapings from all 21 cases of culture- and/or smear-positive cases of *Acanthamoeba* keratitis yielding positive result in PCR. Convinced of the results of specificity of the assay, we evaluated this test on 53 patients with suspected microbial keratitis. To the best of our knowledge this is the first study to evaluate the sensitivity, specificity, and predictive values of a PCR technique vis-à-vis a smear and culture that form the routine diagnostic tests in most ocular microbiology laboratories for the diagnosis of *Acanthamoeba* keratitis.

Our results show that the sensitivity of PCR was similar (87.5%) to that of smear results, which included three methods of smear examination such as calcofluor white, Gram stain, and Giemsa stain (Tables 3 and 4). The long experience of the microbiologists involved in examination of corneal scraping smears in this laboratory may account for the high positivity of smears. Patient samples 20 and 46 (Table 3) were culture negative but smear positive, while sample number 20 was found to be culture negative but PCR positive. It is possible that the testing of last scraping by PCR led to negative results (in 2 samples out of 10) and the sensitivity would have been higher if it was tested on initial corneal scrapings of the patients rather than the last.

On the other hand, this study found higher specificity and predictive values of the PCR assay compared to smear meth-

TABLE 2. Demographic details, predisposing factors, and microbiological diagnosis in 53 patients with microbial keratitis

Characteristic	No. (%) of patients (n = 53)
Demographics	
Males	38 (71.7)
Females	15 (28.3)
Mean age ± SD (yr)	37.92 ± 19.24
Age range (yr)	2–86
Predisposing factors	
Contact lens wear	0
Trauma	29 (54.7)
Diabetes	1 (01.9)
Leprosy	1 (01.9)
Prior surgery	6 (11.3)
Blepharitis	1 (01.9)
Lagophthalmos	2 (03.8)
Spheroidal degeneration	1 (01.9)
Unknown	12 (22.6)
Microbiological diagnosis[a]	
Bacterial	17 (32.0)
Fungal	14 (26.4)
Acanthamoeba	7 (13.2)
Viral (HSV)	2 (03.8)
Bacterial + *Acanthamoeba*	1 (01.9)
Fungal + viral	1 (01.9)
Sterile (unknown)	11 (20.7)

[a] Based on culture of bacteria, fungi, or *Acanthamoeba* and antigen and/or DNA detection of herpes simplex virus (HSV) in corneal scrapings.

TABLE 3. Correlation of microbiological findings and PCR results of corneal scrapings from 53 consecutive patients[a]

Serial no.	Laboratory no.	Direct smear examination result(s)			Culture result(s)	PCR result
		CFW	Gram	Giemsa		
1	400/02	–	–	–	–	–
2	417/02	–	GPC	Cocci	*Streptococcus pneumoniae*	–
3	424/02	–	–	–	*Pseudomonas aeruginosa*	–
4	429/02	FF	FF	FF	*Aspergillus fumigatus*	–
5	432/02	–	GPC	Cocci	*Streptococcus pneumoniae*	–
6	490/02	Microsporidium	Microsporidium	Microsporidium	ND	–
7	503/02	–	–	–	*Staphylococcus epidermidis*	–
8	512/02	–	–	–	*Pseudomonas aeruginosa*	–
9	513/02	–	GPC	Cocci	*Streptococcus pneumoniae*	–
10	569/02	–	–	–	*Staphylococcus epidermidis*	–
11	570/02	–	–	–	—*	–
12	577/02	–	–	Bacilli	*Pseudomonas aeruginosa*	–
13	603/02	Actinomycetes	Actinomycetes	Actinomycetes	*Nocardia asteroides*	–
14	604/02	–	–	–	–	–
15	607/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* spp.	+
16	632/02	FF	FF	FF	UIHF	–
17	652/02	FF	FF	FF	*Aspergillus niger*	–
18	653/02	FF	FF	FF	*Aspergillus niger*	–
19	654/02	FF	FF	FF	UIHF	–
20	661/02	–	*Acanthamoeba* cysts	ND	–	+
21	710/02	–	GPC	Cocci	*Streptococcus pneumoniae*	–
22	753/02	ND	–	ND	–	–
23	788/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* spp.	+
24	862/02	–	–	–	–	–
25	865/02	–	–	–	*α-Hemolytic streptococci*	–
26	877/02	–	–	–	–	–
27	883/02	Actinomycetes	–	–	*Nocardia asteroides*	–
28	886/02	FF	FF	FF	*Bipolaris* spp.	–
29	889/02	–	GPC	Cocci	*Streptococcus pneumoniae*	–
30	918/02	–	GPC	Cocci	*Streptococcus pneumoniae*	–
31	925/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	–	*Acanthamoeba* spp.	+
32	932/02	FF	FF	FF	*Fusarium* spp.	–
33	953/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* spp.	+
34	956/02	FF	FF	FF	*Aspergillus flavus*	–
35	995/02	FF	FF	FF	UIHF, *Streptococcus pneumoniae*	–
36	1001/02	–	GPC, GNB	Cocci, bacilli	*Pasteurella* spp.	–
37	1004/02	–	–	–	–	–
38	1009/02	FF	FF	FF	*Fusarium* spp.	–
39	1014/02	FF	FF	–	UIHF	–
40	1015/02	FF	FF	FF	*Aspergillus flavus**	–
41	1016/02	FF	FF	FF	*Aspergillus flavus*	–
42	1046/02	FF	FF	FF	*Aspergillus flavus*	–
43	1070/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* spp.	+
44	1132/02	–	GPC	Cocci	α-Hemolytic streptococci	–
45	1148/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* spp.	–
46	1177/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	–	–
47	1201/02	–	–	–	–	–
48	1205/02	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* cysts	*Acanthamoeba* spp.	+
49	1210/22	FF	FF	FF	*Aspergillus fumigatus*	–
50	1224/02	–	GPC	Cocci	α-Hemolytic streptococci	–
51	1226/02	–	–	–	—*	–
52	1243/02	–	–	–	*Acanthamoeba* spp. *Staphylococcus epidermidis*	+
53	1253/02	FF	FF	FF	*Fusarium* spp.	–

[a] Abbreviations and symbols: FF, fungal filaments; GPC, gram-positive cocci; GNB, gram-negative bacilli; UIHF, unidentified hyaline fungus; ND, not done; CFW, calcofluor white; –, negative; +, positive; *, positive for herpes simplex virus antigen and/or DNA.

ods (Table 4), although the difference was statistically not significant. Despite repeated testing, no PCR products were seen in the corneal scrapings from patient 45 (smear and culture positive for *Acanthamoeba*) and patient 46 (smear positive for *Acanthamoeba* cysts and culture negative), which we attribute to the possible lack of *Acanthamoeba* DNA in the sample. The possibility of PCR inhibitors was ruled out in these two samples by spiking them with *Acanthamoeba* DNA and retesting them. Low DNA yield in clinical samples is known to affect the success of PCR, especially if only mature cysts are present (9).

Apart from high sensitivity, this study demonstrates high specificity and high PPV and NPV of the PCR assay, all of which are hallmarks of a good diagnostic test. Unlike smear and culture techniques that require familiarity with the morphology of cysts and trophozoites of *Acanthamoeba*, while it does require the proper facilities, PCR does not require such expertise. Any laboratory with a molecular biology set up can

TABLE 4. Summary of smear and PCR testing of corneal scrapings for the diagnosis of *Acanthamoeba* keratitis in comparison to culture-based detection[a]

Diagnostic test result	Culture positive				Culture negative			
	No. of scrapings	SENS (%)	PPV (%)	FN (%)	No. of scrapings	SPEC (%)	NPV (%)	FP (%)
Smear positive	7	87.5	77.8		2	95.6	97.7	4.4
Smear negative	1			12.5	43			
PCR positive	7	87.5	87.5		1	97.8	97.8	2
PCR negative	1			12.5	44			

[a] Abbreviations: SENS, sensitivity; SPEC, specificity; FN, false-negative rate; FP, false-positive rate.

easily adopt the PCR assay used in this study. Moreover, smear examination techniques, especially calcofluor white, are known to miss trophozoites, and a sample with only trophozoites is likely to be labeled as smear negative (12, 13). In this study, a combination of smear and culture provided diagnoses in similar numbers of cases as smear and PCR. There are not many ocular microbiology laboratories that employ multiple smear examination protocol for corneal scrapings. Moreover, the availability of fluorescence microscopes, required for observation of smears stained with calcofluor white, is also not very common owing to its high cost. Under these constraints, clinical diagnosis based on only smear positivity using only Gram or Giemsa stain is likely to be much lower, as is evident from Table 1. Comparatively, the PCR technique can be easily added to the armamentarium of diagnostic methods in a microbiology laboratory. Additionally, the short time taken by the PCR test is a distinct advantage over the culture method. Hence, we strongly believe that the PCR based diagnostic assay, coupled with smear examination, will be very helpful and desirable for rapid diagnosis of *Acanthamoeba* keratitis and be confirmatory in clinically suspected cases with or without culture results.

The high clinical value of PCR in the diagnosis of *Acanthamoeba* keratitis has already been shown by Lehmann et al. (6), who found a sensitivity of 84%, which is similar to ours (87.5%), although they used clinical diagnosis as the gold standard as opposed to culture, which was the gold standard in this study. Evaluation of PCR for *Acanthamoeba* in consecutive cases of suspected microbial keratitis, as done in the present study, has further confirmed the applied value of molecular diagnosis in *Acanthamoeba* keratitis. A novel approach by Mathers et al. (7) of confirming confocal microscopy diagnosis of *Acanthamoeba* keratitis in contact lens wearers using PCR highlights the wide scope of utility of PCR assays. Based on their findings, the authors have speculated that *Acanthamoeba* may be responsible for a large percentage of cases that are commonly diagnosed as contact lens overwear. They have alluded to the possible association of *Acanthamoeba* with many forms of corneal epitheliopathy. Similar associations, which may or may not be related to contact lens wear, are expected to emerge in our setup with improved diagnosis using PCR test for *Acanthamoeba*. Such findings may lead to a revision of the incidence and prevalence data of the ocular or extraocular diseases caused by *Acanthamoeba* species in this part of the world.

Vodkin et al. (15) were the first to use PCR for the genus-specific detection of *Acanthamoeba*, using primer pair ACARNA.for1383 and ACARNA.rev1655, which amplifies 272 bp of 18S rDNA. This primer pair was also tested by Lehmann et al. (6) in their clinical study, along with a second 18S rDNA-based primer pair, P1GP.for2379 and P1GP.Rev2632, which amplifies a 253-bp amplicon. Analyzing complete 18S rDNA sequences of over 80 isolates of *Acanthamoeba*, Schroeder et al. (9) have shown that the above two primer pairs could also amplify rDNA of related amoebae, i.e., *Balamuthia* and *Hartmanella* spp. The primers used in our study (JDP1-JDP2) were designed by them from a large database of 18S rDNA sequences and were shown to be genus specific for *Acanthamoeba*. These primers had failed to amplify DNA from closely related amoebae and from several bacterial, fungal, and human DNAs. Although Schroeder et al. (9) used different PCR conditions for achieving high sensitivity and specificity in their study, in this study we have employed only one set of PCR conditions that provide both high sensitivity and specificity. Stothard et al. (13), using genus- and subgenus-specific oligonucleotide probes, have shown the specific identification of *Acanthamoeba* in both environmental and clinical samples. Therefore, in our opinion, it would be interesting and gainful to integrate such new PCR techniques in ocular microbiology laboratories dealing with large number of patients with microbial keratitis.

Inclusion of PCR for *Acanthamoeba* along with conventional methods of diagnosis of nonviral microbial keratitis is expected to improve the diagnosis of *Acanthamoeba* keratitis in ocular microbiology laboratories that have molecular biology facilities. While awareness regarding bacterial and fungal keratitis is relatively high in a majority of the eye hospitals in India, as reflected in several publications, information regarding *Acanthamoeba* keratitis is grossly inadequate. The reported incidence of *Acanthamoeba* keratitis in India varies from 1 to 3%, and the cases are predominantly in non-contact lens wearers (3, 10, 14). Through this report, we would like to emphasize that with appropriate tests a greater number of cases of *Acanthamoeba* keratitis can be differentiated from bacterial, fungal, or viral keratitis and treated appropriately before it is too late.

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9

Acanthamoeba Keratitis—Pathogenesis

INTRODUCTION

Bacterial and fungal keratitis in Indian patients are well documented in the literature.[1-3] In contrast, *Acanthamoeba* keratitis is a relatively recent development. The largest series describing clinical and laboratory findings of *Acanthamoeba* keratitis in 38 patients was published from our institute.[4] The disease is currently being diagnosed in many eye centres in India. It is that about 1.5 to 4 per cent of laboratory proven infective keratitis in India is caused by *Acanthamoeba*, majority of the cases do not wear contact lens.[4,5]

Diagnostic tests for detection of *Acanthamoeba* in corneal scrapings are simple and can be adapted by any microbiology laboratory with facilities for smear examination and culture. All medium to large size ophthalmology setups can easily incorporate procedures for the diagnosis of *Acanthamoeba* keratitis in the laboratory. However, basic research related to *Acanthamoeba* is confined to large tertiary eye care centres with research facilities. Efforts have been made to achieve molecular typing of *Acanthamoeba* isolated from Indian patients and also to develop molecular diagnostic methods, which are highly sensitive and specific, for the diagnosis of *Acanthamoeba* keratitis.[6] Some studies have focussed on the tissue reaction and pathogenesis of *Acanthamoeba* in the cornea.[7]

At present times, much is known about the epidemiology, risk factors, pathogenesis, genetics, clinical features and treatment of *Acanthamoeba* and the keratitis caused by it. It is beyond the scope of this chapter to discuss recent advances in all aspects. Therefore, this chapter is confined to advances made in recent times with respect to classification, molecular typing, pathogenesis and diagnosis of *Acanthamoeba* keratitis.

Classification and Molecular Typing

Although the genus *Acanthamoeba* was first established in 1931, considerable confusion about its taxonomic classification existed in the literature until recently. Volkonsky divided the existing genus *Hartmanella* into 3 genera i.e., *Hartmanella, Glaeseria and Acanthamoeba*.[8] In 1975, Visvesvara and Balamuth identified definable and demonstrable differences in the trophozoite and cyst stages of *Acanthamoeba* and *Hartmanella*.

Classification of *Acanthamoeba* at the genus level is relatively clear, but since it is an asexual organism, the concept of the species is unclear. Many approaches have been used for the subgenus classification of *Acanthamoeba*, which mainly include classification based on: (i) morphology of the cysts, (ii) isoenzyme electrophoretic patterns, (iii) mitochondrial restriction fragment length polymorphism (mtRFLP), iv) sequencing of nuclear and mitochondrial genes, and (v) riboprinting.

In 1977, Pussard and Pon proposed the classification based on the morphology of cysts. They established 18 different species in 3 distinct groups (Table 9.1).[8]

Table 9.1: Showing classification of *Acanthamoeba* based on morphology of cysts

Group I	*Group II*	*Group III*
A.astronyxis	*A.castellanii*	*A.culbertsoni*
A.comanodoni	*A.rhysodes*	*A.royreba*
A.echinulata	*A.mauritaniensis*	*A.palestinensis*
A.tubiashi	*A.divionensis*	*A.lenticulata*
	A.griffini	*A.pustulosa*
	A.polyphaga	
	A.lugdunensis	
	A.quina	
	A.triangularis	
	A.hatchetti	

The systematic classification of *Acanthamoeba* based on cyst morphology has been deemed ambiguous and vague. It can define an isolate up to the genus level but variations occur in cyst forms within the species and clonal population, this fact makes classification using morphology as very subjective process. Also this system does not show genetic relationship between the strains.[9] Ionic strength of the growth medium is said to alter the shape of the cyst walls, thus substantially reducing the reliability of cyst morphology as a taxonomic characteristic.[10]

In last decade several groups have used analysis of isoenzyme electrophoretic patterns to address intrageneric relationship and to test morphological classification.[11] Zymograms of acid phosphatase, leucine aminopeptidase, malate dehydrogenase, propionyl esterase, glucose phosphate isomerase, phosphoglucomutase and alcohol dehydrogenase suggested changes in the taxonomy within the morphology Group 2 of Pussard and Pon.[11] However, the drawback of this method of classification was that different zymoderms might exist within a species, which suggested that neither isoenzyme pattern nor morphological analysis be used alone for subgenus classification. Several studies divided isolates of *Acanthamoeba* into different groups that often were inconsistent with species and/or morphological group designations.[12]

Since classification at species level had been difficult and the taxonomic designations of a number of strains were in doubt, new approaches for classification were needed. Byers *et al* used electrophoretic patterns obtained with restriction enzyme digest of mitochondrial DNA (mtRFLP) as basis for new approach.[13] They found relatively high degree of molecular diversity among strains classified as a single species. Similar results were obtained by others.[14,15]

Eukaryotes have both cytoplasmic and mitochondrial ribosomes. Both types of ribosomes consist of large subunit (LSU) and small subunit (SSU) ribonucleoproteins. Rns are the nuclear genes coding for 18S rRNA found in the cytoplasmic SSU while rns are mitochondrial genes coding for 16S rRNA found in mitochondrial SSU.[16] Gast *et al* in 1996 have proposed four distinct sequence types based on analysis of complete sequences of nuclear ribosomal subunit RNA genes (Rns) from 18 strains. They were designated as sequence types T1-T4.[12] Tl included *A. castellanii* V006, T2 included *A. palestinensis* Reich, T3 included *A. griffini* S7, while T4, the fourth sequence type included 15 isolates classified as *A. castellanii, A. polyphaga, A. rhysodes* and 10 other isolates of *Acanthamoeba* obtained from keratitis patients. They found that T4 has a worldwide distribution, since isolates from Asia, Europe and North America belonged to this group. Data also indicated that T4 includes representatives of three different species *A.castellanii, A.polyphaga* and *A.rhysodes*. This classification confirmed the inconsistencies of the morphological classification. Nevertheless, even this classification was insufficient for full phylogenetic resolution of branching orders within the T4/sequence type. Booton *et al* analyzed rns sequences (16S rDNA) of 68 strains of *Acanthamoeba*.[17] The phylogeny based on mitochondrial rns sequences was mostly consistent with that observed with nuclear Rns DNA.

Since it is known that nuclear rRNA sequences are useful for identification and differentiation of *Acanthamoeba* isolates, Chung *et al* subjected 23 reference strains of *Acanthamoeba* for classification at the subgenus level by riboprinting i.e., PCR/RFLP analysis.[18] The dendrograms based on riboprints coincided well with grouping based on morphology of cysts and that with dendrogram constructed by Stothard *et al*[10] which is based on rRNA gene sequences.

Pathogenesis

The low incidence of *Acanthamoeba* keratitis, despite its widespread prevalence in nature, can be due to at least two mutually compatible explanations: first that *Acanthamoeba* is a weak pathogen and second there is high degree of innate host resistance against it. The mechanisms involved in corneal tissue damage and invasion by the amoeba are poorly understood, especially those related with early events of amoebae-cornea interaction. Studies on the host immune response to *Acanthamoeba* infection are very limited because only corneal transplantation specimens are available for study and such patients have in most cases been intensively treated with anti-inflammatory agents prior to surgery. Thus, most reports describe the late stages of the disease probably modified by drug therapy.[19-21] Several animal models[22-26] and short-term primary cultures of corneal cells[27] have been used to study *Acanthamoeba* keratitis.

Disadvantages of these methods include lack of reproducibility between experiments and the need to sacrifice animals on a regular basis.[28] Noncorneal cell lines have also been used for *in vitro* studies of the pathogenicity of *Acanthamoeba*,[29] but these studies yield data which are not specific to cornea-pathogen interaction.[14] Recently, primary cultures[30] and immortalized human corneal epithelial cell lines[31] have been developed and they are more characteristic of the *in vivo* situation.

Some authors believe that initial insult to the cornea in form of trauma, chemicals, organic matter, insect or microtrauma because of contact lens wear is required for the infection to occur.[32,33] On the other hand Omana-Molina *et al* in their study on Chinese hamsters have described that *Acanthamoeba* species is capable of producing damage to intact hamster cornea without producing a previous artificial lesion.[34] Once the amoeba is present on the cornea, an important first step in the infectious cascade of *Acanthamoeba* keratitis is its binding to the corneal epithelium. Thus, *Acanthamoeba* keratitis occurs in a sequential manner and is initiated by the pathogens' adherence to the host cells, followed by invasion of the corneal

stroma.[35] Adherence and penetration may be the two step process necessary for *Acanthamoeba* to establish corneal infection.[36] In the initial stages of adhesion, cytoplasmic projections or acanthopodia of the trophozoites come in contact with the superficial cells of the cornea. Soon after trophozoites adhere completely and separate the cell junction of the corneal epithelial cells and eventually desquamate them.[34] Trophozoites can adhere more intensely with the epithelial surface, thus trophozoites are probably more important than the cysts in initiating human corneal disease.[37]

Studying which human corneal constituent acts as substrate for acanthamoebic growth will definitely lead to a better understanding of the pathogenesis of *Acanthamoeba* keratitis.[38] Yang *et al* have demonstrated that corneal epithelium expresses *Acanthamoeba* reactive mannose glycoprotein and the parasites express a mannose-binding protein.[39] Therefore, the authors propose that one mechanism of *Acanthamoeba* adhesion to the corneal surface involves interaction between the mannose binding protein of the amoebae and mannose glycoprotein of the corneal epithelium.[39] Leher *et al* were in agreement with the above investigators and also found that engagement of the mannose receptors induces the release of serine protease which mediates contact independent cytolysis of corneal epithelial cells.[35] Their study implied that the adherence of trophozoites to corneal epithelial cells is essential for initiating the cytolytic machinery of *Acanthamoeba* but is unnecessary once the mannose receptor is engaged. Authors proposed that *Acanthamoeba* trophozoites are capable of mediating both contact dependent and contact independent cytopathic effect. The mannose receptor is crucial for both these processes.[35] Studies on rabbit corneal epithelial cell (SIRC) lines suggested that adherence of *Acanthamoeba* to the monolayer of cells is time and temperature dependent process and interstrain differences in adherence suggest that adherence of *Acanthamoeba* may correlate with observed variation in the rate of progression and virulence *in vivo*.[40]

After adhering to corneal epithelial cell amoebae require cellular elements for its sustenance. The cell surface of the *A.castellanii* is a highly specialized region that is not active in the active transport of solutes, but is involved directly in the uptake of nutrients by endocytosis, membrane fusion events and cell motility.[37] It feeds on complex macromolecules found most commonly in living cells for its nutrition. *Acanthamoeba* feeds directly on the dense cellular epithelial layer causing disruption and eventually there is access to the corneal stroma which provides further nutritional support through

its keratocytes. This plentiful food supply allows the organism to subsist in the stroma for a long period of time.[38]

Moore *et al* suggested that the trophozoites of *A.castellanii* use two methods of penetration in entering human corneas *in vitro*. The first method involves the secretion of material, which mainly includes enzymes, that interferes with the junction of the surface squamous epithelium. *Acanthamoebae* are known to have several enzymes that include ribonucleases, phosphatase, proteinase, α-glucosidase, β-N-acetylglucosaminidase and β-glucuronidase.[36] Plasma membrane of *Acanthamoeba* has enzymes like phospholipase A, lysophospholipase, acetyl Co-A hydrolase, palmitoyl Co-A synthetase,[41] alkaline phosphatase and 5'-nucleotide activities and Mg++ adenosine.[41] Acyl Co-A:lysolecithin acyltransferase, CDP choline: 1,2-diacylglycerolcholinephosphotransferase are present in the microsomal fraction.[41] Thompson and Shultz reported substantial levels of two phospholipases, glucose-6-phosphatase and 5'-nucleotidase in both rough and smooth endoplasmic reticula but found that NADPH cytochrome C reductase and rotenone insensitive NAPH cytochrome C reductase are present only in smooth surface membrane.[42] Moore *et al* suggested that rough ER plays an important role in elaborating substances that break the desmosomes of the squamous epithelium.[36] Because of the enzymatic action, trophozoites separate adjacent surface cells, extend pseudopodia into the separated area and move under the surface of the epithelium without causing damage to overlying cells. This finding may explain the clinical signs of stromal infiltrates without associated epithelial signs.[36] Apart from many enzymes, collagenase was also attributed to pathogenicity of *Acanthamoeba*, since collagenase from the axenic cultures of *A.castellanii* digested collagen shields and purified type I collagen *in vitro*. This finding further suggests that the stromal degradation in *Acanthamoeba* keratitis may be caused by parasite derived collagenase.[43]

Antibodies to free living amoebae have been reported to prevent their adhesion and spread. Antibodies also inhibit phagocytic property of amoebae and promote neutrophil-mediated killing of amoeba.[44]

Based on results of a histopathological study of 30 cases of *Acanthamoeba* keratitis four stage pathogenetic sequence of events after initial breaching of the epithelium by *Acanthamoeba* have been described.[19] They are:

Stage I Initial Infection

Initial infection involves breaching of the surface epithelium. At this stage, there is no inflammatory response, because intact amoebae do

not induce inflammatory response. Hence at this stage opsonization of the parasite by antibody and complement must be occurring.

Stage II Keratocyte Depletion

Keratocyte depletion occurs in the second stage of the infection which is seen in anterior part of stroma. Kertocyte loss is independent of inflammatory cell infiltration and is in consequence of their being consumed by the trophozoites as suggested by the *in vitro* studies of Larkin and colleagues. However, keratocyte loss in deeper stroma (independent of inflammatory response) was also reported to be due to apoptosis.[7]

Stage III Inflammatory Response

The composition of the inflammatory response is predominantly neutrophils with some macrophages. Garner found dearth of lymphocytes and plasma cells and attributed this to absence of stromal vascularization and consequent barrier to invasion by relatively immotile cells.

Stage IV Stromal Necrosis

Garner observed reduced thickness of stromal collagen which was accompanied by acute inflammatory cell infiltration. He attributed lysis of stromal collagen to enzymes released by neutrophil and other collagenolytic activity. There was minimal or no neutrophil infiltration in this stage.[19]

Stewart *et al* in their study showed that macrophages demonstrate a strong chemotactic response to *Acanthamoeba* and can directly kill trophozoites *in vitro*.[45] van Klink *et al* in their experiment on Chinese hamster selectively depleted out macrophages with liposomes containing dichloromethylene diphosphonate (C12MDP-L1P) and found that macrophage depletion affected the incidence, severity and chronicity of keratitis.[46] The absence of macrophages in corneal biopsy specimens and corneal button form penetrating keratoplasy patients was explained by these authors. They pointed out that all the previous histopathologic studies on human corneal specimens and in experimental animals had been done on later stages of the disease and not during the acute phase. They believed that macrophages serve as an important barrier to corneal infection and exert their effect by preventing the initiation of infection and appearance of clinical signs. Thus, they suggested that macrophages

act as a first line of defense and eliminate significant number of *Acanthamoeba* trophozoites.[46]

Neutrophils kill amoeba only when they are activated by lymphokines. In addition, these altered neutrophils cannot act in the absence to complement or antibody. Therefore, combined action of lymphokines, complement and antibody is needed for killing mechanism of neutrophils. Neutrophils killing mechanism involves both the oxidative respiratory system and enzyme which are found in its azurophilic granules such as myeloperoxidase. T-cells and macrophage cytokine augment both oxidative respiratory system i.e., respiratory burst and release of lysosomal enzymes from neutrophils. TNFα is responsible for significant stimulation of the respiratory burst (NADPH oxidase activation) which results in the production of oxygen derived reactive species and the release of azurophilic granules.[44] Thus macrophages and neutrophils are involved in pathogenesis of *Acanthamoeba* keratitis and this indicates that innate immune system plays an important role in controlling the infection.

Diagnosis

Early diagnosis is critical to the outcome of *Acanthamoeba* keratitis. The clinical features, although occasionally pathognomonic, may be misleading. A number of reports have dealt with the clinical diagnosis of *Acanthamoeba* keratitis,[47,49] however, misdiagnosis due to resemblance with viral and fungal infection is common.[4] Corneal or conjunctival swabs are not useful for the diagnosis of *Acanthamoeba* keratitis.[50] Laboratory diagnosis is highly rewarding with corneal scraping or corneal biopsy specimen.

With the availability of confocal microscope, *Acanthamoeba* cysts may be visualized in the cornea of the patient. Tandem scanning confocal microscopy is a non-invasive technique and allows *in vivo* diagnosis of *Acanthamoeba* keratitis. High contrast images of coronal corneal sections containing trophozoites or cysts are visualized on a video monitor. While trophozoites may be mistaken for inflammatory cells, the characteristic morphology of cysts can be well appreciated.[51-53]

Conventional Laboratory Procedures

Diagnosis of *Acanthamoeba* corneal infections is not very difficult since the procedures are within routine laboratory techniques for bacteria and fungi with minor modifications. Two protocols have been described for the investigation of infective keratitis—clinically

viral and nonviral keratitis.[54] The protocol for nonviral keratitis includes a combination of smears and cultures of corneal scrapings which would allow diagnosis of bacterial, fungal and *Acanthamoeba* keratitis. The procedure is described in detail in our earlier publication.[54] While smears stained with calcofluor white or Gram stain provide early diagnosis, culture confirmation on non nutrient agar with *Escherichia coli* may take 1-3 days. The medium, however, should be incubated for up to two weeks before concluding a negative culture.[55]

An alternative to non-nutrient agar has been described. Corneal scrapings may be inoculated in flat-bottomed tissue culture flask containing a suspension of *Escherichia coli* in 1/4 Ringer's solution (3×10^8/mL determined by optical density). The flask is incubated at 37°C and examined daily using an inverted microscope for trophozoites.[55] Samples may be transported in phosphate buffered saline to a distant laboratory without adversely affecting the survival of cysts in the corneal scraping.

Acanthamoeba may grow on media such as blood agar and chocolate agar. However, these media are not recommended for the diagnosis of *Acanthamoeba* although they are included for exclusion of bacterial or fungal aetiology of keratitis.

Corneal biopsy material may be processed similar to corneal scrapings if the corneal stromal infiltrates are deep. Most staining procedures such as calcofluor white, Gram, Giemsa, fluorescein conjugated lectin, haematoxylin and eosin delineate the cyst of *Acanthamoeba* very well showing the characteristic morphology of polygonal, double walled structure with central nucleus.[56-58] Trophozoites, on the other hand, may be difficult to distinguish from inflammatory cells.[59,60] Immunostaining with either indirect fluoroscent antibody[61,62] or immunoperoxidase technique[60,63] have been described. These stains can be used on corneal scrapings as well as corneal tissue sections. Apart from haematoxylin and eosin stain, histopathology sections can also be stained with periodic acid-Schiff, Masson's trichrome stain and Gomori methenamine silver stain for the demonstration of *Acanthamoeba* cysts and trophozoites, although it may be difficult to differentiate trophozoites.

Molecular Methods of Diagnosis

A number of molecular methods for the diagnosis of *Acanthamoeba* keratitis have been described. Some of these methods have been concurrently used for subgenus classification of *Acanthamoeba*. Vodkin *et al* were the first to use polymerase chain reaction (PCR) for the

genus specific detection of *Acanthamoeba* using primer pair which amplified 272 bp of 18S rRNA gene (18S rDNA).[64] This primer pair was tested by Lehmann *et al* in a clinical study.[65] These authors also used a second set of 18S rDNA based primer which amplified 253 bp amplicon. They found PCR to be more sensitive than culture of corneal epithelial samples, and the use of two different primers achieved better sensitivity than a single set.[65] They succeeded in identifying *Acanthamoeba* DNA in tear samples of patients with *Acanthamoeba* keratitis and concluded that PCR of tear samples may complement the results of PCR with corneal epithelial samples,[63] especially in culture negative cases.

Schroeder *et al* have reported a detailed analysis of 18S rDNA sequences of 80 isolates of *Acanthamoeba* and described another primer pair (JDP1-JDP2) that was shown to be highly genus specific.[66] They demonstrated a cross-reaction of the primers used by Lehmann *et al* to that of related amoebae such as *Balamathia* and *Hartmanella* species. JDP1-JDP2 primer pair has been recently evaluated in a clinical study for its sensitivity in the diagnosis of *Acanthamoeba* keratitis. This study compared the PCR assay with conventional microbiological tests for the diagnosis of *Acanthamoeba* keratitis and the results confirmed the efficacy of PCR assay, although the sensitivity was equal to that of smear examination of corneal scrapings.[6] Genus and subgenus specific fluoroscent oligonucleotide probes for detection of *Acanthamoeba* in clinical specimens and cultures have also been described.[67]

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CURRENT PERSPECTIVES ON *Acanthamoeba* KERATITIS IN INDIA: PREDISPOSING FACTORS, MICROBIOLOGY, HISTOPATHOLOGY AND GENETIC CHARACTERIZATION.

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ABSTRACT

This study reports clinical, microbiological, histopathological and molecular features of *Acanthamoeba* keratitis seen at a tertiary eye care center in southern India. Beginning 1995, all patients diagnosed to have *Acanthamoeba* keratitis on the basis of positive smears or culture of corneal scrapings were included in the study. Medical records, microbiological data and histopathological data were retrospectively analyzed. Selected number of isolates, were used for molecular characterization.

Between January 1995 and May 2003, a total of 173 patients were diagnosed to have *Acanthamoeba* keratitis. Only one out of 173 (0.6%) patients had a history of contact lens wear while trauma to cornea and/or washing of eyes with contaminated water was the risk factor in majority of the other patients. Calcofluor white, Gram and Giemsa staining of corneal scrapings established the diagnosis in 89%, 81% and 73% of cases respectively. Monoxenic culture was positive in 81% cases. All patients were treated with 0.02% polyhexamethylene biguanide and /or chlorhexidine. Eighteen out 173 (10%) patients required penetrating keratoplasty/evisceration. Histopathology of corneal buttons revealed necrotising stromal inflammation in most cases. In five cases a rare observation of granulomatous inflammation with a immunophenotype of T cell, CD 68 marker positive and B cell negative was made. Thirteen amoebic isolates from non-contact lens associated keratitis along with several reference strains were analyzed for nucleotide variation in partial or complete 18S rRNA gene.

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Phylogentic analysis showed that all isolates were *Acanthamoeba* carrying T4 genotype signature sequences. Further comparison revealed that partial sequencing was sufficient to distinguish closely related strains of *Acanthamoeba*.

Key words: Genotyping, Granulomatous, Non-contact lens wearers.

INTRODUCTION

Free living amoebae belonging to the genus *Acanthamoeba* are the causative agents of granulomatous amoebic encephalitis (GAE), a fatal disease of the central nervous system, and amoebic keratitis, a painful sight-threatening disease of the eyes (Auran *et al*, 1987). *Acanthamoeba* keratitis (AK) has been described primarily from developed countries with studies suggesting contact lens wear as the greatest risk factor. In contrast, the reports from India and other developing countries are few and have mainly been in non-contact lens wearers (Sharma *et al*, 2000). Studies addressing both clinical and basic aspects of AK in non-contact lens wearers are lacking in the literature.

Early diagnosis and specific therapy is critical to the outcome of AK and laboratory diagnosis is said to be highly rewarding with corneal scrapings (Sharma *et al*,2002). Despite availability of effective medical therapy (Illingworth *et al*,1995) it is well known that in non-responsive severe cases, surgical intervention in the form of penetrating keratoplasty or evisceration is undertaken (Cohen *et al*,1987). The histologic changes observed in *Acanthamoeba* keratitis include, epithelial ulceration, stromal inflammation, necrosis, presence of cysts and trophozoites of *Acanthamoeba* (Garner *et al*, 1993).

In this study, we are presenting our experiences with various aspects of AK seen over a period of eight and half years in a tertiary eye care center in southern India. We describe the clinical, microbiological and histopathological features of the cases seen by us. In addition, we attempted to determine the genotype of some of our isolates.

The sub-genus classification of *Acanthamoeba* has had a confusing evolution wherein the earliest classification was based on the morphology of the *Acanthamoeba* cysts/trophozoites (Pussard *et al*, 1977). This classification method has long been discarded by many researchers and has been replaced by much more reliable and consistent molecular typing methods, especially the DNA sequencing methods (Gast *et al*, 1996, Stothard *et al*, 1998). 18S rDNA typing has mainly been done with *Acanthamoeba* isolates from contact lens wearers and the genotype assigned to most of them is T4.(Gast *et al*, 1996, Stothard *et al*, 1998). In this study, we genotyped amoebic isolates from corneal scrapings of keratitis patients with no history of contact lens wear. Using 18S rRNA gene typing we compared complete and partial sequences of the gene.

MATERIALS AND METHODS

Samples included in the study

A total of 173 cases diagnosed from January 1995 till May 2003 were included in the study.

Medical records of the patients were reviewed for the clinical data. Similarly, the microbiological data of all 173 cases were retrospectively analyzed. Eighteen of 173 cases who underwent penetrating keratoplasy/evisceration were included for histopathology study and the slides were reviewed. Genotyping was carried out with 13 amoebae isolated from corneal scrapings of patients with no history of contact lens wear (table 1).

Table 1. *Acanthamoeba isolate included in the study for 18S rDNA genotyping*

S.No	Laboratory No.	Morphological group	Accession No
1	L402/97	I	AF534143
2	L565/97	II	AF534144
3	L773/96	I	AF534145
4	L1060/96	II	AF534146
5	L407/95	II	AF534147
6	L473/00	II	AF534148
7	L749/98	I	AF534149
8	L1019/99	I	AF534150
9	L1002/99	I	AF534151
10	L1035/99	III	AF534152
11	L1629/99	III	-
12	L98/00	I	AF534153
13	L478/00	I	AF534154

Routine microbiological techniques

Corneal scrapings from all patients had been subjected to smear examination by three methods, viz., potassium hydroxide with Calcofluor White (CFW), Gram and Giemsa stains and culture on media for bacterial and fungal growth along with nonnutrient agar with live *Escherichia coli* overlay for growth of *Acanthamoeba*. Corneal buttons, whenever available for microbiologic studies, were processed for bacteria, fungi and *Acanthamoeba* by culture of tissue homogenate on blood agar, chocolate agar, brain heart infusion broth , Sabouraud dextrose agar and nonnutrient agar with *E.coli*.

Histopathology

Keratectomy/eviscerated material was fixed in 10% buffered formalin. Multiple sections of 5μ thickness were cut from paraffin embedded tissues. Sections were deparaffinized by placing the slides in the oven at 51°C for 1 hour, followed by immersion in xylene and hydration in decreasing ethanol concentration. Hematoxylin - eosin staining, periodic acid Schiff and Gomori's methenamine silver staining were performed on these tissue sections. Immunohistochemistry was performed using monoclonal mouse anti-human antibodies (Dako, Denmark) against, T cell CD 3, Macrophage CD 68 and B cell CD 20 antigen.

Typing of Amoebae

Morphological

Thirteen amoeba isolates were classified based on the trophozoite and the size and shape of cysts into three morphological groups (Visvesvara *et al*, 1991 and Page *et al*, 1967).

18S rDNA based

DNA analysis was done for 13 isolates from corneal scrapings of patients with no history of contact lens wear. All isolates were grown axenically as monolayers in PYG medium. For rDNA analysis, total DNA was extracted from the amoebic cultures using the UNSET lysis buffer method (Hugo *et al*, 1992).

All 13 amoeba isolates were amplified using ASA.S1 (*Acanthamoeba* specific amplimer S1) primers specific for 18S rDNA that were obtained from Dr. Thomas J Byers (Ohio State University, Columbus, Ohio, USA) and consisted of forward primer 5'-GGCCCAGATCGTTTACCGTGAA-3' and reverse primer 5'-TCTCACAAGCTGCTAGGGGAGTCA-3' . These primer sequences correspond to nucleotide positions 928-949 and 1367-1390 bp, respectively, of *A. castellanii* ATCC 50374 18S rDNA (EMBL acc. No.U07413). PCR was carried out as described previously (Pasricha *et al*, 2003). Sequencing of the PCR products was done with DNA sequencer ABI PRISM 3700, following manufacturer's protocol. Seven out of 13 isolates were sequenced for complete 18S rRNA gene by Dr. Gregory C Booton, Ohio State University, Columbus, Ohio, USA. Both the data were analyzed against the same set of reference strains representing all (T1-T12) genotypes for *Acanthamoeba*. Sequences obtained were aligned with those of reference sequences (identified by BLAST search and retrieved from the EMBL database) using CLUSTAL-X (http://www-igbmc.u-strasbg.fr/BioInfo/clustal). Aligned sequences revealed that genotype 7, 8, 9 were very distant from the remainder of *Acanthamoeba* genotypes, therefore, they were not included in further analysis.

The sequence data from 13 amoeba isolates were used to construct tree and infer the genetic identities. To ascertain the confidence values of the rDNA sequence based inferences regarding the genetic identities, sequence data was subjected to bootstrap analysis. For the purpose, the aligned sequences were resampled 100 times using SEQBOOT software and each of the resampled data set was then used to compute genetic distances (DNADIST) and consensus distance tree showing genetic relationships using UPGMA (Unweighted pair group method using Arithmetic averages) followed by CONSENSE programs. All programs were done using PHYLIP package 3.6 (http://evolution.genetics.washington.edu/phylip.html).

In order to compare the capability of partial vs complete sequencing to generate phylogenetically informative data, distances were calculated from the aligned sequences of 7 amoebae isolates for which complete and partial sequences were available. Distances were obtained using DNADIST program using Kimura 2 parameter model and average percent dissimilarity values were calculated without the removal of gaps, intronic regions or unambiguous positions within the sequence.

RESULTS

Predisposing factors

Only one of the 173 cases (0.6%) of *Acanthamoeba* keratitis had history of contact lens wear. Common risk factors associated with AK were history of definite trauma with vegetable matter, stone, dust or washing of eyes with contaminated water.

Microbiological techniques

Complete microbiological data consisting of KOH/CFW, Gram, Giemsa and culture was available in 166 out of 173 patients seen by us. Seven cases with one or more missing data were excluded from the analysis. Acanthamoeba cysts were detected in corneal scrapings by KOH/CFW in 147 (89%) , by Gram stain in 135 (81%) and by Giemsa stain in 122 (73%). Culture on NNA was positive in 135 (81%) of 166 cases. Detailed comparison of KOH/CFW, Gram and Giemsa with culture is tabulated in table 2.

Table 2: Comparison of Calcofluor white, Gram and Giemsa staining with culture in detecting *Acanthamoeba* in corneal scrapings

	KOH/CFW		Gram		Giemsa		Total
	+	-	+	-	+	-	
Culture +	118	17	109	26	95	40	135 (81%)
Culture -	29	2	26	5	27	4	31
Total	147 (89%)	19	135 (81%)	31	122 (73%)	44	166

+ : Positive — : Negative CFW: Calcofluor white

Shaded values: Total of specimens positive by KOH/CFW, Gram and Giemsa and their percentages in parenthesis

Histopathology

Five out of 18 (27.7%) samples that were studied for histopathological features showed presence of granulomatous inflammation. The histopathologic features of these cases included epithelial ulceration with destruction of Bowman's layer in all the cases. The stroma showed inflammatory infiltrates consisting of neutrophils in the anterior two-thirds of stroma. Vascularization of stroma was noted in mid and deep peripheral stroma in two cases. Viable and degenerated cysts of *Acanthamoeba* were seen in the stroma. The deeper stroma and the region around Descemets membrane showed a few aggregates of epitheloid cells, lymphocytes and multinucleated giant cells. Some of the giant cells and occasional keratocytes showed cysts of *Acanthamoeba* in the cytoplasm, suggesting the phagocytosed parasites. When immunophenotyped the inflammatory cells in the corneal stroma were found to be of T cell population. In the granulomatous regions, the cells were positive for T cells, CD 68 and negative for B-cell marker, suggesting a predominance of T lymphocytes with macrophages.

Typing of amoebae

Morphological

All the three groups (based on the morphology of cysts and trophozoites) were represented within the 13 Indian isolates included in this study (table 1).

18S rDNA based

The rDNA based phenogram obtained from partial and complete sequencing of amoebae tested in this study belonged to 'Type T4' sequence (figure 1). The genetic identities were supported by very high bootstrap values that indicate the robustness and reliability of the rDNA sequence data obtained in the study for establishing the genetic affiliation of the analyzed amoeba isolates. Partial rDNA sequences specific to 12 amoeba isolates analyzed in the study are deposited in GenBank (Accession Nos. AF534143-AF534154).

Alignment of 7 Indian isolates with 14 *Acanthamoeba* reference strains (representing all genotypes) revealed 431 and 2961 bases for partial and complete sequence of 18S rDNA respectively. The aligned sequences were uncut, i.e, gaps, introns and unambiguous positions within the sequences were not removed. Average dissimilarity values between the genotypes ranged from 4.55 to 17.04% and 3.73 to 53.3% for partial and complete sequences respectively (table 3). Most of dissimilarity values obtained from partial sequencing were higher when compared to complete sequencing. When using partial sequencing data set overall sequence variation ranged from 0.2 - 3.8% (average 2.48%) and 0 - 3.5% (average 2.18%) between T4 and Indian isolates, (table 3). The values obtained from complete sequencing were comparable. Average dissimilarity values for T3 and T11 when compared to T4 were 5.91% and 5.50% (partial sequencing) and 4.81% and 4.51% (complete sequencing).

DISCUSSION

As reported earlier (Sharma *et al*, 2000) there was only one case among our patients having history of contact lens wear. Trauma was found to be a major risk factor among our patients. In recent years patients with *Acanthamoeba* keratitis are being increasingly diagnosed in India, majority of whom are in non-contact lens wearers (Davamani *et al*, 1998). Routine microbiological methods are often useful in reaching a diagnosis as is shown in this study. We found KOH/Calcofluor staining of corneal scraping to be highly sensitive (89%) in detecting *Acanthamoeba*, followed by Grams staining (81%) and Giemsa staining (73%). Culture was positive in 81% of the cases. Thirty one cases among 166 were culture negative and smear positive (either of three stains), while 17 were smear negative (none of the three staining techniques were positive) and culture positive. Using PCR we have recently reported only a marginal increase in the sensitivity and specificity in the diagnosis of AK (Pasricha *et al*, 2003).

Despite intensive medical therapy with PHMB and/or chlorhexidine some patients require penetrating keratoplasty/evisceration for the control of infection. We had the opportunity to examine 18 such sample constituting 10% of all the cases seen by us. Histologically, the corneal tissues in AK show evidence of epithelial ulceration, polymorphonuclear infiltrates, stromal necrosis along with the presence of trophozoites and/or cysts in the corneal stroma.(Garner *et al*, 1993) Despite the prolonged clinical course of the disease, a few unique observations have been made in *Acanthamoeba* keratitis which include: absence of vascularization, scarcity of lymphocytes (Kremer *et al*, 1994) keratocyte loss through apoptosis and the presence of cysts in the deep stroma, unaccompanied by inflammatory cells(Vemuganti *et al*, 2000). Though acanthamoebic infections of brain usually evoke granulomatous inflammation, this is rarely reported in *Acanthamoeba* keratitis (Meitz *et al*, 1997). We observed granulomatous inflammation in the corneal stroma in five patients presenting with rapidly progressing *Acanthamoeba* keratitis, with extension to the limbus. Granulomatous inflammation is a type of

chronic inflammation characterized by the collection of modified macrophages, namely the epitheloid cells with or without associated multinucleated giant cells and lymphocytes. Though definite pathogenesis of the granulomatous reaction in general remains unknown, the process may have a non-immune or immune aetiology. The presence of T lymphocytes, as found in this study, suggests that granulomatous inflammation in the cornea may be an immune-mediated process, although further studies are warranted to understand the varied presentations of this disease and its clinical implication.

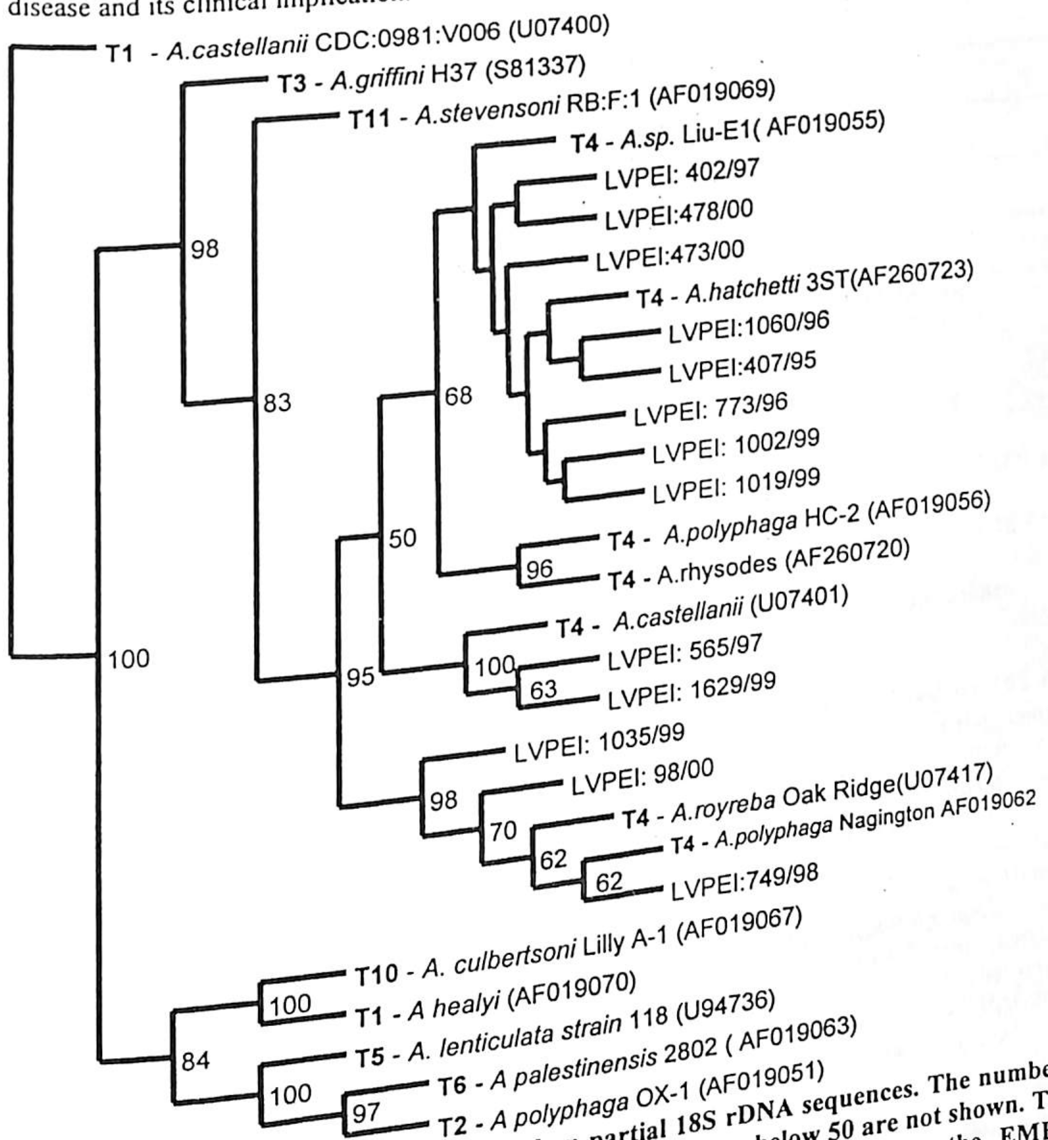

Figure 1. UPGMA phylogenetic tree based on partial 18S rDNA sequences. The numbers at the nodes are the bootstrap values and the value below 50 are not shown. The accession numbers of the reference sequences obtained from the EMBL database are given in parenthesis. Thirteen Indian isolates are shown as LVPEI (L V Prasad Eye Institute) with corresponding laboratory isolation number.

Table 3: Percent average dissimilarity between genotypes using partial and complete sequences of 18S rRNA gene.

	T1	T2	T3	T5	T6	T10	T11	T12	T4	Indian isolates
T2	**17.02*** 9.12#									
T3	**9.96** 6.96	**16.76** 7.54								
T5	**13.22** 36.05	**8.44** 35.66	**12.19** 53.33							
T6	**16.45** 10.17	**4.74** 4.30	**15.94** 8.44	**7.87** 35.74						
T10	**10.99** 11.32	**15.51** 12.24	**12.56** 11.30	**11.82** 38.78	**14.73** 12.27					
T11	**9.34** 7.30	**17.63** 7.85	**7.99** 3.73	**12.20** 34.55	**16.75** 8.65	**11.63** 10.80				
T12	**10.79** 12.15	**16.94** 13.00	**11.24** 12.71	**12.31** 40.46	**16.37** 13.09	**4.55** 7.85	**11.78** 12.23			
T4	**9.36** 6.52	**17.04** 8.00	**5.91** 4.81	**13.16** 35.57	**16.26** 9.31	**12.00** 11.70	**5.50** 4.51	**11.95** 13.45	**2.48** 2.20	
Indian isolates	**9.53** 6.52	**17.33** 8.04	**6.32** 4.73	**13.27** 35.52	**16.28** 9.21	**12.18** 11.71	**5.16** 4.45	**11.87** 13.49	**2.07** 3.59	**2.18** 2.17

* Average percentage dissimilarity between genotypes using partial sequences of 18S rRNA gene.

\# Average percentage dissimilarity between genotypes using complete sequences of 18S rRNA gene.

Shaded values denote the average sequence dissimilarities within T4 and Indian isolates.

We have recently published genotyping of *Acanthamoeba* isolates based on 16S rRNA mitochondrial gene (Ledee *et al*, 2003). In this study, we report results of genotyping using 18S rRNA gene. Our study revealed that there exists a high degree of genetic diversity among the *Acanthamoeba* isolates (figure) and all these carry the 'T4 signatures'. Within themselves, the Indian isolates showed considerable variation suggesting multiple *Acanthamoeba* species. This was emphasized by the fact that representatives of various species of *Acanthamoeba* (T4 reference strains) clustered in T4 genotype. All the three groups (based on morphology) representing various species grouped together in T4 genotype, suggesting again that T4 genotype consists of various species and that the morphological classification cannot be regarded as a tool for speciating *Acanthamoeba*. These results are in conformity with the original work of Gast *et al* which describes the rDNA sequence types and shows that the 'T4 type sequence' characterizes a heterogeneous group of pathogenic isolates of *Acanthamoeba* comprising of many different species.

The results of this study further show that partial sequencing of ASA.S1 provided sufficient interstrain sequence variation to distinguish several clusters of 18S rDNA genotypes and also had ability to distinguish between the closely related T3-T4-T11 clade, since the average

dissimilarity values of both T3 and T11 were >5% , which is considered to be cut-off value to separate any two genotypes. The advantages which partial sequencing has over complete sequencing is that it is less cumbersome, less expensive and consumes much less time both for sequencing and analyzing the data. Thus, in this study we found that partial sequencing of the 18S rDNA served our purpose of genotyping *Acanthamoeba* isolates and hence can be used as a tool both for sub-genus classification and as a marker to detect *Acanthamoeba* in clinical specimens as previously described by us (Pasricha *et al*, 2003).

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LIST OF PRESENATATIONS AND AWARDS

1. Molecular genotyping of clinical isolates of *Acanthamoeba* from Indian patients with keratitis and development of simple PCR based assay for its easy detection. **Gunisha Pasricha,** Debashish Das, Ramesh Aggarwal, Savitri Sharma at **XXV Congress of IAMM** at AIIMS, Delhi 2001. ***IAMM silver jubilee prize for the best paper in Parasitology***

2. 18S and 26S rDNA based genotyping of *Acanthamoeba* isolates and development of multiplex PCR assay for diagnosis of *Acanthamoeba* keratitis. **Gunisha Pasricha,** Debashish Das, Ramesh Aggarwal, Savitri Sharma at the Scientific sessions of **70th Society of Biological Chemists (India)** held at Hyderabad 2001. ***B S Narsinga Rao best poster award.***

3. Genotyping of *Acanthamoeba* from Indian patients with non-contact lens related keratitis. **Gunisha Pasricha,** Debashish Das, Ramesh Aggarwal, Savitri Sharma at **XIth Annual Meeting of Indian Eye Research Group** held at L V Prasad Eye Institute Hyderabad, 2002.

4. 18S and 26S rDNA based genotyping of *Acanthamoeba* isolates and development of multiplex PCR assay for diagnosis of *Acanthamoeba* keratitis. **Gunisha Pasricha,** Debashish Das, Ramesh Aggarwal, Savitri Sharma at **6th ADNAT symposium**, 2002 held at CCMB Hyderabad. ***Best poster award***

5. Granulomatous inflammation in *Acanthamoeba* keratitis-a clinico-pathological study of five cases. **Gunisha Pasricha**, Geeta K Vemuganti, Prashant Garg, Savitri Sharma at **Xth Annual Meeting of Indian Eye Research Group** held at L V Prasad Eye Institute Hyderabad, July 2003.

6. Current perspectives of *Acanthamoeba* keratitis in India: Predisposing factors, Microbiology, Histopathology and Genetic characterization. **Gunisha Pasricha**, Savitri Sharma, Geeta K Vemuganti, Gregory C Booton, Prashant Garg, Debashish Das, Ramesh K Aggarwal at the **Xth International Meeting on the Biology and Pathogenicity of Free-Living Amoebae** at Campus Nainari, Cd Obregon, Sonora, Mexico, October 2003